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

and

E.F.P.P.

The European Foundation for Plant Pathology
Working Group on Biological Control

Biological and Integrated Control of Root Diseases in Soilless Cultures

PROCEEDINGS OF A WORKSHOP

at

Dijon, France
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Edited by C. Alabouvette

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INTRODUCTION

This bulletin contains reviews and short research papers presented at the workshop "Biological and Integrated Control of Root Diseases in Soilless Cultures". This workshop held at Institut National de la Recherche Agronomique in Dijon, France, from 18-21 September 1995, was the fourth joint meeting of the Working Group on Biological Control of Fungal and Bacterial Plant Pathogens of the International Organization for Biological Control, West Palaearctic Regional Section (IOBC/WPRS) and the Working Group on Biological Control of the European Foundation for Plant Pathology (EFPP). The 3-day workshop with 29 contributions was attended by over 35 scientists from 7 European countries, Canada and two Middle Eastern Countries.

The workshop was intended to bring together scientists with interests in biological control of root diseases in modern systems of soilless cultures in greenhouses. The 6 sessions were introduced by a review paper given by a senior scientist followed by short research papers preferably given by young scientists.

The first session focused on the characteristics of root diseases specific to soilless cultures. The following sessions covered screening of biological agents, study of their modes of action, population dynamics of both the pathogens and the biocontrol agents, interactions with the host-plant, and finally all the problems linked with production, formulation and application of biocontrol agents in soilless systems.

The workshop demonstrated the need of studying the epidemiology of root-diseases in soilless cultures. Even when the pathogens are the same as in soil, their distribution and the conditions optimal for their spread and development are quite different in soilless cultures. Therefore understanding the life cycle of the pathogens in relation to cultural practices appears to be a key factor for achieving successful control of the diseases. Great progress has been made in the understanding of the mode of action of the biocontrol agents and of their interactions with the plant at the molecular level. But, in that field nothing is specific to soilless cultures. On the contrary, in cultural systems where the nutrient solution is circulating, population dynamics studies are very important to determine the survival abilities of the biocontrol agents and to choose the best formulation and application procedures. The workshop showed that soilless cultures in greenhouses offer specific opportunities to apply microorganisms to control root-rot and wilt diseases.

Thanks are due to the following organizations for supporting the workshop financially or providing facilities free of charge, and thus helping to keep the cost of participation at a reasonable level :
- International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palaearctic Regional Section (IOBC/WPRS),
- European Foundation for Plant Pathology (EFPP),
- French Society of Phytopathology (SFP),
- Institut National de la Recherche Agronomique (INRA).

The IOBC/WPRS and the EFPP Working Groups are continuing their joint activities. Through the management committee consisting for the IOBC/WPRS of Claude Alabouvette (France), Yigal Elad (Israel) and Nyckle J.Fokkema (Convenor IOBC/WPRS, The Netherlands) and for the EFPP of Geneviève Defago (Switzerland), John Hockenhull (Convenor EFPP; Denmark) and John M.Whipps (United Kingdom), further joint workshops are planned. In addition, the committee is

also involved in the planning stage of the next International Congress of Plant Pathology to be held in Edinburgh in 1998.

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

The organisation of the workshop and the editing of the proceedings would not have been possible without the assistance of Martine Janisz, Patrice Richard, Marie Paule Pasdermadjian and Patricia Janisz to whom the organisers are forever grateful. In addition, thanks must also go to all the other helpful and friendly INRA staff who made the workshop such a success.

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Dijon, June 1996

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TABLE OF CONTENTS

Modes of action of biocontrol agents

Population dynamics

Production, formulation, application of biocontrol agents and integrated control

Detection of plant pathogens and disease risk assessment in soilless cultures

ROOT PATHOGENS IN MODERN CULTURAL SYSTEMS - Assessment of risks and suggestions for integrated control

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Abstract

In the modern systems not only diseases, known in soil cultures, have been observed, but also new diseases, mostly caused by the root pathogens *Fusarium*, *Phytophthora* and *Pythium*. Epidemiological studies have revealed that dispersal of the pathogens depends largely on the irrigation system and the introduction of the pathogens into the systems. Risks of dispersal are higher in systems with overhead- irrigation than in sub irrigation systems, especially when the nutrient solution is recirculated. However, because of the characteristics of the system, risks are lowest in ebb and flow systems. The most prominent problems occur when the nutrient storage container becomes infested. Spread from individual diseased plants is limited.

To prevent serious problems with root pathogens, an integrated disease management system can be developed. At the start of a culture the system must be clean. The use of old contaminated substrates can cause severe problems in a new crop. The water used for the nutrient solution must be pathogen free. Waterbasins contaminated with root pathogens has been observed. Disinfection before use might be necessary. Introduction of the pathogens into the systems via apparently healthy, but contaminated, young plants also has been demonstrated. Techniques to detect pathogens in such material have to be developed. When dispersal occurs, the recalculating nutrient solution should be disinfected. Several disinfection methods, general or selective, are available. Depending on the pathogen one of them can be used. Besides these basic measures, problems can be lessened by using (partially) resistant varieties.

Biocontrol agents can play an important role in suppressing root pathogens in the modern systems, especially because of the limited volume of the substrate, which enables a good interaction between host, pathogen and antagonist. Promising results have been obtained.

Introduction

The last 10- 15 years, new cultural systems, out of the open soil, have been developed especially in glasshouses. Reasons for this change-over were : energy-saving, contamination of the open soil with soil-borne pathogens and especially pressure to reduce the emission of pesticides and nutrients to the environment.

Often these modern systems are called soilless cultures, which in fact implies the absence of a substrate. Real soilless cultures, without a substrate, are nutrient film technique (NFT) and root misting. However in most systems some kind of substrate is used. This substrate can be either natural, viz. soil, sand, lava, expanded clay, coconut fibers etc., or artificial, viz. rockwool, aggrofoam, perlite etc.. The substrates can be placed in various types of "containers", for example pots, buckets, gullies, tables, concrete floors or enfolded in plastic. The essence of the systems is that they are closed, and separated from the subsoil. Frequently the nutrient solution is being drained off, but although this is not a common practice yet, the nutrient solution can be recirculated.

In practice, various systems can be found. However essentially only two different irrigation principles can be distinguished : overhead-irrigation and sub-irrigation, both either continuously or discontinuously. In both irrigation systems the nutrient solution can be drained off or recirculated. An important variation of sub-irrigation is the ebb and flow system, which is by definition discontinuous and recycles the nutrient solution.

Since the introduction of the modern systems several problems caused by soil-borne pathogens have been reported. Not only diseases known in traditional cultural systems occur, such as fusarium wilt of carnation, freesia or tomato, fusarium crown and root rot of tomato and *Phytophthora* and *Pythium* spp. in several crops, but also new problems such as a new *Phytophthora* sp. and *Gnomonia* sp. in roses and a *Cylindrocladium* sp. in *Spathiphyllum*. In Table 1 an overview is given of the most prominent root-pathogens observed in the modern culture systems.

Fungal species	Host plant
Gnomonia sp.	Rose
Phytophthora spp.	Azalea, Campanula, Chamaecyparis, Cucumber, Erica, Gerbera, Hedera, Rose, Saintpaulia, Sinningia, Spathiphyllum, Sweet pepper, Tomato
Fusarium spp.	Carnation, Cucumber, Cyclamen, Freesia, sweet pepper, Tomato
Olpidium spp.	Lettuce, Sweet pepper
Pythium spp.	Anthurium, Chrysanthemum, Cucumber, Lettuce, Spinach, Tomato
Viruses	Cucumber, Eggplant, Lettuce, Sweet pepper

Table 1. Overview of soil-borne pathogens observed in glasshouse crops in modern (recirculation) cultural systems

Dispersal of root pathogens

Epidemiological studies have revealed, that dispersal of the pathogens depends largely on the type of system used, and especially the method of irrigation. There are numerous variations in systems, but only two different irrigation principles can be recognised : sub- and over-head irrigation. An important variation of sub-irrigation is the ebb-and-flow system. Although recirculation systems being developed, the nutrient solution is still frequently drained off. Risks of dispersal of the soil-borne pathogens are higher in recirculation systems than in systems with drainage of the nutrient solution. In systems with overhead-irrigation root pathogens are dispersed more easily than in systems with sub- irrigation, especially when the nutrient solution in the storage container is contaminated. Spread from individually contaminated plants is limited. Risks are lowest in ebb and flow systems (Table 2).

Table 2. Survey of literature concerning dispersal of soil-borne fungi in modern cultural systems

PATHOGEN	CROP			AUTHOR	DISPERSAL
Fusarium oxysporum					
f.sp.*dianthi*	carnation	sub-irrigation	recirculation	Rattink, 1992	+
f.sp.*radicis lycopersici*	tomato	drip-irrigation	recirculation	Rattink, 1991a	±
		drip-irrigation	drainage	Hartman and Fletcher, 1991	±
		sub-irrigation	drainage	Mihuta- Grimm *et al.*, 1990	±
f.sp. *gladioli*	Freesia	sub-irrigation	recirculation	Rattink, unpubl. data	–
f.sp. *cyclaminis*	Cyclamen	ebb and flow	recirculation	Rattink, 1990	+
				Stelder, 1991	+
Phytophthora					
cinnamomi	Azalea, Erica	sub-irrigation	drainage	Wohanka, 1985	–
		sub-irrigation	recirculation	Wohanka, 1985	+
	Erica	overhead	drainage	Braune, 1987	–
		overhead	recirculation	Braune, 1987	+
	Chamaecyparis	overhead	recirculation	Van Kuik, 1992	+
cryptogea	Campanula	ebb and flow	recirculation	Thinggaard, 1990	+
	Gerbera	sub-irrigation	recirculation	Rattink, 1983	+
		ebb and flow	recirculation	Thingggaard, 1990	?
	tomato	NFT	recirculation	Evans, 1979	+
fragariae	strawberry	NFT	recirculation	Benoit, 1986	+
nicotianae	pot plants	ebb and flow	recirculation	Thinggaard and Middelboe, 1989	?
	Saintpaulia	sub-irrigation	drainage	Wohanka, 1988	–
		sub-irrigation	recirculation	Wohanka, 1988	+
		ebb and flow	recirculation	Rattink, unpubl.data	–
	tomato	NFT	recirculation	Van Voorst *et al.*, 1987	+

Table 2. Survey of literature concerning dispersal of soil-borne fungi in modern cultural systems

Pythium					
aphanadidermatun	spinach	sub-irrigation	recirculation	Bates and Stanghellini, 1984	+
				Stanghellini *et al.*, 1984	+
	cucumber	drip-irrigation	recirculation	Paternotte, 1992	+
		drip-irrigation	recirculation	Rattink, unpubl.data	+
		ebb and flow	recirculation	Sanogo and Moorman, 1990	+
		ebb and flow	recirculation	Postma, unpubl. data	+
		ebb and flow	recirculation	Rattink, unpubl. data	+
		NFT	recirculation	Grote and Goehler, 1992	+
		NFT	recirculation	Moulin *et al.*, 1994	+
	poinsettia	ebb and flow	recirculation	Hoitink *et al.*, 1991	+
ultimum	cucumber	NFT	drainage	Evans, 1979	+
		drip-irrigation	recirculation	Chérif and Bélanger, 1991	+
splendens	Anthurium	sub-irrigation	drainage	Boehmer and Papenhagen, 1984	–
spp.	luttuce	sub-irrigation	drainage	FunckJensen and Hockenhull, 1983	–
				Hockenhull and FunckJensen, 1983	–

+ : dispersal positive; - : dispersal negative; ± : conditional dispersal; ? : dispersal not proven

Integrated disease management

Since soil-borne pathogens can be dispersed in the modern cultural systems, introduction of the pathogens into the system should be prevented. Special emphasis should be put on establishing a disease-free system, using disease-free planting material, and pathogen-free irrigation water at the start of each new culture. The use of (partially) resistant cultivars, physical and /or mechanical control methods, biological control agents and strict hygienic measures can help minimise the risks during the growing period.

Disease-free systems

To prevent an initial contamination, the systems must be disease-free at the start of a new crop. This means that either completely new systems, with new substrate (natural or artificial), new irrigation tubes, etc. must be installed or that used systems must be cleaned thoroughly. Not only gullies or tables and the substrate must be disinfected, preferably by heating (steam), but also the greenhouse itself, support material and all other parts of the system, such as storage containers, tubes, sprinklers etc., on the inside and on the outside.

Disease-free planting material

Since spores of pathogens can be spread from diseased plants into the recirculation system, the planting material must be absolutely disease-free. To achieve this, techniques must be available to produce disease-free planting material and to check the sanitary situation during the propagation period. For a number of crops such techniques are available. For example by using in-vitro propagation *Phytophthora*-free plantlets of Gerbera can be produced. By using in-vitro propagation and/or culture indexing, carnation cuttings are delivered free from pathogens such as *Phialophora cinerescens* and *Fusarium oxysporum*. However for a number of other pathosystems, like *F. oxysporum* in cyclamen or Freesia, *Phytophthora* in roses or potplants, reliable sanitary propagation and detection techniques are not used or available.

New modern detection techniques, and certification systems based on them, must be developed for the most important fungi in all crops. Generally visual inspections and traditional detection techniques, like isolation on specific media or serological techniques, are either not reliable, specific or fast enough to detect fungi which are systemically present in low quantities.

Recent research indicates good possibilities for reliable and fast detection of pathogens, present in low amounts in the plant, by the use of electrophoresis techniques, monoclonal antibodies or DNA- probes (Kerssies *et al.*, 1994).

Pathogen-free irrigation water

Frequently dispersal of soil-borne pathogens by water or nutrient solution in closed systems has been demonstrated, especially when the nutrient solution in the storage container becomes infested (Rattink, 1977; 1983; 1991a). Thus water going to be used for the nutrient solution must be absolutely free of root pathogens.

For irrigation, water from several sources can be used, such as tap water, rainwater, or water from wells, ponds, ditches or canals. Tap water, rainwater and water from wells are by definition pathogen-free. However, tap water often is not suitable for glasshouse crops and water from wells often not available. Water from ditches, canals or ponds must be considered suspicious, especially in regions with concentrations of greenhouses, since pathogens can be leached out from contaminated soil via the drainage system to the surface water (Rattink, 1977). These types of water can only be used after disinfection.

However, even the use of well or rain water could be a risk when collected in (open) tanks or water basins, because these are vulnerable to contamination with soil-borne pathogens. A survey has revealed the presence of *F. oxysporum* f.sp. *radicis-lycopersici and F. oxysporum* f.sp. *cyclaminis* in water basins (Rattink and van der Sar, 1990). Strong indications, based upon observations in practice, exist that other fungi, such as *Pythium, Phytophthora* and *Verticillium*, and viruses can be present. It has also been established, that these fungi can survive and remain pathogenic for a very long time in aqueous surroundings (Rattink, 1986).

These data have resulted in changing the old ideas about disinfecting the recirculated nutrient solution to the new idea of disinfecting the irrigation water, which is going to be used for the nutrient solution, before introduction into the system.

Physical and mechanical control methods

Since the introduction of the recirculation systems, research has been done on the development and efficacy of methods to disinfect the nutrient solution.

The effectiveness of the individual methods differs. Some methods ("general"), for example those based on heating, ozone and high UV- irradiation, kill all important pathogens, fungi as well as bacteria and viruses, completely. Other methods ("selective"), like iodine, low UV- irradiation, hydrogen peroxide + "activators" and slow filtration through a sand filter are only effective against bacteria and some fungi (Runia, 1995). The individual practical situation, but particularly the phytopathological problem determines which of the available methods should be choosen.

Other physical control methods are available to prevent, to a large extent, the introduction of root pathogens into the system from storage containers or water basins. Spores of some fungi, especially *Fusarium*- spp., settle under gravity at the bottom of the tanks or basins, when the water or nutrient solution is not disturbed for some time (Rattink, 1990). By using a sedimentation tank or by withdrawing the water from just below the surface, a large part of the spores remain at the bottom of the container and only few are dispersed.

Resistant cultivars

Resistant cultivars can play an important role in an integrated disease management system. However, breeding for resistance takes a long time. After developing a standard test, the resistance mechanism has to be identified and then incorporated by long-term breeding programmes. Therefore the importance of the crop determines the efforts made in this field. In vegetable crops resistance breeding has a long history. Most important crops are nowadays resistant to the most important root pathogens, like races and formae speciales of *F. oxysporum*, *Verticillium* and *Phytophthora*.

Until a few years ago, in floriculture resistance breeding was more or less restricted to carnation and Freesia, to obtain new cultivars resistant to *Fusarium* wilt. The last years more research programmes have been started by government and private research organizations in other crops, such as Chrysanthemum against *Verticillium* and *F. oxysporum* f.sp. *chrysanthemi* and Gerbera and pot plants against *Phytophthora* spp.

Biological control

Biological control of soil-borne fungi in open soil, in glasshouses, is generally disappointing. By contrast, it has been demonstrated during the last 5- 10 years, that biocontrol offers very promising prospects in systems with a limited amount of substrate, either natural or artificial (Rattink, 1989b; 1991b; Postma and Rattink, 1991; 1992; Rattink and Postma, 1995).

Fusarium wilt of carnation can be controlled very effectively by a preventive addition to soil or nutrient solution of the nonpathogenic isolate of *F. oxysporum* nr 618-12. In all experiments disease reduction was 80% or more. A single preventive application reduced an attack by *F. oxysporum f.sp. dianthi* to less than 5% during two consecutive one-year crops in soil and during three consecutive crops on artificial substrate in a recirculation system, regardless repeated inoculation with the pathogen. Also in other crops, such as Freesia, Cyclamen and Chrysanthemum the same nonpathogenic isolate showed positive effects, although not as good as in carnation. An isolate of *Trichoderma harzianum* reduced considerably problems caused by *F. oxysporum* f.sp. *radicis-lycopersici* in tomato. Recent research indicates good possibilities of control of *P. aphanidermatum* on cucumber by *Streptomyces griseo-viridis* or *Pythium oligandrum* (Postma, Rattink, unpublished data).

Data from research on biological control in different pathosystems in closed cultural systems indicate possibilities of translation of the results to other models. However this data also indicate the necessity of testing the available antagonists individually in each pathosystem. The effects depend largely on the interactions between the hostplant and the pathogen and the interactions between pathogen and antagonist.

Especially in closed cultural systems, with a limited amount of substrate, prospects for effective biocontrol of the most important soil-borne fungi in most crops are promising. However, practical application may be delayed by registration procedures.

Conclusion

To reduce the use and emission of pesticides and nutrients to the environment a change over must take place from cultural systems with free drainage of the excess nutrient to closed systems with re-use of the nutrient solution.

These systems pose, especially in connection with soil-borne fungi, new phytopathological problems. The soil-borne pathogens can be spread in the systems. The risks of dispersal depend largely on the irrigation system used.

Serious problems can be avoided by integrated disease management programmes. In these programmes, a number of individual control methods are combined: a disease-free start of the culture (disease-free system, disease-free planting material, pathogen-free irrigation water), hygienic measures, (partially) resistant cultivars, physical and mechanical control methods and biological control. However, research has to be further intensified to optimize the effects of the individual parts of this programme.

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ASSESSMENT OF PATHOLOGICAL RISKS ASSOCIATED WITH RECYCLED NUTRIENT SOLUTION IN SOILLESS CULTURE OF TOMATO

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Soilless culture can be performed either in open or in closed systems. In the open systems as opposed to the closed ones, a fraction of the nutrient solution is drained. This drainage both constitutes a waste of fertilisers and contributes to the water pollution. So development of closed culture systems shoud occur in France in the years to come. However, the growers are concerned about the pathological risks which might be increased in the closed systems compared to the open ones. Indeed, zoospore producing fungi are favoured in aquatic environment such as soilless culture.

In the present study, pathological risks associated with the recycling of the nutrient solution were assessed during a crop of tomato cultivated in rockwool in commercial-like conditions. Three different experimental conditions were compared: open system, closed system and closed system with ultraviolet irradiation of the nutrient solution. Yield of tomato and level of root colonisation by different fungi including *Pythium* spp. and *Fusarium* spp. have been recorded.

Differences could be stressed between the yields from the different experimental treatments. After 8 months of culture, the highest yield (26.96 $kg.m^{-2}$) was obtained from the open system, the lowest one (25.07 $kg.m^{-2}$) was intermediate between the two previous ones. The yield depression in the closed system compared to the open one was associated with a significant increase of the level of root decreased significantly the percentage of roots colonised by *Pythium* spp. but not that of roots colonised by *Fusarium* spp. No symptoms could be detected on the upper part of the plants in any treatments.

The results of this study suggest that even in absence of any major pathological problem, recycling the nutrient solution leads to a yield reduction. This reduction could be related to root losses due to fungi. Further studies are underway to evaluate different methods to control these fungi including UV irradiation and antagonistic micro-organisms.

PYTHIUM APHANIDERMATUM RESPONSIBLE FOR ROOT DAMAGES AND YIELD REDUCTION IN SOILLESS CULTURES OF CUCUMBER

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Abstract

Pythium spp. not only cause damping-off of seedlings but also root rot in older plants. In soilless cultures where the fungus can spread easily with the nutrient solution, root rot can lead to dramatic yield reductions, but usually symptoms on the upper parts of the plants are not characteristic of the disease. The diagnosis of the disease is difficult, and therefore the diagnosis of the *Pythium* species responsible for these symptoms.

An experimental study was conducted to assess the pathogenicity of several *Pythium* species under different experimental conditions chosen in order to favor damping-off symptoms or root rot.

39 *Pythium* isolates obtained from discolored roots were assayed for their ability to cause damping-off on cucumber seedlings in sand-peat and for their pathogenicity in soilless culture of cucumber in rockwool or hydroponic solution. Isolates of *Pythium aphanidermatum*, *P. irregulare*, *P. sylvaticum* and *P. ultimum* were highly pathogenic in sand-peat, but only *P. aphanidermatum* strains were pathogenic in soilless conditions and led to root decay, plant death in rockwool culture and growth reduction in hydroponic culture. One strain of *P. aphanidermatum* significantly reduced the yield of cucumber grown in rockwool under conditions similar to those of commercial cultures.

Introduction

Pythium spp. is an important group of pathogenic fungi causing damping-off of seedlings of numerous plants (Hendrix and Campbell, 1973 ; Van Der Plaats-Niterink, 1981). More recently, it has been shown to be pathogenic on older plants in soil, leading to yield reductions on sugarcane (Lee and Hoy, 1992), turfgrasses (Nelson and Craft, 1991) or wheat (Chamswarng and Cook, 1985). This problem is also particularly important in soilless cultures where nutrient solution can facilitate the fungus development leading to the rot of all the root system.. It has been mentionned on tomato (Jenkins and Averre, 1983), spinash (Bates and Stanghellini, 1984 ; Gold and Stanghellini, 1985), tulips (Westseijn, 1990) and lettuce (Funck-Jensen and Hockenhull, 1983 ; Jenkins and Averre, 1983). In soilless culture of cucumber, symptoms are not so evident ; in clear cases, roots show localized necrotic lesions that spread over the root system which decays. In other cases, the upper parts of plants may wilt during warm and sunny days even in the absence of obvious symptoms of necrotic lesions on the roots (Couteaudier and Lemanceau, 1989; Favrin *et al*, 1988; Stanghellini and Kronland, 1986) that appear uniformly discolored. Plant infection by *Pythium* may lead to yield reductions and/or death of plants. *Pythium aphanidermatum* has been shown to be highly pathogenic under standardized soilless conditions leading to root rot and death of young plants (Favrin *et al.*, 1988 ; Jenkins and Averre, 1983). However, numerous isolates other than *P. aphanidermatum* species are regularly isolated from cucumber roots, and their pathogenicity is still unclear. Moreover, no data are available on the quantitative importance of yield losses due to *Pythium* spp. in commercial cultures.

The purpose of the present study was to determine the pathogenicity on cucumber plants of many isolates of *Pythium* spp. obtained from different origins. As it was impracticable to test all of the strains for their ability to induce yield reduction on mature plants, we developed a test to evaluate the pathogenicity of *Pythium* spp. on young cucumber plants grown in rockwool or in

hydroponic solution. These isolates were also characterized for their ability to cause damping-off. Finally, one isolate of *Pythium* screened for its ability to induce death of plants in rockwool and to reduce growth of cucumber in hydroponic culture, was also tested for its ability to cause yield reduction in a cucumber culture under conditions analogous to those of a commercial crop.

Material and Methods

Microorganisms and inoculations

The 39 isolates of *Pythium* were obtained from discolored roots of cucumber or tomato plants in soilless cultures with or without aerial symptoms. They were maintained on cornmeal agar (CMA, Difco Laboratories, Detroit) at room temperature. Species were identified on CMA and V8-juice agar (20%,v/v) according to the key of Van der Plaats-Niterink (1981).

Inoculation with mycelium was used to compare the pathogenicity of isolates. For the damping-off test, isolates were grown for 48 h on malt agar. For the soilless tests, the inoculation of the plants was made either by introduction of an entire cultured plate on CMA in the hydroponic solution or by introduction into the rockwool of ground mycelium grown for 5 days on malt liquid. To test the ability of *Pythium aphanidermatum* (Edson) Fitzp. to cause reductions in fruit yield, zoospores were used as inoculum. A 5 day-old culture on V8-juice agar was flooded with sterile water enriched with ß-sitosterol (0.002%) to improve the production of zoospores; after 4 h, the sporangia that had formed released zoospores into the solution. Zoospore density was evaluated with a haemacytometer after immobilization of an aliquot of the suspension in a vortex mixer.

Plant growth and inoculation

The method proposed by Bouhot (1975) was applied to assess the ability of the isolates of *Pythium* to cause post-emergence damping-off of cucumber seedlings in a sand -peat mixture. When the cotyledons were open, one 8 mm-diam plug of mycelium cut from a 48-h-old malt agar culture of *Pythium* was added around the hypocotyl of seedlings. The characteristic symptoms of damping-off appeared 5 days later and were evaluated after 6 days. There were four replicates of three plants per isolate in a randomized design. This experiment has been reproduced with similar results.

For the root rot test in rockwool, seeds of cucumber (cv. 'Corona') were germinated in sterile Petri dishes on filter paper wetted with sterile water and after 24 h were sown in 9 ml rockwool plugs and watered daily with nutrient solution (Hydrokani; Hydro Azote spécialités; Vitrolles) (EC=2; pH=5). Each plant was inoculated 7 days after sowing with 9×10 propagules (corresponding to 1×10 mycelial fragments ml^{-1} rockwool). Number of diseased plants was assessed daily over a 3-week period. Each isolate was applied to six replicates of 12 individual plants in a randomized design, and the experiment was repeated.

For the root rot test in hydroponic, young plants (grown 10 days in rockwool plugs as described above) were transplanted to tanks (1 l) containing 900 ml of nutrient solution (Hydrokani; EC=2; pH=5) (one plant per tank). One week later, each tank was infested with a 5-day-old CMA culture of *Pythium*. Tanks of control plants received noninoculated CMA. Two weeks after adding the inoculum, shoots and roots were dried and weighed. Three replicates per isolate were arranged in a randomized block design, and the experiment was repeated twice.

An isolate of *P. aphanidermatum* (Edson) Fitzp. (OP4) pathogenic in the previous tests, was tested for its ability to reduce yield in cucumber grown under greenhouses conditions. Cucumber seeds (cv. Corona) were sown in 10x10x6.5-cm rockwool blocks (one plant per block) and watered daily with a nutrient solution (Hydrokani) maintained at pH 5.5 with a conductivity of 1.8 to 2.5 $mScm^{-3}$. 3 weeks after sowing, the blocks were transplanted to 100x15x6.5-cm rockwool pads (three plants per pad) on gutters (three pads per gutter) that return the drainage to a storage

tank (30 L) so that the plants of each replicate and treatment (3x3 plants) were grown in an independent recirculating system. The composition of the nutrient solution was adjusted according to the stage of crop and to the analysis of the nutrient content of the solution in the rockwool. Plants were inoculated at transplanting time by applying 200 ml of a suspension of $3.25x10^3$ zoospores ml^{-1} (corresponding to $6.5x10^5$ zoospores per plant) uniformly to the surface of the block of rockwool. Plants were grown in five rows, each row corresponding to one block with infested and non infested plants in a randomized block design. The volume of drainage was assessed every 2 days, and the volume of nutrient solution consumed per plant was calculated. The experiment was maintained for 13 weeks after the transfer of the plants onto the rockwool pads. The yield expressed as fruit weight per plant per week was calculated for each treatment. The experiment was repeated twice with similar results.

Statistical analysis

Data were analyzed by ANOVA after arcsin transformation for percentage data and means were separated by Duncan's multiple range test ($p <= 0.05$).

Results and Discussion

In the damping-off test, the number of dead plants was scored 6 days following inoculation with *Pythium* and did not change after this time. In rockwool, symptoms of disease (wilted and dead plants) appeared 6 days after inoculation. The number of dead plants increased during the next 2 weeks (data not shown) and subsequently did not change. In hydroponic culture, isolates were grouped with respect to growth reduction and presence of symptoms on roots compared with control plants, 2 weeks after *Pythium* inoculation.

In the damping-off test, the percentages of dead plants varied between 0 to 100% depending on strains (Table 1). 41% (16) of the isolates were pathogenic under these conditions and belonged to the species *P. aphanidermatum*, *P. ultimum*, *P. intermedium*, *P.* group HS, *P. sylvaticum* and *P. irregulare*. These species have already been recognized as highly pathogenic on several plants including cucumber under such conditions (Hendrix and Campbell, 1973; Van Der Plaats-Niterink, 1981).

On the contrary, only seven isolates (18%), all from cucumber roots, were pathogenic on cucumber grown in rockwool or in hydroponics. Depending on strains, these isolates led to 62 to 91% of dead plants in rockwool, and reduced the growth of roots and shoots by 62 to 78% and 30 to 52% respectively (Table 1). All of them belonged to the species *P. aphanidermatum*.

Species (Number of isolates)	Percentage of dead plants (a) Damping-off test	(b) Rockwool test	Growth reduction (c) Hydroponic test (roots/shoots)
P. aphanidermatum (7)	68 to 100%	62 to 91%	62% to 78% / 30% to 52% *
P. ultimum (1)	100%	0%	- d
P. intermedium (1)	75%	0%	- d
P. group HS (5)	16.7 to 100%	0%	- d
P. sylvaticum (1)	50%	0%	- d
P. irregulare (1)	16.7%	0%	- d
P. flevoense / group F(16)	0%	0%	- d
Isolates nd (7)	0%	0%	- d

Table 1: Pathogenicity of *Pythium* isolates in peat-sand (a), rockwool (b) and hydroponic culture (c). For details, see text. Growth reduction is expressed in comparison with control plants. d: weights are not significantly different from control weights according to Duncan's test ($p = 0.05$). *: root rot.

The experimental conditions chosen to perform the tests in soilless culture seem to enable the distinction between isolates responsible for damping-off from isolates responsible for root decay. This study confirmed the pathogenicity of *P. aphanidermatum* in soilless culture as mentioned by different authors (Bates and Stanghellini, 1984; Favrin *et al*, 1988). However, opposite to previous results (Chérif and Bélanger, 1992; Favrin *et al*, 1988; Jenkins and Averre, 1983; Stanghellini *et al*, 1988), the isolates of *P. irregulare* Buisman, *P. ultimum* Trow var. *ultimum* and *P. intermedium* de Bary used in this study, although inducing damping-off on cucumber seedlings, did not show any pathogenic activity on older plants grown on rockwool or in hydroponic solution. These results do not rule out the hypothesis that other *Pythium* species may be involved in yield reduction in soilless culture of cucumber. However, in all these studies, only one isolate of each species was used in pathogenicity tests. It is difficult to generalize to all the isolates belonging to a given species the behavior of the isolate studied. Moreover, the conditions of the tests described here may have been unfavorable for the expression of pathogenicity of other *Pythium* species. These results support the observations that depending on growing conditions (temperature, pH, electric conductivity,...), cucumbers suffer or are unaffected by damage of *Pythium* (Couteaudier and Lemanceau, 1989; Zinnen, 1988).

One isolate of *P. aphanidermatum* (op4) highly pathogenic in pathogenicity tests showed also a pathogenic activity on mature plants:

The volume of nutrient solution consumed per plant per week is shown in Table 2 as a percentage of the control plants. From the first week of fruit production until the fifth, inoculated plants showed reduced growth as revealed by a significant reduction of water absorption. In inoculated treatment, plants showed rot of all the roots in contrast to white roots in the control but wilts of inoculated plants was never observed until the end of the experiment.

	Weeks of fruit production						
	0	1	2	3	4	5	6
Control	100 a	100 a	100 a	100 a	100 a	100 a	100 a
OP4	93,4 a	78 b	81,5 b	80 b	86 b	86 b	91 a

Table 2: Effect of *P. aphanidermatum* strain OP4 on volume of nutrient solution consumed by plants. Values are given as percentages of control plants (100%). Week 1 corresponded to the first week of fruit production. Data were analysed after arcsin (x-0.5) transformation. For each column, means followed by the same letter are not significantly different at p = 0.05 (Duncan's test).

The cumulative yield (Table 3), expressed as kg per plant, in the inoculated treatment was significantly lower than in the control. This difference in production was already significantly established after 3 weeks of production and was still observed until the end of the experiment.

	Weeks of fruit production									
	1	2	3	4	5	6	7	8	9	10
Control	2,1 a	4,5 a	7,4 a	8,1 a	9 a	10,2 a	11,6 a	13,6 a	15,3 a	15,5 a
OP4	1,8 a	3,9 a	5,8 b	6,5 b	7,4 b	8,4 b	9,8 b	11,5 b	12,9 b	13,2 b

Table 3: Effect of *P. aphanidermatum* strain OP4 on cucumber yield under commercial-like conditions. Week 1 corresponded to the first week of fruit production. For each column, means followed by the same letter are not significantly different at p = 0.05 (Duncan's test).

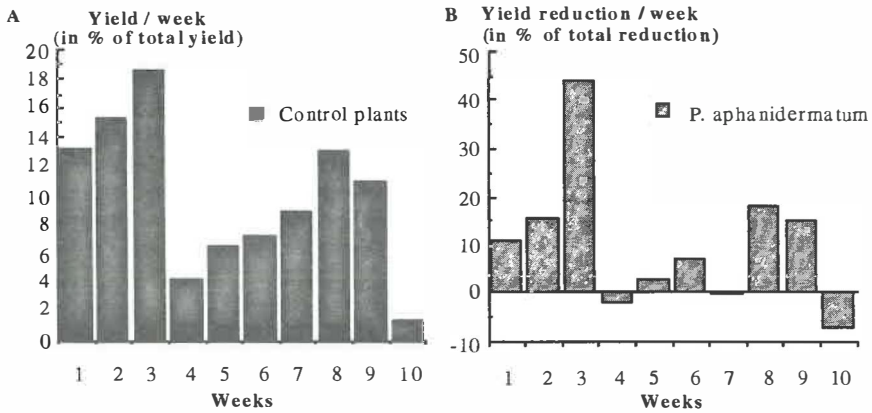

Figure 1: Comparison of yields per week expressed as percentage of total yield of the control plants (A) and yield reductions in *P. aphanidermatum* OP4-treated plants expressed as percentage as the total yield reduction (B).

Moreover, the results obtained indicate that plants have different sensitivity to *Pythium* attack during the production period: figure 1 shows that maximal yield reductions due to *Pythium* are noticed when plants produce the more (weeks 3 and 8), and that 70% of the total yield reduction is recorded during the 3 first weeks. These results confirm several observations (Jenkins and Averre, 1983 ; Couteaudier and Lemanceau, 1989) showing the importance of the plant physiology on the behavior of these fungi called "minor pathogens" (Salt, 1979) or "deleterious microorganisms" (Schippers *et al.* 1987).

To control *Pythium* diseases, different technics using chemicals, UV-irradiation (Stanghellini *et al.*, 1984) or potassium silicate (Chérif and Bélanger, 1992) are mentionned. Several reports have also shown successful control of Pythium damping-off by microorganisms (*Trichoderma* spp., fluorescent *Pseudomonas*...). The root rot pathogenicity test in rockwool described in this paper, is easy to set up and can be applied at a large scale. This biotest allowed us to screen for and select effective antagonists and will allow rapid screening for other effective control methods.

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PYTHIUM F IN SOILLESS CULTURES : DETECTION, COLONIZATION AND INCIDENCE ON TOMATO ROOTS

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Abstract

An epidemiological study has shown that symptomless roots obtained from commercial soilless cultures could be consistently colonized by *Pythium* spp. *Pythium* group F were the most frequent *Pythium* species isolated from these roots. In order to get further informations about this particular plant/pathogen interaction different experimentations have been conducted. i) Pathogenic tests have demonstrated that *Pythium* F isolates were pathogenic to varying degrees on tomato seedlings. Generally root damages were less pronounced than with well-known pathogens such as *P. aphanidermatum* and *P. ultimum*. ii) Another pathogenic test was performed in a tomato culture on rockwool during the fruit production period. It was pointed out that *Pythium* F could induce yield reduction averaging 5,7-7%. *Pythium* F hyphae could also extensively colonize the roots, produce appressoria and penetrate within the root tissues. The fungus could colonize the epidermis, the inner and the outer cortical cells of the roots. iii) Ultrastructural and cytochemical studies have demonstrated that the host plant develop a sequence of defense mechanisms which are efficient to repell the fungal attack in the stelar area of the root.

The frequency and abundance of *Pythium* F in soiless cultures can be of relevance importance, since such minor pathogen can have a key impact in hydroponic cultures during stressed cultural periods.

Introduction

The occurrence of yield losses associated with root rots have been currently noticed in soilless cultures. Surprisingly, yield reductions have also been observed in hydroponic cultures not showing any visible root symptoms (Stanghellini *et al.*, 1994). These observations have led our laboratory to initiate epidemiological studies in commercial greenhouses, during the whole cultural season. Thus, root samples were regularly collected from necrotic but also from symptomless roots, and analysed to determine the fungal contaminations. These studies have convincingly shown the precocity and frequency of root colonization by a *Pythium* spp. complex. This complex was mainly composed of *Pythium* with non-inflated sporangia, grouped as *Pythium* F. These fungi represented at least 75% of all the *Pythium* spp. isolates and were frequently isolated from symptomless roots.

These previous observations are in agreement with numerous reports in the litterature. For instance, in soil, Hodges *et al.* (1985) reported that the root system of golf green apparently healthy and normal in size, consistently yielded *Pythium* species which seemed to induce dysfunction of secondary roots of *Agrostis palustris*.

In soilless cultures, root invasions without symptom are often correlated with yield losses. For instance, *Pythium dissotocum* induced yield reductions as high as 54 % in hydroponically grown lettuce not showing visible damages (Stanghellini *et al.*, 1986). Favrin *et al.* (1988) estimated that such infections might be relatively common in soilless greenhouses, although they have been previously ignored, due to the lack of root symptoms.

These informations have led us to pay particular attention to *Pythium* F, because this group of fungus: (i) is always present and even overwhelming in the cultures, (ii) is frequently and consistently isolated from symptomless roots. In order to enhance our knowledge on the

plant/*Pythium* F relationships, different experimentations regarding both the extent of root colonization and the pathogenic abilities of the fungus have been conducted.

Root colonization by Pythium F: interest of the immunoenzymatic staining procedure

To perform the different epidemiological studies, three different methods have been used: culture plate technics, ELISA tests and an immunocytoenzymatic staining procedure. The first two methods proved useful to detect *Pythium* spp., but they could not account for an effective quantification or an assessment of the extent of root invasion by the fungus.

Therefore, an immuno-enzymatic staining procedure was developped, in order to study *Pythium* F colonization of plant roots growing in commercial greenhouses. The technique was based on the use of polyclonal specific antibodies produced against *Pythium* F. Such antibodies bind specifically to hyphae of *Pythium* F and, to a lesser extent, to hyphae from various *Pythium* species (Rafin *et al*, 1995 a).

This approach provides an efficient tool to directly detect *Pythium* spp. hyphae on roots in addition to distinguish *Pythium* spp. hyphae among other fungi. The extent and the frequency of root colonization, the root areas specifically invaded by the fungi and the *Pythium* hyphae structures on the root surface are easily assess and observed with the technique.

By using, the immunoenzymatic staining procedure, two important results were obtained:

- *a - Pythium spp. location on the roots*: various *in vitro* studies have reported that zoospores are preferentially attracted by the root cap and the elongation zone. Subsequently, further infections were noticed firstly in these areas.By contrast, our observations revealed the occurrence of hyphae in all roots areas. They seemed to be randomly located on the surface of tomato roots grown in commercial greenhouses.
- *b - the level of root colonization by Pythium spp.* : we have observed that during the first three months of the cultural season, the extent of root colonization appeared limited, only short germlings, encysted zoospores and sparse hyphae of *Pythium* spp. By contrast, under conditions of hot stress and physiological changes such as initiation of fruit, an extensive root colonization was noticed (Figure 1). Dense mycelium network constituted with long hyphae and appressoria were frequently observed. It is worth mentionning that high level of root colonization was not always correlated with root lesions. Frequently, numerous hyphae were observed on roots that did not exhibit any visible symptoms. Furthermore *Pythium* F were consistently recovered from these symptomless roots.

These results support the concept that extensive root colonization by *Pythium* spp., and particularly by *Pythium* F, might be relatively common in soilless cultures.

Study of the Pythium F pathogenicity

Pathogenic test on tomato seedlings

It has been reported that *Pythium* F represented at least 75% of all the *Pythium* spp.isolates obtained from tomato cultures. The ability of these *Pythium* F strains to induce root damages are still unknown, so further informations regarding their pathogenicity were an urgently needed complement.

In our laboratory a pathogenic test with 38 *Pythium* F isolates obtained from commercial soilless cultures, was conducted on tomato seedlings (Rafin *et al.*, 1995 b). All the isolates of *Pythium* F were pathogenic to varying degrees on the plantlets (Figure 2). Thirty three percent of the isolates were weakly pathogenic, and induced little necrosis. Forty two isolates caused severe

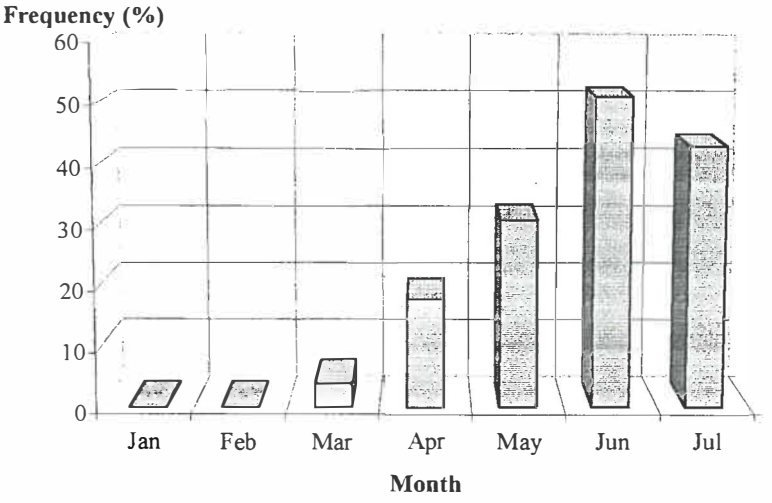

Figure 1 : Assessment of the tomato roots colonization by *Pythium* spp., using the immunoenzymatic staining procedure

The frequency is the percentage of root segments from which *Pythium* spp. hyphae were observed.

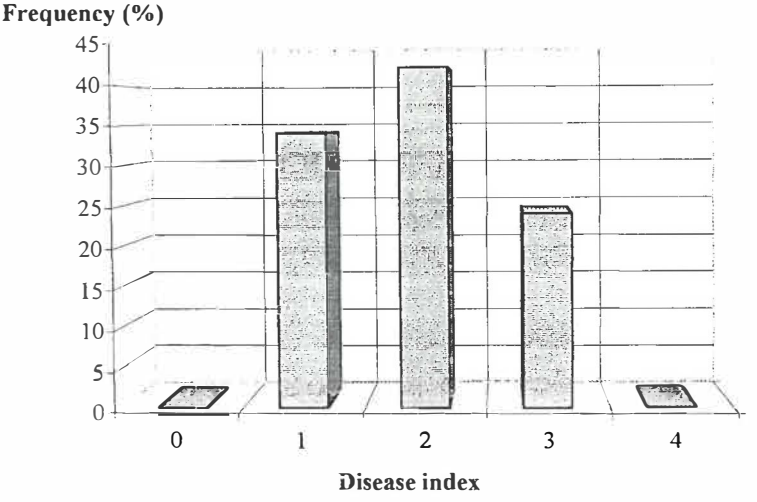

Figure 2 : Pathogenicity of 38 *Pythium* F isolates to seedlings of tomato, using zoospore inoculum

Disease index :
class 0 : healthy root system.
class 1 : little necrosis localized at root apices.
class 2 : more developed necrosis without root reduction.
class 3 : necrosis and slight reduction in root growth.
class 4 : severe necrosis and large reduction in root growth.

root necrosis, but no reduction of root growth. Twenty four percent of the isolates caused root rots accompanied by reductions in root and shoot growth. Although all the *Pythium* F isolates showed pathogenic abilities on tomato seedlings, generally root damages and alterations were less pronounced than with well-known pathogens such as *P. aphanidermatum* or *P. ultimum*.

The present results are in agreement with some experimentations regarding *Pythium* F pathogenic tests. For instance, although *Pythium* F have been described as moderatly pathogenic in soil (van der Plaats-Niterink, 1981), some authors have demonstrated that they can induce severe necrosis on young eucalyptus plants (Linde *et al.*, 1994) and on spinach seedlings. By contrast they have been described as non-pathogenic on rice seedlings.

An epidemiological study conducted by Thinggaard *et al.* (1983) in 8 plant nurseries has revealed the abundant and frequent occurrence of *Pythium* F in the nutrient solution of pot plant grown in ebb and flow bench systems.The authors have also performed a pathogenic test using *Pythium* F zoospores to infect cucumber, tomato, lettuce, cress and *Gerbera* seeds. Pre-emergence damping off was noticed on the five plants considered, the percentage surviving plantlets depended upon the susceptibility of each plant species.

Pathogenic test in a commercial soilless greenhouse

The pathogenic tests previously described were achieved with plantlets or seedlings. However, a facet of the plant/*Pythium* F interactions which is still a matter of speculation is its true pathogenicity on adult plants. The ability of *Pythium* F to induce yield losses, to colonize the root surface and to penetrate within the root tissues of adult plants deserved to be investigated.

Assessment of yield losses: a pathogenic test was conducted in a tomato culture on rockwool during the period of fruit production. In an attempt to reproduce what really occurs in commercial greenhouses, the first infections were performed three months after the beginning of the culture. This corresponds to the stage of fruit initiation, a period generally considered crucial since the level of *Pythium* spp. may dramatically increase in the commercial soilless cultures.

Four months after the first infections, yield losses were estimated. We pointed out a yield reduction averaging 5,7 to 7% (corresponding to 1,5-1,8 kg/m^2). Root samples examined appeared healthy, without apparent necrosis or root rot, excepted a slight yellow discoloration which was occasionally observed.

Roots were inoculated with *Pythium* F, moreover all the *Pythium* spp. isolates identified on the roots were *Pythium* F. So, we may assume that the yield reduction was directly attributable to this fungus.

Root colonization: the immunoenzymatic staining procedure revealed the *Pythium* F abilities to extensively colonize all root areas. Dense mycelium network and appressoria were also frequently noticed. The detection of appressoria was noteworthy because these structures allow fungal penetration within the root.

Pythium F *invasion within the root tissues*: to determine the extent of *Pythium* F ingress throughout the root tissues, the infected root samples were observed by electron microscopy. A specific probe, the exoglucanase gold complex, was used for localizing β-1,4-glucan molecules (Benhamou *et al.*, 1987). This gold complexed probe allows to specifically label the cell walls of both tomato root cells and *Pythium* spp. hyphae, because both structures contain very high amount of cellulose. So by means of this technique, *Pythium* F hyphae can be easily detected within the root tissues.

Several labelled hyphae have been observed in the epidermis, the inner and the outer cortical areas of the roots. Penetration of the host cell walls by *Pythium* F were frequently noticed, but except for alterations induced at sites of potential root wall penetrations; no particular damages were observed in the host cells.

In the present study, *Pythium* F invasion was correlated with light yield reduction. But we may assume that several conditions may increase yield losses induced by the fungus. For instance:
i) *Pythium* F invasions which would occurred early in the cultural season, because it is generally observed that plantlets or young plants are more susceptible to *Pythium* spp. attacks than mature plants.
ii) stress conditions during the cultural season, because Funck-Jensen and Hockenhull (1983) have demonstrated that lettuce roots growing under suboptimal conditions (i.e. poor light exposition), are highly sensitive to *Pythium* F attacks.

Electron microscopy study: process of infection of *Pythium* F isolates within tomato roots

The location of *Pythium* F hyphae in the epidermal and cortical areas of mature plants has been demonstrated for the first time. Then, in order to get further information about the mode of infection of *Pythium* F through the plants, three *Pythium* F isolates with different degrees of pathogenicity were inoculated to tomato roots. The infection process associated with each isolates was investigated and compared.

The most pathogenic *Pythium* F strain induced marked damages in the epidermal and the cortical root areas. The two other isolates caused also cellular alterations in the same root tissues, but induced also several of host reactions. These host responses were mainly physical barriers, such as amorphous osmiophilic material. The exact nature of this last material is still unknown, but its high electron density suggests that it may contain phenolic compounds. The occurrence of necrotic hyphae within these cells supports the concept of phenolic deposition in relation to a fungitoxic environment.

In a recent report (Rey *et al.*, 1996) we have demonstrated that *Pythium* F ingress through cucumber root tissues was halted at the endodermis level. But in the present study, we have noticed that the tomato stelar cells can be also invaded by some *Pythium* F hyphae. Nevertheless, these hyphae are always moribund with disorganized organelles, and hyphae depleted of their protoplasm are also frequently noticed. Thus the host plant responses are efficient to repell the fungal attack in the stelar root area.

Conclusion

For the first time, in the present study, the ability of *Pythium* F to extensively colonize the roots, produce appressoria, penetrate within the roots tissues and induce yield reduction has been described. Thus, *Pythium* F can be described as a "minor pathogen" according to the definition of Salt (1979), because they present the following characteristics:
-1- they are mainly restricted to cortical cells but they can also invade and damage the juvenile root tissues. Generally these alterations are overlooked since young roots are replaced, but they may alter the plant vigour and its ability to resist to further pathogen attack.
-2- *Pythium* F isolates are widely distributed in soilless cultures. They are currently isolated from tomato but also from cucumber roots (unpublished data).
-3- the damages induced by *Pythium* F depend upon cultural and environmental conditions. Under conditions of host stress, the plant defenses may be weakened and allow the rapid development of minor infection, e.g. those caused by *Pythium* F.
-4- usually, colonized plants do not exhibit severe root symptoms, and moreover necrosis is frequently lacking.

