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The effect of root exudates of *Solanum lycopersicum* L. inoculated with biological control agents and/or arbuscular mycorrhizal fungi on soil-borne pathogenic fungi

Diplomarbeit

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Für meine Großeltern

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Abstract

Root exudates play an important role in initiation processes of plant pathogen interactions. Therefore, 3 different *Trichoderma* strains (*Trichoderma viride* strain RE 1-3-4, *T. harzianum* strain T39 and *T. atroviride* strain P1) were screened for their effects on plant growth and root exudation pattern of tomato, plant symbionts (arbuscular mycorrhizal fungi (AMF)) and 3 selected tomato pathogens (*Fusarium oxysporum* f.sp. *lycopersici (Fol)*, *Pyrenochaeta lycopersici* and *Verticillium dahliae*). Plant growth was affected negatively only in the *in vitro* cultures. In order to test the effects of *Trichoderma* on *Fol* and *P. lycopersici* via the alteration of the root exudation pattern, a biotest with a microplate reader was established. This technique was not adequate to measure the growth of *P. lycopersici*, but of *Fol*. In the microplate reader assay the fungal growth of *Fol* in the exudates of the tomato cultures inoculated with *Trichoderma* spp. as well as in the cultures inoculated with *Trichoderma* and/or AMF clear indications are given that *Trichoderma* spp. and AMF, respectively, alter the exudation pattern of tomato plants and that these alterations affect the fungal growth of *Fol*. However, the picture is not clear yet as depending on the tested *Trichoderma* strain, the used substrate and the time of inoculation, inhibitory as well as stimulatory effects on the fungal growth of *Fol* were observed. In the presence of root exudates from mycorrhizal tomato plants the fungal growth of *Fol* was reduced. The inhibitory effect on fungal growth of *Fol* when the different *Trichoderma* strains were applied in combination with the AMF were similar as with AMF alone, only for one treatment (AMF + *T. harzianum* T39) the fungal growth was even lower than for AMF alone. *Trichoderma* spp. did not have any adverse effects on the degree of root colonisation by AMF. In the dual cultures the growth areas of *Fol* and *P. lycopersici* were significantly reduced by the 3 applied *Trichoderma* strains, whereas, no effects on the growth area of *V. dahliae* could be detected. Further studies are needed to elucidate the complex interactions between biological control agents, plants and pathogens.

1. Introduction

The demand of the society for foods with less chemical residues, the great concern for the preservation of the environment (Alabouvette *et al.*, 2006) and the limits of chemical control are some of the reasons for the increase of interest in biological control. Soil-borne fungi are good candidates for the application of biological control agents (BCAs), since alternatives are needed for soil fumigation. Methyl bromide and other fumigants, such as metham sodium, methyl isothiocyanate or dazomet, are very effective but treatments are expensive. Furthermore, they require special machinery and well-trained personnel and cause environmental pollution (Fiume and Fiume, 2003). Methyl bromide is a stratospheric ozone depleter and was therefore listed under the 'Montreal Protocol on Substances that Deplete the Ozone Layer' in 1992. Consequently, the use of methyl bromide will be phased-out according to an agreed schedule (Anonymous, 2000). Thus a demand for alternatives to control soil-borne pathogenic fungi is given. *Trichoderma* spp. have been successfully used as BCAs against different pathogenic fungi (Monte, 2001, Paulitz and Bélanger, 2001). Other promising candidates for plant protection are plant symbionts like arbuscular mycorrhizal fungi, which can have bioprotective effects on their host plants against soil-borne pathogens (Azcón-Aguilar *et al.*, 2002, Singh *et al.*, 2000, Xavier and Boyetchko, 2004, Vierheilig *et al.*, 2007).

Plant-pathogen interactions lead to disease incidence. In soil such negative interactions are triggered by root exudates, therefore, 'interference with the production and activity of exudate stimulants is a promising approach by which to interfere with the pathogen activity and achieve biological control' (Nelson, 1990). The composition of root exudates is influenced by different factors such as plant species, age and physiological state of the plant, soil pH, soil moisture, temperature and the presence of microorganisms (Bais *et al.*, 2006, Bertin *et al.*, 2003, Nelson, 1990).

In Austria tomato is a crop with high economic value. The price of tomato increased 30 % in 2006 compared to the year 2005. In 2006 Tomato was cultivated on 184 ha and 35,321 t of tomato were harvested. This development comes along with an increase in the consumption of vegetables (Grüner Bericht, 2006). Thus, the present

study was conducted with tomato (*Solanum lycopersicum* L.). Due to intensive cultivation soil-borne pathogenic fungi accumulate on fields. For this study three pathogens were chosen, which cause serious yield losses worldwide: *Fusarium oxysporum* f.sp. *lycopersici*, the causal agent of Fusarium wilt, *Verticillium dahliae*, the causal agent of Verticillium wilt, and *Pyrenochaeta lycopersici*, leading to corky root.

Root exudates are primary signals in plant-pathogen interactions (Nelson, 1990), however, little is known about the effect of exudates on the mycelial growth of *F. oxysporum* f.sp. *lycopersici* and *P. lycopersici*. The aim of the present work was to elucidate whether the inoculation of tomato plants with different *Trichoderma* spp. strains and/ or an arbuscular mycorrhizal fungus leads to an alteration of the effect of the root exudates on the growth of the tomato pathogens mentioned above. Apart from the possible indirect effects of *Trichoderma* spp. on *F. oxysporum*, mediated by an altered exudate composition, direct effects of the different *Trichoderma* strains on the pathogens were studied via dual culture tests. Dual culture tests were performed with the three tomato pathogens *F. oxysporum*, *P. lycopersici* and *V. dahliae* and using three *Trichoderma* strains as biocontrol agents.

2. Soil-borne pathogenic fungi and the potential of biological control

2.1. Biological control of plant diseases

According to Cook and Baker (1983) 'Biological control is the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man'. Subsequently, biological control includes avirulent or hypo-virulent individuals or populations within the pathogenic species, antagonistic microorganisms and manipulation of the host plant to resist the pathogen more effectively (Alabouvette *et al.*, 2006). There are several ways to perform biological control, such as mass introduction of antagonists, plant breeding and specific cultural practices aimed at modifying the microbial balance. In this work emphasis will be put on *Trichoderma* spp. as biological control agents (BCAs). Their modes of action will be discussed in section 2.5.

The idea or the beginning of biological control is more than 70 years old but only in the last 20 years the amount of research in this area has increased dramatically. Within the past 10 years over 80 biocontrol products have appeared on the market, most of them are formulations either of the fungi *Trichoderma* or the bacteria *Pseudomonas* and *Bacillus* (Paulitz and Bélanger, 2001). Many of these products are marketed as plant growth promoters, plant strengtheners or soil conditioners, simply because the costs of registration as a biocontrol agent are high due to stringent toxicology testing or the requirements for efficacy testing, which are hard to meet as it is the case in Europe (Alabouvette *et al.*, 2006, Mathre *et al.*, 1999). Efficacy testing includes the evaluation of the product in several experiments, in different geographic regions, for two consecutive years. On the European market there are only a few microbiological products available for the grower, whereas in other countries like the USA, Australia and New Zealand such products are more common. This is due to the fact that biological products fail too often to show a consistent effect under variable environmental conditions (Alabouvette *et al.*, 2006). Biological control is more prevalent in greenhouse and protected structures than in field crops, since environmental conditions such as temperature and relative humidity can be easier controlled and the economic value of greenhouse crops is high (Paulitz and

Bélanger, 2001). Inconsistent effects of BCAs are a problem. Alabouvette *et al.* (2006) suggest that the application of BCAs should be integrated in other farm practices. Furthermore, the whole system, i.e. the interactions between plant, pathogens, antagonists, the environmental conditions and farm practices in a given ecological region, should be the target of research so that the application and use of BCAs can be more efficient. BCAs should not be used as chemical products, because in being biological they should be applied according to their ecological requirements.

In the present work special attention is drawn to three soil-borne pathogenic fungi (*Pyrenochaeta lycopersici*, *Fusarium oxysporum* f.sp. *lycopersici*, *Verticillium dahliae*), *Trichoderma* spp. as BCAs and arbuscular mycorrhizal fungi. Interactions between soil-borne pathogens, antagonists, arbuscular mycorrhizal fungi and plants and the role of root exudates in these interactions will be discussed in the following sections.

2.2. *Pyrenochaeta lycopersici* Schneider & Gerlach

2.2.1. The pathogen

Pyrenochaeta lycopersici Schneider & Gerlach belongs to the kingdom Fungi and the phylum Ascomycota. Before its identification it was referred to as the grey sterile fungus (Gerlach and Schneider, 1964, Punithaligam and Holliday, 1973). The fungus occurs in the field in a sterile mycelial state, only under special conditions, when the infested tomato roots are exposed to light over several weeks, asexual fruiting bodies, pycnidia, within asexual spores, conidia, are formed (Punithaligam and Holliday, 1973, Schneider and Gerlach, 1966). In the literature several methods are described to induce sporulation *in vitro* (Schneider and Gerlach, 1966, Shishkoff, 1993).

The primary infective propagules of *P. lycopersici* in the soil are the microscopic overwintering bodies, the microsclerotia. They are formed in the root cortex and released when roots disintegrate (Kartheuser, 1988). The microsclerotia are resistant to soil degradation, dry heat and desiccation and are still fully pathogenic after 5

years (Ball, 1979, Kartheuser, 1988). This is most likely due to their heavy pigmentation, their small size and their external skin (Ball, 1979).

2.2.2. The disease

P. lycopersici causes corky root or brown root rot of tomato (*Solanum lycopersicum* L.). Glasshouse and field production of tomato in temperate regions and in the Mediterranean region are affected by this causal agent (Abou-Shaar and Hentschel, 1987, Jones *et al.*, 1989, Kartheuser, 1988).

Corky root disease was reported in Canada, Belgium, France, Germany, Italy, the Netherlands, New Zealand, Rumania, Scandinavia, UK, the United States and Lebanon (Jones *et al.*, 1989, Punithaligam and Holliday, 1973, Shishkoff, 1993). The pathogen *P. lycopersici* can be isolated from the roots of tomato, which is the most important host, eggplant (*Solanum melongea* L.), pepper (*Capsicum annuum* L.), tobacco (*Nicotiana tabacum* L.), nightshade (*Solanum* spp.) and other members from the Solanaceae, and from melon (*Cucumis melo* L.), squash (*Cucurbita pepo* L.), cucumber (*Cucumis sativus* L.), safflower (*Carthamus tinctorius* L.), beet (*Beta vulgaris* L.), and spinach (*Spinacia oleracea* L.) (Shishkoff, 1993).

Corky root causes a slow progressive deterioration of the root system of infected plants (Jones *et al.*, 1989, Kartheuser, 1988). In the beginning localised tan lesions appear on the main and secondary roots. With time the lesions become dark brown, furrowed and develop a corky texture (Figure 1). Severely infested plants are more prone to secondary microorganisms, since furrowed fractures provide entry for them (Jones *et al.*, 1989). The lack of fibrous secondary roots with their root hairs leads to a malfunctioning of the uptake of water and nutrients and furthermore to stunting, a lack of vigour and poor yield (Kartheuser, 1988, Punithaligam and Holliday, 1973). Yield losses are ranging between 30 to 50 % and up to 75% on severely infested plots (Abou-Shaar and Hentschel, 1987, Gerlach and Schneider, 1964, Jones *et al.*, 1989, Kartheuser, 1988).

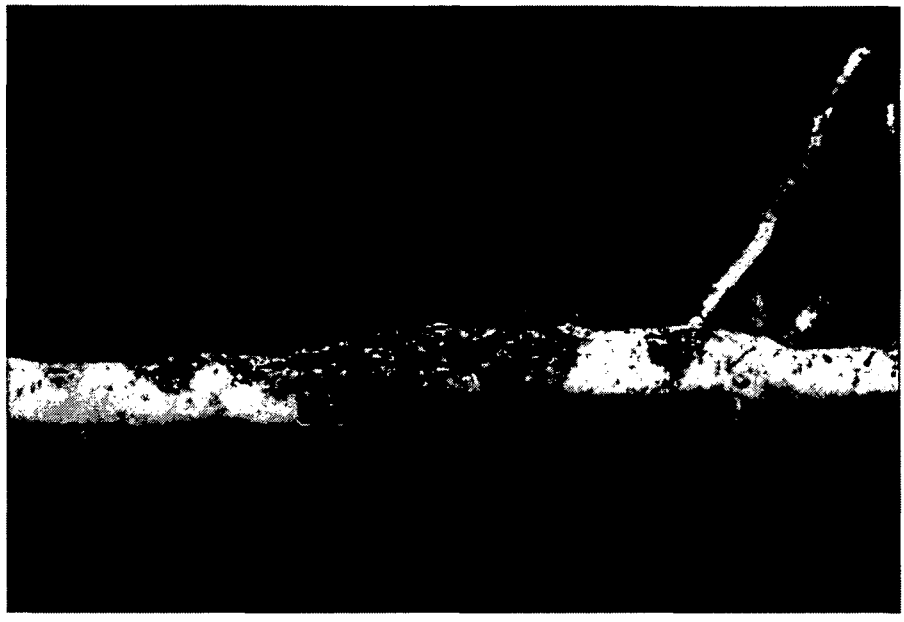

Figure 1: Tomato root infected by *P. lycopersici* (from Aggie Horticulture Network, 2007).

Disease incidence and the accumulation of inoculum in the soil are caused and increased by close succession of tomato (Kartheuser, 1988, Punithaligam and Holliday, 1973). Furthermore, the disease development is highly influenced by low soil temperatures, i.e. the optimum is between 15 and 20°C (Kartheuser, 1988). Since the pathogen grows very slowly disease symptoms develop slowly (Jones *et al.*, 1989) and it is not very competitive to other soil microorganisms (Kartheuser, 1988). Higher soil temperatures reduce the yield losses caused by the pathogen due to the temperature dependent activity of antagonistic microorganisms (Kartheuser, 1988), i.e. at higher soil temperatures *P. lycopersici* is not competitive enough.

2.2.3. Control measurements

P. lycopersici can be controlled by soil solarisation and fumigation. Biological control based on microbial antagonists also provides promising results (Abou-Shaar and Hentschel, 1987, Fiume and Fiume, 2003).

2.3. *Fusarium oxysporum* f. sp. *lycopersici*

2.3.1. The pathogen

Fusarium oxysporum Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans belongs to the kingdom Fungi and to the phylum Ascomycota. The Fungus produces three kinds of asexual spores, microconidia, macroconidia and chlamydospores. Microconidia consist of one or two cells and 'are the most frequently and abundantly produced spores under all conditions, even inside the vessels of the host plant' (Agrios, 1997). Macroconidia are three to five celled and have gradually pointed and curved ends. They appear in sporodochia-like groups on the surface of plants killed by the pathogen. Chlamydospores consist of one or two cells, are thick-walled and round. They are produced within or terminally on older mycelium or in macroconidia. All three types of spores are produced readily in cultures of the fungus and also in the soil, however, only chlamydospores can survive in the soil for several years (Agrios, 1997).

2.3.2. The disease

'Fusarium wilt is a warm-weather disease on tomato, most prevalent on acid, sandy soils' (Jones, 1991). Soil and air temperatures of 28°C are favouring wilt development. The pathogen is soil-borne and it survives between crops in infected plant debris as mycelium and in all its spore forms (Agrios, 1997). Especially in the cooler temperate regions chlamydospores are the most prevalent ones. Dissemination of the pathogen occurs via seed, tomato stakes, soil, infected transplants or infested soil clinging to transplants and farm machinery (Jones, 1991). The soil remains infested for several years. Infection begins with the direct penetration of root tips by germ tubes of spores or by the mycelium, or by the entering through wounds or at the point of formation of the lateral roots (Agrios, 1997). The mycelium grows towards the xylem vessels and travels through them upward to the stem and crown of the plant. In the vessels microconidia are formed which are responsible for further dissemination in the host plant. The first symptoms on older plants are the yellowing of the older leaves (Jones, 1991). Due to vessel clogging older plants start wilting and wilting increases until the water economy of the

host breaks down and the host dies as a direct consequence (Agrios, 1997). The fungus is prevalent in southern locations in the United States and in Europe where it is harmful in the field and in northern areas where it is limited by temperature to greenhouse crops (Jones, 1991).

2.3.3. Control

The only practical measure to control Fusarium wilt is the use of tomato varieties resistant to the pathogen (Agrios, 1997). Soil fumigation and soil solarisation 'reduce the incidence of wilted plants and greatly increases marketable and total yields' (Jones, 1991). 'A crop rotation of 5 to 7 years does not eliminate the pathogen but greatly reduces losses' (Jones, 1991). The use of antagonistic fungi such as non-pathogenic *Fusarium oxysporum* strains and *Trichoderma* strains is promising, but however, not very common in practical control (Agrios, 1997, Paulitz and Bélanger, 2001).

2.4. *Verticillium dahliae*

2.4.1. The pathogen

Verticillium dahliae Kleb. belongs to the kingdom Fungi and to the phylum mitosporic fungi. The pathogen produces short-living conidia and resting structures, microsclerotia, in debris of infected plants (Agrios, 1997, Pohronezny, 1991). Microsclerotia form best under cool conditions (10 – 20°C) and can stay infectious from 8 up to 15 years. *V. dahliae* develops slowly and is a poor soil competitor.

2.4.2. The disease

V. dahliae is a cool-weather disease (daytime high temperatures 20 – 24°C) which appears to be more severe in neutral to alkaline soils (Pohronezny, 1991). This pathogen is very polyphagous because it attacks about 200 plant species, including most vegetables, flowers, fruit trees, strawberries, field crops and forest trees (Agrios, 1997). Symptoms of Verticillium wilt are similar to those of Fusarium wilt. However, symptoms of Verticillium wilt develop more slowly. Infected plants show 'defoliation,

gradual wilting and death of successive branches or abrupt collapse and death of the entire plant' (Agrios, 1997). Infection proceeds usually through wounds on roots, such as those produced during cultivation, in the formation of secondary roots or as a result of nematode feeding or through direct penetration of young roots. Dissemination occurs by contaminated seeds, vegetative cuttings and tubers, wind, surface water and soil. 6 to 50 microsclerotia per gram are sufficient to give 100 percent infection in most susceptible crops. The pathogen is often found in uncultivated areas, i.e. the fungus is native to the soils (Agrios, 1997).

2.4.3. The control

Soil fumigation is very effective but very expensive on large areas (Agrios, 1997, Pohronezny, 1991). Crop rotation can be useful but is limited by the wide host range of the fungus. Soil solarisation is also a possibility to reduce disease incidence.

2.5. *Trichoderma* spp.

2.5.1. Ubiquitous soil colonisers

Trichoderma spp. are ubiquitous soil colonisers. They are general saprophytes with minimal nutrional requirements and have the ability to produce a broad array of secondary metabolites. Furthermore, they grow rapidly, sporulate profusely and are 'easily isolated from soil, decaying wood and other forms of plant organic matter' (Howell, 2003, Klein and Eveleigh, 1998). With regard to this features it is not surprising that *Trichoderma* spp. are promising as well as established candidates for biological control and plant growth stimulants (Monte, 2001, Paulitz and Bélanger, 2001).

Biocontrol achieved by the application and use of *Trichoderma* strains is based on several direct or indirect mechanisms which can operate alone or synergistically (Benítez *et al.*, 2004, Harman, 2006, Harman and Björkman, 1998, Howell, 2003, Monte, 2001).

2.5.2. Direct biocontrol mechanisms of *Trichoderma* strains

2.5.2.1. Competition and rhizosphere competence

As soil saprophytes, *Trichoderma* strains grow rapidly when put in the soil, because they are 'adapted to aggressive colonisation of available nutrient bases and to quiescent persistence as chlamydospores and conidia when nutrients are lacking' (Benítez *et al.*, 2004, Hjeljord and Tronsmo, 1998). Furthermore, they are 'naturally resistant to many toxic compounds, including herbicides, fungicides and pesticides such as DDT and phenolic compounds and can recover very fast after the addition of sublethal doses of some of these compounds' (Benítez *et al.*, 2004). This ability might be due to ATP-binding cassette (ABC) transporters which are ATP-dependent permeases that 'mediate the transport of many different substrates through biological membranes' (Harman *et al.*, 2004). An 'overexpression of ABC-transporter genes decreases the accumulation of toxicants in *Trichoderma* cells' (Harman *et al.*, 2004).
The most useful *Trichoderma* strains for biological control have an additional feature that is referred to as 'rhizopshere competence'. Rhizosphere competence is 'the ability to colonise and grow in association with plant roots' (Harman *et al.*, 2004). This property may not be among the principal mechanisms for efficient biological control but it is certainly a good additional feature of a successful biocontrol agent, since rhizosphere competence is crucial for competition for space and nutrients (Howell, 2003). Nutrients in the rhizosphere originated from plants are e.g. root exudates and necrotic tissue (Hjeljord and Tronsmo, 1998).
There are several examples for the involvement of competition in successful biocontrol of pathogens, e.g. competition has been the driving force in the inhibition of chlamydospore germination of *Fusarium oxysporum* by *Trichoderma harzianum* (Sivan and Chet, 1989). It has been shown that seed treatments with *Trichoderma* isolates reduced sporangia germination of *Pythium ultimum* (damping-off). This has been attributed to competition for germination stimulants (Hjeljord and Tronsmo, 1998). *Trichoderma* was also successfully used in phyllosphere applications against *Botrytis* and *Sclerotinia* spp. on grape, strawberry, apple and cucumber (Elad and Kapat, 1999, Hjeljord and Tronsmo, 1998), i.e. *Trichoderma* competes for necrotic tissues as nutrient source. Early colonisation of fresh wound sites is another form of nutrient competition. Those fresh wound sites are poorly colonised by beneficial or

neutral organisms and vulnerable to pathogen attack, therefore they might be the most promising sites for biological control in the phyllosphere by introduced antagonists. There are some successful examples of *Trichoderma* strains which are effective against pathogens colonising wound sites, e.g. *Botrytis* and *Pythium* (Hjeljord and Tronsmo, 1998).

