Cellular interactions during biocontrol of tomato foot and root rot

Bolwerk, A.

Cellular interactions during biological control of tomato foot and root rot

Annouschka Bolwerk

Cover: Gulf of Naples. In cooperation with Peter Hock.

Printed by: Ridderprint Offsetdrukkerij B.V., Ridderkerk.

Cellular interactions during biological control of tomato foot and root rot

Proefschrift

Ter verkrijging van
de graad Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D. D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 19 januari 2005
te klokke 14:15

door

Annouschka Bolwerk

Geboren te Baarn
in 1978

Promotiecommissie

Promotor:	Prof. Dr. E. J. J. Lugtenberg
Co-promotor:	Dr. G. V. Bloemberg
Referent:	Prof. Dr. M. Lorito
Overige leden:	Prof. Dr. C. A. M. J. J. van den Hondel Prof. Dr. E. van der Meijden Prof. Dr. H.P. Spaink Prof. Dr. J. A. Van Veen

“Cellular interactions during biological control of tomato foot and root rot” by Annouschka Bolwerk

This work was financed by a grant from the Earth and Life Sciences Council NWO and the Earth and Life Sciences Foundation ALW.

Reproduction of color prints was financially supported by the Leiden University Foundation.

Contents

Chapter 1

General introduction

Plant-microbe interactions

The plant rhizosphere

The rhizosphere is defined as the area in close proximity to the root system by which it is influenced (Hiltner, 1904). The exudation of carbon sources such as organic acids, sugars and amino acids (Lugtenberg and Bloemberg, 2004; Vancura and Hovadik, 1965) by the root creates a nutrient-rich environment in which microbial activity is stimulated. Interactions between plants and micro-organisms can be classified as pathogenic, saprophytic and beneficial (Lynch, 1990). Compared to root-free soil the levels of micro-organisms of all major groups, including bacteria, fungi, protozoa, actinomycetes, microalgae and viruses are elevated in the rhizosphere.

Pathogenic interactions

Pathogenic interactions in the rhizosphere involve (i) pathogenic interactions between microorganisms and plant roots causing plant diseases and (ii) pathogenic interactions between microorganisms. Examples of the latter are parasitism of one fungus by another one (referred to as mycoparasitism) and the production of antibiotics that inhibit or kill other microorganisms.

Plant diseases may be caused by nematodes, mites, bacteria, viruses, algae and fungi, of which fungi cause the most important damage. In crops, it is estimated that plant diseases cause 10-20% loss in production (James, 1981). Common fungal pathogens are *Rhizoctonia solani*, *Pythium* spp., *Phytophthora* spp., *Gaeumannomyces graminis*, *Alternaria* spp., *Botrytis cinerea*, *Verticilium* spp. and *Fusarium* spp.

Fusarium oxysporum spp. are saprophytic living fungi and are able to grow and survive for long periods on organic matter in soil and in the rhizosphere of many plant species (Garrett, 1970). Some of these strains are described as pathogenic whereas others are described as non-pathogenic (see below). Among the pathogenic *Fusarium* strains vascular and non-vascular pathogens are identified. Vascular wilt disease is caused by vascular pathogens whereas crown and root rot, stalk rot, head and blights are caused by non-vascular pathogenic *Fusarium* spp. (Summerell et al., 2003).

Both pathogenic and non-pathogenic *Fusarium* strains can penetrate the root and, in contrast to the non-pathogenic strains, pathogenic strains can penetrate the vascular system and cause disease (Olivian and Alabouvette, 1997). *Fusarium* wilt pathogens are highly host-specific and are classified in many different *formae speciales* groups based on the host plant species (Armstrong and Armstrong, 1981). *Fusarium oxysporum* f. sp. *lycopersici* (*F.o.l.*) causes wilt disease in tomato and spreads rapidly through the vascular tissue (Menzies et al., 1990). The fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) causes tomato foot and root rot (TFRR), which is a serious problem for field and greenhouse crops (Jarvis, 1988). In contrast to the wilt causing *F.o.l.*, *F.o.r.l.* is not a vascular pathogen (Rowe, 1980; Menzies et al., 1990). Difficulties in controlling

Fusarium diseases and increasing environmental concern has stimulated research in the biological control of these diseases.

Beneficial interactions

Beneficial interactions can be divided in four classes: (i) Biofertilization involves micro-organisms that increase the availability of nutrients for the plant. The formation of root and/or stem nodules by leguminous host plants upon infection by *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Azorhizobium* increases the availability of nitrogen for the plant (Lugtenberg et al., 1991; Spaink, 1995; van Rhijn and Vanderleyden, 1995). The uptake of phosphate and mineral nutrients by the plant is enhanced upon the interaction of the plant with mycorrhizae, a particular group of soil fungi providing an organic link between the root and bulk soil (Reid, 1990; Okon et al., 1997). (ii) Phytostimulation involves direct plant growth promotion by the production of the phytohormone auxin by micro-organisms (Schippers et al., 1987 and references therein). (iii) Rhizoremediation refers to the degradation of pollutants by microbes present in the rhizosphere of the plant (Anderson, et al., 1993; Kuiper et al., 2004; Schwab and Banks, 1994). (iv) Soils that naturally limit the incidence of plant diseases, even in the presence of a virulent pathogen and susceptible host, are defined as natural suppressive soils. Both biotic and abiotic elements contribute to disease suppression. Although chemical and physical characteristics of the soil affect every living organism, soil organisms or microbial metabolites often determine directly or indirectly soil suppressiveness. The analysis of soil and compost with the natural ability to suppress plant diseases led to the identification of potential biocontrol strains. Biocontrol involves the suppression of plant diseases by (micro)-organisms. Pathogen suppression by these strains operates via diverse mechanisms including competition for nutrients, antibiosis, parasitism and induction of host resistance.

Biocontrol agents

Chemical pesticides have been used on a large scale to protect plants against diseases. Since these substances can have deleterious effects on human health and on the environment and since pathogens can become resistant to chemicals, more and more attention has been paid to biological pesticides. Coating seeds of potato, radish and sugar beet with biocontrol bacteria provided crop protection and increased crop yields (Burr et al., 1978; Geels and Schippers, 1983; Schippers et al., 1995; Suslow and Schroth, 1982). Bacteria identified as plant growth promoting rhizobacteria and biocontrol strains often belong to the following genera. (i) *Bacillus* (Handelsman, 1999; Pusey, 1999; Pusey and Wilson, 1984; Silo-suh et al., 1994), (ii) *Streptomyces* (Emmert and Handelsman, 1999) and (iii) *Pseudomonas* (Geels, F. P. and Schippers, B. 1983a, b; Scher and Baker, 1980; Stutz et al., 1986; Weller and Cook, 1983; Weller et al., 1985). Biocontrol fungi, identified after analysis of natural disease suppressive soils include (i)

nonpathogenic *Fusarium* spp. (reviewed by Fravel et al., 2003; Larkin et al., 1993; Larkin et al., 1996; Louvet et al., 1976; Paulitz et al, 1987; Schneider, 1984; Tamietti and Alabouvette, 1986; Tamietti and Pramotton, 1990; Tousson, 1975); and (ii) *Trichoderma* species with antagonistic activities; isolated both out of soil and compost (Hermosa et al., 2000; Mousseaux, et al., 1998; Simon and Sivasithamparam, 1989; Trillas-Gay et al., 1986).

Pseudomonas

The mechanisms through which *Pseudomonas* spp. control plant diseases involve (i) competition for niches and nutrients, (ii) antibiosis, (iii) predation, and (iv) induction of plant defense responses.

Pseudomonas spp. are efficient root colonizers and can thereby compete for niches and nutrients in the rhizosphere. The *Pseudomonas* bacteria form micro-colonies at the junctions of epidermal plant root cells (Bowen and Rovira, 1976; Chin-A-Woeng et al., 1997), the site where high concentrations of root exudate are thought to be leaking from the root (Rovira, 1956; Chin-A-Woeng et al, 1997; Bloemberg et al., 1997). These intercellular junctions also form the niche for the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) (Lagopodi et al., 2002), which causes tomato foot and root rot (TFRR). The ability of biocontrol strains to reduce the ability of pathogens to propagate in soil involves the competition for nutrient compounds like carbon, nitrogen sources and iron (Handelsman and Stabb, 1996; Loper and Byer, 1991; Lugtenberg et al., 2004).

Pseudomonads produce a wide array of antibiotics including phenazine-1-carboxamide (PCN), 2,4-DAPG, pyoluteorin, pyrrolnitrin and phenazine-1-carboxylic acid (PCA), (Chin-A-Woeng et al., 1998; Keel et al., 1992; Kraus and Loper, 1995; Thomashow and Weller, 1988). Analysis of biosynthetic mutants has shown that antibiotics play an important role in biocontrol by *Pseudomonas* spp. producing these antibiotics. In addition to these small organic molecules volatiles like hydrogen cyanide, which was shown to be a biocidic compound, can be produced by *Pseudomonas* spp. (Voisard et al., 1989).

Pseudomonas spp. producing lytic enzymes such as chitinase, ß-(1,3)-glucanases, cellulases, lipases, and proteases can suppress plant diseases as well (Chatterjee et al., 1995; Dunlap et al., 1997; Dunne et al., 1996a; Dunne et al., 1996b; Dunne et al., 1998; Jijakli and Lepoivre, 1998; RuizDuenas and Matinez, 1996; Shapira et al., 1989). These lytic enzymes can degrade fungal cell wall components such as chitin and glucan, and the biocontrol agent can subsequently utilize the degradation products. Additionally, the enzymes may act synergistically with other antifungal metabolites (Di Pietro et al., 1993; Duffy et al., 1996; Fogliano et al., 2002; Lorito et al., 1994). It should be noted that a thorough genetic analysis about the roles of lytic enzymes, produced by *Pseudomonas* spp., in biocontrol is lacking so far.

Biocontrol strains can trigger physical and chemical defense responses within the host plants. Necrotising agents can cause immunity, which is associated with the accumulation of salicylic acid, and the production of pathogenenis-related (PR) proteins, defined as systemic acquired resistance (SAR). Non-pathogenic

root colonizing microbes or biotic agents such as lipopolysaccharides, siderophores, of flagella (Bakker and Schippers, 1995) can trigger immunity within the host, defined as induced systemic resistance (ISR). The ISR-response involves jasmonic acid and ethylene production (Hoffland et al., 1995; van Loon et al., 1998; Pieterse et al., 1996; Pieterse et al., 2001). By restricting or blocking the ability of the phytopathogenic fungus to establish disease in the host plant, biocontrol microbes provide partial or complete resistance of the host to the disease.

Non-pathogenic Fusarium

In suppressive soils, interactions between pathogenic and non-pathogenic *Fusarium* strains results in disease control. The biocontrol by the non-pathogenic strains involves different modes of action: (i) competition for niches/infection sites, (ii) competition for nutrients, (iii) higher rate of chlamydospore germination and (iv) induction of plant defense responses.

Visualization studies illustrated the root colonization pattern of different non-pathogenic *Fusarium* spp. on different plant hosts (Bao and Lazarovits, 2001; Benhamou and Garand, 2001; Olivian and Alabouvette, 1997; Olivian and Alabouvette, 1999; Olivian et al., 2003). Both pathogenic and non-pathogenic *Fusarium* spp. colonized the root surface and penetrated into the epidermis. The frequency of penetration was lower in case of the non-pathogenic strains and colonization was restricted to the cortex due to defense reactions of the plant. In contrast, pathogenic *Fusarium* spp. were observed to colonize the vessels. Competition for infections sites at the root surface and for root tissue colonization is likely to occur since the root colonization processes by non-pathogenic and pathogenic *Fusarium* spp. have many similarities. The possible role of competition for root tissue colonization is illustrated by studies of Eparvier and Alabouvette (1994) who showed that root colonization reaches a plateau after 14 to 21 days, suggesting that only a limited amount of root tissue can be colonized by *Fusarium* spp..

How non-pathogenic biocontrol *Fusarium* spp. affect pathogenic *Fusarium* spp. strongly depends on the isolate studied. Chlamydospore germination of two different pathogens, *F. oxysporum* f sp. *lycopersici* and *F. oxysporum* f sp. *bacilici*, was significantly reduced at increased glucose concentrations in soil (0.2mg/g) and in presence of one of the three non-pathogenic *Fusarium* spp. tested (*F. oxysporum* Fo47). In contrast, Fo47 was less effective than the other two strains CS-20 and CS-1 in reducing the disease incidence at increasing pathogen concentration. CS-20 was effective at inoculum concentrations 5 to 50 times lower than that of the pathogen, whereas Fo47 was effective at inoculum densities 10 to 100 times higher than that of the pathogen (Larkin and Fravel, 1999). Reduction of chlamydospore germination was also observed for the pathogen *F. oxysporum* WCS816 in presence of the biocontrol agent *F. oxysporum* Fo47b10 (Lemanceau et al., 1993). Additionally, increasing the glucose concentration (up to 10 mg/ml) reduced the inhibition of WCS816 chlamydospore germination by Fo47b10 indicating that glucose is one of the limiting factors for germination.

The control of *F. oxysporum f. sp. lycopersici* Fol8 on tomato plants by Fo47 was analyzed by Fuchs et al. (1997) who tested the effect of separate introduction in time (benomyl and cutting system) or space (split root and layering assay) of the pathogen and biocontrol agent. Their results suggested that ISR is involved in the control of strain Fol8 by Fo47. Fo47 was shown to induce different defense and pathogen related (PR) proteins in tomato that are associated with SAR: ß-1,3-glucanase; ß-1,3-glucosidase; chitinase and PR-1 (Duijff et al., 1998; Fuchs et al., 1997), confirming the ability of Fo47 to induce resistance in tomato plants.

Trichoderma

The biological control by *Trichoderma* spp. involves mechanisms that affect the plant pathogens either directly or indirectly. Direct modes of action include: (i) mycoparasitism and (ii) antibiosis. Indirect modes of action involve (iii) competition nutrients and/or niches, (iv) promotion of plant growth and (v) induction of resistance in plants.

Mycoparasitism acts through the production of extracellular enzymes such as chitinases (Carsolio et al., 1994; De La Cruz et al., 1992; Harman et al., 1993), glucanases (Haran et al., 1995; Lora et al., 1995; Lorito et al., 1994) and proteases. Disruption of an endochinase-encoding gene *ech42* in *T. atroviride* P1, illustrated the role of this enzyme in the control of *Rhizoctonia solani* (Woo et al., 1999). Expression *ech42*, can be triggered by diffusible factors of *R. solani,* and does not require physical contact (Cortes et al., 1998; Kullnig et al., 2000). The expression of another chitinase gene encoded by *nag1* in *T. atroviride* P1 is induced after contact with *R. solani* (Zeilinger et al., 1999). Carbon starvation as well as growth on fungal cell walls induced expression of *ech42*. The latter condition also induced the expression of *nag1* (Mach et al., 1999). In contrast, expression of *nag1* can be reduced by deoxynivalenol (DON), a secondary metabolite produced by different *Fusarium* strains (Lutz et al., 2003). The other direct mode of action involves the production of antibiotics (Ghisalberti and Rowland, 1993; Schirmböck et al., 1994). The membrane affecting compounds of this group can act synergistically with cell wall degrading enzymes (Lorito et al., 1996) and can thereby affect the biocontrol ability of the *Trichoderma* spp.

The indirect modes of action includes competition nutrients and/or niches (Dubos et al., 1982; Simon and Sivasithamparan, 1989; Zimand et al., 1996). Sivan and colleagues (1987) showed that *T. harzianum* was mainly found on the crown and root tip of the tomato root, the sites where *Fusarium* spp. were reduced. Plant growth promotion is described as indirect mechanism of biocontrol by Inbar et al. (1994). Via the constitutive production of chelating substances and the synthesis of reducing metabolites, *T. harzianum* can solubilize plant nutrients from their solid-phase compound under *in vitro* conditions. Both chelation and reduction of metal oxides were described to be involved in the biocontrol of plant pathogens by several rhizobacteria and might contribute to the biocontrol by *T. harzianum* (Altomare et al., 1999).

T. asperellum and the *T. harzianum* strains T39 and T-203 were described to induce resistance in different plants species (De Meyer et al., 1998; Yedidia et al., 1999; Yedidia et al., 2000; Yedidia et al., 2003). The presence of *T. asperellum* was associated with the expression of two defense genes in cucumber encoding phenylalanine ammonia lyase and hydroxyperoxide lyase (Yedidia et al., 2003). Despite the spatial separation of strain T39 and the pathogen, the incidence of the disease was reduced indicating that ISR is involved in the biocontrol by T39 (De Meyer et al., 1998). Strain T203 was found to penetrate cucumber roots and induce defense responses such as increased chitinase and peroxidase activities (Yedidia et al., 1999), strengthening of epidermal and cortical cell walls by appositions (Yedidia et al., 1999) and the induction and accumulation of PR proteins (Yedidia et al., 2000).

Biocontrol agent-plant pathogen interactions

In biological control, the interactions between pathogens and biocontrol organisms can be both antagonistic and parasitic. Pathogens have diverse responses to counteract antagonism, including detoxification, repression of biosynthetic genes involved in biocontrol, active efflux of antibiotics and antibiotic resistance (reviewed by Duffy et al., 2003). Interactions between *F.o.r.l.* and *P. fluorescens* affected gene expression in the biocontrol agent *P. fluorescens*. Fusaric acid (FA), a secondary metabolite produced by *F.o.r.l.* was reported to repress 2,4 diacetylphloroglucinol (2,4-DAPG) biosynthesis (Duffy et al., 1997; Notz et al., 2002; Schnider-Keel et al., 2000). FA also reduces PCN production in *P. chlororaphis* PCL1391 under *in vitro* conditions (van Rij et al., *in press*). Deoxynivalenol (DON), another secondary metabolite produced by different *Fusarium* strains, represses the expression of the exochitinase gene *nag1* in *T. atroviride* P1 (Lutz et al., 2003).

Strategy to visualize interactions during biocontrol

Visualization of microbes using microscopy

In order to obtain more insight in the mechanism of control of TFRR by *Pseudomonas*, *Fusarium* and *Trichoderma* spp. we visualized the interactions between the root, the pathogen *F.o.r.l*. and the three mentioned biocontrol agents. Using scanning electron microscopy (SEM) Chin-A-Woeng and colleagues (1997) visualized and localized *Pseudomonas* bacteria on tomato roots. The drawback of using SEM is that samples should be fixed before root colonization can be studied. Light microscopy is another technique available for the visualization of microbes on plant roots using GUS constructs or histochemical staining. The use of a GUS construct allowed quantification of the nonpathogenic *F. oxysporum* SA70 on roots of tomato plants (Bao et al., 2000; Eparvier and Alabouvette, 1994). Using histochemical staining Bao and Lazarovitz (2001) were able to simultaneously visualize the pathogenic *F. oxysporum* f sp. *lycopersici* and the non-pathogenic *F. oxysporum* SA70 colonizing the root tissue.

Using another technique, confocal scanning laser microscopy (CLSM) Bloemberg et al. (1997; 2000) were able to visualize *Pseudomonas* bacteria on tomato roots without fixing the sample. To this end the bacteria were labeled with an auto-fluorescent protein (AFP). Constitutive promoters upstream the genes encoding the AFPs were introduced in the microbes on plasmids or integrated into the genome. This approach has the advantages that subsequent studies are non-invasive and that the production of AFPs does not require extra substrates or energy. Consequently, life samples can be studied, visualization became less laborious and by using different AFPs Bloemberg and colleagues (2000) could simultaneously visualize up to three different organisms.

Different AFPs and color variants are nowadays available for CLSM studies. The enhanced green fluorescent protein (EGFP), originally isolated from the jellyfish *Aequorea victoria* (Chalfie et al., 1994), the enhanced cyan fluorescent (ECFP) and enhanced yellow fluorescent protein (EYFP) (Ellenberg et al., 1999; Matus et al., 1999; Tsien, 1998; Yang et al., 1998) have different characteristics regarding their excitation and emission spectra. Due to the emission spectra of the Green, Cyan and Yellow fluorescent proteins, the GFP-CFP and the YFP-CFP combinations are most useful for distinguishing the pathogen and biocontrol agent in dual-color images.

Gnotobiotic system

In order to visualize the pathogen and the biocontrol agents on the plant root, tomato seedlings are grown in a gnotobiotic sand system (Fig. 2) described by Simons et al. (1996). Briefly, glass tubes are filled with sand moisturized with a plant nutrient solution (10% vol/wt) (Hoffland et al., 1989) and sterilized. Tomato seeds are sterilized, as described by Simons et al. (1996). Subsequently, germinated tomato seeds were planted. Quartz sand was used because it has the advantage that it can be easily removed from the roots by gentle washing, after which the roots can be examined using CLSM. In contrast, the removal of soil from the root is difficult and subsequent microscopy studies are hampered due to the autofluorescence of the soil particles.

Previously, root colonization by either *Pseudomonas* biocontrol bacteria (Bloemberg et al., 1997; Bloemberg et al., 2000) or the pathogen *F.o.r.l.* (Lagopodi et al., 2002), labeled with an autofluorescent protein markers, were visualized using this gnotobiotic sand system. In these studies the seedlings were coated with bacteria and planted in the gnotobiotic system or seedlings were planted in sand infested with spores of *F.o.r.l.,* respectively.

Disease control can also be studied in this gnotobiotic sand system, which enabled us to analyze the interactions between tomato root, *F.o.r.l.* and the biocontrol agents under disease controlling conditions. Therefore, these studies with autofluorescing organisms will provide more insight in the mechanisms of control of TFRR by the biocontrol agents. Tomato seedlings were grown for seven days in sand infested with *F.o.r.l..* The biocontrol strains were introduced in different ways: (i) coated on seedlings, germinated seeds were incubated in either

a spore (fungi) or bacterial suspension and (ii) mixed though the sand either as spores (non-pathogenic *F. oxysporum*) or as mycelium (*Trichoderma* spp.).

Aim of this thesis

Many genes and traits that are involved in the mechanisms of disease control by *Pseudomonas*, *Fusarium* and *Trichoderma* species have been identified at the molecular level. Insight in how genes and traits of the biocontrol strains affect the pathogen at the cellular level and how they affect disease development will improve our understanding of biocontrol.

The work presented in this thesis focuses on studying the effects of the biocontrol microbes on the pathogen in the tomato rhizosphere, with the aim of deepening our insight in the mechanisms of disease control used by the biocontrol microbes. This study involves (i) the visualization of root colonization by the biocontrol strains using CLSM, (ii) the visualization and quantification of tomato root colonization by the pathogen *F.o.r.l.* in the absence and presence of a biocontrol agent or its mutants using CLSM, and (iii) the analysis of spore germination of the pathogen and biocontrol fungi in tomato root exudate. Using these methods we expect to analyze how previously identified genes and traits affect the pathogen at the cellular level and how this contributes to the observed disease reduction.

Chapter 2

Interactions in the tomato rhizosphere of two *Pseudomonas* biocontrol strains with the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*

Annouschka Bolwerk, Anastasia L. Lagopodi, André H. M. Wijfjes,
Gerda E. M. Lamers, Thomas F. C. Chin-A-Woeng,
Ben J. J. Lugtenberg and Guido V. Bloemberg

Published in Mol. Plant-Microbe Interact. 2003 11: 983-993

Abstract

The fungus *Fusarium oxysporum f. sp. radicis-lycopersici (F.o.r.l.)* causes foot and root rot of tomato plants, which can be controlled by the bacteria *Pseudomonas fluorescens* WCS365 and *Pseudomonas chlororaphis* PCL1391. Induced systemic resistance is thought to be involved in biocontrol by *P. fluorescens* WCS365. The antifungal metabolite phenazine-1-carboxamide (PCN) as well as efficient root colonization are essential in the mechanism of biocontrol by *P. chlororaphis* PCL1391. To understand the effects of bacterial strains WCS365 and PCL1391 on the fungus in the tomato rhizosphere, microscopical analyses were performed using different auto-fluorescent proteins as markers. Tomato seedlings were inoculated with biocontrol bacteria and planted in a *F.o.r.l.*-infested gnotobiotic sand system. Confocal laser scanning microscope (CLSM) analyses of the interactions in the tomato rhizosphere revealed the following. (i) The microbes effectively compete for the same niche, and presumably also for root exudate nutrients. (ii) The presence of either of the two bacteria negatively affects infection of the tomato root by the fungus. (iii) Both biocontrol bacteria colonize the hyphae extensively, which may represent a new mechanism in biocontrol by these pseudomonads. (iv) The production of PCN by *P. chlororaphis* PCL1391 negatively affects hyphal growth and branching, which presumably affects the colonization and infecting ability of the fungus.

Introduction

The fungus *F.o.r.l.* causes tomato foot and root rot (TFRR), which is a serious problem for field and greenhouse crops (Jarvis, 1988). Chemical pesticides do not efficiently suppress TFRR (Benhamou et al., 1994). In contrast, the biopesticides *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 efficiently suppress TFRR (Chin-A-Woeng et al., 1998; Dekkers et al., 2000). The mechanism of biocontrol of TFRR by *P. fluorescens* WCS365 has not been elucidated, but since it was shown that strain WCS365 triggers induced systemic resistance (ISR) in *Arabidopsis thaliana* (Gerrits and Weisbeek, 1996) it is likely that ISR also is the underlying mechanism of biocontrol of TFRR by *P. fluorescens* WCS365 (Dekkers et al., 2000). Strain PCL1119, a mutant derivative of *P. chlororaphis* PCL1391 impaired in the production of the antifungal metabolite phenazine-1-carboxamide (PCN), was not able to suppress TFRR in potting soil, showing that the production of PCN is required for biocontrol (Chin-A-Woeng et al., 1998). Testing of three different competitive root tip colonization mutants of strain PCL1391 showed that efficient colonization of the tomato root system is also essential for suppression of the disease by this strain (Chin-A-Woeng et al., 2000).

Many genes and traits involved in biocontrol have been identified to explain biocontrol at the molecular level (see reviews by Bloemberg and Lugtenberg, 2001; Lugtenberg et al., 2002; Thomashow and Weller, 1996; Whipps, 2001). Reports describing interactions between pathogens and control agents at the cellular level are limited (Benhamou et al.,1993; Benhamou et al.,1997; Benhamou et al.,1999; Chet et al., 1981; Etchebar et al., 1998; Hogan

and Kolter, 2002) and reports on the spatio-temporal analysis at the cellular level of the interactions between the biocontrol agent and the phytopathogenic fungus in the rhizosphere are scarce (Bao et al., 2001; Benhamou et al.,1996). In previous work we have analyzed, using CLSM, the colonization process of the tomato rhizosphere by *Pseudomonas* biocontrol strains (Bloemberg et al., 1997; Chin-A-Woeng et al., 1997; Dekkers et al., 2000) and by *F.o.r.l.* (Lagopodi et al., 2002). In the present paper we report, using microbes differentially labeled with auto-fluorescent proteins, an analysis of the interactions between *F.o.r.l.* and the *Pseudomonas* biocontrol strains WCS365 and PCL1391 in the tomato rhizosphere with the aim to obtain a better understanding of the biocontrol process.

Table 1. Microorganisms and plasmids

Strains, plasmids		**Reference or source**
Bacteria		
WCS365	*Pseudomonas fluorescens*; efficient competitive root colonizer; biocontrol strain of tomato foot and root rot caused by *F.o.r.l.*	Simons et al., 1996; Dekkers et al., 2000
PCL1391	*P. chlororaphis*; efficient competitive root colonizer; biocontrol strain of tomato foot and root rot; produces phenazine-1-carboxamide (PCN)	Chin-A-Woeng et al., 1998; 2000
PCL1119	PCL1391 (*phzB* :: Tn*5luxAB*); does not produce phenazine-1-carboxamide	Chin-A-Woeng et al., 1998
Fungi		
ZUM 2407	*F.o.r.l.* causing tomato foot and root rot	IPO-DLO, Wageningen, The Netherlands
FCL14	*F..o.r.l.* ZUM 2407 containing *gfp* under control of the constitutive *gpdA* promoter	Lagopodi et al., 2002
Plasmids		
pMP4662	pME6010 containing *rfp* under the control of the *lac* promoter; is maintained in *Pseudomonas* without antibiotic pressure; Tc^R	Bloemberg et al., 2000

Results

Biocontrol of tomato foot and root rot by P. fluorescens WCS365 and P. chlororaphis PCL1391 in a gnotobiotic sand-nutrient solution system

To test the biocontrol ability of *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 (Table 1), tomato seedlings were inoculated with cells of either strain and grown in a gnotobiotic system containing sand infested with *F.o.r.l.* spores. Already after four days disease symptoms are visible in 40% of the plants. After seven days of incubation the plants were analyzed for disease symptoms and statistical analysis was performed. The presence of the pathogenic fungus caused disease symptoms in 70-90% of the plants (Tables 2A and 3A) after seven days. The presence of *P. fluorescens* WCS365 on tomato seedlings reduced the percentage of sick plants considerably to 0-15% (Table 2A). Using a chi-squared goodness-of-fit statistical test two different treatments were compared. Comparison of plants grown in sand containing *F.o.r.l.* spores with and without *P. fluorescens* WCS365 showed that strain WCS365 significantly suppressed tomato foot and root rot in the gnotobiotic system (Table 2B).

Table 2. Biocontrol of tomato foot and root rot by *P. fluorescens* WCS365 in a gnotobiotic sand-nutrient solution system

A. Disease symptoms

Micro-organism(s) present	Experiment 1		Experiment 2	
	Healthy	Sick	Healthy	Sick
(i) None	20	0	20	0
(ii) *F.o.r.l.*	6	14	2	18
(iii) *F.o.r.l.* + *P. fluorescens* WCS365	20	0	17	3
(iv) *P. fluorescens* WCS365	20	0	20	0

B. Statistics

Compared treatments	X_2values experiment 1	X_2values experiment 2
(ii) and (iii)	21.54 [c]	22.56 [d]
(iv) and (iii)	0 [e]	3.24 [f]

A. Twenty plants were either grown (i) in the absence of microbes, (ii) in the presence of *F.o.r.l.*, (iii) in the presence of both *F.o.r.l.* and *P. fluorescens* WCS365, or (iv) in the presence *P. fluorescens* WCS365. Seven days after plant inoculation the disease status of the plants was scored. For details of inoculation and growth conditions, see the Materials and Methods section. **B.** Statistical analysis of the biocontrol experiment (panel A) was performed using a chi-squared goodness-of-fit test (Heath, 1995). Critical X_2 value 3.841. For details about the analysis, see the Materials and Methods section.

[c,d] The two compared treatments are significantly different

[e,f] The two compared treatments are not significantly different

Inoculation with *P. fluorescens* WCS365 improved the health condition of plants grown in the presence of *F.o.r.l.* up to the level of the seedlings grown only in the presence of *P. fluorescens* WCS365, which were all healthy (Table 2B). Inoculation of tomato seedlings with *P. chlororaphis* PCL1391 reduced the percentage of sick plants significantly to 6-15% (Table 3A and 3B). In addition PCL1119, a derivative of *P. chlororaphis* PCL1391 in which a promoterless Tn5*luxAB* has been inserted in *phzB* and therefore does not produce PCN, was tested to study the role of PCN production in the biocontrol ability of *P. chlororaphis* PCL1391 in the gnotobiotic system. PCL1119 reduced the percentage of sick plants significantly to 38-60% (Table 3A and 3C). The disease reduction caused by *P. chlororaphis* PCL1119 was significantly less than the reduction caused by *P. chlororaphis* PCL1391 (Table 3C).

Table 3. Biocontrol of tomato foot and root rot by *P. chlororaphis* PCL1391 in a gnotobiotic sand-nutrient solution system

A. Disease symptoms

Micro-organism(s) present	Experiment 1		Experiment 2	
	Healthy	Sick	Healthy	Sick
(i) None	16	0	20	0
(ii) *F.o.r.l.*	4	12	2	18
(iii) *F.o.r.l.* + *P. chlororaphis* PCL1391	15	1	17	3
(iv) *F.o.r.l.* + *P. chlororaphis* PCL1119	10	6	8	12
(v) *P. chlororaphis* PCL1391	16	0	20	0

B. Statistics *P. chlororaphis* PCL1391

Compared treatments	X_2 values experiment 1	X_2 values experiment 2
(ii) and (iii)	15.81 [c]	22.56 [d]
(v) and (iii)	1.03 [e]	3.24 [f]

C. Statistics *P. chlororaphis* PCL1119

Compared treatments	X_2 values experiment 1	X_2 values experiment 2
(ii) and (iv)	4.57 [c]	4.8 [d]
(iii) and (iv)	4.57 [c]	8.64 [d]
(v) and (iv)	7.38 [c]	17.14 [d]

A. Sixteen (exp. 1) or twenty plants (exp. 2) were either grown (i) in the absence of microbes, (ii) in the presence of *F.o.r.l.*, (iii) in the presence of both F.o.r.l. and *P. chlororaphis* PCL1391, (iv) in the presence of both *F.o.r.l.* and *P. chlororaphis* PCL1119 or (v) in the presence *P. chlororaphis* PCL1391. Seven days after plant inoculation the disease status of the plants was scored. For details of inoculation and growth conditions, see the Materials and Methods section. **B.** Statistical analysis of the biocontrol experiment (panel A) was performed using a chi-squared goodness-of-fit test (Heath, 1995). Critical X_2value 3.841. For details about the analysis, see the Materials and Methods section.

[c,d] The two compared treatments are significantly different

[e,f] The two compared treatments are not significantly different

Spatio-temporal analysis of interactions between the biocontrol bacteria P. fluorescens WCS365, P. chlororaphis PCL1391 and F.o.r.l. in the tomato rhizosphere

Using CLSM we visualized *F.o.r.l.* and the *Pseudomonas* biocontrol bacteria simultaneously in the tomato rhizosphere. To distinguish the bacteria and the fungus, GFP-labeled *F.o.r.l.* derivative FCL14 (Lagopodi et al., 2002) and DsRed-labeled (encoded by *rfp*) *P. fluorescens* WCS365 or *P. chlororaphis* PCL1391 harboring the plasmid pMP4662 (Bloemberg et al., 2000) were used.

After inoculation of tomato seedlings with either *P. fluorescens* WCS365 or with *P. chlororaphis* PCL1391 and subsequent plant growth in the gnotobiotic sand system, bacterial cells were detected on the main root and on root hairs. After three days of plant growth single bacterial cells were observed on the 7-cm long roots from the crown to 2.5 cm above the root tip and they were predominantly present along the junctions of the epidermal cells (Fig. 1A). Small microcolonies were formed on the upper half of the root. After six days of growth microcolonies on the upper half of the root was estimated to be increased up to four times in both number and length.

Seedlings coated with either *P. fluorescens* WCS365 or with *P. chlororaphis* PCL1391 grown in *F.o.r.l.*-infested sand were four times examined (three seedlings per condition). Focusing on regions of the main root where either the fungus was most abundantly present, or where both the fungus and the bacteria were present and interactions were visible, showed the following. (i) The colonization pattern of the *Pseudomonas* biocontrol bacteria was unaffected by the presence of the phytopathogenic fungus over fourteen days. (ii) The bacteria reached the root surface earlier and proliferated faster than the fungus. The bacteria were already visible within 24 hours whereas the fungus could only be observed after 48-72 hours. (iii) The bacteria occupied the same niches as the fungus (compare Figs. 1A and 1B). (iv) During the first three days the attachment and initial growth of the fungus along the cellular junctions of the tomato root were not affected by the presence of the biocontrol bacteria. (v) In contrast, after seven days the density of the hyphal network, expressed as the number of fluorescent pixels per cm2 of root, was strongly reduced by the presence of the biocontrol bacteria (Table 4). *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 reduced the hyphal network up to 5 times (compare Fig 1C with 1D and 1E, respectively) whereas the reduction by *P. chlororaphis* PCL1119 was less strong, 3 times (compare Fig 1C with 1F). In two other experiments the density of the hyphae was also reduced, although to a lesser extent (Table 4). (vi) In the close vicinity of the *Pseudomonas* biocontrol bacteria penetration of the tomato root by the fungus was not observed. (vii) Three to four days after planting, the *Pseudomonas* biocontrol bacteria caused an increase of the number of vacuoles within the fungal hyphae (Fig. 1G). (viii) After three days the bacteria attached to, and subsequently colonized the hyphae as was shown by a three dimensional analyses making Z-sections (Fig. 1H). (ix) Ten days after inoculation the bacteria were found predominantly around the hyphae and they had further colonized the hyphae (compare Fig. 2A with 2B).