For all these considerations, the frequency and abundance of *Pythium* F in soilless cultures are of relevance importance. *Pythium* F which were previously described as organisms of minor

pathogenic importance under field conditions, can have a key impact in soilless cultures during stressed cultural periods.

It is generally assume that soilless conditions provide an ideal environment for the proliferation of *Pythium* spp. Stanghellini *et al.* (1994) presented hydroponic cultures as a "solution for zoosporic pathogens", and we may certainly add that it is also an ideal solution for the spread and development of the particular fungal species: *Pythium* F.

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IMPORTANCE OF ROOT DISEASE PROBLEMS IN TOMATO CROPS IN LANGUEDOC-ROUSSILLON (SOUTH FRANCE)

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Soilless cultures of tomato in greenhouses are one of the most important economic feature of Languedoc-Roussillon, with only 150 hectares but almost 225 millions FF turn-over a year.

In crops planted in November and harvested up to the end of next September, grown in mineral or organic culture-bags, a « root disease like » loss of 20% to 30% of plants could be expected before the end of the crop in 50% of the greenhouses. In most cases : the first root problem occurs rather early in the season usually at the harvest of the first truss of fruits when the plant is bearing lot of fruits. The second wave of root problems occurs at the very first hot days in Roussillon. From that the second wave on, the problem would get worst and worst following the vicious circle: plants with root problems receive too much watering that increases root death, etc. In all cases, *Pythium* spp. were isolated from the roots of these dying plants. Very often a « F.o.r.l. type » symptomatology was observed on the same plants as well on susceptible and resistant varieties. In some cases the pathogen was isolated from the necrotic symptom at crown.

In rockwool « corky root type » symptoms could be observed as well. No isolations were made but such symptoms were related with presence of *Humicola fuscoatra* in The Netherlands (RUNIA, personal communication).

All factors that influence the plant physiology may increase the damages and special attention must be paid to the regularity of irrigation and drainage, the temperature of the substrate in the bags, ... Although enhancing factors are well known, the developement of an epidemic situation remains unpredictable. Chemical control of F.o.r.l. or of *Pythium* spp. even applied in a preventive way seems only decrease slightly the importance of the root disease problems.

Screening for effective biocontrol agents

SCREENING FOR EFFECTIVE ANTAGONISTS TO CONTROL ROOT DISEASES IN SOILLESS CULTURES

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Introduction

Biological control of plant diseases has reached a point where biocontrol agents (BCAs) are being or are about to be registered for commercial use in plant production. Most of these agents are based on one or a few antagonistic microorganisms, which have been selected among many potential antagonists, by use of different screening techniques.

Many research programmes over the world are directed towards the development of new BCAs. However, the general findings are that results from tests of potential antagonists in greenhouse or field tests are inconsistent, and that only a few of the antagonists selected by different screening procedures actually work as biocontrol agents in the field. Thus, it seems appropriate to consider in more detail the sources and screening procedures for selecting the best strains. Although these subjects have been addressed by others (Campbell, 1986; Campbell, 1994; Merriman and Russell, 1990; Whipps and Magan, 1987), the literature is limited and in most cases rather general. Furthermore, examples of screening programmes directed towards the selection of antagonists for the use in greenhouse crops are limited. Nevertheless, it might be relevant to discuss what should form the basis of a search for good antagonists to control root diseases, in soilless cultures, in greenhouses.

Plant production in soilless substrates

Plant production in soilless substrates in greenhouses is, in many respects, quite different from the field situation. Soilless substrates are merely there for physical support of the plants and various substrates can be used as for example sphagnum peat moss (either raw or pasteurized), vermiculite, perlite, stone fibre and even expanded polyurethan. Different growing systems are in use: for example drip irrigation where the plants are watered from above, the flood and ebb system where they are watered from below or NFT, in which the roots are constantly exposed to the circulating nutrient solution. The systems can be open, in which case the nutrient solution is drained away, or they can be closed with recirculation of the nutrient solution. The risks for disease outbreak as well as the activity of beneficial microorganisms in the greenhouse are highly dependant on the crop plant and the soilless growing system in use, which will be further discussed below.

Several soilborne pathogens are reported to cause severe diseases in soilless cultures (Paulitz, in press) and among these *Phytophthora* spp., *Pythium* spp. and *Fusarium* spp. are considered to be among the most troublesome (Rattink, this volume).

Pythium problems in soilless cultures

In the following I will mainly focus on *Pythium* disease problems in soilless cultures. The pathogens may be introduced into the systems in different ways and at different times after planting as described by Paulitz (in press) and the diseases will be established and expressed in different ways depending on the growing system and the chosen substrate. From the point where it first becomes established, the pathogen will spread to other parts of the system. *Pythium* attack is often seen on roots in the bottom of pots or rockwool mats (Jensen, unpublished; Rattink, pers. comm.) and in NFT, we have also seen attacks on roots along the edges of narrow gullies (Funck-Jensen and Hockenhull, 1983). Secondary spread by zoospores is considered to be the most important for an epidemic development (Paulitz, in press; Rattink, this volume) and in some systems, spread by hyphal growth from diseased plants is considered to be minimal (Rattink, this volume). However, in our experience with pot plants and the production of herbs, secondary spread of *Pythium* from plant to plant might indeed be very serious leaving patches of dead plants on the greenhouse benches. We have also demonstrated this in growth chamber experiments by looking at the spread of a non zoospore producing isolate of *Pythium ultimum* (Green and Jensen, in prep.).

Isolation of organisms before screening

Potential antagonists may be looked for in different sites. Isolation from soil and plant residues (Knudsen *et al.*, 1995a), from rhizosphere soil (Renwich *et al.*, 1991) and from infection sites at plant surfaces (Weller *et al.*, 1985) have been exploited. Of special interest has been isolation from suppressive soils and this source has proved successful in several cases (Alabouvette *et al.*, 1993; Weller *et al.*, 1985), as has isolation from suppressive sphagnum peat moss, used in plant production, in greenhouses (Tahvonen, 1982; Jensen and Wolffhechel, 1992). Some of the isolates from such sources have now been commercialised (C. Alabouvette, personal communication; and from this volume: Lahdenperä; Tahvonen *et al.*) Isolation of organisms belonging to specific groups known to have members with antagonistic potential might also prove to be a useful strategy. Examples of such groups are the fluorescent pseudomonads (Weller and Cook, 1983), *Bacillus* spp. (Bochow and Dolej, this volume) and *Trichoderma* spp. (Jensen and Wolffhechel, 1995). Isolation of mycoparasites by using special baiting techniques has also been successful (Deacon and Henry, 1978; Lumsden and Lewis, 1989). However, most of the BCAs expected on the market in the near future for use in greenhouse crops have, with a few excepts, originated from quite different ecological niches than those found in modern greenhouses. Although some of these organisms have proven to be successful in controlling diseases in greenhouses, more potent antagonists might be found among the populations of microorganisms naturally present in the special environments found in soilless cultures. Alternatively, if better screening procedures were available, it might prove advantageous to screen many different organisms, isolating randomly and by chance, from various sites in different ecosystems.

Before going into a time consuming screening programme, it might be relevant to take into account whether the isolated organisms could pass a risk assessment procedure in connection with their approval as biocontrol agents. Risk assessment is, however, beyond the scope of this paper.

Screening procedures

In vitro screening: problems and shortcomings

In vitro screening, like dual cultures and other tests on agar, as well as simple tests in soil samples, has often been used as a primary screening to reveal traits like enzyme production, mycoparasitism, competition and antibiosis. In general, the correlation between results of selection *in vitro* and the field performance of the organisms thus selected is poor and many authors, for example Campbell (1986) and Lumsden and Lewis (1989), have stated that screening on agar should be avoided .

Testing for antibiosis on agar in dual culture illustrates some of the problems with the *in vitro* method. On going through the literature, an antagonist like *Trichoderma harzianum* is found to be able to produce more than 20 different secondary metabolites while *Pseudomonas fluorescens* is reported to be able to produce at least 35 different secondary metabolites with antifungal or antibacterial activities (Lübeck, 1994). Production of secondary metabolites may, however, be substrate dependant (Whipps, 1987; Whipps and Magan 1987; Brown *et al.*, 1987). Thus, an antibiotic, produced by an antagonist *in vitro*, might not be produced *in vivo* in the greenhouse and the opposite situation could occur.

Actually there is very little information about what role antibiotics play in the inhibition of plant pathogens under natural conditions and also to what extend these metabolites are produced in the natural environment because most of these metabolites are very difficult to detect *in vivo* (Williams, 1982). There is evidence that the production of some antibiotics like gliotoxin can be induced by the amendment of soil with organic substrates (Williams, 1982; Lumsden *et al., 1992*). It is also believed that such substrates are available for antagonists in the microenvironments around organic particles, in the rhizosphere and in the zone of interaction between the antagonist and the pathogen at infection sites on plant surfaces (Green and Jensen, 1995). There is, however, some evidence that secondary metabolites can be inactivated by clay minerals in soil (Williams, 1982) or be broken down by the indigenous microflora (Williams, 1982). This might also take place in soilless substrates especially substrates containing clay minerals. There will also be an active microflora in the soilless substrate living on root exudates and other organic material from the roots.

From studies carried out with many organisms in pure culture, it is known that the production of secondary metabolites is related to certain stages of growth. It seems clear, that in a natural environment, the antagonist must be at the right place producing the metabolites at the right time if it is to be successful in controlling a pathogen by antibiosis. However, Thomashow *et al.* (1990) have shown that the antibiotic phenazine, produced by fluorescent pseudomonads, was important in the control of take all in wheat under natural conditions and that phenazine also was produced on agar *in vitro*. Howel and Stipanovic (1983), likewise found that gliovirin produced by *Gliocladium virens* (syn. *Trichoderma virens*), plays an important role in the control of *Pythium* damping-off in cotton seedlings. In the case of *Agrobacterium radiobacter* strain K84, the antibiotic agrocin has been shown to be the important antagonistic determinant in the protection of wounds against crown gall (Ryder and Jones, 1990). Based on this type of information, and providing it is known on which substrate an antibiotic is produced, a targeted screening approach can be organized, looking for antibiotic producers on agar. However, such an approach does not take the ecological fitness of the organism into account, and should, therefore, only be used as a supplement to other screening procedures.

A similar discussion is related to *in vitro* screening for antagonists producing cell wall degrading enzymes. Enzymes like chitinases (Lorito *et al.*, 1993) and glucanases (Thrane *et al.*, in prep.) have been shown to inhibit pathogenic fungi in laboratory experiments. It is, however, not well understood what role these enzymes play in the inhibition of fungal pathogens in the soil and rhizosphere. It could be that the antagonist gets an advantage over the pathogen in the competition for organic substrates as suggested by Deacon (1991) or, as suggested by Ahmad and Baker

(1988), an improved rhizosphere competence. Alternatively, it might be due to the direct breakdown of the walls of the pathogen and, Ridout *et al.* (1988) concluded from their experiments with *Trichoderma harzianum* and *Rhizoctonia solani*, that several enzymes must be involved in the breakdown of the cell walls of the pathogen under natural conditions.

Just as for the production of antibiotics, for enzymatic breakdown of cell walls to be able to play a role in the control of a disease in a greenhouse crop, it is important that the genes coding for these enzymes are expressed at the right place and at the right time.

Recently some evidence has come from *in vitro* experiments indicating that there can be a synergistic effect of secondary metabolites and cell wall degrading enzymes in the inhibition of pathogens (Di Pietro *et al.*, 1993). Thus a synergistic effect has been demonstrated following the use of combinations of antagonists to the control soil borne diseases in greenhouses, but, in some cases, the organisms did not show any control at all when applied alone (de Boer *et al.*, this volume). However, exact data verifying that the synergistic effect involves metabolites and enzymes produced by the antagonists in a natural system is limited. Thus, as for antibiotics, in order to be able to design a successful *in vitro* screening procedure it is necessary to know more about the importance of the cell wall degrading enzymes in the soilless substrates and in the rhizosphere.

In recent years induced resistance has been in focus as a mechanism which might be important in biocontrol of soilborne diseases (Zhou and Paulitz, 1994). Again little is known about how antagonists can elicit a resistance reaction in plant roots. It seems highly unlikely that an organism which is able to induce resistance in a plant will be revealed in an *in vitro* screening system unless this system is specially designed to find organisms possessing the factors which are eliciting the resistance responses.

In vivo screening

Most screening programmes have been directed towards selection of antagonists for field application. Based on the examples and the arguments discussed above as well as experience from other *in vitro* studies (ie. concerning mycoparasitism and competition including screening for siderophore producers) it has been generally accepted, that even the primary screening in a screening procedure should be carried out *in vivo* and include plants. This is to help ensure the selection of antagonists with properties which are important in the environment in which the organism is to be used. It has even been argued that there may be no better alternative to direct screening in the field (B. Gerhardson, pers. comm.).

There are several examples of *in vivo* screening systems using a hierarchic approach. A more detailed discussion of these screening systems will be presented elsewhere (Knudsen *et al.*, in prep.). In brief, the primary and secondary screenings are carried out in pots in either growth chambers or greenhouses. Selected organisms are then tested for field performance (Duczek, 1994; Knudsen *et al.*, 1995b; Renwick *et al.*, 1991; Teperi *et al.*, in prep.). Experience from such schemes is, again, varied with some studies showing no correlation between the greenhouse selection and field performance (Kommedahl & Windels, 1978; Duczek, 1994) while in others a good correlation, at least for some groups of antagonistic microorganisms (Knudsen *et al.* 1995b; Teperi *et al.*, in prep.) has been found. Interestingly, in the cases with good correlation, it seems as if the field situation has been closely simulated in the greenhouse/growth chamber screenings. This includes simulation of conditions like temperature and placement in time and space of both antagonists and pathogens. Thus, as stated by Deacon (1991), in a screening procedure the emphasis should be on an ecological approach rather than on the mode of action of the antagonist and the focus should be on the ecology of both the antagonist and the pathogen.

Application of the antagonists to the greenhouse crop

Although sphagnum peat of the light coloured type can possess suppressiveness to *Pythium* spp. due to antagonistic microorganisms (Wolffhechel,1989; Tahvonen, 1982) it is generally believed that soilless substrates have a low microbial buffering effect, because at crop start, the soilless substrates possess a low capacity for supporting microbial activity. Thus, the organic substrates needed for the microflora originate mainly from root exudates, dying cells and dead roots. It is often argued that the low buffering effect at the start of a crop offers good opportunities for the introduction of antagonists (Paulitz, in press; Postma, this volume). However, after introduction of our GUS-transformant of *Trichoderma* harzianum into sterile peat, we have demonstrated, that the antagonist is active only for a few days. From then on it will remain, except for a small, constant turn over of the biomass, as conidia or other resting propagules in the substrate (Green and Jensen, 1995). It has, however, been demonstrated in experiments monitoring the GUS-transformant over short periods, that high GUS activity can be detected in and around wounds in *Pythium* infected roots, indicating a high level of activity of the antagonist (Green *et al.*, in prep). Thus, although the antagonist is present in a high density as living spores in the soilless substrate, this raises the question whether the antagonist, applied at the start of the crop, will be sufficiently active (or can be activated) to ensure an adequate level of control of *Pythium*. Alternatively, the antagonists might be applied with the nutrient solution throughout the cropping period as suggested by Paulitz (in press).

Thus, although this needs further investigation, there seems to be greater possibilities for the successful application of antagonists to greenhouse crops in soilless cultures compared to the field situation as mentioned by Deacon (1991).

Screening for antagonists against pathogenic *Pythium* spp

When setting up a screening procedure it is important to know 1) if the introduction of an antagonist increases the buffering effect and suppresses the primary establishment of inoculum of the pathogen; 2) whether it will limit secondary spread by hyphal growth and/or 3) whether it limits secondary spread by zoospores. However, in order to set up improved screening systems, it is also important to know when the activities of the antagonists are causing disease suppression and where these activities are taking place. In the first case (1), it would be relevant to screen for antagonists which can be activated and either inhibit the germination of the primary inoculum of the pathogen, when it is introduced in the substrate. Alternatively one could screen for antagonists which can protect the roots from primary infection, for example, either by competition or induced resistance mechanisms. A method for producing oospore inoculum to be used in such a screening system is described in Green and Jensen (in prep.).

In case (2) if, for example, secondary spread from diseased roots by hyphal growth is limited because of an increased activity of the antagonist around infected roots, the screening procedure might focus on the selection of antagonists which are effective in competing for the possession of decaying roots. If the suppression of hyphal growth is due to interactions with the antagonist in the substrate, the inability of the fungal pathogen to grow through the soilless substrate in which the test organism has been incorporated, should be the selection criterium. The procedure described in Green *et al.* (in prep.) could be used for such a selection. Another possibility is that control is due to protection of infection sites on healthy roots. If so, a screening system should select organisms which can exclude the pathogen from these niches.

If the third case (3) is likely, selection could be based on the ability of the test organisms to interfere at binding sites of spore cysts on the healthy roots, as suggested by Paulitz (in press). Another possibility could be screening systems for the selection of antagonists which interfere at sites where vesicles are formed and zoospores produced (ie. we have seen vesicles bursting before

zoospores were ready to take off because of bacteria contamination of the nutrient solution in *in vitro* experiments (unpublished data)).

There are thus many possibilities to set up new screening systems. *In vitro* screening has focused mainly on mechanisms and less attention has been paid to the ecology of the pathogen and the antagonist in the cropping systems in which the pathogen is to be controlled. However, for each different cropping system, a more thoroughly investigation is needed. If the pathogen and different organisms known to be effective antagonists are monitored and their interactions studied following their introduction to the system, more information can be obtained about the ecology and mode of action of the organisms at different sites in a natural system. Research in biocontrol is being directed towards such monitoring and interaction studies and many new methods are becoming available for that purpose (Jensen *et al.*, in press; Green and Jensen, 1995). These studies could form the basis for selecting new and better organisms related to the well known and intensively studied antagonists. Thus detailed information can form the basis for deciding which screening methods will be the most relevant to use for selecting new and improved antagonists. In that way screening systems can be designed to select antagonists with the necessary characteristics to enable them to work better in soilless cultures.

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CHARACTERIZATION OF BACTERIAL POPULATIONS FROM THE RHIZOPLANE OF CABBAGE SEEDLINGS GROWN IN DIFFERENT SUBSTRATES

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Summary

Bacteria were isolated from the rhizoplane of cabbage seedlings (*Brassica oleracea)* or from root-free soil and transferred to differentiating media (King's B-medium, chitin-agar, Tween-agar, potato dextrose agar containing conidia of *Fusarium culmorum* or *Phoma lingam*). For each isolate, the reactions on these media were recorded and combined, resulting in different "variants". The number of variants occurring in the different isolations was taken as a measure of variability.

Bacterial populations from the rhizoplane of field-grown plants were more diverse than populations from root-free soil. Furthermore, isolates with chitinase activity, antagonistic properties as well as the ability to fluoresce were more frequent on the rhizoplane.

Among populations from the rhizoplane of plants grown, in the greenhouse, in field soil or in two commercial substrates having suppressive properties towards *Pythium ultimum*, the highest variability was observed in samples from one of the commercial substrates. The rhizoplane of plants grown in the two commercial substrates yielded a higher proportion of antagonists and of isolates with chitinase activity than the rhizoplane of plants grown in field soil. The results strongly indicate that the composition of the bacterial microflora on the rhizoplane is influenced by the potting substrate, and that bacterial antagonists on the rhizoplane are differentially favored depending on the substrate.

Introduction

The suppressiveness of certain compost-amended potting mixes towards soil-borne plant pathogens is well documented. Contrary to the suppression of *Rhizoctonia solani*, which is rather specific, suppression of nutrient-dependent propagules of plant pathogens like *Pythium* spp. is believed to be general and due to high microbial activity with many different kinds of microorganisms being involved (Hoitink *et al.* 1991).

If qualitative and quantitative differences in the microbial populations of potting mixes exist, these differences should be reflected in the microflora of plant roots growing in these substrates. In the present study we used two commercial potting mixes with suppressive properties towards *P. ultimum* (Wohanka, pers. communication; Koch, unpublished) and a field soil in order to evaluate to what extend bacterial populations on the rhizoplane differ depending on the substrate. Further, microbial populations from the rhizoplane of field-grown plants were compared to populations in root-free soil. The usual steps involved in characterization of bacterial populations are isolation, purification and determination. Although highly accurate, this procedure has the disadvantage of being laborious and time consuming. In the present study we therefore used a simple technique which is based on cultivating unpurified bacterial isolates on differentiating media and combining the resulting reactions.

Materials and methods

Raising of plants

Cabbage (*Brassica oleracea)* was planted in August 1993 in the field in a sandy loam or in the greenhouse. In the greenhouse, plants were raised in pots in two commercial substrates (Substrate 1: "Lat Terra Typ K"; 10% volcanic clay, 70% peat, 20% coconut fibers. Substrate 2: "Lignostrat U"; 15% volcanic clay, 30% peat, 40% composted bark, 15% coconut fibers. Industrie-Erdenwerk Archut, Lauterbach, Germany.) or in field soil and watered with sterile tap water.

Isolation of bacteria

Plants in the field were harvested 31, 49, 63 and 77 days after planting. They were dug carefully from the soil, and the roots were thoroughly rinsed under tap water to remove adhering soil particles. The roots were then shaken for 25 min. in sterile saline water which was dilution plated onto 1/10 strength trypticase soy agar (1/10 TSA). In the field experiment, isolates from nonrhizosphere soil were obtained by suspending top soil (upper 15cm, adjacent to plants) in sterile saline and dilution plating onto 1/10 TSA. Bacteria from the roots of greenhouse grown plants (harvesting date: 37 days after planting) were isolated as described above for plants from the field. The number of bacterial propagules in soil and root washings was determined in microtiter plates in TSA using the most probable number technique (Alexander, 1982).

Characterization on differentiating media

Twenty seven single colonies were randomly picked from dilution plates and transferred to a fresh plate on which they were placed in a certain arrangement to serve as a template for a special tool which allowed the replicated bulk transfer to the following media: chitin-agar (Renwick *et al.*, 1991; Shimara and Takiguchi, 1988) Tween-agar (Sands 1990), potato dextrose agar containing conidia of *Fusarium culmorum* or *Phoma lingam* and Kings's B agar (King *et al.* 1954). Because the fluorescence on Kings's B differed considerably, a subdivision was made between strongly and weakly fluorescing isolates. The reactions on the differentiating media were recorded and combined. In this way, each individual isolate could be characterized by assigning it to one of 48 possible combinations (variants) listed in table 1. Isolates which did not grow on one or more of the differentiating media were grouped as not determined (nd).

Results and discussion

The method of differentiation used in the present study is based on using unpurified isolates, which may raise doubts concerning the significance of the results obtained. However, this disadvantage is apparently overcome by the large number of isolates that can be processed. In our study the method proved useful for the characterization of bacterial populations from plant roots and root-free soil. Altogether, more than 2500 single colonies were transferred to differentiating media. Of these, about 15% did not grow on one or more of the media and were omitted from further characterization. The remaining ca. 2100 isolates could be assigned to 39 variants (table 1).

Variant	Tween-esterase	Chitinase	Fluorescence	*in vitro* inhibition *P. lingam*	*F. culmorum*
1#	+	+	++	+	+
2#	+	+	+	+	+
3	+	+	++	+	-
4	+	+	+	+	-
5#	+	+	++	-	+
6#	+	+	+	-	+
7	+	+	++	-	-
8#	+	+	+	-	-
9	+	+	-	+	+
10	+	+	-	+	-
11	+	+	-	-	+
12	+	+	-	-	-
13#	+	-	++	+	+
14	+	-	+	+	+
15	+	-	++	+	-
16	+	-	+	+	-
17	+	-	++	-	+
18	+	-	+	-	+
19	+	-	++	-	-
20	+	-	+	-	-
21	+	-	-	+	+
22	+	-	-	+	-
23	+	-	-	-	+
24	+	-	-	-	-
25#	-	+	++	+	+
26	-	+	+	+	+
27#	-	+	++	+	-
28	-	+	+	+	-
29#	-	+	++	-	+
30	-	+	+	-	+
31	-	+	++	-	-
32	-	+	+	-	-
33	-	+	-	+	+
34	-	+	-	+	-
35	-	+	-	-	+
36	-	+	-	-	-
37	-	-	++	+	+
38	-	-	+	+	+
39	-	-	++	+	-
40	-	-	+	+	-
41	-	-	++	-	+
42	-	-	+	-	+
43	-	-	++	-	-
44	-	-	+	-	-
45	-	-	-	+	+
46	-	-	-	+	-
47	-	-	-	-	+
48	-	-	-	-	-

* + weak, ++ strong fluorescence # variant not observed

Table 1 : Theoretically possible combinations (variants) of traits on differentiating media

At all four sampling dates, the number of bacterial propagules per gram dry weight and the number of variants isolated from the rhizoplane of cabbage grown in the field was higher than in adjacent nonrhizosphere soil (table 2), which is in agreement with previous findings (Campbell and Greaves, 1990; Bazin *et al.* 1990). As an example, the number and frequency of variants at the first sampling date are given in Fig. 1. *In-vitro* antagonists were more often obtained from the rhizoplane than from root-free soil. A similar result was reported by Lindermann *et al.* (1994) who concluded that roots selectively enrich for antagonists from the soil. Furthermore, isolates showing chitinase

activity and fluorescence on King's B medium were more frequently isolated from the rhizosplane (table 3). The higher proportion of fluorescing isolates on roots is probably due to fluorescent pseudomonads which are known to be frequent colonizers of plant roots (Lambert *et al.* 1987).

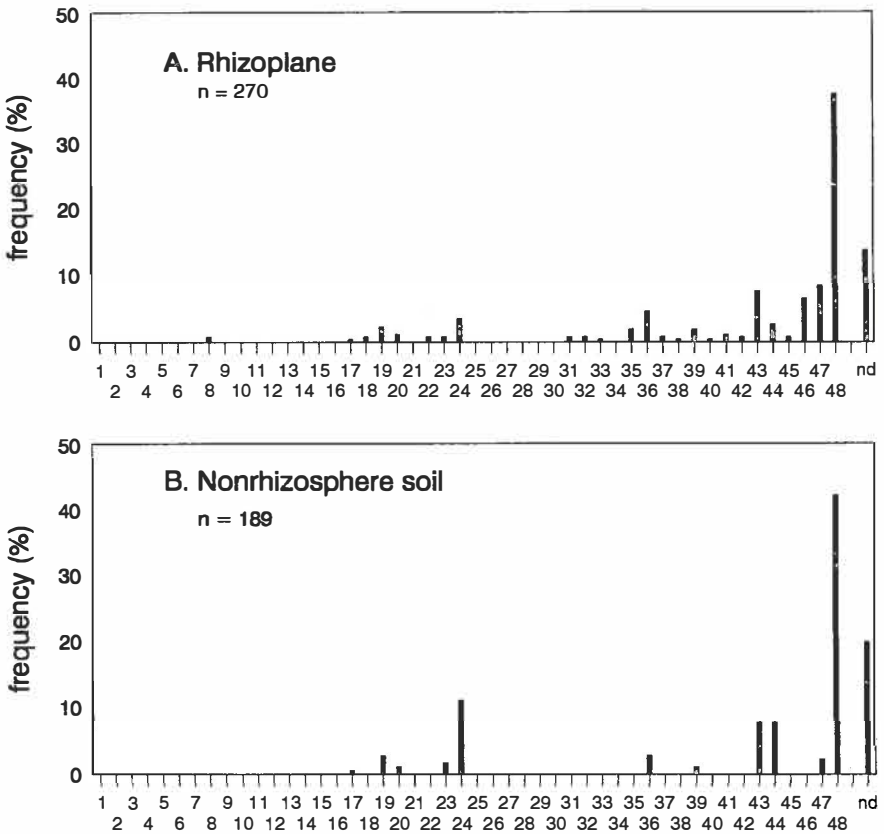

Figure 1 : Occurrence of variants (see table 1) among bacterial populations from the rhizoplane of cabbage seedlings grown in the field (A) and in adjacent nonrhizosphere soil (B) (nd = not determined).

Compared to plants grown in the field in the same soil, the rhizoplane of greenhouse-grown plants yielded lower CFU counts, less variants (table 2) and a reduced proportion of isolates showing chitinase activity and fluorescence on King's B medium (table 3). Apparently, these differences can be attributed to the differing environmental conditions.

Among the plants from the greenhouse, the highest number of bacterial propagules and variants was obtained from the rhizoplane of plants grown in substrate 2 (table 2). The rhizoplane of plants from both commercial substrates yielded a higher proportion of isolates with antagonistic properties and chitinase activity than the rhizoplane of plants grown in field soil (table 3).

Origin	CFU/gram dry weight	Number of variants at sampling dates 31 dap*	37 dap	49 dap	63 dap	77 dap
Field						
Rhizoplane	$2,4 \times 10^8$ **	25	-	21	17	21
Nonrhizo-sphere soil	$2,7 \times 10^7$ **	11	-	10	11	10
Greenhouse (Rhizoplane)						
Field soil	$5,4 \times 10^6$	-	11	-	-	-
Substrate 1	$2,7 \times 10^6$	-	12	-	-	-
Substrate 2	$1,1 \times 10^7$	-	19	-	-	-

* days after planting
** mean of four sampling dates

Table 2 : Number of propagules and variants isolated from the rhizoplane of field- and greenhouse-grown cabbage seedlings and from nonrhizosphere soil (field only)

Origin	Number examined	% isolates exhibiting Antagonism*	Chitinase activity**	Fluorescence***
Rhizoplane	1160	8.6	12.0	29.3
Nonrhizosphere Soil	783	1.9	6.8	11.6
Greenhouse (Rhizoplane)				
Field soil	351	9.1	4.8	1.4
Substrate 1	108	31.4	14.8	2.8
Substrate 2	162	21.0	9.9	4.9

* Against *F. culmorum* and/or *P. lingam*
** Chitin agar
*** King's B

Table 3 : Relationship between origin of isolates and characteristics observed on differentiating media

These results strongly indicate that the composition of the bacterial microflora on the rhizoplane is influenced by the substrate in which the root grows, and that bacterial antagonists on the rhizoplane are differentially favored depending on the type of substrate. Further studies are necessary to test whether a correlation between the number of antagonists on the rhizoplane and the degree of suppressiveness of the respective substrate can be established.

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BIOCONTROL OF *PYTHIUM APHANIDERMATUM* IN CLOSED CULTURE SYSTEMS

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Summary

Pythium aphanidermatum has been responsible for considerable crop loss in glasshouse cucumber. A computer controlled ebb and flow system with 32 separate units was developed to test the effect of antagonists applied at an early stage of growth development. Consistent levels of infection (60 to 80 %) were obtained following infestation of the nutrient solution with a mixture of oospores, mycelium and sporangia of *P. aphanidermatum*. Significant and reproducible reductions in disease incidence (30 to 50%) were obtained with an isolate of *Pseudomonas fluorescens* (WCS365) and with the commercial biocontrol product Mycostop (*Streptomyces griseo-viridis*). However, the *P. fluorescens* isolate had to be added twice to the rockwool blocks and in the nutrient solution, whereas one application of Mycostop to the rockwool blocks was sufficient.

Introduction

The policy of the Dutch government for greenhouse horticulture, is focused on the development of closed culture systems, where the nutrient solutions have to be circulated to minimize environmental pollution. However, root pathogens, particularly those which produce zoospores, have the potential to spread rapidly in an aqueous environment. *P. aphanidermatum* has been responsible for considerable crop loss in cucumber culture on rockwool (Moulin *et al.*, 1994; Paternotte, 1992). In the short term, there are no prospects for breeding resistant cultivars and chemical control is only effective if used as a preventative application. In contrast, substrate systems (e.g. rockwool) offer excellent possibilities for the application of biological control agents. It is easier to introduce and establish the antagonists in substrate systems which commence with a biological vacuum and a limited amount of substrate, compared to a microbiologically well buffered system such as soil.

Young plants are very susceptible to *P. aphanidermatum* and adult plants may become susceptible as a result of environmental stress or agricultural operations (eg. root loss) (Blancard *et al.*, 1994). An effective biocontrol strategy must therefore aim to protect young plants. To test the effect of antagonists applied at an early stage of growth development, a computer controlled ebb and flow system with 32 separate units was developed. Using this system, several bacterial antagonists were tested for their ability to control *P. aphanidermatum* in young cucumber plants. In addition, their ability to establish on the roots was determined by plate counting.

Materials and methods

Ebb and flow system with artificial P. aphanidermatum infection

Rockwool blocks (10 x 10 x 6.5 cm) (Rockwool/Grodan BV, Roermond, The Netherlands) were saturated with a cucumber nutrient solution (pH 5.5; EC 2.4 mS/cm). The nutrient solution contained 972 mg $Ca(NO_3)_2.4H_2O$, 55 mg NH_4NO_3, 557 mg KNO_3, 20 mg Fe-DTPA (7%), 170

mg KH_2PO_4, 616 mg $MgSO_4.7H_2O$, 140 mg $NaNO_3$, 1.7 mg $MnSO_4$, 1.45 mg $ZnSO_4.7H_2O$, 3.35 mg $B_4Na_2O_7.10H_2O$, 0.25 mg $CuSO_4.5H_2O$, and 0.12 mg $Na_2MoO_4.2H_2O$ per litre water, and the pH was adjusted with H_3PO_4. A single cucumber seed (cv. Tyria) (ENZA Zaden BV, Enkhuizen, The Netherlands) was placed in the hole of each rockwool block and covered with vermiculite. Seeds were allowed to germinate in closed containers exposed to day light at 20 °C for five days and were then placed in the ebb and flow system (Fig. 1). Ten cucumber plants were grown in the upper container which was 55 by 75 cm. Each unit started with 30 litres of cucumber nutrient solution, and a small pump was used to give flood (3 to 4 cm high) for two minutes. It took about 20 minutes for the upper container to then be emptied. Depending on the age of the plants, they were flooded three to five times a week. Greenhouse temperature was maintained at 18/25 °C (night/day) and relative humidity was approximately 70 %.

P. aphanidermatum isolates PD 89 and PD 301 (obtained from P. Paternotte) were cultured in 100 ml erlenmeyer flasks containing 20 ml V8-liquid medium (200 ml V8 and 3 g $CaCO_3$ per litre demineralised water, autoclaved at 100 °C). Flasks were incubated for seven days at 25 °C in the dark without shaking, then mycelial mats were washed four times with 30-50 ml sterile demineralised water. The mycelial mats consisting of a mixture of oospores, mycelium, and sporangia were then blended for 30 sec. at high speed in a Waring blender and sieved through cheese cloth. Oospores were counted with aid of a haemocytometer and approximately 5 x 10^6 oospores were added to the 30 litres of nutrient solution seven or eight days after sowing. The number of *Pythium* infected plants (brown stem base, wilted and dead plants) was determined. If necessary, disease development was further encouraged by giving 18 hours flood 19 to 23 days after sowing.

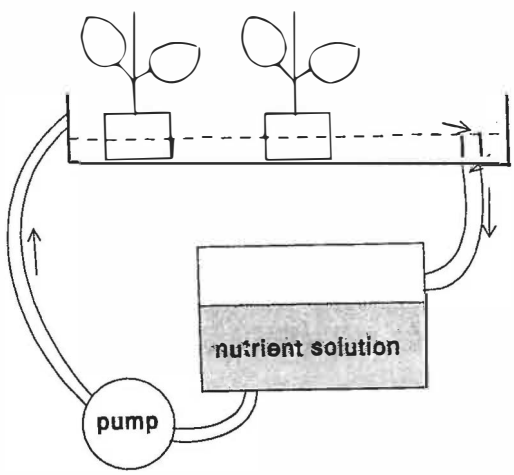

Figure 1 : Ebb and flow system

Biological control experiments

Experiments were carried out in the ebb and flow system infested with *P. aphanidermatum* as described above. An isolate of *P. fluorescens* (WCS365) (Geels and Schippers, 1983; Lugtenberg and de Weger, 1992) and the commercial biocontrol product Mycostop containing *S. griseo-viridis* (Kemira Oy, Espoo, Finland) (Mohammadi, 1992) were added five, seven, or more days after sowing. A spontanic rifampicin-resistant mutant of the *Pseudomonas* isolate was cultured in 100 ml erlenmeyer flasks containing 25 ml King's B broth with 50 mg/l rifampicin incubated for 20 hours at

25 °C. Bacterial cells were washed twice by centrifugation and a suspension of 2.5 x 10^7 cells/ml was prepared in Ringers solution. Mycostop was added as advised by J.C.J. van Adrichem (Aseptafabriek BV, Delft, The Netherlands): 1 g was dissolved in 500 ml tap water at 20 °C. After 30 minutes this suspension was shaken thoroughly and diluted 9.5 litre tap water, giving a final solution of 0.01 % with approximately 4 x 10^4 cfu/ml. Inoculum suspensions were applied to each rockwool block (20 ml) or to the nutrient solution (200 ml).

Populations of bacteria and fungi on cucumber roots were determined by plate counting. WCS365 could be recovered selectively on King's B agar with 100 mg/l rifampicin. For *Streptomyces*, chitin-oatmeal agar with a membrane filter (0.2 µm) was used using the method described by Miller *et al.* (1989). Total bacteria and fungi were enumerated on resp. 10% tryptone-soya agar and potato-dextrose agar.

ANOVA was used and means separated by least significant difference method.

Results

Five to six weeks after sowing there were no symptoms of *Pythium* infection in the uninoculated controls, whereas 60 to 80 % of the plants died or had stem rot after inoculation with *P. aphanidermatum*. Significant ($P < 0.05$) and reproducible disease reductions were obtained with *P. fluorescens* isolate WCS365 and with the commercial

antagonist		added to:	% disease suppression[1)]
Mycostop	1x	block	31*
	2x	block, block	50*
	4x	block, tank, block, tank	59*
WCS365	1x	block	11
	2x	block, block	27, 37
	4x	block, tank, block, tank	50*, 44*

1) 100 x (treatment with antagonist - tr. P.aph alone) / tr. P.aph alone.
Significant disease reductions ($P < 0.05$) are indicated with *.

Table 1 : Biological control of *Pythium aphanidermatum* by the commercial product Mycostop and the *Pseudomonas fluorescens* isolate WCS365

biocontrol product Mycostop (Table 1). Both antagonists reduced the disease incidence by 30 to 50%. However, the *P. fluorescens* isolate had to be added twice on the rockwool blocks and twice in the nutrient solution, whereas one application of Mycostop on the rockwool blocks was sufficient. Population densities of the added antagonists, and the total bacterial and fungal flora on the cucumber roots are summarized in Table 2.

	treatment	logcfu / g fresh root
bacteria		7.5 - 8.5
fungi		3.5 - 4.2
actinomycetes	Mycostop 4x	≤ 3.0
WCS365 (rif^R)	WCS365 4x	4.9 - 5.5

Table 2 : Microbial populations on cucumber roots ± 15 days after sowing

Discussion

The ebb and flow system was an effective research tool to test the effects of biocontrol of root and stem rot in cucumber caused by *P. aphanidermatum*. There were no cross infections between the separate units, disease levels were reproducible, and the system could be used all year round.

Two antagonists showed significant and reproducible biocontrol effects. The commercial product Mycostop, containing *S. griseo-viridis*, is known for its ability to control *P. aphanidermatum* in cucumber (J.C.J. van Adrichem, H. Rattink, pers. comm.). Remarkably, however, is the low population density of *Streptomyces* present on the cucumber roots. The *P. fluorescens* isolate WCS365 was reported for its root-colonizing ability in several crops (Lugtenberg and de Weger, 1992). This is the first report of its ability to control a root disease such as *P. aphanidermatum*.

In future, a greater range of bacterial and fungal isolates, including combinations of micro-organisms, will be tested using this ebb and flow system to improve biocontrol effects. Furthermore, application of antagonists in the ebb and flow system will be compared to application in horticultural practice, and the results with the ebb and flow system will be used to develop a shorter and less laborious bioassay for screening new antagonists.

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We are grateful to H. Roelofsen for building 32 ebb and flow units and to Rockwool/Grodan BV and ENZA Zaden BV for offering rockwool blocks and cucumber seeds, respectively. We thank P. Paternotte, J.C.J. van Adrichem, and B.J.J. Lugtenberg who kindly supplied their *Pythium* isolates and antagonists.

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BIOLOGICAL CONTROL OF FUSARIUM WILT OF RADISH BY COMBINATIONS OF FLUORESCENT PSEUDOMONAS SPP. STRAINS

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Abstract

In this study the hypothesis that compatibility between *Pseudomonas* spp. strains is important to improve biological control by combinations of the strains is tested. Four strains of fluorescent pseudomonads that have the ability to suppress fusarium wilt of radish were tested for their interactions *in vitro*. Some strains were compatible, that is they do not inhibit each other, whereas other combinations were not compatible *in vitro*. To study the relation between interactions *in vitro* and interactions *in vivo*, single strains and combinations of the strains were tested for suppression of fusarium wilt of radish. All bacterial treatments significantly reduced the disease. Treatment with *Pseudomonas fluorescens* strain RS 111 reduced the disease significantly better than the other strains. In combinations with *Pseudomonas fluorescens* strains RE 8 or WCS 358, this disease-suppressive effect of RS 111 was reduced. *In vitro*, strains RE 8 and WCS 358 strongly inhibited RS 111. The combination of RS 111 and *Pseudomonas fluorescens* strain En 401 resulted in the same disease suppression compared to that by RS 111 alone. *In vitro*, RS 111 was not inhibited by En 401. The reduced disease-suppressive effect of RS 111 in combination with WCS 358 or RE 8 might be due to reduced root colonization by RS 111 in the combinations. Combining RE 8 and WCS 358 resulted in a significantly better disease suppression compared to the single strains. However, *in vitro* growth of RE 8 was slightly inhibited by WCS 358. In this case effects of WCS 358 on population densities of RE 8 might be smaller compared to effects of either RE 8 or WCS 358 on RS 111. Thus, in *vitro* interactions between *Pseudomonas* strains to some extend can predict disease suppressive effects by combinations of strains. Population dynamic studies will be conducted to further test this hypothesis.

Introduction

Fusarium wilt diseases cause considerable damage to horticultural and agricultural crops. The fungal pathogen, *Fusarium oxysporum*, infects the roots and colonizes the vascular tissue, leading to wilting of the plant and finally death (Peterson and Pound, 1960). Methods to control the disease are not always reliable. Steam desinfection is expensive and the created microbial vacuum can sometimes lead to devastating disease development. Soil fumigation has negative effects on the environment and will probably be banned in the near future. Therefore other strategies to control the disease, like biological control, have to be developed.

Worldwide, several fusarium wilt-suppressive soils have been described (Alabouvette, 1986; Kloepper *et al.*, 1980; Scher and Baker, 1980). This suppressiveness is of microbial origin (Schippers et al., 1992; Weller, 1988). Especially fluorescent pseudomonads and non-pathogenic strains of *Fusarium oxysporum*, isolated from these soils, have the ability to reduce fusarium wilt. Mechanisms demonstrated to be involved in suppression of fusarium wilt are: competition for substrate, siderophore-mediated competition for iron, and induction of disease resistance (Leeman, 1995; Lemanceau et al, 1992, 1993). Inoculation of a conducive soil with a single strain of a biological control microorganism never reaches the level of suppression observed in naturally suppressive soils, and the positive effects are often inconsistent (Schippers, 1992; Weller, 1988). In

suppressive soils a concerted action of several disease-suppressing microorganisms and mechanisms is postulated to be responsible for the highly consistent disease suppressiveness (Alabouvette, 1986; Lemanceau and Alabouvette, 1991; Schippers, 1992). Therefore, applications of combinations of micro-organisms were tested for their disease controlling abilities. Indeed, in some cases combinations resulted in improved disease control (Lemanceau *et al.*, 1992, 1993; Leeman *et al.*, 1995; Park *et al.*, 1988; Pierson *et al.*, 1994). An important prerequisite for a successful co-inoculation of strains appears to be the compatibility of the co-inoculated microorganisms (Baker, 1990; Li and Alexander, 1988; Raaijmakers, 1994).

In the present study the relation between interactions *in vitro* and disease suppression *in vivo* was investigated for several combinations of strains of fluorescent pseudomonads.

Materials and methods

Microbial cultures and inocula

Pseudomonas putida WCS 358 and RE 8 were isolated from potato rhizosphere and radish root tissue , respectively (Geels *et al.*, 1983a, 1983b; P.A.H.M. Bakker and C. Remkes, unpublished). *Pseudomonas fluorescens* RS 111 and En 401 were isolated from, respectively, the rhizosphere and root tissue of tomato (Van Peer et al, 1990). These four strains significantly reduce fusarium wilt of radish in potting soil bioassays (P.A.H.M. Bakker, C. Remkes and L.C. van Loon, unpublished). The strains were maintained at -80°C in glycerol.

Bacteria were grown for 48 h at 27°C on King's medium B (KB) agar plates (King *et al.*, 1954), and suspensions were prepared in sterile 10 mM $MgSO_4$. The pathogen used was *Fusarium oxysporum* Schlecht f.sp. *raphani* Kendrick & Snyder. It was cultured in aerated 2% malt extract (DIFCO) at 22°C. After 7 days of growth at room temperature, cultures were filtered through glass wool to remove mycelial mats. The microconidia were harvested by centrifugation (8000 rpm, 20 min) and resuspended in 10 mM $MgSO_4$.

In vitro antagonism between Pseudomonas strains

The *Pseudomonas strains* were spot-inoculated on KB agar plates. To test growth inhibition by the spot-inoculated strain, a suspension of the target strain (10^7 cfu/ml) was atomized over the spotinoculated plates after 24 h of incubation at 27°C. Zones of growth inhibition around the spot-inoculated strains were scored after an additional incubation of 24 and 48 h at 27°C. The experiment was designed such that all possible combinations of the 4 strains were tested.

In vivo suppression of Fusarium wilt by (combinations of) pseudomonads

Disease suppression by the single strains and their combinations was tested in a potting soil bioassay (Raaijmakers, 1994; Leeman, 1995). The pathogen was mixed in a potting soil/sand mixture (non autoclaved) to a final concentration of 3.5×10^5 cfu/g mixture. This *Fusarium oxysporum*-infested soil was incubated for 3 days at 20°C. The bacteria were introduced in an autoclaved (2 x 20 min with a 24 h interval) potting soil/sand mixture to a final density of approximately 7×10^7 cfu/g mixture. For the potting soil bioassay fusarium-infested soil, bacterized soil, additional autoclaved soil and non-autoclaved river sand were mixed in a 1:5:9:22.5 ratio. Final densities of *Fusarium oxysporum* and bacteria in this mixture were 10^4 cfu/g and 10^7 cfu/g respectively. Per treatment 9 pots (11 cm high, 14 cm diameter) were filled with this mixture and ten radish seeds (*Raphanus sativus* L.; cultivar Saxa*Nova, S&G Seeds B.V. Enkhuizen) were sown. The plants were grown in

the greenhouse at 20 °C at a photoperiod of 16 hrs. After 19 days fusarium wilt symptoms were scored externally, as well as internally by making cross sections of the root and tuber and examining these for discoloration of the vascular tissues (Leeman, 1995; Raaijmakers, 1994).

Results

In vitro antagonism

The interactions between *Pseudomonas* spp. strains RE 8, WCS 358, RS 111 and En 401 on KB agar plates are summarized in table 1.

Sprayed / Spot	RE 8	WCS 358	RS 111	En 401
RE 8	-	-	++	-
WCS 358	+	-	++	-
RS 111	+	-	-	-
En 401	+	-	-	-

Table 1 . *In vitro* interactions between fluorescent *Pseudomonas spp.* strains RE 8, WCS 358, RS 111 and En 401 on KB agar plates. - ; no inhibition, + ; slight inhibition, ++ ; strong inhibition. The columns show inhibition of growth of the sprayed strains and the rows inhibition by the spot-inoculated strains.

Growth of strain RE 8 was slightly inhibited by WCS 358, RS 111 and En 401. RE 8 itself strongly inhibited growth of RS 111. WCS 358 was not inhibited by any of the other strains, whereas WCS 358 itself inhibited the growth of RE 8 slightly and that of RS 111 strongly. The growth of En 401 was not inhibited by the other strains and En 401 slightly inhibited growth of RE 8. RS 111 was strongly inhibited by strains RE 8 and WCS 358 whereas RS 111 itself slightly inhibited growth of RE 8.

Thus, the combinations of RS 111 and En 401, and WCS 358 and En 401 are considered to be compatible and the combinations of RS 111 and WCS 358 or RE 8, and of RE 8 and WCS 358 or En 401 are considered to be incompatible.

Suppression of fusarium wilt

All bacterial treatments, including the combinations, resulted in a significantly lower percentage of diseased plants compared to the non-bacterized control treatment. Strain RS 111 reduced disease significantly better than any of the other strains. The combination of the strains RS 111 and En 401 did not result in a better disease suppression than the single strain RS 111. The combinations of RS 111 with RE 8 or with WCS 358 resulted in an intermediate disease suppression compared to the disease suppression by each of these strains on their own. The combinations of En 401 with RE 8 or WCS 358 also did not result in a better disease suppression compared to En 401 alone. One combination, RE 8 and WCS 358, did result in significantly higher disease suppression compared to the disease suppression by the single strains (table 2).

Treatment	Percentage diseased plants
Control	87.8 A[1]
WCS 358	51.1 BC
RE 8	55.1 B
RS 111	27.8 EF
En 401	43.5 CD
WCS 358 + RE 8	37.8 DE
WCS 358 + RS 111	34.9 DE
WCS 358 + En 401	38.8 D
RE 8 + RS 111	39.1 D
RE 8 + En 401	41.8 CD
RS 111 + En 401	24.6 F

Table 2 : Percentage of fusarium wilted plants in a potting soil bioassay. Treatments consist out of bacterization of soil with fluorescent *Pseudomonas* spp. strains WCS 358, RE 8, RS 111 and En 401 and their combinations. The strains were mixed through soil as inocula in soil to a final concentration of 10^7 cfu/g soil (in the single and combination treatments). The pathogen was mixed through soil as inoculum in soil to a final concentration of 10^4 cfu/g soil. Plants were score 18 days after sowing. [1] Means followed by the same letter are not significantly different at $P \leq 0.05$, analysis of variance followed by Fisher's least-significant-difference test.

