Institut für Pflanzenkrankheiten
der
Rheinischen Friedrich-Wilhelms-Universität

The interrelationships between rhizobacteria and arbuscular mycorrhizal fungi and their importance in the integrated management of nematodes and soilborne plant pathogens

Inaugural-Dissertation

zur

Erlangung des Grades

Doktor der Agrarwissenschaften

(Dr. agr.)

der

Hohen Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität

zu Bonn

vorgelegt am 07. März 2005

von

Stephan Reimann

aus

Nettetal

Referent: Prof. Dr. R. A. Sikora

Korreferent: Prof. Dr. H. E. Goldbach

Tag der mündlichen Prüfung: 03. Juni 2005

Diese Dissertation ist auf dem Hochschulschriftenserver der ULB Bonn

http://hss.ulb.uni-bonn.diss_online elektronisch publiziert (2005)

D 98

ABSTRACT

The interrelationships between rhizobacteria and arbuscular mycorrhizal fungi and their importance in the integrated management of nematodes and soilborne plant pathogens

A total of 62 bacterial isolates were isolated from surface sterilized AMF (arbuscular mycorrhizal fungi) spores, which were previously extracted from tomato fields in Nakhon Ratchasima Province of Thailand. These spore-associated bacteria were tested for their antagonistic potential against the root-knot nematode *Meloidogyne incognita* and the soilborne plant pathogens *Fusarium oxysporum*, *Pythium aphanidermatum*, *Pythium ultimum* and *Rhizoctonia solani*.

A high percentage of the bacterial isolates (14,5%) reduced nematode penetration of tomato roots in repeated experiments. Two of these antagonistic bacteria were further tested in a long-term experiment, either alone or in combination with *Glomus intraradices*. Single inoculations resulted in low or even no biological control, but both dual inoculations reduced gall and egg mass production.

Twelve bacteria (19,4%) inhibited fungal growth of at least one pathogen *in vitro* and six isolates showed antagonistic potential against all pathogens tested. The seven isolates that inhibited growth of *Fusarium oxyporum in vitro* were tested *in vivo* against the same pathogen. Three of these seven bacteria reduced the reisolation rate of the pathogen and therefore inhibited fungal spread in the tomato stem.

The PHPR *Rhizobium etli* G12, inducing resistance against *M. incognita*, was found to promote establishment of *G. intraradices* on three different tomato cultivars. The bacterium appears to accelerate AMF colonization of the root, and therefore should be regarded a Mycorrhiza helper bacterium (MHB). In *in vitro* studies, *R. etli* stimulated AMF sporulation and hyphal branching, the latter of which is an important prerequisite for penetration of the host roots. This direct effect of the bacterium on fungal morphology is one explanation for the increased mycorrhiza establishment, but a plant-mediated effect cannot be excluded.

The combination of *G. intraradices* with *R. etli* enhanced biocontrol of *M. incognita* on tomato, in comparison to single inoculations. The better mycorrhization of the young transplants and the combination of different modes of action could be the explanations of the increased antagonistic potential.

ABSTRACT

Die Wechselbeziehungen zwischen Rhizosphärenbakterien und arbuskulären Mykorrhizapilzen und ihre Bedeutung in der integrierten Bekämpfung von Nematoden und bodenbürtigen pilzlichen Schaderregern

Insgesamt 62 Bakterienisolate wurden von oberflächensterilisierten AMP-Sporen isoliert, die zuvor aus Tomatenfeldern in der Nakhon Ratchasima Province in Thailand extrahiert wurden. Diese sporenassoziierten Bakterien wurden auf ihr antagonistisches Potential gegen den Wurzelgallennematoden *Meloidogyne incognita* und die bodenbürtigen Schaderreger *Fusarium oxysporum*, *Pythium aphanidermatum*, *Pythium ultimum* und *Rhizoctonia solani* untersucht.

Ein hoher Prozentsatz (14,5%) der Bakterienisolate reduzierte wiederholt die Eindringung des Nematoden in die Tomatenwurzel. Zwei dieser Isolate wurden in einem Langzeitexperiment erneut getestet, entweder allein oder in Kombination mit *Glomus intraradices*. Die Einzelinokulationen führten zu keinem oder nur geringem Bekämpfungserfolg. Demgegenüber führte die Kombination der Mikroorganismen in beiden Fällen zu einer Reduzierung der Gallen- und Eiermassenbildung.

Zwölf der sporenassoziierten Bakterien (19,4%) hemmten das pilzliche Wachstum zu mindest eines Phytopathogens *in vitro* und sechs Bakterienisolate zeigten antagonistisches Potential gegen alle getesteten pilzlichen Schaderreger. Sieben Isolate, die das Wachstum von *Fusarium oxysporum in vitro* gehemmt hatten, wurden gegen das gleiche Phytopathogen *in vivo* getestet. Drei dieser Bakterien reduzierten die Rückisolationsrate des Pathogens und hemmten daher die Ausbreitung des Pilzes im Tomatenspross.

Das Bakterium *Rhizobium etli* G12, welches Resistenz gegen *M. incognita* induziert, förderte die Etablierung von *G. intraradices* an vier verschiedenen Tomatensorten. Durch das Bakterium scheint die Pflanzenwurzel schneller mit Mykorrhiza kolonisiert zu werden und daher sollte es als Mykorrhiza förderndes Bakterium betrachtet werden. In *in vitro* Studien stimulierte *R. etli* die Sporulation des AMP und die Anzahl der Hyphenverzweigungen. Dieser direkte Effekt des Bakteriums auf die Pilzmorphologie ist eine Erklärung für die geförderte Etablierung des AMP, aber ein über die Pflanze vermittelter Effekt kann nicht ausgeschlossen werden.

Die Kombination von *G. intraradices* mit *R. etli* erhöhte den Bekämpfungserfolg von *M. incognita* an Tomate, gegenüber den Einzelinokulationen. Die bessere Mykorrhizierung der Jungpflanzen und die Kombination verschiedener Wirkungsmechanismen könnte eine Erklärung für das erhöhte antagonistische Potential sein.

Table of contents

1 General introduction

Soil is a natural resource and the biotic and abiotic interactions occurring in the soil ecosystem are extremely important topics affecting sustainable agriculture today. In contrast very little is known about the interrelationships between organisms in this ecosystem, because of its complex nature. Spatial and chemical heterogeneity is extremely high and the biodiversity within a few cubic centimeters of soil is equal to that of other important ecosystems, e.g. coral reefs (Sugden *et al.*, 2004; Pal Bais *et al.*, 2004). The importance of soil organisms for plants can be well demonstrated with the symbiotic interrelationship between mycorrhizal fungi and plants. Mycorrhizas are able to form a symbiosis with about 80% of all terrestrial plants. These fungi have well known effects on plant nutrition and seem to have played a mayor role when plants colonized land four hundred million years ago (Penninsi, 2004).

Plant pathologists are interested in protecting crops against harmful organisms and this becomes extremely difficult if they are working with soilborne pathogens. Once a soil is infested with a pathogen the scope of available plant protection tools becomes limited. In the past, the easiest and most efficient treatment was soil decontamination with chemical fumigants such as methyl bromide. However, because of severe environmental side effects, for example ozone depleting properties in the case of methyl bromide, many of these chemical substances are no longer registered for field use (Anonymous, 1987). Integrated pest management (IPM) strategies, which combine multiple pest management methodologies, are the best alternative approach to the use of fumigants (Batchelor and Miller, 1998). The following technologies are important components of IPM programs (Sikora *et al.*, 2005):

- Plant hygiene (Exclusion and quarantine)
- Cultural practices (Crop rotation, multiple cropping, fallow, soil-less culture, tillage, organic amendment, flooding, biofumigation and planting time)
- Choice of planting material (Resistant or antagonistic crops and grafting of susceptible cultivars to resistant root-stocks)
- Physical methods (Solarization and steam treatment)
- Application of selective pesticides
- Biological control with antagonistic organisms

In the soil ecosystem pathogenic and non-pathogenic microorganisms are in competition with each other on a microscopic scale with “survival of the fittest” the rule of law (Sikora and Reimann, 2004). Every soil has an antagonistic potential against specific pests which is defined as the capacity of the soil ecosystem through biotic factors, to prevent or reduce the spread of a pathogen, parasite or deleterious agent (Sikora, 1992). The antagonistic potential can range from extremely low antagonism to total suppressiveness (Baker and Cook, 1982). Therefore a certain level of biocontrol is the rule rather than the exception in agricultural soils.

In the past many soil microorganisms have been isolated and tested for activity against plant pathogens – with varying degrees of success. Some of these biocontrol agents show direct antagonism against a pest or pathogen in the soil. For instance the bacterium *Pasteuria penetrans* can parasitize nematodes and destroys their reproductive capacity, while development of the parasitized nematode is not affected (Stirling, 1991). Different strains of the fungus *Paecilomyces lilacinus* were found to parasitize eggs of root-knot and cyst nematodes (Stirling, 1991; Al-Raddad, 1995) and hence decrease nematode population density. The ubiquitous fungal genus *Trichoderma* includes several species, that produce antibiotics against different plant pathogens and for this reason these fungi have been the most thoroughly researched of the fungal biocontrol agents (Whipps, 2001). Whipps also mentioned that competition for nutrients and space is another possible mode of action for how beneficial fungi can suppress pathogen attack. Antibiosis is the dominant mode of action of bacterial antagonists as well (Chet *et al.*, 1990). The production of siderophores, that efficiently sequester iron and therefore suppress pathogen development, was intensively studied (Raaijmakers *et al.*, 2002).