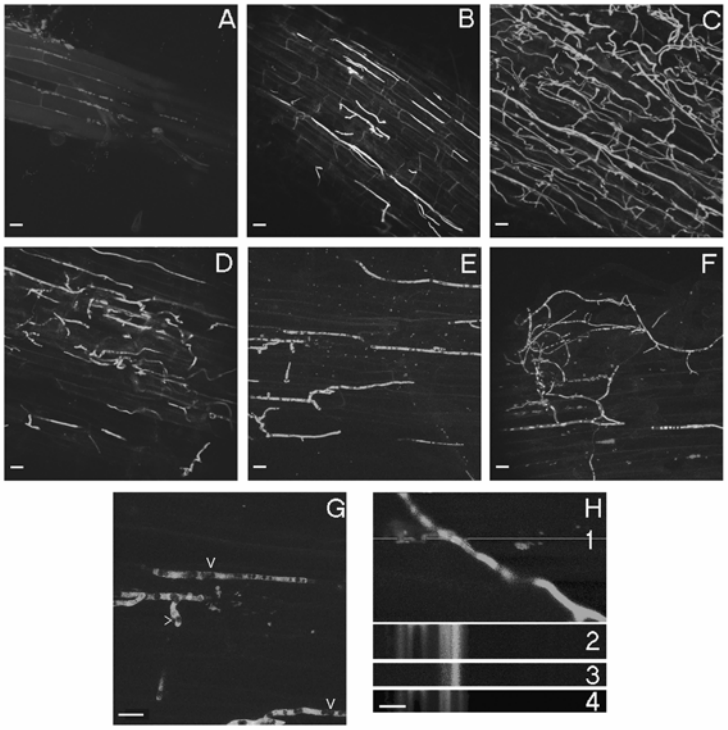


Figure 1. Confocal laser scanning microscopical analysis of tomato root colonization by the phytopathogenic fungus *F. oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) and by *Pseudomonas* biocontrol bacteria. The full color figure is depicted on page 121.
Two-day-old tomato seedlings were inoculated at time zero with cells of either *P. fluorescens* WCS365 or *P. chlororaphis* PCL1391 harboring a reporter plasmid expressing the *rfp* gene, which here appear as red cells. Plants were grown in a gnotobiotic sand system containing spores of *F.o.r.l.* harboring a constitutively expressed *gfp* gene. Walls of tomato root cells appear as red due to autofluorescence. **A,** *P. fluorescens* WCS365 colonizing the intercellular junctions of root cells of an inoculated seedling planted in sterile sand three days after planting. **B,** *F.o.r.l.* hyphae growing along the intercellular junctions of root cells of a sterile seedling three days after planting in sand containing fungal spores. **C,** Hyphal network present in the rhizosphere of a sterile seedling planted in sand containing *F.o.r.l.* spores seven days after planting in absence of biocontrol bacteria; **D,** in presence of *P. fluorescens* WCS365; **E,** in presence of *P. chlororaphis* PCL1391; **F,** in presence of *P. chlororaphis* PCL1119. **G,** Vacuoles (indicated by arrowheads) abundantly present in hyphae in the rhizosphere of seedlings inoculated with *P. chlororaphis* PCL1391 three days after planting. **H**, *P. chlororaphis* PCL1391 attached to fungal hyphae three days after inoculation. The lower part of the panel (2-4) is a cross section in the Z direction at the white line in the upper part (1) showing the attachment. 2. Both the fungus and the bacteria. 3. The GFP signal of the fungus and 4. The DsRed signal of the bacteria. The size bar represents 10 µm in all panels.

Table 4. Reduction of the hyphal network by *Pseudomonas* biocontrol bacteria in a gnotobiotic sand-nutrient solution system

Micro-organism(s) present	Experiment 1		Experiment 2		Experiment 3	
	Fluorescent pixels					
	Nr.	(%)	Nr.	(%)	Nr.	(%)
(i) *F.o.r.l.*	11.016	100	113.846	100	25.337	100
(ii) *F.o.r.l.* + *P. fluorescens* WCS365	2.370	22	55.793	49		
(iii) *F.o.r.l.* + *P. chlororaphis* PCL1391	2.213	20			7.749	31
(iv) *F.o.r.l.* + *P. chlororaphis* PCL1119	3.551	32			13.737	54

The density of the hyphal network in (i) the absence of *Pseudomonas* bacteria, (ii) in the presence of *P. fluorescens* WCS365 (iii) in the presence of *P. chlororaphis* PCL1391 and (iv) in the presence of *P. chlororaphis* PCL1119. The density is expressed as the number of fluorescent pixels per cm^2 of root (as described in the Materials and Methods section) and as a percentage of the network in the absence of *Pseudomonas* bacteria (i).

Spatio-temporal analysis of interactions between P. chlororaphis PCL1391 and F.o.r.l. in the tomato rhizosphere

The observations described below were observed for *P. chlororaphis* PCL1391 but were not seen in presence of *P. fluorescens* WCS365. (i) After seven days *P. chlororaphis* PCL1391 caused an increase of the thickness of part of the hyphae (Fig. 2C). The strain also caused a disturbance of hyphal growth directionality, resulting in (ii) curly growth of hyphae growing along the intercellular junctions of the plant root after nine days (Fig. 2D) and (iii) abrupt changes in the growth direction after ten days (Fig. 2E). (iv) An increase of the frequency of hyphal branching was found after ten days. (v) After thirteen days fork-like branching structures of some hyphae were observed (Fig. 2F).

Analysis of *P. chlororaphis* PCL1119, which does not produce PCN, revealed that hyphal growth, morphology and branching was altered. Compared to its wild type the hyphal network was less strongly reduced (Table 4). The increase of the number of vacuoles within the fungal hyphae was observed 1 day later as compared to its wild type (Table 5). The increase in hyphal thickness, the abrupt changes in the growth direction and the increase of the frequency of hyphal branching were observed 3 days later (Table 5). Curly growth of hyphae was not observed along the cellular junctions (Fig. 2G) and the fork-like branching structures consisted of two branched hyphae, whereas in the presence of its wild type the structures consisted of three branched hyphae (Fig. 2H).

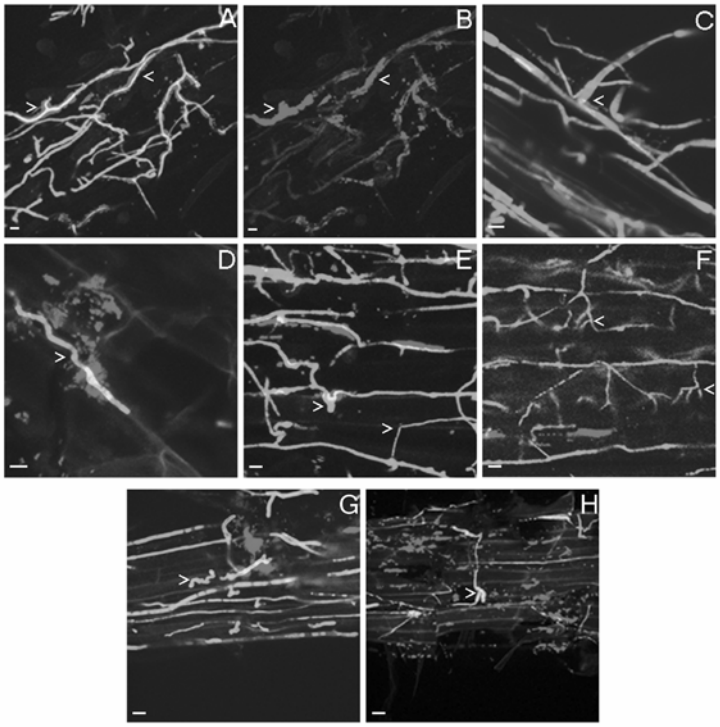


Figure 2. Confocal laser scanning microscopical analysis of effects of the presence of *Pseudomonas chlororaphis* PCL1391 and PCL1119 cells on growth of *F. oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) in the tomato rhizosphere. The full color figure is depicted on page 122.

Two-day-old tomato seedlings were inoculated at time zero with *P. chlororaphis* PCL1391 cells harboring a reporter plasmid expressing the *rfp* gene, which here appear as red cells. Plants were grown in a gnotobiotic sand system containing spores of *F.o.r.l.* harboring a constitutively expressed *gfp* gene. Cell walls of the tomato root appear as red due to autofluorescence. **A,** *P. chlororaphis* PCL1391 cells concentrating around the hyphae and colonizing *F.o.r.l.* hyphae ten days after inoculation. **B,** same picture as 2A without the GFP signal showing that all bacterial cells are attached to the fungal hyphae. **C,** In presence of strain PCL1391 an increase of the diameter of hyphae (indicated by arrowheads) was observed after seven days. **D,** Curly growth of hyphe along the cellular junction of the tomato root was observed in close vicinity of PCL1391 cells, nine days after planting. **E,** In presence of strain PCL1391 abrupt changes in the growth direction of hyphae (indicated by arrowheads) observed after ten days. **F,** Branching of *F.o.r.l.* hyphae resembles fork-like structures (indicated by arrowheads) in presence of strain PCL1391 thirteen days after inoculation. **G,** Hyphal growth in presence of strain PCL1119 in the rhizosphere. **H,** Branching of *F.o.r.l.* hyphae resembles fork-like structures at lower frequency in presence of strain PCL1119 thirteen days after inoculation. The size bar represents 10 µm in all panels.

Table 5. Effects of the *Pseudomonas* bacteria on fungal growth in the gnotobiotic sand-nutrient solution system

Effects on fungal growth in the tomato rhizosphere	*P. fluorescens* WCS365	*P. chlororaphis* PCL1391	*P. chlororaphis* PCL1119
	Observed on day		
Reduction of hyphal network	Day 7 51-78%	Day 7 69-80%	Day 7 46-68%
Vacuole formation	Day 4	Day 3	Day 4
Attachment to fungal hyphae	Day 3	Day 3	Day 3
Increase in hyphal diameter	-	Day 7	Day 10
Curly growth along cellular junction	-	Day 9	-
Abrupt changes in growth direction	-	Day 10	Day 13
Increased branching frequency	-	Day 10	Day 13
Altered branching structures	-	Day 13, 3 branches	Day 13, 2 branches

Analysis of F.o.r.l. growth in the tomato rhizosphere in presence of purified PCN

Tomato plants were grown in the gnotobiotic system containing sand infested with *Fusarium* spores. After three days of growth the plants were gently taken out of the gnotobiotic system, without removing the sand adhering to the root and transferred to PNS-agar plates. At this time point the fungus had attached to the tomato root and started to grow along the cellular junctions of the tomato root. A solution of purified PCN in ethyl acetate was spotted onto the root. As a control ethyl acetate was applied. This had no effect on the growth. CLSM studies of fungal hyphae in the tomato rhizosphere showed that in the direct presence of purified PCN similar alterations in hyphal growth took place as in the presence of *P. chlororaphis* PCL1391 (Table 5). (i) The presence of PCN caused an increase in the number of vacuoles after four hours (Compare the growth in the absence of PCN (Fig. 3A, 3B) with that in the presence of PCN (Fig. 3C)). (ii) An increase in the hyphal diameter (Fig. 3D), (iii) abrupt changes in the growth direction (Fig. 3E), (iv) increased branching frequencies (Fig. 3F) and (v) altered branching structures (Fig. 3G) were observed after one day. (vi) Curly growth was observed after 3 days (Fig. 3H). Analysis of the tomato rhizosphere two cm (or more) from the PCN-inoculation spot showed that hyphal growth was not altered at these sites.

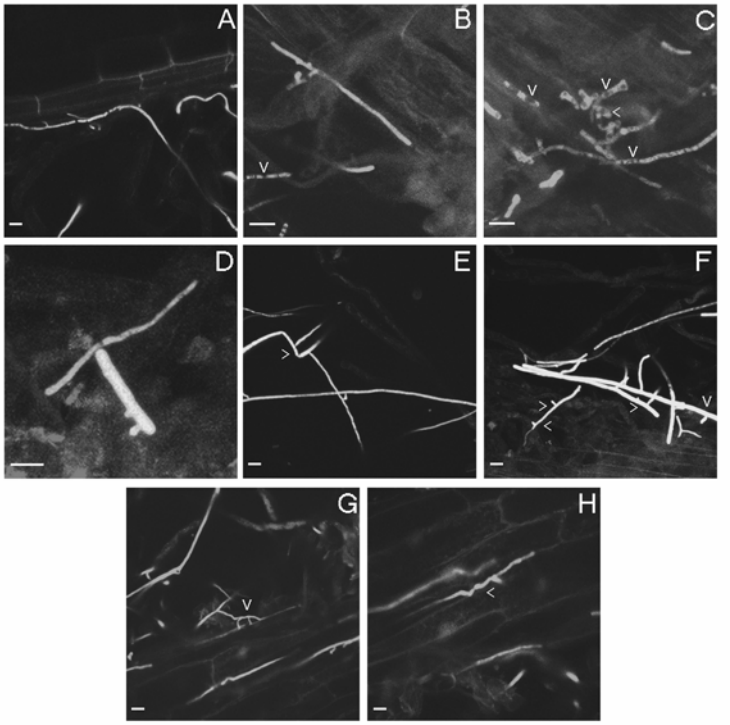


Figure 3. Confocal laser scanning microscopical analysis of effects of the presence of purified PCN on growth of *F. oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) in the tomato rhizosphere. The full color figure is depicted on page 123.
Plants were grown in a gnotobiotic sand system containing spores of *F.o.r.l.* harboring a constitutively expressed *gfp* gene. Cell walls of the tomato root appear as red due to autofluorescence. **A** and **B**, hyphal growth in presence of ethyl acetate. **C**, An increase in the number of vacuoles was observed after four hours in presence of PCN. **D**, An increase in the hyphal diameter was observed after 1 day. E, Abrupt changes in the growth direction was observed after 1 day incubation. **F**, Increased branching frequencies was observed after 1 day. **G**, altered branching structures were observed after 1 day. **H**, Curly growth was observed after 3 days. The size bar represents 10 µm in all panels.

In vitro analysis of interactions between biocontrol strains and F.o.r.l.

To facilitate the interpretation of the effects on fungal growth by the *Pseudomonas* biocontrol strains in the rhizosphere as described above, *in vitro* experiments were performed in which *F.o.r.l.* was grown on LB agar in the vicinity of *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391. The fungus and the bacteria were spotted next to each other on agar and subsequently allowed to grow. Differential interference contrast (DIC) microscopy studies of *F.o.r.l.* growing in the absence of *Pseudomonas* showed straight, radially orientated hyphae (Fig. 4A). The presence of *P. fluorescens* WCS365 cells had no visible effects on growth, branching, and morphology of fungal hyphae, which finally grew over the bacterial clump (data not shown). The presence of *P. chlororaphis* PCL1391 caused a strong inhibition zone of fungal growth. Microscopy studies focused on the inhibition region near the hyphal tips growing towards the *P. chlororaphis* PCL1391 cells (Fig. 4B). The following effects on *F.o.r.l.* hyphae growing towards *P. chlororaphis* PCL1391 were observed. (i) The hyphae lost the radial growth orientation and grew in different directions (compare Fig. 4A with 4B and 4C). (ii) About one percent of the hyphae showed looping growth (Fig. 4D). (iii) Branching of the hyphae was observed about ten times more frequently and closer to the hyphal tip than when bacteria were not present (compare Fig. 4A with Fig. 4E). (iv) Structures very similar to chlamydospores were observed (Fig. 4F), however their size is bigger therefore these chlamydospores could also be swollen bodies. To analyze the role of PCN in the above-described effects on hyphal growth, branching and morphology, we studied growth of fungal hyphae on agar in the vicinity of a PCN-negative mutant *P. chlororaphis* PCL1119. Hyphae growing towards strain PCL1119 were straight and radially orientated as in absence of bacteria (Fig. 4G). To further analyze the role of PCN, hyphae were allowed to grow towards purified PCN. Purified PCN (0.2 mg) spot-inoculated on the agar surface caused an inhibition zone of fungal growth and had similar effects on hyphal growth, branching, and morphology as the presence of cells of *P. chlororaphis* PCL1391: the hyphae (i) lost the radial growth orientation (Fig. 4H and 4I), the hyphae showed (ii) looping growth (Fig. 4J) and (iii) an increased branching frequency (Fig. 4K) whereas (iv) chlamydospore-like structures were observed (Fig. 4L).

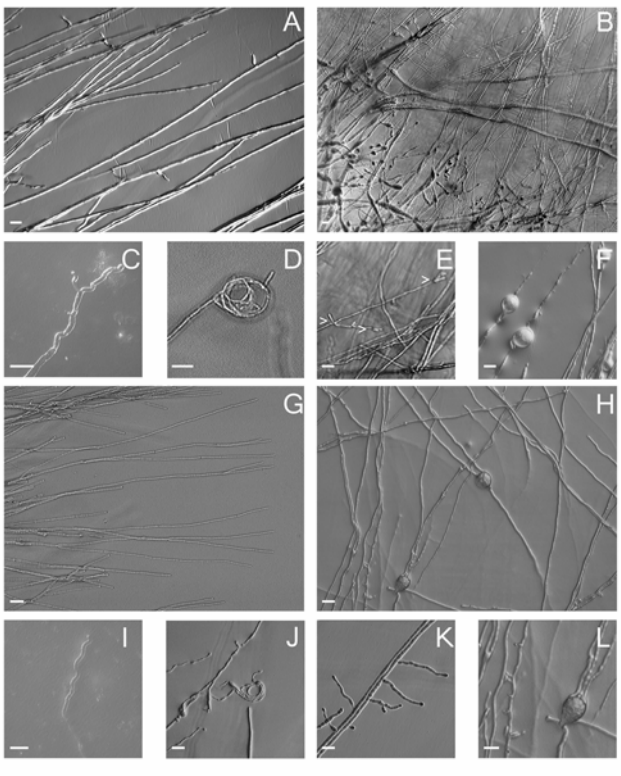


Figure 4. Differential interference contrast microscopy analysis of *in vitro* effects of *Pseudomonas chlororaphis* PCL1391 on hyphal growth and spore formation by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.).* The full color figure is depicted on page 124.
F.o.r.l. was grown in the vicinity of *P. chlororaphis* PCL1391, *P. chlororaphis* PCL1119 or purified PCN, on microscopy glass slides covered with a thin layer of LB agar. Three days after growth *F.o.r.l.* hyphae were examined for effects on growth and spore formation. **A,** Growth of *F.o.r.l.* in the absence of bacteria. **B through F,** Growth of *F.o.r.l.* towards *P. chlororaphis* PCL1391, which is located (outside the picture) in the upper right corner. **B,** Overview of the region close to the inhibition zone caused by PCL1391. **B through D,** Disturbance of hyphal growth directionality. **E,** Frequent branching close to the hyphal tip. **F,** Chlamydospores, observed within the hyphae. **G,** Overview of the region close to PCL1119, which is located (outside the picture) on the right. **H through L,** Growth of *F.o.r.l.* towards purified PCN, which is located (outside the picture) in the upper right corner. **H,** overview of the region close to the inhibition zone caused by PCN. **H through J,** Disturbance of hyphal growth directionality. **K,** Frequent branching close to the hyphal tip. **L,** Chlamydospores, observed within the hyphae. The size bar represents 10µm in all panels.

Discussion

Visualization of biocontrol of tomato foot and root rot by P. fluorescens WCS365 and P. chlororaphis PCL1391 in a gnotobiotic sand-nutrient solution system

TFRR is an important disease caused by the soil-borne pathogen *F. oxysporum* f. sp. *radicis-lycopersici*. The process of colonization and infection of the tomato root has been studied (Charest et al., 1984; Brammall and Higgins, 1988). Recently, more details were revealed using GFP-labeled *F.o.r.l.* (Lagopodi et al., 2002). Coating of seeds or seedlings with the bacteria *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 can efficiently control TFRR in soil infested with *F.o.r.l.* spores (Chin-A-Woeng et al., 1998 and Dekkers et al., 2000).

In the present work we visualized the interactions between the fungus and the bacteria in the tomato rhizosphere in order to get a better understanding of the biocontrol process. The microbes were differentially labeled with autofluorescent proteins to clearly visualize and distinguish them simultaneously. The GFP labeling does not affect the pathogenicity of the fungus (Lagopodi et al., 2002) and DsRed labeling does not cause a genetic or metabolic burden on the bacteria (Bloemberg et al., 2000). Quartz sand was used because it has the advantage that it can easily be removed from the tomato roots by gentle washing, after which the roots can be examined. In contrast, potting soil cannot easily be removed from the roots and subsequent CLSM studies are hampered by autofluorescence of small soil particles (Bloemberg et al., 1997; Chin-A-Woeng et al., 1997; Lagopodi et al., 2002). In addition, the quartz sand system enables us to focus better on the interactions between the two microbes directly involved in biocontrol due to the absence of other rhizosphere microorganisms, which are present in non-sterile soil systems. It should be noted that, presumably due to the absence of competing indigenous bacteria, the use of quartz sand results in a very high disease pressure (70-90%) and very efficient biocontrol (Table 2 and 3).

In potting soil it was it was found that, in contrast to wild type strain PCL1391, mutant PCL1119, did not cause significant biocontrol (Chin-A-Woeng et al., 1998). In the gnotobiotic system we see again a strong effect of PCN production on biocontrol (Table 3) as well as a stronger reduction of the hyphal network (Table 5) and an acceleration of the stress responses in the fungus (Table 5). However, in contrast to in potting soil, there is a significant effect of the PCN-negative mutant PCL1119 on biocontrol in the gnotobiotic system (Table 3). This is likely due to the result of the absence of indigenous bacteria which allows higher levels of PCL1119, which is a derivative of the efficiently root colonizing strain PCL1391. We therefore attribute the significant control activity of strain PCL1119 in the gnotobiotic system to its competition for niches and nutrients.

Colonization of the tomato root by the Pseudomonas biocontrol bacteria and the pathogenic fungus

In the following we will summarize and discuss the major effects of inoculation of seedlings with either of the two biocontrol bacteria grown in quartz sand infested with *F.o.r.l.i* spores. (i) The root colonization behavior of the bacteria was hardly influenced by the presence of the fungus. (ii) The bacteria reached the root surface earlier and multiplied faster than the fungus. Chemotaxis towards root exudate compounds (de Weert et al., 2002) is likely to play a role in the former process. (iii) Bacteria and hyphae colonize the same niches on the tomato root, namely the intercellular junctions (compare Figs. 1A and 1B). This may be due to chemotaxis towards, and utilization of, exudate compounds that are supposed to be exuded preferentially at those niches (Campbell and Greaves, 1990). (iv) After three days no effect of the bacteria on fungal growth was observed, but after seven days the density of the hyphae in the rhizosphere was approximately five-fold reduced by the presence of the biocontrol bacteria (compare Fig. 1C with Figs.1D and 1E). The fact that this effect only was observed in later stages can be explained in a number of ways. (a) In earlier stages the bacterial numbers are lower and therefore have less competitive capacity. (b) After four days forty percent of the plants (in absence of bacteria) show disease symptoms such as small brown lesions on the root. It is likely that cell fluids are leaking at these sites and consequently more nutrients become available for the microbes at the root surface resulting in the fast increasing density of bacteria and hyphae. In the presence of bacteria these sites with high densities of hyphae are absent (see Results section and Fig. 1D, 1E and 1F), suggesting that the presence of bacteria prevents the formation of severe lesions by the fungus. (c) Possibly, the sites where exudates are leaking from the tomato root are vulnerable and can be easily penetrated by the fungus. By colonizing these sites and utilizing the exudate nutrients the bacteria prevent colonization and therefore penetration of the fungus. (d) Another possible factor could be that the bacteria have utilized or degraded a signal required for colonization of the epidermis by hyphae. (v) Hyphae were colonized by the bacterial strains from day three on (Fig. 1H and 2A). (vi) All three bacteria cause an increase in the number of vacuoles in the hyphae (Fig. 1G), which is indicative for acceleration of the aging process of the hyphae (Moore-Landecker, 1996). Possibly this reduces the aggressive action of the fungus towards the plant as well. (vii) Finally, strain PCL1391 caused a number of specific morphological alterations (Table 5), of which many can be explained by the fact that PCL1391 produces the antifungal metabolite PCN (Figs. 2C, 2D, 2E, and 2F), since the purified PCN compound altered the growth and morphology of hyphae both *in vitro* (Figs. 4H, 4I, 4J, 4K and 4L) and *in vivo* (Figs. 3C, 3D, 3E, 3F, 3G and 3H). The lack of PCN production in strain PCL1119 caused a delay in the appearance of the morphological alterations of hyphae (Table 5). We speculate that the production of extracellular enzymes, such as chitinase, protease, lipase and HCN (Chin-A-Woeng et al., 1998) affect growth and morphology of the fungus as well.

Mechanism of action of PCN

Knowledge of the basic principles of hyphal growth and the possible mechanism by which PCN acts may contribute to our understanding of how PCN causes these stress responses. Wessels (1986) reported that polarized growth, i.e. endogenous electrical currents, is the basis of hyphal elongation and branching. When these electrical currents are altered hyphal growth and branching will be influenced. The exact growth-inhibiting mechanism of PCN is unknown. However, Hernandez et al. (2001) reported that phenazine compounds can function as electron shuttles. Such an activity of PCN could affect the endogenous electrical currents in the hyphae, and could thereby affect hyphal growth and branching. As a result, the fungus could be affected in its colonization ability and consequently be reduced in its pathogenicity. Disruption of polarized growth is also described by Gadd et al. (2001). In their study they showed that cadmium reduces the hyphal length and increases branching frequency. They postulate that cadmium affects the mechanisms, which maintain the electrochemical gradients across the apex, which may be involved in polarized growth. Such an action of PCN would explain the following observed *in vivo* effects (Table 5): hyphal swelling (Fig. 2C), disturbance in hyphal growth directionality (Fig. 2D and 2E), increased frequency of branching and altered branching structures (Fig. 2F). Of these, the latter could also be a response of the fungus to the presence of the bacteria, which occupy penetration sites, to search for free penetration sites.

Mechanisms of biocontrol

The present results contribute to our insight in the mechanism of action of the two biocontrol strains. (i) Strains *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 are the best competitive tomato root tip colonizers (Chin-A-Woeng, et al., 1998; Chin-A-Woeng, et al., 2000 and Simons et al., 1996) we have tested so far. Consistent with this is the observation that both strains colonize the root surface fast in comparison with *F.o.r.l.* (see results section). In case of strain PCL1391 colonization is a prerequisite for biocontrol since three (competitive) root tip colonization mutant derivatives tested had no biocontrol activity (Chin-A-Woeng et al., 1998). Similar mutant studies showed that colonization is not or less important for strain WCS365 (Dekkers et al., 2000). The observed fast colonization of the tomato root by the *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 explains the observed decreased occupation of the plant root by *F.o.r.l.* (Fig. 1D and 1E) due to competition with the fungus for niches and nutrients. Considering the results of Dekkers et al. (2000) it is doubtful whether this contributes substantially to biocontrol by strain WCS365. (ii) Induced systemic resistance (ISR) is supposed to play a major role in the mechanism used by *P. fluorescens* WCS365 for biocontrol (Gerrits and Weisbeek, 1996; Dekkers et al., 2000). ISR plays no major role in biocontrol by *P. chlororaphis* PCL1391, since its colonization mutants did not show biocontrol anymore. No differences between the effects on the fungus by strains WCS365 and PCL1391 were observed that could be related to ISR. It cannot be excluded that the ISR effects of strain WCS365 are more or less compensated for the antibiosis effect of strain PCL1391. (iii) Starting at day

three, colonization of *F.o.r.l.* hyphae by cells of both biocontrol bacteria in the tomato rhizosphere was observed. Although experimental evidence is lacking, it seems likely to us that colonization of hyphae by biocontrol bacteria (Fig. 2A, 2B, 2D and 2E) must negatively affect their pathogenic abilities. Colonization of hyphae may therefore be a new mechanism contributing to biocontrol. Since *P. fluorescens* WCS365 shows a chemotactic response towards the culture supernatant of the *F.o.r.l.* (de Weert et al., *personal communication*) it is likely that attraction of the bacteria by fungal secondary metabolites is involved in colonization of the fungus. Colonization of hyphae by biocontrol bacteria is likely to enhance biocontrol in case bacteria produce molecules toxic for the fungus, such as PCN, chitinase, and protease produced by strain PCL1391 (Chin-A-Woeng et al., 1998). Strikingly a correlation between colonization of *Candida albicans* hyphae by *Pseudomonas aeruginosa* and the subsequent killing of the fungus was recently described by Hogan and Kolter (2002). Since such an attack would result in the generation of nutrients, this could explain the high bacterial numbers on the hyphae (Fig. 1B).To analyze the contribution of hyphal colonization to the biocontrol the molecular interactions between the bacteria and fungal hyphae will be studied in the near future. (iv) No indication exists that *P. fluorescens* WCS365 produces antifungal metabolites. In contrast, the production of PCN is essential for the biocontrol ability of *P. chlororaphis* PCL1391 (Chin-A-Woeng et al., 1998). *In vitro* studies showed that both strain PCL1391 as well as purified PCN caused disturbance of hyphal growth directionality (Fig. 4B, 4C, 4D, 4H, 4I and 4J) and increased hyphal branching (Fig. 4E and 4K). This strongly suggests that PCN is the causal agent for the stress responses observed *in vitro.* (v) Analysis of germination of *F.o.r.l.* spores in the culture supernatants of strains PCL1391 and WCS365 showed that these strains significantly reduced spore germination (Fig. 5). These results indicate that both *P. chlororaphis* PCL1391 and *P. fluorescens* WCS365 produce extracellular compounds that inhibit spore germination. It is likely that strains PCL1391 and WCS365 inhibit spore germination in the gnotobiotic sand system as well. As a consequence, the subsequent growth towards, and colonization of, the tomato root will be reduced in this way the inhibition of spore germination can contribute to the control of TFRR.

Overall the results suggest that in case of strain WCS365 in addition to ISR (Gerrits and Weisbeek, 1996) perhaps the colonization of hyphae and possibly to a lesser extent competition for niches and nutrients play a role in the biocontrol of TFRR. In case of strain PCL1391 competition for niches and nutrients, the production of PCN, and possibly the production of extracellular enzymes and the colonization of hyphae play a role in its mechanism of biocontrol of TFRR.

Materials and methods

Microorganisms and growth conditions

The microorganisms used are listed in Table 1. *Pseudomonas* spp. were routinely cultured in King's medium B (King et al., 1954) at 28ºC. When appropriate, tetracycline was added to a final concentration of 80 μg/ml. *F.o.r.l.* was cultured on potato dextrose agar (Difco Laboratories, Detroit) or shaken at 130-160 rpm in Armstrong medium (Singleton et al., 1992) for 2 days at 28ºC.

Purification of phenazine-1-carboxamide

Phenazine-1-carboxamide produced by *P. chlororaphis* PCL1391 was purified as described by Chin-A-Woeng et al. (1998) with minor modifications. *P. chlororaphis* PCL1391 was grown for three days in King's medium B at 28ºC and shaking at 150 rpm. After removal of cells by centrifugation for 20 min at 6,000 rpm, the cell-free supernatant was extracted using an equal volume of toluene. The extracted material was concentrated by evaporation *in vacuo* and dissolved in acetonitrile. The dissolved extracted material was fractionated by HPLC, using an Alltech Hypersil ODS 5 μm 250 x 4.6 mm column (Alltech Associates, Deerdield, IL) and a linear 18-80% (vol/vol) gradient of acetonetrile in water, with 0.1% (vol/vol) trifluoracetic acid and a flow rate of 1 ml/min (Watson et al., 1986; Fernandez and Pizarro, 1997). UV detection was performed with a Pharmacia RSD 2140 diode array detector (Pharmacia, Uppsala, Sweden) with wavelength scanning from 190 to 400 nm. The peak corresponding to PCN was collected, dried *in vacuo* and dissolved in ethyl-acetate to a concentration of 22 mg/ml.

Biocontrol

F.o.r.l. spores were isolated and mixed with quartz sand as described by Lagopodi et al. (2002). *Pseudomonas* spp. were grown overnight in King's medium B (King et al., 1954) at 28ºC under vigorous shaking. Bacterial cells of one ml of overnight cultures culture were washed and resuspended in one ml of phosphate buffered saline (PBS) (Sambrook et al., 1989). The cell suspension was diluted with PBS to an OD 620 nm of 0.1 and used for inoculating tomato (*Lycopersicon esculentum* Mill cv. Carmello) seedlings as described by Simons et al. (1996).

Tomato seeds (kindly provided by Dr. R. Scheffer, Syntenga, Enkhuizen, The Netherlands) were sterilized (Simons et al., 1996) and incubated at 4 ºC for 5 days on plant nutrient solution (PNS) (Hoffland, 1989), solidified with 1.8% agar. The seeds were incubated for 2 days at 28ºC to allow germination. The seedlings were coated with bacteria by incubating the seedlings for 15 minutes in the bacterial suspension prepared as described above.

The spatio-temporal analyses as well as the biocontrol experiments were performed in a gnotobiotic quartz sand system (Simons et al., 1996). The sterile glass tubes were filled with sand moisturized with PNS (10% v/w) and infested with *F.o.r.l.* ($5x10^3$ spores/kg sand). Tomato seedlings were placed 5 mm below the surface of the sand. The plants were grown in climate-controlled growth chambers at 21ºC, 70% relative humidity and 16 hours of light per day. After seven days of

growth the plants were scored by eye as healthy (no disease symptoms) or sick (ranging from plants with pin-point size brown spots on the main root and/or on the crown to dead plants). Sixteen or twenty seedlings were grown per treatment. The difference in health condition (healthy or sick) of plants between two different treatments was statistically analyzed using the chi-squared goodness-of-fit test (Heath, 1995). The degree of freedom was 1 (degree of freedom = 2 conditions tested-1)x(2 classes of plants-1) resulting in the critical X_2 value of 3.841 ($P \leq 0.05$). The null-hypothesis was defined as the lack of significant difference between two conditions tested. To test the null-hypothesis the X_2 value was calculated for the two conditions using the chi-squared goodness-of-fit test. In case the calculated X_2 value was lower than the critical X_2 value, the null-hypothesis was accepted e.g. the two treatments were not significantly different. When the calculated X_2 value was higher than the critical value, the null-hypothesis was rejected e.g. the treatments differ significantly.

A Wilcoxon-Mann-Withney U-test (Sokal and Rohlf, 1981) was used to determine whether the reduction of germination of *F.o.r.l.* spores due to the presence of the culture supernatants of *Pseudomonas* strains was significant.