Discussion

When *in vitro* antagonism is compared to the results of *in vivo* disease suppression, it appears that the *in vitro* test has some predictive value for the disease suppression by combinations of pseudomonads. In these experiments this especially accounts for RS 111, which is strongly inhibited *in vitro* by RE 8 and by WCS 358. The percentage of diseased plants in the combination RE 8 + RS 111 was significantly intermediate compared to the single strain treatments. In the combination RS 111 + WCS 358 the percentage of diseased plants was also intermediate compared to the single strain treatments. The disease-suppressive effect of RS 111 was not reduced by En 401. The latter strain also did not inhibit growth of RS 111 *in vitro*. However, the combination of RE 8 with WCS 358 did give a significant reduction of the percentage diseased plants compared to the single treatments although RE 8 was *in vitro* slightly inhibited by WCS 358. It is possible that WCS 358 has a different effect on population densities of strains RS 111 and RE 8. This will be investigated by studying the population dynamics of the different (combinations of) strains.

In the combination of RE 8 and WCS 358 the reduction of the percentage of diseased plants may be due to synergistic effects resulting from different disease-suppressive actions. It has been demonstrated that WCS 358 suppresses disease by competition for iron (Raaijmakers *et al*, 1995). For RE 8 the disease-suppressive mechanism(s) are under investigation.

It is possible that non-compatibility (like in the combination of RE 8 and WCS 358) results in earlier and greater competition among introduced bacteria in the rhizosphere and, therefore, earlier and more consistent expression of traits involved in competition and disease control (Pierson and Weller, 1994). However, this does not seem to apply to the combinations of RE 8 and WCS 358 with RS 111. Unraveling the mechanisms involved in disease suppression by and non-compatibility of combinations, will offer us tools to improve biological control of soil-borne diseases.

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Plant microorganisms interactions

DISEASE-SUPPRESSIVE ACTIONS OF PSEUDOMONAS BACTERIA : INDUCED RESISTANCE

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Abstract

Disease suppression by non-pathogenic, root-colonizing *Pseudomonas* bacteria can depend on antagonism between the bacteria and soil-borne plant pathogens in the rhizosphere, as well as on effects that appear to be plant-mediated. Specific *Pseudomonas* strains induce systemic resistance in carnation, radish and *Arabidopsis* against both fungi and bacteria. As shown by using defined mutants, this effect of the bacteria can depend on different traits, but is invariably manifested as an induced systemic resistance (ISR) of the plant against a subsequent or "challenge" inoculation. This induced resistance resembles the well-known phenomenon of systemic acquired resistance (SAR), that can be triggered by either pathogens, avirulent forms of pathogens, cultivar-non-pathogenic races or selected chemicals, and brings about an enhanced resistance response against pathogenic fungi, bacteria and viruses, both locally and often systemically, for prolonged periods. Upon primary inoculation, a signal is generated that is transported throughout the plant and induces the resistant state. When, thereupon, plants are challenge inoculated, extant defense mechanisms become active earlier and to a greater extent.

SAR is dependent on endogenously produced salicylic acid (SA) and associated with the accumulation of pathogenesis-related proteins (PRs). In contrast, PRs are not required for the state of ISR to be reached. Although some resistance-inducing *Pseudomonas* isolates can produce SA *in vitro*, evidence for SA as the inducing compound *in vivo* is circumstantial. A model system consisting of *Arabidopsis* as the host and *Pseudomonas syringae* pv. *tomato* as the challenging pathogen shows differential induction and expression of resistance in response to bacterial and chemical inducers. These observations indicate that systemic resistance can be induced by different mechanisms.

Mechanisms of disease suppression by *Pseudomonas* bacteria

Repeated croppings of susceptible plants over large surface areas favour the development of disease epidemics and may lead to crop failure and huge economic losses (Schumann, 1991). Yet, in certain soils disease incidence can be low, in spite of the presence of the pathogen and other conditions usually conducive to disease occurrence. The disease-suppressive effect of such soils has been shown to result from the presence of saprophytic micro-organisms that protect the plants against the pathogenic action of disease-causing micro-organisms. This phenomenon has attracted considerable attention in view of the potential to exploit the disease-suppressive actions of such micro-organisms for environmentally friendly biological disease control (Schippers, 1992).

Among the micro-organisms present in disease-suppressive soils, fluorescent *Pseudomonas* spp. have been shown to be among the most effective in reducing soil-borne diseases (Schippers *et al.*, 1987; Weller, 1988). These non-pathogenic, root-colonizing bacteria are often referred to as plant growth-promoting rhizobacteria (PGPR), because their application stimulates growth and improves plant stand under stressful conditions (Kloepper *et al.*, 1991). Evidence for direct stimulation of plant growth is largely circumstantial. Rather, increased plant productivity can be indirect in that it may rely in large part on the suppression of harmful micro-organisms (Schippers *et*

al., 1987). Such suppression can depend on direct antagonism between bacteria and soil-borne pathogens in the rhizosphere, as well as on indirect effects that appear to be plant-mediated. The mechanisms involved are gradually elucidated and appear to involve multiple bacterial traits as well as specific plant characteristics.

Studies on suppression of fusarium wilt of carnation, caused by *Fusarium oxysporum f.sp. dianthi* (Fod), established competition for iron as the mechanism of disease reduction by *Pseudomonas putida* strain WCS358 (Bakker *et al.*, 1993; Duijff *et al.*, 1994a). Under iron-limiting conditions WCS358 secretes a fluorescent siderophore (pseudobactin 358) in order to complex and take up the scarcely available ferric ion. The siderophore released by Fod under these conditions is less efficient, resulting in a lack of iron for fungal metabolism. The plant, in contrast, does not appear to suffer from iron shortage (Duijff *et al.*, 1994b). Under these circumstances fungal spore germination is inhibited and hyphal growth restrained, effectively lowering the chance that the plants become infected and reducing disease incidence and severity.

That competition for iron is the sole mechanism responsible for the suppression of fusarium wilt by WCS358 is demonstrated by supplying iron. Addition of $FeCl_3$ progressively reduced bacterial antagonism, until at 200 µM suppression was completely abolished. Moreover, a bacterial mutant (JM218), generated by *Tn5* transposon insertion and unable to produce pseudobactin 358, did not reduce disease incidence at any level of iron availability (Duijff *et al.*, 1994a). A different bacterial strain, *Pseudomonas fluorescens* WCS417, was about twice as effective as WCS358 in suppressing fusarium wilt in carnation at low iron concentrations. For this strain too, antagonism was reduced progessively with increased supply of $FeCl_3$. However, the mechanism involved cannot solely be competition for iron because a similar mutant of this strain (S680), lacking siderophore production, was as effective in suppressing disease as the wild type (Duijff *et al.*, 1993). Clearly, a additional iron-dependent mechanism must be responsible for the protection of carnation against fusarium wilt by this mutant.

Competition for iron is a special example of the more general mechanism of competition for nutrients. In the rhizosphere many micro-organisms compete for sugars, organic acids and amino acids in root exudates. Bacteria, such as *Pseudomonas*, are totally dependent on plant-derived metabolites for activity and reproduction. In contrast, most fungi like *Fusarium* can grow saprophytically on complex carbohydrates present in dead and decaying plant material. Competition for nutrients has been shown to be the mechanism by which the non-pathogenic *Fusarium oxysporum* strain Fo47 reduces the incidence of Fod on carnation (Lemanceau *et al.*, 1993) and this strain synergistically enhances the disease-suppressing activity of WCS358 through combination of the mechanisms of competition for iron and for carbon (Lemanceau and Alabouvette, 1993). However, WCS417 is unlikely to act through these mechanisms.

Other possible mechanisms of direct antagonism between bacteria and pathogenic fungi are production of antibiotics (Fravel, 1988) and mycoparasitism (Jeffries and Young, 1994). WCS417 does seem to produce an antifungal factor (AFF) that reduces growth of Fod *in vitro* (Duijff *et al.*, 1993). Moreover, production of AFF seems to increase at lower iron concentrations. The nature of this factor has not been elucidated, however, nor is evidence available to demonstrate that this factor plays a role in disease suppression *in vivo*. Mycoparasitism commonly depends on the secretion of chitinases and/or glucanases, often in conjunction with proteases. Only protease is produced by WCS417 on suitable substrates *in vitro*, making it unlikely that WCS417 has mycoparasitic properties.

Bacterially-induced systemic resistance

A different mechanism was demonstrated when the protective bacterium and the pathogenic fungus were applied to different parts of carnation plants and were confirmed to remain spatially separated.

When the roots were treated with the bacterium and the fungus was introduced in the stem by slashing, disease incidence was reduced substantially in the moderately resistant cultivar Pallas and less consistently in the susceptible cultivar Lena (Van Peer *et al.*, 1991). Thus, WCS417 is acting indirectly and apparently capable of enhancing resistance of carnation against Fod. This induced resistance did not lead to obvious metabolic alterations in the plant, but became manifest associated with an enhanced production of phytoalexins upon subsequent inoculation with Fod. Heat-killed bacteria, as well as purified bacterial lipopolysaccharide (LPS), were as effective in inducing resistance as were live bacteria (Van Peer and Schippers, 1992), confirming that the protective effect is plant-mediated.

For studying the mechanism of bacterially-induced resistance, carnation proved rather unsuitable because experiments required several months and results were not always consistent. Radish was chosen because the related pathogen *Fusarium oxysporum f.sp. raphani* (For) (previously also referred to as *F. oxysporum f.sp. conglutinans*) similarly causes fusarium wilt in this plant species and both *Pseudomonas fluorescens* strains WCS417 and WCS374, but not *Pseudomonas putida* WCS358, induce resistance against For in radish (Leeman *et al.*, 1995a) (Table 1). The protection afforded by these bacteria was demonstrated in bio-assays in which bacteria remain spatially separated from the pathogen. Seedlings were placed horizontally on rock wool cubes with the distal part of the roots on a cube contained in a plastic bag adjacent to another bag with a cube supporting the proximal part of the roots. The roots were laid down through an incision in the bags. The distal part of the roots was treated with a bacterial suspension in talcum powder. Two to three days later the proximal part was inoculated with the fungus in peat mixture. Disease developed within 10 to 14 days and internal browning and external wilting symptoms were scored routinely three weeks after inoculation.

Bacterial strain	Carnation/ *F.oxysporum*	Radish/ *F.oxysporum*	Arabidopsis/ *P.syringae*
P.putida WCS358	competition for iron	competition for iron	ISR
P.fluorescens WCS374	ISR	ISR	-
P.fluorescens WCS417	ISR antibiosis ?	ISR antibiosis ?	ISR

Table 1. Mechanisms of disease suppression of *Pseudomonas* bacteria in different plant species

Bacterially-induced resistance was demonstrated in all cultivars tested, irrespective of their level of genetic resistance to For, provided fungal inoculum pressure was moderate, *i.e.* disease pressure in control plants was between 30 and 70% (Leeman *et al.*, 1995a). The state of induced resistance was not reached immediately after application of the bacteria but developed up to an optimum after about two days. The effect was not dependent on high levels of inducing bacteria: 10^5 cfu g^{-1} talcum were already optimal. Also in radish the bacterial LPS appeared to be the trait responsible for induction. Cell wall extracts were as effective as live bacteria, and bacterial mutants lacking the O-antigenic side-chain of the LPS (OA^-) as well as their cell wall extracts were ineffective, as was strain WCS358 (Leeman *et al.*, 1995b). These results demonstrate that the resistance induced in radish is similar to that in carnation and appears to depend on the same bacterial trait. However, under iron-limiting conditions OA^--mutants still induced resistance (Leeman *et al.*, 1995c), indicating that a determinant expressed only under low-iron conditions can also induce resistance (Fig. 1). Indeed, purified siderophore of WCS374 induced resistance as well as bacterial LPS. Apparently, more than a single bacterial trait can be involved in triggering induced resistance in the plant, but these effects seem complementary rather than additive, and full induction by one factor masks any contribution by others. As shown by *Tn5* mutagenesis, the enhanced

defensive capacity of the plant afforded by the bacteria can depend on different bacterial traits, but is invariably manifested as an induced systemic resistance of the plant against a subsequent or "challenge" inoculation. Under the influence of the bacteria, a signal is produced in the roots that is transported to distant parts and brings about the state of induced resistance. In carnation, this induced resistance is manifest in above-ground parts upon stem inoculation with *Fusarium*. In radish, it likewise extends to the leaves, which show an enhanced resistance against *e.g.* the pathogenic bacterium *Pseudomonas syringae pv. tomato* (Pst) (Hoffland *et al.*, submitted). Because of its systemic nature, bacterially-induced resistance is now referred to as "induced systemic resistance" (ISR) (Kloepper *et al.*, 1992). Neither the nature of the transported signal, nor the mechanism of the enhanced resistance has been clarified.

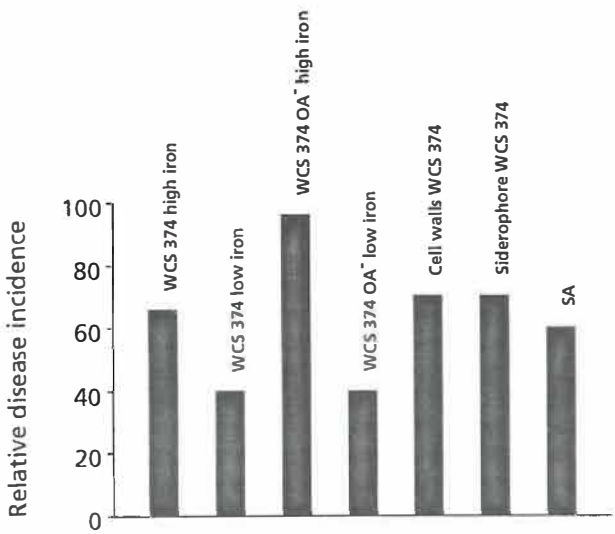

Figure 1. Induced resistance against For in radish upon various treatments, expressed as disease incidence relative to non-treated control plants. Data from representative experiments by M. Leeman.

Systemic acquired resistance

ISR triggered by non-pathogenic, root-colonizing *Pseudomonas* bacteria resembles the well-known phenomenon of acquired resistance, that was first characterized in tobacco reacting hypersensitively to tobacco mosaic virus (TMV). After primary inoculation of a few leaves with TMV, challenge inoculation of other leaves led to necrotic lesions that remained much smaller, and occasionally were less numerous, than on plants that had not been inoculated previously (Ross, 1961). The phenomenon has been termed "systemic acquired resistance" (SAR) and has been extensively studied in tobacco and cucumber. Thus, Kuc (1982) induced resistance in cucumber by either *Colletotrichum lagenarium* or tobacco necrosis virus and found protection against different types of fungi, bacteria and viruses. Mostly, disease symptoms were reduced; sometimes, no symptoms at all became apparent and the plant was fully protected.

Induction and expression of SAR are both non-specific. Thus, SAR can be triggered by either pathogens, avirulent forms of pathogens, cultivar-non-pathogenic races or selected chemicals, and brings about an enhanced resistance response against pathogenic fungi, bacteria and viruses, both locally and often systemically, for prolonged periods. All physical and chemical treatments that

influence the progress of infection after a first inoculation do so similarly after a challenge inoculation. Moreover, no additional metabolic alterations have been detected in resistant plant parts that do not also occur locally at the infection or treatment sites. Apparently, the mechanisms available to reduce pathogen multiplication and spread are the same but upon challenge inoculation, extant defense mechanisms become active earlier and to a greater extent. Non-pathogenic root-colonizing bacteria do not obviously affect host metabolism at their site of application. Nevertheless, they increase the capacity of the plant to respond to challenge inoculation by an increased expression of defensive mechanisms.

Plant defense responses comprise induction of low-molecular-weight antimicrobial phytoalexins, accumulation of high-molecular-weight pathogenesis-related proteins (PRs), and reinforcement of cell walls as physical barriers to pathogen spread. Phytoalexins are produced locally in cells neighbouring infection and their induction can be stimulated upon challenge inoculation (Van Peer *et al.*, 1991). Cell wall rigidification, particularly lignification, likewise is a local event. In contrast, PRs are induced systemically. Eleven families of related PRs have now been recognized (Van Loon *et al.*, 1994). Upon induction in tobacco, high levels of acidic PRs accumulate in leaf intercellular spaces and smaller quantities of basic homologues increase in vacuoles. Essentially similar changes occur in other plant species upon induction, demonstrating high conservation of PRs among plant families. Some of the PRs have glucanase, chitinase and lysozyme activity and/or possess activity against a limited number of fungi and bacteria. None has so far been found to be effective against viruses, however. These observations suggest that PRs contribute to, but are not sufficient to explain, SAR. Nevertheless, they are widely considered to be good markers of SAR.

As for ISR, for SAR to be reached upon primary inoculation a signal must be generated that is transported throughout the plant and induces the resistant state. Also for SAR, the nature of the signal is unknown, but at least in tobacco it appears that ethylene is involved in its release and/or transport, and salicylic acid (SA) is a necessary and sufficient intermediate in the signal-transduction pathway leading to both induction of PRs and induced resistance. Thus, it has been found that many chemicals can induce PRs and SAR, but basically they all seem to act through either ethylene or SA (Van Loon, 1989). Essentially the same seems to hold for some other plant species, *e.g.* cucumber and *Arabidopsis thaliana*, but different plant species appear to be differentially sensitive to those inducers. In *Arabidopsis* the ethylene-releasing chemical ethephon induces a substantial amount of PR-1 mRNA, whereas ethylene itself is hardly inductive (Lawton *et al.*, 1994). Apparently, the accompanying breakdown products hydrochloric acid and phosphoric acid are responsible and cause tissue injury sufficiently to release endogenous elicitors and/or to increase responsiveness of the tissue to ethylene. In tobacco, the injurious acids by themselves do not elicit PRs (Van Loon, 1977), but ethephon likewise is more effective in inducing PRs than the natural ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). None of these effects are seen in transformed plants expressing the *NahG* gene from *Pseudomonas putida*, that causes SA to be converted into catechol (Gaffney *et al.*, 1993; Lawton *et al.*, 1994). Hence, the effects of ethylene/ethephon are dependent on SA. Moreover, ethylene greatly sensitizes the tissue to respond to exogenous SA (Lawton *et al.*, 1994).

In tobacco endogenous SA increases during necrotizing infections and this increase extends to distant leaves that develop SAR (Yalpani *et al.*, 1991). In *NahG* transformants no SAR develops, demonstrating the functioning of SA in the signalling pathway to SAR. Although some of the SA accumulating in distant leaves may have been transported from the primary inoculated ones, several lines of evidence indicate that SA is not the translocated signal. Injection of SA into leaves of non-infected plants induces PRs and resistance locally but only occasionally systemically (Van Loon and Antoniw, 1982). In cucumber SA increased in distant leaves when primary inoculated leaves were detached before they accumulated SA (Rasmussen et *al.*, 1991). Reciprocal grafts of wild type and *NahG* tobacco plants established that wild type scions can develop SAR when the rootstock cannot

accumulate SA, but a *NahG* scion does not express SAR when grafted on a wild type rootstock producing SA (Vernooij *et al.*, 1994). Therefore, distant leaves appear to start producing SA under the influence of a different translocated signal and this SA is necessary for the resistant state to be expressed.

Similarities and differences between ISR and SAR

Whereas SA sprayed onto or injected into leaves is apparently not redistributed, it can be readily taken up by roots and transported in the xylem throughout the entire plant (Van Loon and Antoniw, 1982). In radish, SA applied in talcum powder to distal root parts in µmolar concentration induces resistance against For and Pst (Hoffland *et al.*, 1995 and submitted). Thus, apart from the configuration of the LPS, production of SA in the rhizosphere might be a mechanism by which fluorescent *Pseudomonas spp.* could induce resistance. In fact, SA can be secreted by some bacterial strains under iron-limiting conditions, when it acts as a siderophore (Meyer *et al.*, 1992). *Pseudomonas fluorescens* WCS374 suppresses For in radish better under low-iron than under iron-sufficient conditions (Leeman *et al.*, 1996) (Fig. 1). Moreover, the OA^--mutant suppresses as well as the wild type under low-iron, but is totally ineffective under high-iron conditions. The latter is observed likewise for strain WCS417. Under low-iron, but not under high-iron conditions, WCS374 produces substantial amounts of SA *in vitro*, WCS 417 produces about one-fifth of this amount and WCS358, that does not induce resistance in radish (Table 1), produces none. Hence, there exists a fair correlation between the capacity of these strains to produce SA *in vitro* and their ability to trigger ISR *in vivo*.

Treatment of radish plants with SA induces SAR and PR-homologues of the families PR-1, -2 and -5 (Hoffland et al., 1995). However, none of the bacterial strains triggering ISR lead to the induction of PRs in treated plants, nor is accumulation of PRs seen upon challenge inoculation with For. Similarly, no phytoalexins are induced by the bacteria or are demonstrable when plants have been challenged. ISR clearly differs from SAR in that specific *Pseudomonas* isolates induce systemic resistance in carnation, radish and *Arabidopsis* against both fungi and bacteria, without any visible effect on the host. Phytoalexins and PR-proteins are not induced under these circumstances, indicating that they are not necessary for the induced state to be reached. Although the resistance-inducing *Pseudomonas* isolates can produce SA *in vitro*, evidence for SA as the inducing compound *in vivo* is at best circumstantial. The possible role of bacterially-produced SA in ISR is currently being investigated by screening for mutants impaired in SA production.

To study the mechanism of ISR, a model system consisting of *Arabidopsis* as the host and Pst as the challenging pathogen has been developed. This system is similar to that in radish in that treatment of *Arabidopsis* plants with SA induces SAR and PR-1, -2 and -5 mRNAs, but development of ISR is not accompanied by PR-gene expression or protein accumulation. Remarkably, WCS374 does not induce resistance in *Arabidopsis*, whereas WCS358 is moderately effective (Table 1). The *Arabidopsis* ecotypes Columbia and Landsberg *erecta* are both responsive to the inducing strains, but the ecotype RLD is not although it does develop SAR upon treatment with SA (C.M.J. Pieterse and A.C.M. van Wees, in preparation). Thus, *Arabidopsis* shows differential induction and expression of resistance in response to bacterial and chemical inducers. This system is now being employed to elucidate the interactions between inducing bacteria and plant roots, as well as changes in gene expression associated with the induced state in the plant.

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PYTHIUM F IN SOILLESS CULTURES: IMPACT OF POOR OXYGENATION ON THE PLANT-PATHOGEN INTERACTION

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Abstract

Epidemiological studies conducted in soilless cultures of tomato have demonstrated that minor pathogens belonging to *Pythium* group F were consistently isolated from apparently healthy roots. Ultrastructural and cytochemical studies have pointed out that *Pythium* F hyphae could colonize the cortical area of cucumber roots. Infection of plants with *Pythium* F markedly stimulated the formation of papillae, the deposition of cell wall thickenings, and the accumulation of electron dense aggregates arround fungal hyphae.

The effects of oxygen tension on infection of tomato roots by *Pythium* F under hydroponic conditions were also studied on the basis of induction of biological processes in plant roots related to the oxidative stress. Aeration of the nutritive solution (11-14 % O_2) resulted in a marked increase in plant growth and in a significant decrease in root damage and colonization by the pathogen. Plants receiving the moderate (5,8-7 % O_2) and the low (0,8-1,5 % O_2) oxygen treatments produced higher amounts of conjugated dienes and revealed increased lipoxygenase activity in comparison with well aerated plants, regardless of *Pythium* F infection. Concomitant increases in superoxide dismutase and catalase activities were observed in the low and moderate oxygen treatments after inoculation with the pathogen, but such increases could not be viewed as a part of defense of tomato plants against *Pythium*.

Pythium species are responsible for crown and root rot and yield reduction of several greenhouse-grown crops. Among *Pythium* species, *P. aphanidermatum* and *P. ultimum* have been reported to be the most aggressive, potentially destroying a crop within a few days (Cherif *et al.*, 1994). The majority of the other *Pythium* species are generally considered as "minor" root pathogens that reduce plant growth without causing obvious symptoms (Cook *et al.*, 1972). Recent epidemiological studies, performed by our group, revealed that *Pythium* belonging to group F, with filamentous non-inflated sporongia, were frequently associated with tomato and cucumber roots in soilless cultures resulting in yield reduction even in the absence of root rot symptoms (Rafin, 1993). The frequent infections observed under hydroponic conditions may indicate a higher receptivity of tomato roots to these microorganisms (Drew *et al.*, 1980). Poor aeration of the nutrient solution under these conditions may affect the physiology and function of plant roots and these deleterious effects may then be aggravated in the presence of "minor" root pathogens such as *Pythium* F.

Nevertheless, to bring further insights to this hypothesis it was necessary to investigate the plant-*Pythium* F interaction and understand the etiology of *Pythium* F. In fact several aspects including the ability of *Pythium* F to grow inside the root tissues and its mode of penetration in the root deserved to be investigated. Therefore ultrastructural and cytochemical studies of the plant-*Pythium* F interaction have been performed (Rey *et al.*, this volume).

Inoculation of cucumber or tomato roots with *Pythium* F resulted in a heavy colonization of the cortical area from 24 to 48 h after inoculation. After this time the pathogen was always restricted to the cortical area and never observed in the stelar tissues, in the case of cucumber plants.

Colonization of the epidermis and the cortex by *Pythium* F was associated with the induction of striking host reactions. One of the most noticeable features was the formation of papillae and wall thickenings lining the host cell walls which were usually observed on the opposite side of fungal location. Wall appositions and papillae were never observed in the stele of *Pythium* F-infected roots. Another typical response of *Pythium* F-infected roots was the accumulation of electron dense aggregates and material in the inner cortical cells invaded by *Pythium* F. Hyphae observed in such cells were generally moribund and reduced to empty hyphal shells. The high electron density of this material suggests that it is enriched with fungitoxic phenolic compounds (Cherif *et al.*, 1991).

According to our observations, penetration of plant roots by *Pythium* F, triggered an array of responses such as mechanical defense barriers (papillae, wall appositions...) and others of chemical nature (osmiophilic substances). Such defense responses occurred early in the plant-*Pythium* F interaction and seem to be effective to delay and even stop *Pythium* F at the endodermal level, which may explain the fact that despite the massive infection, the only symptoms that could be noticed was a slight brownish colour at the upper root level. Our ultrasructural observations may explain the earlier observation that *Pythium* F spp. were consistently isolated from apparently healthy roots. Nevertheless, under stress conditions (i.e. thermic stress, poor oxygenation, ...), plant defenses might be delayed to such an extent that the pathogen is allowed to multiply and spread causing cell alterations and appreciable yield losses (Favrin *et al.*, 1988). Little is known about the effects of low levels of oxygen tension on infection of plant roots by *Pythium* under hydroponic conditions. Therefore the next objective was to determine the effects of oxygenation on growth of tomato plants, fungal colonization of the roots and development of *Pythium* root rot.

Tomato plants were grown in the greenhouse under either a high (11-14% O2, bubbling with air) a moderate (6-7% O2, control) or a low (1-1,5%, bubbling with nitrogen) oxygen concentration, and either inoculated or not with *Pythium* F.

Aeration of the nutrient solution with air resulted in an important increase in shoot and root growth of tomato plants as compared to the nitrogen and the control treatments. While aerated roots continued to develop, roots of the other treatments nearly stopped growing until the end of the experiment (Fig. 1). Plants receiving the air treatment more then doubled their aerial weight at the end of the experiment (Fig. 1). Control and nitrogen treated plants showed typical symptoms of root decay within 6 days after inoculation, while aerated plants remained healthy throughout the experiment. By day 10, poorly aerated plants had reached an advanced stage of root decay.

The presence of *Pythium* on and inside infected tomato roots was confirmed by the immunoenzymatic staining procedure (Rafin *et al.*, 1994) (Fig. 2) and isolation of the pathogen on selective medium. Colonization with *Pythium* F occurred very early in the low and moderate oxygen treatments and increased with time (Fig. 2). Colonization of aerated roots started 10 days after inoculation with the pathogen and remained very low indicating the beneficial effects of oxygenation of the nutrient solution.

The causes of aggravation of disease symptoms under poor aeration conditions remain obscure, probably because of the multiplicity of mechanisms. Nevertheless, it is known that oxygen deficiency and pathogen infection can induce a multitude of biological processes in plant tissues, including the formation of active oxygen species and free radicals that are deleterious to the host cells and could contribute to the damages caused by the pathogen as well as serve as antimicrobial agents (Edreva, 1989).

Lipoperoxidation of membrane lipids was estimated by determining lipoxigenase (LOX) activity and analysing conjugated dienes. LOX catalyses the oxidation of free fatty acids such as linoleic and linolenic acids forming the conjugated diene hydroperoxides. Many studies dealing with oxidative stress

were reported in the case of hypersensitive respondr (HR). In most of these host parasite systems the HR has been associated with lipid peroxidation and LOX activity was causally linked to resistance expression. Conversely, oxidative stress in host-fungus systems involving minor pathogens, such as those involving *Pythium* spp., is not well documented. Thus the next objective of the present studies was to delineate some of the biological events, related to the oxidative stress, associated with *Pythium* F-tomato interaction under different oxygen concentrations.

LOX activity was low throughout the experimental period when oxygen concentration was the highest in the nutrient solution (Fig. 3). This activity was only moderately increased by decreasing oxygen concentration (Fig. 3A). After inoculation with *Pythium* F, no major changes in LOX activity were found in the air treatment while drastic increases occurred in the nitrogen treated plants and the controls (Fig. 3B). Infection with *Pythium* F resulted in 3-4 fold increase in LOX activity followed by a slowly declining activity during the remaining period of the experiment.

The patterns of accumulation of conjugated dienes under oxygen stress and *Pythium* infection correlated well with the observed increases in LOX activity (Fig. 4). In absence of *Pythium* F, the amounts of conjugated dienes were lower in aerated roots than those of the other treatments (Fig. 4A). In *Pythium*-infected plants, relatively stable levels of conjugated dienes were detected in aerated plants while a significant increase was observed in the low and moderate oxygen treatments (Fig. 4B). An increasing gradation could be underlined from air treated plants to control and nitrogen treated ones.

The observed increases in conjugated dienes and LOX activity under oxygen stress and *Pythium* infection could not be related to disease resistance since they were coincident with appearance of root decay and aggravation of disease symptoms. Our observations speak in favor of the idea that the increases in LOX activity detected in oxygen stressed tomato roots and infected with *Pythium* F may lead to degradation and disorganisation of membrane lipids which may finally result in root decay.

The effects of oxygenation on enzymatic antioxidants superoxide dismutase (SOD) and catalase (CAT) were also studied. SOD activity was higher in the air treatment than in the low and moderate oxygen treatments (Fig. 5). This difference became significantly different from 4 days onwards until the end of the experiment. After inoculation with *Pythium* F, SOD activity showed no marked change in the air treatment while rapid and significant increases were noticed in the low and moderate oxygen treatments (Fig. 5B).

Results obtained with catalase activity were roughly similar to those obtained with SOD (Fig. 6). In fact, in presence of *Pythium* F, CAT activity remained almost unchanged in the air treatment while increased levels were observed in the other treatments (Fig. 6B).

The highest basal levels in SOD and CAT activities observed in the aerated roots of uninfected plants may reflect an increase in constitutive levels of expression of the two enzymes. It has been shown that high constitutive levels of antioxidants may induce resistance to activated oxygen and this may provide resistance to a particular stress (Spychalla *et al.*, 1990). This may also indicate that well aerated plants are more prepared to defend themselves against *Pythium* invasion than plants grown under poor aeration. The increase in the activity of CAT and SOD after inoculation with *Pythium* F under oxygen stress can be interpreted as evidence for an enhanced demand for detoxification in response to production of reactive oxygen species by infected roots. Nevertheless, such high enzymatic activities were unable to protect the plant against oxidative damage and fungal attack and thus could not be viewed as a part of defense of tomato plants against *Pythium* F.

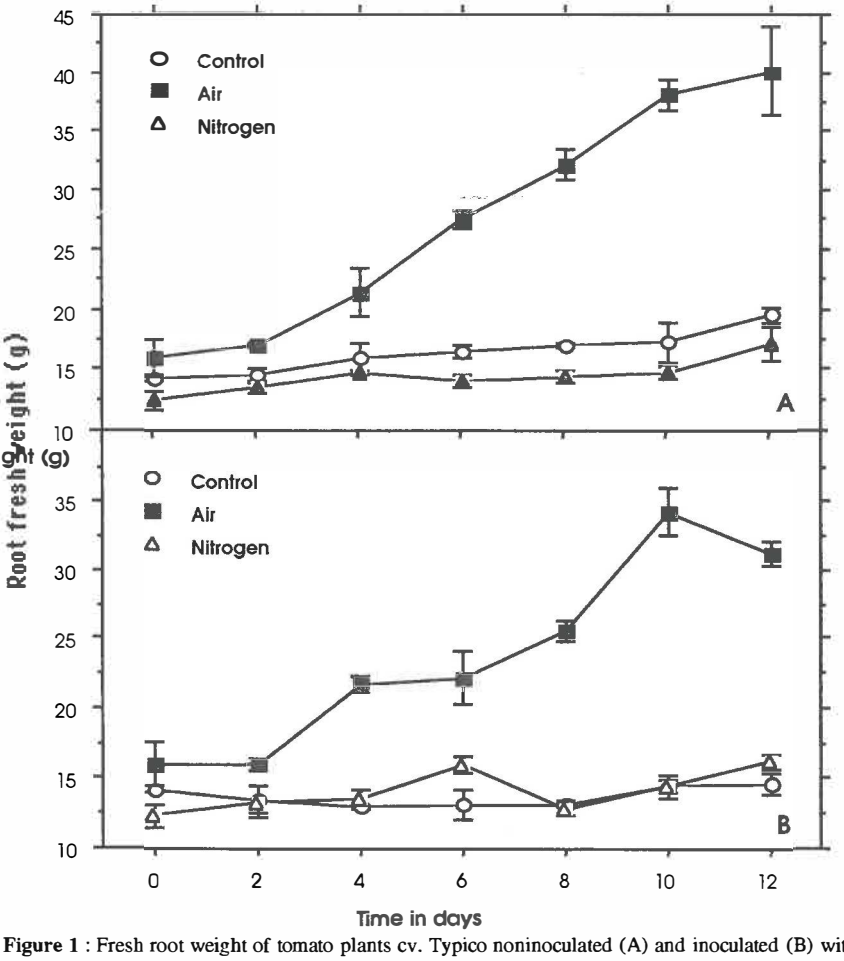

Figure 1 : Fresh root weight of tomato plants cv. Typico noninoculated (A) and inoculated (B) with *Pythium* F707 over time. Values represent the means from three indepedent experiments; bars indicate SD.

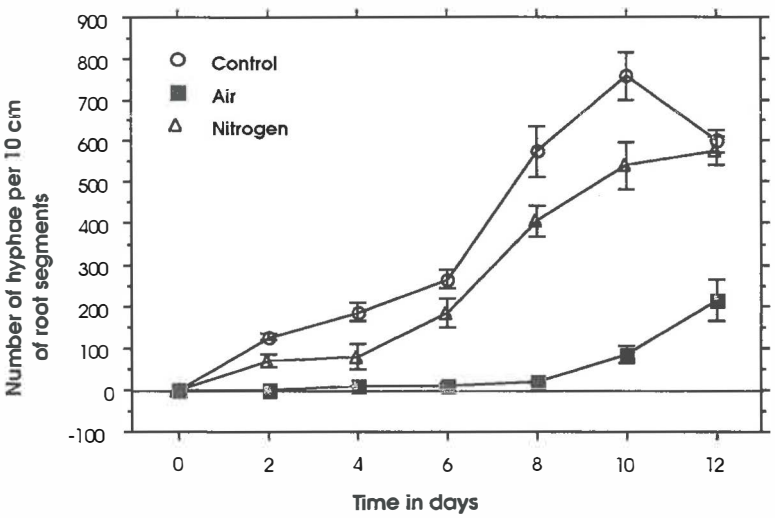

Figure 2 : Colonization over time by *Pythium* F707 of tomato roots of cv. Typico using the immunoenzymatic staining technique. Values represent the means from three indepedent experiments; bars indicate SD.

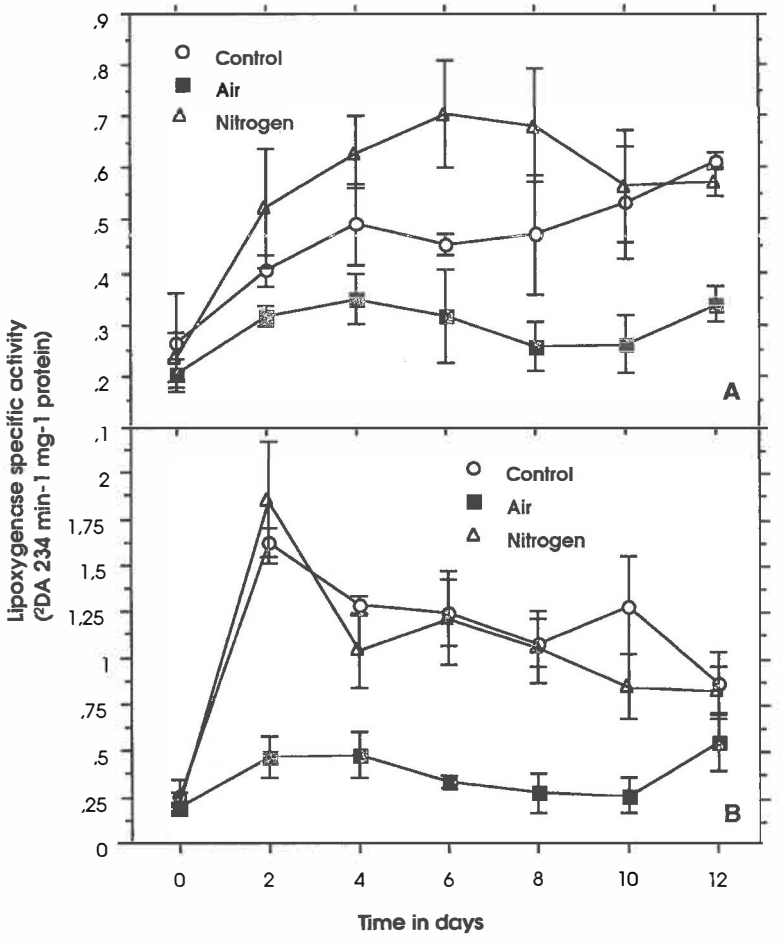

Figure 3 : Variation with time of lipoxygenase activity in *Pythium* F707-noninoculated (A) and -inoculated (B) tomato roots cv. Typico. Values represent the means from three indepedent experiments; bars indicate SD.

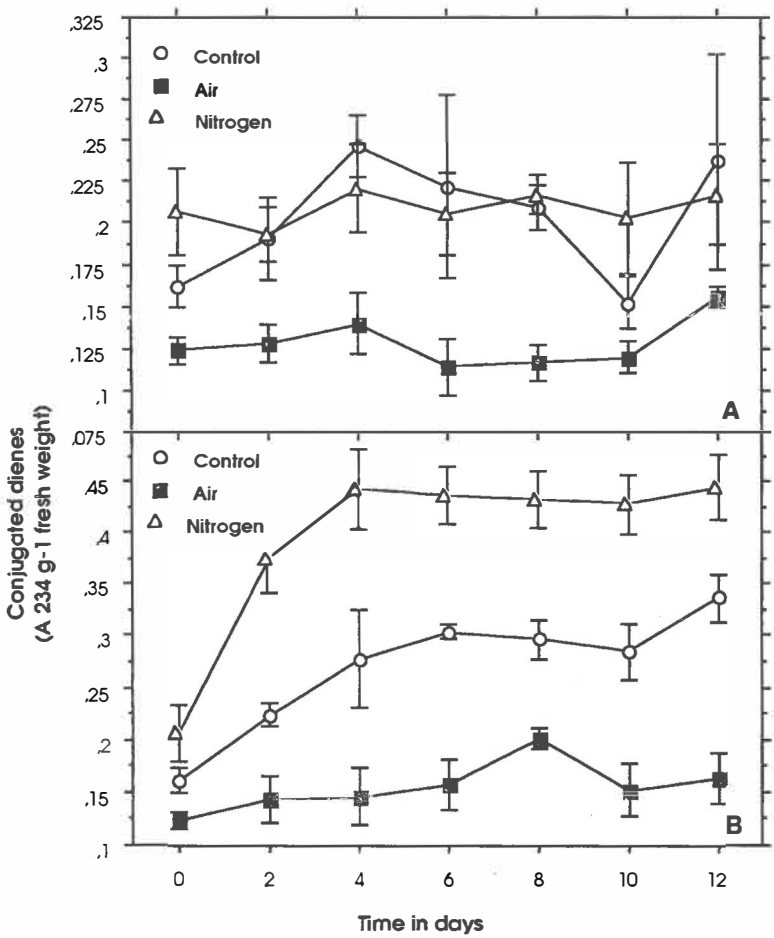

Figure 4 : Variation with time of conjugated dienes in *Pythium* F707-noninoculated (A) and -inoculated (B) tomato roots cv. Typico. Values represent the means from three indepedent experiments; bars indicate SD.

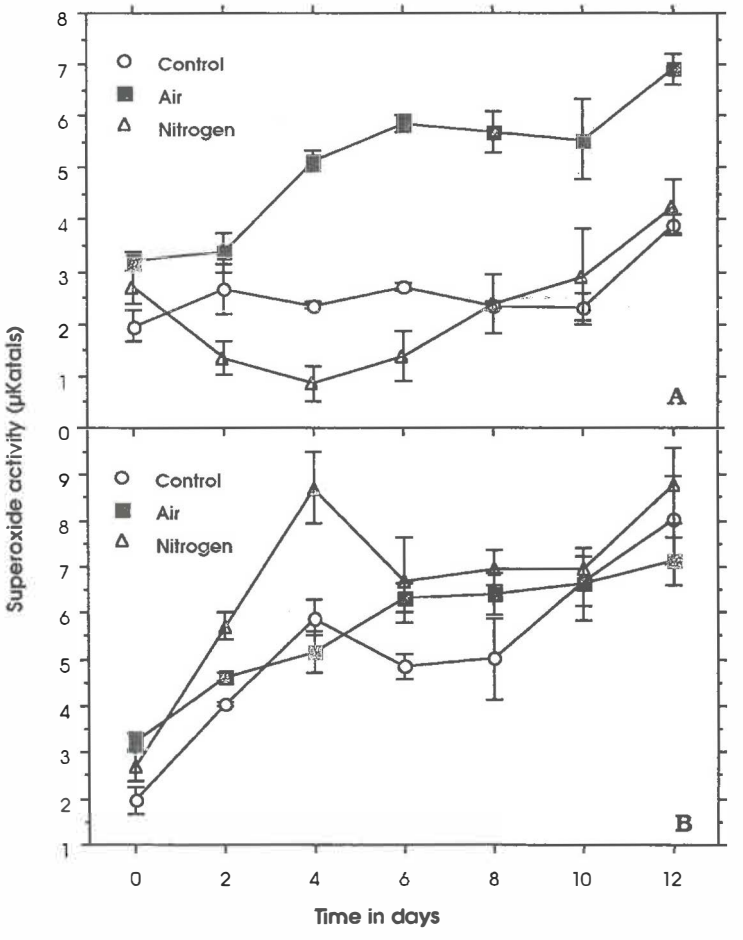

Figure 5 : Variation with time of superoxide dismutase activity in *Pythium* F707-noninoculated (A) and -inoculated (B) tomato roots cv. Typico. Values represent the means from three indepedent experiments; bars indicate SD.

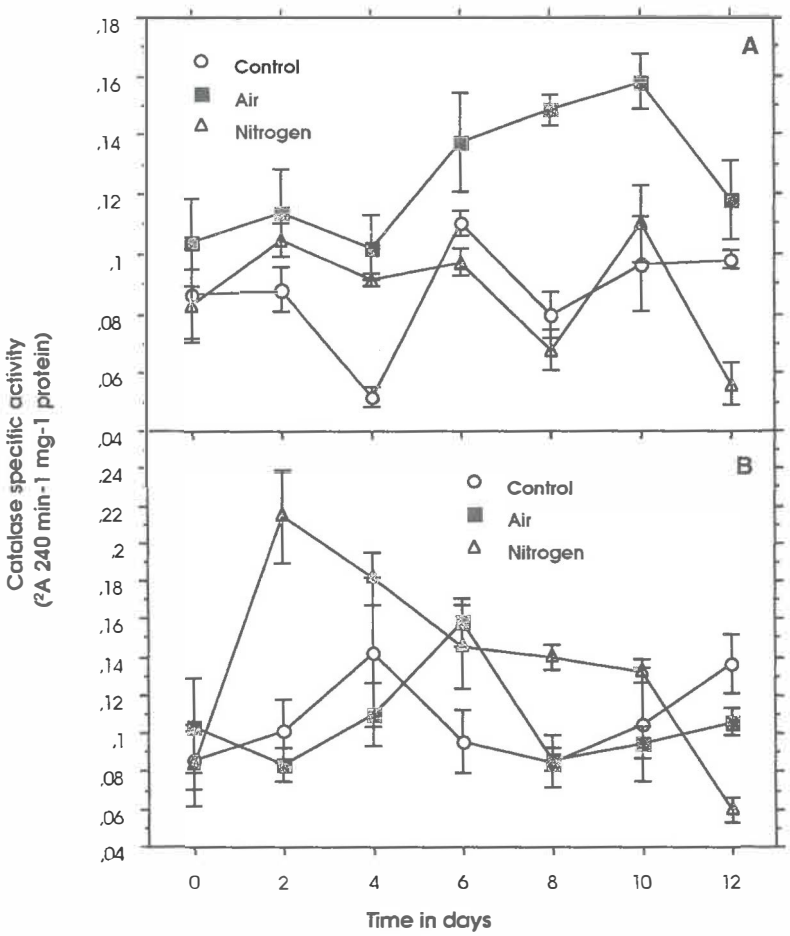

Figure 6 : Variation with time of catalase activity in *Pythium* F707-noninoculated (A) and -inoculated (B) tomato roots cv. Typico. Values represent the means from three indepedent experiments; bars indicate SD.

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COLONIZATION OF TOMATO ROOTS BY *PSEUDOMONAS FLUORESCENS* STRAIN WCS417R AND ITS LPS⁻ MUTANT

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Abstract

Colonization of tomato roots by *Pseudomonas fluorescens* strain WCS417r and a mutant of this strain, lacking the O-antigenic side chain of its cell surface lipopolysaccharides (LPS⁻), was studied. Special attention was paid to colonization of the root interior. Estimations of colony forming units showed that strain WCS417r and its LPS⁻ mutant B4 colonized the rhizoplane of tomato grown in rock wool to the same extent, while colonization of the root interior tended to be higher for WCS417r. Light microscopy observations of sections of roots grown on agar confirmed that both strains colonized the root inter- and intracellularly. Colonization of the root interior was mainly observed if the *Pseudomonas* strains were inoculated at sites of lateral root emergence. Again, WCS417r was seen to colonize the root interior, and especially the cortical intercellular spaces, more frequently than its LPS⁻ mutant. These results indicate a possible involvement of the outermembrane lipopolysaccharides in the colonization of the root interior of tomato by *P. fluorescens* WCS417r.

Introduction

Pseudomonas fluorescens strain WCS417r suppresses fusarium wilt in carnation (Van Peer and Schippers, 1992), radish (Leeman *et al.*, 1995) and tomato (Duijff *et al.*, 1995a) grown on rock wool. This suppression is partly related to the induction of disease resistance by WCS417r. The involvement of the O-antigenic side chain of the outermembrane lipopolysaccharides (LPSs) of strain WCS417r in the induction of resistance against fusarium wilt has been demonstrated in carnation (Van Peer and Schippers, 1992) and in radish (Leeman *et al.*, 1995). Close contact between plant root cells and the resistance-inducing strain might be required for induction of disease resistance. Therefore, the aim of the present study was to characterize the colonization of tomato roots by *Pseudomonas fluorescens* WCS417r and its LPS⁻ mutant, with special attention to the colonization of the root interior.

Material and Methods

Microorganisms and plants

The rifampicin-resistant *Pseudomonas fluorescens* strain WCS417r (Duijff *et al.*, 1995b) and its spontaneous phage-resistant mutant B4, lacking the O-antigenic side chain of the outermembrane LPSs (Leeman *et al.*, 1995), were used as bacterial strains. The test plant was tomato cultivar Monalbo, previously used to demonstrate induction of resistance against fusarium wilt by WCS417r (Duijff *et al.*, 1995a).

Colonization assay with roots grown in rock wool

Tomato seeds were sown in 10 ml rock wool granulate plugs and plants grown in a constant environment with a photoperiod of 16 h at 25°C and a dark period at 22°C. Plants were watered with a standard tomato nutrient solution (TNS) of pH 5.5, resulting in a pH of 5.8 in the rock wool granulate. Ten days after sowing, plants were inoculated with 2 ml of a washed suspension of 10^8 cells ml^{-1} of WCS417r or B4, or with 0.01 M $MgSO_4$ (control), used to prepare the bacterial suspensions.

Ten days after inoculation with the bacteria, roots were sampled to assess colonization of the rhizoplane and the root interior. To determine colonization of the rhizoplane, approximately 200 mg of roots per plant were shaken (Vortex) for 30 s in sterile glass test tubes containing 5 ml 0.01 M $MgSO_4$ and 1 g of glass beads (0.18 mm diameter). The suspensions were diluted and plated on KB^+rif agar (Duijff *et al..*, 1995b) to estimate the number of colony forming units (CFUs) of strains WCS417r and B4, on KB^+ agar (Geels and Schippers, 1983) to estimate the number of CFUs of the total *Pseudomonas* population, and on Bacto peptone (BP) agar supplemented with 100 ppm cycloheximide (BP^+) to estimate the number of CFUs of the total aerobic bacterial population.

To determine colonization of the root interior, approximately 200 mg of roots per plant were immersed for 30 s in 3% NaOCl and rinsed 4 times in sterile distilled water. About 50 mg of disinfected roots were spread gently on BP^+, KB^+ and KB^+rif agar to check the external disinfection. Another 150 mg of the disinfected roots were macerated in 2 ml of sterile 0.01 M $MgSO_4$ with a sterile pestle and mortar and a sample of 200 µl of the macerate was spread on BP^+, KB^+ and KB^+rif agar with sterile glass beads (∅ 4 mm). After incubation for 48 h at 27°C external disinfection of the roots was checked and the number of CFUs from the root macerates determined for interior root colonization.