Other biocontrol agents have no direct effect on the pest or pathogen. Fungal endophytes, that spend almost their entire life cycle in the plant tissue, have been shown to increase plant health (Carroll, 1988). These fungi can increase plant growth, and therefore help the plants to overcome biotic and abiotic stress (West *et al.*, 1988; Latch, 1993). On the other hand they are able to enhance plant resistance against pathogen attack (Sikora, 1992; Hallmann and Sikora, 1994; Fuchs *et al.*, 1997). Some plant health promoting rhizobacteria (PHPR) have been shown to induce resistance toward different plant pathogens and pests (Baker and Paulitz, 1996; Hasky-Günther *et al.*, 1998; Van Loon *et al.*, 1998; Reitz *et al.*, 2001). Induced

resistance is also one possible explanation for better plant growth after inoculation with arbuscular mycorrhizal fungi (Morandi, 1996), but the mechanisms responsible for increased plant health remain unclear (Dehne, 1982; Linderman, 1992).

In the present study, a series of experiments were conducted to investigate the interrelationships between arbuscular mycorrhizal fungi and rhizobacteria in the biocontrol of root-knot nematodes or soilborne pathogens. Therefore a detailed description of the antagonists, nematodes and pathogens used in these studies are presented below.

1.1 Arbuscular mycorrhizal fungi

In 1885 Frank was the first to describe the symbiosis between a fungus and the roots of trees and he coined the word mycorrhiza (fungal root). He himself already recognized the enormous potential and importance of his findings, calling the fungus the “wet nurse” of the tree. Today we know that mycorrhizas are the most widespread associations between microorganisms and higher plants, occurring on roots of more than 80% of all terrestrial plants (Sieverding, 1991). According to fossil records, plants colonizing terrestrial ecosystems four hundred million years ago were mycorrhizal (Remy *et al.*, 1994). Hence mycorrhizal fungi have been involved in the adaptation of plants to unfavorable conditions from the very beginning (Penninsi, 2004).

The most prominent effect of the fungus is improved phosphorus nutrition of the host plant in soils with low phosphorus levels (Koide, 1991). However uptake of nitrogen, zinc, copper and minor nutrients are enhanced as well. Improved plant nutrition is due to (i) increased root surface through extraradical hyphae, (ii) degradation of organic material and (iii) alteration of the microbial composition in the rhizosphere (Marschner, 1998; Hodge and Campbell, 2001). In exchange for the nutrients, the fungus is supplied with photosynthates (about 20% of the plant assimilation capacity) and these assimilates are either respired or released into the soil. As a result of higher carbon drain, microbial rhizosphere composition is altered and therefore the term “mycorrhizosphere” was coined (Rambelli, 1973). As the majority of all plants are mycorrhizal, mycorrhizosphere versus rhizosphere might be the rule rather than the exception (Linderman, 1988). In addition, mycorrhizas increase drought resistance (Augé and Stodola, 1990) and heavy metal tolerance (Brundrett, 1991),

improve soil aggregation (Andrade *et al.*, 1998) and promote overall plant health (Linderman, 1992).

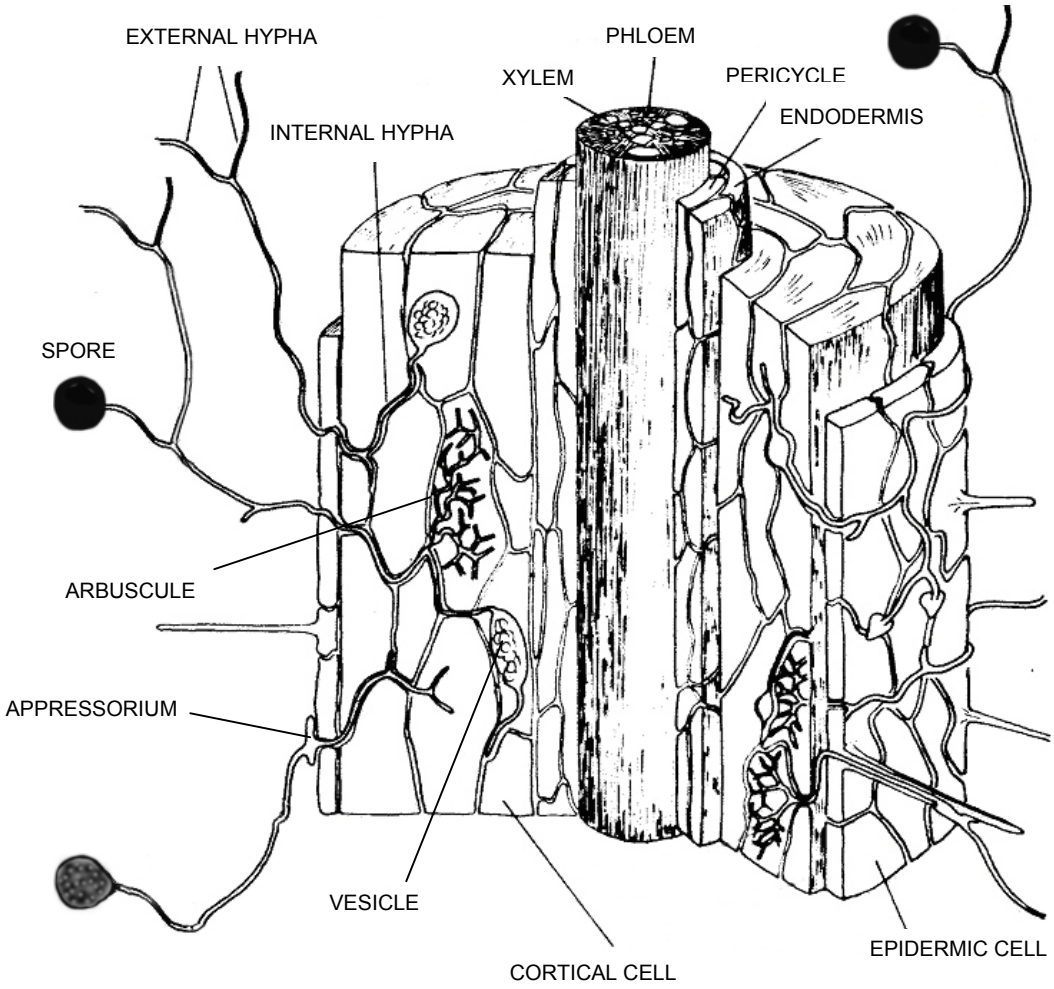

Figure 1.1: Cross-section of a plant root with mycorrhizal features (Azcón-Aguilar and Barea, 1980).

Arbuscular mycorrhizal fungi (AMF) are by far the most abundant of all mycorrhizas. They are characterized by the formation of an extraradical mycelium and branched haustorial structures within the cortical cells termed arbuscules. The latter are the main sites of exchange between the two symbiotic partners (Hock and Varma, 1995). In contrast to ectomycorrhizal fungi, AMF are considered to be obligate biotrophic microorganisms and cannot be cultured axenically. The general differentiation between symbiosis and pathogenicity is not easy and this is particularly true for

mycorrhizas. Depending on environmental conditions, root colonization with arbuscular mycorrhizal can either stimulate or inhibit plant growth (Sanders, 1993) and during the formation of the arbuscular mycorrhizal symbiosis several defence responses have been observed in the plant (Gianinazzi-Pearson *et al.*, 1996; García-Garrido and Ocampo, 2002).

The ability of AMF to suppress root diseases caused by soilborne pathogens has been intensively studied in the last thirty years. Fungal root pathogens are inhibited by mycorrhiza inoculation in the cases of *Fusarium oxysporum* on different crops (Dehne and Schönbeck, 1979; Caron *et al.*, 1986; St-Arnaud *et al.*, 1997; Filion *et al.*, 1999), various *Phytophthora* species (Davis and Menge, 1980; Cordier *et al.*, 1996; Trotta *et al.*, 1996), *Rhizoctonia solani* (Yao *et al.*, 2002) and *Pythium ultimum* (Calvet *et al.*, 1993). Bacterial diseases are also reduced by mycorrhiza establishment on the root (Dehne, 1982). Numerous reports were published on the suppression of nematode penetration and development following AMF inoculation (Saleh and Sikora, 1984; Habte *et al.*, 1999; Talavera *et al.*, 2001; Elsen *et al.*, 2001; Waceke *et al.*, 2001; Diedhiou *et al.*, 2003). Contradictory results also have been reported, which is believed to be due to the heterogeneity of experimental design and varying environmental factors (Trotta *et al.*, 1996).