Confocal laser scanning microscopic analysis of tomato roots

After growth in the gnotobiotic system, tomato roots were carefully taken out of the sand and gently swirled a few times in water in order to wash away the sand particles. Whole roots were placed directly on glass slides in drops of water and examined using a Zeiss Axioplan epifluorescence microscope (Zeiss, Mannheim, Germany) coupled to a Biorad 1024 confocal system (Biorad, Hemel Hempstedd, UK). Images were obtained with a Kr/Ar laser with excitation 488- emission 522/35 nm for EGFP and with excitation 568- 585 nm long pass emission for DsRed. The projections of the individual channels were merged in Photoshop 7.0 software (Adobe, San Jose, CA). The density of the hyphal network was determined using Image J (NIH image Bethesda). First the Biorad image (512x512 pixels) was loaded in Image J and subsequently, the threshold for the fluorescent signal was set at a level at which the background signal was negligible. Analysis of the fluorescent pixels resulted in a total number of fluorescent areas counted and in a mean area size (expressed in the number of fluorescent pixels). By multiplying these factors the total number of fluorescent pixels was calculated. The relative differences in the density of the hyphal network were determined by comparing the total number of fluorescent pixels between different Biorad images per square cm of root surface.

Confocal laser scanning microscopic analysis of tomato roots incubated in presence of purified PCN

After growth in the gnotobiotic system for three days in the presence of *Fusarium* spores, tomato roots were carefully taken out of the sand and placed on 10% PNS agar plates. The root was spot-inoculated with PCN by applying five microliter of a phenazine-1-carboxamide solution (22 mg PCN/ml ethyl-acetate) or ethyl-acetate at one spot on the root. The solution was allowed to diffuse through the sand layer surrounding the root and incubated in climate-controlled growth chambers at 21ºC, 70% relative humidity and 16 hours of light per day. A wet filter disc was placed in the lid and the plate was sealed with parafilm (American National Can, Chicago, Il) to prevent drying of the tomato root. After four hours to three days the roots were examined for hyphal growth.

Differential interference contrast (DIC) microscopical analysis of F.o.r.l. hyphae grown in vitro

Microscopy glass slides were covered with a thin layer (2-3 mm) of Luria Bertani (LB) medium (Sambrook et al., 1989) solidified with 1.8% agar and placed in a plastic petridish. Ten microliters of a two day old *F. oxysporum* f. sp. *radicis-lycopersici* culture were placed in the center of the glass slide at a distance of 2-3 cm from a spot on which ten microliter of an overnight culture of *Pseudomonas* spp. or of a phenazine-1-carboxamide solution (22 mg PCN/ml ethyl-acetate) had been placed. The plates were incubated for 3 days at 28ºC before DIC microscopical using a Zeiss Axioplan 2 (Mannheim, Gemany). Images were processed using Photoshop 7.0 (Adobe, San Jose, CA).

Spore germination

Spores of F.o.r.l. were incubated in culture supernatants of Pseudomonas strains grown in KB or LC overnight at 28ºC. The reaction volume was 500 µl and the final concentration of spores was 4 x 10^5 per ml. The number of germinated and total number of spores were counted using a heamatocytometer and the percentage of germination was calculated. The germination experiments were carried out in triplo and were at least repeated twice.

Acknowledgements

We thank E. Meelis, C. A. M. J. J. van den Hondel, and A. F. J. Ram for valuable discussions and advice. A. Bolwerk was supported by a grant of the NWO Earth and Life Sciences Council, project no. 810.35.003. A. L. Lagopodi was financially supported by a Marie Curie Fellowship of the European Community Program Training and Mobility of Researchers under contract ERBFMBICT982930.

Chapter 3

Visualization of interactions between a pathogenic and a beneficial *Fusarium* strain during biocontrol of tomato foot and root rot

Annouschka Bolwerk, Anastasia L. Lagopodi,
Ben J. J. Lugtenberg and Guido V. Bloemberg

Abstract

The soil-borne fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.)* causes tomato foot and root rot (TFRR), which can be controlled by the addition of the non-pathogenic fungus *Fusarium oxysporum* Fo47 (Fo47) to the soil. To improve our understanding of the interactions between the two *Fusarium* strains on the tomato root during biocontrol, the fungi were labeled using different auto-fluorescent proteins as markers and subsequently visualized using confocal laser scanning microscopy. The results were as follows. (i) An at least 50-fold excess of Fo47over *F.o.r.l.* was required to obtain control of TFRR. (ii) When seedlings were planted in sand infested with spores of a single fungus, Fo47 hyphae attached to the root earlier than those of the *F.o.r.l.*. (iii) Subsequent root colonization by *F.o.r.l.* was faster and to a larger extent than that by Fo47. (iv) Under disease controlling conditions, colonization of the tomato root by the pathogenic fungus was significantly reduced. (v) When the inoculum concentration of Fo47 was increased, root colonization by the pathogen was arrested at the stage of initial attachment to the root. (vi) The percentage of spores of Fo47 that germinates in tomato root exudate *in vitro* is higher than that of the pathogen *F.o.r.l.*. Based on these results the mechanisms by which Fo47 controls TFRR are discussed in terms of (a) rate of spore germination and competition for nutrients before the two fungi reach the rhizoplane, (b) competition for initial sites of attachment, intercellular junctions and nutrients on the tomato root surface and (c) induced systemic resistance.

Introduction

F.o.r.l. is the causal agent of TFRR, which is a serious problem in commercial tomato production (Brayford, 1996; Jarvis, 1988). Biological control of TFRR by *F. oxysporum* strain Fo47 has been described by Alabouvette's group. To be effective Fo47 should be introduced at concentrations ten to a hundred times higher than those of the pathogen (Alabouvette and Couteaudier, 1992; Alabouvette et al., 1993; Paulitz et al., 1987; Roberts and Lohrke, 2003; reviewed by Fravel et al., 2003).

In previous work we have analyzed, using confocal laser scanning microscopy (CLSM), the colonization process of the tomato rhizosphere by *F.o.r.l.* (Lagopodi et al., 2002) and the interactions between *F.o.r.l*. and biocontrol *Pseudomonas* bacteria in the rhizosphere (Bolwerk et al., 2003). These results provided us with new insights in the mechanisms of tomato root infection by *F.o.r.l.* and of biocontrol of TFRR, respectively. To our knowledge reports on simultaneous colonization by both a pathogenic and a non-pathogenic biocontrol *Fusarium* strain are limited (Bao and Lazarovits, 2001; Mandeel and Baker, 1991) and reports on simultaneous visualization of root colonization by both a pathogenic and a non-pathogenic biocontrol *Fusarium* strain are scarce (Bao and Lazarovits, 2001). In the present paper we report the labeling of strains Fo47 and *F.o.r.l.* with different auto-fluorescent proteins followed by an analysis of the tomato root colonization by both fungi simultaneously in relation to disease control, which allowed us to obtain a better understanding of the biocontrol process.

Results

Cloning of the ecfp and eyfp in pGPDGFP and its expression in Fusarium spp.

Construction of the enhanced Green Fluorescent Protein-labeled *F.o.r.l.* derivative FCL14, which was used in CLSM studies, has been described previously (Lagopodi et al., 2002). To be able to distinguish the pathogenic and the non-pathogenic *F. oxysporum* strains (Table 1) when visualizing them simultaneously, we constructed derivatives labeled with the enhanced Cyan Fluorescent Protein and the enhanced Yellow Fluorescent Protein.

In order to express *ecfp* in both *F.o.r.l.* and *F. oxysporum* strain Fo47, the *ecfp* gene was cloned between the *Aspergillus nidulans gpdA* promoter (Punt et al., 1988) and the *trpC* terminator (Mullaney et al., 1985) sequences as follows. Plasmid pGDPGFP (Lagopodi et al., 2002), which contains the *sgfp* gene between the *gpdA* promoter and the *trpC* terminator, was digested with *Nco*I and *Hind*III in order to isolate the *sgfp* gene (Fig. 1). The *sgfp* gene was cloned into a *Nco*I-*Hind*III digested pUC21, which resulted in plasmid pMP4642. Subsequently pMP4642 was digested with *Nco*I and *Bsrg*I in order to remove the *sgfp* gene. The *ecfp* gene was isolated from pMP4516 (Bloemberg et al., 2000) by *Nco*I-*Bsrg*I digestion and cloned into the *Nco*I-*Bsrg*I digested pMP4642, which resulted in plasmid pMP4650. The pMP4650 plasmid was digested with *Nco*I and *Hind*III to isolate the *ecfp* gene. The *Nco*I-*Hind*III *cfp* gene fragment was ligated into the *Nco*I-*Hind*III digested pGDPGFP vector to yield pMP4653 (Fig. 1). The same strategy was used to express *eyfp* in *F.o.r.l.*. The *eyfp* gene was isolated from pMP4518 (Bloemberg et al., 2000) by *Nco*I-*Bsrg*I. Identical cloning steps as used for the *ecfp* cloning resulted in the pUC21 derivative pMP4651 and the pGPDGFP derivative pMP4654 (Fig. 1). *Fusarium* strains were cotransformed as described previously (Lagopodi et al., 2002) using pMP4653 or pMP4654 together with pAN7-1 (Punt et al., 1987). pAN7-1 carries the *Escherichia coli* hygromycin-B (Hm-B) resistance gene *hph*, cloned between the *gpdA* promoter and the *trpC* terminator, which allows selection of transformants on media containing Hm-B. Transformants were subsequently selected as described for transformants expressing *sgfp* by Lagopodi et al. (2002), for (i) high levels of *ecfp* or *eyfp* expression (ten out of twenty Hm-B resistant transformants) (ii) stable *ecfp* or *eyfp* expression (nine out of ten fluorescent transformants) (iii) unaffected growth and (iv) unaffected pathogenicity for *F.o.r.l.* and disease control for Fo47. This resulted in FCL55 (*F.o.r.l.* expressing *eyfp*), FCL64 (*F.o.r.l.* expressing *ecfp*) and FCL31 (Fo47 expressing *ecfp*).

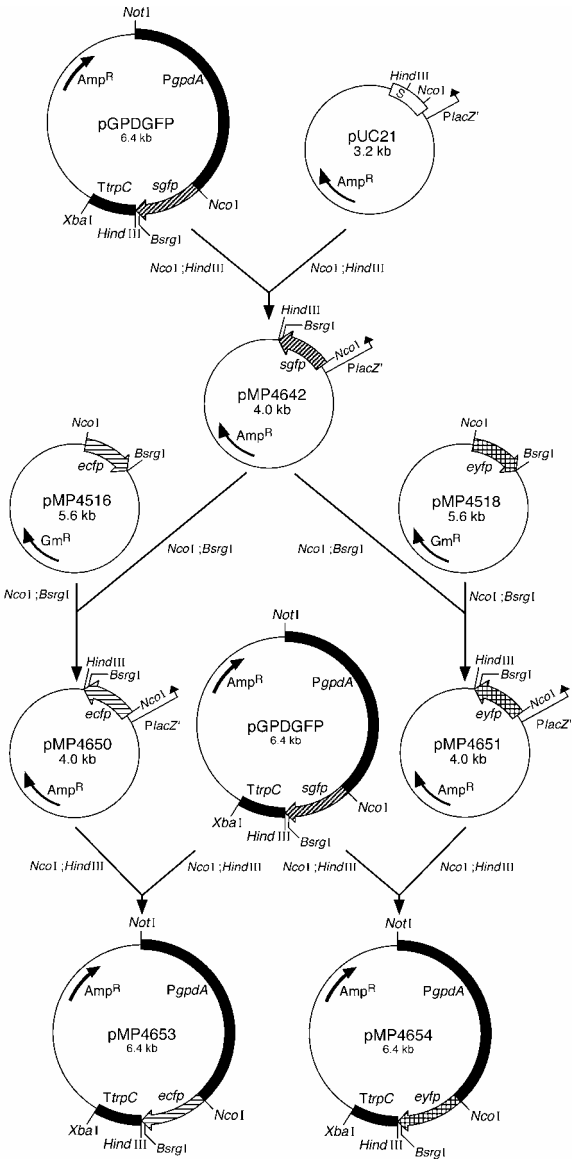


Figure 1. Construction of reporter plasmids to express *ecfp* and *eyfp* in *Fusarium*.
For details on cloning, see results section. Abbreviations: Amp = ampecilin, Gm = gentamycin, P*gpdA* = *gpdA* promoter, P*lacZ'* = *lacZ'* promoter, T*trpC* = *trpC* terminator, *sgfp* = green fluorescent protein, *ecfp* = enhanced cyan fluorescent protein, and *eyfp* = enhanced yellow fluorescent protein.

Table 1. Microorganisms and plasmids

Strains	Relevant characteristics	Reference or source
FUNGI		
ZUM 2407	*Fusarium oxysporum* f. sp. *radicis-lycopersici* causing tomato foot and root rot	IPO-DLO, Wageningen, The Netherlands
Fo47	Non-pathogenic *Fusarium oxysporum,* biocontrol agent, isolated from a Fusarium Wilt Suppressive Soil in France	Alabouvette et al., 1993
FCL14	*F.o.r.l.* ZUM 2407 containing *sgfp* under control of the constitutive *gpdA* promoter	Lagopodi et al., 2002
FCL55	*F.o.r.l.* ZUM 2407 containing *eyfp* under control of the constitutive *gpdA* promoter	This work
FCL64	*F.o.r.l.* ZUM 2407 containing *ecfp* under control of the constitutive *gpdA* promoter	This work
FCL31	*Fusarium oxysporum* Fo47 containing *ecfp* under control of the constitutive *gpdA* promoter	This work
Plasmids		
pUC21	Cloning vector	Promega/Stratagene
pGDPGFP	pAN52-10-S65TGFPn/n derivative containing *sgfp* under the control of the *gpdA* promoter; integrates into the chromosome	Lagopodi et al., 2002
pAN 7-1	*E. coli* hygromycin-B (Hm-B) resistance gene *hph*, cloned between the *gpdA* promoter and the *trpC* from *Aspergillus nidulans*	Punt et al., 1987
pMP4516	pME6010 derivative containing the *ecfp* gene	Bloemberg et al., 2000
pMP4642	pUC21 derivative containing the *sgfp* gene	This work
pMP4650	pUC21 derivative containing the *ecfp* gene	This work
pMP4651	pUC21 derivative containing the *eyfp* gene	This work
pMP4653	pAN52-10-S65TGFPn/n derivative containing *ecfp* under the control of the *gpdA* promoter; integrates into the chromosome	This work
pMP4654	pAN52-10-S65TGFPn/n derivative containing *eyfp* under the control of the *gpdA* promoter; integrates into the chromosome	This work

Control of TFRR by the non-pathogenic strain Fo47 in the gnotobiotic sand system

Plate confrontation assays were performed to test the antagonistic ability of the nonpathogenic Fo47 against the pathogenic fungus *F.o.r.l.*. Both fungi were inoculated next to each other on an agar plate and subsequently allowed to grow. In another experiment the pathogenic fungus was grown on agar plates containing the supernatant fluid of the strain Fo47. Growth inhibition of *F.o.r.l.* was not observed in these experiments (data not shown). In addition to growth, inhibition of spore germination was analyzed in relation to the antagonistic ability of strain Fo47. Spores of *F.o.r.l.* were allowed to germinate in potato dextrose broth (0.1 and 1x) in the presence of the culture supernatant of *F.o.r.l.* or of strain Fo47. Neither the rate of spore germination nor the total percentage of germinated spores was affected by the supernatant fluid of strain Fo47 (data not shown).

To test whether strain Fo47 could protect tomato plants against TFRR in the gnotobiotic sand system (Simons et al., 1996), tomato seedlings were coated with spores of Fo47. This treatment resulted in a decrease of diseased plants, from 100% to 75%. Visualization studies showed that Fo47 colonized only the upper two centimeters, close to the inoculation site, whereas further distribution over the rest of the root was not detected.

In a second strategy to test whether Fo47 can control of TFRR in the gnotobiotic system, tomato seedlings were grown in sand infested with spores of *F.o.r.l.* and Fo47. This strategy was similar to that used by Alabouvette and colleagues (Alabouvette et al., 1992; Alabouvette et al., 1993; Couteaudier, 1992; Lemanceau and Alabouvette, 1990) for biocontrol. Since the inoculum concentration of *F.o.r.l.* was the same in all further experiments ($5x10^4$ spores/kg sand) whereas the inoculum concentration of strain Fo47 varied between $1x10^5$ – $2x10^9$ spores/kg sand, the inoculum size will be indicated further in this paper as (inoculum) ratio. Different ratio's of the pathogenic over the non-pathogenic *Fusarium* strains were analyzed to determine the minimum inoculum concentration of the nonpathogenic strain Fo47 required for significant biocontrol of TFRR in the gnotobiotic system. After seven days of incubation the plants were analyzed for disease symptoms. Healthy plants were scored in disease index (d.i.) 0 and sick plants, with increasing disease severity, were scored in d.i. 1 through 4 (see the Materials and Methods section for details).

The presence of Fo47 alone did not affect the health condition of the plants (Tables 2A and 2B). At inoculum ratio's *F.o.r.l.*: Fo47 of 1:2 and 1:10, a decrease in disease severity was observed as is illustrated by a shift from d.i. 3 to d.i. 2 (Table 2A) and as a shift from d.i. 3 to d.i. 1 and 2 (Table 2B), respectively. Although disease severity was decreased, healthy plants were not observed. Therefore, the inoculum concentration was increased in subsequent experiments and the plants were scored as either healthy or sick.

Table 2. Reduction of TFRR disease symptoms by *F. oxysporum* Fo47 in a gnotobiotic system

A) Disease severity at *F.o.r.l.*:Fo47 ratio 1:2

	Disease index				
	0	*1*	*2*	*3*	*4*
(i) No Fungi	19	0	0	0	0
(ii) *F.o.r.l.* alone	0	0	3	16	0
(iii) Ratio 1:2	0	2	13	4	0
(iv) Fo47 alone	19	0	0	0	0

B) Disease severity at *F.o.r.l.*:Fo47 ratio 1:10

	Disease index				
	0	*1*	*2*	*3*	*4*
(i) No Fungi	16	0	0	0	0
(ii) *F.o.r.l.* alone	0	0	4	11	1
(iii) Ratio1:10	0	7	9	0	0
(iv) Fo47 alone	16	0	0	0	0

Nineteen (**A**) and sixteen (**B**) tomato plants were grown in a gnotobiotic sand-nutrient solution system either (i) in the absence of fungi, (ii) in the presence of *F.o.r.l.* ($5x10^4$ spores/kg sand), (iii) in the presence of both *F.o.r.l.* and Fo47 ($1x10^5$ spores/kg sand [A] or $5x10^5$ spores/kg sand [B]), or (iv) in the presence of strain Fo47 ($1x10^5$ [A] or $5x10^5$ [B] spores/kg sand). The disease index of the plants was scored after seven days of growth. The following disease index scale, ranging from 0 to 4, was used. 0, healthy plants with no visible symptoms of foot and root rot; 1, plants with pin-point size brown spots on the main root and/or pin-point size light brown spots on the crown; 2, plants with brown spots on the main root and extensive brown discoloration of the crown; 3, plants with a wilting appearance and an extensive rot of root and crown; 4, dead plants. For details of inoculation and growth conditions, see the Materials and Methods section.

At an inoculum ratio of 1:50 strain Fo47 reduced the percentage of sick plants from 100% to 58-63% (Table 3A). Comparison of plants grown in sand containing *F.o.r.l.* spores with and without the Fo47 spores, using a chi-squared goodness-of-fit statistical test, showed that strain Fo47 significantly suppressed TFRR in the gnotobiotic system (Table 3B).

Increasing the inoculum concentration of strain Fo47 to 100- fold that of the pathogen did not improve the reduction of TFRR [Compare Tables 3A (ii) and (iii) with (ii) and (iv)]. Increasing the pathogen: biocontrol *Fusarium* ratio to 1:4 $x10^4$, as described by Lemanceau and Alabouvette (1990) for biocontrol in rockwool, resulted in a stronger reduction of diseased plants, from 100% to 42-50% [Table 3A (v)].

Table 3. Control of tomato foot and root rot by Fo47 in a gnotobiotic system

A) Disease severity at *F.o.r.l.*:Fo47 ratio's 1:50, 1:100 and 1:4 x10^4

Fungi present	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	d.i. 0	d.i. 1-4	d.i. 0	d.i. 1-4	d.i. 0	d.i. 1-4	d.i. 0	d.i. 1-4
(i) No Fungi	19	0	19	0	19	0	18	0
(ii) *F.o.r.l.* alone	0	19	0	19	0	19	0	18
(iii) Ratio 1:50	7	12	8	11				
(iv) Ratio 1:100					7	12	7	11
(v) Ratio 1:4 x10^4					11	8	9	9

B) Statistical analysis of disease control at ratio 1:50, 1:100 and 1:4 x10^4 (Z)

Compared treatments	X_2 values experiment 1;3	X_2 values experiment 2;4
(ii) and (iii)	8.58 [y]	10.13 [y]
(ii) and (iv)	8.58 [y]	8.69 [y]
(ii) and (v)	10.13 [y]	13.33 [y]

A) Eighteen or nineteen plants were either grown in a gnotobiotic sand-nutrient solution system (i) in the absence of fungi, (ii) in the presence of *F.o.r.l.* (5x10^4 spores/kg sand) or in the presence of both *F.o.r.l.* (5x10^4 spores/kg sand) and *F. oxysporum* Fo47 (iii) 2.5x10^6, (iv) 5x10^6 or (v) 2x10^9 spores/kg sand. Seven days after inoculation the plants were scored as healthy (disease index 0) or sick (disease index 1-4). For details of inoculation and growth conditions, see the Materials and Methods section. **B**) Statistical analysis of the biocontrol experiment (panel A) was performed using a chi-squared goodness-of-fit test (Heath, 1995) and the calculated X_2 values are given in panel B. Critical X_2 value 3.841. For details about the analysis, see the Materials and Methods section.

[y] The two compared treatments are significantly different, calculated $X_2 > 3.841$

Quantitative and statistical analysis of root surface colonization by F.o.r.l. in presence Fo47

CLSM allows us to differentially and simultaneously visualize *F.o.r.l.* and Fo47 in the tomato rhizosphere under disease reducing and controlling conditions. To distinguish the two fungi, differentially labeled fungi expressing *sgfp*, *ecfp* or *eyfp*, were used. With regard to the emission spectra of the Green, Cyan and Yellow Fluorescent protein the GFP-CFP and the YFP-CFP combinations are most useful for distinguishing the two fungi. Initial CSLM studies indicated that the intensity of fluorescence was stronger for GFP than for YFP. Therefore, the GFP-CFP combination was chosen for subsequent CLSM studies. The GFP-labeled *F.o.r.l.* derivative FCL14 (Lagopodi et al., 2002) and the CFP-labeled *F. oxysporum* Fo47 derivative FCL31 (Table 1) were used.

Tomato seedlings were grown in the gnotobiotic system in sand infested with spores of both *F.o.r.l.* and *F. oxysporum* Fo47 at ratio's of 1:10; 1:50; and 1:100. Using CLSM we visualized and analyzed colonization of the tomato root by *F.o.r.l.* after seven days. Four different stages of root colonization were defined: (i) 'attachment' to root hairs and main root (Fig. 2A and 2B); (ii) growth along one or two plant cells on the main root (Fig. 2B), defined as 'start of colonization'; (iii) growth along three or more adjacent cortical cells, defined as 'colonization' (Fig. 2C and (iv) dense colonization over the total width of the root surface (Fig. 2D), defined as 'heavy colonization'. Note the difference in the amount of biomass present on root cells 'heavily colonized' by *F.o.r.l.*, which is much higher compared to cells 'colonized' by *F.o.r.l.* (compare Fig. 2D with 2C).

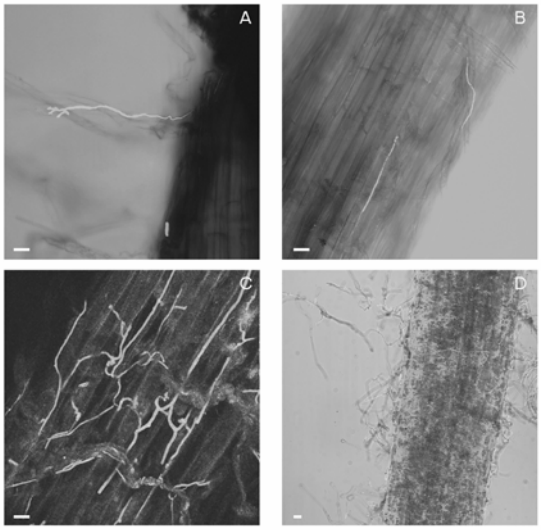


Figure 2. Confocal laser scanning microscopical analysis of tomato root colonization by *Fusarium.* The full color figure is depicted on page 125.
Two-day-old tomato seedlings were grown in a gnotobiotic sand system containing spores of *F.o.r.l.* (FCL14), which harbors a constitutively expressed *sgfp* gene. Walls of tomato root cells appear as gray due to contrast light (panel A, B and D) or reflected light (panel C). Panel A, Initial colonization of the tomato root by *F.o.r.l.* (similar for Fo47) 'attachment' to root hairs. Panels **A through** D: subsequent root colonization stages by *F.o.r.l.* A, 'attachment' to root hair. **B,** hyphe growing along the intercellular junctions of two root cells: 'start colonization'-stage. **C,** *F.o.r.l.* hyphae growing along the intercellular junctions of more than two root cells: 'colonization'-stage. **D,** hyphae growing over the whole root at a very high density and biomass: 'heavy colonization'-stage. The size bar represents 10 µm in all panels.

Additionally, tomato root colonization was quantified by counting the total number of tomato root cells colonized per colonization stage in the length axes (from crown to root tip). Details on how root colonization was counted are described in the Materials and Methods section. In short, when *F.o.r.l.* grew in between two root cells on the intercellular junctions along five cells in the length axes it was counted as five and not as ten. Subsequently, the difference in root colonization by *F.o.r.l.* in the absence and the presence of Fo47 was statistically analyzed by using a Wilcoxon-Mann-Withney U-test. The reduction by Fo47 was analyzed at three different *F.o.r.l.*-Fo47 ratio's (Table 4).

Under the disease reducing condition with an inoculum ratio of 1:10 (Table 2B), the nonpathogenic strain Fo47 reduced all colonization stages of the pathogen (Table 4). However, using a Wilcoxon-Mann-Withney U-test it was shown that this reduction of the colonization stages was not significant except for the 'heavy colonization' stage (Table 4). Under disease controlling conditions with inoculum ratio's 1:50 and 1:100 (Table 3), strain Fo47 significantly reduced *F.o.r.l.* also in the stage of 'colonization'. The 'heavy colonization' stage was even not observed (Table 4). At the ratio 1:100 the pathogen was even significantly reduced in the 'start of colonization' (Table 4) as well. Despite the further reduction of the pathogen on the root by Fo47 (Table 4), the higher inoculum concentration (ratio 1:100) did not significantly improve the disease controlling ability of Fo47 (Table 3).

Table 4. Quantification and statistical analysis of the influence of Fo47 on the number of tomato root cells per root colonized by *F.o.r.l.*[X]

	F.o.r.l. alone	*F.o.r.l.*:Fo47 Ratio 1:10	*F.o.r.l.*:Fo47 Ratio 1:50	*F.o.r.l.*:Fo47 Ratio 1:100
Attachment	22 [a]	16[a]	15[a]	11[a]
Start colonization	37 [a]	31[a]	19[a]	13[b]
Colonization	229 [a]	118[a]	70[b]	50[b]
Heavy colonization	25 [a]	7[b]	0 [b]	0 [b]
Total	313 [a]	172 [a]	104 [b]	74 [b]

X) Tomato root colonization stages of *F.o.r.l.* in the absence and presence of Fo47 were classified and quantified after 7 days of growth as described in the Materials and Methods section and illustrated in Fig. 2B through 2F). It should be realized that the amount of biomass present on root cells 'heavily colonized' by *F.o.r.l.* is much higher compared to cells 'colonized' by *F.o.r.l.* (compare Fig. 2E with 2F). The total number of plant cells per root colonized by *F.o.r.l.* is an average of four roots. The inoculum concentration of *F.o.r.l* was $5x10^4$ spores/kg sand in all cases. The inoculum concentration of *F. oxysporum* Fo47 was 10, 50 or 100 times higher relative to *F.o.r.l.*. The difference in the total number of plant cells colonized by *F.o.r.l.* in presence and absence of strain Fo47 is indicated as not significant [(a)] or significant [(b)] as determined by the Wilcoxon-Mann-Whitney U-test analyzing *F.o.r.l.* colonization data of eight roots.

When a much higher inoculum ratio of *F.o.r.l.*:Fo47 was used (1:4 x10^4) analysis of healthy roots after seven days showed that root colonization by *F.o.r.l.* was reduced to the initial state of attachment of hyphae to the root hairs ranging from zero to two sites on the root. Compared to the root colonization by *F.o.r.l.* in all four colonization stages along more than three hundred root cells in absence of strain Fo47 (Table 4).

Temporal analysis of tomato root surface colonization by F.o.r.l. and strain Fo47

Tomato plants were grown in the gnotobiotic sand system in presence spores of *F.o.r.l.* (5x10^4 spores/kg sand = 5.4x10^1 spores/ml) or Fo47 (2.5x10^6 spores/kg sand = 2.7x10^3 spores/ml), either alone or together at an inoculum ratio of 1:50. Under the latter condition Fo47 significantly controlled the disease (Table 3B) and significantly reduced root colonization of the pathogen in the 'colonization' and 'heavy colonization' stage (Table 4). Visualization of tomato root colonization in time by Fo47 alone (in two separate experiments with two seedlings per condition) showed that after three days of plant growth 'attachment' to and 'start of colonization' of the root by Fo47 occurred at two to five sites on the root, for each of these stages. 'Colonization' of the tomato root surface was observed after four days (Fig. 3), and strongly increased on days six and seven.

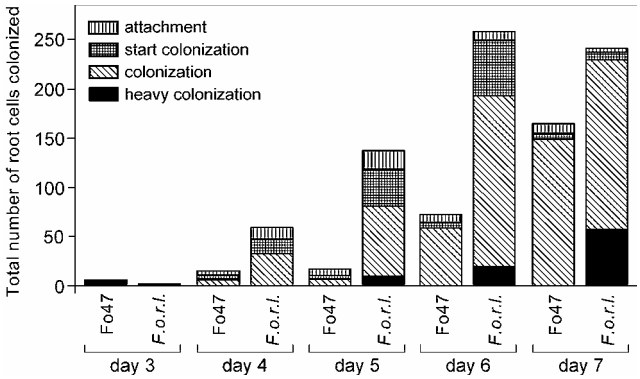


Figure 3. Quantification of tomato root colonization stages by *F.o.r.l.* and *F. oxysporum* Fo47 in time.

Seedlings were grown in Fo47 (2.5x10^6 spores/kg sand), or *F.o.r.l.* (5x10^4 spores/kg sand) infested sand. Plants were scored for tomato root surface colonization after 3, 4, 5, 6 and 7 days of growth. Colonization was classified in four different stages of colonization: 'attachment', 'start of colonization', 'colonization' and 'heavy colonization' as described in the Materials and Method section and shown in Fig. 2. Colonization was quantified by counting the number of plant cells colonized from crown till root tip at the four stages under the following conditions: (i) 'Fo47', root colonization of Fo47 in absence of *F.o.r.l.* and (ii) '*F.o.r.l.*', root colonization of *F.o.r.l.* in absence of Fo47. Per condition two plants were scored and the average of two experiments is depicted in the figure.

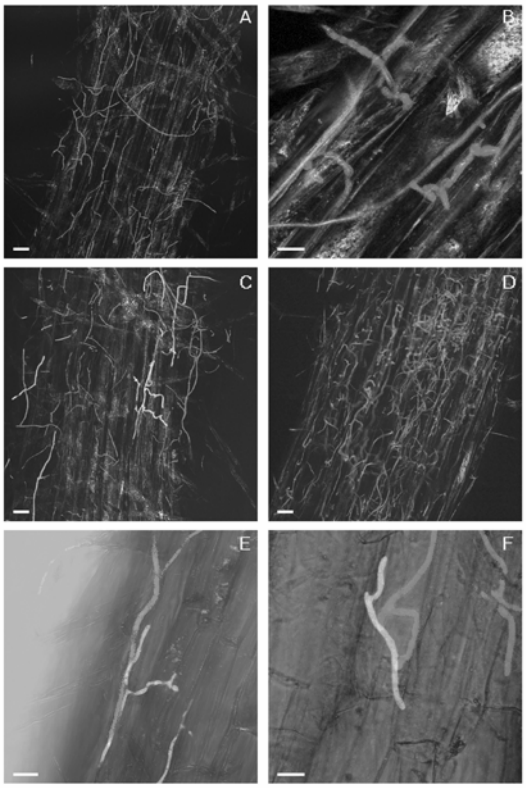


Figure 4. Confocal laser scanning microscopical analysis of tomato root colonization by the pathogenic fungus *F.o.r.l.* and the biocontrol strain Fo47. The full color figure is depicted on page 126.

Two-day-old tomato seedlings were grown in a gnotobiotic sand system containing spores of Fo47 (FCL31) (panel A and B) or spores of both *F.o.r.l.* (FCL14) and Fo47 (FCL31) (panel C-F) at an inoculum ratio of 1:50. *F.o.r.l.* (FCL14) harbors a constitutively expressed *sgfp* gene and appears as green. Fo47 (FCL31) harbors a constitutively expressed *ecfp* gene its emission signal is depicted as red in the shown images. Walls of tomato root cells appear as gray due to reflected light (panel A-D) or contrast light (panel E and F). Panels **A** and **B**, Colonization of the tomato root by Fo47. **A,** Hyphal growth along cellular junctions and crossing root cells. B, Penetration of the tomato root by Fo47 (indicated by an arrowhead). **C,** On healthy roots (disease index 0) Fo47 is dominant. **D,** On sick roots with disease index 1, Fo47 and *F.o.r.l.* are equally present. **E** and **F,** direct cell cell contact between *F.o.r.l.* and Fo47 in the rhizosphere. The size bar represents 10 µm in all panels.

Growth of Fo47 hyphae was not strictly targeted to the cellular junctions (Fig. 4A) and occasional penetration of the tomato root by the non-pathogenic strain Fo47 was observed after three days (Fig. 4B). The density of the hyphal network reached by strain Fo47 after seven days (Fig. 4A) was not as high as the 'heavy colonization' network of the pathogen (Fig. 2D).

For the pathogen *F.o.r.l.* 'attachment' and 'start of colonization' of the root surface after three days was observed at maximally one site on the root surface, for each of these stages. After four days 'colonization' of the tomato root surface was observed along thirty-three tomato cells over the whole main root and strongly increased at days five and six (Fig. 3). Additionally, the pathogenic *Fusarium* heavily colonizes the tomato root surface from day five on. The total root surface area heavily colonized by *F.o.r.l.* further increased at days six and seven (Fig. 3). In contrast to the non-pathogenic strain Fo47, growth of *F.o.r.l.* was mainly targeted to the cellular junctions of the root (Fig. 2C).