Root colonization assay for light microscopy

Tomato seedlings were grown on 1% agar prepared with filter-sterilized (TNS) (pH 5.8). Tomato seeds were disinfected in 5% NaOCl for 20 min, rinsed 4 times in sterile water and fixed with a drop of water agar (1%) on the edge of a TNS agar plate (∅ 10 cm). Agar plates were placed at an angle of 60° and incubated in the dark for 4 days at 25°C. Roots were then spot-inoculated with 1 µl of a suspension of 10^6 cells of WCS417r or its LPS^- mutant B4 at the apex, in the differentiation zone (root hair zone) or at the site of lateral root emergence. After incubation for another 4 days at 25°C, root pieces of about 5 mm long were sampled from the sites of inoculation or from the newly formed root apices, and were embedded in LR White resin as described by Gianinazzi and Gianinazzi-Pearson (1992). Semithin cross sections (0.5 µm) of samples were stained with toluidine blue for light microscopy.

Data analysis

Data were analysed by analysis of variance, followed by mean separation with least significant difference (LSD) (Sokal and Rohlf, 1981).

Results and Discussion

Ten days after treatment with the *Pseudomonas* strain WCS417r or its LPS^- derivative B4, numbers of CFUs on the rhizoplane of tomato did not differ between the two strains (Table 1). However, colonization of the root interior by WCS417r tended to be higher than that by B4. These results

were also reflected in the total *Pseudomonas* population (Table 1). While inoculation with either WCS417r or B4 significantly increased the total *Pseudomonas* population of the rhizoplane to the same level, only inoculation with WCS417r significantly increased the total *Pseudomonas* population of the root interior. This observation confirms Van Peer and Schippers' (1989) previous report of effective colonization of the root interior by strain WCS417r in tomato grown in hydroponic culture.

Observations of semithin root sections allowed more detailed assessment of root colonization by WCS417r and B4. Colonization of the root interior was most abundant if the bacteria were applied where lateral roots emerged (Table 2). These results suggest that the rupture between the cortex of the main root and emerging lateral roots (Charlton, 1991) provide sites for microorganisms to enter the root interior.

Treatment	log(CFUs[1]+1/g root fresh wt)		
	total aerobic bacteria	total pseudomonads	rifampicin-resistant
Rhizoplane :			
Control	7.98 a[2]	6.37 a	< dl3
WCS417R	7.83 a	6.99 b	6.86 a
B4	7.92a	6.92 b	6.75 a
Root interior :			
Control	3.96 p	1.82 p	< dl
WCS417r	3.95 p	2.81 q	2.80 p
B4	3.96 p	2.54 pq	2.00 p

[1] CFUs = colony forming units.
[2] Values within the same column and with different letters differ significantly ($P = 0.05$, n = 6 for rhizoplane, n = 16 for root interior).
[3] < dl = below detection limit.

Table 1 : Colonization of the rhizoplane and root interior of tomato cv. Monalbo in rock wool by *Pseudomonas fluorescens* WCS417r and its LPS⁻ mutant B4 at 10 days after treatment of the roots with the pseudomonads.

Inoculation site	Colonized cells / section	Colonized intercellular spaces / section
Apex	2.0 a[1]	2.2 a
Differentiation zone	2.0 a	1.0 a
Lateral root emergence	5.5 b	3.2 a

[1] Values within the same column and with a different letter differ significantly ($P = 0.1$, n = 6).

Table 2 : Colonization of the root interior of tomato cv. Monalbo by *Pseudomonas fluorescens* WCS417r inoculated on different parts of the root. Samples were taken from the sites of inoculation.

Light microscopy showed that WCS417r colonizes the root interior more frequently than its LPS⁻ mutant B4 (Table 3), confirming estimations of CFUs from plants grown in rock wool (Table 1). However, when the distribution of the two strains in the root tissues was compared, this difference was only significant for the colonization of the intercellular spaces of the root cortex (Table 3). De Weger *et al.* (1988) showed that LPS⁻ mutants of fluorescent pseudomonads were also affected in their ability to colonize the lower parts of potato roots. They also reported an altered cell surface hydrophobicity for these mutants in comparison with their parental strains. A

change in cell surface hydrophobicity might explain the less frequent colonization of intercellular spaces by the LPS$^-$ mutant B4 in comparison with WCS417r. For example, increased surface hydrophobicity of the LPS$^-$ mutant could hamper passive entrance into the root interior by means of water flow through rupture sites where lateral roots emerge. However, this hypothesis needs further investigation.

Treatment	Colonized cells / section	Colonized intercellular spaces / section
WCS417r	5.3 a[1]	1.9 b
B4 (LPS)	3.1 a	0.4 a

[1]Values within the same column and with a different letter differ significantly ($P = 0.1$, n = 20).

Table 3 : Colonization of the root interior of tomato cv. Monalbo by *Pseudomonas fluorescens* WCS417r and its LPS$^-$ mutant B4 estimated from grouped samples taken from the sites of inoculation and the root apices.

In conclusion, *P. fluorescens* WCS417r which is effective in inducing resistance against fusarium wilt in tomato grown on rock wool (Duijff *et al.*, 1995a) colonizes the root interior of tomato grown on rock wool and on agar. Its LPS$^-$ mutant B4 which has a reduced capacity to induce resistance against fusarium wilt in various plant species (Van Peer and Schippers, 1992; Leeman *et al.*, 1995), also seems to be affected in its colonization of the root interior of tomato. We therefore hypothesize that induction of disease resistance by strain WCS417r might be related to efficient colonization of the root interior by this strain. Wether the LPS$^-$ mutant B4 is also affected in its ability to induce resistance against fusarium wilt in tomato remains to be demonstrated.

Acknowledgements

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EFFECT OF FLUORESCENT PSEUDOMONADS ON COLONIZATION OF CUCUMBER ROOT BY *PYTHIUM APHANIDERMATUM*

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Abstract

Pythium aphanidermatum is one of the most damaging fungus in soilless culture of cucumber. It induces root-rot responsible for important yield reduction, even when symptoms are not obvious.

Some strains of fluorescent pseudomonads have been selected for their ability to reduce disease incidence under experimental conditions. To study the mode of action of these bacterial isolates, root colonization by *Pythium aphanidermatum* has been assessed in absence and in presence of either fluorescent pseudomonad strain CH31 or CH1.

Quantification of root colonization of cucumber with *P. aphanidermatum* OP4 in absence and in presence of fluorescent pseudomonad strains was performed by ACP-ELISA. The antiserum used was obtained from rabbit immunized with zoospore cysts. Lack of cross-reaction of this antiserum with non infested roots of cucumber and with fluorescent pseudomonads CH31 and CH1 was checked. Application of the biotest previously developed enabled us to assess the ability of the two strains of fluorescent pseudomonad to suppress root rot due to *P. aphanidermatum* OP4.

Fluorescent pseudomonad CH31 both reduced significantly the root colonization of cucumber with *P. aphanidermatum* OP4 and the severity of Pythium root rot at all the bacterial densities tested. In contrast, fluorescent pseudomonad CH1 had no effect neither on root colonization nor on disease severity.

Introduction

Pythium spp. have been described to cause root rot in several crops in soilless culture, including cucumber (Evans and Adas, 1977 ; Jenkins and Averre, 1983 ; Favrin *et al.* 1988 ; Stanghellini *et al.*, 1988), geranium (Chagnon and Belanger, 1991), lettuce (Funck-Jensen and Hockenhull, 1983 ; Stanghellini and Kronland, 1986), spinach (Bates and Stanghellini. 1984 ; Gold and Stanghellini, 1985), tulip (Weststeijn, 1990) and tomato (Jenkins and Averre, 1983 ; Vanachter *et al.*, 1983). More recently, we demonstrated that infestation of rockwool with *P. aphanidermatum* strain OP4 caused yield losses of cucumber under commercial-like conditions (Moulin *et al.*, 1994a). The use of microbial antagonists such as fluorescent pseudomonads was shown to be an interesting method of controlling this disease on wheat (Weller and Cook, 1986), tulip (Weststeijn, 1990) and cucumber (Paulitz *et al.*, 1992 ; Moulin *et al.*, 1994b). We showed that application of the strain CH31 of fluorescent pseudomonad can avoid yield losses due to *P. aphanidermatum* strain OP4 (Moulin *et al.*, 1994b).

The importance of root rot diseases depends on the number of root infections and the level of root colonization (Last *et al.*, 1969). *Pythium* spp. produce zoospores that are formed in aquatic environments such as soilless cultures (Stanghellini and Rasmussen, 1994). In presence of water, these spores swim and adhere to the roots, encyst, and produce germ tubes that can penetrate the roots (Hendrix and Campbell, 1973 ; Jones *et al.*, 1991). The objective of our study was to evaluate if the disease suppressing-strain CH31 of fluorescent pseudomonad can

reduce the level of root colonization by *P. aphanidermatum*. An ELISA technic was developped to quantify the fungus on and in roots, in the presence and in the absence of the bacteria.

Materials and Methods

Microorganisms and cultural conditions

Cultural conditions for maintenance of *Pythium aphanidermatum* strain OP4 and for zoospores production were as previously described (Moulin *et al.*, 1994a). Zoospore densities were determined under a microscope using a haemacytometer, and adjusted with water. Strain CH31 of fluorescent pseudomonad was previously shown to reduce disease severity due to *P. aphanidermatum* OP4 whereas strain CH1 does not (Moulin *et al.*, 1994b). Bacterial media for cultures, storage and inoculum production were as previously mentionned (Moulin *et al.*, 1994b). Bacterial densities were evaluated using a calibration curve assessed by turbidity (600 nm) and adjusted by dilution with water.

Plant growth and inoculation

In all experiments, cucumber plants (*Cucumis sativus* L., cv. corona) were used. For biological control assays, plants were grown in rockwool plugs (Rockwool-Grodan Roemond, The Netherlands) as previously described (Moulin *et al.*, 1994a). Plants were infested with *P. aphanidermatum* OP4 (1×10^3 zoospores/plant) 7 days after sowing ; fluorescent pseudomonads CH31 and CH1 were introduced at different doses (1×10^5, 1×10^7, 1×10^9 colony forming unit per plant) twice, 2 days before and 2 days after the fungal infestation as previously described (Moulin *et al.*, 1994b). There were 1 plant per rockwool plug and 3 replicates of 12 plants per treatment.

For root colonization assays, plants were grown in nutrient solution under gnotobiotic conditions.The nutrient solution was sterilized by autoclaving (120°C, 20'). Sterilized seeds (NaClO 1,25%, 30' ; 3 washes with sterile water) were germinated on malt (15g/l) agar at 25°C during 48h and seedlings were transferred to 30 ml pots in a system similar to that used by Eparvier and Alabouvette (1994). Plants were infested with *P. aphanidermatum* OP4 (1×10^3 zoospores/plant) 7 days after sowing and fluorescent pseudomonads were introduced (1×10^5, 1×10^7, 1×10^9 cfu per plant) 2 days before the fungal infestation. There were 5 replicates of 3 plants per treatment.

Antigen and antiserum preparation

Zoospore cysts from OP4 strain were used as immunization antigens. They were produced as previously described (Moulin *et al.*, 1994a) and the suspension was concentrated 25 times by centrifugations (13 000 x g, 15 min), collected and stored at -20°C. The protein concentration was determined by the method of Bradford (1976) with the Biorad protein assay (Biorad Laboratories, Richmond, CA, USA). Rabbits received one intravenous injection of 1 ml of antigen (250 µg of proteins) on days 0, 3 and 7 and two intramuscular injections of 1 ml of antigens mixed with 1 ml of Freund's complete and incomplete adjuvant, respectively, on days 18 and 28. Booster injections were then given intramuscularly every 6 weeks with Freund's incomplete adjuvant. Blood was collected from the ear vein, allowed to coagulate for 2h, and the serum was separated by centrifugation (10 000 x g, 10 min), collected and stored in 1.5 ml aliquots at -20°C.

Root systems were dried on a filter paper, weighed, ground in a mortar with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4) in a 1:10 w/v ratio and mixed for

2 min with a high-speed homogenizer (Ultraturax, Janke & Kuntel IKA Werk, Germany). the suspensions were then centrifugated (10 000 x g, 15 min) and the supernatants stored at -20°C.

ACP-ELISA procedure

Suspensions were diluted in PBS to obtain 3 µg protein/ml. Antigens (100 µl/well, 3 wells/sample) were coated into the _microtiter plates overnight at 4°C. Unoccupied binding sites were saturated by the addition of Tris-casein buffer (10mM Tris, 0.5% casein, 0.9% NaCl, pH = 7.6 ; 200 µl/well). Antisera (1/10^4 in Tris-casein buffer, 100 µl/well) and alkaline phosphatase conjutated to goat and anti-rabbit IgG (1/4000 in the same buffer, 100 µl/well) were then added. Absorbance at 405 nm was recorded after substrate addition (*p*-nitrophenylphosphate, Sigma ; 1 mg/ml in 10% diethanolamine-HCl, pH = 9.8, 0.5 mM $MgCl_2$) on a micro-plate reader (Thermomax, Molecular Devices, Mento Park, CA, USA). Three washings were made with Tris-casein buffer between each of these steps (2h).

Statistical analysis

All experiments were duplicated twice. Data were analysed by ANOVA and, according to the experiments, means were separated by Duncan's multiple range test or by Student t-test.

Results

Antiserum response and specificity

	Absorbance (405 nm)			
	(A) Fungal extracts		(B) Root extracts	
Antiserum dilutions	Mycelium	Zoospores	Infected plants	Control
1 x 10^{-4}	2.35 (0.16) [a]	2.29 (0.17) [a]	1.80 (0.11) [a]	0.10 (0.00) [b]
5 x 10^{-4}	0.82 (0.06) [b]	0.92 (0.07) [b]	0.47 (0.01) [a]	0.03 (0.00) [b]
1 x 10^{-5}	0.56 (0.06) [c]	0.59 (0.05) [c]	0.20 (0.00) [a]	0.02 (0.00) [b]

Table 1: Response (absorbance, 405 nm) of antiserum by indirect ACP-ELISA using soluble proteins from mycelium, zoospore cysts of *P. aphanidermatum* OP4 (A), extracts of healthy cucumber roots and roots infected by *P. aphanidermatum* OP4 (B), as coating antigens (3 mg proteins/ml). For each dilution, means (standard error) with the same letter are not significantly different at P = 0.05 according to Student t-test.

The antiserum used showed a similar activity against homologous (zoospore) and heterologous (mycelium) antigens (Table 1A). It reacted strongly and with the same intensity with antigens from mycelium of *P. aphanidermatum* OP4 at different growth stages (Table 2A) and very weakly with antigens from *Pseudomonas* CH31 (Table 2B) and CH1 (not shown). The antiserum also reacted with root systems infested by *P. aphanidermatum* OP4 and weakly with healthy root system (Table 1B).

A	***P. aphanidermatum* OP4**	
Time (days)	Growth (dry weight)	Absorbance (405 nm)
2	0.03	1.78 (0.04) [a]
3	0.15	1.75 (0.08) [a]
4	0.16	1.79 (0.09) [a]
5	0.19	1.79 (0.12) [a]

B	Fluorescent *Pseudomonas* CH31	
Time (hours)	Growth (O.D., 600 nm)	Absorbance (405 nm)
9.5	0.57	0.03 (0.00) [a]
13.5	1.01	0.02 (0.00) [a]
17.5	1.18	0.03 (0.00) [a]
33.5	1.25	0.03 (0.00) [a]

Table 2: Response (absorbance, 405 nm) of antiserum by indirect ACP-ELISA during the growth of *P. aphanidermatum* OP4 (A) and fluorescent *Pseudomonas* CH31 (B) using soluble proteins from mycelium and bacteria respectively as coating antigens (3 mg proteins/ml). Means (standard error) with the same letter are not significantly different at P = 0.05 according to Duncan's multiple-range test.

Effect of fluorescent pseudomonads on root colonization by P. aphanidermatum *OP4*

The kinetic of root colonization of cucumber by *P. aphanidermatum* OP4 was determined during the 6 days following the root infestation (Table 3). When inoculated alone, *P. aphanidermatum* OP4 was detected in the roots as early as 1 day after its infestation. Fungal colonization increased then sharply between the first and the second day and more slowly after. In the presence of fluorescent pseudomonad CH31, root colonization by *P. aphanidermatum* OP4 was delayed and, 2 days after fungal infestation, it was 8.7 times lower than in the absence of the bacteria. Six days after infestation, the bacteria still reduced root colonization by *P. aphanidermatum*.

Days post-infection	Absorbance (405 nm)		
	OP4	OP4 + CH31	Control
1	0.42 (0.25) [a]	0.10 (0.02) [b]	-
2	2.00 (0.10) [a]	0.23 (0.14) [b]	0.10 (0.05) [b]
3	2.25 (0.15) [a]	0.92 (0.24) [b]	-
5	2.54 (0.26) [a]	1.62 (0.28) [b]	-
6	2.78 (0.30) [a]	2.28 (0.18) [b]	0.11 (0.00) [c]

Table 3: Root colonization of cucumber plants by *P. aphanidermatum* OP4 as determined by ACP-ELISA (Absorbance, 405 nm). The fungus was introduced alone at 1 x 10^3 zoospores/plant or in association with fluorescent pseudomonad CH31 introduced 2 days before *Pythium* at 1 x 10^9 cfu/plant. At each date, means (standard error) with the same letter are not significantly different at P = 0.05 according to Duncan's multiple-range test.

The effect of inoculum density of fluorescent pseudomonad CH31 is shown in table 4. Whatever the 3 doses tested (1 x 10^5, 1 x 10^7 or 1 x 10^9 cfu/plant), the bacteria reduced the root colonization by the fungus, to a level non-significantly different from the non-infested control. In contrast, fluorescent pseudomonad CH1 did not decrease the fungal root colonization even at the highest bacterial density tested (1 x 10^9 cfu/plant, not shown).

Treatment	Absorbance (405 nm)
OP4	0.82 (0.16) [a]
OP4 + CH31 (10^9/pl)	0.19 (0.13) [b]
OP4 + CH31 (10^7/pl)	0.27 (0.15) [b]
OP4 + CH31 (10^5/pl)	0.24 (0.18) [b]
Control	0.07 (0.01) [b]

Table 4: Root colonization of cucumber plants by *P. aphanidermatum* OP4, introduced alone or in association with fluorescent pseudomonad CH31 (1 x 10^5, 1 x 10^7 or 1 x 10^9 cfu/plant). For details, see

table 3. Means (standard error) with the same letter are not significantly different at P = 0.05 according to Duncan's multiple-range test.

Effect of fluorescent pseudomonads on severity of root rot caused by Pythium aphanidermatum OP4

The percentage of dead cucumber plants due to *P. aphanidermatum* was recorded daily but only data from the last day (13 days after *Pythium* infestation) are presented (Figure 1). Fluorescent pseudomonad CH31, which was shown to reduce fungal root colonization, also reduced root rot at all densities tested. In contrast, fluorescent pseudomonad CH1 did not suppress root rot at any of the bacterial densities tested (not shown).

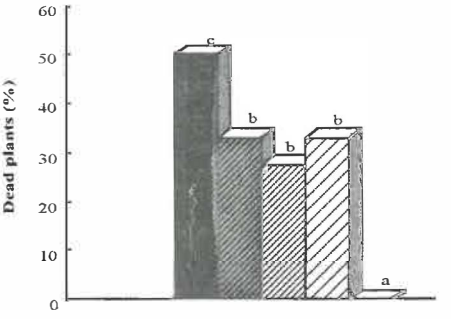

Figure 1: Percentage of dead cucumber plants due to *P. aphanidermatum* OP4 when inoculated alone (■) or in association with fluorescent pseudomonad CH31 (■ :1 x 10^5, ▩ :1 x 10^7 or ▨ : 1 x 10^9 cfu/plant). Means with the same letter are not significantly different at P = 0.1 according to Student t-test. □ : Control plants

Discussion

We developed a method that allows accurate quantification of cucumber root colonization by *Pythium aphanidermatum* based on ACP-ELISA. Several reports mentionned the potentialities of this method to quantify biomass of fungal species such as *Gaeumannomyces* (El Nashaar *et al.*, 1986), *Phytophthora* (Mohan, 1988 ; Harrison *et al.*, 1990) , *Sclerospora* (Kumar *et al*, 1993) or *Fusarium* (Eparvier and Alabouvette, 1994). For *Pythium* spp., Rafin (*et al.*, 1994) described the use of antibodies for detection but not for quantification of this pathogen. Estrada-Garcia (*et al.*, 1989) produced monoclonal antibodies specific of the species *P. aphanidermatum*, but which only recognized transient structures of the fungus such as flagellum and cysts. As both zospores and mycelium are implicated in the root colonization process, we wanted to produce an antiserum that reacted similarly to both fungal structures. The antiserum retained, was obtained after immunization of a rabbit with zoospores of *P. aphanidermatum* OP4. Absence of cross-reaction of this antiserum with fluorescent pseudomonads and non-infected roots was also checked.

Suppression of Pythium root rot by fluorescent pseudomonads was associated with reduced root colonization by *P. aphanidermatum*. Strain CH31 both decreased root colonization of the fungus and root rot severity, whereas strain CH1 neither decreased root colonization nor disease severity. Discrimination of fungal growth at the root surface or within the root tissue was not possible. Our results therefore did not allow us to determine whether the

bacterial suppression of the fungus was expressed during the saprophytic or during the parasitic stage of the fungal growth. However, the method described could be helpful in gaining knowledge of the traits and metabolites of beneficial fluorescent pseudomonads by allowing assessment of the effect of these bacterial characteristics on the level of root colonization by *P. aphanidermatum* OP4.

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Modes of action of biocontrol agents

MECHANISMS OF DISEASE SUPPRESSION IN SOILLESS CULTURES

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Summary

General aspects on mechanisms of disease suppression, with emphasis on soilless culture systems, are discussed. Mechanisms of disease suppression are illustrated with two major fungal diseases in soilless cultures, i.e. Fusarium wilt and root rot caused by *Pythium*. In addition to competition for nutrients and space in rhizosphere or at infection sites, mechanisms active in the plant (i.e. induced resistance, competition in the vessels) are important for an effective biocontrol of a vascular disease like Fusarium wilt, since the pathogen will be present within the plant at a certain stage. To control *Pythium*, which is extremely fast in colonizing and infecting roots, synchronization of the antagonist to the pathogen is considered to be a key factor of success. Other biocontrol mechanisms are suggested.

Introduction

Soilless cultures offer excellent possibilities for the application of biological control agents. It is easier to introduce and establish beneficial microorganisms in substrate systems which commence with a biological vacuum and a limited amount of substrate, compared to a microbiologically well buffered system such as soil. Disease suppression can be obtained by stimulating the general microflora, or by introducing specific antagonists. The success of an antagonist depends on its survival and activity in the substrate system, as well as on the mode(s) of action by which the antagonist suppresses a specific disease. Antagonist and pathogen must be synchronised in time and space. Furthermore, soilless cultures have specific environmental conditions, which can be an advantage for the establishment of specific microorganisms in these systems as compared to a field soil.

In rare cases biocontrol might prove effective without much basic knowledge about the mechanisms. However, if biocontrol is inconsistent or not effective enough for practical application, knowledge about the mechanisms of disease suppression are helpful to improve the system. Time and place of inoculation, and the density of the inoculum can then be optimized. Also for the improvement or selection of more effective strains, knowledge of the mode of action of the antagonist is necessary to develop criteria for selection or (genetic) modification.

Several modes of action of antagonists have been identified. An antagonist may suppress a disease by interference with: (A) the pathogen, by parasitism or by the production of antimicrobial compounds, (B) the plant-pathogen interaction, e.g. through competition for exudates or infection sites, or through other disturbing factors, and (C) the plant, e.g. by induced or acquired resistance or by stimulating root growth (Fig. 1). It is also possible that the antagonist suppresses the pathogen by changing the environmental conditions (D), e.g. nutrient availability. It is difficult, however, to make sharp distinctions between the different modes of action, and effective antagonists might act through several of them.

Many good reviews on this subject are available (Deacon, 1991; Lemanceau and Alabouvette, 1993; Lewis *et al.*, 1989; Whipps, 1992). In the present paper, mechanisms of disease suppression will be illustrated with two important fungal diseases in soilless cultures, i.e. Fusarium wilt and root rot caused by *Pythium*. Fusarium wilt is a vascular disease which causes serious problems in several crops grown in soil or in soilless cultures. *Pythium* root rot is a major threat in

soilless cultures. These two pathogens are different in infection process, disease expression, competitive ability, etc. Therefore, different biocontrol strategies are proposed to be successful in controlling these diseases.

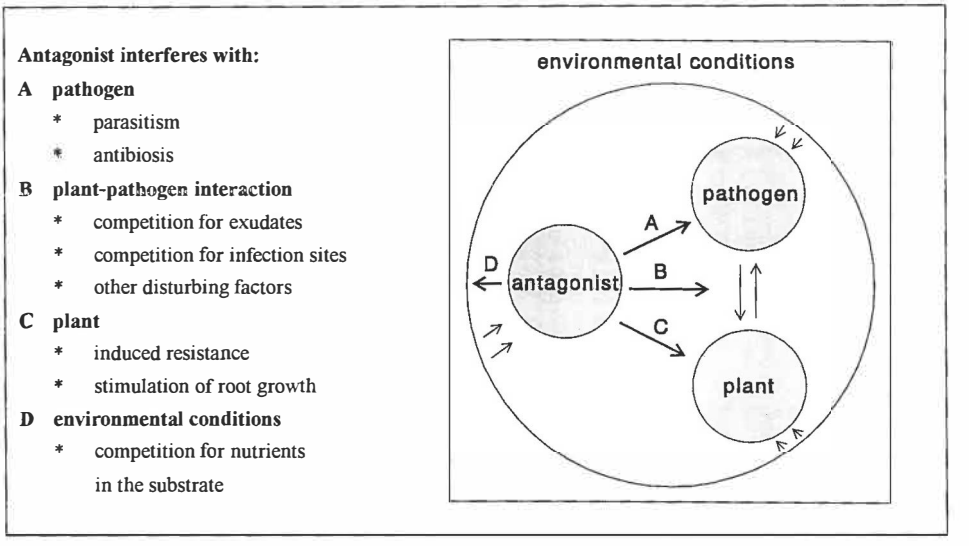

Figure 1. Different types of antagonism

Biological control of Fusarium wilt

Nonpathogenic or less virulent isolates of *Fusarium* spp. are found to be effective antagonists of Fusarium wilt in several crops grown in soil or in soilless cultures: e.g. carnation (Garibaldi *et al.*, 1987; Mattusch, 1990; Postma and Rattink, 1992; Rattink, 1987; Tramier *et al.*, 1987), celery (Schneider, 1984), cucumber (Mandeel and Baker, 1991; Paulitz *et al.*, 1987), cyclamen (Mattusch, 1990), flax (Lemanceau and Alabouvette, 1991; Tamietti and Pramotton, 1987), muskmelon (Mas and Molot, 1977; Meyer and Maraite, 1971; Rouxel *et al.*, 1979), sweet potato (Ogawa and Komada, 1984), tomato (Tamietti and Pramotton, 1987; Alabouvette *et al.*, 1987; Lemanceau and Alabouvette, 1991; Louter and Edgington, 1990), and watermelon (Martyn *et al.*, 1991). Impressive and reproducible biocontrol effects have been found after the application of nonpathogenic *Fusarium* isolates. For example, the application of the nonpathogenic isolate of *F. oxysporum*, 618-12, to soil, rockwool or hydroponics prior to addition of *F. o.* f.sp. *dianthi*, resulted in several experiments in a reduction of the number of wilted carnation plants with 80 % compared to the treatment with the pathogen only (Postma and Rattink, 1992; Postma and Luttikholt, 1993; Rattink, 1987; unpublished data). Best control effects with nonpathogenic *Fusarium* isolates are obtained if the isolate is added to the substrate previous to the pathogen. Moreover, the effect of nonpathogenic *Fusarium* isolates is concentration dependent (Alabouvette and Couteaudier, 1992; Mandeel and Baker, 1991; Rattink, 1987). In general, the optimal ratio between the number of propagules of the nonpathogenic and pathogenic isolate is 100 or more for an effective control.

A wide range of mechanisms has been described for nonpathogenic *Fusarium* isolates. Competition for nutrients in substrate, soil or rhizosphere and competition for infection sites have been suggested as important biocontrol mechanisms of antagonistic *Fusarium* isolates (Alabouvette

and Couteaudier, 1992; Garibaldi *et al.*, 1991; Lemanceau *et al.*, 1992; Mandeel and Baker, 1991; Schneider, 1984). Interesting are the results where the effect of the nonpathogenic *Fusarium* isolate Fo47 was improved by co-inoculation with a *Pseudomonas* isolate C7 (Lemanceau and Alabouvette, 1991). Both organisms were proposed to control *F. o.* f.sp. *radicis-lycopersici* by a different mechanism, respectively competition for nutrients (carbon) and for iron (Lemanceau, 1989).

Induced resistance, which can be invoked at the location where the challenger is present or at a remote distance from the challenger (i.e. localized or systemically induced resistance), can give an additional biocontrol effect. Several studies on nonpathogenic *Fusarium* deals with 'cross protection'. This is confusing, since cross protection can be the result of competition, antibiosis, parasitism or induced resistance *per se*. Induced resistance is often suggested as a mechanism, without having any serious indication about the mode of action. In fact, there are only few studies in which it is proven that nonpathogenic *Fusarium* isolates can induce resistance. Only if spatially separated inoculations have been made, or if host-defence mechanisms have been studied in detail, induced resistance can be characterized as the mode of action. Examples of systemic resistance against Fusarium wilt induced by *Fusarium* isolates are suppression of *F. o.* f.sp. *lycopersici* in tomato by *F. o.* f.sp. *dianthi* and nonpathogenic *Fusarium* isolates (Kroon *et al.*, 1991; Olivain *et al.*, 1994) and *F. o.* f.sp. *cucumerinum* in cucumber by a nonpathogenic *F. oxysporum* isolate (Mandeel and Baker, 1991). These results were obtained in split-root systems.

Systemically induced resistance could not be identified as an important mechanism in the control of *F. o.* f.sp. *dianthi* in carnation by the nonpathogenic isolate 618-12 (Postma and Luttikholt, submitted; Postma and Rattink, 1992). A split-root system as well as separated inoculations of stem and soil were tested. Although systemically induced resistance was not identified to be of significant importance, locally induced resistance could still be involved.

Presence of saprophytic or nonpathogenic *formae speciales* of *F. oxysporum* within intact roots or stems has been described for several hosts: cucumber (Mandeel and Baker, 1991), watermelon (Martyn *et al.*, 1991), tomato (Matta, 1989), and celery (Schneider, 1984). This means that, although rarely mentioned in literature, competition for nutrients or space in the stem between pathogen and antagonist is a mechanism by which nonpathogenic *Fusarium* isolates might control Fusarium wilt. The nonpathogenic *Fusarium* isolate 618-12 was also frequently present in carnation stems after soil applications (i.e. in 80-90% of the plants) (Postma and Luttikholt, 1993; Postma and Rattink, 1992). Moreover, the potential of 618-12 to control Fusarium wilt within carnation stems was shown by inoculating both antagonist and pathogen simultaneously in the stem (Postma and Luttikholt, submitted). Using this inoculation procedure, mechanisms in substrate and roots are excluded, and reproducible and significant disease reductions were found (Fig. 2). The phenomenon was shown in three cultivars, and at different inoculum densities. Also some other nonpathogenic *Fusarium* isolates were able to reduce Fusarium wilt after mixed stem inoculations. Since systemically induced resistance was shown to be of minor importance for isolate 618-12, the suppressive effect in the plant can be due to localized induced resistance or to competition in the stem. A fact in favour of competition is that disease reduction occurred after simultaneous stem inoculation of pathogen and antagonist. For induced resistance, a time interval (usually 2 or 3 days) between inoculations with the inducer and challenger is necessary (Alabouvette and Couteaudier, 1992; Biles and Martyn, 1989; Price and Sackston, 1989). On the other hand, the reaction of the plant tissue around the inoculation site of the nonpathogenic isolate, might be an indication for induced resistance.

The importance of nonpathogenic *Fusarium* in the plant was also shown by Mandeel and Baker (1991). They showed that two nonpathogenic *F. oxysporum* isolates both acted through competition in the rhizosphere, but that the most effective isolate could, in addition, penetrate intact roots and induce systemic resistance in the plant. Presence of the nonpathogenic isolate within the plant was important for biocontrol: in nonwounded roots only isolate C14, which can actively

penetrate the roots, controlled the disease if stems were inoculated with *F. o.* f.sp. *cucumerinum*, whereas both nonpathogenic isolates (C5 and C14) were effective after wounding the roots.

Summarizing, the following mechanisms have been described or suggested for nonpathogenic *Fusarium* isolates to suppress Fusarium wilt:
- competition for nutrients or space in soil or substrate,
- competition for nutrients or space at infection sites,
- competition for nutrients or space in the plant,
- localized induced resistance, and
- systemically induced resistance.

The success of nonpathogenic *Fusarium* isolates in controlling Fusarium wilt can have two reasons: (1) isolates act through several mechanisms, and (2) they share some of the same niches as the pathogen. Nonpathogenic *Fusarium* isolates are highly competitive saprophytes, which can successfully colonize the cortical tissue of roots, and are well adapted for survival in soil (Paulitz *et al.*, 1987).

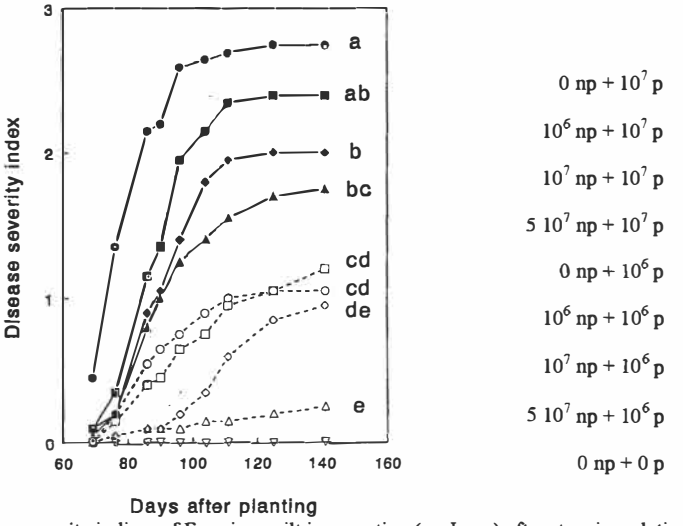

Figure 2. Disease severity indices of Fusarium wilt in carnation (cv. Lena) after stem inoculation with mixtures of a nonpathogenic *Fusarium* isolate 618-12 (np) and a pathogenic isolate of *F. o.* f.sp. *dianthi* (p). Inoculum concentrations are given in number of conidia per ml and disease was expressed by an index ranging from 0 to 5 (healthy-dead). Disease severity levels with the same letter are not significantly different (LSD, $P \leq 0.05$).

Biological control of Pythium root rot

Pathogenic *Pythium* spp. differ in many ways from *F. oxysporum* causing wilt. *Pythium* spp. belong to the Oomycetes. In moist environments zoospores are produced which can actively swim to the roots. *Pythium* is a poor competitor, but shows rapid growth and infection. *Pythium* spp. have a broad host range. All root parts can be attacked, whereas Fusarium wilt infect the vascular tissue.

A lot of research has been directed on biocontrol of *Pythium* spp. of seedlings, i.e. suppression of damping-off. However, *Pythium* can also cause root rot in older plants. *P. aphanidermatum* is a root pathogen which currently causes serious problems in cucumber grown in soilless cultures (e.g. rockwool) (Moulin *et al.*, 1994a; Paternotte, 1992). Antagonistic microorganisms, fungi as well as bacteria, against *P. aphanidermatum* have been described in the literature (Bolton, 1980; Moulin *et al.*, 1994b; Nelson and Craft, 1992; Sivan *et al.*, 1984; Wölk, 1990; Zhou and Paulitz, 1993). However, few data on biocontrol mechanisms are available.

Therefore, this part of the paper will present some hypotheses and speculations on mechanisms that might be important to control *P. aphanidermatum*.

The root pathogen *P. aphanidermatum* produces zoospores which are attracted to roots by a non-specific chemo-attraction through low-molecular-weight root exudates (Estrada-Garcia *et al.*, 1990). The zoospores also show directed movement in electrical fields (electrotaxis towards negative charges) which may act synergistically with chemotaxis in facilitating host location (Morris and Gow, 1993). Zoospores are directed to the zone of elongation, lateral root initials and small lateral roots (Zhou and Paulitz, 1993). Infection of the roots, however, can only occur after encystment of the zoospores. The process of encystment is induced by more or less specific fractions of the root mucilage (Estrada-Garcia *et al.*, 1990). Deacon and Donaldson (1993) presented an overview of specific and non-specific substrates which are important for zoospore attraction, encystment, adhesion and germination. Disturbing the infection process by targeting zoospore taxis and/or germination is therefore proposed as a mechanism to control *Pythium* root rot.

Zhou and Paulitz (1993) demonstrated that the presence of Pseudomonads in root exudates reduced attraction of zoospores and germination of cysts *in vitro* and on roots. They suggest that this was due to utilization of carbon and nitrogen compounds in the root exudates by the bacteria. General competition for root exudates, or bacteria utilising specific compounds that trigger encystment may reduce attraction and encystment of zoospores of *P. aphanidermatum*, so that infection and symptom expression by *P. aphanidermatum* becomes less serious. For this biocontrol strategy, antagonists have to be adapted to the same conditions of the rhizosphere to which the pathogen responds. The antagonists must colonize locations on the root where *Pythium* is expected to encyst and infect (i.e. zone of elongation, lateral root initials and small lateral roots). Colonization of these locations by antagonists can be problematic, since *P. aphanidermatum* is extremely fast in colonizing and infecting the roots. In only a few minutes zoospores are attracted, encyst and attach to for example lateral root initials (see Fig. 3). A crucial question therefore is: do microorganisms exist which colonize these locations which the pathogen prefers?

To overcome this problem of colonization of specific locations by an antagonist, mechanisms of biological control at a remote distance would be a solution. Examples of such mechanisms are (1) systemically induced resistance, (2) production of biosurfactants, and (3) root growth promoting rhizobacteria ('RGPR'). (ad 1) Systemically induced resistance, although documented for Fusarium wilt, has not had much attention in research on *Pythium* root rot. (ad 2) Stanghellini and Tomlinson (1987) found that surfactants could control *Pythium* by lysis of the zoospores. The problem with these surfactants was that they were phytotoxic. The potential of bacteria producing biosurfactants (Bunster *et al.*, 1989) to suppress *P. aphanidermatum* has not yet been evaluated. (ad 3) If the plant can compensate for root loss due to *Pythium*, the plant may become less sensitive to damage caused by *Pythium*. For a series of *Pseudomonas* isolates, a significant correlation was found between root growth of bacteria-treated, pathogen-free plants and bacteria treated, *Pythium*-inoculated plants (Paulitz *et al.*, 1992). In addition, two of the five isolates that suppressed *P. aphanidermatum* in cucumber, stimulated root growth. These results are only indications that root growth promotion might be a way of decreasing *Pythium* problems.

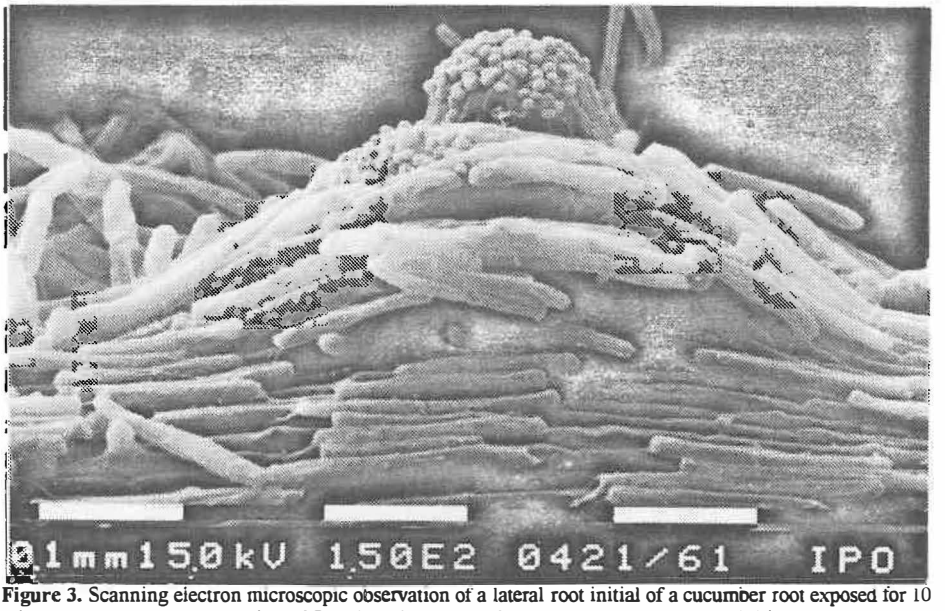

Figure 3. Scanning electron microscopic observation of a lateral root initial of a cucumber root exposed for 10 minutes to a zoospore suspension of *P. aphanidermatum*. Notice cysts crowded on root initial.

Final remarks

Several biocontrol mechanisms have been discussed. The most effective antagonists act through different modes of action, which is shown for the control of Fusarium wilt by Pseudomonads (Duijff *et al.*, 1993) and nonpathogenic *Fusarium* isolates (Mandeel and Baker, 1991). Probably, every additional biocontrol mechanism, also if the effect is small, improves the effect and reliability of the antagonist. In relation to this, it was discussed during this workshop that combinations of antagonists might be additive or even synergistic if they act through different modes of action (see paper of M. de Boer *et al.*).

Antibiosis and (myco)parasitism have not been mentioned as important biocontrol mechanisms of Fusarium wilt and *Pythium* root rot caused by *P. aphanidermatum* in this paper. Some studies showed that nonpathogenic *Fusarium* isolates, although effective antagonists of Fusarium wilt, did not show antibiosis in *in vitro* studies (Louter and Edgington, 1990; Postma and Rattink, 1992). Other studies with effective nonpathogenic *Fusarium* isolates did not present such antibiosis tests. However, even if inhibitory effects *in vitro* are found, it is still not known if antibiosis is an important mechanism *in vivo*. For the control of *Pythium* seed infections by bacteria, Nelson and Craft (1991) showed that this was the result of metabolism of stimulants present in exudates, and not by the production of inhibitors. According to Deacon (1991), antibiosis or contact inhibition *per se* is of no advantage to an antagonist unless the antagonist thereby gains access to some resource; competition can be 'mediated' or 'facilitated' by antibiosis. Also the importance of parasitism *per se* by facultative mycoparasites is unclear, since some of the most aggressive mycoparasites (*Trichoderma* spp., *Gliocladium* spp., *P. oligandrum*) are also strongly competitive for substrates (Deacon, 1991). Effective obligate parasites, such as *Verticillium biguttatum* and *Coniothyrium minitans* which parasitize on *Rhizoctonia solani* and *Sclerotinia sclerotiorum* respectively, have not been described for *Pythium* and *Fusarium*.

During a workshop on soilless cultures, the question has been raised if mechanisms of disease suppression in soilless cultures differ from those in soil. In principle, mechanisms can be the same.

Nevertheless, ecological factors are different, which might influence the relative importance of certain mechanisms of disease suppression. In soilless cultures, the amount of substrate is limited and due to an increased water transport, the rhizosphere effect is expected to be diluted. On the other hand, attachment of antagonists to the root surface (rhizoplane) might be more important then in soil systems. Due to the differences in environmental conditions and horticultural practices between soil and soilless systems, other pathogens will be of importance, and as a consequence other mechanisms of control will play a role.

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PARASITISM OF MACROCONIDIA BY *P. OLIGANDRUM* AND BIOCONTROL OF *FUSARIUM CULMORUM* BY 4 MYCOPARASITIC *PYTHIUM* SPP.

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Summary

The parasitism of *Fusarium culmorum* macroconidia by hyphae of *P. oligandrum* was studied on agar media and on nutrient-free glass slides. Following chance contact by hyphae of *P. oligandrum*, the macroconidia rapidly became devoid of cytoplasm and the mycoparasite produced large numbers of oogonia on the degraded macroconidia. In pot experiments, isolates of 4 mycoparasitic *Pythium* spp.: *P. acanthophoron, P. mycoparasiticum, P. oligandrum* and *P. periplocum,* significantly suppressed disease development in barley seedlings due to *F. culmorum*.

Introduction

Species of mycoparasitic *Pythium* include *P. acanthophoron, P. mycoparasiticum, P. oligandrum* and *P. periplocum*. Particularly *P. oligandrum* (Walther & Gindrat, 1987 and others), but also *P. periplocum* (Hockenhull *et al.*, 1992), have been used in biocontrol experiments. Differences in aggressiveness between species (Laing & Deacon, 1990) and strains (Ribeiro and Butler, 1995) of mycoparasitic *Pythium* have been reported. Studies of mechanisms of mycoparasitism have almost exclusively been devoted to investigation of hyphal interactions (Laing & Deacon, 1991), and to date no work has been published on the parasitism of fungal propagules other than hyphae by mycoparasitic *Pythium* spp.. *Fusarium culmorum*, which ranks as a soil inhabitant, can be a serious pathogen on cereals (Cook, 1968) and some other crops. Macroconidia of *F. culmorum* may infect plants directly or, upon entering the soil, convert to chlamydospores, which are persistant units of survival (Cook, 1968). Therefore, both the survival and inoculum potential of *F. culmorum* can be reduced by destruction of these propagules.

In the current study, the parasitism of *F. culmorum* macroconidia by *P. oligandrum in vitro*, and the capability of 4 mycoparasitic *Pythium* spp. to control disease in barley seedlings caused by *F. culmorum* are investigated. A detailed report of this and allied work is to be published elsewhere (Davanlou *et al.*, submitted).

Materials and methods

Biocontrol of F. culmorum on barley seedlings by four mycoparasitic Pythium spp.

The following species of mycoparasitic *Pythium* were investigated: *P. acanthophoron* (1 isolate), *P. mycoparasiticum* (2 isolates), *P. oligandrum* (2 isolates) and *P. periplocum* (1 isolate). Inocula of the mycoparasites for the biocontrol experiment were produced in flasks containing PCB (liquid from boiled potato (20g) and carrot (20g) made up to 1000 ml). After incubation for 10 days at 25°C, mycelial mats were harvested, homogenized (using a MSE-homogenizer for 30 sec.), resuspended in sterile water and applied at a rate of 0.035 g fresh weight per pot.

Procedures for the production of inoculum of *Fusarium culmorum* (isolate IK 5), inoculation and evaluation are described in Knudsen *et al.* (1992). Briefly, 3 barley seeds were sown in small pots containing moistened sand. One ml of a suspension of macroconidia (10^4 conidia ml^{-1} sterile water) was applied directly to the seeds in each pot. Following simultaneous inoculation with *F. culmorum* and an isolate of mycoparasitic *Pythium*, the pots were incubated at 15°C in cycles of 12 h darkness and 12 h light in a growth chamber. There were 8 replicates in each treatment and 16 replicates in the control. Disease severity was evaluated 19 days after inoculation when each plant in each pot was scored using the scale: 0= healthy, 1= slightly infected plants, 2= severe discolouration of coleoptiles, 3= discolouration of roots and necrosis of coleoptiles and 4= dead seeds (Knudsen *et al.*, 1992). The disease index was computed as the mean of characters of the total number of seedlings in each treatment. The controls were inoculated only with *F. culmorum*.

Mycoparasitism of macroconidia of F. culmorum by P. oligandrum.

A heavy suspension of *F. culmorum* macroconidia ($7x10^6$ conidia/ml) was spread uniformly on Petri plates (9 cm diam.) containing either CMA (Difco Corn Meal Agar, 17g/l) or WA (Difco Bacto-Agar, 10g/l). Two plates of each medium were inoculated with blocks (5mm diam.) of *P. oligandrum* (1 isolate). One block was placed in the centre, and four others were placed equidistantly at the periphery of the agar surface. For comparison, 2 further plates of each medium were inoculated with blocks of an isolate of the common plant pathogenic species, *P. ultimum*, as described above. Controls consisted of Petri plates of each medium inoculated with either macroconidia of *F. culmorum* alone or with blocks of each *Pythium* isolate alone. After 48 h incubation at 25°C, strips of agar (10 mm-width), were cut between the central and peripheral inoculation blocks, stained with Cotton Blue, after which the numbers of oogonia and empty macroconidia in eighteen random fields (area of each field= 2.5 mm^2) were counted under the microscope.

For the microscopic study of parasitism, sterile glass slides were co-inoculated with a heavy suspension of *F. culmorum* macroconidia and blocks (0.9 cm) of *P. oligandrum* (1 isolate). The slides were incubated on wet filter paper in Petri plates at 25°C for 24 h. For comparison, control glass slides were inoculated with macroconidia of *F. culmorum* only.

Results and discussion

Biocontrol of *F. culmorum* on barley seedlings was demonstrated for the first time for *P. acanthophoron* (1 isolate), *P. mycoparasiticum* (1 isolate), *P. oligandrum* (2 isolates) and *P. periplocum* (1 isolate). Under the conditions of the test, all of the species and all except 1 isolate (of *P.mycoparasiticum*) significantly ($P \leq 0.05$ in the F test) controlled *F. culmorum*. This is the first demonstration of the ability of *P. acanthophoron* and *P. mycoparasiticum* to control a plant disease *in planta*.

Mycoparasitism of P. oligandrum on macroconidia of F. culmorum

Pythium oligandrum produced significantly ($P \leq 0.05$) more oogonia in dual culture with *F. culmorum* on CMA and WA than either *P. ultimum* with *F. culmorum* or *P. oligandrum* in single culture (Table 1). Interestingly, *P. ultimum* produced fewer oogonia when associated with macroconidia of *F. culmorum* compared with *P. ultimum* in single culture. This could be because the macroconidia physically prevented *P. ultimum* from making contact with the agar or due to

competition between *F. culmorum* and *P. ultimum* for nutrients. The increase in oogonia production by *P. oligandrum* was clearly associated with the breakdown of the macroconidia (table 1). This indicates that, as a result of mycoparasitism, *P. oligandrum* obtained a supply of nutrients and sterols (Haskins *et al.*, 1964) which enabled it to increase its reproductivity and biomass.

Treatment	CMA		WA	
	Oogonia[2]	Empty conidia[2]	Oogonia[2]	Empty conidia[2]
Po + *Fc*	5.80[a]	18.24[a]	5.48[a]	26.55[a]
Pu + *Fc*	0.66[b]	0[b]	0.47[b]	0[b]
Po (alone)	1.52[b]	-	0.84[b]	-
Pu (alone)	0.86[b]	-	2.11[b]	-
Fc (alone)	-	0[b]	-	0[b]

(1)Petri dishes (9 cm) were heavily inoculated with a suspension of macroconidia of *F. culmorum*.
(2)Mean of two observations, using eighteen fields of 2.5mm^2 for each observation..
(a)Numbers in each column followed by the same letter are not significantly different at the 5% level in the F-test.