With regard to the successful examples mentioned above competition is most likely to be a successful biological control strategy against pathogens. However, this is dependent on 'rapid colonization of the site or nutrient base by *Trichoderma*, so that the site has to be heavily colonised before other microbes become established' (Hjeljord and Tronsmo, 1998). Furthermore, competition does not exclude other control mechanisms and may not be the most effective one.

2.5.2.2. Antibiosis

Trichoderma strains produce volatile and non-volatile secondary metabolites, which inhibit other microorganisms with which they are not physically in contact. This inhibition process is referred to as antibiosis (Hjeljord and Tronsmo, 1998). 'Among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described'. 'In some cases, the production of such metabolites correlates with the biocontrol ability of the strain', i.e. 'purified antibiotics mimic the effect of the whole agent' (Benítez *et al.*, 2004).

The level of antagonism mediated by antibiotics is increased in combination with hydrolytic enzymes (Benítez *et al.*, 2004, Elad and Kapat, 1999, Howell, 2003). However, *Trichoderma* spp. differ in their abilities to produce various antibiotics. Furthermore, the production is also affected by the environment qualitatively as well as quantitatively, and the various pathogens are affected differently by the metabolites (Hjeljord and Tronsmo, 1998).

2.5.2.3. Mycoparasitism

In contrast to antibiosis, where inhibition takes place without physical contact, mycoparasitism is the ability of *Trichoderma* spp. to parasitise or attack other fungi directly (Benítez *et al.*, 2004, Hjeljord and Tronsmo, 1998). Four stages are involved

in mycoparasitism: chemotrophic growth, recognition, attachment and coiling, and lytic activity.

i) Chemotrophic growth

Positive chemotropism is directed growth towards a chemical stimulus. *Trichoderma* is able to detect its host from a distance. Subsequently, the antagonist begins to branch in an atypical way, i.e. the branches are growing towards the target fungus. Amino acids and sugars induce this growth (Chet *et al.*, 1998). This remote sensing is at least partially due to the sequential expression of cell-wall-degrading enzymes by *Trichoderma* spp.. Different strains can follow different patterns of induction, but the fungi apparently always produce low levels of an extracellular chitinase. Diffusion of this enzyme catalyses the release of cell-wall oligomers from target fungi, and this in turn induces the expression of fungitoxic endochitinases, which also diffuse and begin the attack on the target fungus before contact is actually made (Harman *et al.*, 2004).

ii) Recognition

Molecular recognition between the host and the antagonist is an essential event in mycoparasitism. Chemical recognition involves hydrophobic interactions, or interactions between complementary molecules present at the surfaces of the host and the parasite. In such recognition processes lectin-carbohydrate interactions are involved. Lectins are sugar-binding proteins or glycoproteins which agglutinate cells and are involved in the interactions between the cell surface components and their extracellular environment. Recognition initiates differentiation processes in *Trichoderma* which lead to the formation of infection structures (Chet *et al.*, 1998).

iii) Attachment and coiling

Following recognition, *Trichoderma* hyphae attach to the host via the formation of hook-like structures and appressorium-like bodies (Figure 3) and coil around the hyphae of the pathogen (Figure 2). These parasitic interactions occur both *in vitro* and *in vivo*, however, the intensity in soil is lower, probably due to lower concentration of nutrients (Chet *et al.*, 1998). After this typical attachment and coiling event lytic enzyme activity begins.

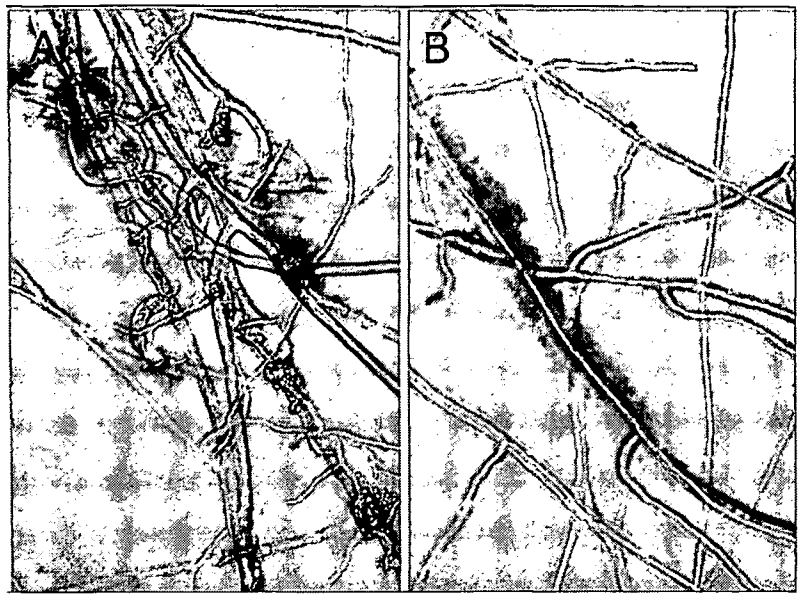

Figure 2: Mycoparasitism of *Rhizoctonia solani* by *Trichoderma virens*: A, parent strain coiling around host hyphae, and B, mycoparasitic mutant with no coiling or penetration of host hyphae (from Howell, 2003).

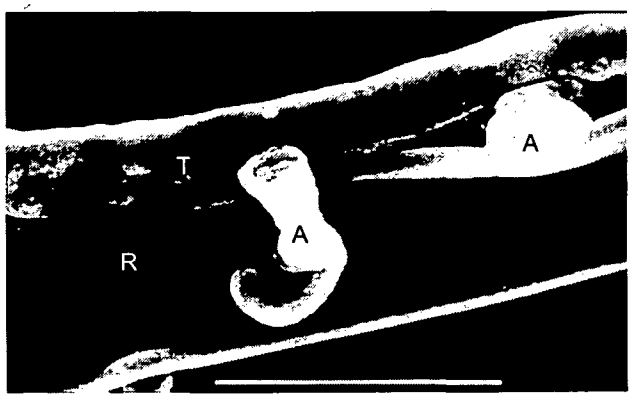

Figure 3: Appressoria-like structures (A) of *Trichoderma* (T) on *R. solani* (R) (from Harman *et al.*, 2004).

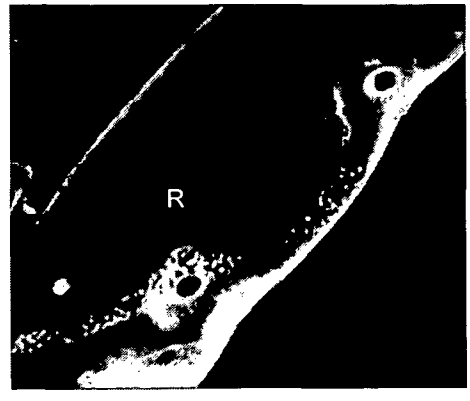

Figure 4: *R. solani* (R) hyphae from which *Trichoderma* has been removed (from Harman *et al.*, 2004).

iv) Lytic activity

Trichoderma spp. have the ability to degrade fungal cell walls due to cell-wall-degrading enzymes, mostly chitinases (1,4-β-acetylglucosaminidase, endochitinases and exochitinases), glucanases (e.g. β-1,3-glucanases) and proteases (Benítez *et al.*, 2004). The production of cell-wall-degrading enzymes (CWDEs) and probably peptaibols (peptides that exhibit antibiotic and membrane channel-forming activities) (Schirmböck *et al.*, 1994) results in dissolution of the cell walls. At the sites of the appressoria, holes can be produced (Figure 4) and therefore provide entry for the *Trichoderma* hyphae into the lumen of the parasitized fungus (Benítez *et al.*, 2004, Chet *et al.*, 1998, Harman *et al.*, 2004).

Mycoparasitism activity of *Trichoderma* spp. is very strain-specific due to the different arrays of expression of CWDEs (Benítez *et al.*, 2004). ABC transporters are probably necessary for the establishment of mycoparasitic interactions because it has been shown that knockout mutants lacking these specific transporters were inhibited by toxins from *B. cinerea*, *R. solani* and *P. ultimum* and were less effective fungal parasites (Harman *et al.*, 2004).

2.5.3. Indirect biocontrol mechanisms of *Trichoderma* strains

2.5.3.1. Induction of defense responses in plants

Induced resistance involves activation of plant defense systems resulting in protection of plants from pathogens or pests. The defense response induced by plant-microbe interactions may remain localized or become systemic. It has been shown that strains of *Trichoderma* added to the rhizosphere protect plants against numerous classes of pathogens, e.g. those that produce aerial infections (viral, bacterial and fungal pathogens). This indicates that mechanisms similar to the hypersensitive response (HR), systemic acquired resistance (SAR) and induced systemic resistance (ISR) in plants are induced by *Trichoderma* spp. (Benítez *et al.*, 2004, Harman *et al.*, 2004). This plant protectional effect is accompanied by an increase in the concentration of metabolites and enzymes related to defensive mechanisms, e.g. phenyl-alanine ammonio-lyase (PAL) and chalcone synthase (CHS). These metabolites are involved in the biosynthesis of phytoalexins (HR

response), chitinases and glucanases, which comprise pathogenesis-related proteins (PR) and enzymes involved in the response to oxidative stress (Benítez *et al.*, 2004). Crucial for induced resistance is the recognition of signals by the host plant (Bailey and Lumsden, 1998). In this process specific *Trichoderma* metabolites, e.g. peptides, proteins and low-molecular-weight compounds, act as elicitors for plant defense mechanisms, indicating that the stimulation by the living organism is not necessary (Benítez *et al.*, 2004, Harman, 2006, Harman *et al.*, 2004). This provides the possibility to use the enzymes as a spray or drench application instead of the whole fungus to induce ISR and generate protection of the plant (Woo *et al.*, 2006).

2.5.3.2. Biofertilisation

Another indirect but more adjunct effect is biofertilisation. Root colonisation by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stress and the uptake and use of nutrients (Benítez *et al.*, 2004, Howell, 2003).

2.6. Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) are the most common underground symbiosis in the roots of a wide variety of host plants. This symbiosis is biotrophic and normally mutualistic (Smith and Read, 1997). Both symbiotic partners derive benefits from this association, i.e. the host plant gains for instance better tolerance for nutrient deficiencies of potassium, phosphorus, calcium and other minerals and an increase in nutrient and water absorption through enhanced absorbance area, whereas, the fungus in return carbon, which means energy (Gupta *et al.*, 2000). AMF are endomycorrhizas which belong to the phylum Glomeromycota (Schüssler *et al.*, 2001) of the order Glomales. Characteristic for the endomycorrhizas is the intracellular penetration of root cortical cells by the symbiotic fungi and formation of highly branched haustoria-like structures named arbuscules. These arbuscules are the site of nutrient exchange between the two symbionts (Gupta *et al.*, 2000). Another interesting aspect of this association is its bioprotective value against soil-borne pathogenic fungi. Reduction of disease symptoms has been described for

fungal pathogens such as *Phytophthora*, *Fusarium*, *Phytium*, *Rhizoctonia*, *Verticillium* and *Pyrenochaeta*, for bacterial pathogens and for nematodes (Singh *et al.*, 2000). The bioprotective effect can be local or systemic (Pozo *et al.*, 2002, Xavier and Boyetchko, 2004). However, the effect seems to depend on several factors such as the host genotype, the AMF and the degree of mycorrhization as reviewed by Vierheilig *et al.* (2007). Local as well as systemic bioprotective effects have been linked to high levels of AM root colonisation. Furthermore, inoculation of the host by AMF should be prior to inoculation with the pathogen (Singh *et al.*, 2000).
Along with the crucial factors mentioned before several mechanisms are said to be involved in mycorrhization-mediated-bioprotection. Some of those are discussed below:

i) Improved plant nutrition

Due to enhanced host nutrition triggered by AMF mycorrhizal plants are more tolerant to pathogens and can compensate for root damage and photosynthate drain by pathogens (Xavier and Boyetchko, 2004).

ii) Competition

AMF may not show competition when it comes to simultaneous colonisation with the pathogen due to its relatively slow growth rate. However, following root entry, competition can occur for infection sites, host photosynthates (carbon) and root space (Xavier and Boyetchko, 2004).

iii) Physiological and biochemical alterations of the host

The host tissue phosphorus levels are typically enhanced when colonised by AMF. This causes a modification of the phospholipid composition and, consequently, the root membrane permeability resulting in a reduction in the leakage of net amount of sugars, carboxylic acids and aminoacids into the rhizosphere. These alterations arrest the chemotactic effect of pathogens to plant roots (Xavier and Boyetchko, 2004). Furthermore, it has been shown that the exudates of mycorrhizal plants have different effects on pathogen propagule germination than exudates of non-mycorrhizal plants (Scheffknecht *et al.*, 2006). When AMF enter host roots they induce a local, weak, and transient activation of the host defence mechanism against pathogens, which comes along with the induction of hydrolytic enzymes. AMF also

induce phytoalexins and elicit induced resistance locally as well as systemically (Xavier and Boyetchko, 2004).

2.7. Root exudates in rhizosphere interactions

2.7.1. The rhizosphere

The rhizosphere is defined as 'the soil zone that surrounds and is influenced by the roots of a plant' (Bais *et al.*, 2006), i.e. by the root activity (Bertin *et al.*, 2003). It is a very important and dynamic area where root growth, exudate production and community development of macro and micro biota take place (Bertin *et al.*, 2003). Strictly speaking the rhizosphere is the place for root-root, root-insect and root-microbes interactions (Bais *et al.*, 2006) and therefore also for interactions between soil-borne pathogens and their host plants. Important mediators for these interactions are root exudates (Bais *et al.*, 2006, Nelson, 1990).

2.7.2. Root exudates

Root exudation comprises 'the secretion of ions, free oxygen and water, enzymes, mucilage and a diverse array of carbon-containing primary and secondary metabolites' (Bais *et al.*, 2006). Exudates can be soluble as well as volatile (Nelson, 1990). Diffusion, ion channel and vesicle transport are the three major ways in which root exudates are released from living root systems (Bertin *et al.*, 2003).

The root exudates can be grouped in low-molecular and high-molecular weight compounds. The low-molecular weight compounds comprise sugars and simple polysaccharides (such as arabinose, fructose, glucose, maltose, mannose, oligosaccharides), amino acids (such as arginine, asparagine, aspartic, cysteine, cystine, glutamine), organic acids (such as acetic, ascorbic, benzoic, ferulic, malic acids), phenolic compounds, alcohols and aldehydes (Bais *et al.*, 2006, Bertin *et al.*, 2003, Nelson, 1990). These low-molecular weight compounds contribute to much of the diversity of root exudates (Bais *et al.*, 2006) and especially the phenolics influence the growth and development of surrounding plants and soil microorganisms (Bertin *et al.*, 2003). In contrast the high-molecular weight compounds such as flavonoids, enzymes, fatty acids, growth regulators, nucleotides, tannins,

carbohydrates, steroids, terpenoids, alkaloids, polyacetylenes and vitamins are less diverse but often compose a larger proportion of the root exudates by mass (Bais *et al.*, 2006, Bertin *et al.*, 2003). Factors such as plant species, age and physiological state of the plant, soil pH, soil moisture, temperature, presence of microorganisms etc. influence the composition of root exudates (Bais *et al.*, 2006, Bertin *et al.*, 2003, Nelson, 1990).

2.7.3. Rhizosphere interactions

Regarding the versatile compounds of root exudates it is not surprising that root exudates elicit various interactions in the rhizosphere (Bais *et al.*, 2006, Bertin *et al.*, 2003, Nelson, 1990). Such interactions are e.g. allelopathy, induced herbivore resistance, induced herbivore defence via predator attraction, phytosiderophores and micronutrient availability, organic acids and phosphorus availability, nodulation of legumes by rhizobia, mycorrhizal associations, plant growth-promoting bacteria and negative plant-microbe interactions like root-pathogen interactions (Bais *et al.*, 2006, Bertin *et al.*, 2003, Nelson, 1990).

Essential for the understanding and for the potential control strategies of soil-borne plant diseases are root-pathogen interactions which are initiated by root exudates. One limiting factor for microbial growth and activity is nutrient availability (Nelson, 1990). The rhizosphere serves plenty of nutrients and can therefore be regarded as an 'oasis in the desert' in bulk soil (Bertin *et al.*, 2003, Nelson, 1990). Pathogenic fungi as well as other microorganisms exist in a state of exogenous dormancy or fungistasis when nutrients are not available. However, fungistasis can be alleviated by appropriate organic and inorganic stimulants, which are components of seed and root exudates released during seed germination and root development. On one hand the understanding of the types of molecules present in seed and root exudates is fairly good but on the other hand the knowledge about how and when specific exudates components interact with root-infecting pathogens is very little (Nelson, 1990).

Consequently, 'since many seed- and root-infecting pathogens are highly dependent on exudate molecules to initiate plant infections, interference with the production and activity of exudate stimulants is a promising approach by which to interfere with the pathogen activity and achieve biological control' (Nelson, 1990).

3. Material and Methods

3.1. Tomato cultures inoculated with *Trichoderma* spp.

To determine whether *Trichoderma* spp. alter the effect of root exudates of tomato on the growth of soil-borne pathogenic fungi, tomato plants were inoculated with three different *Trichoderma* spp. strains. *In vitro* as well as climate chamber tomato cultures were performed.

3.1.1. The fungal material

Trichoderma viride strain RE 1-3-4, *T. harzianum* strain T39 and *T. atroviride* strain P1 were used as fungal material (Table 1). All *Trichoderma* spp. were cultivated on Potato Dextrose agar (PDA, Appendix) at 24°C in the dark.

Table 1: Origin of the different *Trichoderma* spp. strains.

Trichoderma spp.	Origin
Trichoderma viride strain RE 1-3-4	Gabriele Berg, University of Rostock, Germany
Trichoderma harzianum strain T39	Yigal Elad, Agricultural Research Organization, The Volcani Center, Israel
Trichoderma atroviride strain P1	Robert Mach, University of Technology, Vienna

3.1.2. *Trichoderma* inoculum

For the inoculation with *Trichoderma* spp., wheat bran culture germlings (Larkin and Fravel, 1998) were used. Depending on the treatment to the autoclaved (3 times, 121°C) and moistened wheat bran (bran:water, 1:1, wt/vol) *Trichoderma* spp. spore solutions or autoclaved distilled water was added.

For the spore solutions, the different *Trichoderma* strains (Table 1) were incubated on PDA in the dark at 24°C. When sporulation occurred, dishes were flooded with autoclaved distilled water and rubbed with a "Drigalski" spatula. The solutions were passed through 3 layers of cheese cloth (fleece filters, 20-150 µm pore diameter; Laporte, Wels, Austria). Thereafter the spore concentrations were determined using a haemocytometer and were adjusted to the final concentrations, which are presented in Table 2.

The wheat bran was mixed with the spore solutions or sterile water (1:1 bran:solution or water wt/vol) in a beaker. Thereafter the beakers were covered with aluminium foil and sealed with "Nescofilm" (Bando Chemical IND. LTD, Kobe, Japan). The beakers were incubated in the dark at a temperature of 24°C for 14 days for the *in vitro* tomato cultures and for 4 days for the climate chamber tomato cultures.

Table 2: Spore concentrations of *Trichoderma* spp. for wheat bran culture germlings prepared for the *in vitro* and climate chamber tomato cultures.

Experiment	*Trichoderma* strain	Spores/ml
In vitro tomato cultures	T39	4×10^6
	RE 1-3-4	2×10^6
	P1	3×10^6
Climate chamber tomato cultures	T39	1×10^6
	RE 1-3-4	1×10^6
	P1	1×10^6

3.1.3. *In vitro* cultures of tomato inoculated with *Trichoderma* spp.

3.1.3.1. Surface sterilisation of the seeds

Seeds of *Solanum lycopersicum* L. cv. Moneymaker were surface-sterilized in 50% commercial bleach for 10 minutes and afterwards rinsed 3 times for 10 minutes with sterile distilled water.

3.1.3.2. Substrates and potting procedure

Preserving jars (400 ml) were autoclaved and filled with 15 g of autoclaved and dried (drying chamber, 180°C) perlite (Granuperl S 3-6, Knauf Perlite GmbH, Vienna, Austria). Afterwards the jars were placed in a sterile chamber at 180°C. 8 jars were prepared for each treatment (Table 3).