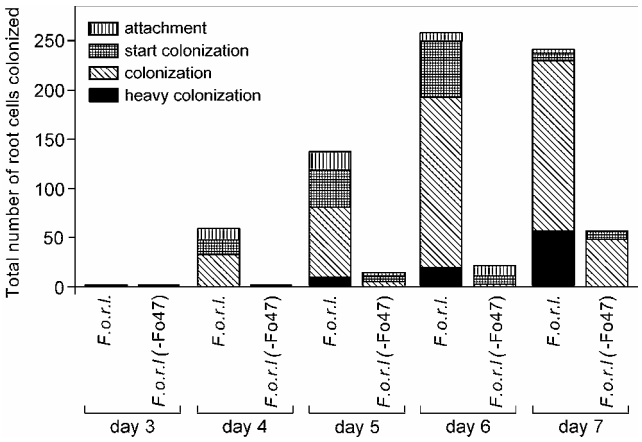


Figure 5. Quantification of tomato root colonization stages by *F.o.r.l.* in absence and presence of *F. oxysporum* Fo47 in time.
Seedlings were grown in *F.o.r.l.* (5x10^4 spores/kg sand), or *F.o.r.l.* and Fo47 infested sand (ratio 1:50). Plants were scored for tomato root surface colonization after 3, 4, 5, 6 and 7 days of growth. Colonization was classified in four different stages of colonization: attachment, start of colonization, colonization and heavy colonization as described in the Materials and Method section and shown in Fig. 2. Colonization was quantified by counting the number of plant cells colonized from crown till root tip at the four stages under the following conditions: (i) '*F.o.r.l.*', root colonization of *F.o.r.l.* in absence of Fo47 and (ii) '*F.o.r.l.*(-Fo47)', the root colonization of *F.o.r.l.* in presence of Fo47. Per condition two plants were scored and the average of two experiments is depicted in the figure.

After inoculation of the sand with a mixture of spores of *F.o.r.l.* and Fo47, Fo47 was observed to be dominantly present on healthy roots (Fig. 4C). With increased disease index of the plants colonization of the tomato root surface by *F.o.r.l.* appeared to be increased relative to colonization by strain Fo47 (compare Fig. 4C with 4D). On healthy roots, *F.o.r.l.* was strongly reduced at all colonization stages till day six (Fig. 5). After seven days *F.o.r.l.* was strongly reduced at the 'colonization' stage. 'Heavy colonization' was not observed during these seven days (Fig. 5). Direct cell-to-cell interactions between *F.o.r.l.* and Fo47 were observed in this period. No stress effects (such as increased branching, swelling of hyphae, undirected growth of hyphae (see Bolwerk et al., 2003) were observed within either of the fungi upon direct interaction (Fig. 4E and 4F).

Spore germination on tomato root exudate

CLSM studies revealed that Fo47 reduced the pathogen already at or before the initial stage of 'attachment' and the subsequent colonization stages under disease controlling conditions (Fig. 3). A high inoculum ratio (1:4 $x10^4$ Fo47 spores/kg sand) arrested *F.o.r.l.* in the stage of 'attachment'. To get more insight in the mechanism causing this strong reduction of *F.o.r.l.*, spore germination of *F.o.r.l.* and strain Fo47 in tomato root exudate was analyzed. The composition of tomato root exudate, with respect to amino acids, sugars and organic acids has been described previously (Lugtenberg and Bloemberg, 2004). It contains glucose (20µM) as the major sugar and citric acid (133 µM) as the main organic acid. After incubation overnight in synthetic root exudate 27% of the *F.o.r.l.* spores was germinated, whereas a significantly higher percentage (47%) of Fo47 spores was germinated (Fig. 6A). Analysis of spore germination in the major sugar and organic acid showed that a significantly higher percentage of Fo47 spores germinated both on glucose and citric acid (4.4% and 10.7%, respectively) compared to *F.o.r.l.* (0.6% and 6.1%, respectively) (Fig. 6A).

Analysis of spore germination in root exudate derived from fresh tomato plant roots, confirmed that a significantly higher percentage of Fo47 spores germinate compared to the spores of *F.o.r.l.*, 49% and 33%, respectively. Over a period of seven days the percentage of spores germinated remained constant, and the difference between Fo47 and *F.o.r.l.* was significant (Fig. 6B).

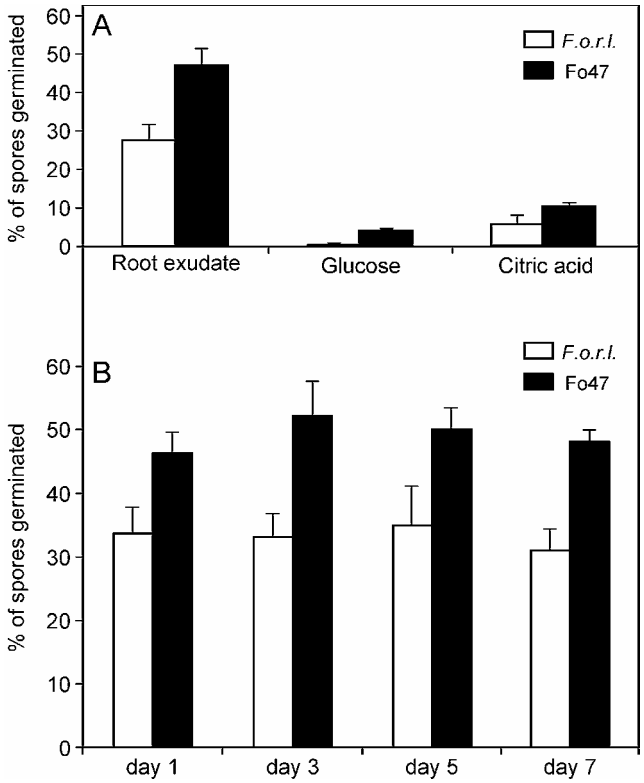


Figure 6. Germination of *F.o.r.l.* and Fo47spores in tomato root exudate and in solutions of its major sugar and organic acid.
Panel **A**. Spores of *F.o.r.l.* and Fo47 were incubated overnight in (i) synthetic root exudate described by Lugtenberg and Bloemberg (2004), (ii) the main exudate sugar glucose (20 µM) or (iii) the main exudate organic acid citric acid (133 µM). The total number of spores and the number of germinated spores were quantified. Subsequently, the percentage of spore germination was calculated. Panel **B**. A time course analysis of the spore germination of *F.o.r.l.* and Fo47 in tomato root exudate derived from plants grown in a hydroponic system. Over a period of seven days, the percentage of spore germination was calculated every two days.

Discussion

Previous visualization studies of root colonization by pathogenic and biocontrol Fusarium strains

The first reports on visualization focused on the colonization of the root tissue by either a pathogenic (Olivian and Alabouvette, 1999; Olivian et al., 2003), or a nonpathogenic *Fusarium* strain (Olivian and Alabouvette, 1997, Olivian et al., 2003) of plants growing in nutrient solutions and using electron microscopy. The use of a ß-glucuronidase construct allowed quantification of the nonpathogenic *F. oxysporum* SA70 on roots of tomato plants grown in soil or potting material (Bao et al., 2000; Eparvier and Alabouvette, 1994). Using histochemical staining Bao and Lazarovitz (2001) were able to simultaneously visualize the pathogenic *F. oxysporum* f sp. *lycopersici* and the non-pathogenic *F. oxysporum* SA70 colonizing the outer and the inner root tissue of plants dipped in a spore suspension and subsequently grown in a liquid modified Murashige and Skoog medium. The process of colonization and infection of the tomato root by *F.o.r.l.* has been studied at the end of the past century (Charest et al., 1984; Brammall and Higgins, 1988) whereas more recently, further details were revealed using GFP-labeled *F.o.r.l.* (Lagopodi et al., 2002). *F.o.r.l.* initially appears to attach to the root hairs, subsequently starts to colonize the main root after which it grows along the intercellular junctions (Lagopodi et al., 2002). At the sites of root penetration hyphae are swollen and heavy colonization of the tomato root is observed at sites where brown lesions are visible on the root (Lagopodi et al., 2002).

Improved visualization of biocontrol of tomato foot and root rot by Fo47 using autofluorescently labeled fungi in a gnotobiotic sand-nutrient solution system

In the present work we visualized, for the first time under disease controlling conditions, tomato root colonization by the pathogenic and the nonpathogenic *Fusarium* strains simultaneously. Tomato seedlings were grown in a sterile gnotobiotic sand system infested with spores of either *F.o.r.l.* or Fo47 or both. This system was previously shown to allow visualization of root colonization by *Pseudomonas* bacteria (Bloemberg et al., 2000; Bloemberg et al., 1997) or *F.o.r.l.* (Lagopodi et al., 2002) and of the interaction between *F.o.r.l.* and biocontrol *Pseudomonas* bacteria in the tomato rhizosphere (Bolwerk et al., 2003). In order to obtain a better understanding of the biocontrol process, root colonization by *F.o.r.l.* and strain Fo47 was visualized, quantified and statistically analyzed. It should be noted that in this gnotobiotic system competing indigenous bacteria are absent.

Interpretation of the results in relation to mechanisms, which could play a role in the control of TFRR by Fo47

Since plate confrontation assays did not show inhibition of the pathogen and since spore germination of *F.o.r.l*. was not affected by the culture supernatant of Fo47, it is unlikely that Fo47 produces antibiotics or extracellular enzymes seriously affecting the growth of the pathogen. Direct interactions in the rhizosphere between *F.o.r.l.* and Fo47 were observed but did not cause stress

effects in either of the two fungi (Fig. 4E and 4F) such as undirected growth, increased branching and hyphal swelling, effects described in *F.o.r.l.* caused by the presence of *P. chlororaphis* PCL1391 (Bolwerk et al., 2003). We therefore conclude that (i) antibiosis and (ii) parasitism and predation as mechanisms for biocontrol of TFRR by Fo47 are unlikely.

Paustian and Schnürer (1987) suggested that C-sources are the growth-limiting factor for fungi in soil. Previously, Couteaudier and Alabouvette (1990) showed that glucose, at concentrations fifty times higher than estimated to be present in tomato root exudate, can be consumed more efficiently by Fo47 than by *F.o.r.l..* In the present paper we have analyzed spore germination in tomato root exudate and its major sugar (glucose) and its major organic acid (citric acid) at concentrations estimated to be present in tomato root exudate (Lugtenberg and Bloemberg, 2004). It was observed that a higher percentage of Fo47 spores germinated on these three components (Fig. 6A).

Analysis of spore germination in root exudate collected from roots of fresh tomato plants revealed that over a period of seven days a higher percentage of spores of Fo47 germinate compared to spores of *F.o.r.l*. (Fig. 6B). This would be advantageous for Fo47 in the tomato rhizosphere within the gnotobiotic system where all nutritional compounds inducing spore germination and supporting hyphal growth are derived from the root exudate. Additionally, the inoculum concentration of Fo47 is fifty times higher than that of *F.o.r.l..* These two factors combined will reduce the nutrients available for spore germination and growth of *F.o.r.l.*. Consequently less *F.o.r.l.* hyphae will reach the root surface to attach to and colonize the tomato root.

Further reduction of the pathogen, once it has reached the root surface, will be caused by occupation of the root surface by the biocontrol strain Fo47. The root colonization process by the two fungi was shown to contain similar stages and niches. As a consequence, competition for niches on the tomato root involves several sites and stages. The first one is the initial attachment to root hairs (Lagopodi et al., 2002; this study). After three days Fo47 has attached to 2-5 sites on the root, whereas *F.o.r.l.* attached to 0-1 site. This is likely to be a result of the higher inoculation concentration of Fo47 and, of faster germination of its spores and will result in a reduction of C-sources available for spore germination and growth by *F.o.r.l.*. Additionally, this results in a reduction of the number of attachment sites available for *F.o.r.l.*. The second site is the growth of fungi along the cellular junctions of the root (Fig. 2C and 4A). The presence of Fo47 at these junctions reduces the sites available for colonization by *F.o.r.l.*. However, root colonization by Fo47 from day four on was slower and to a lower extent compared to that of *F.o.r.l.* despite the fifty-fold higher inoculum concentration (Fig. 3) as shown by the following observations. (a) Five times more root cells were colonized by *F.o.r.l.* than by to Fo47 (colonization was observed along thirty-three and six root cells, respectively) after four days of growth. (b) Colonization by *F.o.r.l.* increased most strongly at day four versus at day six by Fo47. (c) The total root area colonized after seven days of growth is larger for *F.o.r.l.* than for Fo47. (d) The colonization by *F.o.r.l.* is more dense, as indicated by heavy colonization. The

third stage involves the penetration of the root, which is observed for both *F.o.r.l.* (Lagopodi et al., 2002) and Fo47 (Fig 4B), was less frequent for Fo47 than observed for *F.o.r.l.* (Lagopodi et al., 2002) and may be restricted to specific sites of the root that are more frail. We assume that due to the occupation of penetration sites by Fo47, less sites are available for penetration by *F.o.r.l..* Consequently, less lesions are likely to be formed and no additional nutrients will be leaking from the root, thereby preventing the normally extensive growth of *F.o.r.l.* described by Bolwerk et al. (2003). This hypothesis is supported by our observation that under biocontrol conditions 'heavy colonization' is not observed.

The results mentioned above suggest that under biocontrol conditions Fo47 uses the mechanism "competition for niches and nutrients" as a biocontrol strategy. However, it should be noted that in order to be effective Fo47 must be introduced at an at least fifty-fold higher inoculum concentration compared to *F.o.r.l.* (Tables 3 and 4). The observation that root colonization by the biocontrol strain from day four on is less aggressive, slower and to a lesser extent than that of *F.o.r.l.* (Figs. 3 and 4), indicate that Fo47 is not capable of effectively competing with the pathogen for niches and nutrients on the root surface. The higher inoculum concentration is presumably needed to compensate for the poorer root colonization characteristics of Fo47. This is also illustrated by the decrease of root colonization by the pathogen at increasing concentrations of Fo47 (Table 4). In conclusion, our CLSM studies provide strong experimental evidence that the mechanism "competition for niches and nutrients" contribute to the biocontrol by Fo47, as previously suggested by Eparvier and Alabouvette (1994), and that this is the result of excess Fo47 and not of the good colonization properties of Fo47. Since there is a great diversity among strains of *F.o.r.l.*, care should be taken to generalize the interactions described in this work (such as competition for niches and nutrients) for other *Furarium* strains.

A common strategy to introduce a biocontrol agent is seed coating. Coating seeds and seedlings with Fo47 spores resulted in a reduction of the disease incidence, from 100 to 75%. Under these conditions Fo47 hyphae could only be observed just below the crown region. Since Fo47 is not applied to the sand and poorly colonizes the root, it is likely that other mechanisms in addition to competition contribute to the observed disease reduction. This situation resembles a previous observation by Dekkers et al. (2000) in which coating of tomato seeds with mutants of *Pseudomonas fluorescens* WCS365 that are impaired in efficient root colonization were not affected in their ability to protect the plant against TFRR. Biocontrol by strain WCS365 strains is thought to act via induced systemic resistance (ISR). ISR was identified as a potential mechanism of biocontrol by *F. oxysporum* Fo47 (Duijff et al., 1998; Fuchs et al., 1997). The reduced disease incidence in our experiments could therefore be a result of ISR.

To our knowledge this is the first time that the pathogenic and biocontrol fungus have simultaneously been visualized on the tomato root and that colonization of the tomato root surface by these fungi has been quantified. In this report new experimental results obtained under disease controlling conditions are provided

that extend our understanding of the mechanism involved in biocontrol of TFRR by Fo47. (i) Direct antagonism between the biocontrol fungus and pathogen is unlikely to play a role in biocontrol by Fo47. (ii) The preferential germination of Fo47 spores by root exudate components is thought (a) to reduce growth of the pathogen towards the root because more hyphae of Fo47 can compete for nutrients from root exudate and (b) to reduce the number of *F.o.r.l.* hyphae that can compete for attachment sites of the root. (iii) The higher inoculum concentration of Fo47 compensates for the less aggressive growth of Fo47 and consequently contributes to effective competition for niches and nutrients on the tomato root. (iv) ISR plays a role in controlling TFRR.

Materials and methods

Fungal isolates and inoculum production

The microorganisms used are listed in Table 1. *F.o.r.l.* and Fo47 were cultured on potato dextrose agar (Difco Laboratories, Detroit) or shaken at 130-160 rpm in Armstrong medium (Singleton et al., 1992) for 2 days at 28ºC. *F.o.r.l.* spores were isolated as described by Lagopodi et al. (2002). The spores were mixed with quartz sand to a concentration of $5x10^4$ spores/kg ($5.4x10^1$ spores/ml) sand for *F.o.r.l.* and $5x10^4$, $1x10^5$, $3x10^5$, $5x10^5$, $2.5x10^6$, $5x10^6$ and $2x10^9$ spores/kg ($5.4x10^1$, $1.1\ x10^2$, $5.4x10^2$, $2.7x10^3$, $5.4x10^3$, $2.2x10^6$ spores/ml) for Fo47. For analyzing spore-germination on citric acid, *F.o.r.l.* and Fo47 were grown in modified Armstrong medium: instead of sucrose, citric acid is added as single C-source to a final concentration of 1.3 mM.

Transformation of Fusarium

The construction of the plasmids was carried out using standard cloning techniques (Sambrook et al., 1989). Strains Fo47 and *F.o.r.l.* were transformed by a polyethylene glycol/$CaCl_2$-mediated transformation of protoplasts as described by Kistler and Benny (1988) and modified by Mes and colleagues (1999) with additional modifications described by Lagopodi et al. (2002). To select the YFP- or CFP-expressing Hm-B resistant cotransformants, the colonies were directly observed under a Leica MZFLIII stereo microscope equipped with epifluorescence detection (Leica, Bensheim, Germany). Filter sets tailored to the specific chromophores were used (for EYFP, 500/10-nm with excitation 518/16-nm emission and for ECFP, 440/21-nm excitation with 480/36-nm emission).

Control of tomato foot and root rot

Tomato seeds (kindly provided by Dr. Rudy Scheffer, Syntenga, Enkhuizen, The Netherlands) were sterilized (Simons et al., 1996) and incubated at 4 ºC for 5 days on plant nutrient solution (PNS) (Hoffland, 1989) solidified with 1.8% agar. The seeds were incubated for 2 days at 28ºC to allow germination.

The spatio-temporal analyses as well as the disease controlling experiments were performed in a gnotobiotic quartz sand system (Simons et al.,

1996). The sterile glass tubes were filled with sand moisturized with PNS (10% v/w) and infested with spores of *F.o.r.l.* and strain Fo47. Tomato seedlings were placed 5 mm below the surface of the sand. The plants were grown in climate-controlled growth chamber at 21ºC, 40% relative humidity and 16 hours of light per day. Sixteen to nineteen seedlings were grown per treatment. In case of the seed(ling) coating with spores of Fo47, seed(ling)s were incubated in PBS containing Fo47 spores ($1x10^9$ spores/ml) for 15 minutes. After seven days of growth the plants were scored for disease development by eye and classified in disease indexes 0 to 4. These indexes correspond to the following symptoms. 0, healthy plants with no visible symptoms of foot and root rot; 1, plants with pin-point size brown spots on the main root and/or pin-point size light brown spots on the crown; 2, plants with brown spots on the main root and extensive brown discoloration of the crown; 3, plants with a wilting appearance and an extensive rot of root and crown; 4, dead plants.

Confocal laser scanning microscopic analysis of tomato roots

After growth in the gnotobiotic system, tomato roots were carefully taken out of the sand and gently swirled a few times in sterile water in order to wash away the sand particles. Whole roots were placed directly on glass slides in drops of water and examined using an inverted fluorescence microscope (DMIRBE; Leica, Bensheim, Germany) equipped with filter blocks with spectral properties matching those of ECFP, (440/21-nm excitation with 480/36-nm emission; XF114, Chroma, Brattleboro, VT, U.S.A.) or EGFP (470/20-nm excitation with 515-nm long pass emission; I3, Leica, Bensheim, Germany), to which the Leica SP scanhead was attached. Dual color images were acquired by sequential scanning with settings optimal for ECFP (excitation with 457-nm argon laser line, emission detection between 470 and 490-nm), followed by settings optimal for EGFP (excitation with 488-nm argon laser line, detection of emitted light between 500 and 520-nm). Reflected light images were obtained by detection of light at the wavelength used for excitation. The projections of the individual channels were merged in Photoshop 7.0 (Adobe, San Jose, CA, U.S.A.) to facilitate visualization.

To qualify and quantify tomato root surface colonization by the *F.o.r.l.* and Fo47 four tomato roots per treatment were analyzed. Four different stages of root colonization were identified: (i) ‘attachment’ to root hairs; (ii) growth along one-two plant cell on main root, defined as ‘start colonization’; (iii) growth along three or more adjacent cells in length, defined as ‘colonization’; and (iv) dense colonization over the total width of the root surface, defined as ‘heavy colonization’. By using this classification, colonization by the fungi could be categorized.

Quantification of root colonization

All epidermis cells of a tomato root were examined from the crown till the root tip (length-axis) for one of the four colonization stages (see above) of colonization by *Fusarium* hyphae using CLSM. The number of tomato root cells colonized in the length-axis (form crown to root tip) was counted. When a hyphae was growing on the intercellular junction in between two root cells (in length axes)

this was scored as one colonized cell. When five cells in the width-axis on the same length-axis position were colonized it was scored as one colonized cell. In case these five cells in the width-axis were colonized by *Fusarium* in more than one of the four defined stages (for example 'attachment' and 'colonization') the most progressed stage was scored (in this example 'colonization'). Each experiment was performed at least twice.

Statistical analysis

Plants were classed as healthy (disease index 0) or sick (disease index 1 to 4). The difference in health condition (healthy or sick) of plants between two different treatments was statistically analyzed using the chi-squared goodness-of-fit test (Heath, 1995). The degree of freedom was 1 (degree of freedom = 2 conditions tested-1)x(2 classes of plants-1) resulting in the critical X_2 value of 3.841 ($P<0.05$). The null-hypothesis was defined as the lack of significant difference between two conditions tested. To test the null-hypothesis the X_2 value was calculated for the two conditions using the chi-squared goodness-of-fit test. In case the calculated X_2 value was lower than the critical X_2 value, the null-hypothesis was accepted e.g. the two treatments were not significantly different. When the calculated X_2 value was higher than the critical value, the null-hypothesis was rejected e.g. the treatments differ significantly.

Quantification of tomato root colonization was performed by counting the number of root cells colonized by *F.o.r.l.* as described above. To determine whether root colonization by *F.o.r.l.* was significantly reduced by the presence of strain Fo47 after seven days of incubation, four roots per condition (*F.o.r.l..* alone and *F.o.r.l.* in presence of Fo47, e.g. two conditions) were analyzed. Within this analysis eight roots in total were scored from root tip till crown for root colonization by *F.o.r.l..* A Wilcoxon-Mann-Withney U-test (Sokal and Rohlf, 1981) was used to determine whether the difference in root colonization by *F.o.r.l.* in the absence and in the presence of Fo47 was significantly different. This statistical analysis was performed on the three different ratio's of *F.o.r.l*-Fo47 (1:10, 1:50 and 1:100). Each ratio was analyzed at least twice.

Plate confrontation assays

PDA plates were inoculated with agar plugs (four mm) of the fungi, placed four cm apart and incubated at 25ºC and the growth of *F.o.r.l.* was analyzed daily. Additionally, fungal culture supernatant of strain Fo47 and *F.o.r.l.* was also analyzed for its ability to inhibit hyphal growth of *F.o.r.l.*. Hundred microliters of a two times concentrated Armstrong O/N culture was plated on one half of a PDA plate and *F.o.r.l.* was inoculated as a stripe of spores on both halves of the plate. The growth of *F.o.r.l.* was analyzed daily.

Spore germination

Fungal spores of both *F.o.r.l.* and Fo47 were incubated in tomato root exudate (synthetic as described by Lugtenberg and Bloemberg, 2004; and collected from roots of fresh growing tomato plants), 20 µM glucose or 133 µM citric acid overnight at room temperature. Root exudate was isolated as described

previously (Simons et al., 1997). Briefly, 100 ml sterile seedlings were placed in 100 ml PNS and were allowed to grow in a climate-controlled growth chamber at 20ºC, 40% relative humidity and 16 h of daylight. After fourteen days of growth root exudate was collected.

Spore germination on root exudate from fresh tomato plants was analyzed for spores isolated from Armstrong cultures containing sucrose (Singelton et al. 1992). Germination in glucose (20 µM) or citric acid (133 µM) was analyzed for spores isolated form Armstrong cultures containing sucrose (Singelton et al. 1992) or citric acid (1.3 µM), respectively. Spore germination in synthetic root exudate was analyzed for spores isolated from both Armstrong cultures. The reaction volume was 500 µl and the final concentration of spores was 2.5×10^5 per ml.

The number of germinated and total number of spores was counted using a heamatocytometer and the percentage of germination was calculated. The germination experiments were carried out in triplicate and were repeated twice. Using a Mann-Whitney-U test differences between spore germination of the pathogen and biocontrol agent were evaluated.

Acknowledgements

We thank C. Alabouvette for the supply of the nonpathogenic *Fusarium* strain Fo47 and useful discussions. We thank A. H. M. Wijfjes, C. A. M. J. J. van den Hondel, and A. F. J. Ram for the help with fungal transformation. A. Bolwerk was supported by a grant of the NWO Earth and Life Sciences Council, project no. 810.35.003. A. L. Lagopodi was financially supported by a Marie Curie Fellowship of the European Community Program Training and Mobility of Researchers under contract ERBFMBICT982930.

Chapter 4

Biocontrol of tomato foot and root rot by *Trichoderma* spp. and the role of chitinases

Annouschka Bolwerk, Matteo Lorito,
Ben J. J. Lugtenberg and Guido V. Bloemberg

Abstract

Trichoderma atroviride P1, a well studied antagonist of phytopathogenic fungi at the laboratory level, and *T. harzianum* T22, one of the most widely used biofungicides in agriculture, were shown to control tomato foot and root rot (TFRR) caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*). *In vitro* analysis indicated that extra-cellular compounds produced by P1 and T22 strongly inhibit spore germination of the pathogen. *Trichoderma* P1 knock-out mutants illustrated the role of an endochitinase and an exochitinase in the inhibition of spore germination as well as in biocontrol of TFRR. To study the complex interactions between the antagonist and pathogen, growth of the fungi on the tomato rhizoplane alone and under biocontrol conditions was visualized and quantified. These confocal laser scanning microscopy (CLSM) analyses, using strains labeled with auto-fluorescent proteins, revealed the following. (i) When both *F.o.r.l.* and either one of the *Trichoderma* strains were introduced, the disease incidence, the total area as well as the density of tomato root colonization by *F.o.r.l.* were significantly reduced. (ii) The endochitinase and exochitinase are involved in disease suppression and in the reduction of *F.o.r.l.* root colonization by strain P1, which is likely supported by the role of these enzymes in the inhibition of spore germination. (iii) *T. atroviride* P1 and *T. harzianum* T22 colonize the tomato root hairs, which is the initial site of attachment of *F.o.r.l*. in the infection process. (iv) *T. atroviride* P1 and, to a lesser extent *T. harzianum* T22 are poor colonizers of the main root. (v) Growth of T22, and therefore its ability to compete for niches, strongly depends on the nutrient composition of the growth substrate. (vi) P1 and T22 induce plant responses that could contribute to the resistance against phytopathogens such as *F.o.r.l*..

Introduction

Some *Trichoderma* spp. can be used as biocontrol agents of a wide range of phytopathogens (Chet, 1987; Harman et al., 2004; Hjeljord and Tronsmo, 1998) and are commercially applied to control a variety of crop diseases (Koch, 1999). *In vitro* studies focusing on the interaction between *Trichoderma* spp. and phytopathogenic fungi at the cellular level have indicated mycoparasitism as one of the main potential modes of action in biocontrol (Chet et al., 1981; Elad et al., 1983; Elad et al., 1987). *In situ* analysis of the interactions between biocontrol *Trichoderma* strains and phytopathogenic fungi occurring on the plant root are scarce (Lu et al., 2004). By using confocal scanning laser microscopy (CLSM), Lu and colleagues showed that mycoparasitism of *T. atroviride* P1 on *Pythium ultimum* not only occurred in dual cultures but also on cucumber roots. Other mechanisms of action that could be involved in the ability of *Trichoderma* spp. to limit disease development are: (i) The production of cell-wall-degrading enzymes (Brunner et al., 2003; Woo et al., 1999) and antibiotics (Lorito et al., 1996; Schirmböck et al. 1994), (ii) Competition with the pathogen for rhizosphere colonization sites and root exudate nutrients (Sivan and Chet, 1986), and (iii) induced systemic resistance (ISR) (De Meyer et al., 1998; Harman et al., 2004; Yedidia et al., 1999; Yedidia et al., 2000; Yedidia et al., 2003).

Tomato foot and root rot (TFRR), caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*), is a serious problem for field and greenhouse crops (Jarvis, 1988) and can be controlled by selected strains of *Trichoderma* spp. (Datnoff et al., 1995; Marois et al., 1981; Sivan and Chet, 1986; Sivan et al., 1987; Sivan and Chet, 1993). In this paper we focused on the ability of *T. atroviride* P1 and *T. harzianum* T22 to suppress TFRR and on the mechanisms that are involved in this biocontrol. *T. atroviride* P1 is a cold tolerant strain which was initially selected for post-harvest protection (Tronsmo et al., 1989; Tronsmo et al., 1991). Potential biocontrol mechanisms of P1 involve the production of several extracellular enzymes, including glucan 1,3-ß-glucosidases, N-acetyl- ß-glucosaminidases (such as the CHIT73 exochitinase encoded by *nag1*; Lorito et al., 1994), chitobiosidases and endochitinases (such as the CHIT42 endochitinase encoded by *ech42*; Harman et al., 1993). By testing a *ech42* mutant of strain P1 (D11), Woo et al. (1999) found that the production of CHIT42 is essential for full biocontrol activity against *Botrytis cinerea*. Brunner et al. (2003) studied the CHIT73 exochitinase (N-acetyl- ß-glucosaminidase) minus mutant of *T. atroviride* P1 (P1ND1), which showed a reduced biocontrol activity against *Rhizoctonia solani* and *Sclerotinia sclerotiorum* on beans. In addition, this enzyme was found to be important for triggering the expression of other chitinases and fungal cell wall degrading enzymes, including the CHIT42 endochitinase. *T. harzianum* T22 is a rhizosphere competent fungus, which is commercially applied in crop protection (Harman et al., 1996). Biocontrol mechanisms of T22 have been characterized in less detail as compared with P1, but it was shown that T22 produces endochitinases and glucanases during biocontrol (El-Katatny et al., 2000; Lorito et al., *unpublished results*).

We present results on the ability of *T. atroviride* P1 and *T. harzianum* T22 to control TFFR and studied their biocontrol mechanism(s) by analyzing the effects of chitinase production on *Fusarium* spore germination, root colonization, and biocontrol. We visualized the fungi and their interactions in the tomato rhizosphere, making use of strains labeled with different auto-fluorescent proteins. To our knowledge this is the first report which studies the interaction between *Fusarium* and *Trichoderma* biocontrol spp. on the tomato root, under disease controlling conditions.

Results

Control of tomato foot and root rot in potting soil by Trichoderma strains

To test the ability of *T. atroviride* P1 and *T. harzianum* T22 (Table 1) to control TFRR, tomato seeds were coated with *Trichoderma* spores and grown in potting soil infested with *F.o.r.l*. spores. Coating the seeds with either strain P1 or strain T22 significantly reduced the percentage of diseased plants (Fig. 1A). In another experiment the percentage of diseased plants was again significantly reduced from 64% to 36% and 44% due to the presence of strain P1 and T22, respectively. To determine the possible roles of extracellular endochitinase CHIT42 (Harman et al., 1993) and exochitinase CHIT73 (Lorito et al., 1994) in the biocontrol of TFRR by *T. atroviride* P1, the mutants D11 (with the CHIT42 mutation) and P1ND1 (with the CHIT73 mutation) were tested for biocontrol activity.

Table 1. Microorganisms

Strains	Relevant characteristics	Reference or source
Fungi		
ZUM 2407	*Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) causing tomato foot and root rot	IPO-DLO, Wageningen, The Netherlands
FCL14	*F.o.r.l.* ZUM 2407 containing *sgfp* under control of the constitutive *gpdA* promoter	Lagopodi et al., 2002
FCL64	*F.o.r.l.* ZUM 2407 containing *ecfp* under control of the constitutive *gpdA* promoter	Bolwerk et al. *submitted*
P1	*T. atroviride* strain P1 (ATCC 74058)	
T22	*T. harzianum* T22. Commercialized in U.S.A. and Europe	Tronsmo et al., 1989 Stasz et al., 1988
D11	Endochitinase mutant (*ech42*$^-$) of *T. atroviride* strain P1. Containing a hygromycin B-phosphotransferase (*hph*) selectable marker	Woo et al., 1999
P1ND1	Exochitinase mutant (*nag1*$^-$) of *T. atroviride* strain P1. Containing an acetamidase (*amd*S) and hygromycin B-phosphotransferase (*hph*) selectable marker	Brunner et al., 2003
P1 *pki*::*gfp*	*T. atroviride* strain P1 constitutively expressing *gfp*. Under the control of the pyruvate kinase (*pki*) promoter from *T. reesei*	Zeilinger et al., 1999
T22 *pki*::*gfp*	*T. harzianum* T22 constitutively expressing *gfp* under control of the *pki* promoter from *T. reesei*	Lorito et al., *unpublished*

In contrast to the wild type, the mutants did not significantly reduce the disease incidence (Fig. 1B). In another experiment similar results were obtained: the percentage of diseased plants was significantly reduced from 56% to 34% by *T. atroviride* P1 whereas in the presence of D11 and P1ND1 52% and 47% respectively, of the plants were diseased.

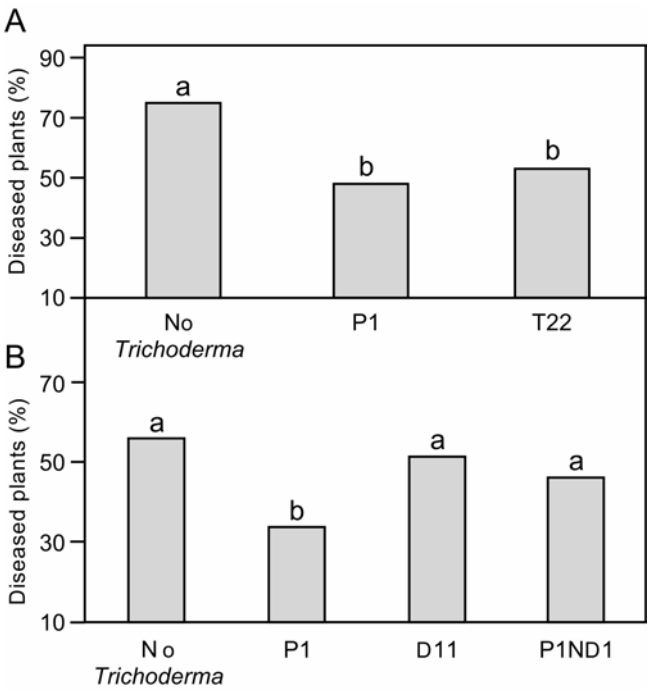


Figure 1. Control of tomato foot and root rot by *Trichoderma* in potting soil
Tomato seeds were grown in potting soil infested with *F.o.r.l.* spores. As a control to determine the disease pressure tomato seeds were coated with methyl cellulose without *Trichoderma*. After three weeks of growth in a daylight greenhouse, roots were analyzed for disease symptoms and the percentage of diseased plants was determined. A variance analysis followed by Fisher's least-significant-difference was used to determine whether the strains could significantly control TFRR. **A.** Biocontrol strains *Trichoderma atroviride* P1 (P1) and *T. harzianum* (T22) were introduced by seed coating as described in the Materials and Methods section. **B.** To analyze the role of the extracellular enzymes produced by strain P1, the endo- and exochitinase mutants D11 and P1ND1were tested for their ability to control TFRR.