Table 1 : Number of empty macroconidia of *Fusarium culmorum*(1) (Fc) and number of oogonia produced by *Pythium oligandrum* (Po) or *Pythium ultimum* (Pu), in single and dual cultures on two media (CMA & WA), recorded 2 days after inoculation.

In the controls, on CMA and WA, the cell contents of the macroconidia of *F. culmorum* stained bright blue with Cotton Blue. By contrast, especially on WA but also on CMA, degraded, empty macroconidia were observed in association with *P. oligandrum* but never with *P. ultimum* (Table 1). At the end of the experiment (48 h), it was observed that some of the non parasitized macroconidia had changed morphologically. Thus some of the macroconidia had become rounded with thickened cell walls resembling the early stage of endoconidial chlamydospore formation as described in Campbell & Griffiths (1974).

Pythium oligandrum grew rapidly over and among the macroconidia on the glass slides. Where hyphae of *P. oligandrum* were in contact with *F. culmorum* macroconidia, coagulation or vacuolation of the cytoplasm of the macroconidia was commonly observed. Following application of Cotton Blue it was seen that the parasitized segments of affected macroconidia had lost their ability to take up the stain. Normal macroconidia, on the other hand, stained bright blue. Growth of hyphae of *P. oligandrum* inside some macroconidia was observed and parasitized macroconidia were often completely degraded and disappeared after a few days. However, normal and fully intact macroconidia as well as partly degraded macroconidia with one or more intact segments, which stained bright blue with Cotton Blue, were frquently observed among the masses of degraded, empty, macroconidia. This indicates that contact between hyphae of *P. oligandrum* and the macroconidia had been a chance event as is the case between hyphae of *P. oligandrum* and hyphae of host fungi (Laing & Deacon, 1991). It has been found that *P. acanthophoron* and *P. periplocum* degrade macroconidia of *F. culmorum* in a similar manner to *P. oligandrum* (Davanlou *et al.*, submitted).

Macroconidia of *F. culmorum* are important in the disease cycle of the pathogen. They can germinate and infect plant parts or, upon entering the soil, convert to endoconidial chlamydospores (Campbell & Griffiths, 1974; Cook, 1968). In this way the population of the pathogen in the soil can increase severalfold in a single season (Cook, 1968; Sitton & Cook, 1981). Chlamydospores infect the roots of cereals and can survive in the soil up to 8.5 years (Sitton & Cook, 1981). The

parasitism of *F. culmorum* macroconidia and chlamydospores (Davanlou *et al.*, submitted) by mycoparasitic *Pythium* spp., and the significance of this interaction for the reduction of the inoculum potential of the pathogen in the soil, are interesting topics for as further study.

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MODES OF ACTION IN BIOCONTROLLING FUNGAL SEEDLING AND ROOT DISEASES BY *BACILLUS SUBTILIS*

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Abstract

Studies of the mode of action of *Bacillus subtilis* isolates (FZB Biotechnik GmbH Berlin collection) have shown a broad range of mechanisms leading to the production of bacterial metabolites which induce phytosanitary effects. These effects are two-fold, proceeding first *via* the hostplant by promoting growth and health, and second by antibiotic effects against pathogens.

Both pro- and antibiotic effects are demonstrated experimentally through the analyses of results obtained with sterile culture filtrates (CF) of *B. subtilis* isolates from different fermentation phases in the model pathosystems chosen: sterile seedling cultures of tomato and *Fusarium oxysporum* f.sp. *radicis-lycopersici*. CF of the logarithmic-, transition- and stationary bacterial fermentation phases have been shown to significantly influence the root length of tomato seedlings. Low concentrations of the log.- and trans.-phases stimulate plant growth. From our results, it seems highly likely that substances with phytohormone-like activity in these filtrates are responsible for the observed effects.

CF, especially from the trans.-phase, do not only stimulate plant growth, but also promote plant tolerance toward the *Fusarium* test pathogen, the highest plant tolerance being observed after pre-cultivations in the trans.-phase filtrates. On the other hand, direct contact of the CF (especially from the stationary fermentation phase) with the test pathogen *F. oxysporum* has led to an antibiotic, inhibiting effect on the pathogen. This is caused mainly by the presence of bacterial-borne peptide antibiotics in the filtrates.

Therefore, it can be concluded that the observed phytosanitary effects of the rhizobacteria *Bacillus subtilis* is based on a complex mode of action along two different lines, one probiotic, the second antibiotic.

Introduction

Bacillus subtilis strains from the FZB Biotechnik GmbH Berlin collection (FZB isolates) have proven to be highly useful biocontrolling agents of seedling diseases of ornamental plants and vegetables, of soil borne fungal root diseases and of *Fusarium*- wilt and *Phytophthora* root diseases of tomatoes (Bochow, 1992; 1994; 1995; Schmiedeknecht, 1993; Schmiedeknecht and Bochow , 1993; Schmiedeknecht et al., 1994; Wandke, 1992). In general, seeds or seedling drenchings are initially treated with bacterial spore suspensions of granulated preparations, in order to introduce the bacteria as first colonizer at the growing roots and in the rhizosphere. At temperatures around 20°C, the bacteria colonize the rhizosphere and rhizoplane and interact with the growing roots (see also Grosch et al. 1995, in this conference).

Different strains of *B. subtilis* will produce various arrays of metabolites, which differ both in the type and amount of each individual metabolite, which in turn results in a variety of expressed phytosanitary and phytoeffective activities. It has turned out that the biocontrolling effect of *Bacillus subtilis* is actually very complex, resulting not only in depressed growth of pathogens, but also in clearly increased plant growth and increased resistance- or tolerance-promoting effects. We have concluded that two different modes of activity are present, as shown in Fig. 1. The first and

most studied mechanism is the production of antibiotics, which are known to inhibit pathogens in soil. The second mechanism is a less-defined probiotic activity based on plant growth- and health-promoting bacterial metabolites.

We have found a number of indications that phytohormones or their precursors play an important role in this second process. This finding appears to be in agreement with Frankenberger and Muhammad Arshad (1995), who described the microbial production and function of phytohormones in soils. Furthermore, we have found that during the bacterial life cycle of the *B. subtilis* isolates investigated, the probiotic mode of action acts first, followed later by the production of antibiotics in connection with *Bacillus* resting spore formation.

In this paper, our experimental results support this hypothesis and are examined and discussed.

Experiments and Results

Our research has been focused on the biochemical analyses of culture filtrates from standardized fermentations of FZB *Bacillus subtilis* isolates (on *Landy*-medium), on biological tests of these culture filtrates, and on different culture filtrate fractions in a special axenic plant and pathosystem. Tomato seedlings, grown in nutrient solution in a phytotron, were used as test plants and *Fusarium oxysporum* f.sp. *radicis-lycopersici* was utilized as the pathogen.

Our initial goal was to determine whether the sterile culture filtrates of *B. subtilis* isolates have either antibiotic effects, probiotic effects, or both, and was accomplished through the stimulation of the plant growth under axenic conditions. Fig. 2 lists the resulting data from our testing of culture filtrates (CF), of 0,1 and 1 % concentration, isolated from the logarithmic-, transition-, and the stationary-fermentation phases of the *B. subtilis* isolate FZB C, on root growth of tomato seedlings after 10 days application under aseptic conditions.

Lower concentrations (0,1 %) of CF, especially from the log.- and the trans.- fermentation phases, contributed to a significant increase of the seedling root growth, as compared to the control. The CF from the stat.-fermentation phase showed no activity, while the higher concentrated (1 %) CF, especially from the stat.-fermentation phase, did remarkably inhibit the root growth. These same CFs from the *B.s.* isolate FZB G (1 % concentration) were also tested on agar plates in order to determine their antibiotic or antifungal activity against *F. oxysporum* f.sp. *radicis-lycopersici* (fig. 3). In contrast to the previously observed probiotic activity, the antagonistic effects of the CFs were greater in the higher concentrated CF, especially in CF from the stat.- fermentation phase. The CFs from the first log.- and trans.-fermentation phases were either low or not antifungally active. Because it is highly likely that the antifungal activity of these CFs is mainly due to a number of known di-, and lipopeptidal antibiotics produced by *Bacillus subtilis*, we lowered the pH to 2,5, separated most of the antibiotics out, and then tested these „peptide-antibiotic-free" CFs again. The results obtained are listed in Fig. 4. This separation indeed reduced to zero, or to a very low degree, the antifungal activity of the CF to that observed from the stat.- fermentation phase. Our results show that during the log.- and trans.- phases, the *B.s.* isolates produced plant growth-promoting substances to a great extent, while in the stat.- fermentation phase of the bacterial growth, antibiotic peptides were produced.

Since the above mentioned antibiotic effects are already well studied, we decided to investigate which substances or group of substances are responsible for the observed plant growth-promoting effects.

The CF of *B.s.* isolates were fractionated and purified by HPLC. Fig. 5 shows the preparative HPLC-purification of samples of CF fractions from the *B.s.* isolates FZB C and FZB G that we found most interesting, namely the ethylacetate fraction (G- fraction), divided into 6 substance groups G1 to G6. All of these G fractions were biotested for their phytohormonal activity. The

cytokinin-activity was tested in comparison to the standard kinetin by the Radish-Cotyledone test and the auxin-activity test, in comparison to IAA, by the Wheat-Coleoptile test.

Fig. 6 shows, that only G3, G5 and G6 were significantly cytokinin active. Highly interesting was the fact that exclusively these same three fractions were remarkably auxin-active as well (Fig 7). However, we have not yet been able to isolate no observe in these fractions either kinetin or IAA. We have as yet been able to observe indications of the presence of precursors of phytohormones, such as the auxin precursor indole-3-pyruvic acid.

We have also investigated the effect of our CFs and our *B. subtilis* fractions in the same pathosystem tomato-seedlings and *Fusarium oxysporum* f.sp. *radicis-lycopersici,* using axenic test conditions. In the first step, CFs from the log.-, trans.- and stat.-fermentation phases of *B.s.* in 0,1 and 1 % concentration were tested. The tomato seedlings were treated for 10 days with a nutrient solution containing CF in defined concentrations. Next, the seedlings were removed, the roots were washed with water, and transferred into tubes containing the same amount of active conidia solution of the test pathogen *F. oxysporum.* After a 7 day incubation period, the percentage of collapsed or pathogen-killed seedlings was calculated (Fig. 8).

Precultivations of the seedlings, especially with CF from the trans.-fermentation phase, have induced a reduction of the number of plants killed by the pathogen up to around 50 % compared with the untreated control. This strongly suggests that there must be one or more substances in the CF which induce plant tolerance against pathogen attack. The same results were observed with tests conducted with our fractions G3, G5 and G6 from CF of the *B.s.* FZB G and C.

Fig. 9 demonstrates the effects of these fractions on the root growth of tomato seedlings after cultivation for 10 days under aseptic conditions, compared with the influence of IAA and kinetin in physiologically active concentrations. The G6 fraction stimulated the testplant root growth significantly in the same degree as IAA and kinetin.

The same G fractions were also tested in the test pathosystem and compared with IAA and kinetin controls. The tomato seedlings were precultivated for 10 days in our nutrient solution containing the different G fractions. Afterwards, the cleaned plantlets were transferred into the inoculum suspension of *F. oxysporum* and incubated for 5 days. Only plants that were treated with the G3 fraction had significantly induced tolerance toward pathogen attack, in contrast to plants treated with IAA or kinetin. It would seem strongly plausible then, that phytohormonal active compounds play a pivotal role in promoting plant health, as observed with tomato plants after their treatment with *Bacillus subtilis* culture filtrates or with living bacteria, but not after treatment with the classic phytohormones IAA or kinetin. To underline this hypothesis, we asepticly pretreated tomato seedlings for 10 days with milli- and nano-molar amounts of pure auxin-precursor indole-3-pyruvic acid (IPyA) and then tested for tolerance against attacks of the pathogen *F. oxysporum* or against an intoxification of 25 ppm fusaric acid. The results are shown in Figs. 11 and 12. Fig. 11 indicates that treating plants with a very low concentration of IPyA makes the seedlings almost completely tolerant to pathogen attack, even when the plant roots were partially cut prior to pathogen inoculation.

Fig. 12 shows that the treatment of plants with IPyA leads to an intoxification from the unspecific fungal toxin fusaric acid.

Conclusions

Our studies of the mode of action of biocontrolling active FZB *Bacillus subtilis* isolates have shown that the biocontrolling effect of the bacteria is based on a complex activity, occuring through two differring modes of action. The effects occur after inoculation and at the start of colonization of the growing seedlings or plant roots.

The first, probiotic mode is characterized by the interaction of the bacteria with the roots of the plant, followed by the promotion of the plant phytohormone metabolism resulting from the extracellular substances of *Bacillus subtilis*. Through these promoting effects, plant growth and plant health are induced. The second mode, the antibiotic mechanism, proceeds as follows: at the end of the vegetative bacterial growth on the roots and in the rhizosphere, the bacteria produce antibiotic substances to protect their ecological niches and by doing so inhibit other plant pathogens.

Our hypothesis can explain variations in the biocontrolling effects of *B. subtilis* that have been observed under practical conditions For instance, if there is a high pathogen inoculum pressure which allows the pathogens to infect the plant *before* the production of antibiotics by *B. subtilis* has been achieved, only the probiotic bacterial activity (plant growth and health promoting) will be observed.

Our future work will focus on investigating *B. subtilis* isolates having both pro- and antibiotic activities, but predominating in the probiotic mode of action.

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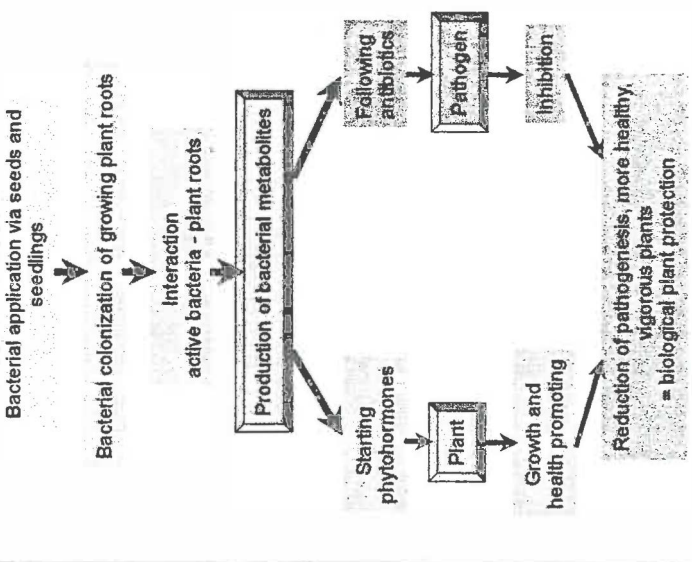

Fig. 1: Experimental based hypothesis on acting mechanisms in the phytosanitary mode of action of FZB *Bacillus subtilis* isolates as biocontrolling agent.

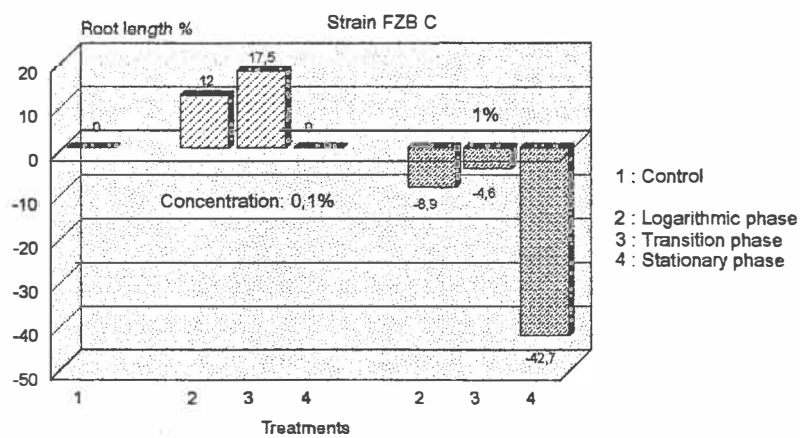

Fig. 2: Influence of tomato seedlings root growth by *Bacillus subtilis* culture filtrates from the log.-, transition- and stationary phase after 10 d under aseptic conditions.

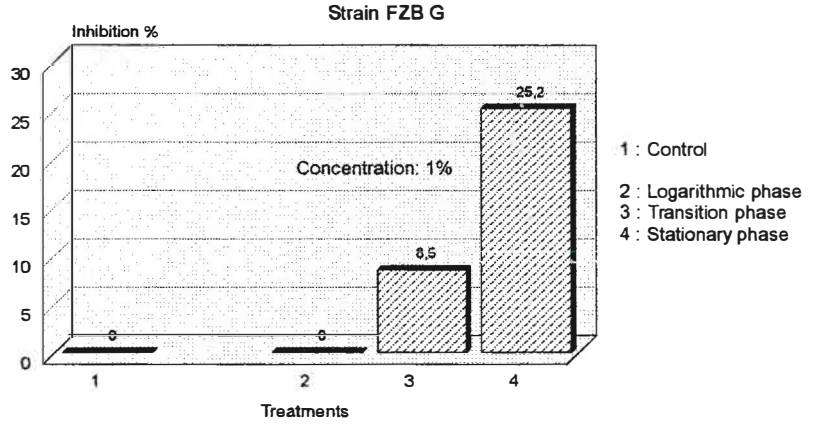

Fig. 3: Inhibition of *Fusarium oxysporum* f.sp. *radicis-lycopersici* by *Bacillus subtilis* culture filtrates from the log.-, transition- and stationary phase.

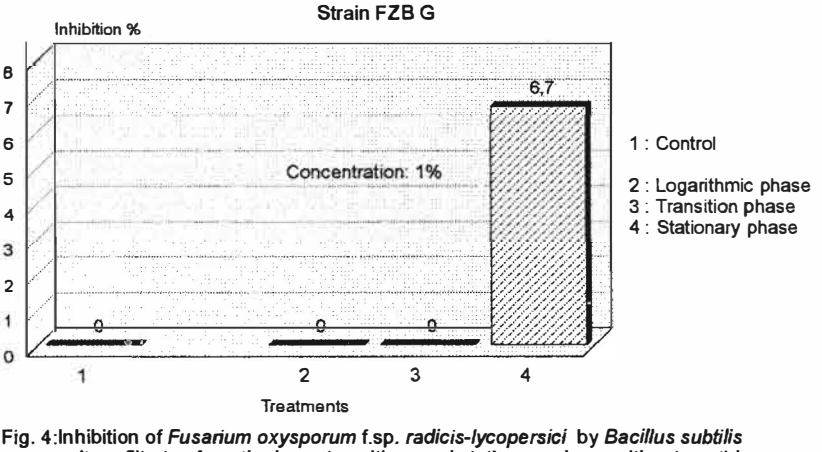

Fig. 4:Inhibition of *Fusarium oxysporum* f.sp. *radicis-lycopersici* by *Bacillus subtilis* culture filtrates from the log.-, transition- and stationary phase without peptide antibiotics.

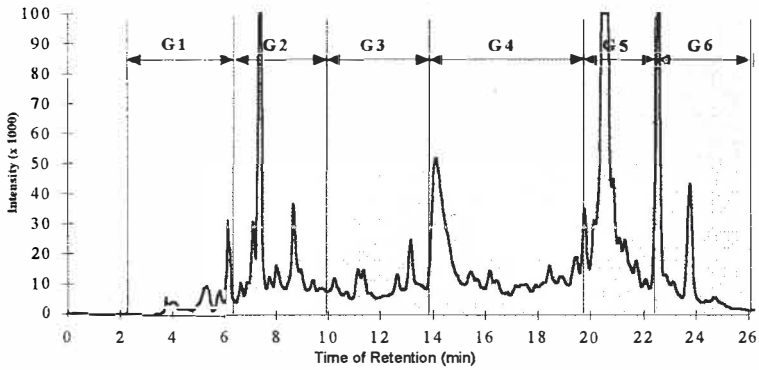

Fig. 5: Preparative HPLC-Purification of the Ethylacetate-Fraction of *Bacillus subtilis* culture filtrates (FZB C, FZB G).

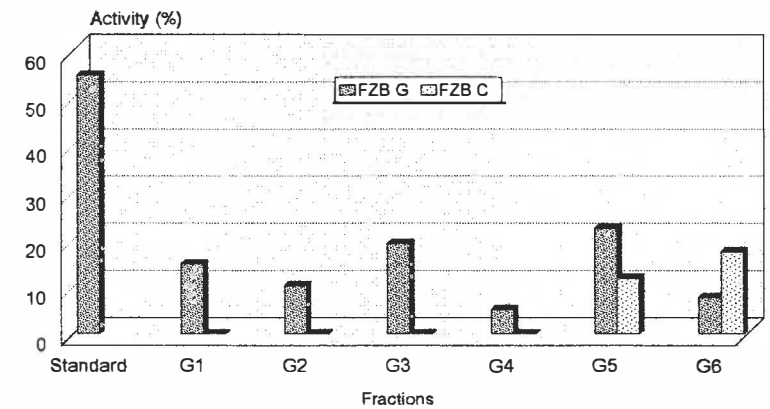

Fig. 6: Cytokinine-Activity of the HPLC-Fractions (G1-G6), measured in Radish-Cotyledone Test.

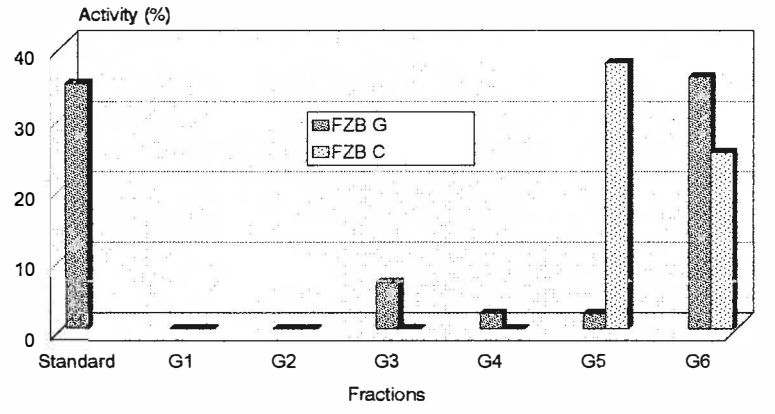

Fig. 7: Auxin-Activity of the HPLC-Fractions (G1-G6), measured in the Wheat-Coleoptile Test.

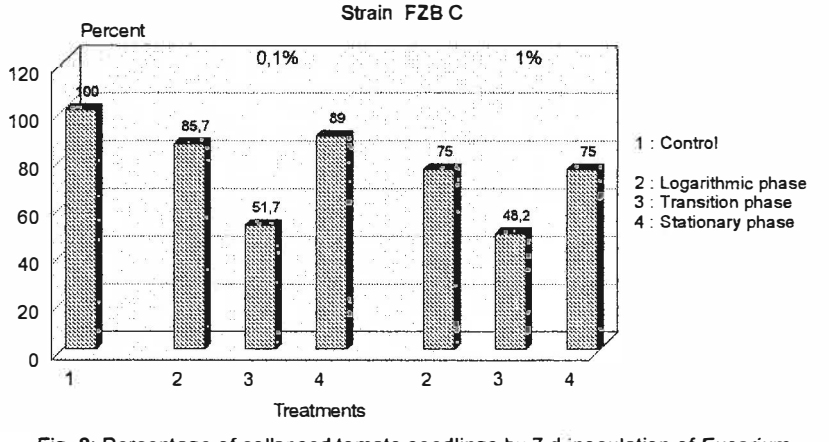

Fig. 8: Percentage of collapsed tomato seedlings by 7 d inoculation of *Fusarium oxysporum* f.sp. *radicis-lycopersici* after 10 d precultivations with *Bacillus subtilis* culture filtrates from the log.-, transition- and stationary fermentation phase.

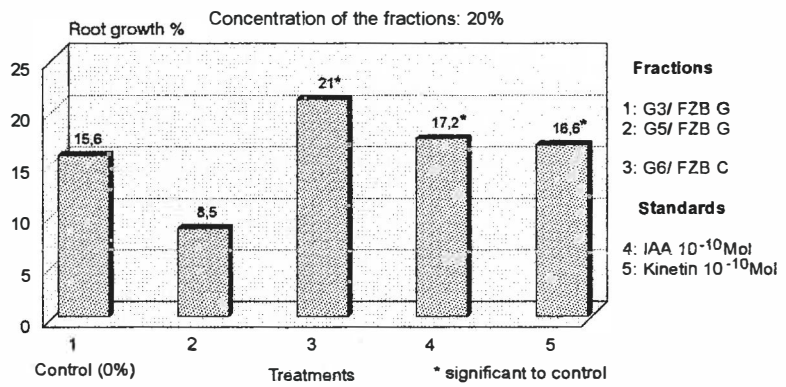

Fig. 9: Effects of *Bacillus subtilis* culture filtrate fractions on the root growth of tomato seedlings after 10 d under aseptic conditions.

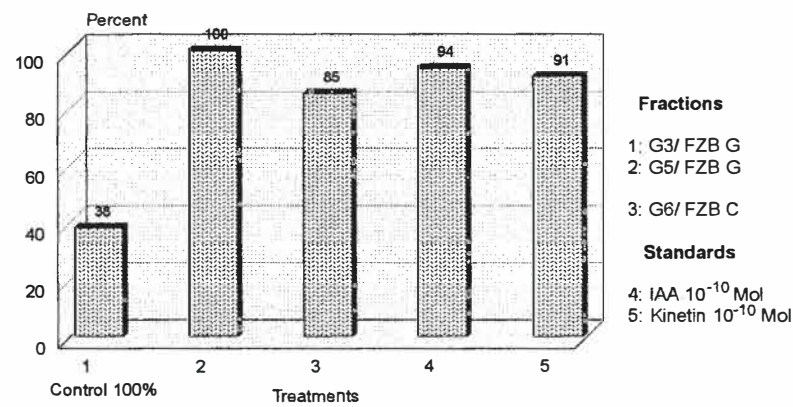

Fig. 10: Percentage of collapsed tomato seedlings by 5 d inoculation of *Fusarium oxysporum* f.sp. *radicis-lycopersici* after 10 d precultivations with *Bacillus subtilis* culture filtrate fractions, compared to IAA and Kinetin applications (standards).

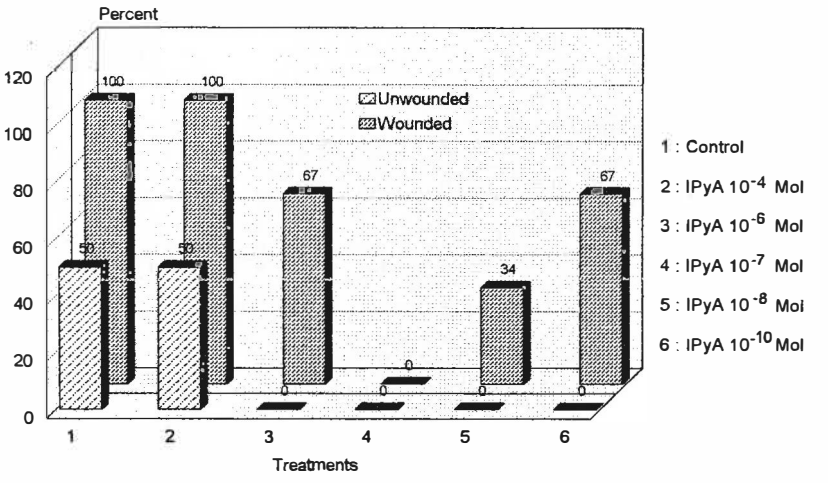

Fig. 11: Percentage of collapsed tomato seedlings by 5 d inoculation of *Fusarium oxysporum* f.sp. *radicis-lycopersici* after 10 d pretreatment with indole-3-pyruvic acid (IPyA).

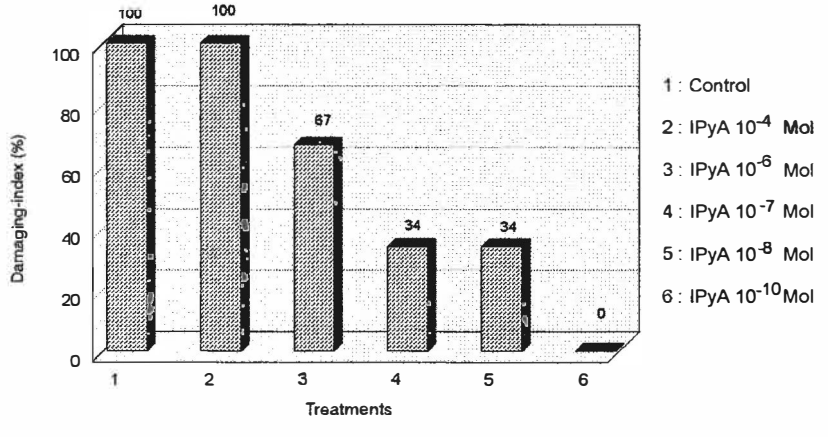

Fig. 12: Damaged tomato seedlings after 5 d contamination with fusaric acid (25 ppm) after 10 d pretreatment with indole-3-pyruvic acid (IPyA).

PSEUDOMONAS FLUORESCENS 63-28, A PGPR STRAIN PRODUCING SEVERAL ANTIFUNGAL METABOLITIES

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Summary

Three PGPR isolates, *Pseudomonas fluorescens* (63-28), *Pseudomonas putida* (G8-32) and *Serratia plymuthica* (R1-GC4) were investigated for their ability to produce antagonistic compounds against three common greenhouse crop fungal pathogens *Pythium ultimum*, *Rhizoctonia solani* and *Phytophthora cryptogea*. The influence of nutrient sources was evaluated on agar-diffusible and volatile antifungal compound production. G8-32 did not show any *in vitro* activity. R1-GC4 and 63-28 showed different profiles of activity, depending on the growth media and the challenged fungus. 63-28 was the most active isolate and several antifungal compounds were extracted from a glucose-nutrient broth culture. Using TLC and HPLC analysis, they were compared to the reference antibiotics pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol, but none of them were identical to these compounds.

Introduction

Greenhouse productions in the province of Quebec have represented a fast growing economical sector in the past few years, and in 1994 the market reached $150 million. Greenhouse crops grown in soilless systems are very susceptible tofungal pathogens such as *Pythium ultimum*, *Rhizoctonia solani*, and *Phytophthora cryptogea*, which cause severe losses due to damping-off or post-emergence root rot.

In the past few years, bacterial microorganisms, which demonstrate a capacity to promote plant growth have been selected. These Plant Growth Promoting Rhizobacteria (PGPR) can be chosen for their direct stimulating effect on the plant, shown by larger root system development and yield increases in gnotobiotic system. They can also be selected for their indirect stimulating activity, through their ability to protect plants against infections by pathogens. The introduction of such microorganisms as biological control agents in soilless cultures should be easier and more successful than in field soils, where the indigenous microbial populations represent a highly competitive obstacle to overcome for the introduced microorganism to successfully colonize root surfaces. Biological control of pathogens by bacteria has been largely studied and different mechanisms have been demonstrated : competition for colonization sites, competition for nutrients such as carbon or iron, enzymatic degradation of fungal pathogen cell wall, production of toxic secondary metabolites or induction of the plant resistance.

In this study, three bacterial strains were used, which were selected previously for their multiple PGPR characteristics, one of them being the control of some fungal pathogens (Reddy et *al.*, 1991; Gagné et *al.*, 1992). The purpose was to assess the importance of secondary metabolite production by these strains. *In vitro* bioassays were set up to evaluate the antagonistic ability of each strain against *Pythium ultimum*, *Rhizoctonia solani*, and *Phytophthora cryptogea*. The production of antibiotic-like molecules and volatile compounds was estimated, as well as the influence of nutrient sources on the production of these compounds. The most effective strain

Pseudomonas fluorescens 63-28 was selected, and some of its compounds were purified for identification purposes.

Materials and methods

The bacteria *Pseudomonas putida* G8-32, *Pseudomonas fluorescens* 63-28 and *Serratia plymuthica* R1-GC4 were originally isolated from soils and roots sampled at various locations in Canada (Kloepper et *al.*, 1991). The fungi *Pythium ultimum*, *Rhizoctonia solani* AG-4, *Phytophthora cryptogea*, and *Cladosporium herbarium* were from the collection of T. Paulitz.

Antifungal metabolite production was evaluated on the following media : 523 (Kado & Heskett, 1970); nutrient agar (NA), complemented with 2% glucose (NA-Glc) or 1% glycerol (NA-Gly); potato dextrose agar (PDA); tryptic soy agar (TSA); King's B medium (KB) (King et *al.*, 1954), complemented with 10 mM (KB10) or 100 mM (KB 100) of $FeCl_3$ (Weller et *al.*, 1988).

Antifungal agar diffusible compounds

A plug of fresh fungal mycelium was inoculated in the middle of the plate and the bacterium was inoculated on three sides around it. Both were inoculated simultaneously or with a delay of 48 h for the fungus, when no inhibition had been detected in the first test. The size of the inhibition zone around the bacterial colonies was measured after 2 to 5 days.

Antifungal volatile compounds

Bacteria were inoculated on each test media. The bottom of the petri dish was then covered with another inverted petri dish bottom containing PDA. The two were sealed with parafilm and incubated for to days at 25°C. A plug of fresh fungal mycelium was then inoculated in the middle of the inverted PDA plate and incubated for 2 to 5 more days. The volatile effect was estimated as a percentage of inhibition of mycelium growth compared to the control without bacteria.

Antibiotic extraction and analysis

P. fluorescens 63-28 was grown in nutrient broth + 2% glucose at 25°C for 5 days. The culture was extracted twice with an equivalent volume of chloroform. The chloroform phases were evaporated to dryness *in vacuo* at 40°C and the final residue was redissolved in acetone. Analysis and separation of distinct compounds were first carried out by thin layer chromatography (TLC) with a migration phase of chloroform-acetone (9:1, v/v). An activity bioassay was performed for detection of the antifungal active spots : a spore suspension of *Cladosporium herbarium* was spread on the dry TLC plate, and incubated for 2 days in a humid chamber. Active spots appeared as white zones on the dark background of *Cladosporum herbarium*. Final purification and UV spectra analysis were realized by HPLC, with a Waters C_{18} columm. Samples were run in a water/acetonitrile gradient (T0-T10 = 50/50, T15 = 0/100, T17 = 50/50) with a flow rate of 1 ml/min. Chromatograms were monitored by UV absorbance (range 230-400 nm) and spectra analyzed with the Waters PDA software system.

Reference antibiotics

Authentic samples of the antibiotics 2,4-diacetylphloroglucinol pyrrolnitrin, and pyoluteorin were kindly provided by B. Nowak-Thompson from USDA-ARS, Corvallis, OR.

Results

Antifungal agar diffusible compounds

Pseudomonas putida G8-32 did not show any inhibition zone production against any of the three fungi tested, whatever the growth medium used.

Serratia plymuthica R1-GC4 produced inhibition zones against *Phytophthora cryptogea* on every media. It produced inhibition zones against *Pythium ultimum* on KB, KB10 and KB100 and on 523 and TSA. Against *Rhizoctonia solani*, R1-GC4 produced inhibition zones on KB, KB10 and KB100; on NA and TSA, inhibition zones were only visible when the fungus had been inoculated 48 h after the bacteria.

Pseudomonas fluorescens 63-28 showed the largest inhibition zone production (Tab. 1). All three fungi were strongly inhibited when tested on PDA, NA-Glc and 523 media. 63-28 did not produced any inhibition zone on NA or NA-Glycerol, pointing out the effect of glucose as an inducer for active metabolite production. On KB, KB10 and KB100 media, inhibition zones against *Phytophthora cryptogea* seemed to decrease as the quantity of iron increased in the media, but the results were not significantly different. Inhibition zones against *Rhizoctonia solani* significantly increased as the iron quantity increased in the media. *Pythium ultimum* was not inhibited on these three media.

Growth medium	Inhibition zone radius around bacterial colony (cm)		
	P. ultimum	*R. solani*	*P. cryptogea*
PDA	0.59 c*	1.03 e	0.3 cde
TSA*	0 a	0.04 a	0 a
NA*	0 a	0 a	0 a
NA-Glc	0.55 c	1.17 ef	0.65 gh
NA-Gly*	0 a	0 a	0 a
523	0.14 b	0.79 d	0.09 ab
KB*	0 a	0.2 ab	0.40 def
KB10*	0 a	0.34 bc	0.50 fg
KB100*	0 a	0.59 d	0.24 bcd

*63-28 inoculated 48h00 before fungi; * Values with different letters within the same columm are sgnificantly different (P = 0.05)

Table 1 : Fungal growth inhibition by agar diffusible compounds of *Pseudomonas fluorescens* 63-28

Antifungal volatile compounds

Serratia plymuthica R1-GC4 showed some cases of inhibition due to volatile production (results not shown).

Pseudomonas fluorescens 63-28 produced volatiles showing a strong antifungal activity (Tab. 2). The inhibition was detected on PDA, NA-Glc or 523, and also on TSA where no agar diffusible effect had been detected. All three fungi were affected. Glucose seemed to be involved in volatile production also, as noticed when NA was compared to NA-Glc.

Bacterial medium*	Mycelium growth inhibition (%)		
	P. ultimum	*R. solani*	*P. cryptogea*
PDA	77.46	76.64	18.63
TSA	42.77	31.16	nd
NA	1.44	0.38	29.71
NA-Glc	45.89	29.48	64.93
523	18.84	47.48	43.08

* Fungi grown on PDA; nd : not determined.

Table 2 : Fungal growth inhibition by volatile compounds of *Pseudomonas fluorescens* 63-28

Chromatography analysis of 63-28 antifungal compounds

The total extract of 63-28 was run on TLC plates and actives spots were detected using the *Cladosporium herbarium* spray bioassay. Four different active spots (FI to FIV) were detected with the Rf 0.89, 0.70, 0.40 and 0.04 respectively. FI, FII and FIII were purified for identification purposes. When compared by high pressure liquid chromatography to the three reference antibiotics 2,4-diacetylphloroglucinol, pyrrolnitrin, and pyoluteorin, the analysis showed that the purified compounds FI, FII and FIII were clearly distinguishable from them, showing different UV spectra and retention times (Tab. 3).

Compound	UV max	Retention time (min)
Pyrrolnitrin	252.0	22.33
2,4-Diacetylphloroglucinol	272.5	13.47
Pyoluteorin	307.5	8.15
FI	255.1	22.21
FII	263.8	14.55
FIII	255.1	10.65

Table 3 : HPLC analysis : comparison of the 63-28 compounds with the reference antibiotics

Discussion

The strain G8-32 did not show any antifungal metabolite production, whereas R1-GC4 and 63-28 showed the production of several agar diffusible compounds as well as volatiles. All three bacterial strains have demonstrated their biological control efficiency against various pathogenic fungi (Reddy et *al.*, 1991; Gagné et *al.*, 1993; Campbell et *al.*, 1992), and the production of secondary metabolites could be part of the mechanism involved for R1-GC4 and 63-28.

In both cases, *Serratia plymuthica* and *Pseudomonas fluorescens* 63-28 showed different inhibitory capacities depending on the fungi, and also variations from one growth medium to the other on the same fungus. These patterns demonstrate that both strains produce more than one active compound, and it was demonstrated later for 63-28, which showed 4 different active spots when the NA-Glc culture extract was bioassayed on the TLC plates.

The production of the various compounds greatly depended on the nutrieny availability. Most of the time, the inhibititory zone around the bacterial colony was detected the next day after inoculation. On NA and TSA, the inhibitory zone around R1-GC4 was detected after 2 or3 days only, meaning that there was a lag phase before the active compounds were synthesized or excreted

into the surrounding medium. In a real situation, such compounds would be less efficient, knowing that an infection by *Pythium* take place in a few hours after the induction.

Iron enhanced the production of the antifugal activity of *Pseudomonas fluorescens* 63-28 against *Rhizoctonia solani*. The decreasing activity against *Phytophthora cryptogea* with the increasing concentration of iron in the medium may be due to a siderophore effect.

With *Pseudomonas fluorescens* 63-28 only, glucose was shown to be involved as a major inducer in secondary metabolite production, resulting in a strong inhibition of all three fungi. PDA contained the same concentration of glucose as NA-Glc, and 523 contained sucrose. The smaller inhibition zones on 523 medium can be explained by th lower concentration of sucrose in the medium (10g/l compared to 20g/l of glucose in PDA and NA-Glc), which gave a potential glucose concentration 4 times lower in 523. There was also a possible delay before glucose became available from the sucrose hydrolysis. The strong inhibitory activity on agar was related to the detection of a minimal of four antifungic spots in the TLC analysis of the NA-Glc extract.

The three major active compounds of 63-28 were purified and compared to the reference antibiotics pyrrolnitrin, pyoluteorin or 2,4-diacetylphloroglucinol. These three antibiotics are some of the major ones produced by *Pseudomonas fluorescens* strains, which have been demonstrated to be involved in the biological control of fungal pathogens such as *Pythium* or *Rhizoctonia* (Howell & Stipanovic, 1979,1980; Levy et *al.*, 1992; Keel et *al.*, 1992). But none of the three 63-28 compounds was identified as one of these antibiotics.

In conclusion, these results show that, when screening for antagonistic activity on agar media, several nutrient sources should be investigated. The screening for antifungal metabolites from *P. fluorescens* 63-28 has demonstrated the ability of this strain to produce many different compounds. It makes this strain particularly interesting as a biological control agent because the variety of antagonistic compounds produced should enable an action against a wider spectrum of pathogens. However, the production of these compounds must be verified *in vivo*, where the nutrient source would be furnished by the root exsudates, which may not contain the proper inducer. Our results showed one exception to the influence of nutrient source on antifungal activity. It was in the case of the inhibition of *Phytophthora cryptogea* by *Serratia plymuthica* R1-GC4, which was observed on every tested media. This could be due to one compound synthesized in a constitutive way, which could have a constant activity *in vitro*.

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BIOCONTROL OF SOIL-BORNE PLANT PATHOGENS BY MEANS OF BIOTECHNOLOGY

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Abstract

The application of microorganisms with antagonistic activity against pathogenic fungi is one of the possibilities to control soil-borne plant diseases. Non-pathogenic *Pseudomonas* species colonize roots of various plant species, and many of them already possess certain types of antagonistic activity against *Fusarium* and *Pythium*. To improve the antagonistic activity of the *Pseudomonas putida* strain WCS358, genes encoding fungal cell wall-degrading enzymes, such as chitinases, glucanases and proteases, will be introduced. Chitinases and glucanases from tobacco show antifungal activity. However, they might not be recognized by the bacterial secretion machinery. Therefore, we are first examining the secretory pathway for proteins in *P. putida*.

P. putida, a Gram-negative bacterium, contains an inner membrane and an outer membrane, which are separated by the periplasm. Proteins synthesized in the cytoplasm can be secreted to the extracellular medium directly across the two membranes in a single step (type I pathway) or in two steps via a periplasmic intermediate (type II pathway). So far, we have demonstrated that genes encoding the components of the type II secretion machinery are present in *P. putida*, even though no proteins could be identified that utilize this pathway for their secretion. Genes encoding components of the type I secretion machinery could not be detected. To investigate whether the type I secretion pathway can be reconstituted in *P. putida*, genes of *P. aeruginosa* encoding alkaline protease and its type I secretion proteins were introduced in *P. putida*. Expression of both the secretion machinery and the alkaline protease resulted in secretion of alkaline protease by *P. putida*.

Introduction

Soil-borne plant pathogens such as *Fusarium* and *Pythium* cause considerable yield losses in a wide variety of crops. Several studies indicate that treatment of fungi with chitinase, alone or in combination with glucanase, inhibits the growth by degradation of the cell walls (Shapira *et al.*, 1989; Arlorio *et al.*, 1992; Oppenheim and Chet, 1992; Benhamou and Chet, 1993; Sela-Buurlage *et al.*, 1993; Koby *et al.*, 1994; Nuero, 1995). Chitinases are produced by plants during fungal attack, as a part of their defence mechanism. Bacteria and fungi produce chitinases to be able to use chitin as a source of carbohydrate. Chitinases are also involved in branching of fungal hyphae. For biocontrol purposes, chitinases can be added as free enzymes to the irrigation water or incorporated into a seed coating. However, free enzymes are not expected to be stable for a long period. Stable enzyme activity can be obtained by introducing a constitutively expressed chitinase gene into susceptible plant species. A disadvantage of this method is that every single susceptible crop needs to be transformed with a chitinase gene. Alternatively, chitinase genes can be introduced into rhizosphere-colonizing bacteria, able to express and secrete the chitinase into the environment. In the present study, we are testing *Pseudomonas putida* WCS358 on its suitability to express and secrete chitinase encoded by the *chiA* gene of *Serratia marcescens*.

P. putida is a Gram-negative bacterium, which has an inner membrane (IM) and an outer membrane (OM). In both membranes, proteins are present that are involved in different secretion systems. Proteins synthesized in the cytoplasm, that make use of the type I secretion system are directly secreted across both IM and OM. The type I secretion is independent of a classical N-terminal signal sequence, but requires a C-terminal secretion signal. A well studied example of a protein that is secreted via the type I pathway is alkaline protease of *P. aeruginosa* (Blight and Holland, 1994). Proteins that make use of the type II pathway, e.g. elastase of *P. aeruginosa*, are first transported over the IM, for which a N-terminal signal sequence is required. In a second step, the protein is transported across the OM. The *chiA* gene product has a classical N-terminal signal sequence, which indicates that chitinase is probably secreted by a type II pathway.

The genes involved in the type II secretion pathway of *P. putida* have been cloned, sequenced and characterized (de Groot *et al.*, 1995). However, they turned out not to be active under the circumstances tested. Type I secretion genes were never detected in Southern blot hybridization experiments using the type I secretion genes from *P. aeruginosa* as probes (de Groot *et al.*, unpublished results). At least 12-14 genes are involved in the type II secretion machinery (Filloux *et al.*, 1990; Bally *et al.*, 1991 and 1992; Akrim *et al.*, 1993), which makes this system rather complex compared with the type I system, where only three genes are involved. Here, we studied the presence of an active type I secretion machinery in *P. putida* by means of heterologous secretion.

Materials and methods

Bacterial strains and growth conditions.

Strains of *Pseudomonas putida* WCS358 (Geels and Schippers, 1983) and *E. coli* were grown in L-broth (Tommassen *et al.*, 1983) at 30°C and 37°C, respectively. Antibiotics were used in the following concentrations: 500 µg/ml chloramphenicol, 40 µg/ml kanamycin, 25 µg/ml nalidixic acid (*P. putida*) and 25 µg/ml chloramphenicol, 25 µg/ml kanamycin (*E. coli*). Isopropyl-ß-D-thiogalactopyranoside (IPTG) was added when required (0.5 mM).

Plasmids and DNA manipulations.

Plasmids pAG302 and pJF1 (Fig.1) carrying *aprA* and *aprD,E,F*, respectively, were constructed from pJUEK72 (Guzzo *et al.*, 1990) by standard recombinant DNA techniques. Plasmids were transferred from *E. coli* to *P. putida* WCS358 by triparental mating, mediated by the helper plasmid pRK2013 (Figurski and Helinski, 1979). Transconjugants were selected on King's B medium (King *et al.*, 1954).

Protease indicator plates.

Protease activity was tested by growing *P. putida* strains on L-broth agar containing 1.5% protifar (Nutricia).

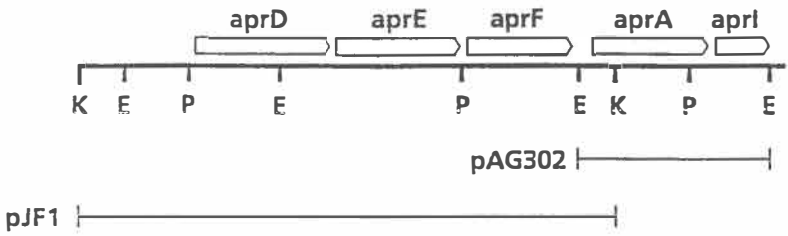

Figure 1 : The *apr* gene cluster of *P. aeruginosa*. The *Kpn*I (K) fragment carrying *aprD,E,F* and the *Eco*RI (E) fragment carrying *aprA* were used to construct pJF1 and pAG302, respectively.

Results

To secrete the alkaline protease (AprA) of *P. aeruginosa*, three gene products are required, AprD, AprE and AprF. They form a channel in the cell envelope through which the alkaline protease is transported into the extracellular medium (Tommassen *et al.*, 1992; Wandersman, 1992). In *P. aeruginosa*, the *aprA* gene is clustered with *aprD*, *aprE* and *aprF* (Fig.1). The *aprD,E,F* cluster and *aprA* were separately cloned into compatible vectors, which were introduced into *P. putida* WCS358. The strains with one plasmid or with a combination of the two plasmids were tested on protease indicator plates. If proteases are secreted, then the protifar around the colony will be degraded and a halo appears. Colonies of cells with only the *aprA* plasmid did not form a halo, indicating that no type I secretion pathway is active in *P. putida* WCS358. Similarly, as expected, no halo was observed around the colonies of the pJF1-containing cells. However, when both the *aprA*- and the *aprD,E,F*-containing plasmid were introduced simultaneously, a halo was formed around the colonies (Table 1).

Genes of *P. aeruginosa* expressed in *P. putida*	Secretion
aprA	-
aprD,E,F	-
aprA and aprD,E,F	+

Table 1 : Secretion of alkaline protease of *P. aeruginosa* in *P. putida*.

Discussion

The results indicate that AprA of *P. aeruginosa* is not recognized by an active secretion machinery of *P. putida*. Additionally, the AprD,E,F secretion apparatus of *P. aeruginosa* was not able to secrete an authentic protease of *P. putida* under the conditions in which the strains were tested. Although we did not manage to detect an active type I secretion machinery, we were able to introduce an active type I secretion apparatus of *P. aeruginosa* into *P. putida*. This type I secretion apparatus can be introduced together with a modified chitinase suitable for secretion via the 1-step pathway.