The *Trichoderma* inoculum was added to the perlite in the jars at a rate of 1 % (wt/vol) (Larkin and Fravel, 1998). Jars were covered with sterile Petri dish lids (Art. Nr. 632 180, Greiner Bio-One GmbH, Kremsmünster, Austria) and shaken thoroughly to mix the perlite properly with the wheat bran. Thereafter, 40 ml of autoclaved (121°C) liquid fertiliser were added to each jar. The liquid fertiliser consisted of 472.30 mg $Ca(NO_3)_2$, 261.40 mg K_2SO_4, 136.00 mg KH_2PO_4, 369.72 mg $MgSO_4$, 8.004 mg NH_4NO_3, 50.000 mg $Fe_6H_5O_7$ x 3 H_2O, 1.30 mg $Na_2BO_4O_7$ x 4 H_2O, 1.500 mg $MnSO_4$ x 4 H_2O, 0.60 mg $ZnSO_4$ x 7 H_2O, 0.450 mg $CuSO_4$ x 5 H_2O, 0.028 mg $Al_2(SO_4)_3$, 0.028 mg $NiSO_4$ x 7 H_2O, 0.028 mg $Co(NO_3)_2$ x 6 H_2O, 0.028 mg TiO_2, 0.014 mg $LiCl_2$, 0.014 mg $SnCl_2$, 0.014 mg KJ, 0.014 mg KBr and 0.070 mg MoO_3 (Steineck, 1951, modified). 3 surface-sterilized seeds of *Solanum lycopersicum* were put in each jar. Finally, the jars were covered with lids of plastic Petri dishes, which were fixed to the jars with adhesive tape.

Table 3: Treatments of the *in vitro* culture experiment.

Substratum	Plant material	Treatment	Number of jars	Duration of cultivation (d)
Perlite	Seeds	Control	8	40
Perlite	Seeds	T39	8	40
Perlite	Seeds	RE 1-3-4	8	40
Perlite	Seeds	P1	8	40

3.1.3.3. Plant growth

Plants were incubated in a growth chamber (16-h-photoperiod, 20°C) for 40 days. They were observed in regular intervals for different developments compared to the control plants.

3.1.3.4. Collection of root exudates

After 20 days the roots of the plantlets were washed under sterile conditions and submerged in autoclaved distilled water for 6 hours. Afterwards the root fresh weight was determined and the exudates were adjusted to a concentration of 20 ml exudate per 1 g root fresh weight with autoclaved distilled water. Then the root exudates were passed through 0.22 µm sterile filters (Steriflip, Millipore, Bedford, U.S.A.) and were stored at -20°C.

3.1.4. Climate chamber cultures of tomato inoculated with *Trichoderma* spp.

3.1.4.1. Preparation of the plant material

For the seedling production surface-sterilized seeds (see section 3.1.3.1) were put in pots with autoclaved perlite and slightly covered with the substrate. Pots were irrigated from below with liquid fertiliser (see section 3.1.3.2) and covered with Petri dish lids until germination to reduce evaporation. After 10 days they were transplanted to the final substrate as mentioned below.

3.1.4.2. Substrates and potting procedure

Perlite and a potting mix of soil and "Leca"-granulate in a ratio of 1:1 were used as substrates. All substrates were autoclaved (121°C) before use. The treatments are shown in Table 4.

Table 4: The different treatments of the tomato plants.

Substratum	Plant material	*Trichoderma* spp.	Number of pots	Cultivation period (d)
Perlite	Seeds	no	6	36
		T39	6	36
		P1	6	36
		RE-1-3-4	6	36
Perlite	Seedlings (10 days old)	no	6	24
		T39	6	24
		P1	6	24
		RE-1-3-4	6	24
Soil + "Leca" 1:1	Seeds	no	6	36
		T39	6	36
		P1	6	36
		RE-1-3-4	6	36
Soil + "Leca" 1:1	Seedlings (10 days old)	no	6	24
		T39	6	24
		P1	6	24
		RE-1-3-4	6	24

For the cultivation of the plants pots with a volume of 630 ml were used. According to Larkin and Farvel (1998) the wheat bran culture germlings were added to the substratum at a rate of 1% of the volume. Corresponding to the treatment (Table 4) 3 seeds or 3 seedlings were placed in one pot.

3.1.4.3. Plant growth

Plants were cultivated, in a growth chamber at 24°C with a 16-hour photoperiod for 36 and 24 days, respectively. Plants cultivated in perlite were irrigated from below with a liquid fertiliser (see section 3.1.3.2), plants cultivated in soil + "Leca" were irrigated with tap water.

3.1.4.4. Collection of the root exudates

After 34 and 24 days, respectively, the roots of the plants were washed thoroughly and submerged in autoclaved distilled water for 6 h. Thereafter, the root fresh weight was determined and the root exudates were adjusted to a final concentration of 20ml/g root fresh weight with autoclaved distilled water. Subsequently, the root exudates were passed through sterile filters (0.22 µm) and stored at -20°C.

3.2. Tomato cultures co-inoculated with *Trichoderma* spp. and arbuscular mycorrhizal fungi

In order to determine whether *Trichoderma* spp. and/or arbuscular mycorrhizal fungi (AMF) alter the effect of exudates of tomato on the growth of soil-borne pathogenic fungi, tomato plants were inoculated with three different *Trichoderma* spp. strains and/or AMF.

3.2.1. Fungal material

Trichoderma viride strain RE 1-3-4, *T. harzianum* strain T39 and *T. atroviride* strain P1 were used as fungal material (Table 1). All *Trichoderma* spp. were cultivated on PDA at 24°C in the dark. For the inoculation of the tomato plants with AMF a commercially available fungal inoculum ("Symbivit", SYMbio-M; Czech Republic) was used. This fungal inoculum consisted of *Glomus intraradices* BEG 98, *G. mosseae* BEG99, *G. claroideum* BEG93, *G. microagregatum* BEG56, *G. caledonium* BEG97 and *G. etunicatum* BEG92.

3.2.2. Preparation of the plant material

Surface-sterilized seeds (see section 3.1.3.1) were placed in pots with autoclaved perlite so that they were slightly covered. Tap water was used for irrigation. Pots were put in a growth chamber at 24°C with a 16-hour photoperiod. Pots were covered with Petri dish lids until germination to reduce evaporation. Plantlets were cultivated for 4 weeks before transplanting.

3.2.3. Substrate and potting procedure

For the greenhouse cultures a substrate was used which consisted of two parts of a mixture of soil, sand and "Leca" in a 3:3:1 ratio and one part of the arbuscular mycorrhizal fungi inoculum ("Symbivit"). The fungal inoculum was either autoclaved or non-autoclaved depending on the treatment (Table 5). The other parts of the substrate were autoclaved. The bottoms of the pots (305 ml) were covered with a layer of filter paper. In each pot one 4-week-old tomato plantlet (3.2.2) was planted. 20 plants were used per treatment (Table 5).

Table 5: The different treatments for the AMF biotest.

Treatment	Abbreviations
Control - autoclaved AMF inoculum	C
Autoclaved AMF + *Trichoderma harzianum* strain T39	T39
Autoclaved AMF + *Trichoderma atroviride* strain P1	P1
Autoclaved AMF + *Trichoderma viride* strain RE 1-3-4	RE
AMF	AMF
AMF + *Trichoderma harzianum* strain T39	AMF + T39
AMF + *Trichoderma atroviride* strain P1	AMF + P1
AMF + *Trichoderma viride* strain RE 1-3-4	AMF + RE

3.2.4. Inoculation with *Trichoderma* spp.

Trichoderma strains were added as a spore solution (1.5-4 x 10^6 spores/ml) at a volume of 5 ml per pot 1 week after potting.

After the application of the conidial suspensions with a pipette directly on top of the substrate, the surfaces of the pots were covered with cotton wool to prevent cross-contamination induced by water drops.

3.2.5. Plant growth

Plants were irrigated from below using a liquid fertiliser, which consisted of the same components as mentioned in section 3.1.3.2., apart from KH_2PO_4 which was not

added to this fertiliser. Plants were cultivated for 8 weeks in a greenhouse (22°C/18°C day/night). In the second week of cultivation plants were grown in a 16-hour-photoperiod (1,000 lux).

3.2.6. Collection of root exudates

For the collection of the root exudates the roots of the 8-week-old plants were washed thoroughly and submerged in autoclaved distilled water for 6 h. Thereafter the root fresh weight was determined and the root exudates were adjusted with autoclaved distilled water to a final concentration of 20ml/g root fresh weight. Afterwards root exudates were passed through sterile filters (0.22 µm) and stored at -20°C.

3.2.7. Staining and measuring of mycorrhizal colonisation

For the determination of the root colonisation, 2-cm-long root samples were taken at a depth of 1 cm. The root pieces were cleared by boiling them for 5 min in 10% KOH. Afterwards they were rinsed three times with tap water and stained according to the method of Vierheilig *et al.* (1998) by boiling them for 3 min in a 5% ink (Shaeffer; black)/household vinegar (= 5% acetic acid) solution. After staining, the percentage of root colonisation was determined using the gridline intercept method (Giovannetti and Mosse, 1980). The coloured roots were spread over a grid under a binocular and 100 crossings of roots with gridlines were checked for colonisation (Figure 5).

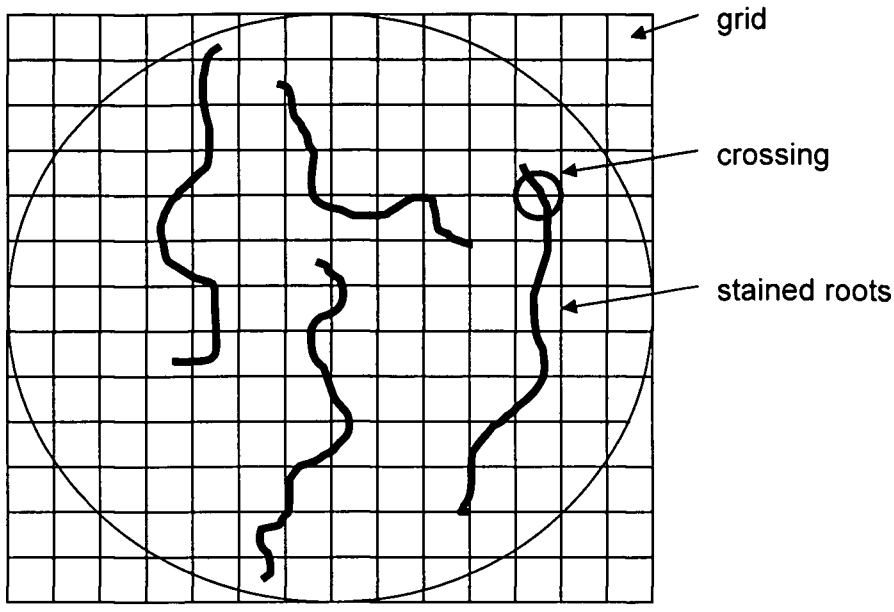

Figure 5: Gridline intercept method. Circle highlights one root-gridline crossing.

3.3. Cultivation of the tomato pathogen *Pyrenochaeta lycopersici*

In order to be able to perform optical density measurements with *P. lycopersici* several tests were done for optimal growth conditions and the production of large amounts of mycelium.

3.3.1. Tests for optimal growth conditions

To determine the optimal cultivation conditions several media, photoperiods and temperatures were tested. The different media and growth conditions are shown in Table 6. Detailed information about the composition of the media is given in Appendix.

Table 6: Different media and growth conditions for *P. lycopersici.*

Medium (Appendix)	Photoperiod	Temperature [°C]
Potato Dextrose Agar (PDA)	dark	18
Potato Dextrose Agar (PDA)	16 h light	24
V8 Agar	dark	18
V8 Agar	16 h light	24
Czapek Dox broth	16 h light	24
Potato Dextrose broth	16 h light	24
V8 broth	16 h light	24
Malt Extract broth	16 h light	24

For solid media cultures Petri dishes were used, whereas for liquid media cultures, Erlenmeyer flasks were filled with 150 ml of liquid medium. The solid media as well as the liquid media were inoculated with one plug (0.64 mm^2) of *P. lycopersici* (strain 62931, DMSZ). The liquid media cultures were incubated on a rotary shaker at a speed of 150 rpm. The growth of the cultures was checked daily.

3.3.2. Tests for microsclerotia and pycnidia induction

To induce pycnidia formation, 12-day-old mycelium grown on acidified PDA (Appendix) was transferred to water agar (Appendix) and incubated in the dark for 3 days. According to Shishkoff (1993) the mycelium was transferred to a double strength V8 agar (Appendix) and incubated at 24°C with a 16-h-photoperiod.

According to Ball (1979) drops of Malt Extract agar were placed on sterilized microscope slides and were inoculated with mycelium of *P. lycopersici.* Thereafter the microscope slides were incubated in sterile humid chambers at 24°C with a 16-h-photoperiod. Autoclaved glass Petri dishes were used as humid chambers. They were provided with filter paper, which was moistened with 5 ml of autoclaved distilled water. After 7 days the cultures were checked for microsclerotia formation.

Mycelia on the margins of the liquid medium cultures of Table 6 were also checked microscopically for microsclerotia formation.

3.4. Experimental set-up to establish a bioassay for the mycelial growth of *Pyrenochaeta lycopersici*

To establish a bioassay for the mycelial growth of P. lycopersici in root exudates of tomato the optimal preparation of the fungal material and different growth media were tested.

3.4.1. Homogenisation of the fungal material

5-day-old mycelia of *P. lycopersici* from liquid media cultures (V8 broth) were used. Several tests were done for a proper preparation of the mycelium. These approaches can be seen in Table 7.

Table 7: Treatments for homogenising the mycelium of *P. lycopersici.*

Method	Time	Speed
Vortexing in water (1.5 ml Eppendorf tube)	1 min	40 Hz
Vortexing in water (1.5 ml Eppendorf tube)	1 ½ min	40 Hz
Vortexing in water (2 ml Eppendorf tube)	1 min	40 Hz
Vortexing in water (2 ml Eppendorf tube)	1 ½ min	40 Hz
Magnetic stirrer + water & 30 glass beads (test-tube)	10 min	1,000 rpm
Magnetic stirrer + water & 60 glass beads (test-tube)	10 min	1,000 rpm
Magnetic stirrer + water + sand (test-tube)	10 min	1,000 rpm
Pestle (1.5 ml Eppendorf tube)	Until material appeared homogenous	

3.4.2. Media test

The optical density (OD) of different growth media (Table 8) was determined. The V8 juice for the media was passed through 2 layers of cheese cloth before use. For the

supernatant production the media were centrifugated at 4,300 rpm for 10 min. The wells of a sterile 96-well-culture plate (Art. Nr. 655 061, Greiner Bio-One GmbH, Kremsmünster, Austria) were filled with 100 µl of the corresponding medium. Then the measurement was carried out at 600 nm (absorption) with a microplate reader (Tecan Spectra Classic).

Table 8: Growth media for optical density measurements.

Growth medium
Water
Czapek Dox broth
Potato Dextrose broth
Potato Dextrose broth supernatant
V8 juice
V8 juice supernatant
V8 broth
V8 broth supernatant
Malt Extract broth

3.4.3. Fungal growth in different media

For fungal growth tests aliquots of 100 µl of the different media (Table 8) were filled in the wells of the microplate using multi-channel pipettes. Subsequently, 25 µl of mycelial solution (500 mg/l) were added into the wells. For the mycelial solution 5-day-old liquid *P. lycopersici* cultures incubated in V8 broth in Erlenmeyer flasks were passed through 3 layers of cheese cloth. The mycelia were homogenised with a pestle (Table 7). Thereafter, the homogenised mycelia were adjusted to the final concentration of 500 mg/ml with autoclaved distilled water. Fungal growth was determined by measuring the optical density at 600 nm with a microplate reader. Microplates were incubated on a rotary shaker (150 rpm) at 24°C with a 16-h-photoperiod for 72 h. Measurements were carried out directly after pipetting and in 24-hour intervals for 120 hours.

3.4.4. Mycelium concentrations

The next step was to determine the appropriate amount of mycelium for the microplate reader growth assay. Therefore, several mycelium concentrations were tested, which were prepared as mentioned in section 0. Thereafter the solutions were passed through a fine sieve (100 µm pore diameter). The used concentrations were 1, 10, 50 and 100 g of mycelium in 1 l water.

3.4.5. Growth of *P. lycopersici* in tomato root exudates

For the experiments with root exudates, mycelium solutions of *P. lycopersici* with a concentration of 10 g/l were used. The control media were V8 broth supernatant and Czapek Dox broth. 2 Microplates were prepared. Each well was filled with 25 µl of mycelial suspension or water and with 100 µl of medium or exudates (tomato cv. Money Maker, 30 d, perlite). 24 wells were prepared for *P. lycopersici* solution and Czapek Dox broth and 24 wells for *P. lycopersici* solution and V8 broth supernatant, respectively. 48 wells were filled with mycelial solution and tomato exudates. Marginal wells were not taken into consideration for the results.

3.5. Fungal growth assay

To test whether the effect of tomato root exudates on the growth of soil-borne pathogens is altered by *Trichoderma* spp. and/or AMF inoculation, a fungal growth assay was performed.

3.5.1. Fungal material

Since the mycelial solutions of *Pyrenochaeta lycopersici* were not appropriate for the fungal growth assay (see section 4.4), the pathogen *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) was used.

The *Fol* isolate 007 was kindly provided by B.J. Cornelissen; Institute for Molecular Cell Biology, Amsterdam. *Fol* was cultivated for 2 weeks on Czapek Dox Agar at 24°C in darkness. Petri dishes of 2-week-old cultures were filled with autoclaved distilled water and rubbed with a "Drigalski" spatula. The solution was passed through

3 layers of cheese cloth. Thereafter the number of microconidia per ml solution was determined using a haemocytometer and adjusted to 1 x 10^7 microconidia/ml water.

3.5.2. Preparation of the microplates and OD measurement

Aliquots of 175 µl of autoclaved distilled water, Czapek Dox medium or root exudates were mixed with 35 µl of spore suspension (*F. oxysporum* f. sp. *lycopersici*, 1 x 10^7 microconidia/ml water) in sterile 96-well-culture plates and incubated at 24°C in the dark while shaking (150 rpm) for 5 days. Fungal growth was assessed by an optical density measurement (absorption, 600 nm) which was carried out every 24 h using a microplate reader (Tecan Spectra Classic).

2 to 4 exudates per treatment (Table 4) of the tomato cultures of the climate chamber inoculated with *Trichoderma* spp. were used. In one plate 6 wells were filled with 1 exudate per treatment and each exudate was repeated 3 times.

4 exudates per treatment (Table 5) of the tomato cultures with *Trichoderma* spp. and AMF co-inoculation were used. In one plate 6 wells were filled with 1 exudate per treatment and each exudate was repeated 3 times.

The wells in the outer ring of the microplate were not taken for the statistical analysis.

3.6. Dual culture tests

To see if *T. viride* strain RE 1-3-4, *T. harzianum* strain T39 and *T. atroviride* strain P1 have antagonistic effects on the three tomato pathogens, *P. lycopersici*, *Fol* and *V. dahliae*, *in vitro* dual culture tests were performed. The tests were based on the experiments of Bell *et al.* (1982) and Pérez *et al.* (2002).

3.6.1. Fungal material

Pyrenochaeta lycopersici (strain 62931, DMSZ) was cultivated on V8 Agar at 24°C at a 16-hour photoperiod. *Fusarium oxysporum* f. sp. *lycopersici* (isolate 007) and *Verticillium dahliae* (kindly provided by Jose M. García-Garrido, EEZ-CSIC, Granada) were cultivated on Czapek Dox agar (CzA) and the 3 *Trichoderma* strains (RE 1-3-4, P1 and T39) on Potato Dextrose agar (PDA) at 24°C in the dark.

3.6.2. Dual cultures of *Pyrenochaeta lycopersici* and *Trichoderma* spp.

A plug (0.64 mm²) of *P. lyopersici* was placed on one margin of a Petri dish with CzA and incubated at 24°C at a 16-hour photoperiod. After 10 days a plug (0.64 mm²) of a *Trichoderma* strain (RE 1-3-4, P1 or T39) or no plug for the control was added on the opposite site of *P. lycopersici*. The dishes were incubated in the dark at 24°C for 7 days and photographed daily. Each *Trichoderma* strain was run in triplicate, which was repeated three times (Pérez *et al.*, 2002).

3.6.3. Dual cultures of *Fusarium oxysporum* f. sp. *lycopersici*, *Verticillium dahliae* and *Trichoderma* spp.

F. oxysporum f. sp. *lycopersici* strain 007 and *Verticillium dahliae* were used for this assay. One plug per fungi (0.64 mm²) was placed on the margin of a Petri dish with CzA. The *Trichoderma* plugs (strain RE 1-3-4, P1 or T39) were added at the same time as the pathogens on the opposite site. For the control treatment no plug was added to the pathogen. Thereafter the dishes were incubated in the dark at 24°C. Each treatment was run in triplicate and repeated three times. Pictures were taken on the first day and after 7 days.

3.6.4. Growth measurement

The growth areas of the pathogenic fungi (cm²) on day 1, when *Trichoderma* spp. was added, and on day 7 were determined using Lucia® software (Laboratory Imaging s.r.o, Prague, Czech Republic). To obtain the increases of the pathogenic fungi the differences of day 7 to day 1 were calculated.

3.7. Statistical analysis

3.7.1. Tomato plants inoculated with *Trichoderma* spp.

The shoot and root fresh weights/plant and the number of harvested plants for root exudates collection were analysed using the Steel-Dwass test (α=0.05).

3.7.2. Tomato plants co-inoculated with *Trichoderma* spp. and/or AMF

The root fresh weights/plant were analysed using the Kruskal-Wallis test and the Steel-Dwass test (α=0.05). Since the data of the degree of root colonisation followed a normal distribution and Bartlett's test revealed homogeneity a one-way ANOVA was performed (α=0.05) to analyse the degree of root colonisation.