Optimizing growth conditions in the gnotobiotic system for the analysis of biocontrol of tomato foot and root rot by Trichoderma spp.

To visualize root colonization of *T. atroviride* P1 and *T. harzianum* T22 and their effect on the tomato root colonization by the pathogen *F.o.r.l.,* a gnotobiotic sand system (Simons et al., 1996) was used. To test the influence of different plant growth media on the root colonization by strains P1 and T22, tomato plants were grown in sand moisturized with either (i) a plant nutrient solution (PNS, Hoffland et al., 1989), (ii) PNS supplemented with 0.01% sucrose, or (iii) a hydroponic solution (HPS, Yedidia et al., 1999). *Trichoderma* was applied as germinated spores or as mycelium and the level of root colonization was determined by CLSM analysis after seven days. PNS appeared to be the least efficient medium for supporting growth of P1 and T22. The addition of sucrose improved hyphal growth of both *Trichoderma* strains and also of *F.o.r.l.* in the tomato rhizoplane, thereby increasing the disease pressure as compared to PNS treatment without sucrose (results not shown). The growth of T22 was further increased when sand was moisturized with HPS, whereas in this medium both the development of strain P1 and the disease pressure were comparable to the condition under which sand was moisturized with PNS without sucrose.

In order to obtain data comparable with previous studies on tomato root colonization by *F.o.r.l.*, which were performed in sand moisturized with PNS (Lagopodi et al., 2002; Bolwerk et al., 2003; Bolwerk et al., *submitted*), the latter medium supplemented with sucrose instead of HPS, was used in all further experiments. For the same reason the inoculum concentration of the pathogen was reduced ten-fold to $5x10^3$ spores/kg sand.

Control of tomato foot and root in the gnotobiotic system

Coating of tomato seedlings with spores of P1 or T22 and subsequent growth in the gnotobiotic system (Simons et al., 1996) containing sand infested with spores of *F.o.r.l.,* reduced the number of sick plants compared to the control. A significant and reproducible reduction of the disease was obtained after coating the seedlings with germinated spores of either of the two biocontrol agents (Fig. 2, open columns). Unexpectedly, the coating treatment with germinated spores of P1 and T22 altered root formation. Under the conditions used, a new main root emerged from the original seed-generated root, which did not grow further and developed a brown staining. In addition, this newly emerged root was in general shorter than the corresponding root of non-treated seedlings. However, when *Trichoderma* was introduced as mycelium mixed through the sand, root formation was not altered and the treatment resulted for both strains P1 and T22 in a significant reduction of the number of diseased plants (Fig. 2, dark columns).

In order to test the effects of the endo- and exochitinases in the control of TFRR, strains D11 (*ech42*$^-$) and P1ND1 (*nag1*$^-$) were tested. It appeared that, in contrast to the wild type P1, neither the *ech42*$^-$ nor the *nag1*$^-$ mutant was able to decrease the disease significantly, no matter whether applied as germinated spores or as mycelium (Fig. 2). In contrast to the wild type strain P1, mutants D11 and P1ND1 did not alter the root formation when applied as germinated spores.

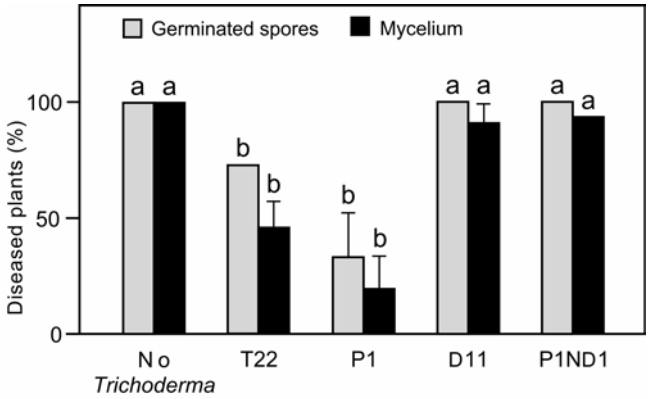


Figure 2. Control of tomato foot and root rot by *Trichoderma* in a gnotobiotic sand system
Tomato seedlings were grown in sand infested with *F.o.r.l.* spores. To determine the disease pressure, tomato seedlings were grown in absence of *Trichoderma*. After seven days of incubation in a growth chamber, roots were analyzed for disease symptoms and the percentage of diseased plants was determined. A chi-squared goodness-of-fit test was used to determine whether the strains tested could significantly control TFFR Biocontrol strains *Trichoderma atroviride* P1 (P1) and *T. harzianum* (T22) were introduced either by coating seedlings with germinated spores or by mixing mycelium with the sand, as described in the Materials and Methods section. To analyze the role of the extracellular enzymes produced by strain P1, the endo- and exochitinase mutants D11 and P1ND1, respectively, were also tested for their ability to control TFRR.

Effect of Trichoderma metabolites on F.o.r.l. spore germination

To determine the effect of extra-cellular enzymes produced by P1 and T22 on spore germination of *F.o.r.l*, conidia were incubated in culture supernatant of the biocontrol strains. After incubation overnight in the presence of the culture supernatant of strain T22 or P1, the percentage of germinated *F.o.r.l*. spores was strongly reduced (Fig. 3A). The inhibition by *T. atroviride* P1 was more efficient as compared to *T. harzianum* T22. Wild type strain P1 inhibited *F.o.r.l.* spore germination significantly stronger than the mutants D11 and P1ND1 did (Fig. 3B).

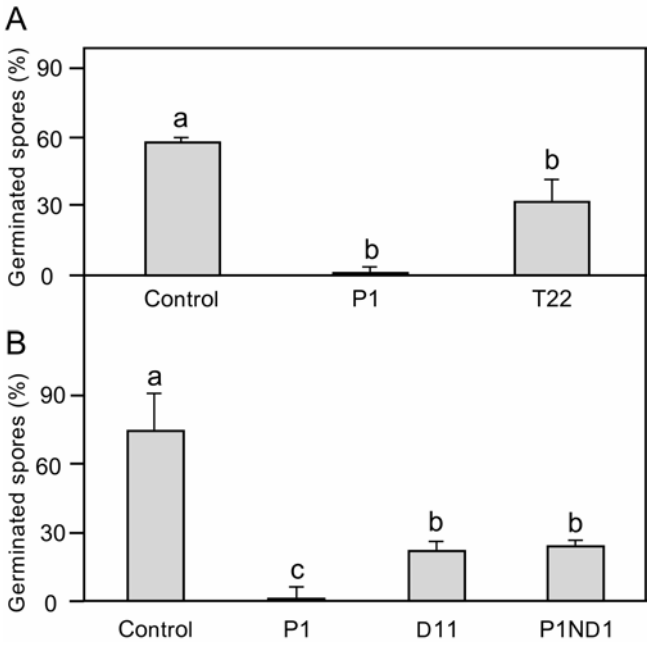


Figure 3. Spore germination of *F.o.r.l.* in the presence of culture supernatants of *Trichoderma* spp.

After overnight incubation at room temperature, the percentage of germinated spores was determined by counting under the microscope using a heamotocytometer. As a control spores were incubated in synthetic medium (SM).The Wilcoxon-Mann-Withney U-test was used to determine whether the treatments reduced spore germination significantly. **A**. *F.o.r.l.* spores were incubated in culture supernatant of the wild type strains *T. atroviride* P1 (P1) or *T. harzianum* T22 (T22) grown in SM. **B**. *F.o.r.l.* spores were incubated in culture supernatant of *T. atroviride* P1 or its chitinase mutants D11 (*ech42*$^-$) or P1ND1 (*nag1*$^-$) grown in SM to test the role of the extracellular enzymes produced by strain P1.

[b]The percentage of germinated spores does differ significantly from [a].

[c]The percentage of germinated spores does differ significantly from [a] and [b].

Tomato root colonization by T. atroviride P1 and T. harzianum T22

Root colonization by *T. atroviride* P1 and *T. harzianum* T22 was studied by CLSM using strains P1 *pki*::*gfp* and T22 *pki*::*gfp* (Table 1), which constitutively express *gfp*. The GFP labeling did not affect the biocontrol ability of the strains in potting soil or in the gnotobiotic system (data not shown).

Coating of seedlings with germinated spores of either P1 or T22 resulted, seven days after inoculation, in the appearance of clumps of hyphae on the main root (Fig. 4A). In close vicinity of these clumps single hyphae were observed on the root surface (Fig. 4A). Only few of these hyphae grew along the intercellular junctions of the tomato root surface.

When instead of coating seedlings with germinated spores, mycelium of strains P1 and T22 was mixed through the sand, hyphae of both strains were observed in between the root hairs (Fig. 4B). Strain T22 was found five times more abundant on the root hairs than strain P1 (two versus ten sites per root). After binding to the root surface, T22 started to grow in between the root hairs (Fig. 4C). Hyphae of T22 were observed on the main root (Fig. 4D). In a few cases these hyphae crossed over tomato root cells and grew along the intercellular junctions (Fig. 4E, 4F and 4G). In the latter case, at least once, a penetration of the root cortex by T22 was observed (Fig. 4G and 4H). In the case of P1, the presence of hyphae on the main root was not observed.

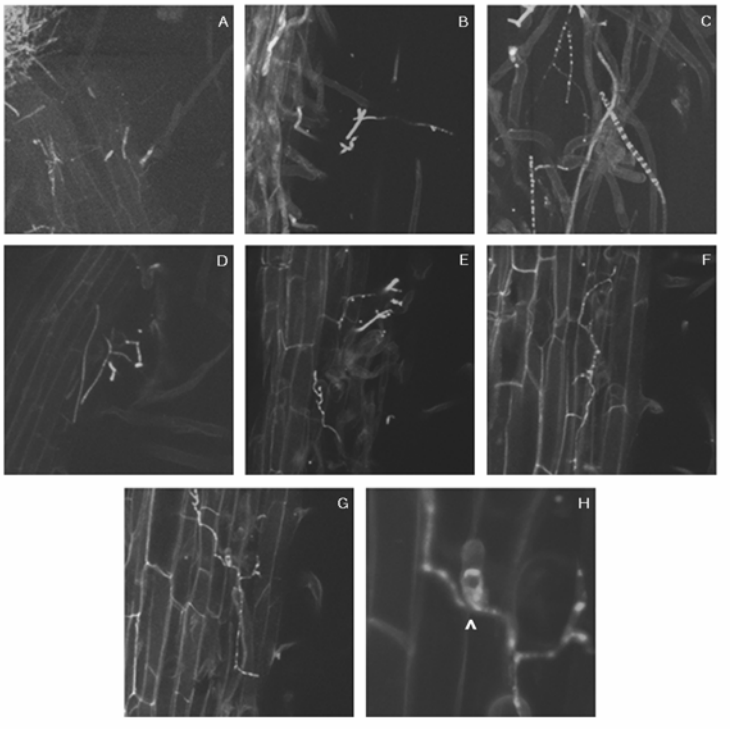


Figure 4. Confocal laser scanning microscopy (CLSM) analysis of tomato root colonization by two *Trichoderma* strains. The full color figure is depicted on page 127.

Two-day-old tomato seedlings were grown in a gnotobiotic sand system. *Trichoderma* strain P1 or T22 was introduced as germinated spores coated on seedlings (panel A) or as mycelium mixed through the sand (panel B through H). After seven days of growth, tomato root colonization by the *Trichoderma* strains was analyzed. Walls of tomato root cells appear red because of auto-fluorescence. **A.** Clump of germinated P1spores with short hyphae. **B.** Initial attachment of P1 hyphae to root hairs. **C.** T22 hyphal growth in the root hair zone. **D.** Hyphae of T22 growing in the rhizosphere but not attached to the root surface. **E to G.** Hyphae of T22 attached to and growing along the main root. **G and H (close-up of G),** Penetration of the tomato root by T22 as indicated with an arrow. Bars represent 10 µm.

Root colonization by F.o.r.l. in the presence of Trichoderma strains P1 and T22

FCL64, the CFP-labeled *F.o.r.l.* derivative (Bolwerk et al., *submitted*), and the GFP-labeled derivatives of strains P1 (Zeilinger et al., 1999) and T22 were used to study the possible interactions between the pathogen and the biocontrol agents. In case *F.o.r.l.* could be observed on the root, the colonization by both *F.o.r.l.* and the *Trichoderma* strains was found to be reduced as compared to treatments with the single strains. In case *F.o.r.l.* was not observed on the root because it was inhibited by the control agent, the root colonization patterns of strains P1 and T22 was found similar to controls. No physical *Fusarium-Trichoderma* interactions were observed after seven days of incubation.

To analyze the effect of the *Trichoderma* strains on the tomato root colonization by *F.o.r.l.,* the GFP-labeled derivative of *F.o.r.l.* - FCL14 (Table 1) - was used. Root colonization by *F.o.r.l.* was quantified by counting the total number of tomato root cells colonized per colonization stage in the length axes (from crown to root tip). Details on how root colonization in the absence and presence of non-labeled *Trichoderma* strains was counted are described in the Materials and Methods section. Both *Trichoderma* strains significantly limited *F.o.r.l*. growth in the stages 'colonization' and 'heavy colonization' (Tables 2A, 2B and C), as was determined by using the Mann-Whitney-U test (Sokal and Rolph, 1981). Not only the severity of root colonization by *F.o.r.l.* but also the total root area colonized by *F.o.r.l.* was significantly reduced by both *Trichoderma* spp. (Tables 2A, 2B and 2C).

Table 2. Quantification of tomato root colonization by *F.o.r.l.* in the presence of *Trichoderma* strains

A. Germinated *Trichoderma* spores coated on tomato seedlings

	F.o.r.l.	*F.o.r.l.*-P1	*F.o.r.l.*-T22
Attachment	1.7[a]	0.3[a]	2.7[a]
Start colonization	6.3[a]	3.7[a]	2.3[a]
Colonization	29.3[a]	0.0[b]	1.7[b]
Heavy colonization	16.0[a]	0.0[b]	0.0[b]
Total	55.3[a]	4.0[b]	6.7[b]

B. Mycelium of *Trichoderma* strain P1 mixed through the sand

	F.o.r.l.	*F.o.r.l.*-P1
Attachment	23.5[a]	1.3[b]
Start colonization	25.5[a]	1.0[b]
Colonization	263.8[a]	11.3[b]
Heavy colonization	9.5[a]	0.0[b]
Total	322.3[a]	13.6[b]

Table 2. –continued

C. Mycelium of *Trichoderma* strain T22 mixed through the sand

	F.o.r.l.	*F.o.r.l.*-T22
Attachment	1.0[a]	16.8[b]
Start colonization	0.8[a]	22.5[b]
Colonization	168.8[a]	33.8[b]
Heavy colonization	83.8[a]	0.0[b]
Total	254.4[a]	73.1[b]

D. Germinated *Trichoderma* spores coated on tomato seedlings.

	F.o.r.l.	*F.o.r.l.*-P1	*F.o.r.l.*-D11	*F.o.r.l.*-P1ND1
Attachment	12.3[a]	12.0[a]	5.5[a]	13.5[a]
Start colonization	4.3[a]	7.8[a]	5.8[a]	12.3[a]
Colonization	126.0[a]	16.0[b]	69.4[a]	77.0[a]
Heavy colonization	78.4[a]	0.0[b]	44.0[a]	78.0[a]
Total	220.9[a]	35.8[b]	124.7[a]	181.1[a]

E. Mycelium of *Trichoderma* strains mixed through the sand

	F.o.r.l.	*F.o.r.l.*-P1	*F.o.r.l.*-D11	*F.o.r.l.*-P1ND1
Attachment	12.3[a]	4.8[a]	12.3[a]	12.5[a]
Start colonization	4.3[a]	1.8[a]	7.0[a]	11.5[a]
Colonization	126.0[a]	17.9[b]	66.0[a]	101.0[a]
Heavy colonization	78.4[a]	0.0[b]	20.6[a]	27.5[a]
Total	220.9[a]	24.4[b]	105.9[a]	152.5[a]

Tomato seedlings were grown in sand infested with *F.o.r.l.* spores. *Trichoderma* strains were introduced either as germinated spores coated on the seedling or as mycelium mixed through the sand (see Materials and Methods section for details). The *Trichoderma* strains analyzed included wild type P1, T22 and the endo- and exochitinase minus mutants of *T. atroviride* P1, D11 and P1ND1, respectively. After seven days of incubation tomato roots were analyzed microscopically from crown till root tip for colonization by *F.o.r.l.*. Four different subsequent stages of root colonization with increasing hyphal density were defined: 'attachment', 'start colonization', 'colonization' and 'heavy colonization'. The total number of plant cells on the length-axis covered by *F.o.r.l.* per stage in the absence of *Trichoderma* (*'F.o.r.l.*') and in the presence of *Trichoderma* (*'F.o.r.l.-Trichoderma* strain) is presented. By using the Mann-Whitney-U test, it was determined for each colonization stage whether the total number of root cells covered by *F.o.r.l.* in the absence and the presence the *Trichoderma* strains differed significantly.

[a] The two compared treatments do not differ significantly.

[b] The two compared treatments do differ significantly.

When the chitinase-negative derivatives of strain P1, mutants D11 and P1ND1, were applied as germinated spores or as mycelium a reduction of the root colonization by *F.o.r.l.* in the 'colonization' and 'heavy colonization' stage was still observed (Tables 2 D and E). In contrast to what was observed for the wild type strain P1, the inhibitory effect on *F.o.r.l.* was not significant.

In another experiment, in which root colonization by *F.o.r.l*. was more intense and the disease pressure was higher, the following observations were made. (i) Wilting in addition to the extensive brown coloration of the crown and the main root was observed. (ii) Only the later two stages of root colonization ('colonization' and 'heavy colonization') by the pathogen were found. (iii) The pathogen colonized the whole root, whereas under low disease pressure root regions without *F.o.r.l.* could be detected. Under these high disease pressure conditions the wild type strain P1 still significantly reduced both the severity (a reduction in the colonization stages: 'colonization' and 'heavy colonization') and the total root colonization by the pathogen. In contrast, root colonization by the pathogen in the presence of D11 and P1ND1 under high disease pressure conditions was reduced in terms of density but not in terms of total root coverage in comparison with the control without *Trichoderma*. The effect of mutants D11 and P1ND1 on the root colonization by *F.o.r.l.* (severity and total root area covered) was significantly lower than the effect of wild type strain P1, when either germinated spores or mycelium was applied (data not shown).

Discussion

The mechanisms involved in the biocontrol of plant diseases by *Trichoderma* spp. have been studied at the molecular level (Harman et al., 1993; Harman et al., 2004; Lorito et al., 1994; Woo et al., 1999; El-Katatny et al., 2000). To improve our understanding of the biocontrol mechanisms of *Trichoderma* spp., we studied the interactions between the pathogen *F.o.r.l.,* two antagonistic *Trichoderma* strains and the plant root under disease controlling conditions using CLSM. Such *in vivo* studies focusing on the interactions at the cellular level are scarce (Lu et al., 2004) and, to our knowledge, have never been published under biocontrol conditions. *F.o.r.l*., the causal agent of TFRR, can be significantly controlled by *T. atroviride* P1 and *T. harzianum* T22, both in soil (Fig. 1A) as well as in the gnotobiotic sand system (Fig. 2A). For *T. atroviride* P1 we observed that extra-cellular endo- and exochitinases produced by this strain are involved in the control of TFRR, both in soil (Fig. 1B) as well as in the gnotobiotic system (Fig. 2B).

To visualize root colonization of *T. atroviride* P1 and *T. harzianum* T22 and the effect of these strains on the root colonization of the pathogen *F.o.r.l.,* a gnotobiotic sand system was used (Simons et al., 1996). In this system the influence of the endogenous microflora and background of small autofluorescent soil particles interfering with microscopic analysis is excluded. Analysis of fungal growth in different plant media showed that, in contrast to the growth of strain P1

and the disease development, the growth of T22 was improved when sand was moisturized with HPS as compared to PNS. Comparison of the mineral composition of PNS and HPS showed that the latter contains additional minerals (K^+, Ca^{2+} and NH_4NO_3) and the concentration of trace elements is up to 5000 times higher. This suggests that the growth of strain T22, in contrast to the growth of P1 and *F.o.r.l.*, strongly depends minerals and trace elements.

We will discuss the results of the present work in relation to possible mechanisms of biocontrol used by *Trichoderma* strains P1and T22.

Inhibition of spore germination

Analysis of *F.o.r.l.* spore germination in culture supernatants of either strain P1 or T22 indicated that these *Trichoderma* strains produce extra-cellular compounds that limit spore germination (Fig. 3A), the initial stage of *F.o.r.l.* growth prior to attachment to and colonization of the tomato root. Testing the culture supernatant of the endo- and exochitinase minus mutants of *T. atroviride P1*, D11 and P1ND1 respectively, indicated that both the CHIT42 endochitinase and the CHIT73 exochitinase contribute to the inhibition of spore germination by the wild type P1 (Fig. 3B). We cannot exclude that other factors also contribute to the observed reduction of *F.o.r.l.* spore germination in vitro in the presence of P1 culture filtrate. Cell wall degrading enzymes are also produced by strain T22. The presence of endochitinases and glucanases in T22 culture filtrate, which was demonstrated by El-Katatny et al. (2000), could be involved in the observed inhibition of *F.o.r.l.* spore germination (Fig. 3A).

The results predict that, by reducing the number of *F.o.r.l.* spores germinated in sand, *Trichoderma* will allow less hyphae to grow and to reach the tomato root for attachment to and subsequent colonization of the root. Such a reduction of root colonization was shown by CLSM analysis. By comparing root colonization by *F.o.r.l.* in the absence and the presence of the *Trichoderma* strains P1 and T22 under biocontrol conditions we showed a significant reduction of both the density of root colonization as well as the total root area colonized by *F.o.r.l.* (Table 2A, 2B and 2C). Analysis of the root colonization by F.o.r.l. in the presence of the endo- and exochitinase mutants of strain P1 indicated that both enzymes contribute to the reduction (in both density well as total area) observed in the presence of P1 (Table 2A, 2B, 2D and 2E). These CLSM studies support the hypothesis that the chitinases produced by *T. atroviride* P1 limit spore germination, which results in a reduced root colonization by *F.o.r.l.*

Competition for niches and nutrients

When *T. atroviride* P1 and *T. harzianum* T22 were applied as germinated spores on seedlings we found hyphae on the main root in close proximity of and below a clump of germinated spores (Fig. 3D). These hyphae are thought to have migrated downwards with the growing root originating from the clumps of germinated spores. When the *Trichoderma* strains were applied as mycelium mixed through the sand, hyphae of T22 were observed in between the root hairs five times more frequent than hyphae of P1 (Fig. 3B). Moreover, in contrast to those of P1, hyphae of T22 grew between the root hairs along the main root (Fig. 3C). By reducing the number of available sites where initial attachment by *F.o.r.l.*

can take place, namely on the root hairs (Lagopodi et al., 2003), the *Trichoderma* strains would be able to reduce *F.o.r.l.* growth already at this early stage of root colonization.

In contrast to those of strain P1, hyphae of T22 were also observed on the main root (Fig. 3C). Only in a few cases strain T22 colonized the same niche as F.o.r.l. on the main root, i.e. the intercellular junctions (Fig. 3E, 3F and 3G). Consequently, competition for niches by T22 would mainly concern the initial sites of attachment.

Based on the root colonization behavior of the *Trichoderma* strains, P1 and to a lesser extent strain T22 must be considered as poor colonizers of tomato roots. Despite the differences in root colonization patterns observed when the *Trichoderma* strains are introduced as germinated spores or as mycelium, the reduction of root colonization by the pathogen is comparable. Based on these observations we conclude that it is unlikely that competition for niches plays a major role in the control of TFRR (Fig. 2) by the two *Trichoderma* spp.. Nevertheless, the growth of T22, and therefore its ability to compete for niches and nutrients, strongly depends on the nutrient and mineral composition of the media used in the gnotobiotic system.

Antibiosis and mycoparasitism

Some *Trichoderma* spp. are known to produce antibiotics which can inhibit fungal growth (Lorito et al., 1996; Schirmböck et al. 1994). However, the production of antibiotics by *T. atroviride* P1 and *T. harzianum* T22 has not been demonstrated. Also in our work we could not find indications that antibiosis is involved in the control of TFRR by either P1 or T22 (Bolwerk et al., *unpublished*).

Direct mycoparasitism of and attachment to fungal hyphae by *Trichoderma* spp. was till recently only demonstrated in dual cultures (Chet et al., 1981; Elad et al., 1983; Elad et al., 1987). Lu et al. (2004) were able to visualize growth of *T. atroviride* P1 towards and attachment to *P. ultimum* on cucumber roots. In our present work neither direct mycoparasitism nor attachment to *F.o.r.l.* hyphae was observed by either of the *Trichoderma* strains.

Plant defense responses

We observed that when seedlings were coated with either one of the two biocontrol agents *T. atroviride* P1 and *T. harzianum* T22 stimulated the development of a new root from the original seed-generated root, which did not grow further and developed a brown staining. Interestingly, D11 or P1ND1, the mutants of P1 impaired in the production of chitinases, did not cause these effects. Chitinases and glucanases are described as pathogenesis-related proteins in tomato (Duijff et al., 1998; Fuchs et al., 1997; Joosten and De Wit, 1988). Benhamou et al. (1990) studied tomato roots infected with *F.o.r.l.* and showed that chitinase accumulation was spatially related with pathogen infection. Possibly, chitinases and glucanases produced by *T. atroviride* P1 and *T. harzianum* T22 could trigger plant defense responses via a positive feedback mechanism or by acting as avirulence proteins (Harman et al., 2004; Lorito et al, *unpublished results*). Proteome analysis of P1 and T22 identified Avr homologues that together

with oligosaccharides, low molecular weight compounds and enzymes such as xylanase are known to induce resistance in plants (reviewed by Harman et al., 2004). The induction of plant defense mechanisms by T22 may be stimulated by the ability of the fungus to penetrate the root cortex (Fig. 3G and 3H).

Conclusions

In this work new results are reported that extend our understanding of the mechanisms involved in biocontrol of TFRR by strains *T. atroviride* P1 and *T. harzianum* T22. (i) Extra-cellular compounds produced by both strain P1 and T22 strongly reduce *F.o.r.l.* spore germination (Fig. 3). This could be an important mechanism of contribution to the suppression of hyphal growth by *Trichoderma* observed on tomato roots (Table 2), which results in the control of the disease (Fig. 1 and Fig. 2). The production of endo- and exochitinases by P1was shown to contribute to the inhibition of spore germination (Fig. 3), reduction of *F.o.r.l*. root colonization (Table 2), and disease control by the wild type strain P1 (Fig. 1 and Fig. 2). The glucanases and chitinases produced by T22 could play a role in the biocontrol of TFRR by *T. harzianum* T22 through inhibition of spore germination. (ii) The number of hyphae attaching to the root by T22 is strongly influenced by the presence and concentration of minerals and trace-elements. When T22 occupies more initial sites of attachment to the root surface it would be able to compete more effectively with the pathogen for niches. This observation may be relevant for its application as biofungicide. (iii) Mycoparasitism of the *Trichoderma* strains on *F.o.r.l.* on the tomato root is a rare event or does not occur at all. (iv) T22 and P1 are able to induce plant responses stimulating the growth of new roots and possibly induce plant resistance to phytopathogens.

Materials and Methods

Fungal isolates and inoculum production

F.o.r.l. strains were cultured on potato dextrose agar (PDA, Difco Laboratories, Detroit) for 7 days at 25ºC or in liquid Armstrong medium (Singleton et al., 1992) shaken at 130-160 rpm for 2 days at 28ºC. *Trichoderma* strains were grown on synthetic medium (SM, Lorito et al., 1994) without colloidal chitin, which was solidified with 1.8% agar when appropriate. *Trichoderma* spores were collected in water from PDA plates on which *Trichoderma* was inoculated and incubated for three days in the dark followed by three days with 10 hours of light per day at 25ºC. The spore suspension was filtered through two layers of Miracloth. *Trichoderma* mycelium (of strain P1 and T22) was collected from SM cultures that were grown for three days at 150 rpm at 25ºC and filtered over one layer of Miracloth. To collect sufficient mycelium of the chitinases mutants D11, P1ND1 and the wild type strain P1(as a control) were grown in potato dextrose broth (PDB, Difco Laboratories, Detroit) for three days at 150 rpm at 25ºC and filtered over one layer of Miracloth.

Control of TFRR in potting soil

Tomato seeds (kindly provided by Dr. Rudy Scheffer, Syntenga, Enkhuizen, The Netherlands) were coated with either methyl cellulose or methyl cellulose containing 10^9 *Trichoderma* spores/ml. *F.o.r.l.* spores were isolated as described by Lagopodi et al. (2002). Tomato seeds were placed in non-sterile potting soil infested with *F.o.r.l*. spores (5 x 10^6 spores/l soil). For each strain, ninety-six plants were tested in eight trays of twelve plants. The plants were grown for three weeks in a day-light greenhouse, in which the temperature varied between 20 ºC and 26 ºC. Roots were examined by eye for root and foot rot symptoms, e.g. the presence of rotting and lesions. Roots lacking any symptoms were classified as healthy.

Spore germination

Spores of *F.o.r.l.* were incubated in filter sterilized (product information on filters) culture supernatants of *Trichoderma* strains grown in SM at 25ºC for 48 hours. The reaction volume was 500 µl and the final concentration of spores was 4 x 10^5 per ml. After overnight incubation at 25 ºC the number of germinated spores and the total number of spores were counted by using a heamatocytometer and the percentage of germinated spores was calculated. The germination experiments were carried out in triplicate and were performed at least twice.

Control of TFRR in the gnotobiotic system

Tomato seeds were sterilized (Simons et al., 1996) and incubated at 4 ºC for 5 days on plant nutrient solution (PNS) (Hoffland, 1989) solidified with 1.8% agar. The seeds were incubated for 2 days at 28ºC to allow germination. The composition of PNS is as follows: per liter 1.18 g $Ca(NO_3)_2 \cdot 4\ H_2O$; 0.50 g KNO_3 , 0.48g $MgSO_4 \cdot 7\ H_2O$, 1ml B5 solution (containing per liter 0,75 mg KI, 3.0 mg H_3BO_3, 10 mg $MnSO_4 \cdot H_2O$, 2 mg $ZnSO_4 \cdot 5\ H_2O$, 0.25 mg $Na_2MoO_4 \cdot 2\ H_2O$, 0.025 mg $CuSO_4 \cdot 5\ H_2O$ and 0.025 mg $CoCl_2 \cdot 6\ H_2O$), 1/100 V/V 100 mM KH_2PO_4 are dissolved in of water. The composition of HPS (Yedidia et al., 1999) is as follows: 0.24 g MgSO4·7 H2O, 0.17 g K2SO4, 0.04 g KH2PO4, 0.344 g CaSO4 ·2 H2O, 0.64 g NH4NO3, 1 ml of a microelements solution (containing per liter 0.05g FeCl3, 0.728 g KCl, 1.546 g H3BO3, 0.846 g MnSO4·H2O, 0.375 g ZnSO4·7 H2O, 0.125 g CuSO4·5 H2O, 0.81 g H2MoO4 and 0.001g CoCl2·6 H2O) per liter of water.

The biocontrol experiments were performed in a gnotobiotic quartz sand system (Simons et al., 1996). The sterile glass tubes were filled with sand moisturized with PNS (10% v/w) plus sucrose (0.01%) to stimulate growth of *Trichoderma*. The spores were mixed with quartz sand to a concentration of 5x10^3 spores/kg sand. The concentration of *F.o.r.l.* spores was ten times reduced compared to previous experiments (Bolwerk et al, 2003; Bolwerk et al, *submitted*), in which no sucrose was added, to obtain a sufficient disease pressure. Sand was infested with mycelium of *Trichoderma* (2g/ kg sand) or *Trichoderma* was introduced by coating tomato seedlings with germinated spores. Coating was done by incubating the seedlings for 1 hour in a suspension of 10 ml SM with *Trichoderma* spores (10^7 spores/ml), which had been allowed to germinate for 3

days at 25ºC. Subsequently, seedlings were placed 5 mm below the surface of the sand and were grown in a climate-controlled growth chamber at 21ºC, 40% relative humidity and 16 hours of light per day. Seventeen to twenty seedlings were grown per treatment. After seven days of growth, the plants were scored as healthy or diseased (with symptoms varying from pin-point size brown spots to dead plants).

Confocal laser scanning microscopic analysis of tomato roots

After growth in the gnotobiotic system, tomato roots were carefully taken out of the sand and gently swirled a few times in sterile water in order to wash away the sand particles. Whole root systems were placed directly on glass slides in drops of water and examined by using an inverted fluorescence microscope (DMIRBE; Leica, Bensheim, Germany) equipped with filter blocks with spectral properties matching those of ECFP, (440/21-nm excitation with 480/36-nm emission; XF114, Chroma, Brattleboro, VT, U.S.A.) or EGFP (470/20-nm excitation with 515-nm long pass emission; I3, Leica, Bensheim, Germany), to which the Leica SP scanhead was attached. A Zeiss Axioplan epifluorescence microscope (Zeiss, Mannheim, Germany) coupled to a Biorad 1024 confocal system (Biorad, Hemel Hempstedd, UK) was used to obtain images with a Kr/Ar laser with excitation 488- emission 522/35 nm for EGFP and with excitation 568-585 nm long pass emission for the auto-fluorescence of the plant. The projections of the individual channels were merged in Photoshop 7.0 (Adobe, San Jose, CA, U.S.A.) to facilitate visualization.

Analysis of tomato root colonization by the pathogen

To determine the density of the tomato root colonization by *F.o.r.l.* and to quantify the surface area colonized by *F.o.r.l.* four tomato root systems with comparable sizes per treatment were analyzed in detail. Root cells colonized by *Fusarium* hyphae was examined and classified as one of four different stages of root colonization defined as following: (i) 'attachment' to root hairs; (ii) growth along one or two adjacent plant cells on the main root, referred to as 'start colonization'; (iii) growth along three or more adjacent cells in length, referred to as 'colonization'; and (iv) dense colonization over the total width of the root surface, referred to as 'heavy colonization'. By using this classification, colonization by *F.o.r.l* could be quantified and statistically analyzed. These stages occur in chronological order and with increasing intensity of root colonization from (i) to (iv). Each root was examined from the crown till the root tip (lengths-axis) for each one of the four stages of root colonization by *F.o.r.l*. using CLSM. The number of tomato root cells colonized in the length-axes was counted. When five cells in the width-axes on the same length-axis position were colonized it was counted as one cell in the length axis. In case these five cells in the width-axis were colonized by the pathogen in more than one of the four defined stages (for example 'attachment' and 'colonization') the most progressed stage was scored (in this example 'colonization'). The total number of root cells colonized per stage in the absence and in the presence of the *Trichoderma* biocontrol strains P1 and T22 were compared. Each experiment was performed at least twice.

Statistical analysis

Data of the biocontrol experiments in potting soil were statistically analyzed as described previously (Chin-A-Woeng et al., 2000) using a variance analysis followed by Fisher's least-significant-difference test ($P<0.05$), which was performed with SPPS software (SPPS Inc., Chicago, Il., U.S.A.).

Data of the biocontrol experiments in the gnotobiotic system were statistically analyzed by using a chi-squared goodness-of-fit test (Heath, 1995) as described before (Bolwerk et al., 2003). The null-hypothesis was defined as the lack of significant difference between two conditions tested. To test the null-hypothesis the X_2 value was calculated for the two conditions. In case the calculated X_2 value was lower than the critical X_2 value, the null-hypothesis was accepted e.g. the two treatments were not significantly different.