Mechanisms of how AMF contribute to plant health have been intensively studied, leading to development of several hypothesis (Linderman, 1994). The most important are:

- increased nutrient uptake results in higher resistance of the plant to pathogen invasion or a compensation of the symptoms
- competition for photosynthates or space
- plant morphological changes and barrier formation
- changes in biochemical compounds related with plant defence
- increased percentage of microbial antagonists in the rhizosphere

1.2 Rhizobacteria

The rhizosphere was defined one century ago (Hiltner, 1904) as the narrow zone of soil subject to the influence of living roots. It is characterized by intense bacterial activity as a result of leakage from the root or root exudation (Lynch, 1990). Bacteria isolated from this ecosystem are commonly referred to as rhizobacteria. These microorganisms play a decisive role in nutrient uptake and the adaptation of plants to adverse soil conditions (Marschner, 1998). On the other hand the rhizosphere is the front-line between plant roots and soilborne pests. Therefore it seems logical that microorganisms which colonize the same niche could be ideal biocontrol agents (Weller, 1988). Rhizobacteria and their effects on plant growth and health have been intensively studied. Approximately 10% of all isolated bacteria affect plant growth and/or health. Consequently, they were named either plant growth promoting rhizobacteria (PGPR) or plant health promoting rhizobacteria (PHPR) according to their mode of action (Sikora, 1992), in order to distinguish them from neutral strains.

Colonization of roots with mycorrhizal fungi modifies root soil aggregation and water distribution in the soil through extramatrical hyphae (Andrade *et al.*, 1998). In addition, root exudation is altered by the fungus while acting as a carbon sink and by its own hyphal exudation. This leads to changes in both the qualitative and quantitative release of exudates into the rhizosphere and therefore to changes in the microbial composition (Hodge, 2000). Hence Rambelli (1973) already suggested in 1973 the term mycorrhizosphere to describe the soil surrounding and influenced by mycorrhizae. This term is now widely accepted.

Meyer and Lindermann (1986) observed that rhizosphere leachates of mycorrhizal plants inhibited sporulation of the plant pathogen *Phytophthora cinnamomi*, while leachates of non-mycorrhizal plants had no effect. They hypothesized that either sporulation-inducing microorganisms were missing or that the number of sporulation-inhibiting microorganisms increased. Secilia and Bagyaraj (1987) found more pathogen-antagonistic actinomycetes in the rhizosphere of mycorrhizal plants than in the rhizosphere of the non-mycorrhizal controls. In another study 1 out of 8 bacteria isolated from the mycorrhizosphere had antagonistic potential against several soilborne pathogens *in vitro* and against *Phytophthora parasitica* also *in vivo* (Budi *et al.*, 1999). These findings support the hypothesis that the mycorrhizosphere may be

rich in PHPR, but information about the antagonistic potential of microorganisms from the mycorrhizosphere is still scarce.

1.3 Spore-associated bacteria

Researchers have faced many problems trying to establish AMF under aseptic conditions. Initially it was difficult to obtain bacteria-free spores and later the sterile spores failed to germinate or penetrate sterile plants (Mosse, 1962). Based on these findings, Mosse supposed that AMF are highly dependent on other soil organisms for establishment and survival. After successfully establishing AMF multiplication under sterile conditions *in vitro* with Ri T-DNA transformed carrot roots (Bécard and Fortin, 1988) this hypothesis was no longer accepted. However, recently interest in spore-associated bacteria has increased, because bacteria obtained from AMF spores supported fungal growth up to complete spore production *in vitro* in the absence of a host (Hildebrandt *et al.*, 2002). Walley and Germida (1996) observed bacterial contamination even after intense surface sterilization.

Based on their findings, Mayo *et al.* (1986) and Xavier and Germida (2003) reported that spore-associated bacteria seem to play a mayor role in AMF spore germination. Budi *et al.* (1999) isolated one *Paenibacillus spp.* from a mycorrhizosphere exclusively formed by spore-associated bacteria and have shown that this bacterium has a broad spectrum of antagonistic activity against soilborne pathogens *in vitro* and *in vivo*.

1.4 Combination of AMF and PHPR

Inconsistent results and insufficient antagonistic activity of a single biocontrol agent are the main reasons for the lack of impact of biocontrol in agricultural disease management. One possible mean for increasing the efficacy of biocontrol would be the application of multiple antagonists with different modes of action (Stirling, 1991; Sikora, 1992; Weller and Thomashow, 1994). Reports of additive or additive effects between different antagonists in biological control of pathogens are encouraging, indicating that a combination of biological control agents could be a powerful tool for IPM in the future (Mao *et al.*, 1998; Raupach and Kloepper, 1998; Gamalero *et al.*, 2004).

In studies combining PHPR and AMF, different interactions between these microorganisms were observed. Some bacterial antagonists had negative side-effects on AMF establishment (Burla *et al.*, 1996), while others had no effect (Edwards *et al.*, 1998; Barea *et al.*, 1998; Vázquez *et al.*, 2000) or even increased mycorrhiza colonization (Meyer and Linderman, 1986). On the other hand population densities of different health promoting pseudomonades were decreased on roots of mycorrhizal plants (Ames *et al.*, 1984; Paulitz and Linderman, 1989). In conclusion, a better understanding how PHPR and AMF interact in the soil is needed in order to develop strategies that provide optimum levels of plant protection against soilborne pests and pathogens.

1.5 Root-knot nematode

Root-knot nematodes (*Meloidogyne* spp.) belong to the superfamily *Heteroderidae* and their common name refers to the characteristic galls or root-knots, associated with nematode infestation. Over 50 species have been described in agriculture around the world. They cause severe damage and high yield loss on field crops and vegetables, as well as on fruit and ornamental trees. Root-knot nematodes (RKN) are unquestionably the most economically important plant parasitic nematodes (Ferraz and Brown, 2002).

Meloidogyne incognita is the most prominent and most widely distributed representative of this genus. It has a broad host range, reproducing on more than 2000 plant species. Four races of *M. incognita* have been identified by their host spectrum. This tropical nematode has an optimum temperature range between 25 to 30°C and prefers light-textured sandy soils with a pH of 4.0 to 8.0. All vegetables, tomato in particular, are heavily damaged by this root-knot nematode in the tropics and under greenhouse conditions in temperate regions (Sikora and Fernández, 2005).

Infective second-stage juveniles (J2) invade plant roots just behind the root tip. They migrate intercellularly in the cortex until they reach the vascular cylinder. With their heads in the periphery of the vascular tissue, the juveniles induce the formation of their feeding sites, the so called giant cells (Hussey, 1985). Concurrent with the formation of giant cells, root tissues in the vicinity of the nematode undergo hyperplasia or hypertrophy. Together with the swelling female these changes at the

cell level become apparent outside the root as typical galls or root-knots. Following establishment in the root, the J2 moult three times in quick succession to the adult stage. The rare, vermiform males leave the root and *M. incognita* usually reproduces via mitotic parthenogenesis (Ferraz and Brown, 2002). Sedentary "flask-shaped" females continue to feed and start the production of ovoid-shaped eggs. At the end up to 500 eggs are released into the soil inside a gelatinous matrix. First-stage juveniles moult within the egg and the resulting J2 hatch under favorable conditions. Emerging juveniles often reinvade the nearby root or surrounding tissue and therefore, damage increases exponentially leading to big fleshy galls (Dropkin, 1989).

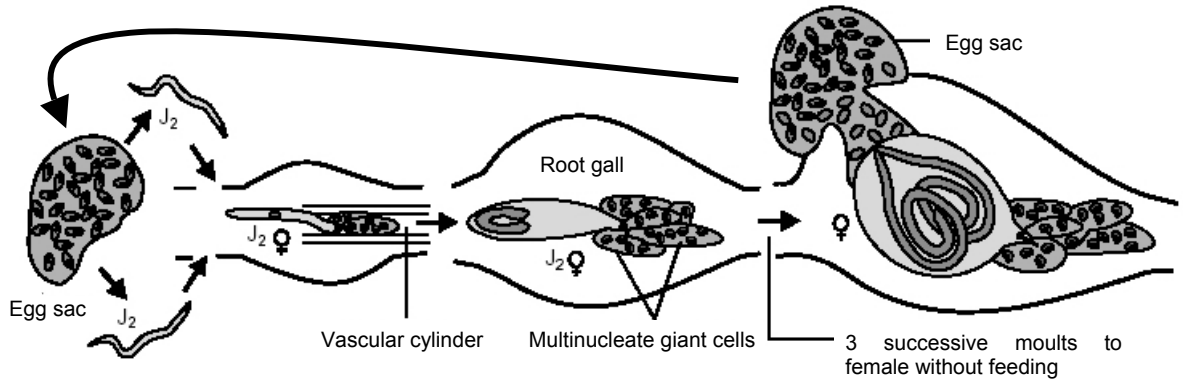

Figure 1.2: Life cycle of root-knot nematodes (Jung and Wyss, 1999).

The presence of galls on the root system disturbs important root functions like uptake and transport of water and nutrients. In addition RKN and their surrounding newly formed tissues act as a metabolic sink. Consequently shoot growth is suppressed and wilting or nutritional deficiencies like chlorosis appear. When seedlings are infected numerous plants die and those plants that survive are greatly reduced in flowering and yield potential. The nematode damaged root system also facilitates invasion by plant pathogenic bacteria and fungi. These disease complexes can increase damage relative to single pathogen attack (Sikora and Fernández, 2005).