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INVOLVEMENT OF INDUCED SYSTEMIC RESISTANCE IN THE CONTROL OF FUSARIUM WILT OF TOMATO BY *FUSARIUM OXYSPORUM* STRAIN FO47 AND *PSEUDOMONAS FLUORESCENS* STRAIN WCS417R

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Abstract

Nonpathogenic *Fusarium oxysporum* strain Fo47 and *Pseudomonas fluorescens* strain WCS417r significantly suppressed fusarium wilt in tomato grown on rock wool. The relative importance of induced systemic resistance in this disease suppression was investigated by comparing the reduction of disease incidence by each strain in a split-root system with that in a non split-root system. If the pathogen and biocontrol strain were applied together in the non split-root system, the reduction of disease incidence was more effective with Fo47 than with WCS417r. However, if antagonism by the biocontrol strains was excluded by spatially separating them from the pathogen in a split-root system, reduction of disease incidence was more effective with WCS417r than with Fo47. These results indicate that induced systemic resistance is mostly responsible for the suppression of fusarium wilt in tomato achieved by the *P. fluorescens* strain WCS417r, but not for that by the nonpathogenic *F. oxysporum* strain Fo47.

Introduction

Nonpathogenic *Fusarium oxysporum* (Lemanceau *et al.*, 1992) and fluorescent pseudomonads (Van Peer *et al.*, 1991; Duijff *et al.*, 1995) significantly suppress fusarium diseases in soilless cultures. Microbial antagonism (Lemanceau *et al.*, 1993; Duijff *et al.*, 1995) and induced resistance (Van Peer *et al.*, 1991; Duijff *et al.*, 1995) have been proposed as the main mechanisms explaining this disease suppression.

The aim of the present work was to assess the involvement of induced systemic resistance (Kloepper *et al.*, 1992) in the suppression of fusarium wilt in tomato by the nonpathogenic *Fusarium oxysporum* strain Fo47 and by *Pseudomonas fluorescens* strain WCS417r.

Material and Methods

Microorganisms and plants

The rifampicin-resistant *Pseudomonas fluorescens* strain WCS417r (Duijff *et al.*, 1995) and the nonpathogenic *Fusarium oxysporum* strain Fo47 (Alabouvette, 1986) were used as the biocontrol strains in this study. The tomato wilt pathogen was *Fusarium oxysporum* f. sp. *lycopersici*, race 0, strain Fol32 (Olivain *et al.*, 1995). Tomato cultivar Monalbo, that is moderately resistant against fusarium wilt (Olivain *et al.*, 1995) was used as the test plant.

Biocontrol experiment

Tomato seedlings were grown for three weeks in rock wool plugs, when, from the half of the number of plants, the root system and the hypocotyl were split. These plants were grown with each half of their root system in a separate rock wool plug for another week before being transplanted to rock wool cubes. Plants with a non split-root were cultivated for the same period of time. The non split-root plants were planted in 500 ml rock wool cubes. The split root-plants were planted with each half of their root system in two separate 365 ml rock wool cubes. Two days after planting, plants were treated with a cell suspension of WCS417r or a microconidial suspension of Fo47, resulting in a final concentration in the rock wool cubes of 2×10^7 cells of WCS417r or 1×10^6 microconidia of Fo47 per ml of nutrient solution. Control plants were treated with 0.01 M $MgSO_4$, used to prepare the bacterial and conidial suspension. Three days after treatment with the biocontrol strains, plants were inoculated with a microconidial suspension of the wilt pathogen Fol32, resulting in a final concentration of 1×10^4 microconidia per ml of nutrient solution.

Plants were grown in a glasshouse with a photoperiod of 16 h at 25°C and a dark period at 22°C. The pH of the nutrient solution measured in the rock wool cubes was 5.7 ± 0.2.

The percentage of plants showing fusarium wilt symptoms (disease incidence) was assessed from 6 replicates of 6 plants each. The experiment was ended 11 weeks after inoculation with Fol32 when disease incidence no longer increased.

Root and stem colonization

At different times, samples of roots and stem were taken to estimate the colonization by the biocontrol strains. Root samples of three plants per treatment were shaken (Vortex) for 30 s in glass test tubes containing 5 ml of 0.01 M $MgSO_4$ and approximately 1 g of glass beads (0.18 mm diameter). The suspensions were diluted and plated on selective agar media to estimate the microbial population densities by numbers of colony forming units (CFUs). Bacto peptone (BP) agar supplemented with 100 ppm cycloheximide, KB^+ agar (Geels and Schippers, 1983), KB^+rif agar (Duijff *et al.*, 1995) and Komada agar (Komada, 1975) were used to estimate the population densities of the total aerobic bacteria, the total fluorescent pseudomonads, strain WCS417r and *Fusarium oxysporum*, respectively. After incubation for 48 h at 27°C the numbers of CFUs of the bacteria were determined and 24 h later that of the fungi.

To investigate the internal colonization of the stem by WCS417r or Fo47 the stem was surface-disinfected (flamed after dipping in 95% ethanol) and sections were cut at 1, 10 and 20 cm above the split of the stem. These sections were placed on KB^+rif, Komada, and, as a control, on BP agar. After incubation for 72 h at 27°C, the agar plates were checked for bacterial and fungal growth.

Data analysis

Data were analyzed by analysis of variance, followed by mean separation with least significant difference at $P = 0.05$ (Sokal and Rohlf, 1981).

Results and Discussion

If the pathogen and biocontrol strain were applied together in the non split-root system, both Fo47 and WCS417r reduced disease incidence significantly (Table 1). However, reduction of disease incidence was more effective with Fo47 than with WCS417r. If the biocontrol strain was spatially

separated from the pathogen in the split-root system, WCS417r significantly reduced disease incidence and Fo47 did not (Table 1). These results indicate that induction of systemic resistance plays a more important role in the control of fusarium wilt in tomato by WCS417r than by Fo47. Most likely, the pH of 5.7 of the nutrient solution was too low for strain WCS417r to reduce fusarium wilt by means of antagonism, e.g. siderophore-mediated competition for iron. Duijff *et al.* (1995) demonstrated that at pH 5.5 siderophore production by strain WCS417r was low, and that at this pH the suppression of fusarium wilt of carnation by WCS417r was more effective by induction of resistance than by antagonism. The more effective disease reduction by nonpathogenic *Fusarium oxysporum* Fo47 in the non split-root system compared with that in the split-root system indicates that also other mechanisms than induced systemic resistance plaid a role in the suppression of fusarium wilt of tomato by Fo47. Competition for carbon has been demonstrated to be involved in the biological control of fusarium wilt in soilless cultures by strain Fo47 (Lemanceau *et al.*, 1993). Probably, in the present study this mechanism contributed to the disease reduction by Fo47, and the combination of induced resistance and competition for carbon resulted in a significant disease suppression by Fo47.

	Relative disease incidence (%)	
Treatment	non split	split
Control	100b[1]	100b
WCS417r	69a	61a
Fo47	42a	72ab

[1] Values with different letters differ significantly ($P = 0.05$).

Table 1 : Suppression of fusarium wilt in tomato grown either in a non split-root or a split-root system in rock wool by *Pseudomonas fluorescens* WCS417r or by nonpathogenic *Fusarium oxysporum* Fo47, 79 days after inoculation with the pathogen *Fusarium oxysporum* f. sp. *lycopersici*. Disease incidence is expressed as the percentage of disease incidence in the infested control. Original values of disease incidence in the infested controls were 94% and 53% for the non split-root and the split-root plants, respectively.

Strain WCS417r colonized tomato roots at a high density and root colonization by WCS417r was consistent until 33 days after inoculation. Afterwards, it decreased (Fig. 1). Until 33 days after treatment with WCS417r, about 25% of the total population of *Pseudomonas* bacteria and about 2% of the total aerobic bacterial population on the root surface consisted of strain WCS417r. Also nonpathogenic *Fusarium* Fo47 colonized tomato roots at a high density (Fig. 1). Its root colonization started to decrease 20 days after inoculation. However, this decrease was relatively less than that of WCS417r. Such a good root colonization by the two biocontrol strains might be a prerequisite for an effective disease control by these strains. The role of root colonization in biological control was demonstrated by Bull *et al.* (1991) for a *Pseudomonas* strain which root colonization correlated linearly with its disease suppressing activity.

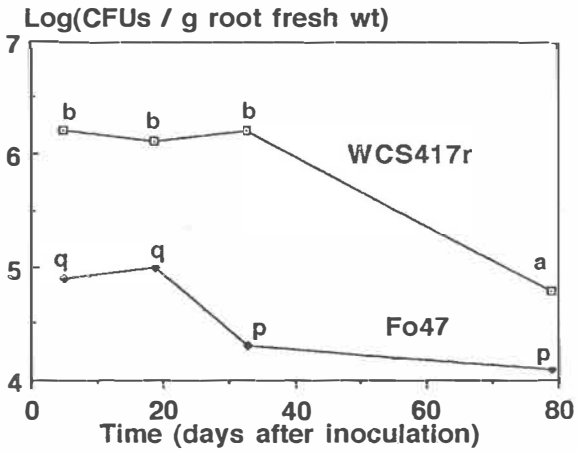

Figure 1 : Root colonization of tomato by *Pseudomonas fluorescens* WCS417r and nonpathogenic *Fusarium oxysporum* Fo47 at different times after inoculation. Values for the same strain and with different letters differ significantly in time ($P = 0.05$).

In the split-root system cross-contamination by the biocontrol strains of the rock wool cubes and roots inoculated with the pathogen did not occur. Moreover, strain Fo47 and strain WCS417r were not isolated from the tomato stem tissue. Thus, the split-root system was suitable to keep pathogen and biocontrol strain spatially separated and to demonstrate induction of systemic resistance against fusarium wilt by the biocontrol strains.

In conclusion, fusarium wilt in tomato grown on rock wool at pH 5.7 was significantly suppressed by *P. fluorescens* strain WCS417r and by nonpathogenic *F. oxysporum* strain Fo47. Induced systemic resistance seems mostly responsible for the suppression achieved by WCS417r. Induction of disease resistance by Fo47 was less effective than that by WCS417r and other mechanisms contributed to the disease suppression by Fo47 as well.

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Population dynamics

POPULATION DYNAMICS OF BIOCONTROL AGENTS AND PATHOGENS IN SOILLESS SYSTEMS

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Abstract

The study of population dynamics can provide useful information about the biology and ecology of both the biocontrol agent (BCA) and pathogen. The survival of the BCA in the substrate before and after pathogen/plant introduction and the threshold level of BCA inoculum and BCA activity necessary for biocontrol can be determined by measuring BCA population densities over time. The relationship between inoculum density of the pathogen and disease incidence (ID/DI) and disease thresholds (economic, damage and action) can be elucidated by examining the population dynamics of the pathogen. Techniques for measuring pathogen populations are discussed, including selective media, baiting, filtration, ELISA and DNA based PCR/RFLP techniques. BCA fungi can be quantified with similar methods, including GUS-*B* glucuronidase transformation. BCA bacteria can be measured using antibiotic marked strains, ELISA and immunofluorescence colony staining. Gene reporter systems such as GUS, lux, and ice nucleation protein can also provide details about the expression and activity of traits important in biocontrol, such as antibiotic production. Because of the inherent complexity of the experimental design and the large number of treatments required, few studies have examined the dynamics of both the BCA and pathogen and their effect on each other over a range of population densities over time. Modelling of these interactions could contribute to the knowledge about the efficiency of the BCA, the performance of the biocontrol agent under a number of disease scenarios, and the maximum attainable level of disease control. Several models are addressed, including simple efficiency models, examining the effect of the BCA on inoculum density/disease incidence curves, and the use of hyperbolic saturation and negative exponential (multiple infection) models.

Introduction

Studying the population dynamics of the pathogen and the biocontrol agent (BCA) can give important insights into their ecology and biology, especially their interactions that result in reduction of plant disease. Population dynamics looks at the changes in the population densities of the biocontrol agent and pathogen over time and how changes in one affect the other. This is an important facet to study in any biocontrol system, whether in the soil or on the foliage, and the literature is full of examples. However, some of these fundamental questions have not been adequately answered for biocontrol systems in soilless or hydroponic culture. Although closed structures may offer a more amenable environment for biological control compared to field agriculture, much less biocontrol work has been done in soilless systems. In recent years, much of the research on population dynamics has been overshadowed by more exciting work on the mechanisms and the molecular and biochemical basis of the antagonistic reaction. I will cover some information that can still be gained with population dynamics studies, including some examples of population studies of PGPR (plant growth-promoting rhizobacteria) in peat-based media and *Pythium aphanidermatum* in nutrient film technique (NFT) systems. Some of the current and future methods that could be used in soilless systems will be discussed including their strong points and

shortcomings. Finally, I will give some examples of how mathematical modelling of populations can be used to answer questions about the interactions between biocontrol agents and pathogens.

Questions addressed by studies of population dynamics

The BCA

The survival of the biocontrol agent (BCA) in the formulation or substrate prior to pathogen or plant introduction is a crucial component in the development of any biocontrol system. If the BCA cannot survive in populations high enough to effect disease control when used by the grower, it will not be commercially accepted. Many factors can affect survival, including moisture, temperature, time and formulation. Our lab has studied the effects of these factors on the survival of PGPR strains in peat media. For example, *Pseudomonas fluorescens* strain 63-28 survived above the threshold level of 10^4 bacteria per gram for only 3 months in peat at 25 and 45% moisture (w/w). Peat is normally shipped at 45%. But 63-28 could survive 6 months or longer at moisture levels of 100-200%. At storage conditions of -20° C, it survived for 12 months, but when stored outside in the summer with temperatures of 30-35° C, it only survived 1 month. At 22° C, it survived for 6 months or longer. *Serratia plymuthica* R1GC4, another PGPR, could survive better than 63-28 under stressful conditions. These studies have shown that moisture is the main limiting factor for the survival of PGPR, but it would not be economically feasible to ship peat media at higher moisture levels. Therefore, we have investigated the addition of adjuvants in the culture media and peat to enhance the survival of the bacteria. But studies of population densities of the BCAs may only give part of the picture. The threshold level of activity of the BCA should also be considered. The total populations may not be as important as the physiological activity of the BCA and whether it is producing antibiotics or siderophores in the right time, place and amount to give antagonism.

The Pathogen

One basic question that needs to be answered in any disease system is the relationship between inoculum density and disease incidence (ID/DI relationships). But from a grower's standpoint, pathogen or disease thresholds may be more useful. What are the inoculum and disease thresholds for soilless systems? What is the economic threshold - the level of inoculum or disease where the cost of control equals the potential crop loss, i.e. the point at which it is profitable to control the disease? What is the damage threshold - the level of inoculum necessary to cause economic loss on the crop? For root pathogens like *P. aphanidermatum* on cucumbers, a significant amount of root loss can occur before the yield is reduced. What is the action threshold? This is the disease level where the biocontrol agent must be applied to keep the disease from reaching the damage threshold. These are basic concepts of integrated crop protection that may be useful in soilless systems. However, if the BCA is to be used as a prophylactic from the beginning of the crop cycle, these thresholds may not be as important for grower decision making. Recently, Menzies et al. (1995) studied the effect of introduced inoculum levels of *P. aphanidermatum* in recirculating hydroponic nutrient solutions with cucumber. When as few as 22 zoospores/100 L were added, plant dry weight, fruit weight and fruit number at harvest were significantly reduced. When 2 X 10^4/ml were added, yield (weight) and number of cucumbers were reduced by over 50%. A significant negative relationship was seen between the log inoculum density added and the harvest parameters. However, the populations of the pathogen were not monitored after they were added, and the plants may have been able to tolerate and compensate for fairly high zoospore numbers that were probably present in most treatments, once secondary sporulation had occurred.

The Pathogen and the BCA Interaction

The pathogen and the BCA interact together, but the logistical problem of working with complex factorial experiments has limited the study of their interactions. Often, researchers consider the BCA and pathogen separately. An ID/DI curve is first established to find the level of the pathogen that gives approximately 50% disease. Then various population densities of the BCA are tested to find the lowest inoculum dose that gives control. This gives an idea of how the BCA performs under average conditions. But what about with high or low levels of the pathogen? Raaijmakers et al. (1995) found a quadratic relationship between the percent disease incidence of Fusarium wilt of radish in the inoculated control and the absolute percent disease reduction caused by inoculation with *Pseudomonas* spp. In other words, the maximum disease reduction was seen with inoculum densities of the pathogen that gave 50% disease in the inoculated controls. At higher disease levels, the control was less; at lower disease levels, there was not much disease to reduce. Again, this emphasizes that biocontrol is very dependent on the population level of the pathogen and this type of work should be done with soilless systems. With soilless systems there is another factor to consider. Soilless systems are assumed to start out pathogen-free, but the pathogen somehow gets introduced through contamination. In the field, the assumption is that the inoculum is already present at the start of the crop cycle. For example, what is the effect of the time of introduction of the pathogen into a previously pathogen-free recirculating system? Does an early introduction of the pathogen have the same effect as a later introduction?

Technics and methods for studying population dynamics

Detection of the Pathogen

Culture plating. Dilution-plating on selective media is the most widely used conventional technique for following the population dynamics of pathogens. Most culture media for population studies are based on the selective inhibition of unwanted contaminants by use of antibacterial antibiotics and antifungal compounds such as benomyl, PCNB (pentachloronitrobenzene) and pimaricin. Many selective media have been formulated for *Phytophthora*, *Pythium*, and *Fusarium* (Dhingra and Sinclair, 1995; Singleton et al, 1992). Other media are based on the selective enhancement of the target organism or differentiation based on colony morphology. Dilution plating methods have an advantage in soilless systems, since there are fewer total microbial populations compared to soil, and less of a problem with interfering contamination. However, plating and culturing have drawbacks, including not knowing the activity of the pathogen or what propagule is actually measured. In addition, it is difficult to quantify pathogens that are slow growing or in low numbers. The threshold of detection may be too high to measure zoosporic pathogens in recirculating systems.

Baiting. This technique increases the selectivity of recovery by using a natural substrate that will be selectively colonized by the target pathogen. This works well with zoosporic pathogens such *Pythium aphanidermatum*. Rankin (1992) used millet seed to bait and quantify the populations of *P. aphanidermatum* in rockwool, using Pythium selective media and a standard curve derived from known zoospore concentrations. Most-probable-number techniques could also be used for quantification. Baiting is more sensitive than dilution plating and can be used to target zoospores, by spatially separating the sample from the bait in water. The sensitivity can be further increased by using filtration to concentrate propagules from recirculating systems, although high levels of organic matter may reduce the efficiency of filtration. Ali-Shtayeh et al. (1991) concentrated samples of

irrigation water onto filters to test for the presence of *Pythium* and *Phytophthora* using ELISA test kits.

Serology ELISA (Enzyme-Linked Immunosorbent Assay) kits based on monoclonal antibodies that detect and quantify *Pythium, Phytophthora*, and *Rhizoctonia* are now marketed by Neogen Co., Lansing, MI. These genus-specific kits have been tested in container nurseries (Benson, 1991) and irritation water (Ali-Shtayeh et al. 1991). Dip stick and dot-blot applications of ELISA have also been developed and are easier to carry out in the field without special equipment. They are faster, more sensitive than dilution plating and can detect the pathogen in host tissue. These assays are also quantitative. These assays are very useful for disease diagnosis, but may not be as useful for population dynamic studies because of the cost per sample and the large number of samples needed. Another drawback, until recently, has been the lack of specificity at the species level. Recently, species-specific monoclonal antibodies have been developed for *Phytophthora cinnamomi* (Cahill and Hardham, 1994) and *Pythium ultimum* (Avila et al. 1995).

DNA techniques. The development of PCR (Polymerase Chain Reaction) combined with RFLP (Restriction Fragment Length Polymorphisms) has revolutionized the field of pathogen detection. PCR can amplify specific DNA sequences of a pathogen in a sample. Then these sequences can hybridize with DNA probes of known strains or can be detected by digesting the amplified DNA with endonucleases. The digested DNA is separated on gels and identified based on RFLP patterns. Within the last five years, research in this area has exploded, with PCR primers being developed for the detection of soil borne pathogens such as *Gaeumannomyces graminis* (Henson et al. 1993), *Verticillium* (Hu et al. 1993), *Monosporascus* spp. (Lovic et al. 1995), and *Phytophthora* (Lee et al. 1993). The two main applications of this technology have been in the area of population genetics and disease diagnosis. The extreme sensitivity of the method makes it possible to detect the pathogen with just a few cells in a sample. The ability to distinguish among individual strains or races makes it possible to look at genetic relationships among isolates. Primers can be derived from conserved or variable regions of the DNA, resulting in genus specific, species specific, or even strain specific detection methods. For example, Edel et al. (1995) were able to distinguish strains of *Fusarium oxysporum* using amplified IGS (intergenic spacer) regions of the rDNA. A species-specific probe for *Pythium ultimum* has been developed from the amplified region of the ITS (internal transcribed spacer) region of ribosomal DNA (Levesque et al. 1994) This same technology is being used to develop reverse dot-blot kits to detect other *Pythium* species on one filter. However, the use of these techniques for population dynamic studies is hindered because it is difficult to quantify the amount of DNA originally present in the sample after amplification has taken place. PCR is presently not amenable for population dynamics studies because of the labor involved in amplifying the samples, the cost of materials, and the limitations in the number of samples that can be processed in one day. However, these costs will come down as the technology develops. Another limitation with the technique has been the need to use RFLP to analyze the amplified DNA. New technologies may eliminate the need for RFLP. Recently, a method for the detection of PCR amplification products using emitted fluorescence from species-specific probes has been reported (Knorr et al., 1995).

Detection of the Biocontrol Agent

Fungal Biocontrol Agents. Some of the methods described above can also be used to detect and quantify fungal biocontrol agents. Strains can also be mutated for tolerance to fungicides such as benomyl (Ahmad and Baker, 1987) and detected with a selective medium. Transformation of *Trichoderma harzianum* with the GUS (ß-glucuronidase) reporter gene from *Escherichia coli* enabled Green and Jensen (1995) to follow the population growth and activity of the fungus in the

rhizosphere. Expression of this enzyme can be detected with a substrate that turns a blue color and can be quantified spectrophotometrically or visualized microscopically.

Bacterial Biocontrol Agents One of the most common methods of detection is to use marked strains resistant to antibiotics such as naldixic acid or rifampicin. These can then be quantified on media containing these antibiotics. However, some bacteria may be nonculturable under certain conditions and their populations may be underestimated. Serological techniques such as ELISA or immunofluorescence can also be used and do not require culturing of the bacterial strain. However, the most exciting work is on measuring not only the populations of BCA bacteria, but also their activity by using gene reporter systems. This information can be used to indirectly measure phenotypes that may be involved in antagonism, such as antibiosis or siderophores. By fusing a promoterless reporter gene to a promoter of a genomic region involved in biocontrol activity, the expression of the reporter gene can be measured to give an indirect indication of the expression of the biocontrol phenotype. Some commonly used systems are the ß-glucuronidase gene, the ice nucleation gene and the lux gene. For example, to measure siderophore expression, Loper and Lindow (1994) fused a promoterless ice nucleation gene (inaZ) to the iron-regulated promoter of pyoverdine production in *Pseudomonas syringae*. They showed expression of iron-responsive ice nucleation activity in the rhizosphere and phyllosphere of beans, and the addition of Fe^{+3} decreased transcription of the reporter gene. By comparing the expression of an intact ice-nucleation gene not under control of the iron promoter, they showed that iron was not as limiting in microsites as had been hypothesized. The production of the antibiotic phenazine in *Pseudomonas aureofaciens* in the spermosphere was measured with an ice-nucleation gene reporter system by Georgakopoulos et al. (1994). They showed that phenazine production was expressed by bacteria on the seed surface 12-36 hrs after planting. There was high variability among the population for the expression of the phenazine gene, indicating that not all bacteria in the population produced the antibiotic. Higher levels of expression were seen on wheat seeds than cotton seeds. Expression of phenazine production on seeds was not affected by initial inoculum level, matric potential or soil type. This indicates that the level of nutrients in seed exudates may play a greater role in phenazine production than edaphic factors.

However, caution must be taken that the reporter gene systems do not change the ecological fitness of the strain. For example Mahaffee et al. (1995) compared three methods of monitoring bacterial populations in a field experiment. They introduced a wild type strain of *Pseudomonas fluorescens*, a spontaneous rifampicin resistant mutant, and a strain engineered with the bioluminescence (lux) gene into a cucumber field with seed treatments. The wild type strain was monitored with immunofluorescent colony staining (IFC). The rif mutant and the lux strain were measured by spiral plating. The highest populations were measured with the wild type strain detected with the IFC method, but much lower populations of the rif or lux strains were detected, suggesting lower ecological fitness.

Modelling population dynamics of pathogens and biocontrol agents

Useful information about the interactions between pathogens and biocontrol agents can be obtained by mathematical modelling. Although this has not been done specifically for soilless systems, some examples for soilborne pathogens will demonstrate its potential.

Biocontrol Efficiency

A simple example of modelling is to calculate the efficiency of the BCA in biocontrol or how much inoculum of the BCA is necessary to control one propagule of the pathogen. Adams (1990) asked

this questions with some mycoparasites. He selected some examples from the literature, looked at the inoculum density of the BCA and the inoculum density of the pathogen, and calculated the efficiency ratio - the population density of the BCA/population density of the pathogen. These values varied from 1.6 for the *Sporidesmium sclerotivorum-Sclerotinia minor* system, to 15 for the *Pythium nunn-Pythium ultimum* system, to 50,000 for *Trichoderma* spp.-*Rhizoctonia solani*.

Effect of the Biocontrol Agent on Inoculum Density/Disease Incidence (ID/DI) Curves

Other researchers have looked at the effect of BCAs on the inoculum density/disease incidence curve, a relative indication of the efficiency of the pathogen inoculum. Schneider (1984) looked at the effect on non-pathogenic isolates of *Fusarium oxysporum* on *F. oxysporum* f. sp. *apii* in celery fields. He recognized that the typical ID/DI curve resembled the hyperbolic saturation curve of a first order enzyme kinetic equation plot of substrate concentration vs. enzyme velocity. Using a Lineweaver-Burk inverse plot, the Km (substrate concentration that gives 1/2 the maximum velocity, Vmax) can be calculated from the x intercept. In the same way, an inverse plot of ID/DI curves could give a value of ID that gives 1/2 the maximum level of disease. Schneider demonstrated that some of the non-pathogenic *Fusarium oxysporum* increased the ID level of the pathogen needed to cause 1/2 the maximum level of disease. Thus, the BCA reduced the efficiency of the pathogen. Mandeel and Baker (1991) looked at the relationships between *Fusarium oxysporum* f. sp. *cucumerinum* and two isolates of non-pathogenic *Fusarium oxysporum*, C14 and C5. They performed factorial experiments with varying inoculum levels of the pathogen and the BCA. They examined the effects of the BCA on the pathogen by adding increasing levels of C14, and showed that the slope of the ID/DI curve of *F. o. cucumerinum* was significantly reduced. The effect of the pathogen on the BCA could be ascertained by measuring root colonization by C14. This strain could colonize the cortex and could be distinguished from the pathogen on selective media. Increasing levels of the pathogen decreased the efficiency of the C14 inoculum to colonize the root. The authors concluded that the BCA and pathogen were competing for infection sites, although other experiments indicated that systemic induced resistance could also be a mechanism. Johnson (1994) proposed a negative exponential model to describe the relationship between pathogens and biocontrol agents. This is a derivation of the multiple infection model used in plant pathology, proposed by Van der Planck (1975). This model assumes that the efficiency of the pathogen is highest at lower inoculum densities. As the inoculum density increases multiple infections of the same individual occur, so the slope of the line decreases and an asymptote (the maximum proportion of the host infected) is reached. The same reasoning can be used with biocontrol agents and pathogens. As the inoculum density of the BCA increases, there is a greater chance of one pathogen propagule being attacked or antagonized by more than one BCA unit. Therefore, the efficiency of the BCA decreases at high inoculum densities. Johnson used this $\log_n 1/1-Y$ transformation and a gompit transformation on the data of Mandeel and Baker. This showed that the maximum level of disease control with C14, a root cortex colonizer, was much less than with C5, which did not colonize the root. By linearizing these curves, he calculated the efficiency of the two BCAs and found they were similar. He concluded that the degree of biocontrol depends on the density of the BCA, the density of the pathogen, the efficiency of the BCA in attacking the pathogen, and the maximum level of the pathogen that can be attacked, i.e. the maximum level of disease control expected under ideal conditions with the highest levels of the BCA.

However, none of these models take into account the factor of time. Gilligan (1990) fitted non-monotonic models to the progress curves of pathogens and BCAs from several biocontrol systems in the literature, including *Pythium ultimum-Laetisaria arvalis*, *P. ultimum-Pythium nunn*, and the colonization of planting media by *Trichoderma viride*. In the non-monotonic model, there is a rise in the population density of the pathogen or BCA over time, followed by a fall to an

asymptote. None of the BCAs affected the rate parameters of the models, but did affect the asymptotic and locational parameters.

Conclusions

Many important questions can be answered by studying the population dynamics of the biocontrol agent and the pathogen in soilless systems. How well can the BCA survive and persist in formulations and soilless media? How much BCA needs to be applied and at what time to give maximum disease control? How does the pathogen spread in space and time in the soilless system? What are the disease thresholds for soilless systems? Can a decision-based control program be developed to base application of the BCA on attaining a certain population density of the pathogen? Finally, by looking at the dynamics between the BCA and pathogen, can we predict the efficiency of the BCA and the maximum level of disease control that can be attained? The answers to these questions await further research.

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POPULATION DYNAMICS OF BIOCONTROL AGENT *BACILLUS SUBTILIS* IN CLOSED HYDROPONIC PLANT CULTIVATION SYSTEMS AFTER APPLICATION OF DIFFERENT CELL NUMBERS

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Abstract

Hydroponic systems are increasingly important in glasshouse cropping. However, root pathogen control is a crucial point in closed hydroponic cultures and is yet a problem far from being resolved. Since fungicides are often not available or authorized for pathogen control; the possibilities of biological control have been investigated.

A major problem in the control of root pathogens by microbiological agents is the colonization of root systems by introduced microorganisms. Thereby, environmental conditions appear to be important factors affecting root colonization.

In this study, we investigated the colonization of the tomato root system by *Bacillus subtilis,* in closed hydroponic systems, after inoculation of *B. subtilis* at different densities. The introduced biocontrol agent *B. subtilis* FZB A, isolated from the soil, is supposed to have an antifungal and plant health promoting effect against different soil borne pathogens.

In a closed Nutrient Film Technique (NFT) system the colonization of roots of tomato seedlings in rockwool cubes was studied after bacterization with 20 ml of spore suspension of *B. subtilis* containing 10^7 ; 10^6 or 10^5 cfu ml^{-1}. *B. subtilis* was also added to the nutrient solution so that 1 ml of nutrient solution contained 10^7 , 10^6 or 10^5 cfu at the beginning of the vegetation period. Population dynamics were determined on the roots and in the circulating nutrient solution.

In addition, the colonization of upper and lower parts of the root systems of tomato plants grown in quartz sand was estimated, after bacterization of seeds and seedlings.

Constant colonization of the ectorhizosphere in all treatments could be observed for 10 weeks. With the introduction of lower bacterial densities to the seedlings, it was necessary to increase the bacteria in the nutrient solution to reach higher and constant root colonizations. An intensive and constant root colonization was only found after introducing higher cell numbers to the seedlings. In all treatments, the density of *B. subtilis* in the nutrient solution decreased during the course of the experiment.

The roots of the plants were colonized uniformly, showing that the bacterial populations replicated along with root growth provided that enough water was present around the roots. This can be an advantage in soilless plant cultivation since root colonization is a supposition for disease suppression by rhizobacteria.

Introduction

The cultivation systems and techniques in horticulture have changed considerably over the last decades. As a result there is a rising interest in the application of hydroponics and substrate cultures (Van Assche and Vangheel, 1994). Current glasshouse horticulture is based on high inputs of pesticides, despite increased application of biological control methods (Welles, 1992). Also, fungi are still chemically controlled.

Changes of the root medium led to changes of the micro and meso fauna and flora in the rhizosphere and on the hydroplant (Van Assche and Vangheel, 1994). In hydroponic systems, the chance of epidemics increases, if pathogens penetrate into the system, where they multiply and spread, due to the absence of potential antagonists (Van Assche and Vangheel, 1989).

The biological control of plant diseases and the microbial ecology of the rhizosphere in hydroponic systems has received scant attention (Van Peer and Schippers, 1989).

Therefore, especially the possibilities of biological control of root pathogens through introduction of antagonistic organisms in closed hydroponic cultures have to be investigated.

It is generally assumed that root colonization by introduced bacteria is essential for biocontrol of root pathogens (Weller, 1988; Digat, 1988). Understanding the dynamics of root colonization by specific microorganisms of the rhizoplane is essential for the development of biological control of root pathogens.

Various studies implicate that bacteria such as *Pseudomonas sp.* or *Bacillus sp.* may play a role in the reduction of diseases. The introduced biocontrol agent *Bacillus subtilis* in this report, isolated from the soil, has an antifungal and plant health promoting effect against different soil borne pathogens such as *Rhizoctonia solani, Fusarium spp.* or *Alternaria radicina* in soils or horticultural substrates (Hentschel and Bochow, 1990; Obieglo *et al.* 1990; Bochow, 1995).

The aim of this study was to prove the ability of *B. subtilis* to colonize roots and to survive in hydroponic systems, and to find out whether this depends on the introduced bacterial densities and on the mode of application. The colonization of tomato roots was studied in a closed hydroponic system with recirculating nutrient solution and in a sand culture system where water percolation was excluded.

Materials and methods

Plant cultivation in hydroponic systems

Tomato seedlings (Lycopersicon lycopersicum (L.) Karst. ex Farw. cv. Counter) were grown in rockwool cubes and transferred to a closed hydroponic system (NFT) at the start of the five-leaf-stage. Ten uniformly sized tomato seedlings were planted in each of three separately closed systems per treatment, set up in a glasshouse. The experiment was finished 10 weeks after planting.

In a second glasshouse experiment, the plants were transferred to 7 l-pots containing quartz sand as substrate. Two plants in the two-leaf-stage were planted in each of the 19 pots for each treatment. *B. subtilis* was reisolated at five dates after planting. The quartz sand was moistened before the plants were transferred into the pots. Nutrient solution was supplied by cotton wicks.

In both trials, the nutrient solution was composed according to Sonneveld and Straver (1988).

Plant bacterization

In experiment I, the tomato plants grown in rockwool cubes were inoculated with *B. subtilis* strain FZB A and in treatment I/6 with strain FZB E at the start of the four-leaf-stage. The strains were obtained from FZB GmbH (Research Centre for Biotechnology Berlin, Germany) in granulated form. Final adjustment of bacterial spore suspensions was therefore very simple. Each plant was inoculated with 20 ml bacterial suspensions (10^5, 10^6, 10^7 or 10^9 cfu ml^{-1}) eight days before planting in the closed hydroponic system. In addition, *B. subtilis* was added to the nutrient solution six days after planting so that 1 ml of the nutrient solution contained final concentrations of 10^7, 10^6, 10^5 cfu (Tab. 1). The spores of *B. subtilis* were heated (15 min at 60°C) before bacterization of the nutrient solution.

In two treatments, only the seedlings were inoculated with *B. subtilis* spore suspensions at concentrations of $2x10^8$ or $2x10^{10}$ cfu ml^{-1}. Furthermore, the population density of the *B. subtilis* strain FZB E was studied after inoculation of tomato seedlings and nutrient solution in treatment I/6 with a concentration concentration of $2x10^8$ cfu $plant^{-1}$ and 10^7 cfu ml^{-1} of nutrient solution.

In experiment II, seedlings were planted in pots at the two-leaf-stage and bacterized at the start of the four-leaf-stage, six days after planting with, 20 ml spore suspensions of *B. subtilis* strain FZB A ($2x10^5$ or $2x10^7$ cfu $plant^{-1}$). The course of root colonizations with two different *B. subtilis* strains (FZB A and FZB E) was investigated after seed bacterization. Ten tomato seeds were inoculated with 10 µl spore suspensions at a concentration of 10^{10} cfu ml^{-1} to establish a population size of $1x\ 10^7$ cfu $seed^{-1}$.

Experimental details are given in table 1 for both experiments presented here.

	Experiment I (closed hydroponic system)	Experiment II (pots containing quartz sand)
Vegetation period	06.07 (planting) - 14.09.1994	09.11 (planting) - 19.01.1995
Application of *Bacillus subtilis* (strain FZB A)	**I/1** $2x10^8$ cfu $plant^{-1}$ 10^7 cfu ml^{-1} nutrient solution	**II/1** 10^7 cfu $seed^{-1}$
	I/2 $2x10^7$ cfu $plant^{-1}$ 10^6 cfu ml^{-1} nutrient solution	**II/2** 10^7 cfu $seed^{-1}$ (strain FZB E)
	I/3 $2x10^6$ cfu $plant^{-1}$ 10^5 cfu ml^{-1} nutrient solution	**II/3** $2x10^5$ cfu $plant^{-1}$
	I/4 $2x10^8$ cfu $plant^{-1}$	**II/4** $2x10^7$ cfu $plant^{-1}$
	I/5 $2x10^{10}$ cfu/$plant^{-1}$	
	I/6 $2x10^8$ cfu $plant^{-1}$ (strain FZB E) 10^7 cfu ml^{-1} nutrient solution	

Table 1 : Experimental details

Bacterial colonization of rhizosphere and nutrient solution

Strain *B. subtilis* FZB A is a selected streptomycin-resistant mutant, while strain FZB E is chloramphenicol resistant. Colonization of the rhizosphere with *B. subtilis* was determined before and after bacterization of the nutrient solution in the NFT-system at the beginning and at several times throughout the course of the experiment. Root samples (1 g from each replication) were shaken vigorously for 45 min in glass tubes containing 10 ml of sterile 0.3 % NaCl-solution and five glassbeads. Serial dilutions were plated on selective media NI (Merck, 7881) with 900 mg l^{-1} streptomycin-sulphate (SIGMA S-9137) or 5 mg l^{-1} chloramphenicol. On each petri dish, 50 µl of bacterial suspensions were placed (five replications). Cells of *B. subtilis* were counted after incubation at 30 °C for 24 hours.

Cell numbesr of *B. subtilis* in the circulating nutrient solution were determined asdescribed above.

The colonization of *B. subtilis* on different root sections was studied on tomato plants grown in pots. Root samples were collected (approximately 1 g fresh weight) from the upper part, from two different points in the middle and from the lower part of the root systems (especially root tips) of four tomato plants, in order to determine the density of *B. subtilis*. The quartz sand was systematically removed from the root samples.

The numbers of reisolated *B. subtilis* cells were determined before and after heating (5 min at 90°C) of the dilution samples. The vegetative cells of *B. subtilis* were killed through heating.

Fresh and dry weights of root samples were recorded and the *B. subtilis* populations were calculated per root dry weight.

Results

Colonization of roots and nutrient solution in NFT

The colonization of roots with *B. subtilis* strain FZB A was determined during the vegetation period (10 weeks) of tomato in a closed hydroponic system (NFT). Table 2 shows the density of *B. subtilis* on the root surface before bacterization of the nutrient solution (six days after planting) and eight days after the second application of *B. subtilis*.

As shown in table 2, *B. subtilis* could not be detected on root tips of tomato plants six days after planting when inoculated with $2x10^6$ cfu plant^{-1}, suggesting that a low number of *B. subtilis* cells applied to seedlings is inefficient to colonize the growing root in this hydroponic system. The density of *B. subtilis* even after application of $2x10^7$ cfu plant^{-1} was very low on root tips at this stage. Additional introduction of bacteria in the nutrient solution was necessary to reach higher and constant root colonization in both treatments. The population density on the root surface of treatments I/1, I/2 and I/3 were significantly different (LSD = 1.35, p = 0.05) before and after the bacterization of the nutrient solution.

In all treatments, a constant colonization of the ectorhizosphere could be observed for 10 weeks during the vegetation period (Fig. 1).

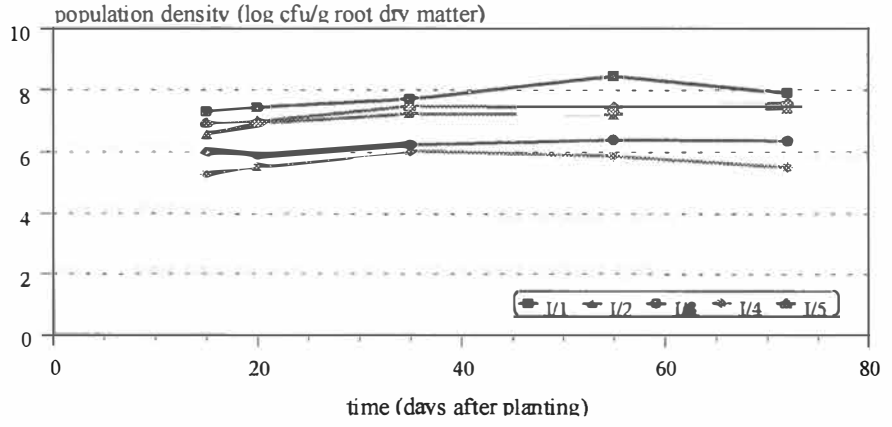


Figure 1 : Population density of *B. subtilis* (strain FZB A) on the tomato root surface in a closed hydroponic system as dependent on the applied cell number (I/1 2×10^8 cfu plant^{-1} + 10^7 cfu ml^{-1} nutrient solution (n.s); I/2 2×10^7 cfu plant^{-1} + 10^6 cfu ml^{-1} n.s.; I/3 2×10^6 cfu plant $^{-1}$ + 10^5 cfu ml^{-1} n.s.; I/4 2×10^8 cfu plant^{-1}; I/5 2×10^{10} cfu plant^{-1})

The population density on the root was significantly different (LSD = 1.25; p = 0.05) in experiment I between treatment 1 compared to 2, 3, 4;treatment 3 compared to 4, 5 and treatment 4 compared to 5, 6.

Figure 2 (A) and (B) show that no significant differences exist between the *B. subtilis* strains FZB A and E concerning the population densities on the root surface and in the nutrient solution.

The density of *B. subtilis* on different parts of the root system was determined in experiment II employing tomato plants grown in pots with quartz sand.

Treatment	6 d[1]	15 d	20 d	35 d	55 d	63 d	72 d	mean
I/1	5.9	7.32	7.45	7.72	8.45	-	7.9	7.89
I/2	2.06	6.57	6.93	7.25	7.22	-	7.4	7.08
I/3	nd	5.98	5.93	6.23	6.4	-	6.35	6.14
I/4	5.83	5.29	5.52	6.03	5.88	-	5.5	5.75
I/5	6.21	6.95	7.0	7.49	7.45	-	7.58	7.29
I/6[2]	5.11	7.82	7.75	8.01	-	7.48	-	7.71

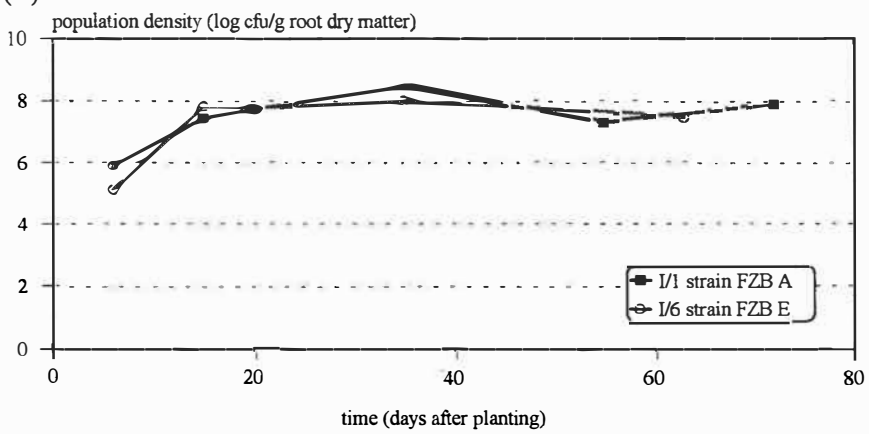
[1] colonization before bacterization of the nutrient solution
[2] *B. subtilis* strain FZB E. nd - not detectable

Table 2 : Colonization of the root surface (log cfu/g root dry matter) after application of different cell numbers of *B. subtilis* strain FZB A and E to seedlings and nutrient solution in a closed hydroponic culture.

After planting. colonization of the root surface with *B. subtilis* was studied at five different periods in time (3rd. 4th. 5th. 8th and 9th week). The density of *B. subtilis* on the lower (root tips) and upper parts of the root system were determined from the third week onwards and the middle part of the root system was included in the measurements thereafter. The results indicate that colonization of the tomato root surface with *B. subtilis* FZB A and FZB E is possible after bacterization of seeds as well as seedlings (Tab. 3 and Fig. 4).

In all treatments the density of *B. subtilis* in the nutrient solution declined during the course of the experiment (Fig. 3).

(A)



(B)

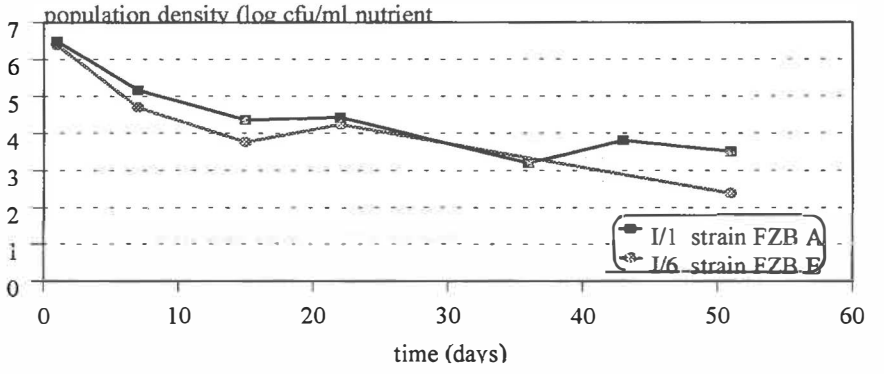


Figure 2 : Population densities of two B. subtilis strains on the root surface of tomato plants (A) and in the nutrient solution (B) of a closed hydroponic system (NFT). after application of the same cell numbers (2×10^8 cfu plant^{-1} + 10^7 cfu ml^{-1} of nutrient solution).

Figure 4 shows that the mean density of *B. subtilis* on the root surface of plants bacterized at the beginning of the four-leaf stage was lower after application of $2x10^5$ cfu plant^{-1} when compared to $2x10^7$ cfu plant^{-1} in all experiments. Differences in the population density of both treatments decreased with time. but in treatment II/3 the density rapidly declined at the 9th week (Fig. 4). *B. subtilis* was detectable only on the lower part of the root system (root tips) at this time (Tab. 3). During the first 5 weeks of the growing season the differences in mean density of *B. subtilis* on roots were not significant between all treatments (LSD = 1.57. p = 0.05).

In treatment II/2. *B. subtilis* could not be reisolated from the lower part of the root system (root tips) from the 5th week on and also could not be determined in treatments II/1 and II/3 from the upper and middle parts of the root systems in the 9th week (Tab. 3).

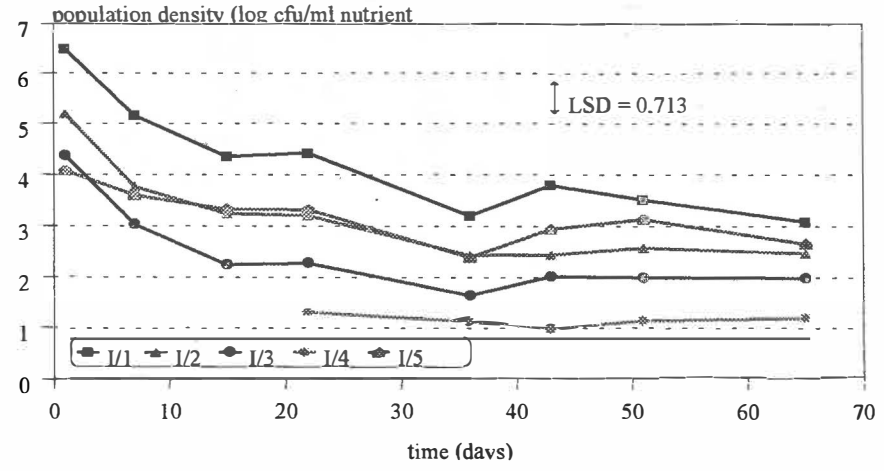


Figure 3 : Population density of *B. subtilis* (strain FZB A) in the nutrient solution (n.s) of a closed hydroponic system depending on the applied cell number (I/1 2×10^8 cfu plant^{-1} + 107 cfu ml-1 n.s; I/2 2×10^7 cfu plant $^{-1}$ + 10^6 cfu ml^{-1} n.s; I/3 2×10^6 cfu plant $^{-1}$ + 10^5 cfu ml^{-1} n.s; I/4 2×10^8 cfu plant^{-1}; I/5 2×10^{10} cfu plant^{-1})

Figure 5 shows the population density on different root sections during the first five weeks after planting. The colonization was uniform and independent on the treatments during this time.

Table 3 shows the population density of *B. subtilis* after heating of the samples. The reisolated part of vegetative cells of *B. subtilis* was especially high within the first five weeks.

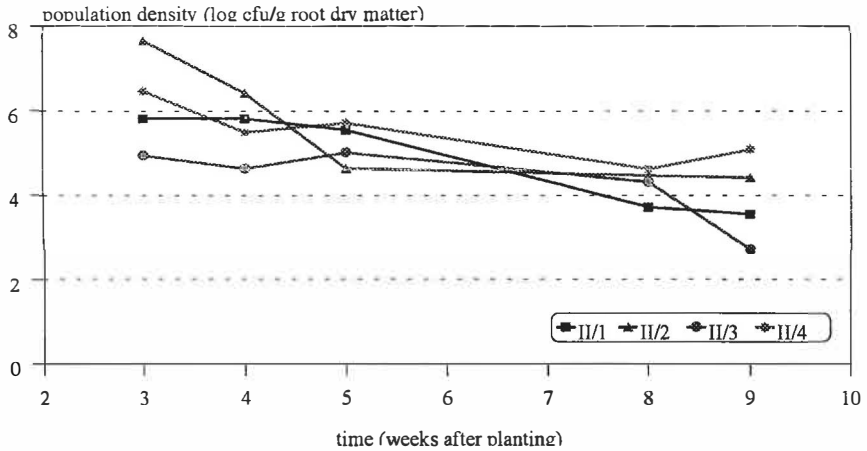


Figure 4 : Mean population density of *B. subtilis* (strain FZB A in treatments II/1 1×10^7 cfu seed^{-1}. II/3 2×10^5 cfu plant^{-1} and II/4 2×10^7 cfu plant^{-1}; FZB E in treatment II/2 1×10^7 cfu seed^{-1}) on the tomato root surface after bacterization of seeds and seedlings.