The quantification of tomato root colonization was performed by counting the number of plant cells colonized by the fungi (see above). A Wilcoxon-Mann-Withney U-test (Sokal and Rohlf, 1981) was used to determine whether the reduction in root colonization by *F.o.r.l.* due to the presence of the *Trichoderma* strains was significant.

Acknowledgements

We thank S. Woo for the supply of and information on the *Trichoderma* strains T22, P1 and mutants D11 and P1ND1. A. Bolwerk was supported by a grant of the NWO Earth and Life Sciences Council, project no. 810.35.003. This study was supported by grants to M. Lorito by FIRB-MIUR 2002; PON-MIUR 2002; EU TRICHOEST; EU 2E-BCAs, EU FAIR 98PL-4140; MIUR-MIPAF 2003; MIUR PRIN 2004.

Chapter 5

Summary

The plant rhizosphere

Organic compounds are exuded by the plant root in the rhizosphere and can be used as nutrient sources by micro-organisms inhabiting this area. The microbial community in the rhizosphere includes viruses, bacteria, mycorrhizae and other fungi. In addition, nematodes and protozoa can be present. The composition of the microflora and microfauna differs (i) from one soil type to another, (ii) from one plant species to another and (iii) from the surrounding soil where less nutrients are available. The composition of the root exudate is influenced by multiple factors such as the plant species, root region, abiotic and biotic factors of the surrounding soil. Major soluble components of tomato root exudate have recently been described by Lugtenberg and Bloemberg (2004) and contains organic acids, sugars and amino acids. Citric acid (133 μM) and glucose (20 μM) are the main organic acid and sugar, respectively.

The outcome of the interactions between micro-organisms present in the rhizosphere and the plant root can be divided into different classes: deleterious, neutral and beneficial (Lynch, 1990). Here we focus on the interactions between the plant root with bacteria and fungi. Deleterious interactions involve plant pathogens causing diseases, which is a serious problem in crop production (James, 1981). Beneficial interactions involve mycorrhizae and plant growth promoting microbes. The latter organisms can (a) increase the availability of nutrients for the plants (biofertilization) (Reid, 1990; Okon et al., 1997), (b) promote plant growth by the production of the phytohormone auxin by micro-organisms (phytostimulation) (Schippers et al., 1987 and references therein), (c) degrade pollutants in the rhizosphere (rhizoremediation) (Anderson, et al., 1993; Kuiper et al., 2004; Schwab and Banks, 1994), and (d) suppress plant diseases, a process which is called biocontrol (Burr et al., 1978; Geels and Schippers, 1983; Schippers et al., 1995; Suslow and Schroth, 1982).

Control of plant diseases

Diseases in agricultural crops severely affect the yield and this has serious consequences for the product quality, world food supply, prices and economy. Obviously, loss of crop production may have negative economic consequences for both the growers and the retail industry. And since the human population grows strongly, the demand for food is constantly increasing. These factors contributed to the battle against plant diseases, using different agricultural practices and/or pesticides.

Cultural practices involve soil tillage, crop rotation, planting date, heat treatment and the removal of diseased plants in order to create unfavorable conditions for disease development. However, most of these methods are labor-intensive and therefore economically unattractive.

The use of chemical pesticides is widespread and intensive (Cook, 2000). Although many of these pesticides are effective, they are unspecific in the sense that they often negatively affect the surrounding environment. Additionally, they

can have deleterious effects on human health. These risks increased the concern among the human population.

The use of biological pesticides is a promising alternative for the use of the chemical pesticides. In the U.S.A. alone, over one hundred products have been registered. In Europe, registration is more difficult, especially because of the lack of transparent criteria. Disease control by the application of micro-organisms is established by attacking the pathogen or by interfering with the growth of the pathogen or with its disease development. Biocontrol organisms are usually isolated from naturally suppressive soils and also from plant roots of healthy plants growing is such soils (Montesinos, 2003). Since biocontrol bacteria and fungi are natural enemies, these organisms can proliferate in the plant rhizosphere and their effect on the surrounding environment is minimal.

As mentioned previously, many biological products are commercially available for crop protection (Fravel and Larkin, 1996). The application of biocontrol agents is not always effective (Koch, 1999). To improve the consistency of biocontrol, an extensive fundamental knowledge on the interactions between the plant root, pathogen and biocontrol agent is needed. Understanding the mechanisms involved in disease control, one is able to optimize the conditions and the (commercial) product for efficient biocontrol, both in time and place.

The development and application of molecular and biochemical methods has led to the identification of genes and traits involved in the biocontrol by various organisms. The use of autofluorescent proteins (AFPs) and confocal laser scanning microscopy (CLSM) allowed non-destructive, *in situ* studies on root colonization by *Pseudomonas* biocontrol bacteria (Bloemberg et al., 1997) as well as on colonization and infection by the plant pathogen *F.o.r.l.* (Lagopodi et al., 2002). The use of different AFPs allowed simultaneous visualization of up to three different *Pseudomonas* bacteria (Bloemberg et al., 2000).

Commercial application of biocontrol agents

The selection of a suitable biocontrol agent involves different aspects (Koch, 1999; Montesinos, 2003; Spadaro and Gullino, 2004) that can be divided into the following classes (i) biotic, (ii) abiotic, (iii) production and formulation, (iv) legal, and (v) social.

(i) Depending on the plant species and the pathogen to be controlled a first selection of a potential biocontrol agent can be made. The efficacy of biocontrol by this agent is determined by (a) the biocontrol traits of the agents that contribute to the disease control, (b) the interactions between the plant root, pathogen and biocontrol agent and (c) the ecology of a biocontrol agent with respect to its survival and fitness in different growth substrates and conditions. (ii) The latter indicates the influence of abiotic factors of the growth system such as potting substrate, temperature, pH and water content. To be commercially applicable, the agent should be (iii) produced at a large scale and subsequently formulated. The mass production via fermentation should be rapid, efficient and inexpensive. A long shelf-live of the product will make it economically more

attractive. (iv) The government should clear directions and criteria for registration allows the use of the product. (v) Finally, perception of growth and consumers strongly influences the use of the product. To this end the product should be effective and safe.

Biocontrol of tomato foot and root rot (TFRR)

Known mechanisms of biocontrol are (i) competition for niches and nutrients, (ii) antibiosis, (iii) parasitism and predation, and (iv) induced systemic resistance. Depending on the biocontrol agent, pathogen, plant species, abiotic and biotic features of the soil, the contribution of these mechanisms is likely to differ.

In this thesis we analyzed the effect of different biocontrol agents on the pathogen *F.o.r.l.* which causes foot and root rot of tomato. Disease control can be studied in a gnotobiotic sand system described by Simons et al. (1996). Previously, this system was shown to be useful to visualize *Pseudomonas* biocontrol bacteria (Bloemberg et al., 1997; Bloemberg et al., 2000) or the pathogen *F.o.r.l.* (Lagopodi et al., 2002), labeled with AFP markers. Quartz sand was used because it has the advantage that it can be easily removed from the roots by gentle washing, after which the roots can be examined using CLSM. In contrast, the removal of soil from the root is difficult and subsequent microscopy studies are hampered due to the autofluorescence of the soil particles.

Using CLSM and combining different AFPs allowed simultaneous visualization of both the pathogen and biocontrol agent on the tomato root under disease controlling conditions in the gnotobiotic system. The biocontrol strains tested belong to the genera *Pseudomonas*, *Fusarium oxysporum* and *Trichoderma*. For these analyses tomato seedlings were grown for seven days in the gnotobiotic sand system of which the sand was infested with *F.o.r.l..* The biocontrol strains were introduced in different ways: (i) germinated tomato seeds were incubated in either a bacterial or germinated spores (*Trichoderma* spp.) suspension and subsequently sown or (ii) germinated tomato seeds were sown in sand infested with spores of the non-pathogenic *F. oxysporum* or with mycelium of the *Trichoderma* spp. in addition to *F.o.r.l.*.

To obtain more insight in the effect of the biocontrol strains on the stage of spore germination by the pathogen, spore germination by *F.o.r.l.* was studied in an *in vitro* setup. Spore germination was analyzed in tomato root exudate and in culture supernatants of the biocontrol strains. These *in vivo* and *in vitro* studies contributed to our understanding of how disease control was established by the above-mentioned biocontrol agents, which is schematically presented in Table 1.

Mechanisms involved in the biocontrol of TFRR by *Pseudomonas* spp.

P. chlororaphis PCL1391 produces the antifungal metabolite (AFM) phenazine-1-carboxamide (PCN). Analysis of the PCN-biosynthetic mutant *P. chlororaphis* PCL1119 indicated that the production of PCN is required for biocontrol of TFRR in potting soil (Chin-A-Woeng et al., 1998; Table 1). In chapter 2 we reported the analysis of interactions of the pathogen with strain PCL1391, PCL1119 or with purified PCN at the cellular level, which indicated that PCN caused stress effects within the pathogen both on plate and in the tomato rhizosphere, affecting hyphal morphology (Fig. 2C and 3D), growth directionality (Chapter 2, Fig. 2D, 2E, 3E and 3H) and branching (Chapter 2, Fig. 2F, 3F and 3G).

Previously, Chin-A-Woeng and colleagues (2000) demonstrated that efficient colonization of the tomato root system is also essential for suppression of the disease by this strain PCL1391 (Table 1). Root colonization is considered to be the delivery system for AFMs, thereby inhibiting the pathogen over the total root. In contrast to strain PCL1391, it was shown that extensive root colonization for *P. fluorescens* WCS365 is not essential for biocontrol (Dekkers et al., 2000; Table 1). Apparently for WCS365 presence at the top part of the root is sufficient to cause biocontrol. Gerrits and Weisbeek (1996) showed that strain WCS365 triggers induced systemic resistance (ISR) in *Arabidopsis thaliana.* Therefore, ISR is thought to be involved in the control of TFRR by *P. fluorescens* WCS365 (Table 1).

Cells of both strains PCL1391 and WCS365 colonize the root faster than the pathogen does (Chapter 2) and they occupy the same main niche as *F.o.r.l.*, i.e. the cellular junctions of the tomato root cells (Chapter 2, Fig. 1A and 1B), where root exudate is thought to be excreted (Chin-A-Woeng et al., 1997; Lagopodi et al., 2002; Chapter 2). It is likely that as a consequence, the *Pseudomonas* bacteria can effectively compete for both niches and nutrients (Table 1), which results in a reduction of root colonization of *F.o.r.l.* by up to 80% (Chapter 2, compare Fig. 1C with 1D, 1E or 1F).

It appeared that in addition to root colonization, cells of *Pseudomonas* strains PCL1391 and WCS365 are able to colonize hyphae (Chapter 2, Fig. 2A and 2B). Several pilot experiments indicated that these *Pseudomonas* biocontrol bacteria could grow on the exudates and culture supernatants of *F.o.r.l.* (de Weert and Kamilova, *personal communication*). Consequently, bacteria that attach to the hyphae (Chapter 2, Fig. 1H) may feed on the hyphae, which will result in the observed extensive hyphal colonization (Chapter 2, Fig. 2A). By feeding of the bacteria on the hyphae, the pathogenicity of the fungus is likely to be negatively affected, this parasitism might therefore be a new mechanism of biocontrol.

Compared to strain PCL1391, its mutants PCL1119 caused similar stress effects within the pathogen (Chapter 2, Fig. 2G and 2H) in the gnotobiotic system, although with a delay of three days (Chapter 2, Table 5). We therefore hypothesize that the production of extra-cellular metabolites other than PCN, such as chitinase, hydrogen cyanide and protease (Chin-A-Woeng et al., 1998), can cause stress effects as well and that PCN accelerates the occurrence of these stress effects within the pathogen (Chapter 2). Additionally, the production of chitinase and

protease enables strain PCL1391 to attack the cell wall of the fungus and subsequently utilize the released compounds.

Analysis of germination of *F.o.r.l.* spores in the culture supernatants of *P. chlororaphis* PCL1391 and the GacS mutant (PCL1123) showed that secondary metabolites produced by strain PCL1391 can limit spore germination by the pathogen in an *in vitro* setup (Table 1). Reduction of spore germination by *P. fluorescens* WCS365 could only be observed in minimal medium and was less strong than the inhibition by *P. chlororaphis* PCL1391. It is likely these compounds limit spore germination in the gnotobiotic sand system as well and could contribute to the control of TFRR (Table 1).

Mechanisms involved in the biocontrol of TFRR by *Fusarium oxysporum* Fo47

Temporal analysis of spore germination in tomato root exudate indicated that a higher percentage of Fo47 spores germinated compared to *F.o.r.l*. spores, both in solutions of the major exudate sugar (glucose) as well of its organic acid (citric acid) (Fig. 6). Consequently, Fo47 can start faster with the utilization of the nutrients and subsequent proliferation (Chapter 3 and Table 1). In addition, the inoculum concentration of Fo47 used for biocontrol is fifty times higher compared to *F.o.r.l.*. As a result Fo47 hyphae will reach the root earlier and in higher numbers compared to the pathogen, a prediction which was confirmed by the CLSM visualization studies (Chapter 3, Fig. 3).

Analyses of tomato root colonization and disease development of seedlings sown in sand infested with spores of both *F.o.r.l.* and Fo47 showed that an at least 50-fold excess of the biocontrol fungus over the pathogen was required to obtain control of TFRR. Root colonization by Fo47 and *F.o.r.l.* involves the same niches at the root (Chapter 3, Fig. 2A, 2C, 4A and 4B). Nevertheless, root colonization by Fo47 is slower, less aggressive and to a lesser extent than that of the pathogen despite its fifty-fold higher inoculum (Chapter 3, compare figure 2D with 4A). This high Fo47 inoculum is thought to be required to compensate for the difference in root colonization efficiency and would be necessary to allow Fo47 to effectively compete for niches and nutrients when both fungi reach the rhizoplane after spore germination in the rhizosphere (Table 1). This notion is confirmed by the observed decrease of root colonization by the pathogen at increasing concentrations of Fo47 (Chapter 3, Table 4). Our results indicate that the preferential spore germination and the higher inoculum concentration enables Fo47 to reduce both the severity and total root colonization by *F.o.r.l.* (Chapter 3, Fig. 5), thereby contributing to the disease control.

Analysis of tomato root colonization and disease development after coating seedlings with Fo47 showed that, despite the lack of distribution over the root Fo47, was able to reduce the disease incidence (Chapter 3). This suggested that competition for niches and nutrients plays a less profound role when Fo47 spores are coated on seedlings and that under these conditions another mechanism contributes to the reduction of diseased plants (Chapter 3). This

situation resembles a previous observation by Dekkers et al. (2000), namely that colonization mutants of *P. fluorescens* WCS365, a strain supposed to control TFRR via induced systemic resistance (ISR), still control TFRR. Our results with Fo47 therefore provide evidence that ISR contributes to the disease reduction by Fo47 (Table 1). Previously, Fuchs and colleagues (1997) illustrated the ability of strain Fo47 to protect tomato plants against *F. oxysporum f. sp. lycopersici* Fol8 when introduced separately in time or space, an observation which also indicated that *F. oxysporum* Fo47 is able to trigger ISR in tomato plants.

Mechanisms involved in the biocontrol of TFRR by *Trichoderma* spp.

Analysis of germination of *F.o.r.l.* spores in the culture supernatants of *T. harzianum* T22 and *T. atroviride* P1 showed that both strains can inhibit spore germination by the pathogen in an *in vitro* setup (Table 1 and Chapter 4, Fig. 4). *T. atroviride* P1 produces different enzymes such as glucanases and chitinases and mutant analysis showed that both the endochitinase and exochitinase contribute to the observed inhibition of spore germination. For strain T22 an endochitinase and a glucanase are likely to be involved in the inhibition of the germination of *F.o.r.l.* spores.

It is likely that the extracellular enzymes produced by strains P1 and T22 inhibit spore germination in the gnotobiotic sand system as well. As a consequence, the subsequent growth towards, and colonization of, the tomato root will be reduced. CLSM analysis confirmed this hypothesis. In the rhizosphere *T. harzianum* T22 and *T. atroviride* P1 were able to significantly reduce the severity and total root colonization by *F.o.r.l*. (Table 4). Analysis of the chitinase mutants of strain P1, showed the involvement of the endo- and exochitinase in the observed reduction of the pathogen (Chapter 4, Table 4).

The rhizosphere competence of strain T22 is strongly influenced by the composition of minerals and concentrations of trace elements present in the system (Chapter 4). Therefore, it is hypothesized that the ability of *T. harzianum* T22 to effectively compete for niches and nutrients strongly depends on the nutrient composition. In our studies, which take place in sand moisturized with PNS (Hoffland et al., 1989) containing 0.01% sucrose, the main niche occupied by P1 and T22 are the root hairs (Chapter 4, Fig. 4A through 4C) whereas growth on the main root was also observed in a few cases for strain T22 (Chapter 4, Fig. 4D through 4G). Since T22 and P1 are poor root colonizers in the gnotobiotic sand system used (Chapter 4, Fig. 4), competition for niches and nutrients is thought to play only a minor role in the observed control of TFRR by both T22 and P1 in this system (Chapter 4).

Upon seedling coating and subsequent growth in the gnotobiotic system, *T. atroviride* P1 and *T. harzianum* T22, were observed to stimulate the development of new roots. The endo- and exochitinase mutants, in contrast to the wild type strain P1, did not alter root formation. In tomato different pathogenesis-related proteins are described including chitinases and glucanases (Duijff et al., 1998; Fuchs et al., 1997; Joosten and De Wit, 1988). Additionally, chitinase

deposition in the plant root was spatially related with pathogen infection Benhamou et al. (1990). Since *T. atroviride* P1 and *T. harzianum* T22 produce chitinases and glucanases, we hypothesize that these extra-cellular enzymes could induce defense responses within the plant via a positive feedback mechanism or by acting as a-virulence proteins (Harman et al., 2004; Lorito et al, *unpublished results*). Avr homologues could be identified by proteome analysis within P1 and T22. Besides Avr homologues, oligosaccharides, low-molecular-weight compounds and enzymes such as xylanase are known to induce resistance in plants (reviewed by Harman et al., 2004). It is likely that the ability of strain T22 to penetrate the root cortex (Fig. 3G and 3H) supports this type of induction of disease resistance. The ability of *Trichoderma* spp. to induce a defense responses within tomato plants was described previously by De Meyer and colleagues (1998) who showed that *T. harzianum* T39, spatially separated from *Botrytis cinerea*, reduced disease symptoms in tomato plants.

Pathogen defense

Antibiosis is described for many biocontrol agents. Not surprisingly pathogens have developed defense strategies (reviewed by Duffy et al., 2003). (i) Different secondary metabolites of pathogens have been described that negatively affect the expression of biocontrol genes. Interactions between *F.o.r.l.* and *P. fluorescens* affected gene expression in the biocontrol agent *P. fluorescens*. Fusaric acid (FA), a secondary metabolite produced by *F.o.r.l.* was reported to repress 2,4 diacetylphloroglucinol (2,4-DAPG) biosynthesis (Duffy et al., 1997; Notz et al., 2002; Schnider-Keel et al., 2000). FA also reduces PCN production in *P. chlororaphis* PCL1391 under *in vitro* conditions (van Rij et al., 2004). Deoxynivalenol (DON), another secondary metabolite produced by different *Fusarium* strains, represses the expression of the exochitinase gene *nag1* in *Trichoderma atroviride* P1 (Lutz et al., 2003). (ii) Biodegradation (Mitchell et al., 2002) and detoxification can be other strategies to confer resistance. The detoxification by enzymatic degradation is described by Kinderlerer et al. (1993), by hydroxylation the toxicity of hydrocarbon compounds can be minimized. HCN tolerance can be established by detoxifyication through the conversion of HCN to formamide (Osbourn et al., 1996). (iii) Active efflux of antibiotics through membrane-bound pumps such as ABC transporters is another strategy of pathogens to reduce the inhibitory effects by the biocontrol strains (reviewed by Duffy, 2003). Schoonbeek and colleagues (2002) demonstrated the biological importance of an ABC transporter, which can be considered as a trait that confers multidrug resistance, in the sensitivity of *Botrytis cinerea* to antibiotics produced by *Pseudomonas* and its capability to cause disease symptoms.

Table 1. Mechanism involved in the biocontrol of TFRR by *Pseudomonas*, *F. oxysporum* and *Trichoderma spp.*[a]

Biocontrol agent / Mechanism	PCL1391	WCS365	Fo47	P1	T22
Inhibition of spore germination	+ [2]	+ [2]	- [3]	+ [4]	+ [4]
Competition for nutrients before reaching root			+ [3]	- [4]	- [4]
Competition for niches and nutrients in rhizoplane	+ 2	+ [2]	+ [3]	- [4]	- [4]
Antibiosis	+ [2, b]	- [2]	- [3]	- [4]	- [4]
Root colonization	+ [b]	- [d]	+ [3]	- [4]	- [4]
Hyphal colonization, parasitism	+ [2]	+ [2]	- [3]	- [4]	- [4]
Induction of plant defense responses	- [c]	+ [d]	+ [3, e]	+ [4]	+ [4]

[a] The possible mechanisms involved in the biocontrol of TFRR by *P. chlororaphis* PCL1391, *P. fluorescens* WCS365, *F. oxysporum* Fo47, *T. atroviride* P1 and *T. harzianum* T22 are presented in this table. The studies that contributed to the insight of the mechanisms involved are denoted between parentheses. These studies are described within the chapters of this thesis or in previous publications by others. Mechanisms contributing to the control of TFRR by the different biocontrol agents are denoted in black. Mechanisms that are not involved in the control of TFRR by the different biocontrol agents are denoted in gray. References: [b, c and d] Mutant analysis: Chin-A-Woeng et al., 1998 [b] and 2000 [c], [d] Dekkers et al., 2000; Duijf et al., 1998;Fuchs et al., 1997: [2, 3 and 4] Chapter within this thesis

Improving efficiency of biocontrol of TFRR

Fogliano and colleagues (2002) reported the biocontrol by *T. atroviride* P1 could be improved by the addition of *P. syringae* pv. *syringae* B359. The degrading enzymes produced by strain P1 were shown to act synergistically with the lipodepsopeptides produced by strain B359 in the control of a *Botrytis cinerea* on apple fruit. This study supports the concept of combining two different biocontrol agents to improve consistency is commercial products (Koch et al., 1999; Spadaro et al., 2004).

In case of the control of TFRR, efficiency can be improved by combining two agents described in this thesis, *P. fluorescens* WCS365 and *T. harzianum* T22. The advantage of using strain WCS365 is that its biocontrol activity is hardly dependent on mechanisms that can be negatively influenced by the system or the pathogen, such as extensive root colonization and antibiosis. *P. fluorescens* WCS365 is thought to trigger defense responses in the tomato plant. *Trichoderma* is thought to induce defense responses in the plant as well in addition to the potential direct action of *T. harzianum* T22 on spore germination of the pathogen as mechanism of control. In this way both the host and the pathogen are affected. In addition, *Trichoderma* species are already commercially used which would make registration of the cocktail less difficult. Moreover, strain WCS365 does not produce antibiotics, which makes registration easier.

Concluding remarks

The application of biocontrol agents is an attractive alternative for chemical control of plant diseases. Unfortunately, biological control is not always effective. To improve the consistency of biocontrol, an extensive fundamental knowledge on different aspects is needed. The visualization studies of the interactions between the plant root, the pathogen, and biocontrol agent deepened our insight on these aspects. (i) The ecology of a biocontrol agent with respect to its survival and fitness in different growth substrates and conditions. The development of *T. harzianum* T22 in the tomato rhizosphere was shown to be strongly dependent on the mineral composition of the gnotobiotic system (Chapter 4). (ii) The biocontrol traits of the agents that contribute to the disease control. Our studies indicated that hyphal colonization and parasitism by *Pseudomonas* may represent a new mechanism of biocontrol by these bacteria (Chapter 2). Moreover, the preferential spore germination by the non-pathogenic *F. oxysporum* Fo47 strongly contributes to the control of TFRR (Chapter 3). Inhibition of spore germination by *F.o.r.l.* due to the production of extra-cellular enzymes is considered as the main mechanism of biocontrol by both *Trichoderma* strains analyzed (Chapter 4). (iii) The interactions between the plant root, the pathogen and biocontrol agent. The visualization studies on the interactions in the rhizosphere described in this thesis, contributed to a better understanding of how the biocontrol agents affect the pathogen, *F.o.r.l.*. *P. fluorescens* WCS365, *P. chlororaphis* PCL1391 and *F. oxysporum* Fo47 reduce the severity and total root colonization by *F.o.r.l.* through the competition for niches and nutrients and preferential spore germination in case of Fo47 (Chapters 2, 3 and 4). *P. chlororaphis* PCL1391 causes different stress effects within *F.o.r.l.* during root colonization through the production of PCN. Both *Trichoderma* strains T22 and P1 reduce the severity and total root colonization by *F.o.r.l.* by inhibiting spore germination and possibly hyphal growth through the production of extracellular enzymes such as chitinase and glucanases. The inhibition of spore germination could also be a mechanism of biocontrol by the *Pseudomonas* strains WCS365 and PCL1391.

Future perspectives

The complexity of the interactions between the biocontrol agent and the pathogen influence the efficacy of biological control of plant diseases. Analysis of the gene expression in the plant, the pathogen and the biocontrol bacterium in presence of each other on the tomato root will provide more information on the regulation of pathogenicity and biocontrol traits and of the reaction of the plant. To explore the role of hyphal colonization in the control of TFRR by *Pseudomonas* bacteria, attachment to- and subsequent colonization of- the hyphae should be studied in more detail, focussing on gene expression profiles, stress responses of the pathogen and biocontrol ability of the bacteria. The development of an ISR-test system for tomato will contribute to a broader screening potential of biocontrol agents of TFRR.

References

Alabouvette, C. and Couteaudier, Y. 1992. Biological control of Fusarium wilts with non-pathogenic *Fusaria*. In: biological control of plant diseases, Plenum press, New York. 415-426.

Alabouvette, C., Lemanceau, P., and Steinberg, C. 1993. Recent advances in biological control of Fusarium wilts. Pestic. Sci. 37: 365-373.

Altomare, C. Norvell, W. A., Björkman, T., and Harman, G. E. 1999. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. Appl. Env. Microbiol. 65: 2926-2933.

Anderson, T. A., Guthrie, E. A., and Walton, B. T. 1993. Bioremediation in the rhizosphere. Environ. Sci. Technol. 27: 2630-2636.

Armstrong, G. M. and Armstrong, J. K. 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. In: Nelson, P. E., Tousson, T. A., Cook, R. J. (eds.) *Fusarium*: disease, biology and taxonomy. University Park, PA, USA: State University Press, 391-399.

Bao, J. R., Velema, J., Dobinson, K. F., and Lazarovits, G. 2000. Using GUS expression in a nonpathogenic *Fusarium oxysporum* to measure fungal biomass. Can. J. Plant Pathol. 22: 70-78.

Bao, J. R. and Lazarovits, G. 2001. Differential colonization of tomato roots by nonpathogenic and pathogenic *Fusarium oxysporum* strains may influence Fusarium wilt control. Phytopathology 91: 449-456.

Benhamou, N., Joosten, M. H. A. J., De Wit, P. J. G. M. 1990. Subcellular localization of chitinase and its potential substrate in tomato root tissue infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Plant Physiol. 92: 1108-1120.

Benhamou, N., Chet, I. 1993. Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*: Ultrastructure and gold cytochemistry of the mycoparasitic process. Phytopathology 83:1062-1071.

Benhamou, N., Lafontaine, P. J., and Nicole, M. 1994. Induction of systemic resistance to *Fusarium* crown and root rot in tomato plants treated with chitosan. Phytopathology 84: 1432-1444.

Benhamou, N., Bélanger, R. R., and Paulitz, T. C. 1996. Pre-inoculation of Ri T-DNA-transformed pea roots with *Pseudomonas fluorescens* inhibits colonization by *Phytium ultimum* Trow: an ultrastructural and cytochemical study. Planta 199: 105-117.

Benhamou, N., Rey, P., Chérif, M., Hockenhull, J., and Tirilly, Y. 1997. Treatment with the mycoparasite *Pythium oligandrum* triggers induction of defense-related reactions in tomato roots when challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Phytopathology 87: 108-122.

Benhamou, N., Rey, P., Picard, K., and Tirilly, Y. 1999. Ultrastructural and cytochemical aspects of the interaction between the mycoparasite *Pythium oligandrum* and soil born plant pathogens. Phytopathology 87: 108-122.

Benhamou, N. and Garand, C. 2001. Cytological analysis of defense-related mechanisms induced in pea root tissues in response to colonization by non-pathogenic *Fusarium oxysporum* Fo47. Phytopathology. 91: 730-740.

Bloemberg, G. V., O'Toole, G. A., Lugtenberg, B. J. J., and Kolter, R. 1997. Green Fluorescent Protein as a marker for *Pseudomonas* spp. Appl. Environ. Microbiol. 63: 4543-4551.

Bloemberg, G. V., Wijfjes, A. H. M., Lamers, G. E. M., Stuurman, N., and Lugtenberg, B. J. J. 2000. Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. Mol. Plant-Microbe Interact. 13: 1170-1176.

Bloemberg, G. V. and Lugtenberg, B. J. J. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. 4: 343-350.

Bolwerk, A., Lagopodi, A. L., Wijfjes, A. H. M., Lamers, G. E. M., Chin-A-Woeng, T. F. C., Lugtenberg, B. J. J., and Bloemberg, G. V. 2003. Interactions in the tomato rhizosphere of two *Pseudomonas* biocontrol strains with the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Mol. Plant-Microbe Interact. 11: 983-993.

Bowen, G. D.and Rovira, A. D. 1976. Microbial colonization of plant roots. Ann. Rev. Phytopathol.. 14: 121-144.

Brammall, R. A. and Higgins, V. J. 1988. A histological comparison of fungal colonization in tomato seedlings susceptible or resistant to *Fusarium* crown and root rot disease. Can. J. Bot. 66: 915-925.

Brayford, D. 1996. *Fusarium oxysporum* f. sp. *radicis-lycopersici*, IMI descriptions of fungi and bacteria no. 1270. Mycopathologica 133: 61-63.

Brunner, K., Peterbauer, C. K., Mach, R. L., Lorito, M., Zeilinger, S., and Kubicek, C. P. 2003. The Nag1 *N*-acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinases induction by chitin and of major relevance to biocontrol. Cur.. Genet. 43: 289-295.

Burr T. J., Schroth, M., N., and Suslow, T. 1978. Increased potato yields on treatment of seed pecies with strains of *Pseudomonas fluorescens* and *P. putida*. Phytopathology, 68: 1377-1383.

Campbell, R. and Greaves, M. P. 1990. Anatomy and community structure of the rhizosphere. Pages 11-34 in: The rhizosphere. J. M. Lynch, ed. John Wiley & Sons, West Sussex, England.

Carsolio, C., Gutierrez, A., Jimenez, B., Van Montagu, M., and Herrera-Estrella, A. 1994. Primary structure and expression pattern of the 33-kDa chitinase gene from the mycoparasytic fungus *Trichoderma harzianum*. Proc. Natl. Acad. Sci. USA 91: 10903-10907.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. Science 263: 802-805.

Charest, P. M., Quelette, G. B., and Pauze, F. J. 1984. Cytological observations of early infection process by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants. Can. J. Bot. 62: 1232-1244.

Chatterjee, A., Cui, Y., Lui, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Inactivation of *rsmA* leads to overproduction of of extracellular pectinases, cellulases and proteases in *Erwinia carotovora* subs. *carotovora* in the absence of the starvation/cell density-sensing signal, N-(3-oxohexanyol)-L-homoserine lactone. Appl. Environ. Microbiol. 61: 1959-1967.

Chet, I. 1987. *Trichoderma*: application, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi, p. 137-160. *In* I. Chet (ed.) Innovative approaches to plant disease control. Wiley, New York, N. Y.

Chet, I., Harman, G. E., and Baker, R. 1981. *Trichoderma hamatum*: Its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. Microb. Ecol. 7:29-38.

Chin-A-Woeng, T. F. C., de Priester, W., van der Bij, A. J., and Lugtenberg, B. J. J. 1997. Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. Mol. Plant-Microbe Interact. 10: 79-86.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., van der Bij, A. J., van der Drift, K. M. G. M., Schripsema, J., Kroon, B., Scheffer, R. J., Keel, C., Bakker, P. A. H. M., Tichy, H.-V., de Bruijn, F. J., Thomas-Oates, J. E., and Lugtenberg B. J. J. 1998. Biocontol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Mol. Plant-Microbe Interact. 11: 1069-1077.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., Mulders, I. H. M., Dekkers, L. C., and Lugtenberg B. J. J. 2000. Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato root rot. Mol. Plant-Microbe Interact. 12: 1340-1345.

Cook, R. J. (2000). Advances in plant health management in the 20th century. Annu. Rev. Phytopathol. 38: 95-116.

Cortes, C., Gutierrez, A., Olmedo, V., Inbar, J., Chet, I., and Herrera-Estrella. 1998. The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. Mol. Gen. Genet. 260: 218-225

Couteaudier, Y., and Alabouvette, C. 1990. Quantitative comparison of *Fusarium oxysporum* competitiveness in relation to carbon utilization. FEMS Microbiol. Ecol. 74: 261-268.

Couteaudier, Y. 1992. Competition for carbon in soil and rhizosphere, a mechanism involved in biological control of Fusarium wilts. In: biological control of plant diseases, Plenum press, New York. 99-104.

Datnoff, L. E., Nemec, S., and Pernezny, K. 1995. Biological control of Fusarium crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices.* Biol. Control. 5: 427-431.

Dekkers, L. C., Mulders, I. H. M., Phoelich, C. C., Chin-A-Woeng, T. F. C., Wijfjes, A. H. M., and Lugtenberg B. J. J. 2000. The *sss* colonization gene of the tomato- *Fusarium oxysporum* f. sp. *radicis-lycopersici* biocontrol strain *Pseudomonas fluorescens* WCS365 can improve colonization of other wild-type *Pseudomonas* spp. bacteria. Mol. Plant-Microbe Interact. 13: 1177-1183.

De La Cruz, J. A., Hidalgo-Gallego, A., Lora, J. M., Benitez, T., Pintor-Toro, J. A., and Llobell, A. 1992. Isolation and characterization of three chitinases from *Trichoderma harzianum*. Eur. J. Biochem. 206: 859-867.

De Meyer, G., Bigirimana, J., Elad, Y. and Höfte, M. 1998. Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinearea*. Eur. J. Plant Pathol. 104: 279-286.

De Weert, S., Vermeiren, H., Mulders, I. H. M., Kuiper, I., Hendrickx, N., Bloemberg, G. V., Vanderleyden, J., De Mot , R., and Lugtenberg , B. J. J. 2002. Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens.* Mol. Plant-Microbe Interact. 15: 1173-1180.

Di Pietro, A. Lorito, M., Hayes, C. K., Broadway, R.M. and Harman, G. E. 1993. Endochitinase from *Gliocladium virens*: isolation, characterisation, and synergistic antifungal activity in combination with gliotoxin. Phytopathology 83: 313-318.

Dowling, D. N. and O'Gara, F. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. TIBTECH 12: 133-141.

Dubos,B., Jailloux, F. and Bulit, J. 1982. Protection du vignoble contre la pourriture grise:les propriétés antagonistes du *Trichoderma* a l'égard du *Botrytis cinerea*.Les colloques de l'ÍNRA. 11: 205-219.