Many different strategies of nematode management are used with varying degrees of success. First of all, prevention of field contamination with RKN is the easiest and most effective means of nematode control. Once a field is infested, farmers may choose between chemical, cultural and biological methods (Trudgill and Block, 2001).

Chemical nematicides are either fumigants or non-fumigants, with the former providing excellent nematode control. However, the majority of the chemical nematicides are highly toxic to humans and some are also harmful for the environment when improperly used (Sikora and Fernández, 2005). Cultural measures to control RKN include crop rotation, use of resistant cultivars, fallow, flooding, incorporation of organic amendments and soil solarization or steam treatment (Sikora *et al.*, 2005). In the last decades biological control of root-knot nematodes, using antagonistic bacteria and fungi, received increasing interest. Promising reports about biocontrol agents are numerous (Stirling, 1991; Sikora, 1992; Siddiqui and Shaukat, 2002) but to date very few products are commercially available. Integrated nematode management technologies, combining different methodologies, seem to be the most promising means for nematode control (Sikora *et al.*, 2005).

1.6 Soilborne plant pathogens

Tomato production is threatened by numerous foliar and soilborne pathogens. Damage caused by latter pathogens is often not recognized because of the unseen nature of many soilborne root diseases (Campbell and Neher, 1996). Additionally, several reports on the interactions of soilborne plant pathogens with plant parasitic nematodes support the observation that damage caused by nematodes can sometimes increase damage due to secondary pathogens (Mayol and Bergeson, 1970; Golden and Van Gundy, 1975; Wardle *et al.*, 2004). Some of the most important tomato root pathogens and their control have been outlined by Jones *et al.* (1991) and Aochi & Baker (1985). A brief description of the diseases, subject to the experiments of this study here, are presented below.

1.6.1 Fusarium wilt

Fusarium oxysporum Schlechtend.: Fr. f.sp. *lycopersici* (Sacc.) Snyder & Hansen is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable producing areas. The disease caused by this fungus is characterized by yellowing of the older leaves, browning of the vascular system, wilting in a later stage and finally death of the whole plant. Chlamydospores of the pathogen remain in infested soils for several years and invasion occurs through wounds on the root surface. Favorable conditions for disease development are high air and soil

temperatures (28°C), optimum soil moisture for plant growth, low soil pH and weakened plants. Three different physiological races are reported and therefore breeding for resistance is very difficult.

1.6.2 Rhizoctonia diseases

Rhizoctonia diseases are prevalent throughout the world. *Rhizoctonia solani* (Kühn) causes various diseases on different plant species including damping off, root rot, basal stem rot, stem canker and fruit rot. The fungus survives as actively growing mycelium because of its excellent saprophytic growth properties and only occasionally sclerotia are formed. Germinating seedlings are killed before or soon after emergence, while infection of older plants occurs only under unfavorable conditions for the plant or on wounded roots, e.g. after root-knot nematode attack.

1.6.3 Pythium diseases

Several *Pythium* species may attack tomato plants during their early stages of growth causing seed rot, damping off, stem and fruit rot. *P. aphanidermatum* and *P. ultimum* can be regarded as the most prominent members of this genus. The former prefers tropical conditions (>30°C), while latter develops best in temperate climate (<20°C). Oospores of *Pythium* species germinate after a period of dormancy under favorable conditions and either zoospores are released or vegetative mycelia are produced. Zoospores or mycelia are able to penetrate tomato roots directly, but wounds enhance penetration and infection.

1.6.4 Disease control

The simplest way to control all of these pathogens is to avoid infesting the soil with contaminated seeds, plants or agricultural equipment. The use of resistant tomato cultivars is an effective management technique when fields are already infested, but very few cultivars are resistant to all pathogens or races of one pathogen. Soil fumigation is a technique used to control soilborne pathogens. Many of these chemical agents, like the efficient and very frequently used methyl bromide, have been banned and there is a lack of effective alternatives. Steam treatments are only economically relevant if small amounts of soil have to be treated. Cultural techniques like crop rotation will reduce the inoculum density, but non-hosts are rare. Prevention

of nematode attack is also important in that it reduces damage caused by secondary invasion of soilborne pathogens.

1.7 Objectives

The present study was conducted to examine the interrelationships between PHPR and AMF, with special emphasis on their importance for biocontrol. The following aspects were studied:

- What types of bacteria can be found on the surface of AMF chlamydospores?
- Do these spore-associated bacteria have direct or indirect effects on root-knot nematodes or soilborne plant pathogens?
- What is the antagonistic potential of AMF and bacteria alone and in combination?
- How do PHPR that are antagonistic to root-knot nematodes affect mycorrhizal establishment?

1.8 References

AL-RADDAD, A.M. (1995) Interaction of *Glomus mossae* and *Paecilomyces lilacinus* on *Meloidogyne javanica* of tomato. Mycorrhiza 5, 233-236

AMES, R.N., REID, C.P.P. and INGHAM, E.R. (1984) Rhizosphere bacterial population responses to root colonization by a vesicular arbuscular mycorrhizal fungus. New Phytol. 96, 555-563

ANDRADE, G., MIHARA, K.L., LINDERMAN, R.G. and BETHLENFALVAY, G.J. (1998) Soil aggregation status and rhizobacteria in the mycorrhizosphere. Plant Soil 202, 89-96

ANONYMOUS (1987) Montreal protocol on substances that deplete the ozone layer. United Nations Press, New York, USA

AOCHI, L. and BAKER, L. (1985) Integrated Pest Mangement for Tomatoes. University of California, Oakland, California, USA

AUGÉ, R.M. and STODOLA, A.J.W. (1990) An apparent increase in symplastic water contributes to greater turgor in mycorrhizal roots of drougthed *Rosa* plants. New Phytol. 115, 285-295

AZCÓN-AGUILAR, C. and BAREA, J.M. (1980) Micorrizas. Investigación y Ciencia 47, 8-16

BAKER, K.F. and COOK, J.R. (1982) Biological control of plant pathogens. APS Press, St. Paul, Minnesota, USA

BAKER, R. and PAULITZ, T.C. (1996) Theoretical basis for microbial interactions leading to biological control of soilborne plant pathogens . In: Hall, R. (ed.): Principles and Practice of Managing Soilborne Plant Pathogens. St. Paul, Minnesota, USA, 50-79

BAREA, J.M., ANDRADE, G., BIANCIOTTO, V., DOWLING, D., LOHRKE, S., BONFANTE, P., O'GARA, F. and AZCÓN-AGUILAR, C. (1998) Impact on arbuscular mycorrhizal formation of *Pseudomonas* strains used as inoculants for biocontrol of soilborne fungal plant pathogens. Appl. Environ. Microbiol. 64, 2304-2307

BATCHELOR, T. and MILLER, J. (1998) Report of the Methyl Bromide Technical Options Committee - 1998 Assessment of alternatives to methyl bromide. UNEP Nairobi Ozone Secretariat, Nairobi, Kenia

BÉCARD, G. and FORTIN, J.A. (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol. 108, 211-218

BRUNDRETT, M.C. (1991) Mycorrhizas in natural ecosystems. Adv. Ecol. Res. 21, 171-313

BUDI, S.W., VAN TUINEN, D., MARTINOTTI, G. and GIANINAZZI, S. (1999) Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soil borne fungal pathogens. Appl. Environ. Microbiol. 65, 5148-5150

BURLA, M., GOVERDE, M., SCHWINN, F.J. and WIEMKEN, A. (1996) Influence of biocontrol organisms on root pathogenic fungi and on the plant symbiotic micro-organisms *Rhizobium phaseoli* and *Glomus mosseae*. Z. Pflkrankh. Pflschutz. 103, 156-163

CALVET, C., PERA, J. and BAREA, J.M. (1993) Growth response of mariegold (*Tagetes erecta*) to inoculation with *Glomus mosseae*, *Trichoderma aureoviride* and *Pythium ultimum* in a peat-perlite mixture. Plant Soil 148, 1-6

CAMPBELL, C.L. and NEHER, D.A. (1996) Challenges, opportunities and obligations in root disease epidemiology and management. In: Hall, R. (ed.): Principles and Practice of Managing Soilborne Plant Pathogens. St. Paul, Minnesota, USA, 20-49

CARON, M., RICHARD, C. and FORTIN, J.A. (1986) Effect of preinfestation of the soil by a vesicular-arbuscular mycorrhizal fungus, *Glomus intraradices*, on Fusarium crown and root rot of tomatoes. Phytoprotection 67, 15-19

CARROLL, G.C. (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiosis. Ecology 69, 2-9

CHET, I., ORDENTLICH, A., SHAPIRA, R. and OPPENHEIM, A. (1990) Mechanisms of biocontrol of soil-borne pathogens by rhizobacteria. Plant Soil 129, 85-92

CORDIER, C., GIANINAZZI, S. and GIANINAZZI-PEARSON, V. (1996) Colonisation patterns of root tissues by *Phytophthora nicotinae* var. *parasitica* related to reduced disease in mycorrhizal plants. Plant Soil 185, 223-232

DAVIS, R.M. and MENGE, J.A. (1980) Influence of *Glomus fasciculatus* on Phytophthora root rot of citrus. Phytopathology 70, 447-452

DEHNE, H.W. (1982) Interaction between vesicular-arbuscular mycorrhizal fungi and plant pathogens. Phytopathology 72, 1115-1119