3.7.3. Fungal growth

The ODs of *Fol* in different exudates after 120 hours were analysed using the Kruskal-Wallis test and the Steel-Dwass test (α=0.05).

3.7.4. Dual culture tests

The increases of the areas of the pathogenic fungi were analysed using a one-way ANOVA (α=0.05) and the Student-Newman-Keuls test as post-hoc test (α=0.05).
All statistical analyses were performed using SAS, Enterprise Guide Version 2.0.0.417 (SAS Institute Inc, Cary, NC, USA) and KyPlot Version 2.0 beta 15 (32bit) (Koishi Yoshioka, 1997 – 2001).

4. Results

4.1. Tomato cultures inoculated with *Trichoderma* spp.

To determine whether *Trichoderma* spp. alter the effect of root exudates of tomato on the growth of soil-borne pathogenic fungi, tomato plants were inoculated with three different *Trichoderma* spp. strains. *In vitro* as well as climate chamber tomato cultures were conducted.

4.1.1. *In vitro* cultures of tomato inoculated with *Trichoderma* spp.

In vitro cultures of tomato plants are shown in Figure 6 - Figure 9. The plants of the control treatment as well as the plants inoculated with *Trichoderma* viride RE 1-3-4 appeared healthy and vigorous (Figure 6 and Figure 7) after 20 days of cultivation, whereas the plants of the *T. harzianum* strain T39 and *T. atroviride* strain P1 treatment had only developed cotyledons. Some of the plantlets of treatment T39 and P1 were brownish and necrotic, after 20 days of cultivation (Figure 10 and Figure 11). Due to the small and necrotic plantlets of treatment T39 and P1 the plant material could not be used for root exudate extraction.

Figure 6: Control treatment after 20 days of cultivation.

Figure 7: *T.* viride strain RE 1-3-4 treatment after 20 days of cultivation.

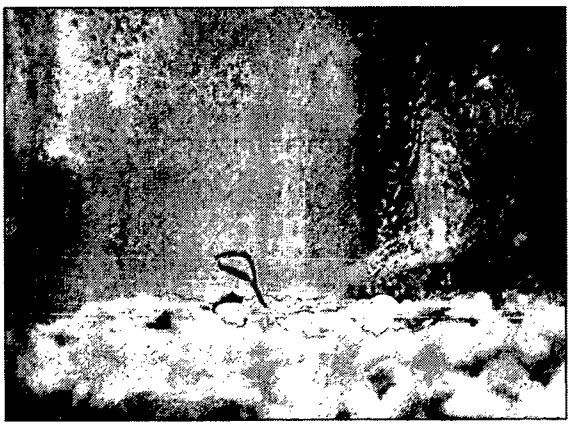

Figure 8: *T. atroviride* strain P1 treatment after 20 days of cultivation.

Figure 9: *T. harzianum* strain T39 treatment after 20 days of cultivation.

Figure 10: *T. harzianum* strain T39 treatment after 20 days of cultivation.

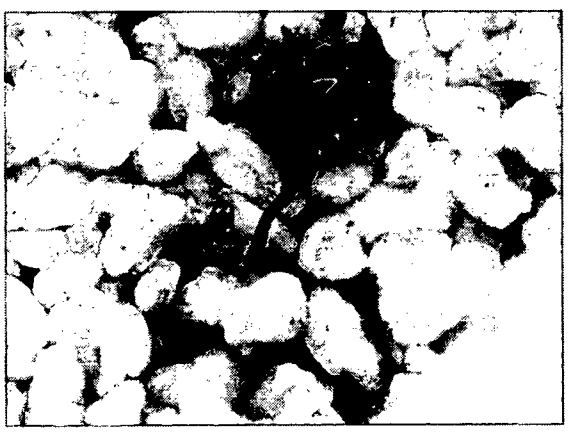

Figure 11: *T. atroviride* strain P1 treatment after 20 days of cultivation.

4.1.2. Climate chamber cultures of tomato inoculated with *Trichoderma* spp.

4.1.2.1. Plant material of the seed treatments at the day of exudates collection

In Table 9 the shoot and root fresh weights of the seed treatments at the day of exudates collection are presented. No significant differences were revealed by the Steel-Dwass test (α=0.05).

Table 9: Shoot fresh weights and root fresh weights of the tomato plants of the seed treatments 36 days after inoculation with *Trichoderma* spp. (strains P1, T39, RE 1-3-4). Different letters indicate significant differences according to Steel-Dwass test (α=0.05).

Substratum	Treatment	Number of plants	Shoot fresh weight/plant [g] ± standard deviation	Root fresh weight/plant [g] ± standard deviation
Soil	Control	14[a]	3.76 ± 0.30[a]	0.74 ± 0.16[a]
	P1	15[a]	3.43 ± 0.80[a]	0.63 ± 0.43[a]
	T39	16[a]	2.09 ± 0.25[a]	0.24 ± 0.03[a]
	RE 1-3-4	16[a]	2.23 ± 1.53[a]	0.26 ± 0.23[a]
Perlite	Control	15[a]	2.43 ± 0.82[a]	0.43 ± 0.14[a]
	P1	14[a]	1.37 ± 0.73[a]	0.24 ± 0.15[a]
	T39	13[a]	0.68 ± 0.31[a]	0.09 ± 0.06[a]
	RE 1-3-4	11[a]	1.02 ± 0.92[a]	0.14 ± 0.14[a]

4.1.2.2. Plant material of the seedling treatments at the day of root exudates collection

In Table 10 the shoot and root fresh weights for the seedling treatments at the day of exudates collection are shown. No significant differences were revealed by the Steel-Dwass test (α=0.05).

Table 10: Shoot fresh weights and root fresh weights of the tomato plants from the seedling treatment 24 days after inoculation with *Trichoderma* spp. (strains P1, T39, RE 1-3-4). Different letters indicate significant differences according to Steel-Dwass test (α=0.05).

Substratum	Treatment	Number of plants	Shoot fresh weight/plant [g] ± standard deviation	Root fresh weight/plant [g] ± standard deviation
Soil	Control	18[a]	2.57 ± 0.03[a]	0.61 ± 0.19[a]
	P1	14[a]	2.79 ± 0.73[a]	0.73 ± 0.17[a]
	T39	12[a]	1.40 ± 0.20[a]	0.23 ± 0.13[a]
	RE 1-3-4	15[a]	1.72 ± 0.88[a]	0.27 ± 0.17[a]
Perlite	Control	18[a]	0.85 ± 0.26[a]	0.16 ± 0.03[a]
	P1	12[a]	1.19 ± 0.44[a]	0.21 ± 0.08[a]
	T39	11[a]	0.29 ± 0.20[a]	0.06 ± 0.02[a]
	RE 1-3-4	10[a]	0.65 ± 0.26[a]	0.10 ± 0.04[a]

4.2. Tomato cultures co-inoculated with *Trichoderma* spp. and arbuscular mycorrhizal fungi

In order to determine whether *Trichoderma* spp. and/or AMF alter the effect of exudates of tomato on the growth of soil-borne pathogenic fungi, tomato plants were inoculated with three different *Trichoderma* spp. strains and/or AMF.

The means of the root fresh weights/plant for the *Trichoderma* spp. and AMF co-inoculation assay are presented in Figure 12. No significant differences (Kruskal-Wallis-Test, α=0.05) between the treatments could be detected.

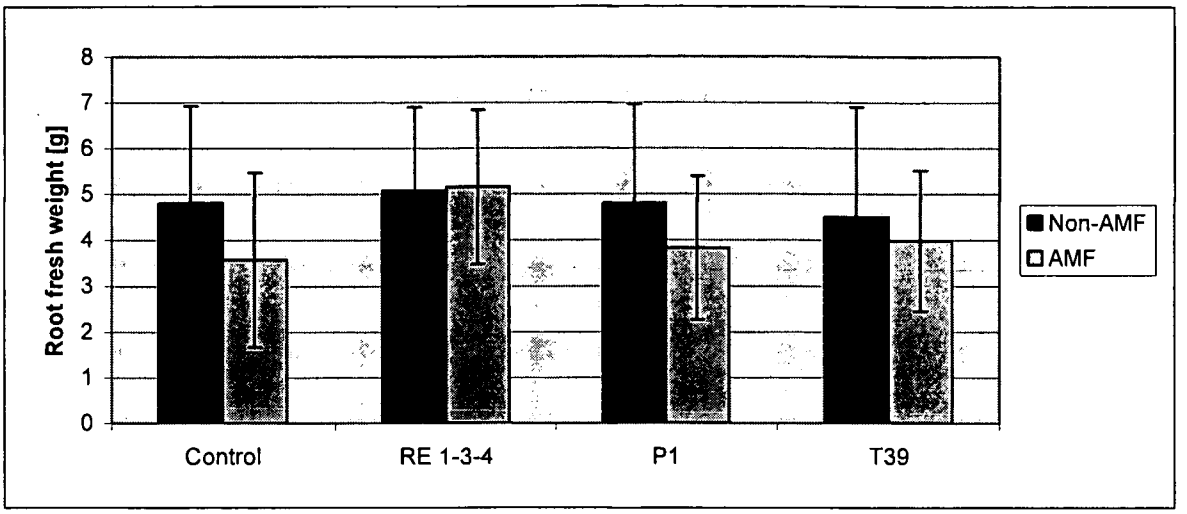

Figure 12: Root fresh weight/plant for the 8-week-old tomato cultures with *Trichoderma* and AMF co-inoculation. Vertical bars indicate standard deviation. Differences are not significant (Kruskal-WallisTest, α=0.05).

In Figure 13 the root colonisations for the AMF and AMF + *Trichoderma* spp. treatments are shown. There were no significant differences in the degree of root colonisation between the AMF treatment and the AMF + *Trichoderma* spp. treatments (ANOVA, α=0.05).

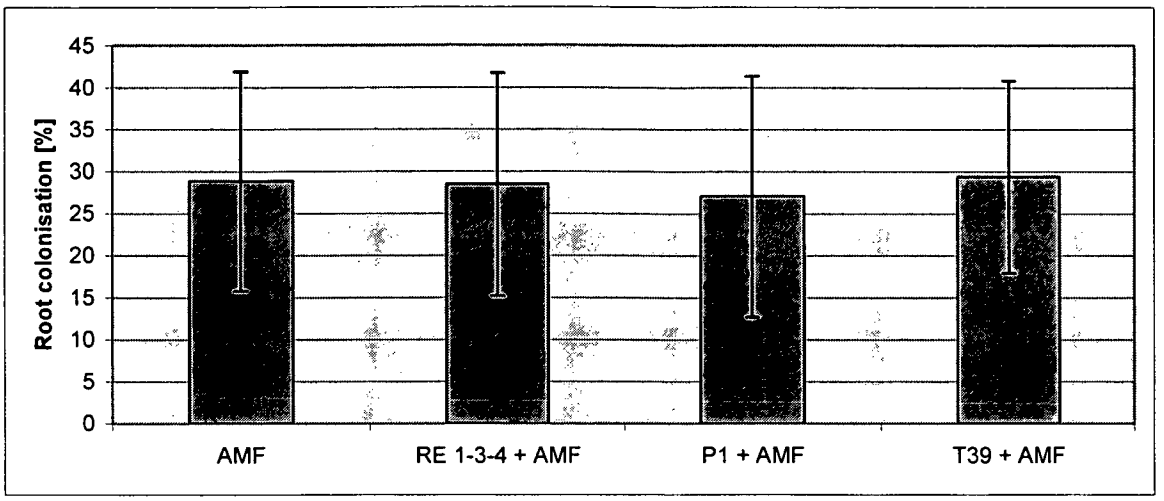

Figure 13: Root colonisation in % for AMF and the *Trichoderma* spp. + AMF treatments. Vertical bars indicate standard deviations.

4.3. Cultivation of the tomato pathogen *Pyrenochaeta lycopersici*

In order to be able to perform optical density measurements with *P. lycopersici* several tests were done for optimal growth conditions and the production of large amounts of mycelium.

4.3.1. Optimal growth conditions

The optimal growth conditions for *P. lycopersici* were tested. The results for the solid media cultures are presented in Table 11. According to Table 11 the best growth condition for a fast mycelium production was V8 Agar at a 16-hour photoperiod (24°C), since mycelial growth occurred after 3 days of incubation.

Table 11: Results for solid media cultures with different growth conditions for *P. lycopersici*.

Medium (Appendix)	Photoperiod	Temperature [°C]	Fungal development
Potato Dextrose Agar (PDA)	dark	18	growth after 5 d
Potato Dextrose Agar (PDA)	16 h light	24	growth after 5 d
V8 Agar	dark	18	growth after 5 d
V8 Agar	16 h light	24	growth after 3 d

The liquid media cultures were more suitable to produce large amounts of mycelium in 5 days whereas *P. lycopersici* would take at least 2 weeks to cover 90% of a Petri dish. Furthermore, it was hard to separate the mycelium properly from solid media.
The culture characteristics of the fungus cultivated in liquid media varied depending on the used growth medium. When *P. lycopersici* was grown in Czapek Dox broth, it consisted of very firm mycelial balls with spikes around them (Figure 14). When V8 broth and Potato Dextrose broth were used, the mycelial balls were less firm and were easier to squeeze (Figure 15 and Figure 16). The Malt Extract broth (Figure 17) was not an adequate growth medium because the amount of mycelium was less than for the other growth media.

Figure 14: Liquid *P. lycopersici* culture in Czapek Dox broth (10 days old).

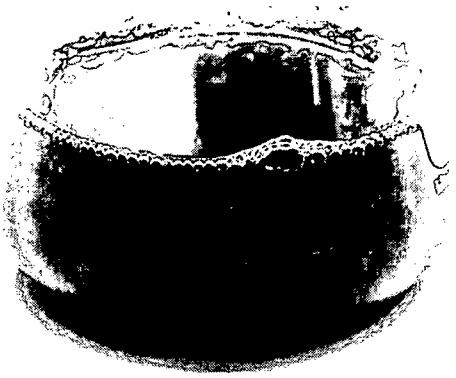

Figure 15: Liquid *P. lycopersici* culture in V8 broth (6 days old).

Figure 16: Liquid *P. lycopersici* culture in Potato Dextrose broth (6 days old).

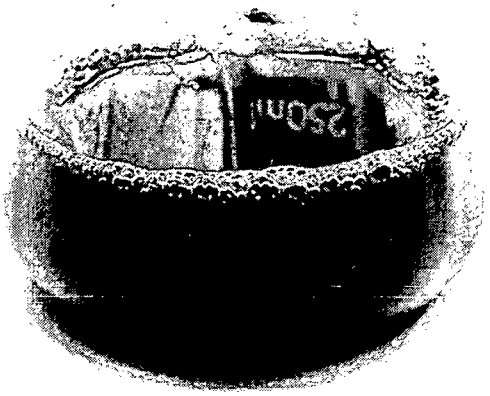

Figure 17: Liquid *P. lycopersici* culture in Malt Extract broth (6 days old).

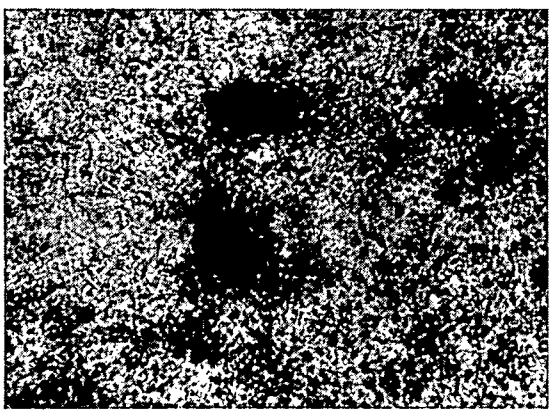

Figure 18: Microsclerotia of *P. lycopersici*.

4.3.2. Microsclerotia and pycnidia induction

Neither pycnidia nor microsclerotia were formed using the methods of Shishkoff (1994) and Ball (1979), respectively. However, microsclerotia formation could be detected on the margins of Czapek Dox broth liquid cultures after 12 days of incubation. Figure 18 shows black aggregations of mycelium, the microsclerotia, under the microscope.

4.4. Establishment of a bioassay for the mycelial growth of *P. lycopersici*

To establish a bioassay for the mycelial growth of *P. lycopersici* in root exudates of tomato the optimal preparation of the fungal material and different growth media were tested.

4.4.1. Homogenisation of the fungal material

The different treatments for homogenising the mycelium displayed in Table 12 were rather ineffective, since most of them did not disintegrate the mycelium to a satisfactory level. A satisfactory level of disintegration was reached when the mycelial fragments appeared homogenous under the microscope. Only disintegration with a pestle was satisfactory.

Table 12: Results for homogenising the mycelium of *P. lycopersici.*

Method	Time	Speed	Appropriate disintegration
Vortexing in water (1.5 ml Eppendorf tube)	1 min	40 Hz	no
Vortexing in water (1.5 ml Eppendorf tube)	1 ½ min	40 Hz	no
Vortexing in water (2 ml Eppendorf tube)	1 min	40 Hz	no
Vortexing in water (2 ml Eppendorf tube)	1 ½ min	40 Hz	no
Magnetic stirrer + water & 30 glass beads (test-tube)	10 min	1,000 rpm	no
Magnetic stirrer + water & 60 glass beads (test-tube)	10 min	1,000 rpm	no
Magnetic stirrer + water + sand (test-tube)	10 min	1,000 rpm	no
Pestle (1.5 ml Eppendorf tube)	Until material appeared homogenous		yes

4.4.2. Media test

According to Broekaert *et al.* (1990) in the absorbance range of 0 to 0.6 units there exists a straight-line relationship between absorbance at 595 nm and dry weight of microplate cultures. Therefore a medium for the positive control should have a low absorbance level.

As shown in Figure 19 V8 juice exceeded the 0.6 level. Malt Extract broth (MB), Potato Dextrose broth (PDB), V8 juice supernatant and V8 broth were around the 0.4

level. The other media, Czapek Dox broth (CzB), PDB supernatant, V8 broth supernatant and water showed levels between 0.04 and 0.1 units.

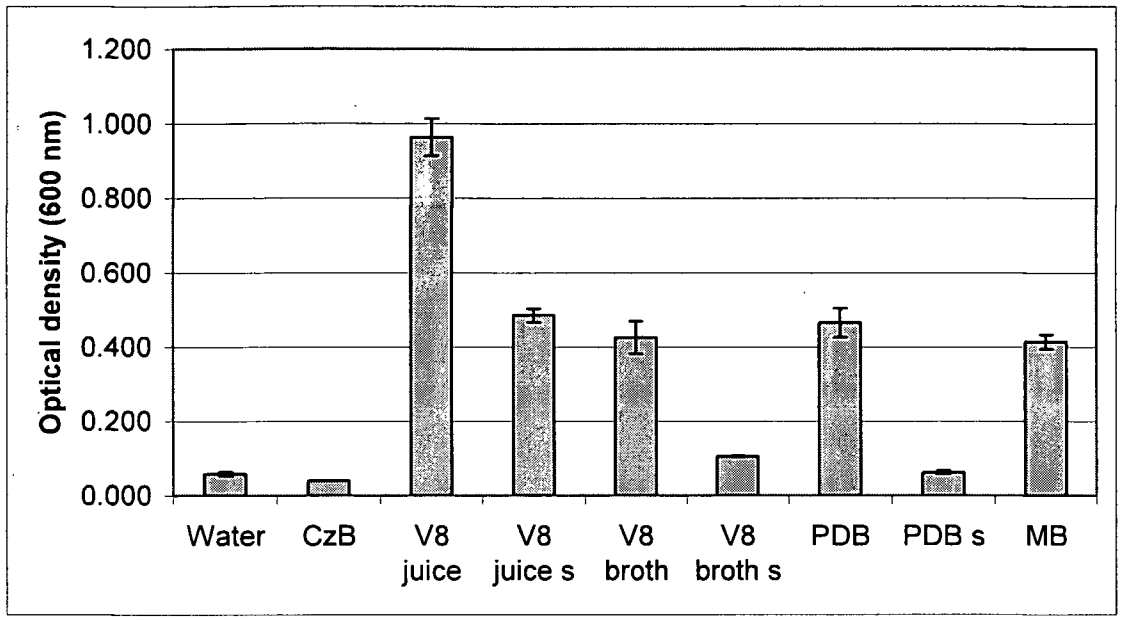

Figure 19: Optical density (600 nm) of different growth media (Appendix). Columns are the average of 6 measurements (n=6). Bars indicate standard deviations. CzB = Czapek Dox broth, V8 juice s = V8 juice supernatant, V8 broth s = V8 broth supernatant, PDB = Potato Dextrose broth, PDB s = Potato Dextrose broth supernatant, MB = Malt Extract broth.

4.4.3. Fungal growth in different media

In Figure 20 the development of the mycelium of *P. lycopersici* in different growth media is shown. V8 broth, V8 broth supernatant and PDB ranged around the 0.6 level in the first 72 hours. After 96 hours a striking rise of the curves compared to the development in the first 72 hours could be seen. MB was around the 0.2 level and a rise to 0.4 units occured after 96 h. In the first 48 hours V8 broth supernatant, PDB supernatant, CzB and water ranged between 0.04 and 0.12 units. The curves of V8 broth supernatant and PDB supernatant, respectively, show a high increase of the optical density after 72 hours from 0.3 and 0.2 units, respectively, to 1.8 units. After 72 hours there was a sharp growth increase expressed in an enhanced OD. After

120 hours of incubation the wells were totally filled with the fungus. A rise of the OD of the wells with CzB as growth medium occured after 96 hours and reached 0.6 units 120 h after incubation. The inclination of the curve was much lower as for V8 broth supernatant and PDB supernatant, indicating a slower growth. The water control did not change over the 120 h of incubation.