Duijff, B. J., Pouhair, D., Olivain, C., Alabouvette, C., and Lemanceau, P. 1998. Implication of systemic induced resistance in the suppression of Fusarium wilt of tomato by *Pseudomonas fluorescens* WCS417r and by nonpathogenic *Fusarium oxysporum* Fo47. Eur. J. Plant Pathol. 104: 903-910.

Duffy, B. K., Simon, A., and Weller, D. M. 1996. Combination of *Trichoderma koningii* with fluorescent Pseudomonads for control of take-all on wheat. Phytopathology. 86: 188-194.

Duffy, B. K. and Défago, G. 1997. A *Fusarium* pathogenicity factor blocks antibiotic biosythesis by Pseudomonas fluorescens biocontrol strains. Phytopathology 87: S2.

Dunlap C., Delaney, I., Fenton, A., Lohrke, S., Moënne-Loccoz, Y., and O'Gara, F. 1996. The biotechnology and application of *Pseudomonas* inoculants for the biocontrol of phytopathogens, pages 441-448. In: Stacey, G., Mullin, B., Gresshoff, P. M. (eds.) Biology of plant microbe interactions. St Paul, MN, USA: International Society for Molecular Plant-Microbe Interactions.

Dunlap I., Crowley, J. J., Moenneloccoz, Y., Dowling, D. N., de Bruijn, F. J., and O'Gara, F. 1997. Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilla* W81 is mediated by an extracellular proteolytic activity. Microbiology UK 143 Part 12: 3912-3931.

Dunne, C. I., Delaney, I., Fenton, A., Lohrke, S., Moënne-Loccoz, Y., and O'Gara, F. 1996a. The biotechnology and application of *Pseudomonas* inoculants for the biocontrol of phytopathogens, p. 441-448. In: Stacey, G., Mullin, B., and Gresshoff, P. M. (eds.), Biology of Plant-Microbe Interactions. International Society for Molecular Plant-Microbe Interactions, St. Paul.

Dunne, C. I., Delaney, I., Fenton, A., and O'Gara, F. 1996b. Mechanisms involved in biocontrol by microbial inoculants. Agronomie 16: 721-729.

Dunne, C. I., Moënne, Y., McCarthy, J., Higgins, P., Powell, J., Dowling, D., and O'Gara, F. 1998. Combining proteolytic and phloroglucinol-producing bacteria for improved biocontrol of Pythium-mediated damping-off of sugar beet. Plant Pathology 47: 229-307.

Elad, Y., Chet, I., Boyle, P., and Y. Henis. 1983. Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii*-scanning electron microscopy and fluorescence microscopy Fungi, structure, soilborne plant pathogens, biological control. Phytopathology. 73:85-88.

Elad, Y., Sadowsky, Z., and Chet, I. 1987. Scanning electron microscopical observations of early stages of interaction of *Trichoderma harzianum* and *Rhizoctonia solani*. Trans. Br. Mycol. Soc. 88: 259-263.

El-Katatny, M. H., Somitsch, W., Robra, K.-H., El-Katatny, M. S. and Gübitz, G. M. 2000. Production of chitinase and –1,3-glucanase by *Trichoderma harzianum* for control of the phytopathogenis fungus *Sclerotium rolfsii*. Food Technol. Bioctechnol. 34: 173-180.

Ellenberg, J., Lippincott, S.J., and Presley, J. F. 1999. Dual-color imaging with GFP variants. Trends Cell Biol. 9: 52-56.

Emmert, E. A. B. and Handelsman, J. 1999. Biocontrol of plant disease: a (Gram-) positive perspective. FEMS Microbiol. Lett. 171: 1-9.

Eparvier, A. and Alabouvette, C. 1994. Use of ELISA and GUS-transformed strains to study competition between pathogenic and non-pathogenic *Fusarium oxysporum* for root colonization. Biocontrol Science Technol. 4: 35-47.

Etchebar, C., Trigalet-Demery, D., Gijsegem, F., Vasse, J., and Trigalet, A. 1998. Xylem colonization by an HrcV^{-} mutant of *Ralstonia solanacearum* is a key factor for the efficient biological control of tomato bacterial wilt. Mol. Plant-Microbe Interact. 11: 869-877.

Fernandez, R. O. and Pizarro, R. A. 1997. High performance liquid chromatography analysis of *Pseudomonas aeruginosa* phenazines. J. Chromatogr. 771: 99-104.

Fogliano, V., Ballio, A., Gallo, M., Woo, S. Scala, F., and Lorito, M. 2002. *Pseudomonas* lipopeptides and fungal cell wall-degrading enzymes act synergistically in biological control. Mol. Plant-Microbe Interact. 15: 323-333.

Fravel, D. R., and Larkin, R. P. 1996. Availability and application of biocontrol products. In: Canaday, C. H. (Ed.), Biological and cultural tests for control of plant diseases, vol. 11. APS Press, St. Paul, pp 1-7.

Fravel, D., Olivain, C. and Alabouvette, C. 2003. *Fusarium oxysporum* and its biocontrol. New Phytopathol. 157: 493-502.

Fuchs, J.-G., Moënne-Loccoz, Y., and Défago, G. 1997. Nonpathogenic *Fusarium oxysporum* strain Fo47 induces resistance to Fusarium wilt in tomato. Plant disease. 81: 492-496.

Gadd, G. M., Ramsay, L., Crawford, J. W., and Ritz, K. (2001). Nutritional influence on fungal colony growth and biomass distribution in response to toxic metals. FEMS Microbiol. Lett. 204: 311-316.

Galli, E., Silver, S., and Witholt, B. 1992. *Pseudomonas*: molecular biology and biotechnology. Washington DC, UK: American Society for Microbiology.

Garrett, S. D. 1970. Pathogenic root-infecting fungi. London, UK: Cambridge university press.

Geels, F. P. and Schippers, B. 1983a. Selection of antagonistic fluorescent *Pseudomonas* spp. and their root colonization persistence following treatment of seed potatoes. Phytopathology Z 108: 193-206.

Geels, F. P. and Schippers, B. 1983b. Reduction of yield depression in high frequency potato cropping soil after seed tuber treatments with antagonistic fluorescent *Pseudomonas* spp.. Phytopathology Z 108: 207-214.

Gerrits, J. P. L. and Weisbeek, P. K. 1996. Induction of systemic acquired resistance by saprophytic *Pseudomonas* spp. in the model plant *Arabidopsis thaliana*. Pages 13-14 in NWO-LNV Priority Program Crop Protection Progress Report. Lunteren, The Netherlands.

Ghisalberti, E. L. and Rowland, G. Y. 1993. Antifungal metabolites from *Trichoderma harzianum*. J. Nat. Prod. 56: 1799-1804.

Haas, D. Blumer, C. and Keel, C. 2000. Biocontrol ability of fluorescent pseudomonads genetically dissected: importance of positive feedback regulation. Curr. Oppin. Biotechn. 11: 290-297.

Handelsman, J. and Stabb, E. V. 1996. Biocontrol of soil-borne plant pathogens. Plant Cell 8: 1855-1869.

Handelsman, J., Raffel, S. J., Mester, E. H., Wunderlich, L. and Grau, C. R. 1999. Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. Appl. Environm. Microbiol. 56: 713-718.

Haran, S. Schickler, H., Oppenheim, A., and Chet, I. 1995. New components of the chitinolytic system of *Trichoderma harzianum*. Mycol. Res. 99: 441-446.

Harman, G. E., Hayes, C. K., Lorito, M., Broadway, R. M., Di Pietro, A., Peterbauer, C. K., and Tronsmo, A. 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. Phytopathology. 83: 313-318.

Harman, G. E., Latorre, B., Agosin, A., San Martin, R., Riegel, D. G., Nielsen, P. A., Tronsmo, A., Pearson, R. C. 1996. Biological and integrated control of Botrytis bunch rot of grape using *Trichoderma* spp. Biological Control. 7: 259-266.

Harman, G. E., Howell, C. H., Viterbo, A., Chet, I., and Lorito, M. 2004. *Trichoderma* species- opportunistic, avirulent plant symbionts. Nature Rev. Microbiol. 2:43-56.

Heath, D. 1995. The chi-squared test for a difference between two population proportions. Pages 216-218 in An introduction to experimental design and statistics for biology. University College London, London, U.K.

Hermosa, M. R., Grondona, I., Iturriaga, E. A., Diaz-Minguez, J., M., Castro, C., Monte, E., and Garcia-Acha, I. 2000. Molecular characterization and identification of biocontrol isolates of Trichoderma spp. Appl. Environm. Microbiol. 66: 1890-1898.

Hernandez, M. E., and Newman, D. K. 2001. Extracellular electron transfer. Cell. Mol. Life Sci. 58: 1562-1571.

Herrera-Estrella, A., and Chet, I. 1999. Chitinase in biological control. In: Chitin and chitinases. Birkhäuser Verlag Basel, Switserland. Pages 171-184.

Hiltner, L. 1994. Uber neue erfahrungen und probleme auf dem gebiet der bodembakteriologie und unter besonderes berucksichtigung der grundugungen und brauche. Arb. Dtsch. Landwirt. Ges. Berl. 98: 59-78.

Hjeljord, L. and Tronsmo, A. 1998. *Trichoderma* and *Gliocladium* in biological control: an overview, p129-151. *In* Harman, G. E., and Kubicek, C. P., (ed.), *Trichoderma* and *Gliocladium*, vol. 2. Enzymes, biological control and commercial application. Taylor and Francis Ltd., London, United Kingdom.

Hoffland, E., Pieterse, C. M. J., Bik, L., and Pelt, J. A. 1995. Induced systemic resistance in radish is not associated with the accumulation of pathogenisis-related proteins. Physiol. Mol. Plant Pathol. 46: 309-320.

Hogan, D. A., Kolter, R. 2002. *Pseudomonas-Candida* interactions: An ecological role for virulence factors. Science 296: 2229-2232.

Inbar, J., Abramshy, D., Cohen, D., and Chet, I. 1994. Plant growth enhancement and biocontrol activity of *Trichoderma harzianum*. Microbiology. 142: 2321-2331.

James, W. C. 1981. Estimated losses of crops from plant pathogens. In: Handbook of Pest Management in Agriculture. Pimentel, D. (ed.)Vol. 1: 79-94. Boca Raton, FL: CRC Press.

Jarvis, W. R. 1988. *Fusarium* crown and root rot of tomatoes. Phytoprotection 69: 49-64.

Jijakli, M. H. and Lepoivre, P. 1998. Characterization of an exo-beta-1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. Phytopathology. 88: 335-343.

Joosten, M. H. A. J. and De Wit, P. J. G. M. 1988. Identification of several pathogenisis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (Syn. *Fulvia fulva*) as ß-1,3-glucanase and chitinases. Plant Physiol. 89: 945-951.

Kinderlerer, M., Kaneko, I., Komiyama, M., Takatsuki, A. and Koshino, H. 1998. Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from mycotoxins. Cloning and characterization of *Tri*101. J. Biol. Chem. 273: 1654-1661.

King, E. O., Ward, M. K., and Raney, D. E. 1994. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44: 301-307.

Kistler, H. C. and Benny, U. K. 1988. Genetic transformation of the fungal wilt pathogen, *Fusarium oxysporum*. Curr. Genet. 13:145-149.

Koch, E. 1999. Evaluation of commercial products for microbial control of soil-borne plant diseases. Crop Protection 18: 199-125.

Kuiper, I., Lagendijk, E.L., Bloemberg, G.V., and Lugtenberg, B.J.J. 2004. Rhizoremediation: A beneficial plant-microbe interaction. Mol. Plant-Microbe Interact. 17:6-15

Kullnig, C. Mach, R. L., Lorito, M., and Kubicek, C. P. 2000. Enzyme diffusion from *Trichoderma atroviride* (= *T. harzianum* P1) to *Rhizoctonia solani* is a prerequisite for triggering of *Trichoderma ech42* gene expression before mycoparasitic contact. Appl. Env. Microbiol. 66: 2232-2234.

Lagopodi, A. L., Ram, A. F. J., Lamers, G. E., Punt, P. J., van den Hondel, C. A. M. J. J., Lugtenberg, B. J. J., and Bloemberg, G. V. 2002. Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis and using the green fluorescent protein as a marker. Mol. Plant-Microbe Interact. 15: 172-179.

Larkin, R. P., Hopkins, D. L., and Martin, F. N. 1993. Effect of successive watermelon plantings on *Fusarium oxysporum* and other microorganisms in soils suppressive and conductive to fusarium wilt of watermelon. Phytopathology 83:1097-1105.

Larkin, R. P., Hopkins, D. L., and Martin, F. N. 1996. Suppression of fusarium wilt of watermelon by nonpathogenic *Fusarium oxysporum* and other microorganisms recovered from a disease suppressive soil. Phytopathology 86: 812-819.

Larkin, R. P. and Fravel, , D. R. 1999. Mechanisms of action and dose-response relationships governing biological control of Fusarium wilt of tomato by nonpathogenic *Fusarium* spp. Phytopathology. 89: 1152-1161.

Lemanceau, P. and Alabouvette, C. 1990. Biological control of *Fusarium* diseases by the association of fluorescent *Pseudomonas* and non-pathogenic *Fusarium,* p. 45-50. In: Plant growth promoting rhizobacteria report, Keel C., Knoller B., and Defago G. (eds.). Interlaken, Switzerland.

Lemanceau, P., Bakker, P. A. H. M., de Kogel, W. J., Alabouvette, C., and Schippers, B. 1993. Antagonistic effect of nonpathogenic *Fusarium oxysporum* Fo47 and Pseudobactin 358 upon pathogenic *Fusarium oxysporum* f. sp. *dianthi*. Appl. Env. Microbiol. 59: 74-82.

Loper, J. E. and Byer, J. S. 1991. Siderophores in microbial interactions on plant surfaces. Phytopathology. 74: 1454-1460.

Lora, J., De La Cruz, J. A., Benitez, T., and Pintor-Toro, J. A. 1992. A putative catabolite-repressed cell wall protein from the mycoparasitic fungus *Trichoderma harzianum*. Mol. Gen. Genet. 247: 639-645.

Lorito, M., Hayes, C. K., Di Pietro, A., Woo, S. L., and Harman, G. E. 1994. Purification, characterization, andsynergistic activity of a glucan 1,3-ß-glucosidase and an N-acetyl- ß-glucosaminidase from *Trichoderma harzianum*. Phytopathology. 84: 398-405.

Lorito, M., D'Ambrosio, M. D., Woo, S. L., Kubicek, C. P., Harman, G. E., Hayes, C. K., and Scala, F. 1996. Synergistic interaction between cell wall degrading enzymes and membrane affecting compounds. Mol. Plant-Microbe Interact. 9: 206-213.

Louvet, J. Rouxel, F. and Alabouvette, C. 1976. Recherches sur la résistance des sols aux maladies. I-Mise en évidence de la nature microbiologique de la résistance d'un sol au développement de la fusariose vasculaire du melon. Annales de Phytopathologie 8: 425-436.

Lu, Z., Tombolini, R., Woo, S., Zeilinger, S., Lorito, M. And Jansson, J. K. 2004. In vivo study of *Trichoderma*-pathogen-plant interactions with constitutive and inducible GFP reporter systems. Appl. Environm. Microbiol. 5: 3073-3081

Lugtenberg B. J. J., de Weger, L. A., and Bennett, J. W. 1991. Microbial stimulation of plant growth and protection from disease. Curr. Opin. Biotechnol. 2: 457-464.

Lugtenberg B. J. J., de Weger, L. A., and Schippers, B. 1994. Bacterization to protect seed and rhizosphere against disease. BCPC Monograph. 57: 293-302.

Lugtenberg, B. J. J., Kravchenko, L. V., and Simons, M. 1999a. Tomato seed and root exudate sugars: composition, utilization by Pseudomonas biocontrol strains and role in the rhizosphere colonization. Environ. Microbiol. 1: 439-446.

Lugtenberg, B. J. J., Dekkers L. C., Bansraj, M., Bloemberg, G. V., Camacho M., Chin-A-Woeng, T. F. C., van den Hondel, C., Kravchenko, L., Kuiper, I., Lagopodi, A. L., Mulders, I., Phoelich, C., Ram, A., Tikhonovich, I., Tuinman, S., Wijffelman, C., and Wijfjes, A. 1999b. *Pseudomonas* genes and traits involved in tomato root colonization. In: De Wit, P. J. G. M., Bisseling, T., and Stiekema, W. J. (eds.) In: IC-MPMI congress Proceedings: biology of plant-microbe interactions, vol. 2. St Paul, MN, USA: International Society for Molecular Plant-Microbe Interactions.

Lugtenberg, B. J. J., Dekkers, L. C., and Bloemberg, G. V. 2002. Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu. Rev. Phytopathol. 39: 461-490.

Lugtenberg, B. J. J. and Bloemberg, G. V. 2004. Life in the rhizosphere. In: The Pseudomonads. Vol. I: Genomics, life style and molecular architecture. Ramos, J-L. (ed.). Kluwer/Plenum Publishers, New York, USA.

Lugtenberg, Ben, Guido Bloemberg, Annouschka Bolwerk, Daan van den Broek, Francisco Cazorla-Lopez, Thomas Chin-A-Woeng, Kevin Eijkemans, Fain D. Kamilova, Irene Kuiper, Ine H. M. Mulders, Evert T. van Rij, and Sandra de Weert. Microbial control of tomato foot and root rot. In: 11th IC-MPMI Congress Proceedings: Biology of Plant-Microbe Interactions. *In press*

Lutz, M. P., Feichtinger, G., Défago, G., and Duffy, B. 2003. Mycotoxigenic *Fusarium* and deoxynivalenol production repress chitinase gene expression in the biocontrol agent *Trichoderma atroviride* P1. Appl. Environ. Microbiol. 69: 3077-3084.

Lynch, J. M. 1990.Introduction: some consequences of microbial rhizosphere competence for plant and soil. In; The rhizosphere. Lynch, J. M. (ed.). Wiley and Sons, Chichester, pages 1-10.

Mandeel, Q. and Baker, R. Mechanisms involved in biological control of Fusarium wilt of cumcumber with strains of nonpathogenic *Fusarium oxysporum*. Phytopathology. 1991: 81:462-469.

Marois, J. J., Mitchell, D. J., Sonoda, R. M. 1981. Biological control of Fusarium crown rot of tomato under field conditions. Ecol. Epidemiol. 71: 1257-1260.

Matus, A. 1999. GFP in motion CD-rom-introduction: GFP illuminates everything. Trends Cell Biol. 9:43.

Menzies, J. G., Koch, C., and Seywerd, F. 1990. Additions to the host range of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Plant Dis. 74: 569-572.

Mes, J. J., Wit, R., Testerink, C. S., de Groot, F., Haring, M. A., and Cornelissen, B. J. C. 1999. Loss of avirulence and reduced pathogenicity of a gamma-irradiated mutant of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Phytopathology 89: 1131-1137.

Mitchell, T. K., Chilton, W. S., and Daub, M. E. 2002. Biodegradation of the polyketide toxin cercosporin. Appl. Environ. Microbiol. 68: 4173-4181.
Montesinos, E. 2003. Development, registration and commercialization of microbial persticides for plant protection. Int. Microbiol. 6: 245-252.

Moore-Landecker, E. 1996. Fundamentals of the fungi. 4th ed. Upper Saddle River, Prentice Hall, NJ 07458, U.S.A.
Mousseaux, M. R., Durroese, R. K., James, R. L., and Knudsen, G. R. 1998. Efficacy of *Trichoderma harzianum* as a biological control of *Fusarium oxysporum* in container-grown Douglas-fir seedlings. New Forests 15: 11-21.

Mullaney, E. J., Hamer, J. E., Roberti, K. A., Yelton, M. M., and Timberlake, E. 1985. Primary structure of the *trpC* gene from *Aspergillus nidulans*. Mol. Gen. Genet. 199: 37-45.

Notz, R., Maurhofer, M., Dubach, H., Haas,D., and Défago, G. 2002. Fusaric acid-producing strains of *Fusarium oxysporum* alter 2,4-diacetylphloroglucinol biosynthetic gene expression in *Pseudomonas fluorescens* CHA0 in vitro and in the rhizosphere of wheat. Appl. Environ. Microbiol. 68: 2229-2235.

Okon Y., Bloemberg, G. V., and Lugtenberg, B. J. J. 1997. Biotechnology of biofertilization and phytostimulation. In: Agricultural Biotechnology. Altman, A. (ed.). Dekker, New York.

Olivain, C. and Alabouvette, C. 1997. Colonization of tomato root by a non-pathogenic strain of *Fusarium oxysporum*. New Phytopathol. 137: 481-494.

Olivain, C. and Alabouvette, C. 1999. Process of tomato root colonization of tomato root by a pathogenic strain of *Fusarium oxysporum* f. sp. lycopersici in comparison with a non-pathogenic strain. New Phytopathol. 141: 497-510.

Olivian, C., Trouvelot, S., Binet, M.N., Cordier, C., Pugin, A., and Alaboevette, C. 2003. Colonization of flax roots and early physiological responses of flax cells inoculated with pathogenic and nonpathogen strains of *Fusarium oxysporum*. Appl. Environ. Microbiol. 69: 5453-5462.

Osbourn, A. E. 1996. Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell. 8: 1821-1831.

Paulitz, T. C., Park, C. S., and Baker, R. 1987. Biological control of Fusarium wilt of cucumber with nonpathogenic isolates of *Fusarium oxysporum*. Can. J. of Microbiol. 33:349-353.

Paustian, K. and Schnürer, J. 1987. Fungal growth response to carbon and nitrogen limitation: application of a model to laboratory and field data. Soil Biol. Biochem. 19: 621-629.

Pieterse, C. M. J., van Wees, S. C. M., Hoffland, E., van Pelt, J. A., and van Loon, L. C. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is dependent of salicylic acid accumulation and pathogenisis related gene expression. Plant Cell 8: 1225-1237.

Pieterse, C. M. J., van Pelt, J. A., van Wees, S. C. M., Ton, J., Leon-Kloosterziel, K. M., Keurentjes, J. J. B., Verhagen, B. W. M., van Knoester, M., dSI, and Bakker, P. A. H. M. 2001. Rhizobacteria-mediated induced systemic resistance: triggering, signaling and expression. Eur. J. Plant Pathol. 107: 51-61.

Punt, P. J., Dingemanse, M. A., Jacobs-Meijsing, B. J. M., Pouwels, P. H., and Van den Hondel, C. A. M. J. J. 1998. Isolation and characterization of the glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*. Gene 69: 49-57.

Pusey, P. L. and Wilson, C. L. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtillis*. Plant Disease 68: 753-756.
Pusey, P. L. 1999. Use of Bacillus subtillus and related organisms as biofungicides. Pesticide Science 27: 133-140.

Ramette, A., Frapolli, M., Défago, G., and Moënne-Loccoz, Y. 2003. Phylogeny of HCN synthase-encoding *hcnBC* genes in biocontrol fluorescent Pseudomonads and its relationship with host plant species and HCN synthesis ability. Mol. Plant-Microbe Interact. 16: 525-535.

Reid, C. P. P. 1990. Mycorrhizas. In: The Rhizosphere. Lynch, J. M. (ed.). Wiley and Sons, Chichester, pages: 281-315.

Rhodes, D. J. and Powell, K. A. 1994. Biological seed treatments- the development process. BCPC Monograph 57: 303-310.

Roberts, D. P. and Lohrke, S. M. 2003. United States Department of Agriculture-Agricultural Research Service research programs in biological control of plant diseases. Pest Manag. Sci. 59: 654-664.

Rovira, A. D. 1956. A study of the development of the root surface microflora during the initial stages of plant growth. J. Appl. Bact. 19: 72-79.

Rowe, R. C. 1980. Comparative pathogenicity and host ranges of Fusarium oxysporum isolates causing crown and root rot in greenhouse and field-grown tomatoes in North America and Japan. Phytopathology. 70: 1143-1148.

RuizDuenas, F. J. and Matinez, M. J. 1996. Enzymatic activities of *Trametes versicolar* and *Pleurotus eryngii* implicated in biocontrol of *Fusarium oxysporum* f sp. *lycopersici*. Current Microbiology 32: 151-155.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Cold, Spring Harbor Laboratory, NY, U.S.A.

Sands, D. C. and Rovira, A. D. 1971. *Pseudomonas fluorescens* biotype G, the dominant fluorescnt pseudomonad in South Australian soils and wheat rhizospheres. J. Appl. Bact. 34: 261-275.

Scher, F. M. and Baker, R. 1980. Mechanism of biological control in a *Fusarium*-suppressive soil. Pytopathology 72: 1567-1573.

Schippers, B., Scheffer, R. J., Lugtenberg, B. J. J. and Weisbeek, P. J. 1995. Biocoating of seeds with plant growth-promoting rhizobacteria to improve plant establishment. Outlook on Agriculture 24:179-185.

Schippers, B., Bakker, A. W., and Bakker, P. A. H. M. 1987. Interactions of deleterious and benificial rhizosphere microorganisms and the effect on croppong practices. Ann. Rev. Phytopathol. 25: 339-358.

Schirmböck, M., Lorito, M., Wang, Y.-L-., Hayes, C. K., Arisan-Atac, I., Scala, F., Harman, G. E., and Kubicek, C. P. 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanism involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Appl. Environ. Microbiol. 60: 4364-4370.

Schneider, R. W. 1984. Effects of nonpathogenic *Fusarium oxysporum* on celery root infectin by *F. oxysporum* f. sp. *apii* and a novel use of the lineweaver–burk double reciprocal technique. Phytopathology 91: 1231-1237.

Schnider-Keel, U., Seematter, A., Maurhofer, M. Blumer, C., and Duffy, B. 2000. Autoinduction of 2,4 diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. J. Bacteriol. 182: 1215-1225.

Schwab , A. P. and Banks, M. K. 1994. Biologically mediated dissipation of polyaromatic hydrocarbons in the root zone, p 132- 141. In: T. A. Anderson and J. R. Coats (eds.) Bioremediation through Rhizosphere Technology. American Chemical Society, Washington DC.

Shapira, R., Ordentlich, A., Chet, I., and Oppenheim, A. B. 1989. Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. Phtopathology 79: 1246-1249.

Spadaro, D., and Gullino, M. L. 2004. Sate of the art and future prospects of the biological control of post-harvest fruit diseases. Int. J. Food Microbiol. 91: 185-194.

Silo-suh, L. A., Lethbridge, B. J., Raffel, S. J., He, H., Clardy, J., and Handelsman, J. 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Appl. Environm. Microbiol. 60: 2023-2030.

Simon, A. and Sivasithamparam, K. 1989. Pathogen-suppression: A case study in biological suppression of *Gaemmanomyces graminis* var. *tritici* in soil. Soil. Biol. Biochem. 21: 331-337.

Simons, M., van der Bij, A. J., Brand, I., de Weger, L. A., Wijffelman, C. A., and Lugtenberg, B. J. J. 1996. Gnotobiotic system for studying rhizosphere colonization by plant-growth promoting *Pseudomonas* bacteria. Mol. Plant-Microbe Interact. 7: 600-607.

Singleton, L. L., Mihail, J. D., and Rush, C. M., eds. 1992. Methods for research on soilborne phytopathogenic fungi. American Phytopathological Society Press, St. Paul, MN, U.S.A.

Sivan, A., and Chet, I. 1986. Possible mechanisms for control of *Fusarium* spp. by *Trichoderma harzianum*. British crop protection conference-Pests and Diseases. Pages 865-872

Sivan, A., Ucko, O., and Chet, I. 1987. Biocontrol of Fusarium crown rot of tomato by *Trichoderma harzianum* under field conditions. Plant Disease 71: 587-592.

Sivan, A., and Chet, I. 1993. Integrated control of fusarium crown and root rot of tomato by *Trichoderma harzianum* in combination with methyl bromide or soil solarization. Crop protection. 12: 380-386.

Sokal, R. R., and Rohlf, F. J. 1981. Biometry: The principles and practice of statistics in biological research. 2nd ed. W. H. Freeman and Co. New York.
Spaink, H. P. 1995. The molecular basis of infection and nodulation by rhizobia: the in and outs of sympathogenisis. Ann. Rev. Phytopathol. 33: 345-368.

Stasz, T. E., Harman, G. E., and Weeden, N. F. 1988. Protoplast preparation and fusion in two biocontrol strains of *Trichoderma harzianum*. Mycologia. 80: 141-150.

Stutz, E. W., Defago, G., and Kern, H. 1986. Naturally occurring fluorescent pseudomonads involved in the suppression of black root rot of tobacco. Phytopathology 76: 181-185.

Summerell, B. A., Salleh, B., and Leslie, J. F. 2003. A utilitarian approach to *Fusarium* identification. Plant Dis. 87: 117-128.

Suslow T. V. and Schroth, M. N., 1982. Rhizobacteria of sugarbeets: effects of seed application and root colonization on yield. Pathypathology 72: 199-206.

Tamietti, G. and Alabouvette, C. 1986. Résistance des sols aux maladies : XIII-Rôle des *Fusarium oxysporum* non pathogènes dans les mécanismes de résistance d'un sol de Noirmoutier aux fusariose vasculaires. Agronomie 6 : 541-548.

Tamietti, G. and Pramotton R. 1990. La réceptivité des sols aux fusariose vasculaires: rapports entre résistance et microflore aotuchtone avec référence particulière aux *Fusarium* non pathogènes. Agronomie 10 :69-76.

Thomashow, L. S. and Weller, D. M. 1996. Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. In: Plant-Microbe Interactions. Chapmann & Hall, New York. 187-235.

Thomashow, L. S. and Weller, D. M. 1998. Role of phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaemmanomyces graminis* var. *Tritici*. J. Bacteriol. 170: 3499-3508.

Tousson, T. A. 1975. Fusarium-suppressive soils. In: Bruehl G. W. (ed.) Biology and control of soil borne plant pathogen. St. Paul, MN, USA: American Phytopathological Society, 145-151.

Tronsmo, A. (1989). *Trichoderma harzianum* used for biological control of storage rot on carrots. Norw. J. Agric. Sci. 3: 157-161.

Tronsmo, A. (1991). Biological and integrated controls of *Botrytis cinerea* on apple with *Trichoderma harzianum*. Biol. Control. 1:59-62.

Trillas- Gay, M. I., Hoitink, H. A. J., and Madden, L. V. 1986. Nature of suppression of Fusarium wilt of radish in a container medium amended with composted hardwood bark. Plant Disease 70: 1023-1027.

Tsien, R.Y. 1998. The green fluorescent protein. Annu. Rev. Biochem. 67: 509-544.

Vancura, V. and Hovadik, A. 1965. Root exudates of plants II. Composition of root exudates of some vegetables. Plant and Soil. 22: 21-32.

Van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J. 1998. Systemic resistnace induced by rhizosphere bacteria. Ann. Rev. Phytopathol. 36: 453-483.

van Rij, E.T., Wesselink, M., Chin-A-Woeng, T.F.C., Bloemberg, G.V., and Lugtenberg, B.J.J. 2004. Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. Mol. Plant-Microbe Interact. 17:557-566.

Van Rhijn, P. and Vanderleyden, J. 1995. The *Rhizobium*-plant symbiosis. Microbiol. Rev. 59: 124-142.

Voisard, C., Keel, C., Haas, D., and Defago, G. 1989. Cyanide production by Pseudomonas fluorescens helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J. 8: 351-358.

Watson, D., MacDermot, J., Wilson, R., Cole, P. J., and Taylor, G. W. 1986. Purification and structural analysis of pyocyanin and 1-hydroxyphenazine. Eur. J. Biochem. 159:309-313.

Weller, D. M. and Cook, R. J. 1983. Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. Phytopathology 73: 710-713.

Weller, D. M., Zhang, B. X., and Cook, R. J. 1985. Application of rapid screening test for selection of bacteria suppressive to take-all of wheat. Plant Disease 69: 710-713.

Wessels, J. G. H. 1986. Cell wall synthesis in apical hyphal growth. Int. Rev. Cytol. 104: 37-79.

Whipps, J. M. 2001. Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52: 487-511.

Woo, S. L., Donzelli, D., Scala, F., Mach, R., Harman, G. E., Kubicek, C. P., Del Sorba, G., and Lorito, M. 1999. Disruption of the *ech42* (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. Mol. Plant-Microbe Interact. 12: 419-429.

Woo, S. L. 2003. Identifying biocontrol genes in Trichoderma spp. and mechanisms for activating biocontrol processes. 8th International congress of Plant Pathology, Christchurch, New Zealand Abstracts, 268.

Yang, T. T., Sinai, P., Green, G., Kitts, P. A., Chen, Y.T., Lybarger, L., Chervenak, R.,Patterson, G.H., Piston, D. W., and Kain, S. R. 1998. Improved fluorescence and dual color detection with enhanced blue and green variants of the green fluorescent protein. J. Biol. Chem. 273: 8212-8216.

Yedidia, I., Benhamou, N., and Chet, I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum* strain. Appl. Env. Microbiol. 65: 1061-1070.

Yedidia, I., Benhamou, N., Kapulnik, Y., and Chet, I. 2000. Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T203. Plant Physiol. Biochem. 38: 863-873.

Yedidia, I., Shoresh, M., Kerem, Z., Benhamou, N., Kapulnik, Y., and Chet, I. 2003. Concominant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* (T203) and accumulation of phytoalexins. Appl. Env. Microbiol. 69: 7343-7353.

Zeilinger, S. Galhaup, C., Payer, K., Woo, S. L., Mach, R. L., Fekete, C., Lorito, M., and Kubicek, C. P. 1999. Chitinase expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genetics Biol. 26: 131-140.

Zimand, G., Elad, Y., Gagulashvily, N., and Chet, I. 1996. Effect of the biocontrol agent *Trichoderma harzianum* T-39 on the pathogenicity of *Botrytis cinerea*. Phytoparasitica. 23: 241-242.

Zimand, G., Elad, Y., and Chet, I. 1996. Effect of *Trichoderma harzianum* on *Botrytis cinerea* pathogenicity. Phytopathology. 86:1255-1260.

Samenvatting

Samenvatting

Planten kunnen groeien omdat ze koolzuurdioxide (CO_2) uit de lucht en water en voedingstoffen uit de grond kunnen opnemen via de wortels. Omgekeerd scheiden plantenwortels ook organische verbindingen uit, welke een voedselbron zijn voor micro-organismen in de directe omgeving van de plantenwortels (de rhizosfeer). Een aantal van deze verbindingen functioneren als signaalstof om interacties tussen organismen en plantenwortels in de rhizosfeer te beïnvloeden. De samenstelling van de microbiële gemeenschap is sterk afhankelijk van bodemsoort, bodemsamenstelling en plantensoort. Schimmels en bacteriën die behoren tot de microbiële gemeenschap kunnen worden onderverdeeld in drie functionele groepen. Deze kunnen respectievelijk een negatieve, een neutrale of een positieve invloed hebben op de plantengroei.

Ziekteverwekkers van planten veroorzaken grote problemen in de productie van bloemen en plantengewassen: kwaliteit en opbrengst gaan achteruit met alle economische gevolgen voor de agrarische, retail en transport sector van dien. Daarnaast is er ook een probleem ten aanzien van onze primaire levensbehoefte: de groei van de wereldbevolking gaat samen met een toenemende vraag naar voedsel. Het bestrijden van plantenziekten is daarom van groot sociaal en economisch belang.