DEHNE, H.W. and SCHÖNBECK, F. (1979) Untersuchungen zum Einfluss der endotrophen Mycorrhiza auf Pflanzenkrankheiten I. Ausbreitung von *Fusarium oxysporum* f.sp. *lycopersici* in Tomaten. Phytopathol. Z. 95, 105-110

DIEDHIOU, P.M., HALLMANN, J., OERKE, E.C. and H.W. DEHNE (2003) Effects of arbuscular mycorrhizal fungi and a non-pathogenic *Fusarium oxysporum* on *Meloidogyne incognita* infestation of tomato. Mycorrhiza 13, 199-204

DROPKIN, V.H. (1989) Introduction to plant nematology. John Wiley & Sons, New York, USA

EDWARDS, S.G., YOUNG, J.P.W. and FITTER, A.H. (1998) Interactions between *Pseudomonas fluorescens* biocontrol agents and *Glomus mosseae*, an arbusculas mycorrhizal fungus, within the rhizosphere. FEMS Microbiol. Lett. 116, 297-303

ELSEN, A., DECLERCK, S. and DE WAELE, D. (2001) Effects of *Glomus intraradices* on the reproduction of the burrowing nematode (*Radopholus similis*) in dixenic culture. Mycorrhiza 11, 49-51

FERRAZ, L.C.B.B. and BROWN, D.J.F. (2002) An Introduction to Nematodes: Plant Nematology. Pensoft, Sofia, Bulgaria

FILION, M., ST-ARNAUD, M. and FORTIN, J.A. (1999) Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere micro-organisms. New Phytol. 141, 525-533

FRANK, B. (1885) Über die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze. Ber. Dtsch. bot. Ges. 3, 128-145

FUCHS, J.G., MOENNE-LECCOZ, Y. and DEFANO, G. (1997) Nonpathogenic *Fusarium oxysporum* strain Fo47 induces resistance to Fusarium wilt in tomato. Plant disease 81, 492-496

GAMALERO, E., TROTTA, A., MASSA, N., COPETTA, A., MARTINOTTI, M.G. and BERTA, G. (2004) Impact of two fluorescent pseudomonades and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. Mycorrhiza 14, 185-192

GARCÍA-GARRIDO, J.M. and OCAMPO, J.A. (2002) Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. J. Exp. Bot. 53, 1377-1386

GIANINAZZI-PEARSON, V., DUMAS-GAUDOT, E., GOLLOTTE, A., TAHIRI-ALAOUI and GIANINAZZI, S. (1996) Cellular and molecular defence-related root responses to invasion by mycorrhizal fungi. New Phytol. 133, 45-57

GOLDEN, J.K. and VAN GUNDY, S.D. (1975) A disease complex of okra and tomato involving the nematode *Meloidogyne incognita* and the soil inhabiting fungus *Rhizoctonia solani*. Phytopathology 65, 265-273

HABTE, M., ZHANG, Y.C. and SCHMITT, D.P. (1999) Effectiveness of *Glomus* species in protecting white clover against nematode damage. Can. J. Bot. 77, 135-139

HALLMANN, J. and SIKORA, R.A. (1994) Occurrence of plant pathogenic nematodes and non-pathogenic species of *Fusarium* in tomato plants in Kenya and their role as mutualistic synergists for biological control of root-knot nematodes. Int. J. Pest Manag. 40, 321-325

HASKY-GÜNTHER, K., HOFFMANN-HERGARTEN, S. and SIKORA, R.A. (1998) Resistance against the potato cyst nematode *Globodera pallida* systemically induced by the rhizobacteria *Agrobacterium radiobacter* (G12) and *Bacillus sphaericus* (B43). Fundam. Appl. Nematol. 21, 511-517

HILDEBRANDT, U., JANETTA, K. and BOTHE, H. (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl. Environ. Microbiol. 68, 1919-1924

HILTNER, A. (1904) Über neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache (On recent insights and problems in the area of soil bacteriology under special consideration of the use of green manure and fallowing). Arbt. Dtsch. Landwirt. Ges. 98, 59-78

HOCK, B. and VARMA, A. (1995) Mycorrhiza structure, function, molecular biology and biotechnology. Springer, Berlin, Germany

HODGE, A. (2000) Microbial ecology of the arbuscular mycorrhiza. FEMS Microbiol. Ecol. 32, 91-96

HODGE, A. and CAMPBELL, C.D.F.A.H. (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413, 297-299

HUSSEY, R.S. (1985) Host-parasite relationships and associated physiological changes. In: Sasser, J.N. and Carter, C.C. (eds.): An Advanced Treatise on *Meloidogyne*. Volume I: Biology and Control. Raleigh, North Carolina, USA, 143-153

JONES, J.P., STALL, R.E. and ZITTER, T.A. (1991) Compendium of Tomato Diseases. APS Press, St. Paul, Minnesota, USA

JUNG, C. and WYSS, U. (1999) New approaches to control plant parasitic nematodes. Appl. Microbiol. Biotechnol. 51, 439-446

KOIDE, R.T. (1991) Transley Review No. 29: Nutrient supply, nutrient demand and plant response to mycorrhizal infection. New Phytol. 117,

LATCH, G.C.M. (1993) Physiological interactions of endophytic fungi and their hosts. Biotic stress tolerance imparted to grasses by endophytes. Agric. Ecosys. Environ. 44, 143-156

LINDERMAN, R.G. (1988) Mycorrhizal interactions with the rhizosphere microflora: The mycorrhizosphere effect. Phytopathology 78, 366-371

LINDERMAN, R.G. (1992) Vesicular-arbuscular mycorrhizae and soil microbial interactions. In: Mycorrhizae in Sustainable Agriculture. Madison, USA, 45-70

LINDERMAN, R.G. (1994) Role of VAM fungi in biocontrol. In: Pfleger, F.L. and Linderman, R.G. (eds.): Mycorrhizae and Plant Health. St. Paul, Minnesota, USA, 1-25

LYNCH, J.M. (1990) The rhizosphere. John Wiley & Sons, Chichester, Sussex, GB

MAO, W., LEWIS, J.A., LUMSDEN, D. and HEBBAR, K.P. (1998) Biocontrol of selected soil-borne diseases of tomato and pepper plants. Crop Protection 6, 535-542

MARSCHNER, H. (1998) Mineral Nutrition of Higher Plants. Academic Press Limited, London, Great Britain

MAYO, K., DAVIS, R.E. and MOTTA, J. (1986) Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. Mycologia 78, 426-431

MAYOL, P.S. and BERGESON, G.B. (1970) The role of secondary invaders in *Meloidogyne incognita*. J. Nematol. 2, 80-83

MEYER, J.R. and LINDERMAN, R.G. (1986) Response of subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth-promoting bacterium, *Pseudomonas putida*. Soil Biol. Biochem. 18, 191-196

MEYER, J.R. and LINDERMAN, R.G. (1986) Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. Soil Biol. Biochem. 18, 191-196

MORANDI, D. (1996) Occurrence of phytoalexins and phenolic compounds in endomycorrhizal interactions, and their potential role in biological control. Plant Soil 185, 241-251

MOSSE, B. (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J. Gen. Microbiol. 27, 509-520

PAL BAIS, H., PARK, S.-W., WEIR, T.L., CALLAWAY, R.M. and VIVANCO, J.M. (2004) How plants communicate using the underground information superhighway. Trends Plant Sci. 9, 26-32

PAULITZ, T.C. and LINDERMAN, R.G. (1989) Interactions between fluorescent pseudomonades and VA mycorrhizal fungi. New Phytol. 113, 37-45

PENNINSI, E. (2004) The secret life of fungi. Science 304, 1620-1622

RAAIJMAKERS, J.M., VLAMI, M. and DE SOUZA, J.T. (2002) Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek 81, 537-547

RAMBELLI, A. (1973) The rhizosphere of mycorrhizae. In: Marks, G.L. and Koslowski, T.T. (eds.): Ectomycorrhizae. New York, USA, 299-343

RAUPACH, G.S. and KLOEPPER, J.W. (1998) Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88, 1158-1164

REITZ, M., HOFFMANN-HERGARTEN, S., HALLMANN, J. and SIKORA, R.A. (2001) Induction of systemic resistance in potato by rhizobacterium *Rhizobium etli* strain G12 is not associated with accumulation of pathogenesis-related proteins and enhanced lignin biosynthesis. Z. Pflkrankh. Pflschutz. 108, 11-20

REMY, W., TAYLOR, T.N., HASS, H. and KERP, H. (1994) Four hundred million-year-old vesicular arbuscular mycorrhizae. Proc. Natl. Acad. Sci. USA 91, 11841-11843

SALEH, H.M. and SIKORA, R.A. (1984) Relationship between *Glomus fasciculatum* root colonization of cotton and its effect on *Meloidogyne incognita*. Nematologica 30, 230-237

SANDERS, F.E. (1993) Modelling plant growth responses to vesicular arbuscular mycorrhizal infection. Adv. Plant Pathol. 9, 135-166

SECILIA, J. and BAGYARAJ, D.J. (1987) Bacteria and actinomycetes associated with pot cultures of vesicular-arbuscular mycorrhizas. Can. J. Microbiol. 33, 1069-1073