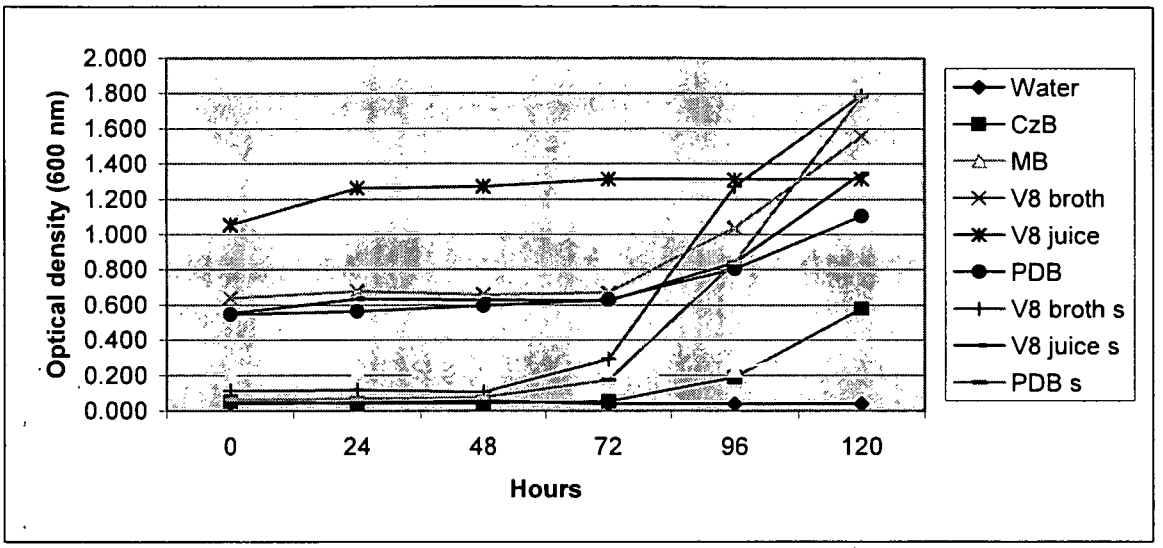

Figure 20: Optical density (600 nm) in 24-h intervals for *P. lycopersici* in different growth media (Appendix). CzB = Czapek Dox broth, MB = Malt Extract broth, PDB = Potato Dextrose broth, V8 broth s = V8 broth supernatant, V8 juice s = V8 juice supernatant, PDB s = Potato Dextrose broth supernatant.

4.4.4. Mycelium concentrations

Figure 21 shows that the OD of the highest concentration of the fungus (100 g/l) ranged around 0.2 units at the beginning of the experiment (0 h), whereas the lower concentrations (10 g/l and 1 g/l) ranged around 0.1 units when incubated in V8 supernatant. Furthermore, there was no striking difference between the two lower concentrations. Consequently, the 10 g/l concentration was used for the incubation of *P. lycopersici* in root exudates (section 4.4.5).

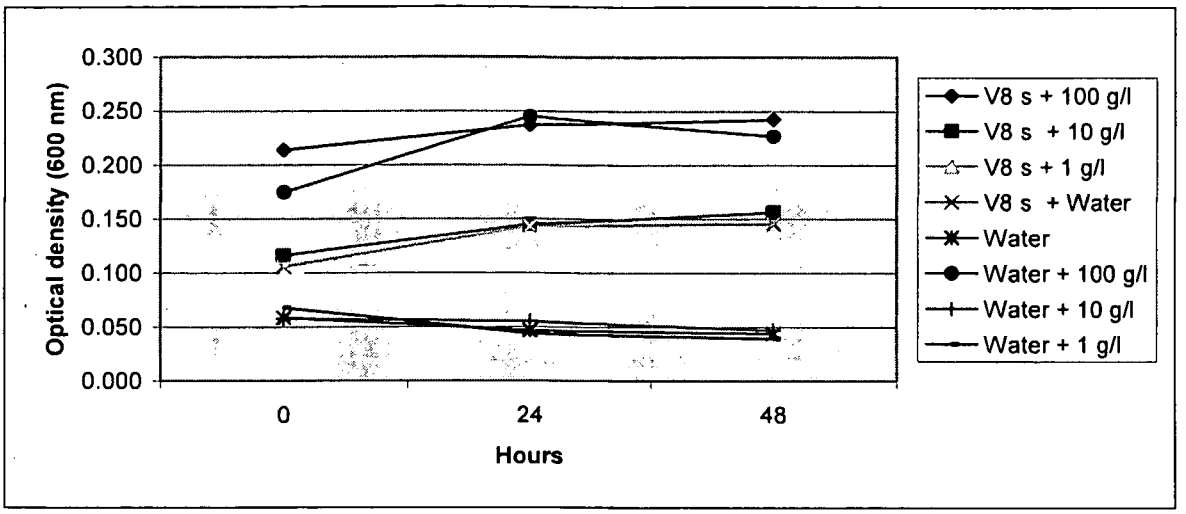

Figure 21: Optical density (600 nm) for different mycelium concentrations of *P. lycopersici* in autoclaved distilled water or V8 supernatant. V8 s = V8 broth supernatant.

4.4.5. Growth of *Pyrenochaeta lycopersici* in tomato root exudates

Figure 22 shows the development of *P. lycopersici* in root exudates compared to 2 growth media. Furthermore, it is shown whether the ODs of the exudates and the growth media alone change during incubation. A rise of the growth curve of *P. lycopersici* could be observed between 96 and 120 hours of incubation in V8 broth supernatant. The ODs of the media (Czapek Dox broth and V8 broth supernatant) and water treatments did not or hardly changed within 120 h of incubation. Furthermore, no alteration of the OD could be observed in the *P. lycopersici* + CzB treatment as well as in the *P. lycopersici* + root exudate treatment within 120 hours. The exudates and water treatment showed no significant rise of the OD as well.

Since no growth of *P. lycopersici* occurred in root exudates after 120 hours of incubation *Fusarium oxysporum* f.sp. *lycopersici* was used for the fungal growth assay.

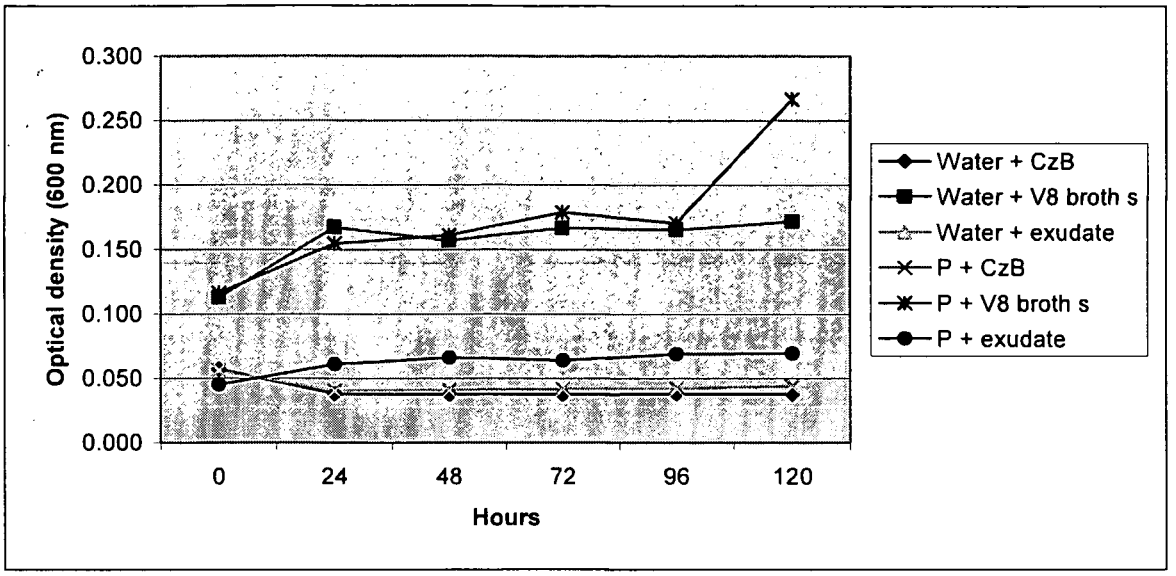

Figure 22: Optical density (600 nm) of *P. lycopersici* incubated in root exudates, Czapek Dox broth or V8 broth supernatant for 120 h. P = *P. lycopersici,* CzB = Czapek Dox broth, V8 broth s = V8 broth supernatant.

4.5. Fungal growth assay

To test whether the effect of tomato root exudates on the growth of soil-borne pathogens is altered by *Trichoderma* spp. and/or AMF inoculation a fungal growth assay was performed.

4.5.1. Root exudates from tomato cultures inoculated with *Trichoderma* spp. and seeds as plant material

In order to examine if there are any differences in the growth of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) in the root exudates from tomato cultures with *Trichoderma* spp. inoculation, optical density measurements were performed. The results for the fungal growth assay are presented in Figure 23 and Figure 24.

At the beginning of the experiment (0 h) the ODs of the exudates from tomato plants grown in soil and inoculated with the different *Trichoderma* strains were around 0.07

units (Figure 23). The control, the soil + P1 and the soil + T39 treatments showed a clear inclination of the OD compared to the other treatments within 120 hours. The control treatment showed a greater increase than the soil + T39 treatment. However, after 120 hours they reached similar OD levels around 0.17 units. The soil + P1 treatment reached the highest OD of all treatments after 120 hours (0.22). Water and the soil + RE 1-3-4 treatment had the same OD development as well as the lowest OD compared to the other treatments.

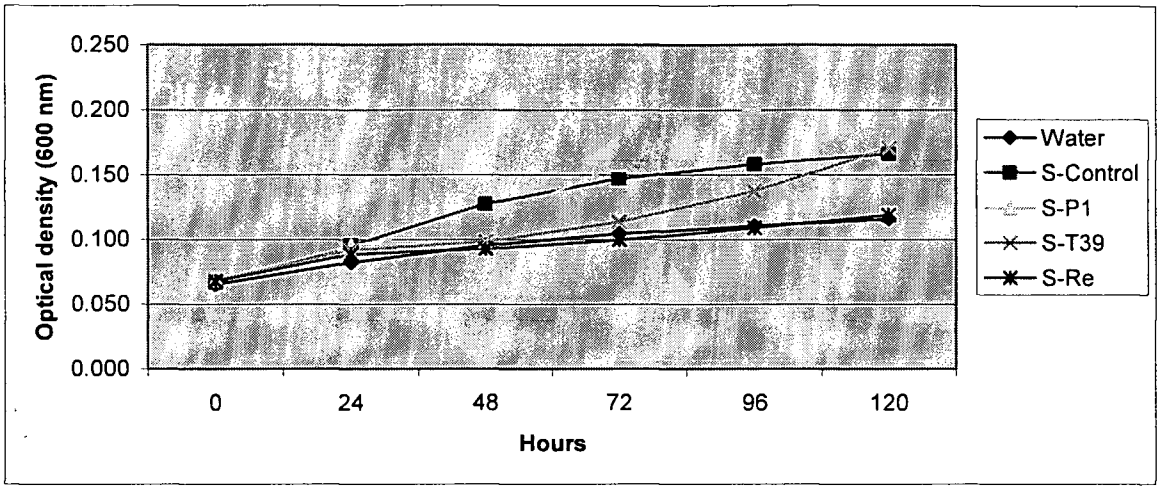

Figure 23: Optical density (600 nm) for *Fol* incubated in the different exudates of the seed treatments (soil) for 120 hours. Means are presented. S-Control = soil control, S-P1 = soil + P1, S-T39 = soil + T39, S-Re = soil + RE 1-3-4.

In Figure 24 the ODs for the exudates of the seed treatments in perlite are presented. At the beginning of the experiment (0 h) the ODs range around 0.07 units. After 24 hours of incubation the OD of *Fol* in the perlite control and the perlite + *Trichoderma* strain P1 exudates ranged around 0.11 units. The ODs of the other exudates were lower and ranged around 0.08 units. After 48 h the ODs of *Fol* in the perlite control and in the perlite + strain P1 treatments remained nearly stable around 0.11 units. The treatments perlite + RE 1-3-4 and perlite + strain T39 showed the lowest growth development of all treatments. *Fol* incubated in water was less inclining than the

perlite control and the perlite + P1 treatments but at the end, after 120 hours of incubation, they reach equal levels of ODs (approximately 0.12 units).

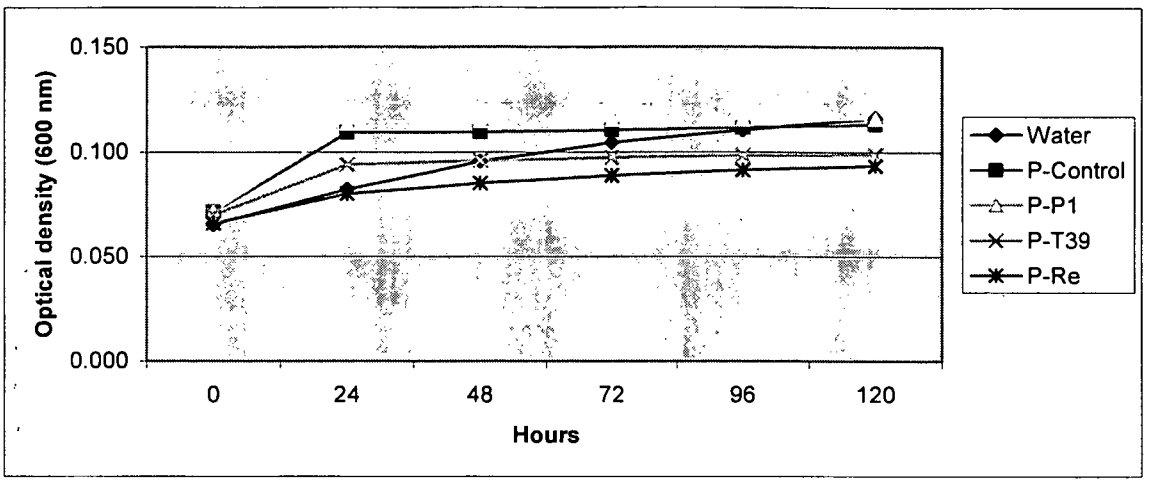

Figure 24: Optical density (600 nm) for *Fol* incubated in the different exudates of the seed treatments (perlite) for 120 hours. P-Control = perlite control, P-P1 = perlite + P1, P-T39 = perlite + T39, P-Re = perlite + RE 1-3-4.

In Figure 25 the results of the incubation of *Fol* in the different exudates after 120 hours are presented. The significant differences according to the Steel-Dwass test are indicated by different letters. ODs of the perlite control, the perlite + strain P1 and the soil + strain RE 1-3-4 were not significantly different from water as growth medium. Perlite + T39 and perlite + Re 1-3-4 treatments were significantly lower than water. All of the soil treatments differed significantly from each other apart from the control and the T39 treatment, which were not significantly different from each other. All of the perlite treatments were significantly lower than their soil counterparts. Furthermore, the perlite control and the perlite + P1 treatment and the perlite + T39 and perlite + RE 1-3-4 treatments, respectively, did not differ significantly.

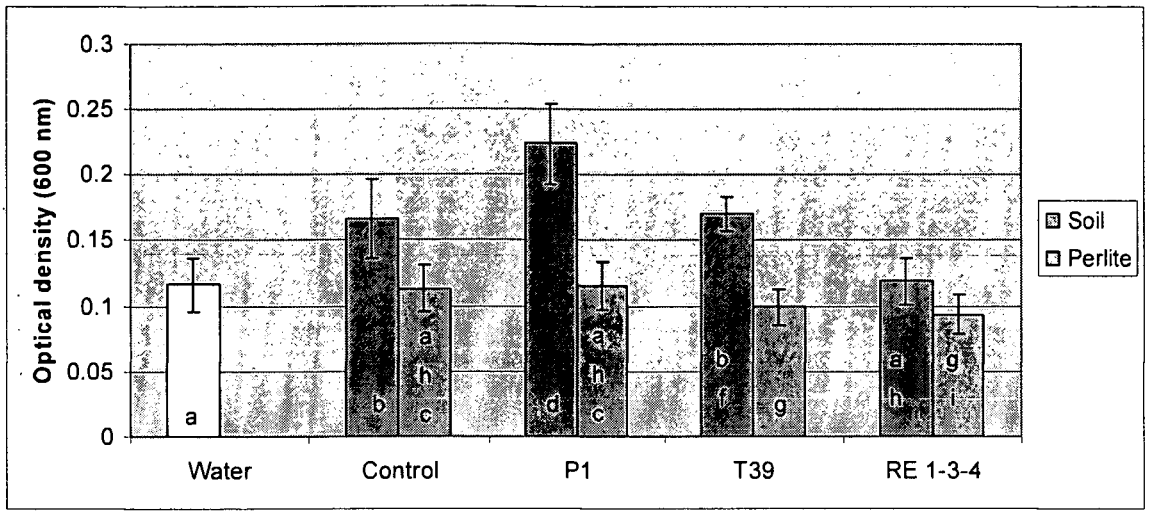

Figure 25: Optical density (600 nm) for *Fol* incubated in the different exudates of the seed treatment after 120 hours. Different letters indicate significance (α=0.05). Re = RE 1-3-4.

4.5.2. Root exudates from tomato cultures with *Trichoderma* spp. inoculation and seedlings as plant material

The OD development of *Fol* incubated in the exudates of the tomato cultures with *Trichoderma* spp. application and seedlings as plant material is shown in Figure 26 and Figure 27, respectively.

At the beginning (0 h) the ODs ranged around 0.06 units (Figure 26). After 24 hours the OD of the soil control had increased more than the other treatments. A difference of at least 0.04 units between the soil control and the other treatments occurred after 48 hours of incubation. The soil control treatment increased further until 0.22 units after 120 hours of incubation. Regarding the ODs after 72 hours, there was a clear difference between the soil control (0.18), the soil + P1 treatment (0.13) and the other treatments, which had only half of the OD as the soil control had. The soil + P1 treatment inclined further until 0.18 units after 120 hours. After 96 hours the growth activity of *Fol* in the soil + T39 treatment exudates increased more than before and reached an OD of 0.16 units after 120 hours. The water and the soil + RE 1-3-4 treatment did not show a clear increase of the OD during 120 hours of incubation.

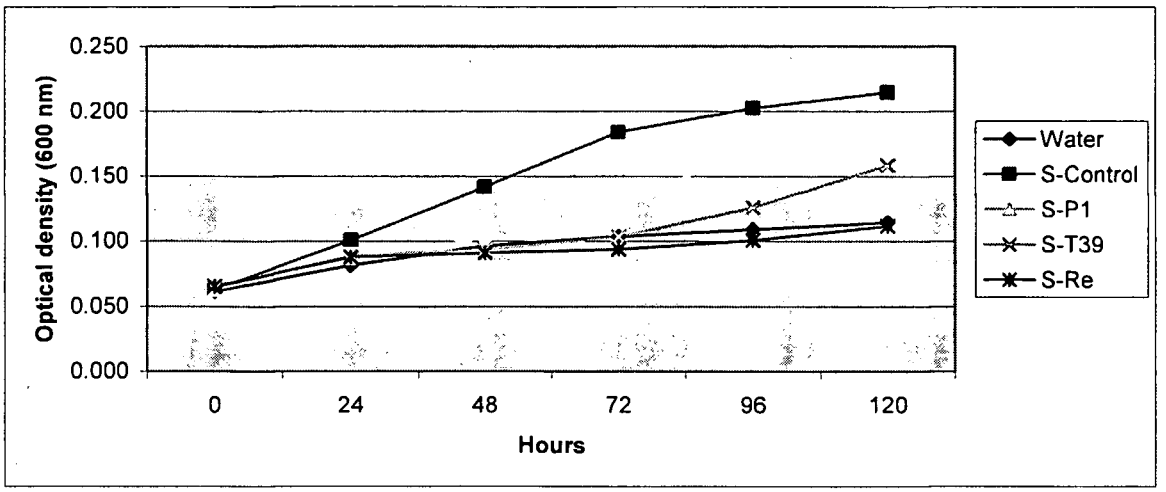

Figure 26: Optical density (600 nm) of *Fol* incubated in the different exudates of the seedling treatments (soil) for 120 hours. Means are presented. S-Control = soil control, S-P1 = soil + P1, S-T39 = soil + T39, S-Re = soil + RE 1-3-4.

Water, the perlite control, the perlite + P1 and the perlite+ RE 1-3-4 treatment did not show a high increase of the OD during 120 hours of incubation (Figure 27). However, the OD of *Fol* in water was higher than in the other treatments.