De toepassing van chemische middelen kan bescherming bieden tegen de meeste plantenziekten. Echter, er zijn vele nadelen verbonden aan het gebruik ervan. Het product en zijn werking zijn vaak niet specifiek. Ook andere organismen kunnen dus negatief worden beïnvloed door het gebruik van chemicaliën. Naast het effect op de directe omgeving kunnen chemische bestrijdingsmiddelen zich verspreiden via het (grond-)water waardoor ook het milieu en de mens wordt blootgesteld aan deze verbindingen. De risico's die de verspreiding en blootstelling met zich meedragen heeft de discussie over de wenselijkheid van de toepassing van dergelijke producten versterkt.

Door de toenemende behoefte aan alternatieven en onderzoek naar biologische middelen om plantenziekten te bestrijden, wordt een toenemend aantal biocontroleproducten ontwikkeld. Biocontrole staat voor de vermindering van de plantenziekten door de toepassing van andere (vaak micro-) organismen. De volgende mechanismen van biocontrole door microorganismen zijn beschreven. (i) Competitie voor niches (habitat, leefomgeving) en nutriënten tussen de biocontrolestam en de pathogeen. (ii) De productie van antibiotica door de biocontrolestam die de pathogeen remmen in zijn groei of zelfs doden. (iii) Door productie en secretie van bepaalde enzymen kunnen biocontrolestammen de pathogeen aanvallen en vervolgens groeien op (resten van) de pathogeen. Dit mechanisme wordt 'parasitisme en predatie' genoemd. (iv) Een laatste mechanisme van biocontrole omvat de inductie van verdedigingssystemen in de plant door de biocontrolestam waardoor resistentie tegen de pathogeen wordt opgebouwd. Afhankelijk van de biocontrolestam, de pathogeen, de plantensoort, abiotische en biotische factoren van de grond is de bijdrage van deze mechanismen verschillend.

In dit proefschrift hebben we de interacties tussen de tomatenplant, de pathogeen *Fusarium oxysporu*m f. sp. *radicis lycopersici* (*F.o.r.l.*) en verschillende biocontrolestammen op cellulair niveau bestudeerd. *F.o.r.l.* veroorzaakt het rotten van tomatenwortels, hieronder aangegeven als TWR. De biocontrolebacteriën die bestudeerd zijn behoren tot de soort *Pseudomonas*, de biocontroleschimmels tot de soorten *Fusarium oxysporum* en *Trichoderma.* Deze organismen geven significante reductie van de ziekte in potgrond en in een model systeem. In dit gnotobiotisch zand systeem, beschreven door Simons en collega's (1996), zijn sporen van *F.o.r.l.* door het zand gemengd, waarna hierin gekiemde tomaten zaden (zaailingen) geplant worden. Na zeven dagen groeien werden de wortels geanalyseerd op ziekte-symptomen. In aanwezigheid van de pathogeen en in afwezigheid van biocontrolestammen was 90-100% van de planten ziek. Biocontrolestammen werden op verschillende manieren geïntroduceerd: *Pseudomonas* bacteriën werden gecoat op de zaailingen; sporen van *Fusarium oxysporum* werden, net als die van de pathogeen, door het zand gemengd; mycelium van *Trichoderma* werd door het zand gemengd of zaailingen werden gecoat met gekiemde sporen van *Trichoderma*.

Middels het gnotobiotisch zand systeem kan tevens wortelkolonisatie door *F.o.r.l.* (Lagopodi et al., 2002) en *Pseudomonas* bacteriën (Bloemberg et al., 1997; Bloemberg et al., 2000) zichtbaar worden gemaakt. Daarvoor wordt gebruik gemaakt van autofluorescente eiwitten (AE) en confocale laser scanning microscopie (CLSM). We moesten hiervoor de experimenten in een gnotobiotisch zand systeem uitvoeren. Redenen waren dat zand, in tegenstelling tot potgrond, gemakkelijk van de wortels verwijderd kan worden en dat zand niet interfereert met microscopie studies. Door gebruik te maken van verschillende AE's werd het mogelijk de pathogeen en biocontrolestam tegelijketijd te visualiseren onder ziekte-reducerende condities in het gnotobiotisch systeem. Door het effect van de biocontrolestammen op de pathogeen onder deze condities te bestuderen is meer inzicht verkregen in de mechanismen die bijdragen aan de biologische controle van TWR door *Pseudomonas, Fusarium oxysporum* en *Trichoderma.*

In hoofdstuk 2 wordt beschreven dat *P. chlororaphis* PCL1391 en *P. fluorescens* WCS365 de wortel sneller koloniseren dan *F.o.r.l.* PCL1391 en WCS365 hebben dezelfde niche als de pathogeen, de ruimte tussen wortelcellen waar nutriënten waarschijnlijk worden uitgescheiden (Chin-A-Woeng et al., 1997; Lagopodi et al., 2002). Hierdoor kunnen PCL1391 en WCS365 concureren voor niches en nutriënten waardoor de wortelkolonisatie van de pathogeen afnam met 80%. PCL1391 en WCS365 kunnen ook de hyphen (= schimmeldraden) van *F.o.r.l.* koloniseren. Pilot experimenten hebben aangetoond dat deze bacteriën op schimmel exudaat kunnen groeien (De Weert en Kamilova). Het koloniseren van en het groeien en voeden op de hyphen zal mogelijk de virulentie van *F.o.r.l.* reduceren en zou dus een nieuw mechanisme van biocontrole kunnen zijn. Daarnaast bleek dat PCL1391 en WCS365 de kieming van *F.o.r.l.* sporen reduceren middels componenten uitgescheiden in het groeimedium. Indien deze componenten ook op de tomatenwortel worden geproduceerd en uitgescheiden,

wordt de pathogeen al in het primaire stadium van spoorkieming geremd, wat kan bijdragen aan de controle van TWR.

P. chlororaphis PCL1391 produceert de antischimmel component phenazine-1-carboxamide (PCN) die noodzakelijk is voor de biocontrole van TWR in potgrond (Chin-A-Woeng et al., 1998). In dit proefschrift hebben we aangetoond dat PCN stress reacties veroorzaakt bij *F.o.r.l*. op de plantenwortels. De morfologie, groeirichting en vertakkingspatroon van de hyphen waren aangetast, wat het negatieve effect van PCL1391 op de virulentie van *F.o.r.l.* kan verklaren.

In hoofdstuk 3 wordt beschreven dat het percentage sporen van *F. oxysporum* Fo47 dat in tomatenwortelexudaat kiemt hoger is dan dat van *F.o.r.l..* Dit heeft tot gevolg dat Fo47, net als *F.o.r.l*. aanwezig als sporen in het zand, sneller de exudaat nutriënten kan gebruiken om naar de wortel te groeien. Wanneer de concentratie van *F. oxysporum* Fo47 50 maal hoger was dan die van de pathogeen vond er significante biologische controle van de ziekte plaats (een reductie van 100% naar 60% zieke planten). Deze twee factoren dragen ertoe bij dat Fo47 de wortel eerder en in hogere aantallen bereikt, zoals waargenomen tijdens CLSM studies. De aansluitende kolonisatie van de wortel door Fo47 is daarentegen langzamer, minder agressief en resulteert in een lagere dichtheid dan wordt bereikt door *F.o.r.l*.. De hogere inoculatie concentratie is dan ook waarschijnlijk nodig om het verschil in wortelkolonisatie gedrag te compenseren, waardoor Fo47 effectief kan concureren voor niches en nutriënten op de wortel. Samen met de betere kieming van sporen heeft dit tot gevolg dat de wortelkolonisatie door de pathogeen significant gereduceerd wordt.

De wortelkolonisatie door Fo47 nadat het gecoat was op de zaailingen, in plaats van gemengd door het zand, beperkte zich tot de bovenste 2 cm van de wortel, vlakbij de plek van inoculatie. Ondanks de geringe verspreiding over en kolonisatie van de wortel nam de ziektedruk af van 100% naar 75%. Deze observatie impliceert dat de rol van competitie voor niches en nutriënten minimaal is onder deze condities. De situatie is vergelijkbaar met die gevonden na analyse van wortelkolonisatie mutanten van *P. fluorescens* WCS365 welke nog steeds biocontrole gaven, waarschijnlijk via het induceren van verdedigingsmechanismen in de plant (Dekkers et al., 2000). Fuchs en collega's (1997) hebben aangetoond dat Fo47 bij gescheiden introductie (in tijd en ruimte) tomatenplanten bescherming bood tegen *F. oxysporum* f. sp. *lycopersici* Fol8. Daarbij kan Fo47 de productie van pathogenese gerelateerde eiwitten (PR-eiwitten) in tomatenplanten stimuleren (Duijff et al., 1998). Het is daarom waarschijnlijk dat, wanneer Fo47 is gecoat op de zaailingen, de inductie van verdedigings mechanismen in tomatenplanten door Fo47 bijdraagt aan de ziekte reductie.

In hoofdstuk 4 worden *in vitro* studies beschreven die aangaven dat extracellulaire eiwitten van *T. atroviride* P1 en *T. harzianum* T22 de kieming van *F.o.r.l.* sporen significant reduceren. Wanneer deze eiwitten ook in de rhizosfeer worden geproduceerd kan dit de groei van de pathogeen naar de wortel, en daardoor de wortelkolonisatie, verminderen. Deze hypothese wordt bevestigt door de CLSM studies die aantoonden dat in de aanwezigheid van *Trichoderma* stammen P1 en T22, de wortelkolonisatie door *F.o.r.l*. (met andere woorden de

aantasting van de wortel) gereduceerd was in zowel oppervlakte als in intensiteit. Door de reductie van de wortelkolonisatie zal het infectieproces geremt worden, hetgeen bijdraagt aan de waargenomen biologische controle van TWR door P1 en T22.

De productie van endo- en exochitinases door P1 is betrokken bij (i) de controle van TWR (in potgrond en zand), (ii) de reductie van de wortelkolonisatie door *F.o.r.l.* en (iii) de reductie van kieming van *F.o.r.l.* sporen. Deze resultaten versterken de hypothese dat de remming van spoorkieming van *F.o.r.l.* door de productie van de chitinases door *T. atroviride* P1, een direct effect hebben op de ziekte reductie. De productie van de extracellulaire eiwitten chitinase en glucanase wordt beschouwd als een mechanisme dat bijdraagt aan de biocontrole door T22.

In het gnotobiotisch zand systeem koloniseren *T. atroviride* P1 en *T. harzianum* T22 de tomatenwortel matig: P1 en T22 hyphen waren voornamelijk aanwezig tussen de wortelharen. T22 koloniseerde in enkele gevallen de hoofdwortel. Competitie voor niches en nutrienten zal daarom een minimale rol spelen in de biocontrole door P1. De competitie voor aanhechtingsplekken (primaire stadium wortelkolonisatie) kan een rol spelen bij de biocontrole door T22.

De toepassing van biocontrolestammen is een aantrekkelijk alternatief voor het gebruik van chemische bestrijdingsmiddelen. Echter, de biocontrole is niet altijd effectief. De combinatie van verschillende biocontrolestammen kan de biologische controle van plantenziekten verbeteren. Tevens is een gedegen kennis van de volgende aspecten nodig om de betrouwbaarheid te vergroten. (i) De mechanismen die een rol spelen in biocontrole, (ii) biologische eigenschappen van de biocontrolestam, de pathogeen en het infectieproces en (iii) de interacties tussen plantenwortel, pathogeen en biocontrolestam. Deze interacties zijn echter zo complex dat er naast de verkregen inzichten beschreven in dit proefschrift, vervolg onderzoek gedaan moet worden. Zo kunnen componenten van de pathogeen de productie van (a) het antischimmel component geproduceerd door *P. chlororaphis* PCL1391 (van Rij et al., 2004) en (b) één van de chitinases geproduceerd door *T. atroviride* P1 (Lutz et al., 2003), reduceren. Het is echter niet bekend of PCL1391, P1 of één van de andere biocontrolestammen de productie van eiwitten betrokken bij de virulentie van *F.o.r.l.* beïnvloeden.

Wanneer een gedegen kennis van de boven beschreven aspecten verkregen is, kunnen potentiële biocontrolestammen geselecteerd worden voor commerciële toepassing. Daarbij is het van belang dat de stammen op grote schaal en tegen lage kosten geproduceerd kunnen worden. Registratie is noodzakelijk voordat de biocontroleproducten toegepast kunnen worden. Echter, in Europa zijn de richtlijnen onduidelijk waardoor tot op heden slechts enkele producten beschikbaar zijn. Uiteindelijk zullen de biocontroleproducten niet alleen bijdragen aan een vermindering van gewasverlies maar ook aan duurzame landbouw.

Curriculum vitae

Annouschka Bolwerk werd geboren op 14 augustus 1978 te Baarn. Na het behalen van het VWO-diploma aan Het Baarnsch Lyceum te Baarn in 1996, begon zij met de studie Biologie aan de Vrije Universiteit te Amsterdam. Binnen deze studie werd de richting moleculaire biologie gekozen. Van september 1998 tot oktober 1999 heeft zij een onderzoeksstage uitgevoerd bij de afdeling Moleculaire Celfysiologie aan de Vrije Universiteit onder leiding van dr. Rob van Spanning. Vervolgens heeft zij van december 1999 tot juli 2000 een tweede onderzoeksstage uitgevoerd bij TNO Voeding op de afdeling 'Toegepaste Microbiologie en Gentechnologie' te Zeist onder leiding van dr. Jan Jore en dr. Mariët van der Werf. Het doctoraal diploma werd behaald in augustus 2000. In september 2000 startte zij met het in dit proefschrift beschreven promotieonderzoek in het Instituut Biologie van de Universiteit Leiden, in de sectie Moleculaire Microbiologie onder begeleiding van dr. Guido Bloemberg en prof. dr. Ben Lugtenberg. Sinds september 2004 is zij werkzaam als postdoc bij dezelfde onderzoeksgroep.

Publications

Bolwerk, A., Lagopodi, A. L., Wijfjes, A. H. M., Lamers, G. E. M., Chin-A-Woeng, T. F. C., Lugtenberg, B. J. J., and Bloemberg, G. V. 2003. Interactions in the tomato rhizosphere of two *Pseudomonas* biocontrol strains with the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Mol. Plant-Microbe Interact. 11: 983-993.

Ben Lugtenberg, Guido Bloemberg, Annouschka Bolwerk, Margarita Camacho, Thomas Chin-A-Woeng, Linda Dekkers, Lev Kravchenko, Irene Kuiper, Ellen Lagendijk, Anastasia Lagopodi, Ine Mulders, Monique Radjkoemar-Bansraj, Sietske Tuinman, Sandra de Weert and André Wijfjes. 2002. Improving Root Colonization by *Pseudomonas* Inoculants. In: Sally A. Leong, Caitilyn Allen, and Eric W. Triplett (eds.). Biology of Plant-Microbe interactions. Vol. 3.

Bolwerk, A., Lagopodi, A. L., Wijfjes, A. H. M., Lamers, G. E. M., Lugtenberg, B. J. J., and Bloemberg, G. V.. Interactions between *Pseudomonas* biocontrol strains and *Fusarium oxysporum* f. sp. *radicis-lycopersici* in the tomato rhizosphere. *In*: Igor Tikhonovich, Ben Lugtenberg and Nikolai Provorov (eds.).11th IC-MPMI Congress Proceedings: Biology of Plant-Microbe Interactions. Vol. 4: 323-325.

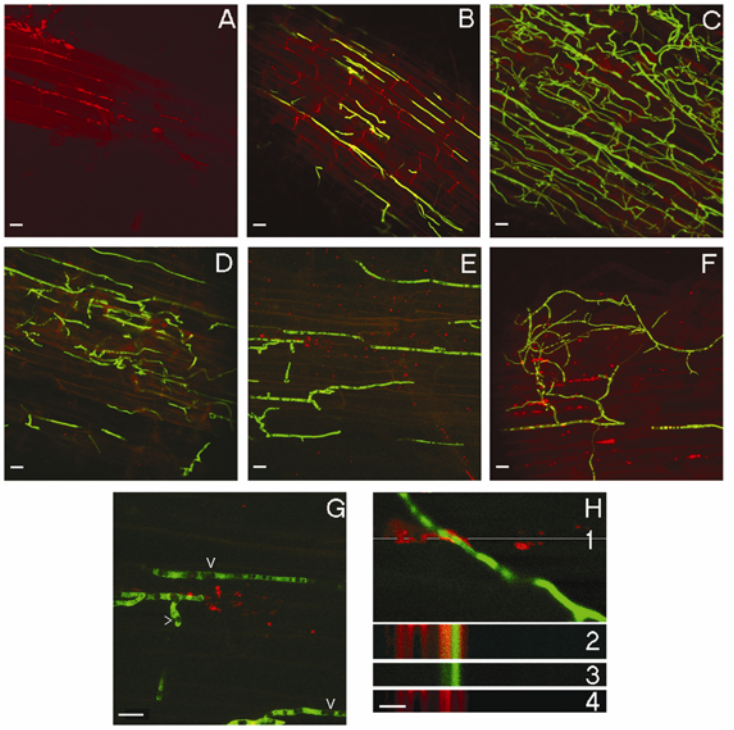
Ben Lugtenberg, Guido Bloemberg, Annouschka Bolwerk, Daan van den Broek, Francisco Cazorla-Lopez, Thomas Chin-A-Woeng, Kevin Eijkemans, Faina D. Kamilova, Irene Kuiper, Ine H. M. Mulders, Evert T. van Rij, and Sandra de Weert. Microbial control of tomato foot and root rot. *In*: Igor Tikhonovich, Ben Lugtenberg and Nikolai Provorov (eds.).11th IC-MPMI Congress Proceedings: Biology of Plant-Microbe Interactions. Vol. 4: 305-308.

Bolwerk, A., Lagopodi, A. L., Lugtenberg, B. J. J., and Bloemberg, G. V. Visualization of interactions in the tomato rhizosphere between a pathogenic and biocontrol *Fusarium* strain. *Submitted*

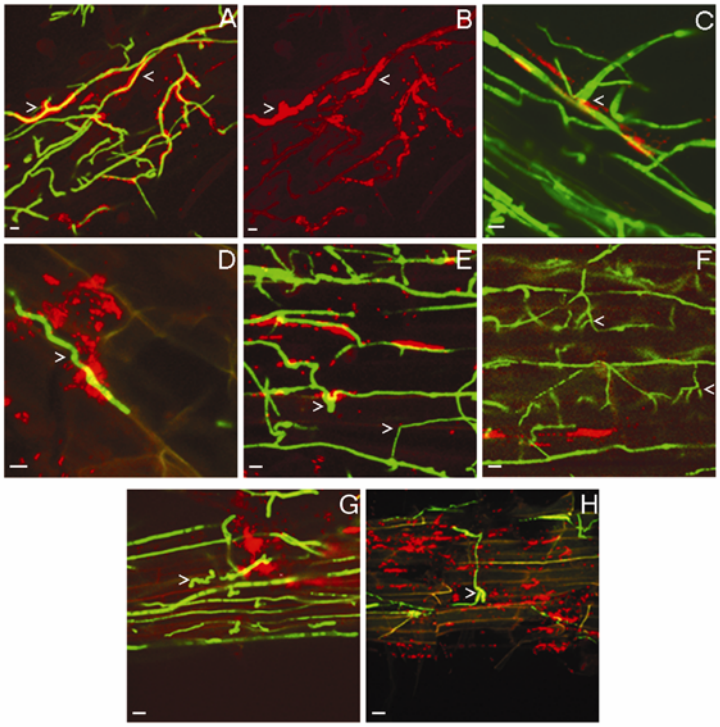
Bolwerk A., Lugtenberg, B. J. J., Lorito, M., and Bloemberg, G. V.. Biocontrol of tomato foot and root rot by *Trichoderma* spp. and the role of chitinases. *Submitted*

Bolwerk A. and Lugtenberg, B. J. J.. Visualization of interactions of various microbial control agents with the tomato root and with the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. In: PGPR: Biocontrol and Biofertilization. Zaki A. Siddiqui (ed.). *In press*

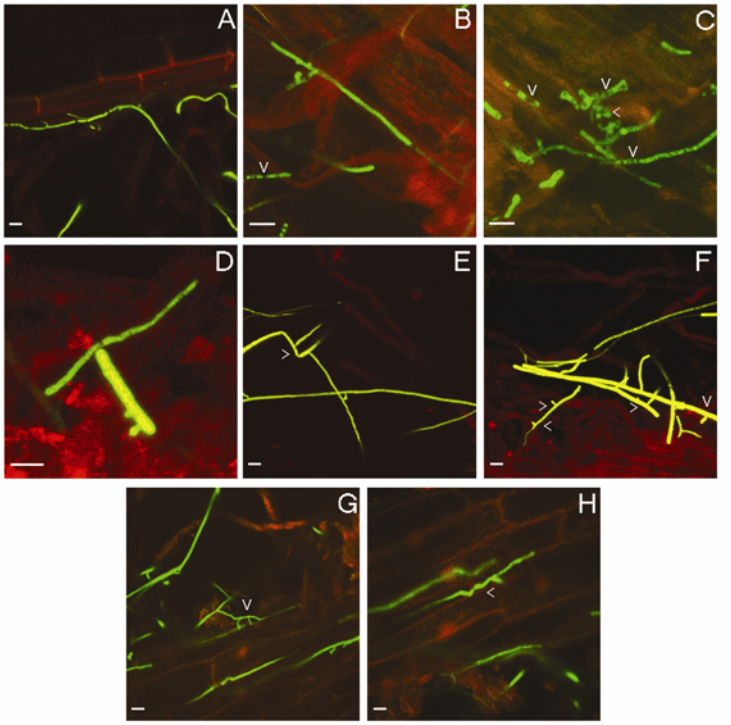
Full color figures



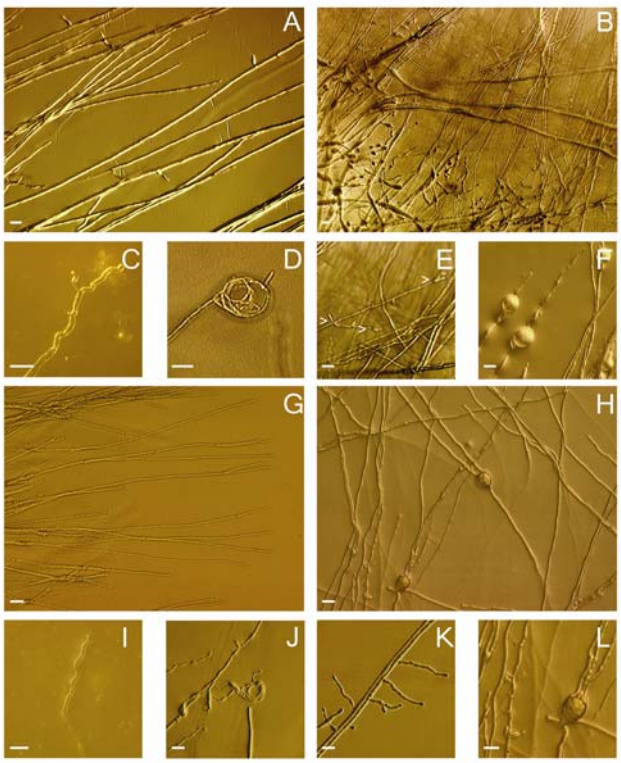
Chapter 2, Figure 1. Confocal laser scanning microscopical analysis of tomato root colonization by the phytopathogenic fungus *F. oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) and by *Pseudomonas* biocontrol bacteria.
Two-day-old tomato seedlings were inoculated at time zero with cells of either *P. fluorescens* WCS365 or *P. chlororaphis* PCL1391 harboring a reporter plasmid expressing the *rfp* gene, which here appear as red cells. Plants were grown in a gnotobiotic sand system containing spores of *F.o.r.l.* harboring a constitutively expressed *gfp* gene. Walls of tomato root cells appear as red due to autofluorescence.
A, *P. fluorescens* WCS365 colonizing the intercellular junctions of root cells of an inoculated seedling planted in sterile sand three days after planting. **B,** *F.o.r.l.* hyphae growing along the intercellular junctions of root cells of a sterile seedling three days after planting in sand containing fungal spores. **C,** Hyphal network present in the rhizosphere of a sterile seedling planted in sand containing *F.o.r.l.* spores seven days after planting in absence of biocontrol bacteria; **D,** in presence of *P. fluorescens* WCS365; **E,** in presence of *P. chlororaphis* PCL1391; **F,** in presence of *P. chlororaphis* PCL1119. **G,** Vacuoles (indicated by arrowheads) abundantly present in hyphae in the rhizosphere of seedlings inoculated with *P. chlororaphis* PCL1391 three days after planting. **H**, *P. chlororaphis* PCL1391 attached to fungal hyphae three days after inoculation. The lower part of the panel (2-4) is a cross section in the Z direction at the white line in the upper part (1) showing the attachment. 2. Both the fungus and the bacteria. 3. The GFP signal of the fungus and 4. The DsRed signal of the bacteria. The size bar represents 10 µm in all panels.



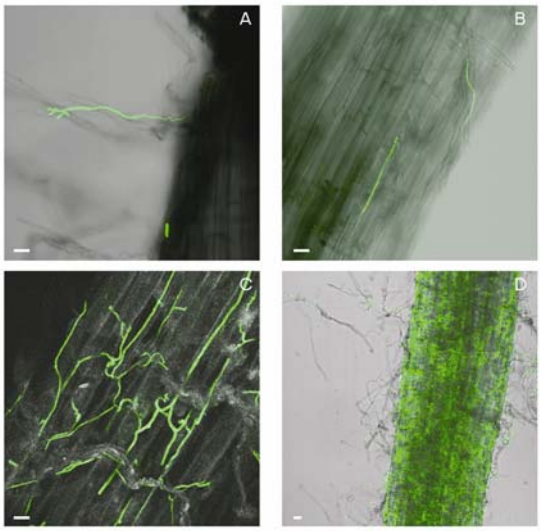
Chapter 2, Figure 2. Confocal laser scanning microscopical analysis of effects of the presence of *Pseudomonas chlororaphis* PCL1391 and PCL1119 cells on growth of *F. oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) in the tomato rhizosphere.
Two-day-old tomato seedlings were inoculated at time zero with *P. chlororaphis* PCL1391 cells harboring a reporter plasmid expressing the *rfp* gene, which here appear as red cells. Plants were grown in a gnotobiotic sand system containing spores of *F.o.r.l.* harboring a constitutively expressed *gfp* gene. Cell walls of the tomato root appear as red due to autofluorescence. **A,** *P. chlororaphis* PCL1391 cells concentrating around the hyphae and colonizing *F.o.r.l.* hyphae ten days after inoculation. **B,** same picture as 2A without the GFP signal showing that all bacterial cells are attached to the fungal hyphae. **C,** In presence of strain PCL1391 an increase of the diameter of hyphae (indicated by arrowheads) was observed after seven days. **D,** Curly growth of hyphe along the cellular junction of the tomato root was observed in close vicinity of PCL1391 cells, nine days after planting. **E,** In presence of strain PCL1391 abrupt changes in the growth direction of hyphae (indicated by arrowheads) observed after ten days. **F,** Branching of *F.o.r.l.* hyphae resembles fork-like structures (indicated by arrowheads) in presence of strain PCL1391 thirteen days after inoculation. **G,** Hyphal growth in presence of strain PCL1119 in the rhizosphere. **H,** Branching of *F.o.r.l.* hyphae resembles fork-like structures at lower frequency in presence of strain PCL1119 thirteen days after inoculation. The size bar represents 10 µm in all panels.



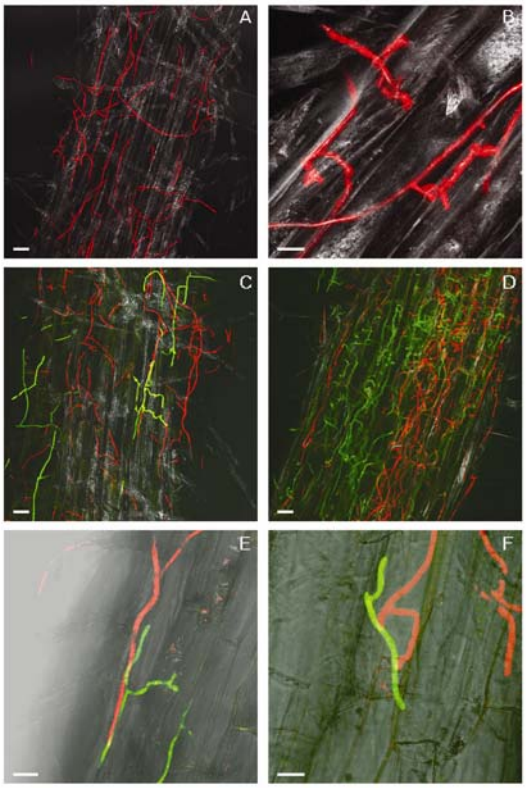
Chapter 2, Figure 3. Confocal laser scanning microscopical analysis of effects of the presence of purified PCN on growth of *F. oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) in the tomato rhizosphere.
Plants were grown in a gnotobiotic sand system containing spores of *F.o.r.l.* harboring a constitutively expressed *gfp* gene. Cell walls of the tomato root appear as red due to autofluorescence. **A** and **B**, hyphal growth in presence of ethyl acetate. **C**, An increase in the number of vacuoles was observed after four hours in presence of PCN. **D**, An increase in the hyphal diameter was observed after 1 day. E, Abrupt changes in the growth direction was observed after 1 day incubation. **F**, Increased branching frequencies was observed after 1 day. **G**, altered branching structures were observed after 1 day. **H**, Curly growth was observed after 3 days. The size bar represents 10 µm in all panels.



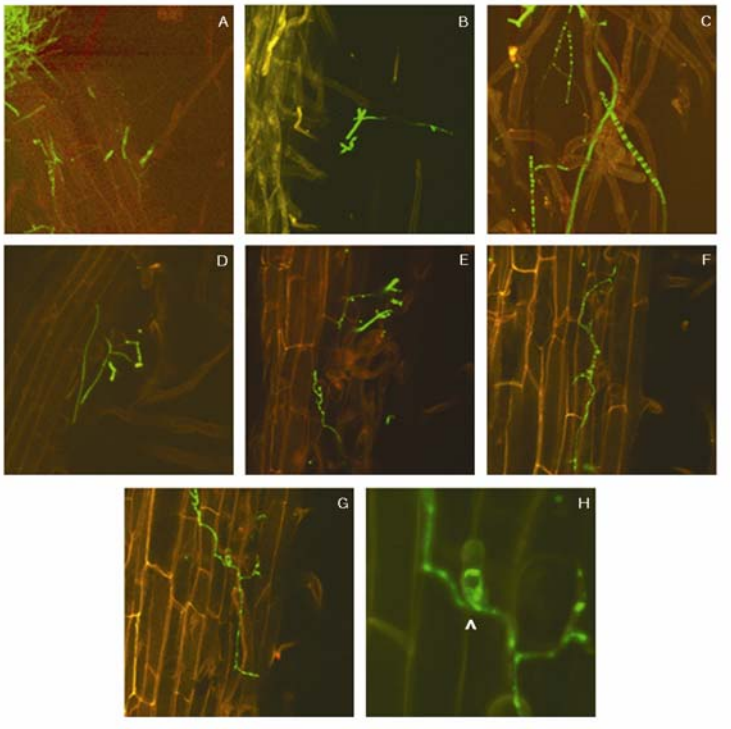
Chapter 2, Figure 4. Differential interference contrast microscopy analysis of *in vitro* effects of *Pseudomonas chlororaphis* PCL1391 on hyphal growth and spore formation by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.).*
F.o.r.l. was grown in the vicinity of *P. chlororaphis* PCL1391, *P. chlororaphis* PCL1119 or purified PCN, on microscopy glass slides covered with a thin layer of LB agar. Three days after growth *F.o.r.l.* hyphae were examined for effects on growth and spore formation. **A,** Growth of *F.o.r.l.* in the absence of bacteria. **B through F,** Growth of *F.o.r.l.* towards *P. chlororaphis* PCL1391, which is located (outside the picture) in the upper right corner. **B,** Overview of the region close to the inhibition zone caused by PCL1391. **B through D,** Disturbance of hyphal growth directionality. **E,** Frequent branching close to the hyphal tip. **F,** Chlamydospores, observed within the hyphae. **G,** Overview of the region close to PCL1119, which is located (outside the picture) on the right. **H through L,** Growth of *F.o.r.l.* towards purified PCN, which is located (outside the picture) in the upper right corner. **H,** overview of the region close to the inhibition zone caused by PCN. **H through J,** Disturbance of hyphal growth directionality. **K,** Frequent branching close to the hyphal tip. **L,** Chlamydospores, observed within the hyphae. The size bar represents 10µm in all panels.



Chapter 3, Figure 2. Confocal laser scanning microscopical analysis of tomato root colonization by *Fusarium.*
Two-day-old tomato seedlings were grown in a gnotobiotic sand system containing spores of *F.o.r.l.* (FCL14), which harbors a constitutively expressed *sgfp* gene. Walls of tomato root cells appear as gray due to contrast light (panel A, B and D) or reflected light (panel C). Panel A, Initial colonization of the tomato root by *F.o.r.l.* (similar for Fo47) 'attachment' to root hairs. Panels **A through** D: subsequent root colonization stages by *F.o.r.l.* A, 'attachment' to root hair. **B,** hyphe growing along the intercellular junctions of two root cells: 'start colonization'-stage. **C,** *F.o.r.l*. hyphae growing along the intercellular junctions of more than two root cells: 'colonization'-stage. **D,** hyphae growing over the whole root at a very high density and biomass: 'heavy colonization'-stage. The size bar represents 10 µm in all panels.



Chapter 3, Figure 4. Confocal laser scanning microscopical analysis of tomato root colonization by the pathogenic fungus *F.o.r.l.* and the biocontrol strain Fo47.
Two-day-old tomato seedlings were grown in a gnotobiotic sand system containing spores of Fo47 (FCL31) (panel A and B) or spores of both *F.o.r.l.* (FCL14) and Fo47 (FCL31) (panel C-F) at an inoculum ratio of 1:50. *F.o.r.l.* (FCL14) harbors a constitutively expressed *sgfp* gene and appears as green. Fo47 (FCL31) harbors a constitutively expressed *ecfp* gene its emission signal is depicted as red in the shown images. Walls of tomato root cells appear as gray due to reflected light (panel A-D) or contrast light (panel E and F). Panels **A** and **B**, Colonization of the tomato root by Fo47. **A,** Hyphal growth along cellular junctions and crossing root cells. B, Penetration of the tomato root by Fo47 (indicated by an arrowhead). **C,** On healthy roots (disease index 0) Fo47 is dominant. **D,** On sick roots with disease index 1, Fo47 and *F.o.r.l.* are equally present. **E** and **F,** direct cell cell contact between *F.o.r.l.* and Fo47 in the rhizosphere. The size bar represents 10 µm in all panels.



Chapter 4, Figure 4. Confocal laser scanning microscopy (CLSM) analysis of tomato root colonization by two *Trichoderma* strains.
Two-day-old tomato seedlings were grown in a gnotobiotic sand system. *Trichoderma* strain P1 or T22 was introduced as germinated spores coated on seedlings (panel A) or as mycelium mixed through the sand (panel B through H). After seven days of growth, tomato root colonization by the *Trichoderma* strains was analyzed. Walls of tomato root cells appear red because of auto-fluorescence. **A.** Clump of germinated P1spores with short hyphae. **B.** Initial attachment of P1 hyphae to root hairs. **C.** T22 hyphal growth in the root hair zone. **D.** Hyphae of T22 growing in the rhizosphere but not attached to the root surface. **E to G.** Hyphae of T22 attached to and growing along the main root. **G and H (close-up of G),** Penetration of the tomato root by T22 as indicated with an arrow. Bars represent 10 µm.