SIDDIQUI, I.A. and SHAUKAT, S.S. (2002) Mixtures of plant disease suppressive bacteria enhance biological control of multiple tomato pathogens. Biol. Fertil. Soils. 36, 260-268

SIEVERDING, E. (1991) Vesicular-Arbuscular Mycorrhiza Management in Tropical Agrosystems. Schriftenreihe der GTZ, No. 224. Deutsche Gesellschaft für Technische Zusammenarbeit GmbH, Eschborn, Germany

SIKORA, R.A. (1992) Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. Annu. Rev. Phytopathol. 30, 245-270

SIKORA, R.A., BRIDGE, J. and STARR, J.L. (2005) Management Practices: An overview of integrated nematode management technologies. In: Luc, M., Sikora, R.A. and Bridge, J. (eds.): Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. Melksham, Great Britain,

SIKORA, R.A. and FERNÁNDEZ, E. (2005) Nematode Parasites of Vegetables. In: Luc, M., Sikora, R.A. and Bridge, J. (eds.): Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. Melksham, Great Britain,

SIKORA, R.A. and REIMANN, S. (2004) Suppressive soils, the edge of chaos and multitrophic strategies for biocontrol of pests and diseases in soil ecosystems. In: Sikora, R.A., Gowen, S., Hausschild, R. and Kiewnick, S. (eds.): Multitrophic interactions in soil. Gent, Belgium, 251-258

ST-ARNAUD, M.C., HAMEL, C., VIMARD, B., CARON, M. and FORTIN, J.A. (1997) Inhibition of *Fusarium oxysporum* f.sp. *dianthi* in the non-VAM species *Dianthus caryophyllus* by the co-culture with *Tagetes patula* companion plants colonized by *Glomus intraradices*. Can. J. Bot. 75, 998-1005

STIRLING, G.R. (1991) Biological control of Plant Parasitic Nematodes. CAB International, Wallingford, Great Britain

SUGDEN, A., STONE, R. and ASH, C. (2004) Ecology in the underworld. Science 304, 1613

TALAVERA, M., ITOU, K. and MIZUKUBO, T. (2001) Reduction of nematode damage by root colonization with arbuscular mycorrhiza (*Glomus* spp.) in tomato-*Meloidogyne incognita* (*Tylenchida*: *Meloidogynidae*) and carrot-*Pratylenchus penetrans* (*Tylenchida*: *Pratylenchidae*) pathosystems. Appl. Entomol. Zool. 36, 387-392

TROTTA, A., VARESE, G.C., GNAVI, E., FUSCONI, A., SAMPÒ, S. and BERTA, G. (1996) Interactions between the soilborne root pathogen *Phytophthora nicotinae* var. *parasitica* and the arbuscular mycorrhizal fungus *Glomus mosseae* in tomato plants. Plant Soil 185, 199-209

TRUDGILL, D.L. and BLOCK, V.C. (2001) Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. Annu. Rev. Phytopathol. 39, 53-77

VAN LOON, L.C., BAKKER, P.A.H.M. and PIETERSE, C.M.J. (1998) Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36,

VÁZQUEZ, M.M., CÉSAR, S., AZCÓN, R. and BAREA, J.M. (2000) Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azosporillum*, *Pseudomonas*, *Trichoderma*) and their effects on mycorrhizal population and enzyme activities in the rhizosphere of maize plants. Appl. Soil Ecol. 15, 261-272

WACEKE, J.W., WAUDO, S.W. and SIKORA, R.A. (2001) Response of *Meloidogyne hapla* to mycorrhiza fungi inoculation. Afric. J. Sci. Tec. 2, 63-70

WALLEY, F.L. and GERMIDA, J.J. (1996) Failure to decontaminate *Glomus clarum* NT4 spores is due to spore wall-associated bacteria . Mycorrhiza 6, 43-49

WARDLE, D.A., BARDGETT, R.D., KLIRONOMOS, J.N., SETÄLÄ, H., VAN DER PUTTEN, W.H. and WALL, D.H. (2004) Ecological linkages between aboveground and belowground biota. Science 304, 1629-1633

WELLER, D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26, 379-407

WELLER, D.M. and THOMASHOW, L.S. (1994) Current challenges in introducing beneficial microorganisms into the rhizosphere. In: O'Gara, F., Dowling, D.N. and Boesten, B. (eds.): Molecular Ecology of Rhizosphere Microorganism Biotechnology and the Release of GMOs. Weinheim, Germany, 1-18

WEST, C.P., IZEKOR, E., OOSTERHUIS, D.M. and ROBBINS, R.T. (1988) The effect of *Acremonium coenophialum* on the growth and nematode infection of tall fescue. Plant Soil 112, 3-6

WHIPPS, J.M. (2001) Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52, 487-511

XAVIER, L.C.J. and GERMIDA, J.L. (2003) Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. Soil Biol. Biochem. 35, 471-478

YAO, M.K., TWEDDELL, R.J. and DÉSILETS, H. (2002) Effect of two vesicular-arbuscular mycorrhizal fungi on the growth of micropropagated potato plantlets and on the extent of disease caused by *Rhizoctonia solani*. Mycorrhiza 12, 235-242

2 Isolation and identification of bacteria associated with spores of arbuscular mycorrhizal fungi

2.1 Introduction

Many studies have been conducted in the past to determine the composition of bacterial communities in different habitats (Hallmann *et al.*, 1997; Mahaffee and Kloepper, 1997; Sturz *et al.*, 1999; Krechel *et al.*, 2002). By characterizing the bacterial spectrum in a certain tissue or ecosystem researchers hope to gain information about interactions between these bacteria and the other organisms involved. Plant pathologists have concentrated on the ability of bacteria to compete with deleterious organisms living in the same niche to improve plant health and ultimately yield (Weller, 1988).

Arbuscular mycorrhizal fungi (AMF) form a symbiosis with 80% of all plants and are able to increase plant nutrition (Marschner, 1998) and plant health (Dehne, 1982). In addition, AMF establishment in the root causes changes in the microbial community of the rhizosphere (Oswald and Ferchau, 1968; Meyer and Linderman, 1986; Andrade *et al.*, 1998; Marschner *et al.*, 2001) by altering root exudation. Therefore plant health and growth promotion after AMF establishment in the plant may be due to shifts in microbial composition favoring beneficial organisms. However, very few investigations have been conducted to shed light upon this interrelationships (Barea *et al.*, 2002).

Research has shown that the spores of mycorrhizal fungi are colonized by bacteria (Mosse, 1962; Walley and Germida, 1996) that can influence spore germination and growth of arbuscular mycorrhizal fungi (Bianciotto and Bonfante, 2002; Hildebrandt *et al.*, 2002; Xavier and Germida, 2003). Budi *et al.* (1999) demonstrated that these bacteria are involved in the formation of the mycorrhizosphere and show high antagonistic potential against different soilborne pathogens. Mycorrhizal spores are easy to extract from the soil and spore-associated bacteria can be easily isolated. Therefore, studies were conducted to determine the antagonistic potential of spore-associated bacteria on plant health and to obtain more knowledge about the importance of these interrelationships in the mycorrhizosphere.

The objectives of this study were (i) isolation and (ii) identification of bacteria from AMF spores in order to determine the composition of bacterial communities associated with mycorrhizal spores.

2.2 Materials and methods

2.2.1 AMF spores extraction

In this study six soil samples were taken from different tomato fields in the Nakhon Ratchasima Province of Thailand. AMF spores were isolated from these soil samples by a modified extraction procedure (Gerdemann and Nicolson, 1963). Samples were thoroughly suspended in a substantial volume of water and the heavy particles were allowed to settle for 1 min. The solution was decanted through series of sieves (750, 500 and 50 µm) and the sieving procedure was repeated until the water was clear and only heavy particles remained in the bucket. The solid fraction on the 50 µm sieve was transferred with a water jet to a centrifuge tube. The vials were agitated and then centrifuged for 5 min at 900 g in a horizontal rotor. The supernatant was poured of carefully. The pellet was then resuspended in a 50% sucrose solution and afterwards the vial was centrifuged again for 1 min at 900 g. This time the liquid supernatant with the AMF spores was transferred to a 50 µm sieve and thoroughly washed with tap water. Spores were collected in vials with water and stored in a fridge at 4°C until further needed.

2.2.2 Isolation and storage of spore-associated bacteria

The AMF spores (2.2.1) were poured into a Petri dish and individual spores were removed to another Petri dish with a Pasteur pipette in the smallest amount of water possible. In this dish they were sterilized with a 2% Chloramine-T solution for 15 min. Afterwards spores were transferred to a sterile Petri dish with a sterile pipette and washed three times with sterile water in order to remove the Chloramine-T. Finally spores were transferred to a sterile filter paper and allowed to dry. A sterile needle was dipped in sterile water and then single spores were picked and placed on Petri dishes containing nutrient agar. The dishes were stored at 28°C for two days in an incubator. Bacterial colonies produced on these plates were streaked out on nutrient agar again for purification.

Pure bacteria isolates were transferred to 2-ml vials (Eppendorf) containing sterile Tryptic Soy Broth (TSB) with 1.5% glycerine (v/v) and stored at -20°C. For long term storage, the isolates were transferred to Kryo-vials (Mast-Diagnostica, 126392) and kept at -80°C.