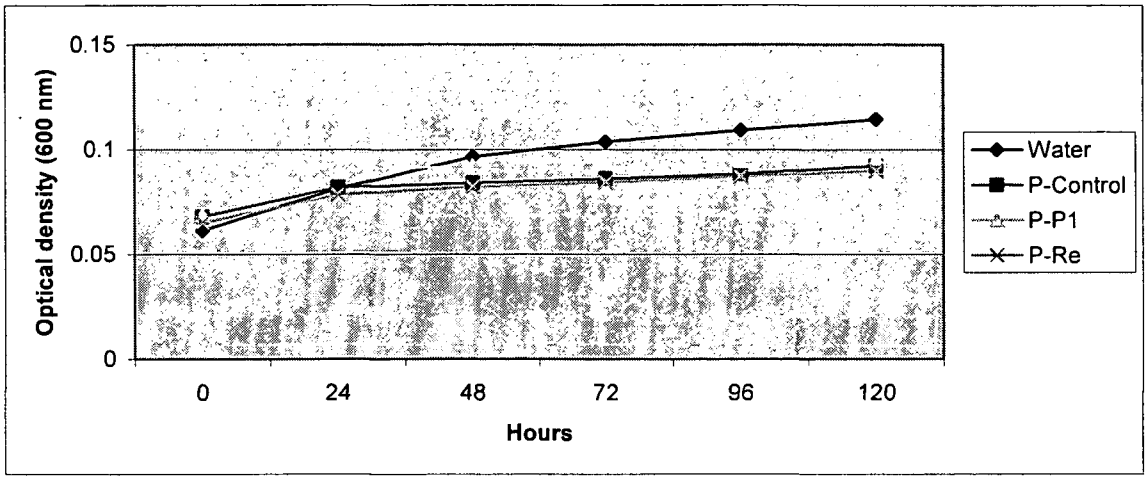

Figure 27: Optical density (600 nm) of *Fol* incubated in the different exudates of the seedling treatments (perlite) for 120 hours. Means are presented. P-Control = perlite control, P-P1 = perlite + P1, P-Re = perlite + RE 1-3-4.

The optical density of *Fol* in root exudates after 120 hours of incubation can be seen in Figure 28. All the soil treatments were significantly different. The OD was the highest in the soil control followed by the soil + P1, + T39 and + RE 1-3-4 treatment. All of the soil treatments were significantly higher than perlite treatments. The difference between the soil + RE 1-3-4 treatment and the water was not significant. All of the perlite treatments had a lower OD than water. There was no significant difference between the perlite control and the perlite + RE 1-3-4 treatment. The OD of the perlite + P1 treatment was significantly higher than the perlite control and the perlite + RE 1-3-4 treatment but still significantly lower than the OD of *Fol* in water.

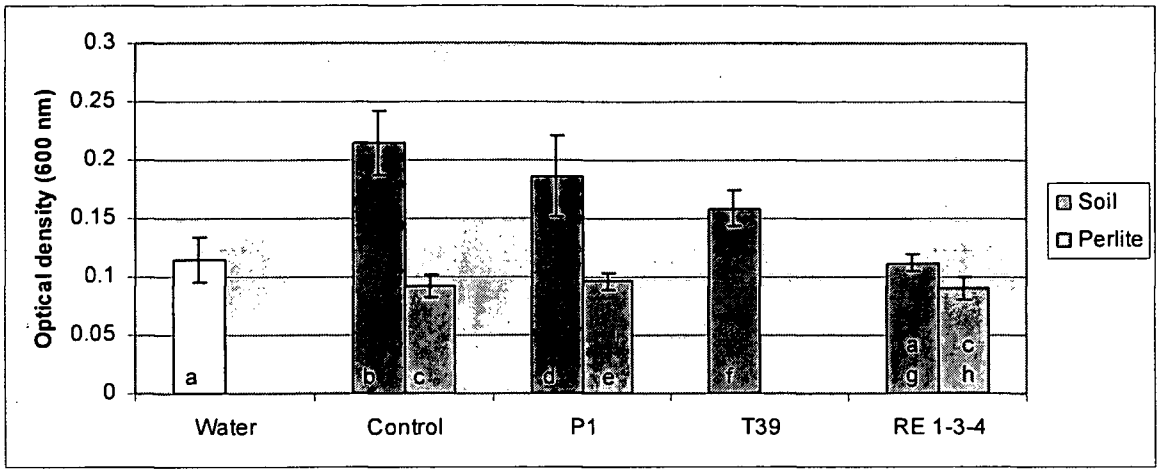

Figure 28: Optical density of *Fol* incubated in the different exudates of the seedling treatment after 120 hours. Different letters indicate significance (α=0.05). RE = RE 1-3-4.

In Figure 29 the ODs of Fol incubated in the different exudates of the seedling and seed treatment in soil after 120 hours of incubation are presented. Apart from the *T. viride* strain RE 1-3-4 treatments, the differences between the seeds and seedlings treatments are significantly different (α=0.05) for the control as well as for the other *Trichoderma* treatments.

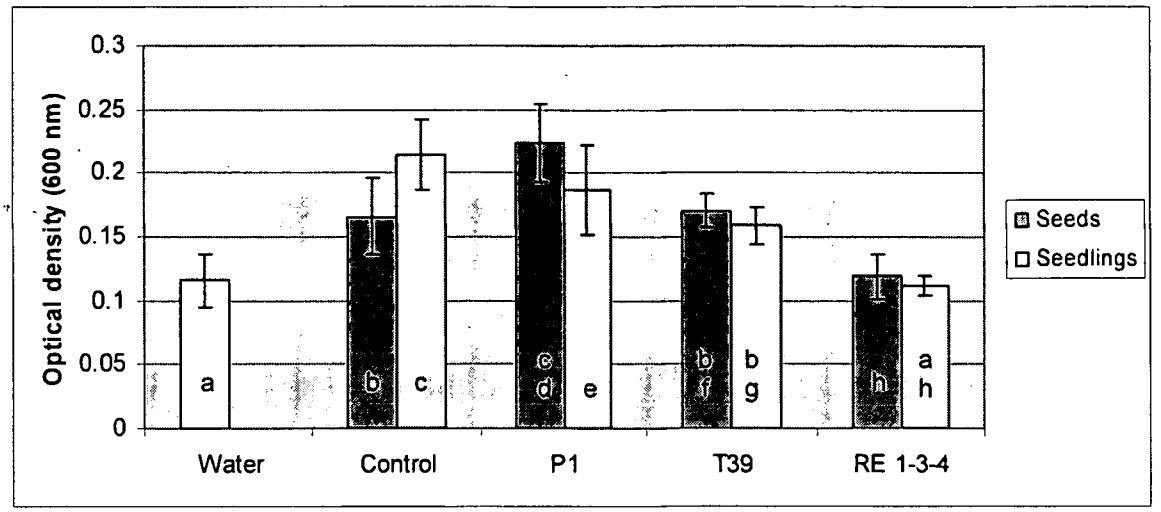

Figure 29: Optical density of *Fol* incubated in the different exudates of the seedling and seed treatments in soil after 120 hours. Different letters indicate significant differences (α=0.05).

4.5.3. Root exudates from tomato cultures with *Trichoderma* spp. and arbuscular mycorrhizal fungi co-inoculation

In Figure 30 the ODs of *Fol* incubated in exudates of the AMF and *Trichoderma* co-inoculation assay are shown. At the beginning (0 h) the ODs ranged around 0.060 units. The curves of the RE 1-3-4, RE 1-3-4 + AMF, AMF, P1 + AMF, T39 + AMF treatments and of water showed an increase within the first 24 hours and remained nearly constant for the rest of the experiment. The P1, the control and the T39 treatments had the highest increase of the ODs compared to the other treatments after 24 hours. The control and the P1 treatment had a similar growth development for 72 hours. Afterwards the OD of P1 was higher than the OD of the control treatment. P1 and the control increased more than T39.

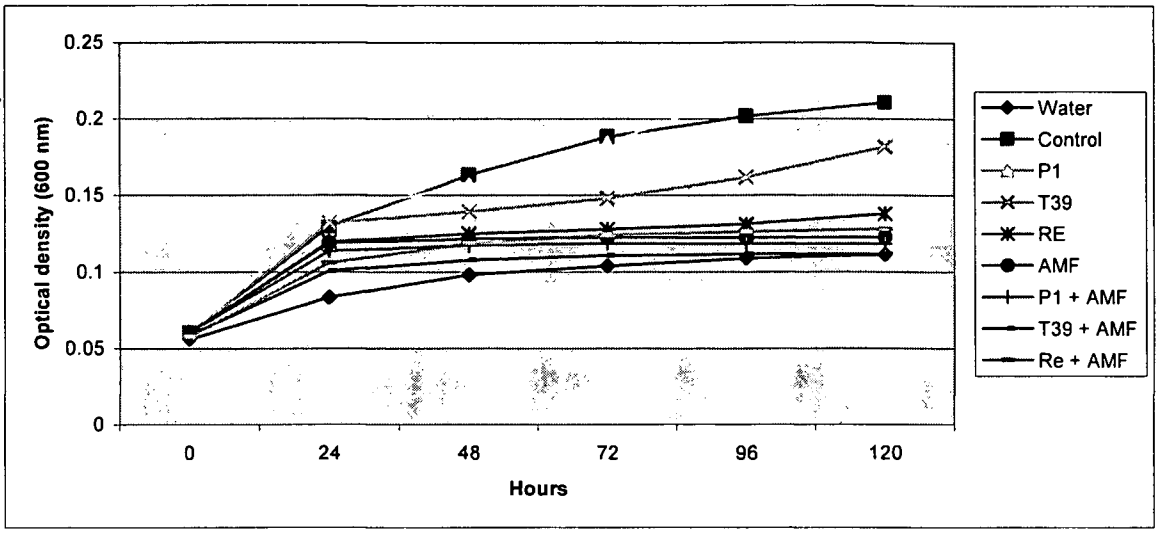

Figure 30: Optical density (600 nm) of *Fol* incubated in exudates of tomato plants co-inoculated with AMF and *Trichoderma* and in water for 120 hours. RE = RE 1-3-4, AMF = arbuscular mycorrhizal fungi, Re + AMF = RE 1-3-4 + AMF.

In Figure 31 the means of the optical densities of *Fol* after 120 hours of incubation in root exudates of the *Trichoderma* spp. and AMF co-inoculated plants are shown. There was a significant difference between the AMF and non-AMF treatments for the control, *Trichoderma* strain P1 and T39, i.e. the growth of *Fol* was in the AMF-treatments significantly lower than in the non-AMF treatments. Furthermore, the ODs for the AMF + P1 and AMF + T39 exudates were not significantly different from the OD of water. No significant difference could be determined between the AMF + RE 1-3-4 and non-AMF + RE 1-3-4 exudates. The ODs of both were significantly higher than the OD of water. However, the OD of the non-AMF + RE 1-3-4 exudates was the lowest of all non-AMF treatments. There was no significant difference between the AMF and the AMF + P1 and AMF + RE 1-3-4 treatments. The AMF + T39 treatment was significantly lower than the AMF control treatment.

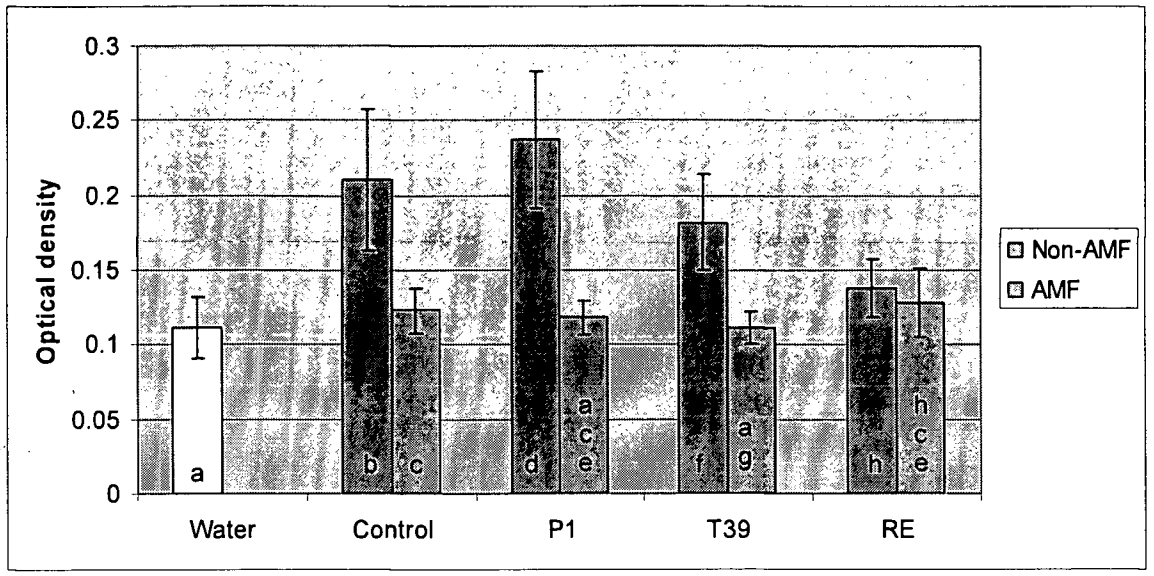

Figure 31: Means of optical density (600 nm) of *Fol* after 120 hours for the *Trichoderma* spp. and AMF co-inoculation exudates. Different letters indicate significance (α=0.05). Vertical bars indicate standard deviations (n=72). RE = RE 1-3-4.

4.6. Dual culture tests

To see if *T. viride* strain RE 1-3-4, *T. harzianum* strain T39 and *T. atroviride* strain P1 have antagonistic effects on the three tomato pathogens, *P. lycopersici*, *Fol* and *V. dahliae*, *in vitro* dual culture tests were performed.

4.6.1. Dual cultures of *Pyrenochaeta lycopersici* and *Trichoderma* spp.

In order to detect inhibitory effects of *Trichoderma* spp. on *P. lycopersici* dual culture tests were performed. The pathogen and the antagonistic fungus were incubated together for 7 days on a Petri dish.

Figure 32 and Figure 33 show the control treatment of *P. lycopersici* on day 1 and on day 7, respectively. Figure 34 to Figure 36 show the 3 *Trichoderma* and *P. lyopersici* treatments on day 7. Figure 34 - Figure 36 show that *Trichoderma* spp. grew less

densely but faster than *P. lycopersici*. All *Trichoderma* strains reached the pathogen within 7 days of incubation.

The increase of the area of the pathogenic fungus in 7 days of incubation was determined and is shown in Figure 37. The increase of *P. lycopersici* in the control treatment was significantly higher (Student-Newman-Keuls test, α=0.05) than the 3 treatments with *Trichoderma* spp.. No statistically significant difference exists between the 3 *Trichoderma* treatments. The increase of the area of the control treatment of *P. lycopersici* was 3.78 (± 2.33) cm^2, 3.25 (± 0.86) cm^2 and 2.3 (± 1.83) cm^2 higher than the RE 1-3-4 treatment, the T39 treatment and the P1 treatment, respectively.

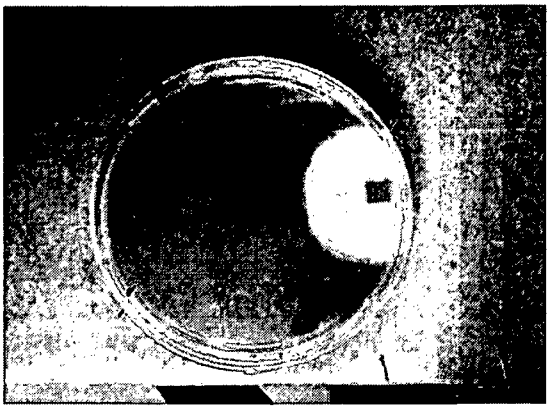

Figure 32: Control treatment of *P. lycopersici* on day 1.

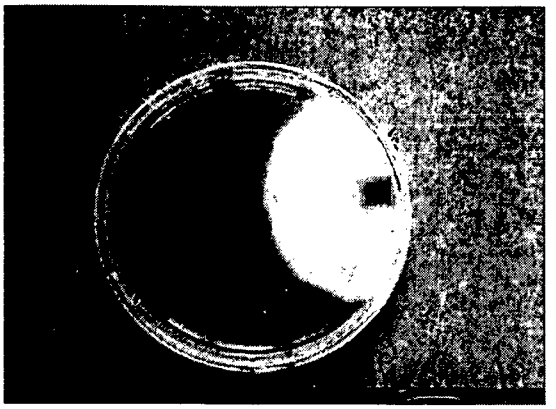

Figure 33: Control treatment of *P. lycopersici* on day 7.

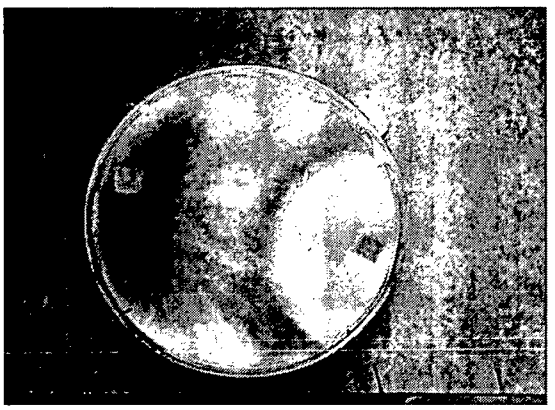

Figure 34: Dual culture of *P. lycopersici* and *Trichoderma viride* strain RE 1-3-4 on day 7.

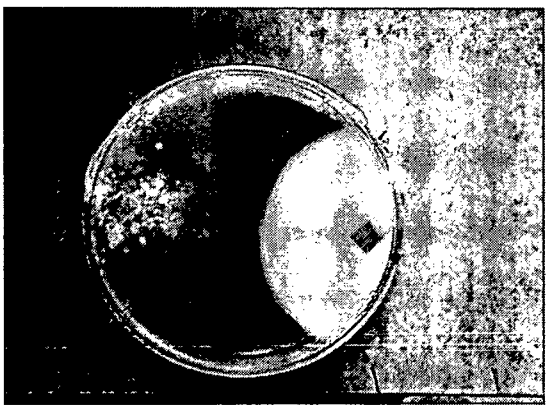

Figure 35: Dual culture of *P. lycopersici* and *Trichoderma harzianum* strain T39 on day 7.

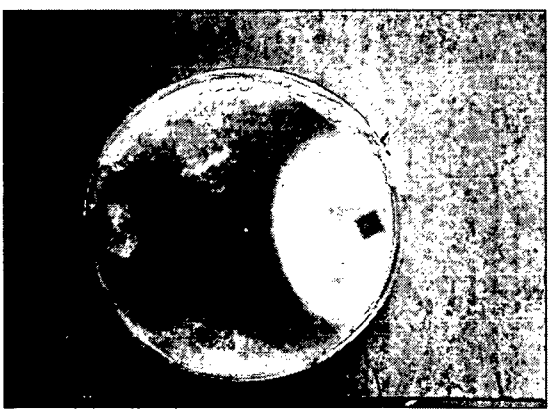

Figure 36: Dual culture of *P. lycopersici* and *Trichoderma atroviride* strain P1 on day 7.

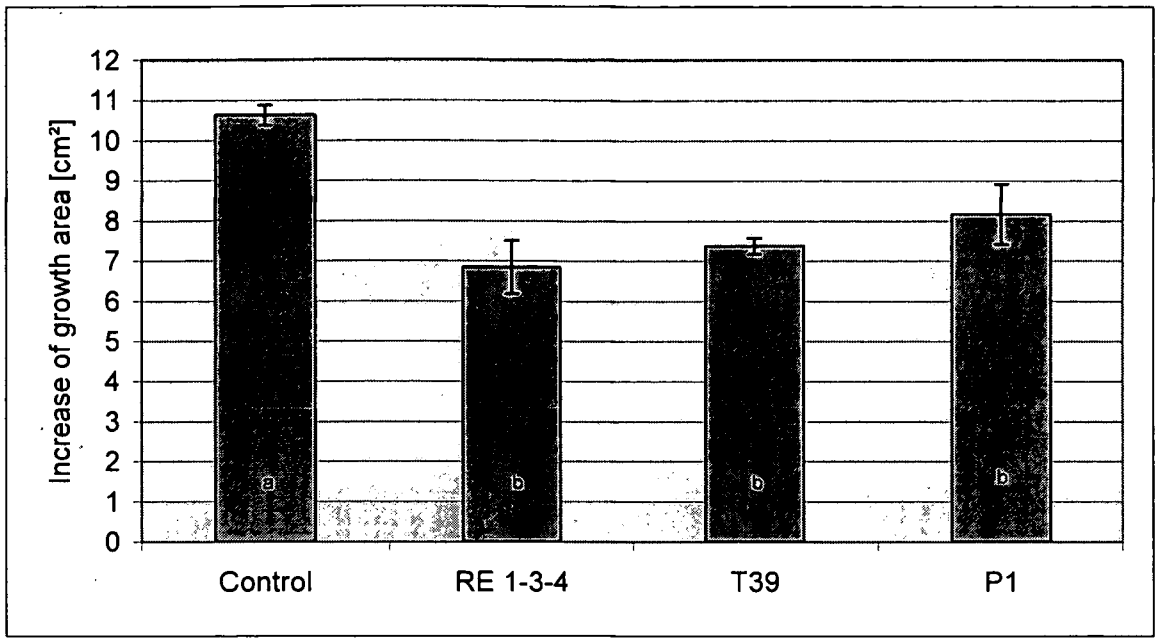

Figure 37: The increase of the area of *P. lycopersici* after 7 days of incubation in dual culture with *Trichoderma* spp. Vertical bars indicate standard error. Different letters indicate statistical significance (ANOVA, α=0.05).

4.6.2. Dual cultures of *Fusarium oxysporum* f. sp. *lycopersici* and *Trichoderma* spp.

The pictures of the control treatment of *Fol* on day 1 and on day 7 are shown in Figure 38 and Figure 39, respectively.