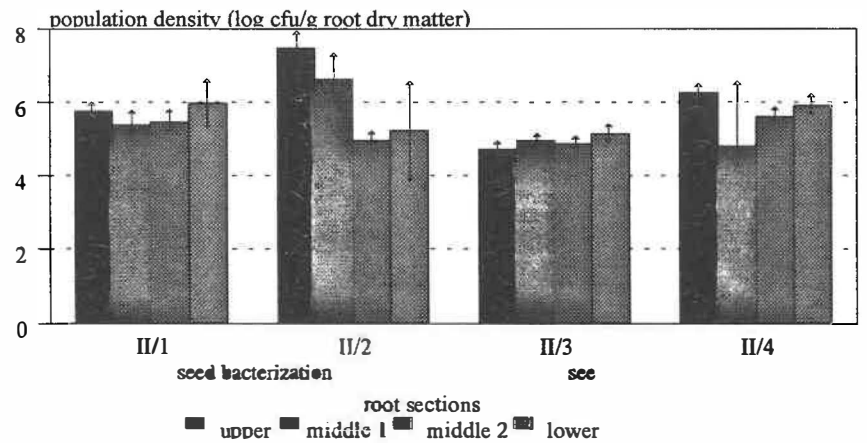


Figure 5 : Population density of *B. subtilis* (strain FZB A in treatments II/1 1×10^7 cfu seed^{-1}. II/3 2×10^5 cfu plant^{-1} and II/4 2×10^7 cfu plant^{-1}; FZB E in treatment II/2 1×10^7 cfu seed^{-1}) on different sections of the root surface in three replications. five weeks after planting.

Time week	Treatment	Upper part			Middle section 1			Middle section 2			Lower root sys-tem (root tips)		
		non heated	heated	vegetati ve cells (%)	non heated	heated	vegetati ve cells (%)	non heated	heated	vegetati ve cells (%)	non heated	heated	vegetati ve cells (%)
3rd	II/1	6.05	5.75	**50**	*			*			5.35	5.19	**30**
	II/2	7.94	5.38	**99**							5.43	-	**100**
	II/3	4.48	5.33	**0**							5.35	5.17	**66**
	II/4	6.56	6.46	**20**							6.32	5.53	**83**
4th	II/1	4.82	4.47	**54**	4.82	4.53	**48**	4.51	4.51	**0**	6.40	5.22	**93**
	II/2	6.16	5.16	**90**	6.94	5.03	**98**	5.06	5.06	**0**	5.33	4.42-	**87**
	II/3	4.69	5.05	**0**	4.93	4.77	**30**	4.39	-	**100**	4.10	5.09	**100**
	II/4	5.78	5.73	**10**	nd	-	-	5.26	5.64	**0**	5.24		**28**
5th	II/1	5.71	5.71	**0**	5.60	5.29	**51**	5.72	5.40	**52**	3.37	-	**100**
	II/2	4.94	5.09	**0**	4.86	4.71	**29**	4.78	4.88	**0**	nd	-	-
	II/3	4.88	4.63	**43**	4.96	4.71	**43**	5.07	4.94	**25**	5.20	4.60	**75**
	II/4	6.11	5.93	**35**	5.10	5.23	**0**	5.76	5.73	**6**	5.03	4.68	**55**
8th	II/1	4.09	3.61	**66.6**	4.05	4.05	**0**	-	-	-	3.95	4.05	**0**
	II/2	4.99	4.99	**0**	4.32	4.65	**0**	4.70	4.70	**0**	-	-	-
	II/3	3.84	3.84	**0**	4.76	-	**100**	4.30	4.30	**0**	4.40	-	**100**
	II/4	5.20	4.87	**52.9**	3.89	4.20	**0**	-	-	-	-	-	-
9th	II/1	nd	-	-	nd	-	-	nd	-	-	4.16	-	**100**
	II/2	4.78	4.78	**0**	4.24	4.24	**0**	4.45	4.45	**0**	nd	-	-
	II/3	nd	-	-	nd	-	-	nd	-	-	3.32	-	**100**
	II/4	5.51	5.51	**0**	5.12	5.12	**0**	4.43	4.43	**0**	4.02	4.02	**0**

*(The density of *B. subtilis* on the middle part of the root system was not determined three weeks after planting).
nd - not detectable

Table 3: Density of *B. subtilis* (log cfu g^{-1} root dry matter) on the surface of different root sections and percentages of vegetative cells.

Discussion

Our investigations of the population density of *B. subtilis* on roots of tomato plants growing in a closed hydroponic system showed a constant and intensive colonization in all treatments of experiment I for 10 weeks. However. the population density on the root surface was significantly different according to the introduced cell number. The application of a lower bacterial cell number to seedlings led to an insufficient colonization. and an additional introduction of bacteria into the nutrient solution was necessary to reach the desired constant root colonization (Fig. 1. Tab. 2). Bacterization of the solution with 10^7 cfu ml^{-1} nutrient solution achieved a nearly 100-fold higher density on the root surface compared with a non bacterization of the solution (Tab. 2. Fig. 1).

The bacterization of seedlings only with a high cell number of *B. subtilis* (treatment I/5) resulted in a similar density as the bacterization of seedlings and nutrient solution (treatment I/1 or I/6). Part of the applied cell number to seedlings in treatment I/5 disappeared in the nutrient solution after planting (Fig. 3). It seems that more cells of *B. subtilis* could not be established on the root surface.

Van Peer and Schippers (1989) studied the colonization of roots with *Pseudomonas spp.* recovered from roots varying in numbers between 2.6×10^5 and 2.4×10^6 cfu g^{-1} root fresh weight. In our experiments *B. subtilis* was reisolated. and for example in treatment I/1 the numbers varied between 1.58×10^4 and 5.63×10^7 cfu g^{-1} root fresh weight. Species of the genus *Bacillus* are

usually not considered as typical rhizosphere inhabitants (Campbell. 1990; Freier *et al.*. 1990). The composition of the microbial community in hydroponic systems has been shown to be less complex than in soils (Van Peer and Schippers. 1989; Price. 1976). Therefore. the conditions appear to be positive for the development of *B. subtilis* in hydroponics since in addition. the recirculating nutrient solution enables the rapid movement of an introduced bacteria in this system. From the view point of the suppression of the activity of other microorganisms the early introduction of a high number of beneficial bacterial cells can be seen as an advantage. but it has yet to be proved whether disease suppression can be achieved.

The results of the density of *B. subtilis* in the nutrient solution showed a rapid decrease of the cell number in all treatments immediately after bacterization (Fig. 3). The densities were dependent on the applied cell number. The conditions for *B. subtilis* in the circulating nutrient solution were probably not favourable so the vegetative cells moved to the roots. In this respect attention has to be drawn to the fact that the applied spore cells of *B. subtilis* were heat-activated in order to accelerate germination.

The first experiment included investigations on the population density of a second *B. subtilis* strain (FZB E. treatment I/6) in the hydroponic system. Figure 2 (A) and (B) show no clear cut differences in the population density between *B. subtilis* strains FZB A (treatment I/1) and E (treatment I/6) on the root surface or in the nutrient solution after application of the same cell number ($2x10^8$ cfu $plant^{-1}$ and 10^7 cfu ml^{-1} nutrient solution). However. the cell number of strain FZB E decreased rapidly in the nutrient solution at the end of the culture time. and could not be recovered from the root surface 9 weeks after inoculation. These results strongly suggest different properties of the *B. subtilis* strains employed concerning their persistence in the hydroponic system as well as their abilities to colonize tomato roots.

In a second experiment. the colonization of different sections of the root system with *B. subtilis* was investigated. Strains FZB A and E colonized the growing roots after seed inoculation (Fig. 4). *B. subtilis* FZB A varied in numbers between 6.6×10^5 and 3.6×10^3 cfu g^{-1} root dry weight and FZB E varied between 2.63×10^4 and 4.36×10^7 cfu g^{-1} root dry weight. *B. subtilis* colonized various sections of the growing root system at a relatively uniform level during five weeks after planting (Fig. 5). Turner and Backman (1991) have shown that seed bacterization of peanuts with *B. subtilis* also resulted in a consistent colonization of the roots in soil.

B. subtilis strain FZB E. however. could not be recovered from the lower root system (root tips) five weeks after planting (Tab. 3). It seems that this strain colonized especially the upper and middle part of the root systems. Field experiments with peanuts. bacterized with *B. subtilis*. showed that colonization was greatest in the upper taproot region. and population decreased towards the tip of the taproot (Turner and Backman. 1991).

The colonization of root and rhizosphere is strongly affected by the percolation of water (Davies and Whitbread. 1989). It has been shown that the movement of water influences the distribution of rhizobia and pseudomonads in the soil and rhizosphere (Bahme and Schroth. 1987; Hamdi. 1971). In this respect conditions in a recirculating nutrient solution should be optimal.

B. subtilis strain FZB A could not be detected on the upper and middle part of the root system 9 weeks after planting (Tab. 3). It appears possible that *B. subtilis* was displaced by other microorganisms.

The application of different cell numbers ($2x10^5$ or $2x10^7$ cfu $plant^{-1}$. treatments II/3 and II/4) of *B. subtilis* to seedlings did not result in a significantly different density on the root surface (five weeks after planting). However. the application of a lower cell number of *B. subtilis* did not allow for the reisolation from the upper and middle part of the root system 9 weeks after planting. It seems that populations of *B. subtilis* decreasing below a certain density (treatments II/1 and II/3) are unable to survive and to compete with other microorganisms. Therefore. it is necessary to achieve a constant and intensive colonization of the root system and to treate plants with a high cell number of bacteria. To realize the disease reducing potential and the promotion of plant growth by

antagonistic rhizobacteria it is necessary to increase the distribution and density of the antagonists on the whole root system (Bahme *et al.*. 1988).

In the literature. failure of etablishing biocontrol agents in the soil have been reported frequently (Davies and Whitebread. 1989; Suslow. 1982; Kloepper and Schroth. 1981). A reason for this may be that in most cases plant growth promoting and antagonistic microorganism have been selected without paying attention to their ability to colonize the root system (Schroth and Hancock. 1981; Suslow. 1982).

Our results suggest that seed bacterization with *B. subtilis* allows for a rapid colonization of the growing roots. A combination of seed treatment with seedling bacterization could give even more security for attaining a more uniform and reliable alteration of the rhizosphere. but this is yet to be proven.

In experiment II. the proposed production of vegetative cells of *B. subtilis* through the heating of samples was investigated. The results showed that the percentages of vegetative cells were different between and within treatments ranging from 0 to 100% (Tab. 3). Most vegetative cells were recovered from roots during the first five weeks. Therefore. it appears that *B. subtilis* was more active during this time but so far we have little information on which ecological conditions are important for achieving high biological activity.

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COMPARISON OF THE SURVIVAL KINETICS OF A STRAIN OF *PSEUDOMONAS PUTIDA* IN THE RHIZOSPHERE OF TWO DIFFERENT PLANT SPECIES

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Abstract

Fluorescent pseudomonads suppress various soilborne diseases. Their efficiency in biological control is related to both their antagonistic activities and their rhizosphere competence. Knowledge about bacterial traits promoting root colonisation is lacking. A previous study has shown that the fluorescent pseudomonads associated to roots of flax and tomato differed in their ability to utilise specific organic compounds. A strain of *P. putida* (LR228) representative of the fluorescent pseudomonads associated to roots of flax has been chosen to compare its survival in the flax rhizosphere with that in the tomato rhizosphere.

The plants were grown in non gnotobiotic conditions in soil. A spontaneous mutant resistant to rifampicine (LR228r4) was used to study the survival kinetics.

P. putida LR228r4 survived significantly better in the flax rhizosphere than in the tomato rhizosphere. Twenty days after bacterial inoculation, the density of LR228r4 was 6.2 times higher in the flax rhizosphere than in tomato rhizosphere. Since the carrying capacity of both rhizosphere were similar, the difference of rhizosphere competence of LR228r4 between the two plant species could be related to the presence in the flax rhizosphere of organic compounds favourable to LR228r4.

This hypothesis is currently being evaluated. Further studies are also required to determine if these organic compounds are the same as the ones which previously allowed the discrimination between the fluorescent pseudomonads associated to flax from those associated to tomato.

Introduction

Fluorescent pseudomonads suppress various soilborne diseases (Weller, 1988). Their efficiency in biological control is related both to their antagonistic activities and to their rhizosphere competence (Cook *et al.*, 1995). Bacterial antagonism has been associated to different modes of action including nutrient and iron competition, antibiosis and induced resistance (Thomashow and Weller, in press). Knowledge about bacterial traits promoting root colonisation is lacking (De Weger *et al.*, 1995).

In order to make progress in that field, we recently compared the ability of populations of fluorescent pseudomonads, from an uncultivated soil and from the roots of two plant species (flax and tomato) cultivated in this same soil, to use different organic compounds and to dissimilate nitrogen (Clays-Josserand *et al.*, 1995 ; Lemanceau *et al.*, 1995). Numerical analysis of the results enabled us to group in clusters isolates showing a high level of similarity. Some clusters only included flax isolates and others only tomato isolates These results indicate that populations associated with uncultivated soil and with each plant species differed and then suggest that each plant species selected specific populations of fluorescent pseudomonads.

The aim of the present study was to determine if an isolate representative of those selected by flax is better adapted to flax rhizosphere than to the rhizosphere of tomato. So the survival kinetics of an isolate of *P. putida*, belonging to a cluster including only flax isolates, were compared both in the flax and in the tomato rhizosphere.

Materials and methods

P. putida LR228 and selection of a rifampicin resistant mutant

Strain of *P. putida* LR228 was previously isolated from the rhizosphere of flax cultivated in the soil from Dijon (Lemanceau *et al.*, 1995). This strain was representative of isolates selected by flax cultivated in this soil.

Rifampicin mutants of LR228 were obtained by plating a bacterial suspension of this strain to King's medium B (King *et al.*, 1954) containing 250 µg ml^{-1} of rifampicin. Specific growth rates of LR228 and of 10 rifampicin spontaneous resistant mutants were compared in liquid King's medium B at 25°C by measuring the optical density of the cell suspensions during 30 hours every 2 hours. The mutant LR228r4 showing the closest specific growth rate to the wild-type was chosen for further studies. The stability of the rifampicin mutation in LR228R4 was tested by subculturing this strain on King's B agar (KB) 15 times for 48 h at 25° and by comparing the number of colony forming units (CFU) on KB and on KB supplemented with 250 µg ml^{-1} of rifampicin after the 5th, 10th and 15th subcultures. Growth of LR228r4 in competition with the parental strain LR228 was studied as previously described by Glandorf *et al.* (1992). The mutant LR228r4 was also compared with the parental strain for its ability to use 49 carbohydrates, 49 organic acids and 49 amino acids with the API-50-CH, API-50-OA and API-50-AA strips (BioMerieux, La Balme les Grottes, France).

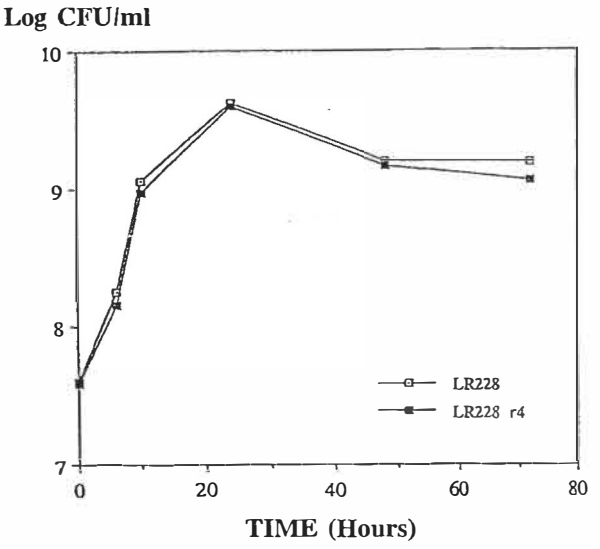
Plant growth conditions and bacterial inoculation

Plants were grown in non-gnotobiotic conditions in soil microcosms previously described by Steinberg *et al.* (1989) containing 10 g of dry soil of Dijon. Flax seeds were surface sterilised in a 1.5% solution of NaClO for 20 min, washed three times with distilled sterile water and were germinated on sterile filter at 28°C during 24 h and 48 h for flax and tomato seeds, respectively. Five seedlings of flax or tomato were transferred in each microcosm. Plant were grown in a growth chamber (18 h light period at 25°C and 8 h dark period at 23°C). The soil was kept at pF=2.

Bacterial inoculants were produced on KB agar supplemented with 150 µg/g of rifampicin, scraped from the medium and suspended in sterile distilled water, pelleted by centrifugation (6000xg, 20min), and washed twice and resuspended in sterile distilled water. Bacterial density was adjusted to provide approximately 10^6cells g^{-1} of dry soil.

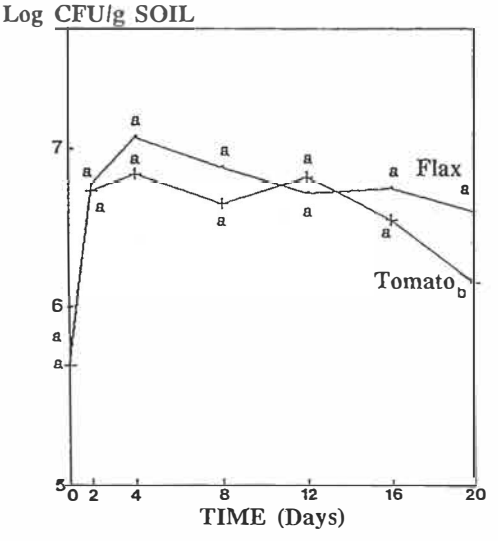
Bacterial enumeration

The densities of fluorescent pseudomonads in the rhizospheric soil was determined by dilution plating. The soil from each microcosm was suspended in 90 ml of sterile distilled water by mixing with a Waring blender for 1 min at high speed. A serie of 10-fold dilution was prepared from that suspension. An aliquot (100 µl) of each appropriate dilution was plated on either modified KB (Geels and Schippers, 1983) for the numeration of total fluorescent pseudomonads or on modified KB supplemented with rifampicine (150 µg ml-1) for the numeration of LR228r4. Five microcosms were sampled for each experimental treatment and at each time interval. The number of CFU were counted after 48 h of incubation at 25° on 3 Petri dishes per replicate. Since populations of bacteria approximate a log normal distribution (Loper *et al.*, 1984), values were logarithmically transformed before analysis. Transformed values of microbial numeration were analysed by analysis of variance and followed by the test of Student.



Values, for each sampling time, with the same letter are not significantly different at P=0.05.

Figure 1 : Competition between the parental strain *P. putida* LR228 and the rifampicin mutant LR228r4 in liquid KB.



Values, for each sampling time, with the same letter are not significantly different at P=0.05.

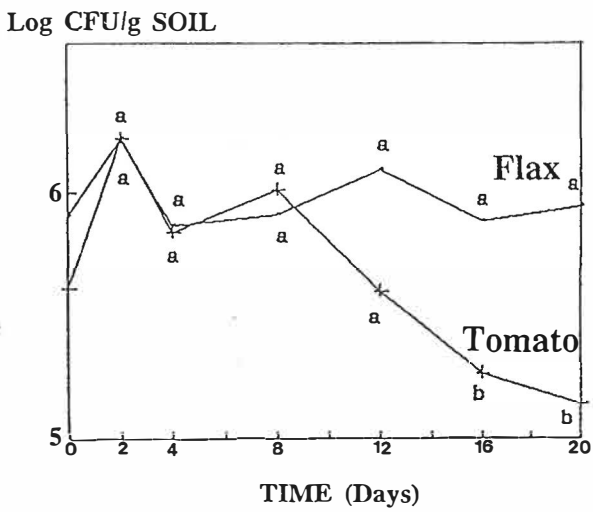
Figure 2 : Population kinetics of the total populations of fluorescent pseudomonads in the rhizosphere of flax and of tomato cultivated in Dijon soil.

Results

LR228r4 showed a similar specific growth *in vitro* and used the same organic compounds as the parental strain (data not shown). Assay for competition indicated that the initial ratio (CFU ml^{-1}) of LR228r4 and the parental strain LR228 was maintained in KB liquid medium throughout the experiment (Fig. 1).

Total populations of fluorescent pseudomonads in both rhizosphere of non inoculated flax and tomato were similar (Fig. 2). However, density of fluorescent pseudomonads in tomato rhizosphere showed a slight decrease during the last two numeration (16 and 20 days after inoculation) and was significantly lower that that in flax rhizosphere after 20 days of plant growth (Fig. 2).

In both rhizosphere of flax and tomato inoculated with LR228r4, the density of the mutant increased during the 2 first days, after inoculation, to $1.7x10^6$ CFU ml^{-1} and decreased during the 2 following days (Fig. 3). These variations were followed by a stabilisation at a level close to 10^6 CFU ml^{-1} in the flax rhizosphere. In the tomato rhizosphere, after a short stabilisation (4 days), the density of LR228r4 decreased and reached a value as low as $1.4x10^5$ the 20^{th} day after inoculation. Twenty days after bacterial inoculation, the density of LR228r4 was 6.2 times higher in the flax rhizosphere than in tomato rhizosphere (Fig. 3).



Values, for each sampling time, with the same letter are not significantly different at P=0.05.

Figure 3 : Survival kinetics of *P. putida* strain LR228r4 in the rhizosphere of flax and of tomato cultivated in Dijon soil.

Discussion

The strain LR228r4, representative of those specifically selected by flax roots survived significantly better in flax rhizosphere than in tomato rhizosphere. These results are in agreement with those obtained by Malterre (1994) with the same strain and the same plant species but grown in nutrient solution with glass beads. Seventy-two hours after the bacterial inoculation, the density of the strain LR228r4 was 7.3 times higher in the flax rhizosphere than in the tomato rhizosphere.

This difference cannot be ascribed to a difference of the carrying capacity for fluorescent pseudomonads in both rhizospheres. Indeed, densities of total populations of fluorescent

pseudomonads of non inoculated plants were similar in both rhizospheres. These data suggest that specific compounds present in the flax rhizosphere but absent in the tomato rhizosphere could favour the survival of the strain isolated from the flax rhizosphere.

Further studies based on the use of isogenic mutants of the strain LR228r4 are underway to determine if those specific compounds are those previously showed to allow discrimination between isolates from flax and from tomato rhizosphere.

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LIMITATION OF DRAIN WATER CIRCULATING MICROORGANISMS USING A MODIFIED WET CONDENSATION HEATER

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Abstract

Recycling drain water can avoid pollution as well as save fertilizers, but is a potential source of pathogenic microorganisms for the crops. Therefore, drain waters must be disinfected for recycling purposes. An experimental system was set up in a greenhouse to test the relevance of a modified wet condensation heater to reduce or to eradicate microbial populations in drain water in addition to providing heating and CO_2.

The efficiency of the modified heater was tested :
(i) intrinsically: various kinds of microorganisms (bacteria and fungi) and one virus were introduced into the disinfection circuit. Results varied depending on both the type of microorganisms and the flow rate: fungi were destroyed after 30s at 59° and most of the bacteria except some spore-forming bacteria were destroyed after 45s at 63°. Virus particles were more difficult to destroy, but their number was greatly reduced, limiting the probability of plant infection.
(ii) *in situ*: population densities assessed in various compartments of the circuit showed that bacteria survived and colonized the treated water but their number was generally reduced.
(iii) on plant productivity: tomatoes were grown on rockwool slabs inoculated or not with non-pathogenic microorganisms. Crops were grown in an open system, a closed system and a closed system with disinfection. *F.oxysporum* f.sp. *radicis lycopersici* and *Clavibacter michiganense* were introduced in irrigation water tanks. No significant difference was observed in plant productivity nor any symptom on the plants despite the presence of the pathogenic microorganisms in the water.

It is suggested that recirculating drain water can generate a suppressive medium analogous to some extent to soil suppressiveness. The use of biocontrol agents to enhance this suppressiveness is also suggested.

Introduction

Avoidance of soil-borne pathogens was one of the main objectives underlying the development of hydroponics. This technology has been used to grow ornamental plants as well as high cash value vegetable crops in nutrient solution, either with or without the use of a mechanical support for the root system. Until now, most of the hydroponic systems are open systems, that means that the unused nutrient solution is discharged either to pits or fields around the greenhouses, and when available in the main sewage. Generally, 20% of the total nutrient solution is drained out and run to waste resulting in both an economic waste of fertilisers and groundwater pollution problems.

As an example, 1 hectare of tomato cultivated in soilless culture for around 8 months (March to October in the south west of France) will need 10,000 to 15,000 m^3 of nutrient solution to produce 450 tonnes of tomato. But 2,000 to 3,000 m^3 of the irrigating solution will be drained, that is 8 tonnes of fertilisers, from which 50% are nitrogen fertilisers. To avoid these problems, drain water can be collected and reintroduced into the irrigation system, but there is a risk of recirculating and spreading pathogenic microorganisms. Gaillard (1994) observed the presence of up to 10^5 CFU ml^{-1} drain water of either *Clavibacter michiganense* subsp. *michiganense*, *Fusarium oxysporum*

f.sp. *radicis lycopersici* and *Pythium* sp in the drain water of a tomato culture. Jenkins and Awerre (1983) reported the transmission of four *Pythium* species in the nutrient solution of a hydroponic system. Tomlinson and Faithfull (1979) reported that symptoms of lettuce big vein virus affected 99% of young plants grown in hydroponics. In a recent review, Stanghellini and Rasmunsen (1994) reported that among the various pathogenic microorganisms isolated from roots of hydroponically grown vegetable crops, most of them were likely to be spread by infested nutrient solution. Among them, zoosporic fungi were even more likely to be spread. These microorganisms can develop rapidly in the substrate for various complementary reasons: abundance of a genetically uniform host, physical environment with constant and favourable conditions for microbial growth, system that allows for a rapid and uniform dispersal, absence of a natural microbial buffer. For these reasons, the reintroduction of infectious agents, as secondary inoculum, is generally more detrimental than a primary inoculum which could be restricted to a few plants. An alternative to reduce or eradicate this secondary inoculum could be the disinfection of drain water. Different processes have been tested: ozonisation, chlorination,, ultrafiltration, UV irradiation, heat treatment (Ewart and Chrimes, 1980, Goldberg *et al* 1992, McPherson *et al* 1995, Runia 1994a, 1994b). All of them were rather efficient when tested under laboratory conditions (intrinsic efficiency). Nevertheless, their use is not easy to manage under the conditions of commercial cultures. The choice of a disinfection system will depend on both the constraints of soilless culture, the basic structures of the greenhouse and the intrinsic efficiency of the process.

The use of biocontrol agents (BCAs) has proved to be efficient in reducing disease incidence in soilless culture (Alabouvette *et al* 1993). Eparvier *et al* (1991) showed that among BCAs, non pathogenic *Fusarium oxysporum* and fluorescent *Pseudomonas* strains were able to survive for long period in rockwoll, a widely used substrate for soilless cultures. Therefore, recirculation of drain water could be combined with the use of BCAs introduced into the substrate slabs before the bedding of seedlings.

We describe here the results of an experiment conducted in a greenhouse in the South of France. The aim of this experiment was to set up a simple system that allows to recirculate drain water from tomato cultures with a minimal risk of recirculating pathogenic microorganisms and that takes into account the basic structures of the greenhouse.

Material and Methods

A classical wet condensation heater was modified to ensure the thermodisinfection of irrigation water or recycled nutrient solution, simultaneously with water heating and CO_2 production. Details of the technical modifications made to the heater have already been described. (Steinberg *et al* 1994).

1 - The intrinsic efficiency of the modified heater was tested using known quantities of bacteria, fungi and viruses. These microorganisms originated from (i) pure cultures of both pathogenic and non pathogenic bacteria and fungi, and pure suspension of Tobacco Mosaic Virus (TMV) and (ii) extracts of used culture substrata containing a mixture of microorganisms, some of which being phytopathogenic. They were successively introduced into the disinfection circuit above the heater owing to an inoculation valve, with the gas furnace on. The suspensions were continuously injected for 2-5 min using a pump and according to the flow rate of the circuit to reach a concentration similar to the one observed by Gaillard (1994) in the drain water of tomato cultures. As controls, identical injections of microbes were done with the gas furnace off. Samples were taken downstream from the heater by means of a tap designed for that purpose. The number of microorganisms was determined by the plate count technique using yeast peptone agar medium (YPA) for bacteria and malt agar medium (MA) for fungi. When bacteria and fungi where mixed in

the samples, cycloheximide (100mg l^{-1}) and citric acid (250mg l^{-1}) were added to YPA and MA to inhibit the growth of fungi and bacteria respectively. The results are the average of 6 samples. Student-t tests were used to compare the results. The concentration of TMV was assessed by counting the number of necrotic lesions produced by diluted suspensions of the samples, compared to the one produced by standard concentrations.

2 - The wet condensation heater was integrated in the water circuit of a greenhouse (fig.1). Modifications brought to the circuit were: (i) introduction of a drain water tank connected to the heating tank, and a treated water tank connected to the irrigation station, (ii) fresh water entered the circuit through the heating tank (fig. 1). Fresh water was preheated in a heat exchanger that was cooling the treated water, downstream the heater. The in situ efficiency was tested by following the bacterial population dynamics in the different tanks along the circuit : heating tank, drain water tank, and treated water tank. Three samples of water were weekly collected from each of the tanks. Bacterial density was then assessed using the plate count method on YPA.

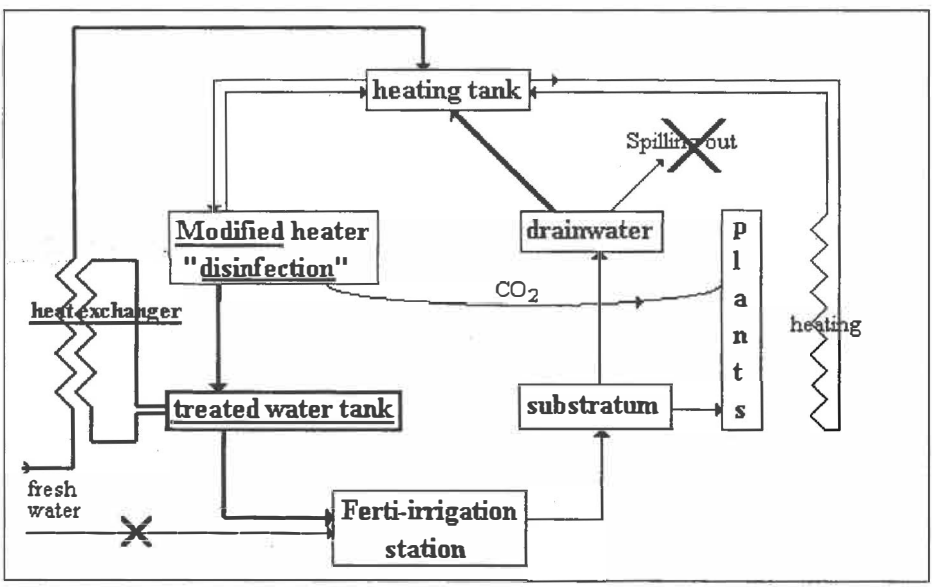

Figure 1 : Recirculating system for studying the efficacy of the heater in controlling microorganisms *in situ*. Modifications made to the original open system have been underlined or drawn in bold lines.

3 - Incidence of recirculating drain water was then checked by assessing the yield of a tomato crop after 4 months of cultivation. In addition to comparison between a closed, a closed with disinfection and an open system, a comparison between systems with and without introduced BCAs was set up resulting in 6 treatments: 1: closed system with BCA, 2: closed system without BCA, 3: closed system + disinfection with BCA, 4: closed system + disinfection without BCA, 5: open system + disinfection with BCA, 6: open system + disinfection without BCA. Each treatment consisted of 8 slabs of three plants each. The BCAs were strain Fo47 of F. oxysporum and strain C7 of a Pseudomonas fluorescens (Eparvier et al 1991). They were introduced into the substratum to obtain 10^6 conidia ml^{-1} of rockwool for Fo47 and 10^8 CFU ml^{-1} for C7, just before bedding the seedlings.

Two pathogens were introduced into the irrigation water 15 days after the bedding of the seedlings: F.oxysporum f.sp. radicis lycopersici, strain Forl28 was introduced to obtain 10^3 conidia ml^{-1} of irrigation water and Clavibacter michiganense subsp. michiganense, strain 1462 was introduced to obtain 10^6 CFU ml^{-1} of irrigation water.

During the course of the experiment, samples were taken from the drain water of each treatment and checked for the presence of bacteria on YMA and fungi on MA.

Results and Discussion

1 - The intrinsic efficiency of the heater to disinfect the circulating water depends on the time during which the water stays in the combustion chamber. A flow rate of $1m^3h^{-1}$ involves the water staying in the combustion chamber for 2 min. The temperature reached by the water at the top of the combustion chamber was also dependent of the flow rate. In average, the temperature of the water at the top of the combustion chamber was 74, 70, 63, 59, and 54° for flow rates of 1, 2, 3, 4, and 5 m^3h^{-1} respectively. The microbial densities in the heated water were always signifidantly different at the 1% level from the densities in the control. Therefore the heater efficiency was expressed as the percentage of microorganisms killed by the heat treatment.

Fungi were destroyed whether they originated from pure culture or substratum extracts except in the case of Hortifiber® extract at a flow rate of $5m^3h^{-1}$ (efficiency =99.7%) corresponding to a temperature lower than 59°. As the recommended exposure to destroy fungi in irrigation water is 90°C for 2 min (Runia *et al* 1988), the efficiency of the heater was attributed to a combined effect of both the temperature and the UV radiations from the flame. UV radiations are known to kill microorganisms by acting on their nucleic acid. Rotem and Aust (1991) showed that UV radiations affected the survival of various fungal species at various degrees. Stanghellini *et al* (1984) succeeded in controlling root rot of spinach induced by *Pythium aphanidermatum* with a UV dose of $90mJ.cm^2$ in a recirculating hydroponic system. Runia (1994a) showed that the infectivity of *F.oxysporum* conidia was reduced to 99.9% with similar doses ($84mJ.cm^2$). Unfortunately, in our trial, UV energy was not quantified.

In the case of bacteria, the heater efficiency varied according to the flow rate and the kind of microorganisms introduced (fig.2).

The combined action of heat and UV radiation must have also occurred on bacteria. Depending on the genus, some of them were very sensitive such as *P.fluorescens* while others could survive the treatment. The persistence of *B.polymyxa* and to a lower extent of *C.michiganense* could be explained by the fact that these bacteria are Gram-positive bacteria with a stronger cell wall which renders them less sensitive to UV radiation or high temprature. Moreover, *B. Polymyx*a can form resting spores that are more resistant than vegetative cells. The survival of *E. carotovora* cells is more difficult to explain as they are Gram-negative bacteria. Before entering the combustion chamber in the heater, the water is preheated (Steinberg *et al* 1994), therefore, synthesis of heat shock proteins responsible for heat resistance could have been induced. This hypothesis has to bo tested (Lindquist and Craig, 1988).

Concerning the bacteria from substratum extracts, the weak efficiency of the heater could be explained by at least two non excluding hypotheses: (i) soil-borne bacteria are much more diverse in their susceptibility to high temperature than bacteria originating from a cultivated clonal population. (ii) soil-borne bacteria are probably protected from both UV radiations and the heat shock by some soil particles such as clay particles that are known to protect microorganisms in soil (Stotzky 1966).

TMV survived the treatment when the temperature was 70° or lower (fig.2). Because viruses do not multiply on their own, the efficiency of the treatment was assumed to greatly reduce the probability of a virus infection. Nevertheless, the heater failed to completely eliminate the risk because both temperature and UV-dose were probably too weak. Runia (1994a) succeeded in

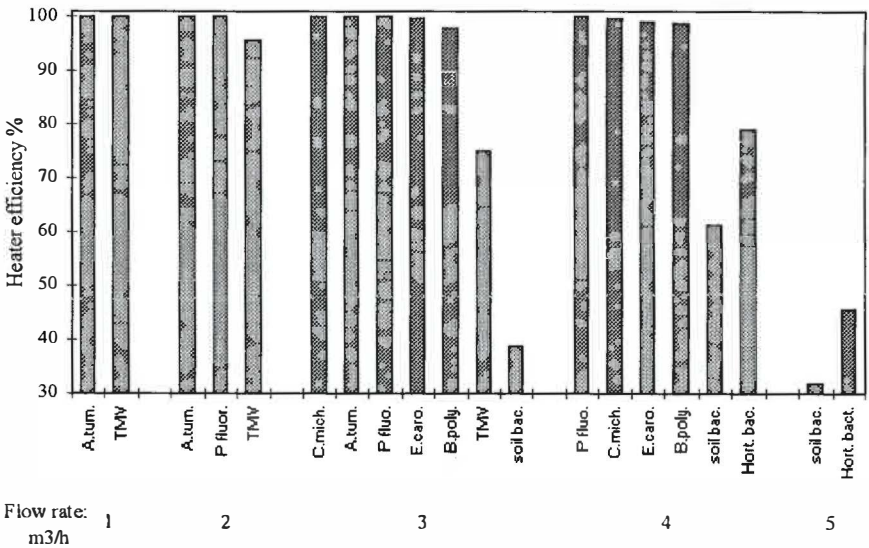

Figure 2 : Heater efficiency in controlling the number of bacteria and virus particles introduced above the heater. Heater efficiency is expressed as a percentage of particles eliminated from the treated water compared with the respective cold control. Experiments have been carried out at 5 flow rates. The temperature of the water at the top of the combustion chamber were of 74, 70, 63, 59, and 54° when the flow rates were of 1, 2, 3, 4, and 5 m^3h^{-1} respectively.

A.tum = *Agrobacterium tumefasciens*, TMV = tobacco mosaic virus, P.fluo = *Pseudomonas fluorescens*, C.mich = *Clacibacter michiganense* subsp *michiganense*, E.caro = *Erwinia carotovora*, B.poly = *Bacillus polymyxa*, soil bac = bacteria from a soil extract, Hort.bac. = bacteria from an Hortifiber extract.

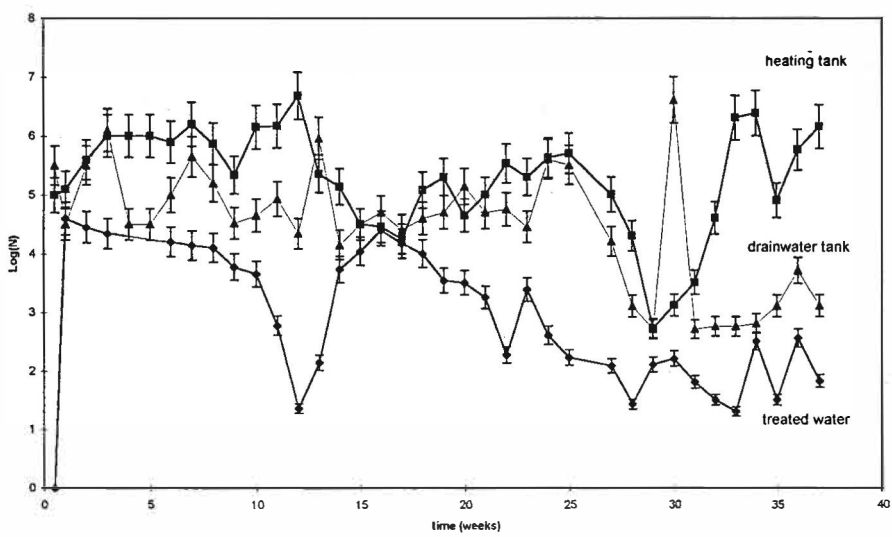

Figure 3 : Population dynamics of bacteria in the 3 tanks of the circuit: drain water tank, heating water tank and treated water tank.

reducing virus infectivity by 99.9% with UV-doses (150-277mJ.cm^2) higher than that required for fungi.

2 - In the second step of the study, the heater was connected to the irrigation circuit of a greenhouse and the dynamics of the bacterial population were followed in the treated water tank, the drain water tank and the heating tank. These dynamics are shown figure 3. It was almost impossible to keep the treated water tank sterile since it was recolonized only 3 days after the beginning of the experiment. The curves show that bacteria from the drain water tank multiplied in the heating tank (the latter being 10 times larger than the former) but this number was greatly reduced in the treated water owing to the action of the heater.
The partial efficiency of the heater, previously shown, explained the presence of microorganisms in the treated water. Moreover, technical problems such as a delay in the closing of the security valves were noticed. These valves must close when the temperature of the water in the combustion chamber decreases below a given threshold. This delay, although short (few seconds) allowed non treated water or insufficiently treated water from the heating tank to flow into the treated water tank. Although technically easy to solve, this kind of problems shows the weakness of a system that would rely on a 100% sterility within the greenhouse.

3 - Since it was not possible to obtain fully disinfected water to irrigate the culture, it was decided to compensate the lack of 100% efficiency of the heater by introducing BCAs into the culture substratum. These microorganisms, selected for their antagonistic activity (Alabouvette et al 1987) and their ability to survive in rockwool (Eparvier et al 1991) were assumed to occupy the niches provided by the growing roots, and thus, limit infection by a pathogen surviving the treatment.

Surprisingly, as shown in table 1, whatever the treatment (1 to 6), no significant difference (p=0.05) was observed in the plant productivity.

Treatment	1	2	3	4	5	6
Average yield/plant (kg)	2.29	2.85	2.67	2.83	2.87	3.27
Number fruits/plant	34	32	33	33	32	31

table 1 : productivity of the tomato crop after 4 months of cultivation in different irrigation systems (=treatment): 1: closed system with BCA, 2: closed system without BCA, 3: closed system + disinfection with BCA, 4: closed system + disinfection without BCA, 5: open system with BCA, 6: open system without BCA. Each treatment consisted of 8 rockwool slabs with three plants. Difference were not statistically significant (p=0.05)

The presence of microorganisms was checked in the drain water. In all cases except treatment 3, bacteria and *F. oxysporum* were detected but it was not possible to determine whether they were the pathogenic strains or the BCAs. In the case of treatment 3, bacteria and *Penicillium* sp. colonies were detected.

Since no symptoms of neither *C.michiganense* nor *F.oxysporum* f.sp.*radicis lycopersici* were noticeable on tomatoes grown in the open system without BCAs, the inoculation procedure of the pathogenic strains must be discussed. Both pathogens were introduced as a suspension of microorganisms originating from pure cultures. This procedure may be responsible for a reduced aggressivity of the pathogens. McPherson *et al* (1995) inoculated directly the slabs by inserting agar discs containing the pathogens, or by dropping infected seeds into the irrigation water tank. The pathogenic inoculum was then less shocked, progressively released into the medium and therefore more aggressive.

In the case of closed systems (1, 2) the pathogens were detected but did not cause any symptoms. McPherson *et al* (1995) also reported a partial recovery from root disease in closed system after a few weeks of cultivation of tomato and cucumber inoculated with *Phytophthora cryptogea* and *Pythium. aphanidermatum* respectively. One explanation could be that the pathogens have partly lost their intrinsic virulence. Another explanation could be that recirculated water carries besides pathogenic microorganisms, non pathogenic ones that could act as antagonists. Therefore, recirculating drain water could lead to a kind of suppressive system analogous to some extent to soil suppressiveness. In that case, introducing non pathogenic microorganisms selected for their rhizosphere competence into the substratum could enhance the supressiveness of the medium. In that case, as in supressive soil no obvious relationship can be established between the presence of pathogenic microorganisms and the pathological risk.

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Concentration of oxygen and organic matter in a recirculating watering system

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In a commercial greenhouse with 23 000 *Crossandra infundibuliformis* watered by NFT system, the concentration of oxygen and organic matter (measured as COD = Chemical Oxygen Demand) as well as the microflora were measured in the water reservoir. The water reservoir was normally holding 4-6 000 liters of water and the plants were watered for 1 hour every other day.

In the first experiment there was an increase in COD during the growth period (4 months) from app. 40 to app. 80 mg O_2/liter. After watering there was a mean increase of 5% compared to before watering. There were no variations in COD between periods of high radiation and periods of low radiation.

During watering and refilling of the reservoir the oxygen content of water rose to 90-100% relative content and fell to 0-5% within a few hours after watering. At the end of the trial the oxygen content was kept high by continuous pumping during 3 days resulting in a decrease in COD of only 4 mg oxygen/liter.

In a second experiment, the water in the reservoir was desinfected with per-acetic acid. For 3 days the water remainded sterile, then there was an increase in the content of bacteria and after 5 days the content of fungi increased, dominated by *Trichoderma sp.*. After watering on the 6th day the oxygen content of the water decreased as in the first experiment, and during the following 3 days there was a rise in the rate of decrease.

The relatively constant amount of organic matter in the recirculating water indicates, that organic matter from mineralizing of sphagnum and root exudates are digested by the microflora in the pot and only a very small amount diffuse into the water. This is supported by the small decrease in organic matter by high oxygenation. The soil in the pots and the water could therefore be considered as 2 different ecosystems, that differ in the amount of organic substrate. This opens for the possibility of manipulating the microflora in the water by adding specific organic matter.

Production, formulation, application of biocontrol agents and integrated control

POSSIBLE STRATEGIES TO IMPROVE THE EFFICACY OF MICROBIAL INOCULANTS AND INOCULATION METHODS

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Abstract

After a short survey of the factors influencing the efficacy of the inoculation process, possible strategies for improvement are discussed.

Increasing the number of microorganisms in the inoculants requires optimization of their production and shelf-life. To control the microbial activity, culture and storage conditions must be improved, and the possibility of using microbial associations should be considered. To be efficient, an inoculant must be delivered close to the target and be present at the right time. Several methods can be used to prevent microbial loss during inoculation and allow good survival or growth after inoculation. The last factor to be considered is the practical acceptability of inoculant use. Compatibility with available agricultural equipment, usual crop treatments and acceptable reliability should be considered.

Introduction

In the last several decades, important scientific investments have been made for the use of microorganisms in agriculture. However, successful applications are scarce, except for legume inoculation with rhizobial inoculants. Plenty of potentially interesting new technologies for pest and disease biocontrol, plant growth stimulation, soil bioremediation, remain at the laboratory or greenhouse level without practical use in agriculture and forestry. One of the most important limitations of this technology transfer, is probably due to the poor efficacy of the inoculation process (inoculant and inoculation method). To be efficient at the farm level, an inoculation process must combine three factors : (1) a potentially efficient inoculant, (2) an efficient method of inoculation, and (3) a good compatibility with existing farming practices. This paper deals with the possible strategies to improve them, in order to produce new technologies using microrganisms acceptable by farmers.

How to improve the potential efficacy of inoculants ?

A potentially efficient inoculant must contain microorganisms of an efficient selected strain, with the required number of viable cells at the time of use, having the required biological activity. Strains have been generally carefully selected, however, more effort must be made to improve the number and activity of microorganisms in the inoculant.

To obtain the expected biological effect, a certain inoculation rate (number of viable and active microorganisms per seed, per plant) must be applied. As an example, in soybean, optimum nodulation will require, at least 10^6 *Bradyrhizobium japonicum* per seed at sowing (Catroux, 1991). This inoculation rate is roughly equivalent to 0.1 to 0.4 l of *B. japonicum* liquid culture per hectare and can be produced economically as an inoculant. For the use of *Pseudomonas*, assessments of the required inoculation rate are more difficult to perform. Numbers up to 10^7 per seed and 10^5-10^8 per g of soil were reported (Germida & de Freitas, 1994 ; Seong *et al.*, 1991). These amounts correspond to a wide range of inoculant culture, 0.1 to 100 l per hectare, reaching

an uneconomical level. Thus, there is a general need to increase the inoculant microbial concentration, in order to decrease the cost of manufacturing, and/or increase the inoculation rate and the shelf life..

Except for rhizobia, few data has been reported on the optimization of microorganism cultivation for inoculant production. Nevertheless, bacterial concentration can be increased from 10^9 to more than 10^{10} cells per ml using a central composite experimental design (Cliquet *et al*;, 1992) or other optimization techniques. In this way cultures of $2x10^{10}$ viables cells per ml of *B. japonicum* (Cliquet *et al.*, 1992) and *Pseudomonas* (Nicot *et al.*, 1995, unpublished results) have been produced.

In addition to the production of highly concentrated cultures, attention must be focussed on the shelf life of the microorganisms in the inoculants, as they will be transported and stored before use. Further to the possible effect of the shelf life, packaging and storage conditions of inoculants, the presence of contaminants must also be considered carefully. Contaminants will decrease the shelf life (Catroux, 1991 ; Date & Roughley, 1977) and thus must be controlled or eliminated. When the conservation has been optimized, inoculants can be stored easily for more than one year. Our studies have shown that an uncontaminated commercial french soybean inoculant stored at room temperature, lost more than 0.5 and 1 log unit per year per g of peat and per ml of liquid inoculant respectively (unpublished results).

Microbial activity of inoculants are rarely considered for quality control. For example, rhizobial inoculants are controlled only by plate counts performed on culture media or in plant tests, thus in optimal growth conditions. The real activity of bacteria for nodulation in adverse conditions such as in the field may not be assessed properly with these methods. In order to take this point into account, we compared several characteristics of soybean inoculants stored for one to 8 years at room temperature. We found, in inoculants from the same manufacturer, that the percentage of *B. japonicum* resistant to desiccation and "active" for nodulation and grain production in a field trial, decreased when storage increased. These results can be interpreted as an over estimation of the microbial activity by classical plate and plant test numerations. Additionally, it was demonstrated that manufacturing technology influences the microbial activity, indicating that this parameter has to be considered to maintain the activity during inoculant storage.

Microbial activity may also be improved using "microbial cocktails", that is combinations of microorganisms with complementary actions in the rhizosphere. Such examples were reported for rhizobia and *Azospirillum* (Ganry *et al.*, 1985 ; Sarig *et al.*, 1986), for rhizobia and *Bacillus* (Halverson & Handelsman, 1991), for rhizobia and *Pseudomonas* (Nishjima *et al.*, 1988 ; Polonenko *et al.*, 1987), for *Fusarium* and *Pseudomonas* (Lemanceau & Alabouvette, 1993) and for *Laccaria* and *Pseudomonas* (Duponnois & Garbaye, 1991). Although the corresponding technology has not yet be experimented on a large scale, this type of association might be very promising in the future.

How to decrease losses of microorganisms during inoculation ?

Inoculant losses during inoculation can be very important, mainly due to microbial death by desiccation. Decreases of 1 log cfu per g or ml inoculant, were reported for *B. japonicum* inoculated on dry granules (Fouilleux et al., 1994) and on soybean seeds (Burton, 1976). This is not surprising, as seeds can develop moisture potentials as low as -50 to -100 MPa, submitting a moist inoculant under very fast drying. These losses can be reduced using hydrophilic protectants such as arabic gum, cellulose derivatives, polyvinyl pyrrolidone, etc. Another possible way is to use dry inoculants since they are, by nature, more resistant to desiccation as we have shown (Fouilleux *et al.*, 1994).