2.2.3 Identification of bacteria

All isolates were identified by their fatty acid profile with the help of the Fatty Acid Methyl Ester-Gas Chromatography (FAME-GC). The standardized extraction of whole cell fatty acids was carried out as described by Sasser (1990).

The bacteria, stored at –20°C, were transferred to TSB agar and incubated for 24h at 28°C. Bacteria isolates were then plated out in four fractions on standardized TSB agar and incubated at 28°C for 24h. The colonies in the third quadrant (and if needed in the second) were collected until a sterile inoculation loop was completely filled. Bacterial cells were transferred to the bottom of a heat resistant glass vial with a Teflon-lined cap.

The following solutions were used for the extraction procedure:

Reagent 1: Saponification

NaOH (ACS grade, Merck)	45	g
Methanol (HPLC grade, Merck)	150	ml
Aqua bidest.	150	ml

Reagent 2: Methylization

6.0 N HCl (Merck)	325	ml
Methanol (HPLC grade, Merck)	275	ml

Reagent 3: Extraction

Hexan (HPLC grade, Merck)	200	ml
Methyl tert-Butyl Ether (Merck)	200	ml

Reagent 4: Washing

NaOH (ACS grade, Merck)	10.8	g
Aqua bidest.	900	ml

The extraction procedure in four steps:

1.) Saponification *(bacteria destruction and reaction of fatty acids with sodium)*

Bacteria were covered with 1 ml of Reagent 1 and subsequently shaken on a Vortexer for 8 sec. Afterwards the vial was heated in a water bath (100°C) for 5 min,

shaken again and in the end incubated for 25 min at 100°C in the same water bath. Then the vial was cooled down for 15 min at room temperature.

2.) Methylization *(sodium is replaced from fatty acid salts by a methyl group)*

After cooling 2 ml of Reagent 2 were added to the solution. The mixture was shaken on the Vortexer for 8 sec and then heated in a water bath (80°C) for 10 min. Afterwards the vial was cooled down rapidly in cool tap water.

3.) Extraction *(transfer of fatty acids into organic phase)*

1.25 ml of Reagent 3 were filled into the vial and shaken 10 min in an overhead shaker. The lower aqueous phase was removed with a Pasteur pipette and discarded.

4.) Washing *(purification of organic phase from dirt and remaining free fatty acids)*

The 3 ml of the basic washing Reagent 4 were added to the solution. Subsequently the vial was shaken in the overhead rotary shaker for 5 min. Two phases appeared and phase separation was improved by addition of one drop of sterile saturated NaCl-solution. Two third of the upper phase were transferred into GC vials which were capped with a gum septum and then stored at –20°C until analysis.

Chromatographic separation of the fatty acids was achieved with the Hewlett Packard GC-System 5890 (series II). The 5% silicon capillary column with a diameter of 0.2 mm was 25 m long. A calibration mix with a standardized mixture of fatty acids was analysed at the beginning of every analysis cycle and continuously repeated after every 10 samples.

Bacteria isolates were identified from their fatty acid profiling with the help of Sherlock MIS (Microbial Identification System, Version 3.9, MIDI Inc., Newark, USA). The profiles of the unknown samples were compared with the profiles in the database. The output listed genus and species of the most similar isolate from the database and a similarity index (SI). Latter is a value describing the probability that the bacteria of the sample is the same as the one in the database. According to this index bacteria were assigned on the species ($SI>0.6$) and genus level ($0.2<SI<0.6$) or declared as an unknown species.

2.3 Results and discussion

Fifty-three isolates were identified to the genus or species level (see annex), while eight bacteria could not be identified because of a very low similarity index. None of the bacteria in the database matched with SR 17. In Tab. 2.1 the genera of the isolated bacteria are listed. Numbers of each isolate, their gram staining reaction and the percentage of total bacteria examined are shown as well.

Table 2.1: Genera, gram stain reaction (+/-) and percentage of the bacteria investigated.

Genus	Gram	no. of isolates	% of total bacteria
Acinetobacter	-	12	19.2
Arthrobacter	+	5	8.0
Bacillus	+	9	14.4
Brevundimonas	+	1	1.6
Burkholderia	-	1	1.6
Cellulomonas	+	1	1.6
Corynebakterium	+	1	1.6
Micrococcus	+	2	3.2
Moraxella	-	12	19.2
Paenibacillus	+	3	4.8
Pseudomonas	-	3	4.8
Rathayibacter	+	1	1.6
Rhodococcus	+	1	1.6
Sphingobacterium	+	1	1.6
* not identified		9	14.4
Sum total		**62**	

Among the 18 genera identified, *Acinetobacter* (19.2%) and *Moraxella* (19.2%) were dominant. These two genera belong to the main genus *Neisseria* and are widespread in nature (Baumann, 1968; Madigan *et al.* 2001). They can be found in many biologically active soils and bodies of water and the main difference between the two genera is based on their oxidase reaction: *Moraxella* is oxidase-positive while *Acinetobacter* is oxidase-negative (Baumann *et al.*, 1968a; Baumann *et al.*, 1968b). *Moraxella* strains have been previously isolated from mycorrhizal fungi (Gazanelli *et al.*, 1999). Both genera are known to be involved in biodegradation and have been intensively studied for biotechnical applications (Desouky Abdel-El-Halem, 2003).

Strains of *Acinetobacter* can be used in phosphate removal from waste water, because they are able to accumulate high levels of phosphorus under aerobic conditions (Auling *et al.*, 1991). Some bacteria of the same genus are highly resistant to heavy metal toxicity and play an important role in the removal of heavy metals from contaminated sludge (Francisco *et al.*, 2002). Different strains of *Moraxella* and *Acinetobacter* isolated from soil also have the ability to degrade herbicides (Villarreal *et al.*, 1991; Martin *et al.*, 1999). All these features are important, when considering that AMF are involved in phosphate nutrition and degradation of organic matter (Hodge and Campbell, 2001). Furthermore heavy metal resistance in plants (Gildon and Tinker, 1983) and decreased herbicide injury were observed after AMF inoculation (Siqueira *et al.*, 1991). These findings suggest that spore-associated bacteria are involved in these mycorrhiza interrelationships with the abiotic environment.

The percentage of *Pseudomonas* spp. (4.8%) was relatively low when compared with their usually high frequency of occurrence in the rhizosphere. These results are in agreement with previous reports (Vancura *et al.*, 1990; Waschkies *et al.*, 1994; Marschner *et al.*, 1997; Vázquez *et al.*, 2000) who observed less pseudomonades in the rhizosphere of mycorrhizal than of non-mycorrhizal plants. Andrade *et al.* (1997) isolated strains of *P. putida* from the hyphosphere of mycorrhizal plants, while the well known root colonizer *P. fluorescens* was absent. The three pseudomonades isolated from AMF spores in the present study also belonged to the species *P. putida*.

The isolation procedure could have affected the low recovery of gram-negative pseudomonades. Xavier and Germida (2003) observed a decrease of pseudomonades when spores of *Glomus clarum* were surface sterilized with 5% Chloramine-T for 30 min prior to bacteria isolation. However, the same authors also observed an inverse relationship between decontamination time and survival of gram-negative bacteria. In the present study, almost 50% of all bacteria isolated were gram-negative and therefore the effect of spore-decontamination on bacterial composition was minimal.

Xavier and Germida (2003) and Andrade *et al.* (1997) reported that bacilli are the most dominant genera associated with mycorrhizal spores and in the

mycorrhizosphere respectively. The percentage of *Bacillus* spp. was also high in this study. Other bacterial genera isolated from the spores, like *Burkholderia*, *Arthrobacter* and *Paenibacillus*, were previously described in or on arbuscular mycorrhizal fungal mycelia and spores (Bianciotto *et al.*, 1996; Hildebrandt *et al.*, 2002; Xavier and Germida, 2003).

The antagonistic potential of these 62 spore-associated bacteria against different soilborne diseases was subject to the studies that are presented in Chapter 3 and 4.