The dual cultures of *Fol* and the 3 different *Trichoderma* strains are shown in Figure 40, Figure 41 and Figure 42. The *Trichoderma* spp. strains grew less densely than the pathogen. *T. viride* strain RE 1-3-4 showed macroscopically hardly any mycelium between the *Trichoderma* inoculation plug and the pathogen (Figure 40). However, in the close vicinity of *Fol* macroscopically detectable mycelium of RE 1-3-4 appeared. Furthermore, *Fol* showed different zones at the margin of the mycelium. In Figure 41 appeared a ring between *Fol* and *T. harzianum* T39 where no greenish mycelial dots of T39 occured. In contrast *T. atroviride* strain P1 colonised the whole area from the P1 inoculation plug to the pathogen.

The increase of *Fol* in the control treatment is significantly higher than for the *Trichoderma* spp. treatments (Student-Newman-Keuls test, α=0.05). No significant differences were detected between the 3 *Trichoderma* treatments. The increase of the area for the control treatment of *Fol* was 7.36 (± 3.04) cm^2, 5.62 (± 2.02) cm^2 and 5.34 (± 2.09) cm^2 higher than the RE 1-3-4, the T39 and the P1 treatment, respectively.

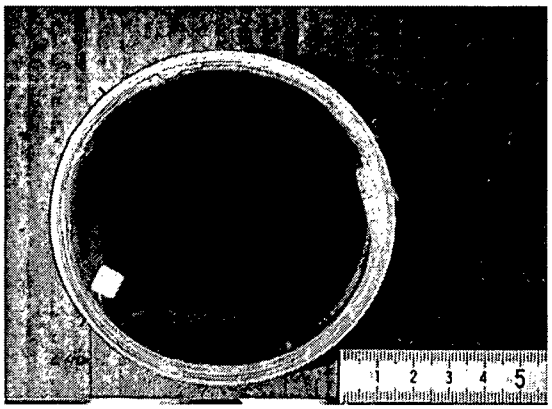

Figure 38: Control treatment of *Fol* on day 1.

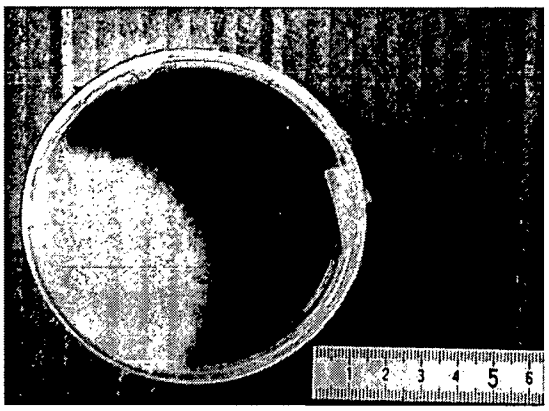

Figure 39: Control treatment of *Fol* on day 7.

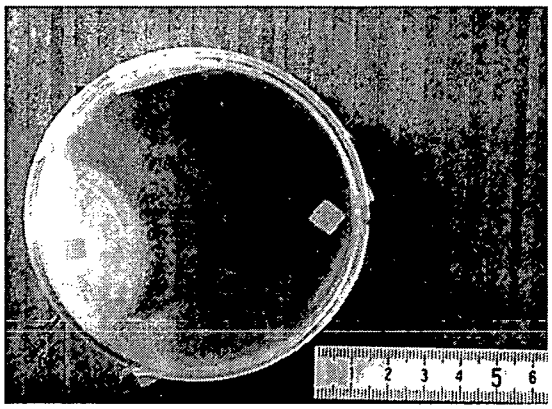

Figure 40: Dual culture of *Fol* and *Trichoderma viride* strain RE 1-3-4 on day 7.

Figure 41: Dual culture of *Fol* and *Trichoderma harzianum* strain T39 on day 7.

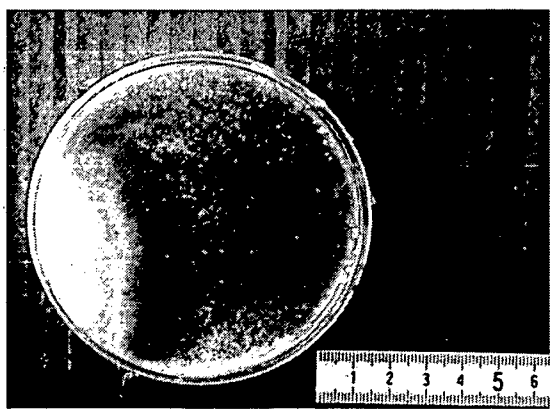

Figure 42: Dual culture of *Fol* and *Trichoderma atroviride* strain P1 on day 7.

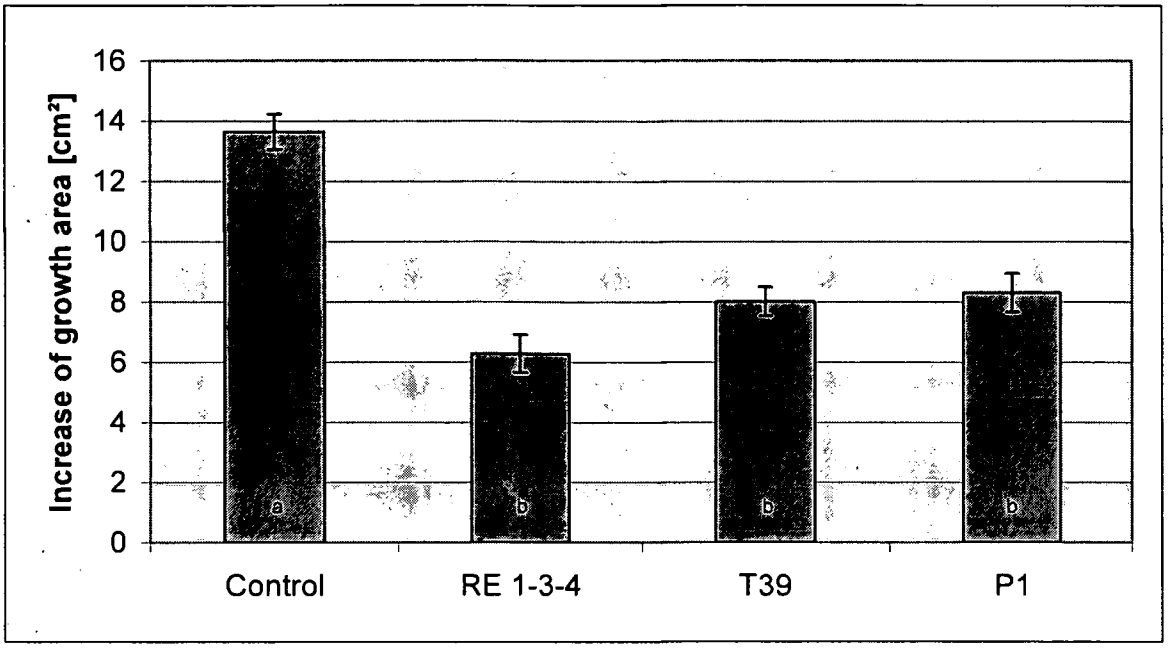

Figure 43: The increase of the area of *Fol* after 7 days of incubation in dual culture with *Trichoderma* spp. Vertical bars indicate standard error. Different letters indicate statistical significance (ANOVA, α=0.05).

4.6.3. Dual cultures of *Verticillium dahliae* and *Trichoderma* spp.

The pictures of the control treatment of *V. dahliae* on day 1 and on day 7 are shown in Figure 44 and Figure 45, respectively. The dual cultures of *V. dahliae* and the 3 different *Trichoderma* strains on day 7 are shown in Figure 46, Figure 47 and Figure 48. *T. viride* strain RE 1-3-4 showed macroscopically hardly any mycelium between the *Trichoderma* inoculation plug and the pathogen (Figure 46), but in the close vicinity of *V. dahliae* mycelium of *T. viride* could be seen macroscopically. *T. harzianum* strain T39 colonised the whole area between the pathogen and the *Trichoderma* inoculation plug (Figure 47). Furthermore, isolated greenish mycelial dots could be seen. *T. atroviride* strain P1 colonised the whole area from the P1 inoculation plug to the pathogen (Figure 48).

The increase of the area of V. dahliae in the different treatments is shown in Figure 49. There is no significant difference between the control and the *Trichoderma* treatments (Student-Newman-Keuls test, α=0.05).

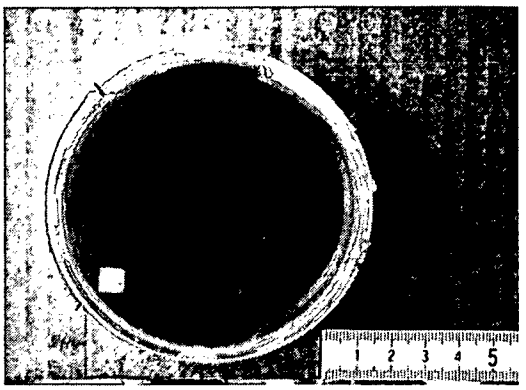

Figure 44: Control treatment of *V. dahliae* on day 1.

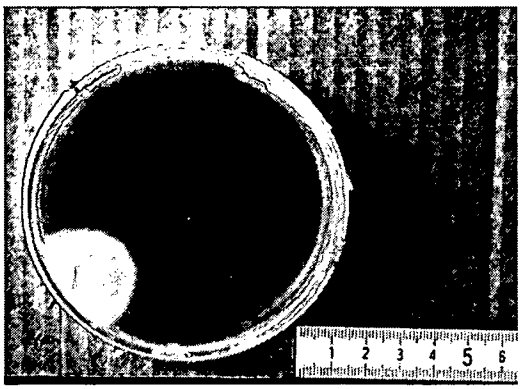

Figure 45: Control treatment of *V. dahliae* on day 7.

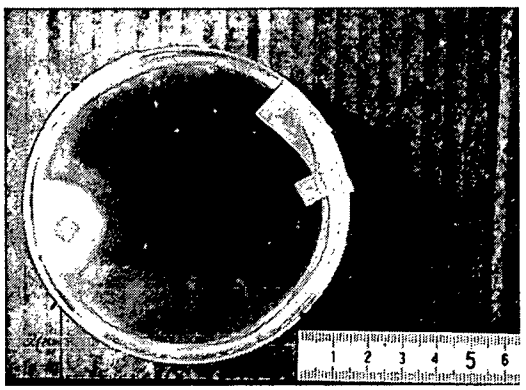

Figure 46: Dual culture of *V. dahliae* and *Trichoderma viride* strain RE 1-3-4 on day 7.

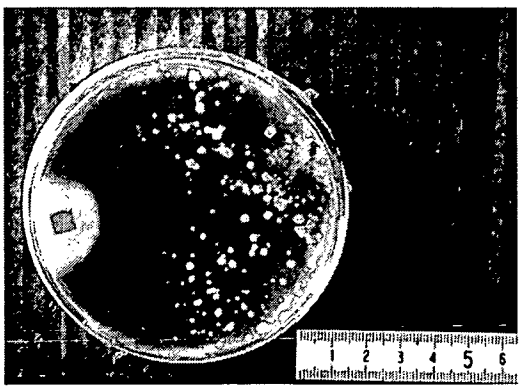

Figure 47: Dual culture of *V. dahliae* and *Trichoderma harzianum* strain T39 on day 7.

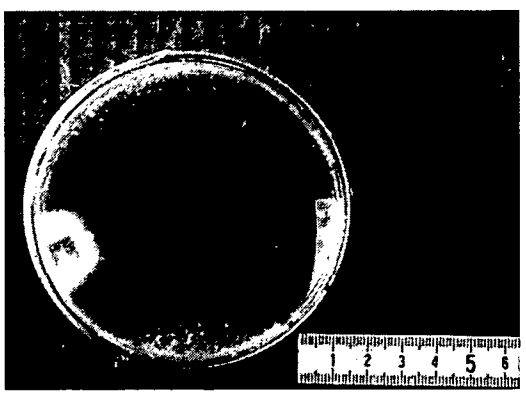

Figure 48: Dual culture of *V. dahliae* and *Trichoderma atroviride* strain P1 on day 7.

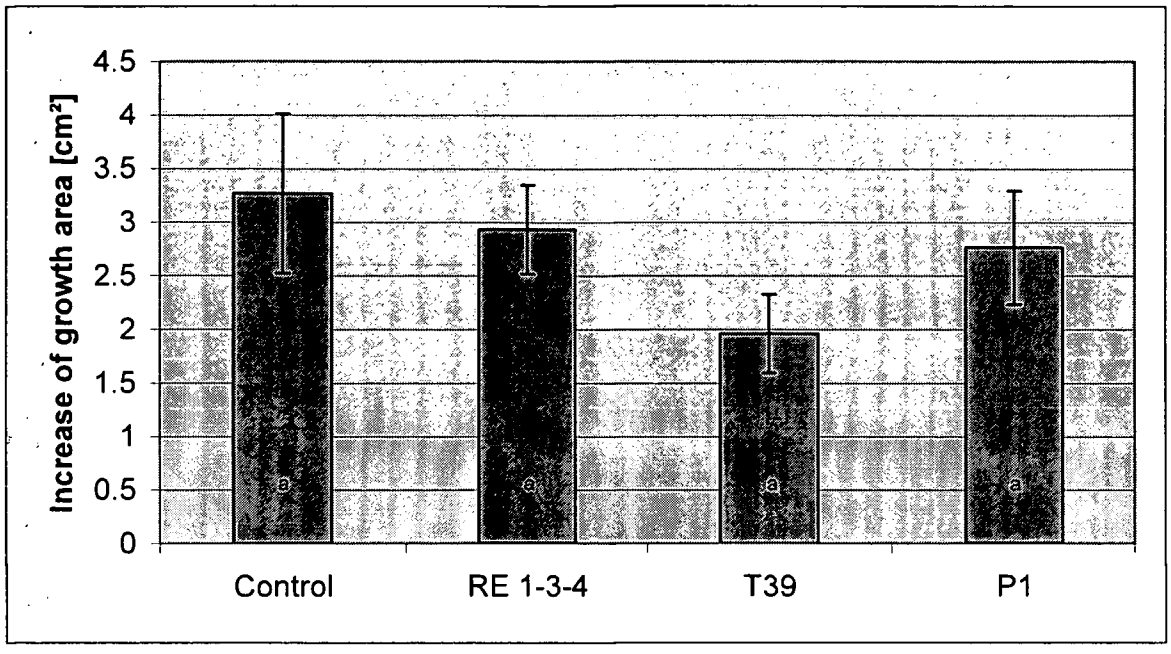

Figure 49: The increase of the growth area of *V. dahliae* after 7 days of incubation in dual culture with *Trichoderma* spp. Vertical bars indicate standard error. Different letters indicate statistical significance (ANOVA, α=0.05).

5. Discussion

Root exudates play an important role in initiation processes of plant-pathogen interactions. However, far too little is known about how exudates interact with root-infecting pathogens. In this work the effect of root exudates of tomato inoculated with three different *Trichoderma* strains and/or arbuscular mycorrhizal fungi (AMF) on *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) and *Pyrenochaeta lycopersici* was studied. Furthermore, direct effects of *Trichoderma* spp. on three soil-borne pathogenic fungi (*Fol*, *P. lycopersici* and *Verticillium dahliae*) were studied in *in vitro* dual culture tests.

Tomato cultures inoculated with *Trichoderma* spp. – Effects on plants

To determine whether *Trichoderma* spp. alter the effect of root exudates of tomato on the growth of soil-borne pathogenic fungi, tomato plants were inoculated with *Trichoderma viride* strain RE 1-3-4, *T. harzianum* strain T39 or *T. atroviride* strain P1. *In vitro* as well as climate chamber tomato cultures were performed.

In the *T. harzianum* strain T39 and *T. atroviride* strain P1 treatments of the *in vitro* cultures, some plants had only developed cotyledons and were brownish and necrotic, whereas, in the climate chamber cultures such effects could not be detected. *T. viride* strain RE 1-3-4 did not show any adverse effects. There have been reports that isolates of *Trichoderma* spp. can cause plant diseases under conducive conditions, i.e. saprophytic colonisation of senescing or necrotic tissue turns into pathogenicity on living tissue (Hjeljord and Tronsmo, 1998). *T. viride*, for instance, isolated from a healthy tomato root was pathogenic to seedlings of cucumber, pepper and tomato in laboratory and greenhouse experiments. Hjeljord and Tronsmo (1998) concluded that the pathogenicity of *Trichoderma* spp. depends on: (1) the isolate of the species-group, and (2) the environmental conditions.

The conditions in the *in vitro* cultures might have been favourable for the development of disease incidence. This could be due to the lack of fresh air, since the jars were covered, or due to the lack of nutrients for the *Trichoderma* isolates. Another cause might be the production of phytotoxic compounds such as gliotoxin,

viridin, viridol and gliovirin by *Trichoderma* spp. (Bailey and Lumsden, 1998). Gliotoxin and viridin can inhibit the germination and root growth of seeds but this effect depends highly on the plant species due to differences in tolerance to these toxins. Moreover, the activity of gliotoxin and viridin seems to be dependent on the pH. Furthermore, differences exist between different *Trichoderma* strains, since some produce 'predominantly gliotoxin or viridin whereas other strains [produce] no detectable quantities of either compound' (Bailey and Lumsden, 1998). However, 'the modes of action resulting in the toxicity of these compounds to plants are still under study' (Bailey and Lumsden, 1998). It has to be pointed out that 'the phytotoxic effects of gliotoxin and viridin are limited compared [to] their antifungal activities' (Bailey and Lumsden, 1998). Another secondary metabolite of *Trichoderma* spp. is viridiol, a dihydro-derivative of viridin, which 'has very little antibiotic activity but has considerable herbicidal activity' (Bailey and Lumsden, 1998). This phytotoxicity is active against a broad range of plant species including tomato. However, the degree depends on the amount of fungal inoculum. Typical symptoms caused by viridiol are reduced seedling emergence and reduced shoot and root weights. Viridiol is very unstable, i.e. 'the accumulation of this substance in the soil [peakes] 5 to 6 days after inoculation and [declines] to undetectable levels by 2 weeks' (Bailey and Lumsden, 1998). Furthermore, direct contact with the *Trichoderma* inoculum is necessary to cause adverse effects on plants (Bailey and Lumsden, 1998). Currently, there are no reports about adverse effects of the strains T39 and P1 on tomato plants and about the production of phytotoxic compounds or the performance under stress, i.e. the lack of nutrients. T39 is even commercially available as Trichodex® against *Botrytis* of vegetables and grapevines (Monte, 2001). However, adverse effects of *T. harzianum* strain T39 and *T. atroviride* strain P1 were detected in my *in vitro* culture experiment indicating the importance of toxicology testing under different environmental conditions. It is feasible that the environmental conditions were responsible for the adverse effects in these experiments, since in the climate chamber tomato cultures neither the number of plants nor the shoot and root fresh weight/plant were significantly affected.

Apart the ability of some *Trichoderma* spp. to control pathogenic fungi some strains can also enhance plant growth, which is indicated by increased root and shoot fresh weights (Harman *et al.*, 2004). However, the results of this work did not show any

significant increase in the root or shoot fresh weights/plant neither in the *in vitro* nor in the climate chamber cultures.

Due to the adverse effects observed in the *in vitro* cultures the climate chamber cultures were performed with seeds as well as seedlings as starting material, and with soil and perlite as substrates, in order to have enough plant material for root exudates extraction. The root exudates of the climate chamber cultures were used for the fungal growth assay, which was performed with microconidia of *Fol.* The results of this assay are discussed in section 'Fungal growth assay'.

Summing up, *in vitro* cultures of tomato inoculated with *T. harzianum* strain T39 and *T. atroviride* strain P1 showed degenerated plants. However, tomato plants of the climate chamber cultures were not adversely affected by any of the *Trichoderma* strains, i.e. in the present study the adverse effects were probably caused by conducive environmental conditions.

Tomato cultures co-inoculated with *Trichoderma* spp. and arbuscular mycorrhizal fungi – Effects on AMF colonisation and plants

In order to determine whether *Trichoderma* spp. and/or AMF alter the effect of exudates of tomato on the growth of soil-borne pathogenic fungi, tomato plants were inoculated with three different *Trichoderma* spp. strains and/or AMF.

There have been inconsistent reports about the effects of *Trichoderma* spp. on mycorrhizal fungi (Hjeljord and Tronsmo, 1998). Adverse as well as positive effects were observed. In this study no significant difference (ANOVA, α=0.05) in the root colonisation between the treatments with AMF and AMF + *Trichoderma* could be detected, i.e. in this study *Trichoderma* spp. had no adverse effect on the root colonisation of AMF. On the contrary in the study of Green *et al.* (1999) the root colonisation of cucumber by *Glomus intraradices* was significantly reduced in the presence of *T. harzianum.* McAllister *et al.* (1994a, b) found for *Trichoderma koningii* that when inoculated before or at the same time as *G. mosseae,* mycorrhizal formation was reduced. However, when *T. koningii* was applied 2 weeks after *G. mosseae* no inhibition could be seen. In my study *Trichoderma* spp. was applied 1 week after the AMF. This might be the reason for no adverse effects on the degree of colonisation. Another reason could be the used AMF inoculum which consisted of

several different AMF and therefore the failure of some AMF to colonise the roots was compensated by the other AMF. This is feasible since these interactions are highly strain specific (Hjeljord and Tronsmo, 1998).