Losses of inoculant can be due to matter losses during inoculation. If inoculants are not properly sticked onto the seeds, they can be lost in air, when air-seeders are used for sowing. Losses can also be due to the transport of inoculant out of the root zone by the seed cotyledons (Jauhri, 1988). Additionnally, a bad localization of the inoculant will decrease the amount of available microorganisms for the seed or root-target. The use of adhesives and the delivery of the inoculant in the seed-bed can easily decrease such losses and improve inoculant efficacy.

How to improve inoculant survival and target colonization ?

Until now, attempts to improve inoculation efficacy were directed mainly on the increase of inoculation rates and decrease of losses during inoculant delivery. Other possibilities can be considered, such as the improvement of inoculant survival and target colonization after inoculation in soils.

The effect of pesticides on rhizobial colonization of legume roots and consecutive nodulation has been reported (Alexander, 1985). Fungicides and a resistant rhizobial strain were used successfully to outcompete the indigenous microflora in the rhizosphere colonization. Such a principle could be adapted to biological control and other inoculations, using more acceptable inhibitors of the indigenous microflora than pesticides.

The use of nutrient addition to increase the population of a microorganism introduced in soil has been reported previously for rhizobia (Germida, 1988 ; Pena-Cabriales & Alexander, 1983) and other microorganisms (Bashan, 1986 ; Van Elsas *et al.*, 1992 ; Lewis & Papavizas, 1985 ; Papavizas *et al.*, 1987). However, the amount of carbon source added per hectare is generally uneconomical. We have used the principle of nutrient supply with the inoculant in a different way, localizing the inoculant and the nutrient on clay granules used as carrier. 10 kg clay granules per ha were amended with 1 kg organic nutrient and stored dry until sowing. Soybean inoculants, peat-based or liquid, were mixed just before use. Using amended granules in laboratory incubations in non-sterilized soils, we observed a decrease of the immediate bacterial losses after inoculation, a growth of the inoculant in soil close to one log unit per g or ml of inoculant, and a better survival under soil drying conditions (unpublished results). Such technology could be used to "boost" the inoculant efficacy through the increase of the number and activity of microorganisms after inoculation.

How to improve the practical acceptability of inoculation by farmers ?

Microbial inoculation is a relatively new method for farmers, and biological products are more "fragile" than usual pesticides and fertilizers. Thus, inoculation must be adapted to existing agricultural practices such as seed disinfection with chemical products which are potentially harmful for the inoculant microorganism. Inoculation must also be compatible with available equipment. For example, liquid inoculants will not be purchased by farmers if they do not have spraying equipment able to deliver the inoculant in the seed bed.

Additionally, the inoculation process must be as reliable as other products used in agriculture, especially in adverse climatic conditions as dry soil at sowing, cold spring, etc.

Finally, registration procedures must consider more flexible statistical confidence limits than the classical 95% level used by scientists. Farmers can be interested by lower limits providing the inoculation is beneficial on average, over a several year period.

Conclusion

"Successful use of biocontrol agents in soil requires a high density and high activity at the right place and time" (van Veen, 1995). This good summary of the prerequisite conditions for an efficient inoculation process seems evident. However, improvements remain to be done if we want to develop the use of microbial inoculant on a large scale.

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DEVELOPMENT OF A FORMULATION OF *GLIOCLADIUM ROSEUM* FOR BIOLOGICAL SEED TREATMENT

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Abstract

In order to develop a biological product for practical application on cereals, we are working on methods to improve fermentation and formulation techniques as well as methods to prolong shelf life of an antagonistic isolate of *Gliocladium roseum*. Fermentation experiments are being conducted in order to increase the yield of viable propagules as well as to improve tolerance to desiccation. Wheat bran, peat, oat and maize meal individually or in combination are used as substrates. Survival of the antagonist after storage at temperatures of 4, 15 and 20° C is being investigated. The efficacy of the various formulations on the disease index of *Fusarium culmorum* and on plant germination is tested in a bioassay. The correlation between doses of antagonistic propagules on the seed and the disease index is examined by washing propagules from the seed and plating on PDA. Dried and stored conidia are separated from fermentor biomass and compared with fresh conidia with regard to biocontrol efficiency and conidia germination in a time course. Promising formulation of the antagonist will be tested in field trails on wheat and barley.

Introduction

An antagonistic isolate of *Gliocladium roseum* Bain has been isolated from barley roots and tested in field trials. Results have clearly shown, that seed treatment with freshly harvested conidia of *G. roseum* controlled *Fusarium culmorum* (W.G. Sm) Sacc. as effectively as fungicide treatment (Knudsen *et al.*, 1994; Knudsen *et al.*, 1995).

In order to develop a biological product for seed treatment, the formulated antagonist must fulfil a number of requirements: (1) appropriate fungal structures must be produced rapidly at high and reproducible levels, (2) these structures must be able to withstand drying and storage and should be activated at sowing, (3) disease control efficiency should be high and stable under varying environmental conditions, and (4) the antagonist should be harmless to the germinating seed.

Experiments are now in progress to develop a product based on the antagonistic fungus *Gliocladium roseum* for seed treatment of cereals against *Fusarium culmorum*.

Methods

The antagonist *Gliocladium roseum* (IK 726) was isolated from roots of barley plants infected with *Fusarium culmorum* and the pathogen, *F. culmorum* (IK 5), was isolated from barley roots (Knudsen, 1992).

Fresh conidia of *G. roseum* were harvested from potato dextrose broth shake cultures, after 8 days. Material of the antagonist for storage was produced on a mixture of peat and wheat bran. The antagonist was grown on this substrate for 15 days (modified from Sivan *et al*, 1984), air-dried for 2 days, milled and stored at 4°, 15° and 20°C, respectively. Viability of the stored preparations was

Treatment	Disease Index	Disease control %	Plant emerge	Washing test cfu/seed
Fusarium control	1.42 A[1)]	-	94 A	-
Fresh *G. roseum*, 1 x 10^6	0.03 D	98	96 A	1.7 x 10^3
Fresh *G. roseum*, 1 x 10^7	0.02 D	99	93 A	6.5 x 10^3
Peat/bran+*G.r.*, 4°C, 1 x 10^6	0.36 C	74	94 A	6.5 x 10^2
Peat/bran+*G.r.*, 4°C, 1 x 10^7	0.05 D	97	85 A	3.8 x 10^3
Peat/bran+*G.r.*, 20°C, 1 x10^6	0.70 B	51	85 A	3.2 x 10^2
Peat/bran+*G.r.*, 20°C, 1 x10^7	0.06 D	96	94 A	3.0 x 10^3

1) Means followed by different letters are significant different at P = 0.05 (Duncan test).

Table 1. Control of *F. culmorum* with fresh conidia of *G. roseum* and with conidia stored for 6 weeks at 4°C and 20°C.

Treatment	Disease Index	Disease Control %	Washing test cfu/seed	Plant emerge	Dry weight mg/plant
Fusarium control	1.74 A	-	-	81 AB	11 C
Fusarium + *G. roseum* 1 x 10^4	1.08 B	38	4.2 x 10^1	78 B	12 BC
Fusarium + *G. roseum* 1 x 10^5	0.46 C	74	5.1 x 10^2	76 B	12 BC
Fusarium + *G. roseum* 1 x 10^6	0.06 D	97	8.4 x 10^3	82 AB	13 AB
Fusarium + *G. roseum* 1 x 10^7	0.02 D	99	3.9 x 10^4	81 AB	13 AB
G. roseum 1 x 10^4	0.04 D	98	4.2 x 10^1	93 A	13 ABC
G. roseum 1 x 10^5	0.02 D	99	5.1 x 10^2	85 AB	14 A
G. roseum 1 x 10^6	0.02 D	99	8.4 x 10^3	85 AB	13 AB
G. roseum 1 x 10^7	0.00 D	100	3.9 x 10^4	86 AB	13 ABC
nontreated control	0.00 D	100	-	82 AB	13 AB

1) Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test.

Table 2. Influence of dosage of *Gliocladium roseum* conidia on the control of *Fusarium culmorum* on barley seeds and on germination and plant dry weight of plant not inoculated with *F. culmorum*.

Although the germination rate of dried and stored conidia is reduced compared to freshly harvested spores, results from growth chamber experiments indicates, that the stored conidia which do germinate, are as active in controlling *F. culmorum* as are the fresh conidia.

tested frequently by plating. Conidia were separated from the fermented biomass and sprayed on water agar. Germination was recorded after 4, 6, 12 and 24 h.

The efficacy of conidia after storage and the relationship between the doses of conidia on the seeds, disease control and plant growth were tested in bioassays (modified from Knudsen *et al.*, 1992). Barley seeds were inoculated with a suspension of *F. culmorum* conidia and dried for 24 h. Seeds were then coated with suspensions of either fresh or dried conidia of *G. roseum* (8 ml suspension/4 g seeds) and sown in small pots containing sand. The number of conidia per seed was estimated by washing seed samples and plating. Disease severity was evaluated after 19 days at 15°C. The disease index (DI) was as follows: 0=healthy plants 1=slightly brown coleoptile/roots, 2=moderately brown coleoptile and roots, 3=severe browning of the coleoptile and roots, 4=dead plants (Knudsen *et al.*, 1992).

Results

Production of *G. roseum* in a mixture of peat and wheat bran gave approximately 1 x 10^9 cfu per g dried material. When preparations were stored at 4°C, the viability was stable for 23 weeks (Fig. 1). However, at 15° and 20°C, viability tended to decrease slightly after 4-8 weeks and after 23 weeks viability was reduced by factor 10.

Germination of conidia was significantly affected by the production method and storage conditions (Fig. 2.). Germination was 90% for freshly harvested conidia and 60% and 39% for conidia stored at 4° and 20°C, respectively. Drying conidia and high storage temperatures decreased the germination rate.

Stored conidia gave more than 90% disease control (Table 1) and at high inoculum levels (10^7 conidia/ml), they were as effective as freshly harvested conidia. However, at lower levels (10^6 conidia/ml) control by stored conidia was unsatisfactory, especially by those stored at 20°C. The low disease control was correlated with a low number of cfu per seed.

A dosage of 8 x 10^3 *G.roseum* conidia per seed gave almost full control of the pathogen (Table 2). This dosage applied to healthy seeds did not affect plant emergence or plant dry weight compared to the nontreated, healthy control.

Discussion

Production of the antagonistic fungus *G. roseum* on a mixture of peat and bran gives a relatively high cfu per g dried material, which is necessary if the preparation is to be applied directly to the seeds. Production on solid substrates is a relatively inexpensive production method. The peat/bran formulation of *G. roseum* contains conidia which can tolerate drying and storage for several month at 4°C. However, shelf life must be improved at higher temperatures, especially if the antagonist is stored on the seeds.

The present data show that a minimum of 10^3 *G. roseum* conidia per seed is sufficient for the effective control of the pathogen. Such a dosage has no negative side effects on germination or the dry weight of the plants. A possible growth promoting effect by *G. roseum* is to be examined.

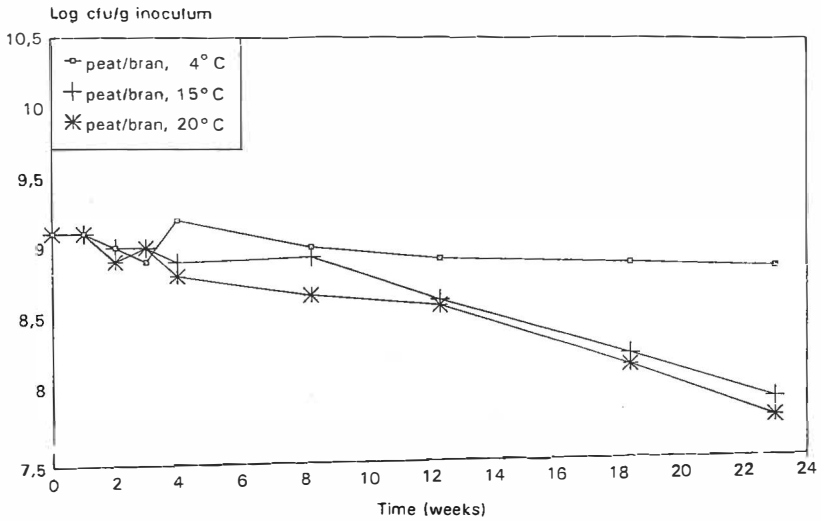

Figure 1 : Survival of *Gliocladium roseum* in a mixture of peat and bran and the effect of storage at 4°C, 15°C and 20°C on survival, measured as cfu/g inoculum.

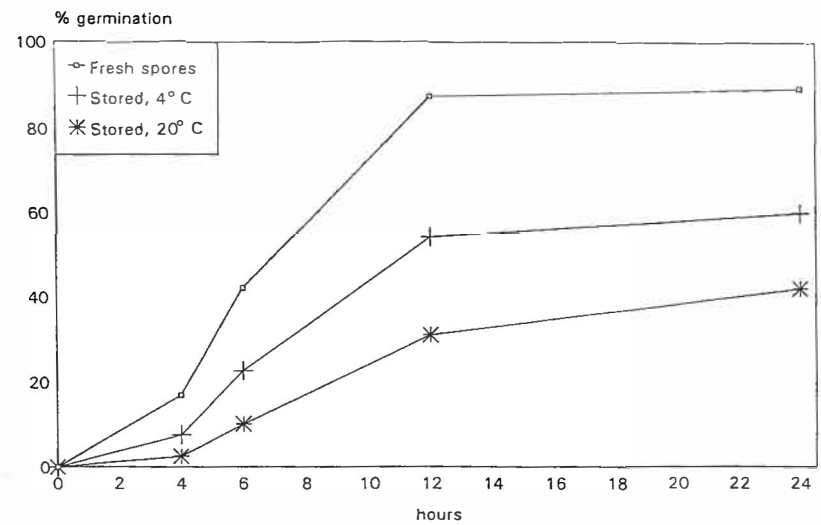

Figure 2 : Percentage germination of fresh and dried *G. roseum* conidia. The dried conidia were stored in peat/bran at 4°C and 20°C for 6 weeks.

tested frequently by plating. Conidia were separated from the fermented biomass and sprayed on water agar. Germination was recorded after 4, 6, 12 and 24 h.

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The present data show that a minimum of 10^3 *G. roseum* conidia per seed is sufficient for the effective control of the pathogen. Such a dosage has no negative side effects on germination or the dry weight of the plants. A possible growth promoting effect by *G. roseum* is to be examined.

Although the germination rate of dried and stored conidia is reduced compared to freshly harvested spores, results from growth chamber experiments indicates, that the stored conidia which do germinate, are as active in controlling *F. culmorum* as are the fresh conidia.

The effect of promising formulations of the *G. roseum* on *F. culmorum* will be tested in field experiments on wheat and barley.

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GLIOCLADIUM CATENULATUM AS A BIOCONTROL AGENT AGAINST ROOT DISEASES IN PEAT BAG CULTURE AND HYDROPONICS

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Abstract

Gliocladium spp. obtained from various Finnish field soils were tested for suppression of *Rhizoctonia solani* and *Pythium ultimum* damping-off of cabbage and sugarbeet in pot experiments. The best isolate,*C. catenulatum* , was used in powder form in modern peat bag cultures of cucumber and in hydroponic cultures with nutrient film technique of lettuce both in experimental and in commercial greenhouses. Some *C. catenulatum* and *C. roseum* isolates controlled soil-borne damping-off in peat substrate with an efficiency rate of 60-90 %. The control efficiency was maintained even after one month of storing of the treated peat substrate. Wetting of the cucumber seedling pot immediately after planting with the powder preparation of *C. catenulatum* increased the number of healthy plants at the end of the growing season. Mixing *C. catenulatum* powder into peat substrate used for lettuce seedlings increased the yield of lettuce in hydroponic culture12-17 % and the weight of roots by 20-30 %.

Introduction

Many fungal diseases are harmful in modern greenhouse production where plants are grown in peat or rockwool plastic bags and in hydroponics. The most common disease fungi are *Pythium* spp., but *Fusarium* and *Phytopthora* can also be harmful (Stanghellini and Rasmussen 1994). The use of fungicides is not advisable because undesirable of pesticide residues in vegetables. Therefore it is necessary to find alternatives to control diseases in modern greenhouse cultivation.

The Nordic countries, Denmark, Finland, Norway and Sweden, had a common biological disease control project in 1990 - 93 for seed-borne diseases of cereals. The aim of the Finnish part of the project was to find fungal antagonists against *Fusarium culmorum* (Tahvonen and Teperi 1995). The best antagonist candidates were tested also in peat substrate to control *P. ultimum* and *Rhizoctonia solani* damping-off diseases. The final objective was to find biocontrol agents for modern growth substrates used in greenhouses.

Material and methods

The best *Gliocladium* spp. isolates against *F. culmorum* in the parallel investigation (Tahvonen and Teperi 1995) were tested against soil-borne *P. ultimum* of sugarbeet and *R. solani* of cauliflower in peat substrate. Spores and mycelium of one Petri dish of *Gliocladium* isolate were mixed in one litre of peat one day or one month before sowing. Test plants were grown 3-4 weeks before analysis of disease content.

The best isolate,*G. catenulatum* , was made by Kemira Ltd. into a powder form with 10^8 cfu/g. This preparation was used on cucumber by wetting seedling pots immediately after planting on peat substrate (bag culture) and mixing the preparation in peat before seeding of lettuce pots for hydroponic culture (nutrient film technique). Yield, size of the roots and damage caused by *Pythium* spp. were analyzed at the end of the growing season.

Results and discussion

Gliocladium isolates controlled effectively or very effectively damping-off diseases caused by P. ultimum and R. solani. The best isolates gave 90-100 % control as compared to healthy soil. Soil treatment increased the growth of sugarbeet and cauliflower seedlings. There were no differences between G. catenulatum and G. roseum in the control of disease and in growth promoting effect (Table 1). These results are in agreement with previous results with the same material on cereals (Tahvonen and Teperi 1995). Gliocladium spp. are well known as biocontrol agents in literature (Papavizas 1985), but G. catenulatum is mentioned only a couple of times. Remarkable biocontrol objects have been Botrytis, Fusarium and Pythium.(Alekseeva et al. 1992, Reyes and Dirks 1985, Simay 1988).

The best isolate, *G. catenulatum* , was effective against both *Pythium* and *Rhizoctonia*. In many other effciency tests it was the best an average (data not presented). Therefore this isolate was used later as a control agent in cucumber and lettuce experiments.

Pythium ultimum on sugar beet

Time between inoculation and sowing		*C.catenulatum*		*C. roseum*		Untreated	
		mean	range	mean	range	diseased mean	healthy mean
One day	% healthy plants	65.9	80-69	70.1	61-70	38	96
	dry weight (g/seedling)	1.54	1.70-0.96	1.60	1.85-1.48	1.39	1.59
One month	% healthy plants	57.8	70-44	58.9	67-51	37	85
	dry weight (g/seedling	1.13	1.22-1.10	1.12	1.85-1.06	0.70	1.07

Rhizoctonia solani on cauliflower

Time between inoculation and sowing		*C.catenulatum*		*C. roseum*		Untreated	
		mean	range	mean	range	diseased mean	healthy mean
One day	% healthy plants	67	81-48	71	79-63	44.8	88.8
	dry weight (g/seedling)	1.54	1.72-1.39	1.59	1.78-1.39	1.39	1.59
One month	% healthy plants	70.6	80-38	72.2	81-60	20.8	81.6
	dry weight (g/seedling	0.74	0.85-0.62	0.77	0.93-0.93	0.47	0.70

Table 1 : Effect of *Gliocladium catenulatum* (n=15) and *C. roseum* (n=5) isolates on *Pythium* damping-off of sugarbeet and *Rhizoctonia* damping-off of cauliflower, when inoculated in peat substrate, one day or one month before sowing.

Treatments	Emergence %	Dry weight g/seedling	Healthy % seedlings
Untreated, healthy	94.4	0.79	92.0
Untreated, diseased	79.2	0.49	36.0
Spore suspension	91.2	0.74	71.2
1 g/l	92.0	0.80	80.0
0.5 g/l	92.8	0.78	75.2
0.1 g/l	88.0	0.68	57.6
0.05 g/l	89.6	0.69	56.0
0.01 g/l	84.8	0.48	36.8
0.001 g/l	82.4	0.47	28.0

Table 2 : Effect of *Gliocladium catenulatum* powder preparation on *Rhizoctonia solani* damping-off of cauliflower. *Gliocladium* preparation mixed in peat before sowing.

The powder preparation of the best isolate,*G. catenulatum* , was as effective as the spore suspension made from pure fungal culture on PDA agar in the control of *Rhizoctonia* damping-off (Table 2). The concentration of the powder preparation for satisfactory disease control was 0.05 g/l peat (=$5x10^6$ cfu/l). The same result was obtained also in the control of *Pythium* on sugarbeet (data not sown in table).

The wetting treatment of cucumber pots after planting decreased significantly *Pythium* symptoms determined at the end of the growing season. Although the growing time was short (August-September), the yield in the first test was 6 % higher in the *Gliocladium* treatment than in the untreated control. In this experiment was used peat where cucumber had been grown earlier in the same summer , when as a new peat substrate was used in second test (Table 3).

		Untreated	*Gliocladium*
Greenhouse 1	Relative yield	100	106
	Disease index (0-3)	0.69	0.28
Greenhouse 2	Relative yield	100	100
	Disease index (0-3)	1.64	0.78

Table 3 : Effect of *Gliocladium* on the yield and *Pythium* infection of roots in two commercial greenhouses. Wetting of seedling pots with *Gliocladium* preparation after planting

	Untreated	*Gliocladium*
Weight of lettuce heads (% of the control))		
Test 1	100	112
Test 2	100	114
Test 3	100	117
Test 4*	100	114
Dry weight of the roots (% of the control)		
Test 1	100	135
Test 2	100	124
Test 3	100	123

* = commercial greenhouse

Table 4 : Effect of *Gliocladium* on the growth of lettuce in the hydroponic culture. *Gliocladium* preparation mixed with peat used in pots before sowing.

G. catenulatum increased significantly the growth of lettuce in hydroponics. The yield increases were 12-17 % in. The growth of roots was stimulated by 24-35 %. There were no *Pythium* disease symptoms in roots. It is not clear by which mechanism growth is promoted, whether it is growth stimulation or a result of disease control.

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ACTIVITY OF MYCOSTOP BIOFUNGICIDE IN ROCKWOOL

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Abstract

Mycostop biofungicide containing a selected Streptomyces strain provides effective plant disease control for many horticultural crops. Initially, the product was supposed to act only in organic substrates, but later studies have also proved its efficiency in rockwool. Mycostop has effectively controlled *Fusarium oxysporum* of gerbera grown in rockwool. The additional flower yield reached even to 30% in a very susceptible variety under artificial infection. The yield increases in commercial greenhouses under natural disease pressure were about 10%. Repeated treatments during the long growing period are even more necessary than in peat. The most suitable application method is to spray or drench the rockwool with Mycostop, although in practice drip irrigation is the most convenient means of delivery. It is not recommended to presoak rockwool in Mycostop suspension because of the slightly negative effect on the yield in the beginning of the harvest. Mycostop dipping or spraying substantially increased the percentage of rooted plantlets and promoted the growth of micropropagated gerberas transplanted to rockwool plugs. Pythium of cucumber is among the target pathogens of Mycostop. Moreover, a clear yield increase has often been observed in cases with no visible disease symptoms. This indicates that, in addition to a direct control effect, a growth promoting factor may also be involved. By using Mycostop seed dressing, the number of marketable cucumber plants was 10% higher than in the untreated control in large-scale rockwool trials.

Introduction

Mycostop biofungicide, based on a selected *Streptomyces griseoviridis* strain, provides a new tool for restricting plant diseases, particularly in horticultural crops. The first experience of the product came from peat, but there soon arose a need for rockwool tests: growers are increasingly changing over to synthetic substrates, which also have disease problems like *Pythium* root rot. *Fusarium* fungi also seem to cause a lot of damage to many crops in rockwool. Since Mycostop had proved effective against those pathogens in peat cultivation, it made sense to determine whether the bioagent can be active in rockwool, too.

A short summary of the key results of the Mycostop trials on rockwool is presented in the following paper. Cucumber and gerbera were used as target crops.

Seedling production of cucumber

Seed treatment with Mycostop significantly reduced the amount of poor-quality cucumber seedlings in large-scale rockwool trials conducted by Dr. R. Tahvonen in the greenhouse of Martens Vegetable Research Station in Finland. Instead, dressing the seeds with benomyl had no significant effect on the health of the young plants (Fig. 1).

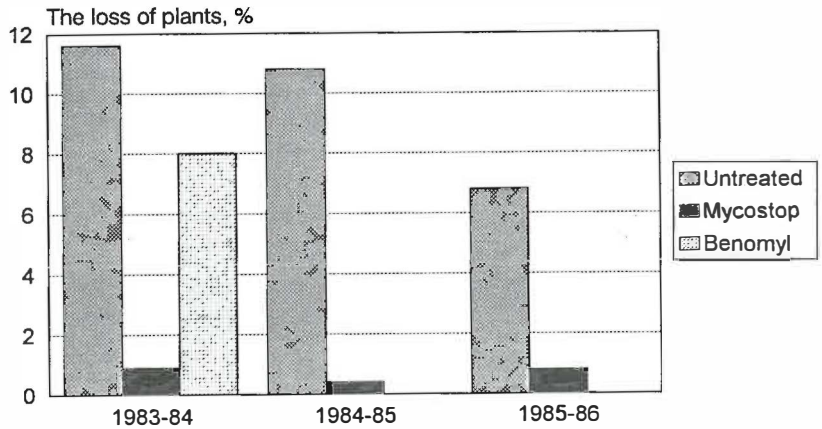

Figure 1 : The effect of Mycostop seed treatment on the quality of cucumber seedlings in rockwool. In 1983-85 seed dressing was carried out with a suspension made of the preliminary preparation and, in 1985-86, with the commercial powdery product.

Root disease control on cucumber

Experimental greenhouse trials

Mycostop decreased *Fusarium oxysporum* wilt symptoms in naturally infected cucumber; The disease index (0-3) was reduced from 1.79 in the untreated to 0.91 in Mycostop spraying. The corresponding yield increase was 8% and the application rate was 3mg/plant (106 cfu) per treatment, which in professional cultivation means 45 g/ha.

In another experiment Mycostop sprayings were compared with the combined soaking and spraying method. In the soaking application before planting, the rockwool blocks were soaked in nutrient solution which included Mycostop (0.01 g/l). Although there was no indication of any root diseases in the trial, differences in yields during the three-month harvest period were observed. Sprayings provided a 9% increase of the total yield of cucumber, but soaking plus sprayings showed a 4% decrease. Positive effect was never seen when the application interval was too short. In a series of four experiments in the absence of artificial infection, the mean increase in the number of harvested cucumber fruits was 9.5% as a consequence of monthly treatments, whereas two-week treatments gave only 0.7% additional yield.

Commercial greenhouse trials

The trials conducted via the Vegetable Section of the Finnish Glasshouse Growers'Association, showed the effectiveness of Mycostop sprayings in cucumber rockwool cultivation in practice. The yield increase was 6%, when a relatively healthy crop was involved, and 11% in the case of more severe root disease infection. Similar results were achieved in Danish commercial cultivations, when Mycostop was

given by drip irrigation. After monthly application of 5mg or 7 mg per plant, the yield increases were 4 and 9% respectively (unpublished).

In most cases, Mycostop application has been used after transplanting. However, an interesting observation was made in a commercial rockwool trial in Holland. It showed that Pythium disease established in the substrate during the previous crop was sufficiently controlled when the planting holes in re-used rockwool slabs were treated with Mycostop prior to new cucumber transplanting (van Adrichem, Aseptafabriek, oral communication).

Micropropagated gerberas

The application of Mycostop has also been tested in four experiments concerning direct rooting of micropropagated gerberas. In this method the shoots were transferred from the propagation medium to rockwool plugs without any special rooting stage in vitro. Mycostop dipping and spraying with highly diluted suspensions substantially increased the percentage of rooted plantlets (Table 1). Besides the posivive effect on rooting, clear growth-promotion was also evident.

Mycostop treatments	Relative percentage of rooting	
	Trial 1 (Cv. Fame)	Trial 2 (Cv. Party)
Water control	100	100
Mycostop 100 mg/l	107	-
Mycostop 10 mg/l	116	102
Mycostop 1 mg/l	123	104
Mycostop 0.1 mg/l	-	110

Table 1 : Effect of Mycostop dipping on the rooting of micropropagated gerbera shoots in rockwool plugs.

Root disease control on gerbera

Control of root disease caused by *Fusarium oxysporum* of gerbera has succeeded by using Mycostop in both experimental greenhouses and in commercial cultivations. The flower yield benefit was as high as 30% on a very susceptible variety under artificial infection (Lahdenpera, 1991). In commercial greenhouse trials in Holland with natural disease pressure, the yield increases were around 10% when 5 or 10 mg of Mycostop were used, whereas 1mg proved to be too low a dosage. Spraying or drenching the surface of rockwool blocks provides a suitable application method. However, later in the growing season, when gerbera stands are bigger, it is impossible to spray the surface of the substrate block. That is why delivery of the microbial fungicide via drip irrigation was tested in a trial where Mycostop spraying and injection were compared. Injection simulated the application through drip irrigation. Mycostop spraying and injection increased the flower yield by 7% and 12% respectively (Mohammadi and Lahdenpera, 1994). Repeated treatments are needed to protect the crop during the long cultivation period. Presoaking of the rockwool blocks in Mycostop suspension showed a slightly negative effect on the early yield.

Conclusions

Initially the use of Mycostop biofungicide containing Streptomyces griseoviridis microbes was supposed to be active only in peat, but later studies brought out the possibilities of using this bioagent also in inorganic growing media. Mycostop can be used for decreasing the damage caused by several wilt and root pathogens of different horticultural crops even in rockwool.

The most successful application method for Mycostop seems to be spraying or drenching. In practice, this can be replaced by the delivery of the microbe through drip irrigation system. Moreover, seed treatment is also a recommended way to control pathogens with Mycostop. Tahvonen and Avikainen (1987) proved that e.g. Brassica seedlings can be effectively protected against damping-off caused by Alternaria fungi if Mycostop seed dressing is used in peat cultivation. In rockwool trials of cucumber, the increase in marketable plants was about 70 seedlings more per 1000 seeds compared with untreated seeds, which already means an economically significant result. In another cucumber seedling production trial (Mohammadi & Lahdenpera, 1994) the emergence of cucumber increased with the spraying of the rockwool blocks, while delivery by subirrigation tended to have a negative effect on germination. Because a slightly negative effect on the yield of both cucumber and gerbera during the first yielding months was observed, presoaking of rockwool blocks in Mycostop suspension is notrecommended either.

Determining the right application rate for Mycostop is not as easy as for chemical fungicides because environmental factors greatly affect the efficacy of microbes. However, the following instructions for using Mycostop in rockwool cultivation can be given: In general, the sufficient application rate is 5-10mg per plant, but under a very slight disease pressure, 1-3mg is enough. Treatments are needed once a month or every other month, depending on the patogen incidence. Due to Mycostop's preventive character, the first treatment must be made at the time of transplanting. In some special cases, such as micropropagated plantlets, a highly diluted suspension should be used.

Since clear yield increases have often been observed in cases with no visible disease symptoms, a growth promoting factor can be involved. This may be explained by the excretion of the earlier observed indole-3-acetic acid (Tuomi *et al.*, 1994) or by the ability to control minor pathogens.

These streptomycetes, in spite of being typical soil microbes, have also proved to be active antagonists in rockwool, where organic nutrient sources are very poor, consisting of root exudates and old root cells. Microbial analyses proved that the streptomycetes are able to survive in rockwool. Even 6 weeks after Mycostop treatment, the population of Streptomyces was at the original level or 1/10 of that. However, rockwool is apparently not very favourable for the proliferation of Streptomyces. Consequently, repeated treatments may be even more necessary than in peat. On the other hand, in the beginning of rockwool cultivation, actinomycetes probably colonize the roots of the plants more easily than in peat or field soil, because in new rockwool the streptomycetes need not compete with other microbes.

Already in 1992, the registration for commercial application of Mycostop in rockwool cultivation was obtained in Finland. According to customer inquiries, the feedback has in general been very positive. Nowadays the sales permit has been extended to several other countries for plant protection in vegetables and ornamentals in greenhouses.

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THE EFFECT OF INITIAL MOISTURE OF YELLOW LUPIN SEEDS ON THEIR GERMINATION, EMERGENCE AND ON FUNGI GROWTH UNDER IMBIBITIONAL CHILLING STRESS

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Abstract

Seeds of yellow lupin cv. Juno of initial water content adjusted to 8, 13 and 20% were exposed to imbibitional chilling stress at 3-4°C for 24h. Germination and emergence of air dry and conditioned seeds after stress were similar, significantly better than germination of excessive dried seeds. The degree of root infection by fungi was independent from initial seed moisture. Exudates of seeds were beter than PDA medium for the growth of saprophytic fungi. Pathogenic fungi were isolated only on PDA and from the seeds with the lowest water content.

Introduction

The sensitivity of seeds to imbibitional stress is controlled by three factors: the initial moisture content of the seed, the temperature of the soil and the rate at which water is taken up. The gratest reduction of seed vigor of different legume species was observed when seeds with low initial water content were imbibed in the medium at a low temperature with high availability of water (Taylor et al., 1992). Some authors (Górecki et al., 1990, Majchrzak 1992) suggested that temperature is a factor much more important for seeds and seedlings survival under imbibitional chilling stress than initial seed moisture content.

As the seed hydrates, solutes such as sugars, amino acids, ions, and proteins may leak into the surrounding medium. These solutes are able to capable to stimulate spore germination and germ tube growth of seed-rotting fungi (Short and Lacy 1975). In the field, seed exudates might stimulate the growth of fungi and bacteria, which will invade the seed and lead to its deterioration. High solute leakage results in an increase of inoculum potential, and the dead tissue of cotyledons can act as an initial site for infection (Matthews et al., 1986). The relationship between nutrient exudation of yellow lupin seed and pathogen activity has not been critically examined.

The aim of our study was to determine the effect of initial water content in yellow lupin seeds under short imbibitional chilling stress on the stimulation of the growth of fungal pathogens by seed exudates, as well as on seed germination and emergence.

Materials and methods

Seeds of yellow lupin (*Lupinus luteus* L., cv. Juno) with 95% laboratory germination capability and average level 3.3 (in 9 degree scale) of fungal infection were used in the experiment. Water content in seeds was adjusted to three levels: about 8% - excessive dried seeds, 13% - air-dried seeds, and 20% - conditioned seeds. Seeds were dried in an air dryer at 35°C for 7 days and conditioned in water vapour at 18°C for 4 days. Air-dried seeds were stored before use at room temperature and relative humidity of 60-65%. About 350 grams of seeds with different initial water content imbibed in 1000 ml of redestilled, sterilized water at temperature 3-4°C for 24h. After the stress, lots of 4x50 non-dressed seeds and seeds dressed with Funaben T (thiram) were put on filter paper and in

sterilized soil for about 2 weeks at temperature 8-10°C (growth chamber) and 20°C (greenhouse). Germination and emergence as well as healthiness of roots were determined as percentage on a 9 degree scale (1- no infection, 9 - all roots infected). PDA, agar and agar plus seed exudates were used for evaluation of the fungal growth which was carried out after 6 and 20 days of incubation. Statistical differences were tested by Tukey test at p=95%.

Results and discussion

Short imbibitional chilling stress caused a significant decrease in germination of both dressed and non-dressed yellow lupin seeds (Table 1). Excessive dried seeds germinated in the significant lowest per cent. However, there were no differences in germination of air-dry and conditioned seeds. In many laboratory experiments (Górecki et al., 1990, Mathews et al., 1986, Taylor et al., 1992) with legume plants such as pea, bean, field bean, soybean and lupins that phenomena has been observed.

Seed treatment	Initial water content in seeds			Mean
	8%	**13%**	**20%**	
non-dressed	55.8 a	80.1 a	85.6 a	73.8 a
dressed	53.3 a	77.6 a	79.0 a	69.9 a
Mean	54.5 b	78.8 a	82.3 a	71.8

Table 1 : Viability of seeds on filter paper after imbibitional chilling stress (in %)
Values in the same line (column) followed by different letters are significantly different

The average number of plants emerged from the sterilized soil was lower by 6% than their average germination determined on filter paper (Table 2). The air-dry and conditioned seeds as well as seeds dressed with thiram emerged significantly better than excessive dried seeds and non-dressed ones. There was no effect of seed dressing on the emergence of air-dry and conditioned seeds. Dressing of seeds with 8% of water content increased the number of emerged plants by 27,6% compared to non-dressed seeds. The infestation of emerged plants by fungi was not observed at all (data not shown).

Rapid water uptake causes cell death in outer layers of embryo and extensive damage and loss of solutes which impaired cell function (Matthews et al., 1986). Water-soluble nutrients (Short and Lacy 1975) as well as volatile compounds lost by seeds during imbibition can promote the growth of seed and root-rotting pathogens (Norton and Harman 1985). Because the dried seeds are more sensitive to imbibitional stress than conditioned ones (Taylor et al., 1992)) it is possible to get more rapid growth and extent of fungi infestation of seedlings grew out from seeds with initial low water content. The infestation degree of yellow lupin seedlings with fungi in greenhouse was independent from initial seed moisture content (Table 3). In our previous study (Sadowski et al., 1995a,b) we did not find any significant effect of pre-sowing seed moisture on the healthiness of lupin and pea plants, also under field conditions. Seed dressing significantly decreased the extent of fungi infection, especially of excessive dried seeds. Air dry and conditioned seeds were much more infected with bacteria than excessive dried ones (data not shown).

Seed treatment	Initial water content in seeds			Mean
	8%	**13%**	**20%**	
non-dressed	29.4 a	74.0 a	78.8 a	60.7 b
dressed	57.0 b	71.1 a	85.4 a	71.1 a
Mean	43.2 b	72.5 a	82.1 a	65.9

Table 2 : Number of yellow lupin plants emerged in sterilized soil after imbibitional chilling stress (in %)
Values in the same line (column) followed by different letters are significantly different

Seed treatment	Initial water content in seeds			Mean
	8%	**13%**	**20%**	
non-dressed	4.05 a	3.82 a	3.49 a	3.79 a
dressed	1.96 b	2.87 a	2.36 a	2.40 b
Mean	3.01 a	3.35 a	2.93 a	3.10

Table 3 : Degree of root rot infestation of sprouts on filter paper after imbibitional chilling stress (in 1-9 scale*)
* 1 no infection, 9 all sprouts infected
Values in the same line (column) followed by different letters are significantly different

Seed treatment	Initial seed water content		
	8%	**13%**	**20%**
PDA			
non-dressed	8	44	97
dressed	0	0	0
Agar			
non-dressed	0	0	85
dressed	0	0	0
Agar + seed exudates			
non-dressed	27	100	100
dressed	0	0	0

Table 4 : Number of *Penicillium* spp. isolates from seeds exposed to imbibitional chilling stress after 6 days of incubation depending upon the medium

Non-dressed and dressed seeds were placed on Petri dishes filled with PDA, agar and agar plus exudates of seeds with different initial water content exposed to imbibitional chilling stress. Evaluation of seeds carried out on the 6th day of incubation showed only isolates of genus *Penicillium* (Table 4). The highest number of *Penicillium* spp. was present on all media when conditioned seeds were used.

Seed treatment / Initial water content	Number of isolates			
	Penicillium spp.	Mucor spp.	Rhizopus spp.	Alternaria spp.
PDA				
Non-dressed				
8%	63	30	23	8
13%	66	24	21	3
20%	97	24	18	0
Dressed				
8%	96	12	6	0
13%	100	5	4	0
20%	97	8	8	0
Agar				
Non-dressed				
8%	64	10	10	0
13%	85	0	0	0
20%	85	3	2	0
Dressed				
8%	75	20	7	0
13%	85	0	0	0
20%	95	0	0	0
Agar + seed exudates				
Non-dressed				
8%	81	35	24	0
13%	100	10	10	0
20%	100	23	12	0
Dressed				
8%	97	15	6	0
13%	100	6	4	0
20%	100	0	0	0

Table 5 : Fungi isolated from lupin seeds exposed to imbibitional chilling – stress after 20 days of incubation depending upon the medium

After 20 days of incubation isolates of *Mucor* spp. and *Rhizopus* spp. were also detected (Table 5). Pathogenic fungi - *Alternaria* spp. were rearly isolated only on PDA and on seeds contained 8% of water - 13 isolates and 13% of water - 3 isolates. Majchrzak (1992) also observed higher activity of not typical pathogens under chilling stress. For air dry and conditioned seeds the difference in the number of isolates on PDA and agar plus seed exudates was small. Neverthless, seed exudates were better media for the growth of saprophytic fungi than agar alone, where mycelium was weak, flat and hardly visible.

In conculsion, seed conditioning (seed priming) of air-dry yellow lupin seeds was not a profitable treatment. There was no significant difference in the viability of air-dry and conditioned seed exposed to imbibitional chilling stress, but the excessive dried seeds germinated and emerged significantly less. The effect of the pre-sowing seed moisture on the healthiness of lupin seedlings in sterilized soil was not significant. Seed exudates added to agar were better than PDA medium for

fungal growth. The higher initial water content in seeds, the greater number of *Penicillium* spp. isolates were observed.

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THE EFFECT OF *LAETISARIA ARVALIS* AND COMPOST ON *PYTHIUM ULTIMUM* ROOT ROT IN A TULIP FORCING SYSTEM

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Summary

Tulip flowers are produced in boxes filled with substrate, generally potting soil. To avoid unnecessary waste it is desirable to re-use the substrate. Used substrates however, may contain infected root residues from a former culture. *Pythium spp.* surviving in these root residues can cause serious damage in the next crop. The symptoms of the disease vary from a completely rotted root system with hardly any growth of the plant to a less severe infection of the roots resulting in a loss of quality of the flowers. Therefore, treatment of the substrate to eliminate *Pythium* is essential. Steam-sterilization can be applied as an environmentally friendly measure to realize a *Pythium*-free substrate. A disadvantage of this method is the complete destruction of the indigenous microflora resulting in a conducive substrate. In case of re-infestation by Pythium, the fungus rapidly occupies the root residues giving rise to severe damage of the next tulip crop.

In order to prevent occupation of root residues by *Pythium ultimum* in a sterilized substrate amendment with the antagonist *Laetisaria arvalis* or organic household waste compost was studied. The substate used was riversand, because the roots can be harvested undamaged and close observation of the root system is possible. Both the amendment with compost and *L. arvalis* gave a reduction of disease severity.

Colonization of the root residues to prevent an increase of *Pythium* root rot seems possible by both the application of a specific antagonist, like *L. arvalis*, and an organic medium inhabited by various microorganisms like compost.

Introduction

Tulip flowers are produced in greenhouses. Flower production is conducted in boxes filled with a growth substrate, generally potting soil. *Pythium*, root rot, is a serious problem in this growing system. The fungus attacks the roots resulting in a loss of flower quality and in case of severe infection no flowers are produced at all.

Usually growing substrates are used only once to avoid the risk of damage caused by *Pythium* surviving in root residues of a possibly infected former culture. In order to reduce the amount of waste material, however, it is desirable to re-use growing substrates. Treatment of used substrates before planting a next culture is necessary to eliminate *Pythium* left from a former culture. Due to constraints on chemical disease control alternative methods to control *Pythium* are studied. Steam-sterilization is an environmentally friendly measure to obtain a *Pythium*-free substrate. As a consequence, however, also the complete indigenous microflora is destroyed resulting in a conducive substrate. In case *Pythium* is introduced in such a substrate the fungus has the opportunity to colonize root residues, giving rise to severe damage in the next tulip crop.

The possibilities to restore suppressiveness by amendment of a steam-sterilized substrate with composted organic household waste or the antagonist *Laetisaria arvalis* was studied.

Materials and methods

Inoculum

Pythium ultimum isolate P17, originally obtained from diseased tulip roots was used. Inoculum was produced on Czapek Dox Agar supplemented with sunflower oil, which stimulates production of oospores. The colonized agar was suspended in water with a blender and diluted to the required concentration of oospores and hyphal swellings (cfu=colony forming units). Growing substrate was infested with 10^5 and 10^6 cfu per liter just before planting bulbs.

Production of sclerotia of L. arvalis

L. arvalis was grown on autoclaved oatseeds (200 ml oat seed, 100 ml water) or a autoclaved cornmeal-perlite mixture (15 g perlite, 32 g cornmeal, 50 ml water) for 4 weeks. Sclerotia were harvested by wet sieving and air-dried. Sclerotia were added to the substrate at a rate of 0.5 g per liter one week before infestation and planting of bulbs.

Composted organic household waste (compost)

The compost used originated from fruit, vegetable, and garden wastes. The material was composted aerobically in a Dutch composting facility. After the composting process the compost was cured for 10 weeks at 20°C and a moisture level of 50% (w/w). Compost was added to the growing substrate at a rate of 1% (v/v) one week before infestation and planting of bulbs.

Growth substrate

Experiments were conducted in riversand, because the roots can be harvested undamaged in this substrate and close observation of the root system is possible. A steam-sterilized used substrate was simulated by autoclaved riversand containing 10 grams of tulip root residues and standard tulip fertilization. The sterilized riversand was amended with compost (1% v/v) or sclerotia of *L. arvalis* (0.5 g/l) one week before planting tulipbulbs. Infestation of the amended riversand was carried out just before planting at the *Pythium*inoculum levels of 10^5 and 10^6 cfu/l.

Bulbmaterial

Bulbs (circumference $\geq$ 12 cm) of the tulip cultivar Gander, which is susceptible to *P. ultimum* were used. The bulbs received a cold treatment at 2°C or 5°C. The outer brown skin of the bulbs was removed just before planting and the bulbs were disinfected in 0.025% etridiazole (70% wp) + 0.1% carbendazim (50% wp) for 15 minutes. Bulbs were planted in square boxes (15 bulbs /20x20x10 cm box) and placed in the greenhouse at a temperature of 17°C. Tulip plants were harvested when the plants of the non-infested controls were flowering. Root fresh weight was determined and root rot severity was estimated on a 0-5 basis with 0= symptomless to 5=all roots decayed.

Statistical analysis

The experiment was performed twice with five replicates per treatment in a randomized block design. Separations of means were based on least significant difference (LSD) at p=0.05.

Results and discussion

In the infested controls at both inoculum densities serious root rot developed. Amendment with compost or *L. arvalis* of the autoclaved riversand containing tulip root residues reduced root rot considerably. At the inoculum level of 10^5 cfu/l only mild symptoms were observed in both amended treatments. At the level of 10^6 cfu/l *L. arvalis* suppressed *P.ultimum* more than the compost amendment (Table 1).

Treatment	Inoculum* (cfu/l)	Root rot severity **
Control	10^5	3.0 c
(no treatment)	10^6	4.0 d
Compost	10^5	0.6 a
	10^6	2.3 b
L. arvalis	10^5	0.5 a
	10^6	1.0 a

* The treatments without *P.ultimum* showed no root rot symptoms and are not mentioned in the table.
**Numbers followed by the same letter are statistically not different (LSD, p=0.05)

Table1 : Effect of 1% (v/v) compost and *Laetisaria arvalis* (0.5 g/l) on *Pythium*-root rot severity of tulips in autoclaved riversand with root residues.
0=symptomless to 5=all roots decayed.

The effect of the treatments on root fresh weight was similar. At the high inoculum level, however, there was no difference between disease reduction by *L. arvalis* and compost. At the level of 10^5 cfu/l both amendments resulted in root fresh weights weights not different from the non-infested controls. At the density of 10^6 cfu/l root fresh weights of the amended treatments did not reach the level of the pathogen-free treatments, but the values were significantly higher than those of the 10^6 cfu/l infested control (Fig. 1).

Various studies have indicated that *Pythium* can saprophytically colonize fresh crop residues and use the material as a food base to establish and reproduce in the soil environment (Lifshitz and Hancock, 1983; Martin and Hancock,1985; Wall, 1984). The fungus, however, is considered a poor competitor and, therefore, is not able to utilize crop residues that are previously colonized by other microorganisms (Hancock and Lifshitz, 1983). A sterilized substrate containing fresh root residues can be extremely conducive to *P. ultimum* as shown by our results. Introduction of *L. arvalis* into the conducive substrate previous to *Pythium* -infestation prevented the development of severe root rot. *L. arvalis* has been described as an specific antagonist of *P. ultimum* and *Rhizoctonia solani* in other studies (Burdsall *et al.*, 1980; Hoch and Abawi, 1979; Lewis and Papavizas, 1992; Martin *et al.*, 1983; Martin *et al.*, 1986). Amendment with compost containing a wide range of microorganisms also resulted in suppression of root rot. The suppressive capacity of composted organic household waste is in accordance with results described by Schüler *et al.*, 1989.

When substrates are re-used and sterilized to eliminate *Pythium* from a former culture both the application of a specific antagonist, like *L.arvalis*, and an organic medium colonized by various microorganisms like compost can restore suppressiveness.

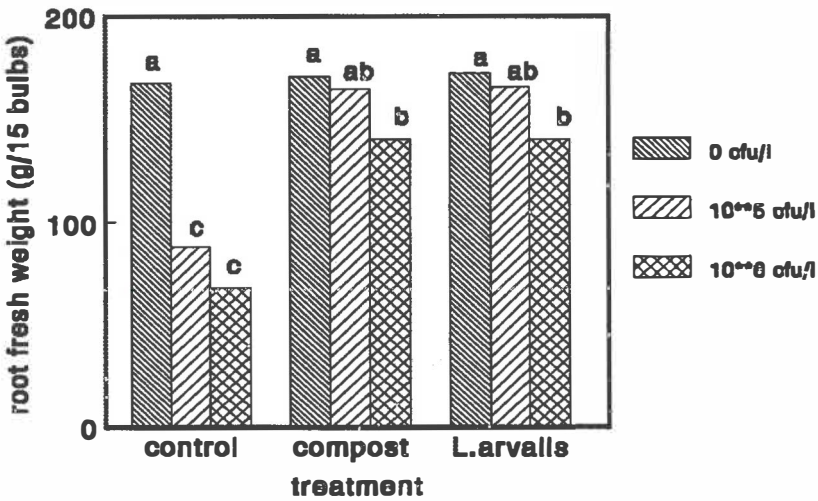

Figure 1 : The effect of 1% (v/v) compost and *Laetisaria arvalis* (0.5 g/l) on root fresh weights of tulips in autoclaved riversand with root residues. Bars with the same letter are statistically not different (LSD, p=0.05).

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