As far as the root fresh weight/plant is concerned no significant differences (Kruskal-Wallis test, α=0.05) were found between the treatments of the *Trichoderma* spp. and AMF co-inoculation assay. These findings indicate that neither the AMF and *Trichoderma* treatments alone nor the AMF and *Trichoderma* spp. co-inoculation treatments enhanced or reduced plant root growth significantly. However, plant growth promotion has been reported before for *Trichoderma* spp. (Bailey and Lumsden, 1998, Harman and Björkman, 1998, Harman *et al.*, 2004) and AMF alone (Utkhede, 2006).

The root exudates of tomato cultures co-inoculated with *Trichoderma* spp. and AMF were used for the fungal growth assay, which is discussed in section 'Fungal growth assay'.

To summarize, neither the AMF and *Trichoderma* treatments alone nor the AMF and *Trichoderma* spp. co-inoculation treatments enhanced or reduced plant root growth significantly in the present work. The degree of root colonisation was not significantly affected by *Trichoderma* spp..

Cultivation of the tomato pathogen Pyrenochaeta lycopersici

In order to be able to perform optical density measurements with *P. lycopersici* several tests were done for optimal growth conditions and the production of large amounts of mycelium.

The best growth condition for fast mycelium production on solid medium was V8 Agar at a 16-hour photoperiod. However, for the bioassay for mycelial growth large amounts of mycelium were needed. Consequently, liquid media cultures were more suitable, since a sufficient amount of mycelium could be produced within 5 days, whereas, *P. lycopersici* would take at least 2 weeks to cover 90 % of a Petri dish. Furthermore, it was hard to separate the mycelium properly from solid media. Therefore 5-day-old liquid media cultures of *P. lycopersici* in V8 broth incubated in a 16-hour photoperiod at 24°C were taken for the experiments to establish a bioassay for the mycelial growth of *P. lycopersici.*

Establishment of a bioassay for the mycelial growth of Pyrenochaeta lycopersici

According to Broekaert *et al.* (1990) there is a straight-line relationship between absorbance and dry weight of fungi in microplate cultures. Strictly speaking, a microplate reader assay is a reliable method to determine fungal growth in different liquids like root exudates. In addition to spores as starting material, this method is also valid for mycelial fragments. Ludwig and Boller (1990) came to the same results. Furthermore, this technique is fast and requires a small culture volume (200 µl/well).
To establish a bioassay for the mycelial growth of *P. lycopersici* in root exudates of tomato the optimal preparation of the fungal material and different growth media were tested. First of all the mycelium of the liquid media cultures had to be disintegrated to a satisfactory level, which could be reached by using a pestle. According to Broekaert *et al.* (1990) 'in the absorbance range of 0 to 0.6 units, a straight-line relationship eixsts between absorbance at 595 nm and dry weight of microplate cultures'. Hence, the medium for the positive growth control should have a low absorbance level. Regarding the 0.6 units level of Broekaert *et al.*(1990), 0.4 units are still too high, simply because the adding of mycelial suspension would raise the level. Therefore, Malt Extract broth, Potato Dextrose broth, V8 juice supernatant and V8 broth were no appropriate growth media, since they ranged around 0.4 units. Czapek Dox broth and V8 broth supernatant showed levels between 0.04 and 0.1 units. Consequently, these media are adequate for the fungal growth assay of *P. lycopersici* as far as the level of the optical density is concerned. However, their value for fungal growth has also to be taken into consideration.
For the fungal growth test in the different media, aliquots of 100 µl of growth medium and 25 µl of mycelial solution (500 mg/l) were incubated in a microplate for 120 hours and optical density measurements were carried out in intervals of 24 hours. A rise of the ODs, i.e. mycelial growth, occurred 72 hours after incubation in V8 broth supernatant and Potato Dextrose broth supernatant. In Czapek Dox broth growth occurred 24 hours later. This indicates that the growth rate of *P. lycopersici* is very low. These findings are inconsistent with the development of *P. lycopersici* in another test which was performed at an even higher mycelium concentration of 10 g/l but with equal aliquots as the other test. V8 broth supernatant as well as Czapek Dox broth were used as positive controls. Furthermore, the mycelial fragments were incubated

in root exudates and in water. However, no rise of the OD could be observed within 120 hours of incubation, apart from V8 broth supernatant, where a rise of 0.1 units could be observed within the last 24 hours. These findings indicate that no growth or hardly any growth occurred within 120 hours. This does not imply that *P. lycopersici* does not react to exudates at all. It is possible due to the slow development of *P. lycopersici* (Jones *et al.*, 1989) that a rise of the OD would occur after 120 hours of incubation. However, after 120 hours the wells start drying out and as the microplates cannot be kept under complete sterile conditions, contamination with other microorganisms is likely. Another source of error might be the production of the mycelial suspensions. The used disintegration as well as the used quantification methods do not yield mycelial suspensions with homogeneous mycelial fragments as special devices would do.

Summing up, the microplate technique does not seem to be adequate for measuring the development of *P. lycopersici* in root exudates due to its slow growth. Hence, the tests of the collected root exudates were carried out with microconidia of *Fusarium oxysporum* f.sp *lycopersici* (*Fol*).

Fungal growth assay

To test whether the effect of tomato root exudates on the growth of soil-borne pathogens is altered by *Trichoderma* spp. and/or AMF inoculation a fungal growth assay was performed. Therefore, 35 µl of microconidia solution of *Fol* (1 x 10^7 microconidia/ml water) were incubated in 175 µl of the different root exudates in a microplate. For this assay the root exudates of the tomato cultures inoculated with *Trichoderma* spp., and *Trichoderma* spp. and/or AMF, respectively, were taken. Growth was determined measuring absorbance (600 nm) with a microplate reader every 24 hours for five consecutive days. As already mentioned before this assay is based on the findings of Broekaert *et al.* (1990) and Ludwig and Boller (1990) that there is a straight-line relationship between absorbance and dry weight of fungi in microplate cultures. Thus, this assay should give an idea about alterations in the growth development of *Fol* in root exudates of tomato due to *Trichoderma* spp. or AMF or due to a combination of both.

It is very likely that alterations occur, since the composition of root exudates is highly influenced by factors such as plant species, age and physiological state of the plant, soil pH, soil moisture, temperature and the presence of microorganisms (Bais *et al.*, 2006, Bertin *et al.*, 2003, Nelson, 1990). There are several findings that some *Trichoderma* strains (Benítez *et al.*, 2004, Harman *et al.*, 2004) as well as AMF (Azcón-Aguilar *et al.*, 2002, Pozo *et al.*, 2002, Singh *et al.*, 2000, Xavier and Boyetchko, 2004) can activate defence mechanisms, which is an alteration of the physiological state of the plant. Furthermore, effects of root exudates of tomato on the germination of the microconidia of *Fol* have been reported by Steinkellner *et al.* (2005) and Scheffknecht *et al.* (2006). But nothing is known about the fungal growth of Fol in exudates. With regard to my experiments there were virtually no findings so far that *Trichoderma* spp. change the root exudation pattern of plants.

Measuring the fungal growth with a microplate reader can only give a small idea of the processes involved in infecting the host. Olivain and Alabouvette (1999) studied the colonisation process of tomato roots by a pathogenic *Fol* strain in comparison to a non-pathogenic strain. Within 48 hours after inoculation the pathogenic strain rapidly colonised the root surface and built a dense network of hyphae. 24 hours after inoculation the pathogen had already penetrated the epidermis of the root. The built-up barriers in the hypodermis and in the cortex failed to prevent the centripetal growth of the pathogen. 7 days after inoculation *Fol* was highly active in the stele. In the present fungal growth assay only the development outside of the root can be simulated. The further development of the mycelium of *Fol* cannot be predicted with this fungal growth assay. However, Olivain and Alabouvette (1999) showed that the pathogenic and the non-pathogenic *Fol* strain competed for colonisation of the root surface and the root tissue. Furthermore, Olivain and Alabouvette (1999) point out that the pathogenic as well as the non-pathogenic strain 'are more abundant where root exudation is important, indicating potential competition for nutrients'. This indicates that mycelial growth is crucial for successfully colonising the root surface and that mycelial growth is directed towards root exudates, and, therefore, this fungal growth assay can give an idea about crucial events in the infection process.

In the present work, the fungal growth test with the exudates of the climate chamber cultures revealed significant differences in the mycelial growth of *Fol*. In the tomato cultures where 12-day-old seedlings were used as starting material all *Trichoderma* strains significantly reduced the growth of *Fol* compared to the control. When seeds

were used as starting material *T. harzianum* strain T39 and *T. viride* strain RE 1-3-4 reduced the growth of *Fol*, whereas, *T. atroviride* strain P1 enhanced the growth of *Fol*. This behaviour could be detected in the soil treatments. In the perlite treatments the situation was different. The differences between the perlite treatments, if there were any, were smaller than for the soil treatments. In the seed treatment strain P1 showed equal levels as the control and *Fol* incubated in water, whereas, strain T39 and RE 1-3-4 showed lower levels of the OD of *Fol* than the control. In the seedling treatment strain RE 1-3-4 did not differ from the control, whereas, strain P1 showed a higher OD of *Fol* than the control did. The ODs of *Fol* in all exudates were lower than in water. Consequently, the influences of *Trichoderma* spp. on the growth of *Fol* depend on the *Trichoderma* strain, the time of inoculation and on the substrate.

It has to be mentioned that the data of Broekaert *et al.*(1990) and Ludwig and Boller (1990) showed very small relative standard deviations of 2 to 6 %, whereas, in this assay the relative standard deviations ranged between 5 and 18 %. This is probably due to the used microplate reader, which measures only at one spot per well and is therefore more prone to extremes. In the experiments of Broekaert *et al.*(1990) and Ludwig and Boller (1990) mircoplate readers were used, which measured at 5 spots per well and calculated the average. To obtain more homogenous results it would be better to use such a device. Nevertheless, standard deviations in the results of the present work are still very low.

The findings of this assay indicate that the used *Trichoderma* strains are able to affect the growth of *Fol* indirectly by influencing the exudate pattern of the host plant and thus, are possibly involved in the bioprotectional effect of *Trichoderma* spp. towards *Fol*. This could be due to defence mechanisms activated by metabolites of *Trichoderma* spp. (Bailey and Lumsden, 1998, Benítez *et al.*, 2004, Harman, 2006, Harman *et al.*, 2004) or a possible cross-talk between *Trichoderma* spp. and the host to release more nutrients (Nelson, 1990). The lower growth rate of Fol in the perlite treatments compared to the soil treatments could be due to the availability of nutrients in the substrate, since steaming of soils and potting mixes is known to release nutrients that can be utilised by microorganisms (Green and Jensen, 2000) and can influence the exudation pattern as well. More generally speaking, a high growth rate of *Fol* could be caused by components in the root exudates which are favourable for mycelial development, i.e. components that indicate the pathogen that the host is worth attacking and components that provide *Fol* with carbon. If a high

growth rate of *Fol* is beneficial or adverse for the tomato plant, can only be guessed. A high growth rate would be certainly adverse if this leaded to increased infection of the plant. On the other hand a high growth rate would be beneficial if the antagonistic fungus was a better competitor than the pathogen and reduced as a consequence fungal inoculum of the pathogen. A low growth rate might be caused by inhibitory compounds released by the plant and *Trichoderma* spp. might be the trigger for the release of such compounds. However, it was clearly demonstrated that *Trichoderma* spp. alter the exudation pattern and as a consequence of these alterations fungal growth of *Fol* is affected. Apart the *Trichoderma* strain, the time of inoculation and the substrate had an influence on the growth development of *Fol*. But little is known about the interactions of *Trichoderma* spp. and *Fol* via the plant, consequently, further studies are needed to elucidate the exact role of alterations of the root exudation by *Trichoderma* spp. in the bioprotectional effect against *Fol*.

The fungal growth test with the exudates of the tomato cultures inoculated with *Trichoderma* spp. and AMF confirms that the root exudation of tomato plants is altered by mycorrhization and the application of *Trichoderma* spp.. Whereas microconidia germination was enhanced in the presence of root exudates from mycorrhizal tomato plants (Scheffknecht *et al.*, 2006), in the present work the fungal growth of *Fol* was reduced by the presence of root exudates from mycorrhizal tomato plants. This indicates that the alterations in the exudation pattern through mycorrhization affect microconidia germination and fungal growth of *Fol* differently. The inhibitory effect on fungal growth of *Fol* when the different *Trichoderma* strains were applied in combination with the AMF were similar as with AMF alone, indicating that the alterations of the exudation pattern due to mycorrhization are more important than the effects caused by alteration of the root exudation by the *Trichoderma* strains. There was only one exception: Fungal growth in root exudates from the treatment *Trichoderma* strain T39 + AMF was even more reduced than in the AMF treatment. This could mean that *Trichoderma* strain T39 and AMF alter the root exudation in a similar way, both microorganisms enhancing the exudation of inhibitory compounds. Further studies are needed to elucidate these complex interactions.

To summarize, the inoculation of tomato with *Trichoderma* spp. alter the exudation pattern in a way that the fungal growth of *Fol* is affected. The observed effects

depend on the *Trichoderma* strain, the used substrate and the time of inoculation. The fungal growth of *Fol* was reduced by the presence of root exudates from mycorrhizal tomato plants. The inhibitory effect on fungal growth of *Fol* when the different *Trichoderma* strains were applied in combination with the AMF were similar as with AMF alone, only for one treatment (AMF + *T. harzianum* T39) the fungal growth was even lower than for AMF alone.

Dual culture tests

To see if *T. viride* strain RE 1-3-4, *T. harzianum* strain T39 and *T. atroviride* strain P1 have direct antagonistic effects on the three tomato pathogens, *P. lycopersici*, *Fol* and *V. dahliae*, *in vitro* dual culture tests were performed.

All *Trichoderma* strains reduced the growth of *P. lycopersici* significantly. No differences were detected between the *Trichoderma* strains. Pérez *et al.* (2002) found that the ability of different *T. harzianum* isolates to reduce the *in vitro* development of *P. lycopersici* is based on the production of non-volatile metabolites, such as β-1,3-endoclucanases, proteases and endochitinases. The secretion of chitinases appears to be more important than the other metabolites in the biological control of *P. lycopersici.* However, since the production of secondary metabolites is highly strain-specific the secretion pattern could be different in the present work.

The growth area of *Fol* was significantly reduced by all 3 *Trichoderma* strains. Differences between the 3 *Trichoderma* strains were not significant. These results indicate that beside the indirect effects of *Trichoderma* spp. on the growth of *Fol* in root exudates the 3 tested *Trichoderma* strains are able to influence the growth of *Fol* on Czapek Dox Agar directly. The growth reduction could be due to the production of secondary metabolites of the *Trichoderma* strains, however, further studies are needed to elucidate the responsible mechanisms involved in the growth reduction of *Fol* in dual culture tests. Nevertheless, the observed effects on the growth rate of *Fol* in crude root exudates and the direct effects in the dual culture tests caused by *Trichoderma* spp. indicate the versatility in the modes of action of *Trichoderma* spp..

No differences in the growth development of *V. dahliae* between the *Trichoderma* treatments and the control were detected. On the one hand this might be due to the small growth rate of *V. dahliae* so that it would take longer until differences would be

clear-cut. On the other hand *V. dahliae* is known to be a poor soil competitor (Pohronezny, 1991) and therefore, might not be appropriate for dual culture tests.

It has to be pointed out that the results of the dual culture tests have to be seen with caution. Whipps (1987) found that the used medium for dual cultures affected the growth rates of pathogenic fungi as well as the production of and the response to volatile and non-volatile antibiotic compounds and hyphal interactions. However, the performance of the fungi on the media was related to their inherent properties and to their natural ecological behaviour. Consequently, Whipps (1987) suggested that antagonists should be tested on several media simulating different nutrient conditions. Although *in vitro* dual culture tests cannot provide the same conditions as in the field, they are adequate for a general screen for the effectiveness of antagonists. Nevertheless, it has to be borne in mind that promising candidates of the *in vitro* tests have to be tested *in vivo* as well.
Summing up, in the dual cultures the growth areas of *Fol* and *P. lycopersici* were significantly reduced by the 3 applied *Trichoderma* strains, whereas, no effects on the growth area of *V. dahliae* could be detected.

The present work shows that interactions between BCAs, pathogens and plants are very complex, therefore, the findings of the present work can only give a small idea of the interactions taking place in the field. Finally, further studies are needed to elucidate these complex interactions, in order to develop effective and environmentally friendly control measurements against important plant diseases.

6. Summary

Root exudates play an important role in initiation processes of plant pathogen interactions. Therefore, 3 different *Trichoderma* strains (*Trichoderma viride* strain RE 1-3-4, *T. harzianum* strain T39 and *T. atroviride* strain P1) were screened for their effects on plant growth, root exudation pattern, plant symbionts (AMF) and tomato pathogens.

Plant growth was affected negatively only in the *in vitro* cultures, probably due to adverse environmental conditions. Positive effects were not detected.

In order to test the effects of *Trichoderma* on *Fol* and *P. lycopersici* via the alteration of the root exudation pattern, it was tried to develop a biotest with a microplate reader. This technique was not adequate to measure the growth of *P. lycopersici*, but of *Fol*. Therefore only the effects on the growth rate of *Fol* were tested.

In the growth assay of *Fol* in the exudates of the tomato cultures inoculated with *Trichoderma* spp. as well as in the cultures inoculated with *Trichoderma* and/or AMF clear indications are given that *Trichoderma* spp. alter the exudation pattern of tomato plants and that these alterations affect the fungal growth of *Fol*. However, the picture is not clear yet as depending on the tested *Trichoderma* strain, the used substrate and the time of inoculation, inhibitory as well as stimulatory effects on the fungal growth of *Fol* were observed. The effects on *Fol* observed in the dual culture tests were all inhibitory, but these findings are only valid for Czapek Dox Agar as growth medium.

Trichoderma spp. did not have any adverse effects on the degree of root colonisation by AMF. In the microplate reader assay the fungal growth of *Fol* was reduced by the presence of root exudates from mycorrhizal tomato plants. The inhibitory effect on fungal growth of *Fol* when the different *Trichoderma* strains were applied in combination with the AMF were similar as with AMF alone, only for one treatment (AMF + *T. harzianum* T39) the fungal growth was even lower than for AMF alone.

In the dual cultures the growth areas of *Fol* and *P. lycopersici* were significantly reduced by the 3 applied *Trichoderma* strains, whereas, no effects on the growth area of *V. dahliae* could be detected.

Interactions between BCAs, pathogens and plants are very complex, therefore, the present work can only give a small idea of the interactions taking place in the field. Consequently, further studies are needed to elucidate these complex interactions.
In conclusion:

- Inoculation of tomato with *Trichoderma* spp. altered the root exudation pattern indicated by a change in the fungal growth development of *Fusarium oxysporum* f.sp. *lycopersici* incubated in these exudates.
- Inoculation of tomato with arbuscular mycorrhizal fungi altered the root exudation pattern indicated by a change in the fungal growth development of *Fusarium oxysporum* f.sp. *lycopersici* incubated in these exudates.
- The microplate technique is not an adequate method to measure the growth development of *P. lycopersici* in root exudates.
- Co-inoculation of tomato with *Trichoderma* spp. and AMF had the same effect on the fungal growth development of *Fol* in the exudates as AMF alone, with only one exception: the combination of AMF + *T. harzianum* strain T39.
- *Trichoderma* spp. had no effect on the degree of root colonisation by AMF.
- In the dual cultures the growth areas of *Fol* and *P. lycopersici* were significantly reduced by the 3 applied *Trichoderma* strains, whereas, no effects on the growth area of *V. dahliae* could be detected.

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Appendix

Acidified Potato Dextrose Agar (APDA)

31.2 g	Potato Dextrose Agar
3 – 5 drops of	25 % lactic acid
800 ml	dH_2O

Czapek Dox Agar (CzA)

2.4 g	$NaNO_3$
0.8 g	K_2HPO_4
0.4 g	KCl
0.4 g	$MgSO_4 \times 7\ H_2O$
0.008 g	$FeSO_4 \times 7\ H_2O$
24 g	Saccharose
12 g	Agar
800 ml	dH_2O

Czapek Dox Broth

2.4 g	$NaNO_3$
0.8 g	K_2HPO_4
0.4 g	KCl
0.4 g	$MgSO_4 \times 7\ H_2O$
0.008 g	$FeSO_4 \times 7\ H_2O$
24 g	Saccharose
800 ml	dH_2O

Malt Extract Agar (MA)

32 g	Malt Extract
16 g	Agar
800 ml	dH_2O

Malt Extract Broth

32 g	Malt Extract
800 ml	dH_2O

Potato Dextrose Agar (PDA)

31.2 g	Potato Dextrose Agar
800 ml	dH_2O

Potato Dextrose Broth

800 ml	Infusion from potatoes
16 g	Glucose
12 g	Agar

Potato infusion: Scrubbed and sliced potatoes (200 g) were boiled in water for 1 hour. Afterwards they were passed through a fine sieve. New potatoes should be avoided.

V8 Agar

160 ml	"BioBio - Gemüsesaft" (Zielpunkt Warenhandel GmbH & Co KG, Vienna, Austria)
2,4 g	$CaCO_3$
12 g	Agar
800 ml	dH_2O

Double V8 Agar

320 ml	"BioBio - Gemüsesaft"
1.6 g	$CaCO_3$
12 g	Agar

V8 Broth

160 ml	"BioBio - Gemüsesaft"
2,4 g	$CaCO_3$
800 ml	dH_2O

Water Agar (WA)

12 g	Agar
800ml	dH_2O