2.4 References

ANDRADE, G., LINDERMAN, R.G. and BETHLENFALVAY, G.J. (1998) Bacterial associations with the mycorrhizosphere and hyphosphere of the arbuscular mycorrhizal fungus *Glomus mosseae*. Plant Soil 202, 79-87

ANDRADE, G., MIHARA, K.L., LINDERMAN, R.G. and BETHLENFALVAY, G.J. (1997) Bacteria from rhizosphere and hyphosphere soils of different arbuscular mycorrhizal fungi. Plant Soil 192, 71-79

AULING, G., PILZ, F., BUSSE, H.J., KARRASCH, S., STREICHAN, M. and SCHON, G. (1991) Analysis of the polyphosphate-accumulating microflora in phosphorus-eliminating, anaerobic-aerobic activated sludge systems by using diaminopropane as a biomarker for rapid estimation of *Acinetobacter* spp. Appl. Environ. Microbiol. 57, 3585-3592

BAREA, J.M., AZCÓN, R. and AZCÓN-AGUILAR, C. (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie van Leeuwenhoek 81, 343-51

BAUMANN, P., DOUDOROFF, M. and STANIER, R.Y. (1968a) Study of the *Moraxella* group. 1. Genus *Moraxella* and the *Neisseria catarrhalis* group. J. Bacteriol. 95, 58-73

BAUMANN, P., DOUDOROFF, M. and STANIER, R.Y. (1968b) A study of the *Moraxella* group. 2. oxidase-negative species (genus *Acinetobacter*). J. Bacteriol. 95, 1520-1541

BAUMANN, P. (1968) Isolation of *Acinetobacter* from soil and water. J. Bacteriol. 96, 39-42

BIANCIOTTO, V., BANDI, C., MINERDI, D., SIRONI, M., TICHY, H.V. and BONFANTE, P. (1996) An obligately endosymbiotic fungus itself harbors obligately intracellular bacteria. Appl. Environ. Microbiol. 62, 3005-3010

BIANCIOTTO, V. and BONFANTE, P. (2002) Arbuscular mycorrhizal fungi: a specialized niche for rhizosphere and endocellular bacteria. Antonie van Leeuwenhoek 81, 365-371

BUDI, S.W., VAN TUINEN, D., MARTINOTTI, G. and GIANINAZZI, S. (1999) Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soil borne fungal pathogens. Appl. Environ. Microbiol. 65, 5148-5150

DEHNE, H.W. (1982) Interaction between vesicular-arbuscular mycorrhizal fungi and plant pathogens. Phytopathology 72, 1115-1119

DESOUKY ABDEL-EL-HALEM (2003) *Acinetobacter*: environmental and biotechnological applications (Minireview). Afr. J. Biotechnol. 2, 71-74

FRANCISCO, R., ALPOIM, M.C. and MORAIS, P.V. (2002) Diversity of chromium-resistant and -reducing bacteria in a chromium-contaminated activated sludge. J. Appl. Microbiol. 92, 837-843

GAZANELLI, G., MALATESTA, M., PIANETTI, A., BAFFONE, W., STOCCHI, V. and CITTERIO, B. (1999) Bacteria associated to fruit bodies of the ectomycorrhizal fungus *Tuber borchii* Vittad. Symbiosis 26, 211-222

GERDEMANN, J.W. and NICOLSON, T.H. (1963) Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. Trans. Br. Mycol. Soc. 46, 235-244

GILDON, A. and TINKER, P.B. (1983) Interactions of vesicular-arbuscular mycorrhizal infections and heavy metals in plants. II. The effects of infection on uptake of copper. New Phytol. 95, 263-268

HALLMANN, J., QUADT-HALLMANN, A., MAHAFEE, W.F. and KLOEPPER, J.W. (1997) Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43, 895-914

HILDEBRANDT, U., JANETTA, K. and BOTHE, H. (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl. Environ. Microbiol. 68, 1919-1924

HODGE, A. and CAMPBELL, C.D.F.A.H. (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413, 297-299

KRECHEL, A., FAUPEL, A., HALLMANN, J., ULRICH, A. and BERG, G. (2002) Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode *Meloidogyne incognita* (Kofoid & White) Chitwood. Can. J. Microbiol. 48, 772-786

MADIGAN, M.T., MARTINKO, J.M. and PARKER, J. (2001) Mikrobiologie. Spektrum Akademischer Verlag, Heidelberg, Germany

MAHAFFEE, W.F. and KLOEPPER, J.W. (1997) Temporal changes in the bacterial communities of soil, rhizosphere and endorhiza associated with field grown cucumber (*Cucumis sativus*, L.). Can. J. Microbiol. 34, 210-223

MARSCHNER, H. (1998) Mineral Nutrition of Higher Plants. Academic Press Limited, London, Great Britain

MARSCHNER, P., CROWLEY, D.E. and HIGASHI, R.M. (1997) Root exudation and physiological status of a root-colonizing fluorescent pseudomonad in mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum* L.). Plant Soil 189, 11-20

MARSCHNER, P., CROWLEY, D.E. and LIEBEREI, R. (2001) Arbuscular mycorrhizal infection changes 16 S rDNA community composition in the rhizosphere of maize. Mycorrhiza 11, 297-302

MARTIN, M., MENGS, G., ALLENDE, J.L., FERNANDEZ, J., ALONSO, R. and FERRER, E. (1999) Characterization of two novel propachlor degradation pathways in two species of soil bacteria. Appl. Environ. Microbiol. 65, 802-806

MEYER, J.R. and LINDERMAN, R.G. (1986) Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. Soil Biol. Biochem. 18, 191-196

MOSSE, B. (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J. Gen. Microbiol. 27, 509-520

OSWALD, E.T. and FERCHAU, H.A. (1968) Bacterial associations of coniferous mycorrhizae. Plant Soil 28, 187-192

SASSER, M. (1990) Identification of bacteria through fatty acid analysis. In: Klement, Z., Rudolph, K. and Sands, D.C. (eds.): Methods in Phytobacteriology. Budapest, Hungary, 199-204

SIQUEIRA, J.O., SAFIR, G.R. and NAIR, M.G. (1991) VA-mycorrhizae and mycorrhiza stimulating isoflavanoid compounds reduce plant herbicide injury. Plant Soil 134, 233-242

STURZ, A.V., CHRISTIE, B.R., MATHESON, B.G., ARSENAULT, W.J. and BUCHANAN, N.A. (1999) Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soilborne plant pathogens. Plant Pathology 48, 360-369

VANCURA, V., OROZCO, M.O., GRAUOYÁ, O. and PRIKRYL, Z. (1990) Properties of bacteria in the hyphosphere of a vesicular-arbuscular mycorrhizal fungus. Agric. Ecosys. Environ. 29, 421-427

VÁZQUEZ, M.M., CÉSAR, S., AZCÓN, R. and BAREA, J.M. (2000) Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azosporillum*, *Pseudomonas*, *Trichoderma*) and their effects on mycorrhizal population and enzyme activities in the rhizosphere of maize plants. Appl. Soil Ecol. 15, 261-272

VILLARREAL, D.T., TURCO, R.F. and KONOPKA, A. (1991) Propachlor degradation by a soil bacterial community. Appl. Environ. Microbiol. 57, 2135-2140

WALLEY, F.L. and GERMIDA, J.J. (1996) Failure to decontaminate *Glomus clarum* NT4 spores is due to spore wall-associated bacteria. Mycorrhiza 6, 43-49

WASCHKIES, C., SCHROPP, A. and MARSCHNER, H. (1994) Relations between grapevine replant disease and root colonization of grapevine (*Vitis* sp.) by fluorescent pseudomonades and endomycorrhizal fungi. Plant Soil 162, 219-227

WELLER, D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26, 379-407

XAVIER, L.C.J. and GERMIDA, J.L. (2003) Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. Soil Biol. Biochem. 35, 471-478

3 Spore-associated bacteria and *Glomus intraradices* in the biocontrol of *Meloidogyne incognita*

3.1 Introduction

Mycorrhizal establishment has been shown to reduce reproduction of plant parasitic nematodes and the damage they cause (Sikora, 1995; Jaizme-Vega *et al.*, 1997; Nagesh *et al.*, 1999; Elsen *et al.*, 2001; Talavera *et al.*, 2001; Diedhiou *et al.*, 2003). Numerous suggestions have been made to explain the enhanced resistance/tolerance of mycorrhizal plants against nematodes. Altered root exudation in the mycorrhizosphere, resulting in a higher percentage of antagonistic microorganisms, could be one explanation for increased plant health and numerous reports support this hypothesis (Linderman, 1994; Azcón-Aguilar and Barea, 1996; Marschner *et al.*, 2001; Reimann and Sikora, 2003).

The term rhizosphere was coined by Hiltner (1904), describing a dynamic ecosystem directly influenced by the root. It is characterized by intense bacterial activity resulting from root carbon exudation. Mycorrhizal fungi were discovered by Frank (1885) and are known to live in symbiosis with roots of more than 80% of all plants (Penninsi, 2004). During formation of this symbiosis, both root morphology (Gamalero *et al.*, 2004) and root exudation are intensively altered (Bansal and Mukerji, 1994), resulting in changes of microbial composition, in the zone referred to as mycorrhizosphere (Rambelli, 1973).

Whether the observed changes in the microbial composition in the mycorrhizosphere are due to changes caused by mycorrhizal establishment or bacteria associated with the spores of mycorrhizal inoculum is open to question. Mosse (1962) was the first to show that it was difficult to obtain sterile spores of AMF and the problem still exists today (Budi *et al.*, 1999; Bianciotto and Bonfante, 2002). Chloramine-T for example failed to eliminate bacteria from spores of *Glomus clarum* even after long-term sterilization (Walley and Germida, 1996). Spore-associated bacteria are involved in AMF spore germination (Mayo *et al.*, 1986; Xavier and Germida, 2003) and the formation of the mycorrhizosphere (Budi *et al.*, 1999). The antagonistic potential of spore-associated bacteria against soilborne pathogens and pests needs to be more

fully explored in order to obtain information on the plant health promoting effect of the mycorrhizosphere.

In the following investigations spore-associated bacteria were tested for their antagonistic potential against the root-knot nematode *Meloidogyne incognita* on tomato. The most effective isolates were then combined with *Glomus intraradices* in the biocontrol of this root-knot nematode.

3.2 Materials and methods

3.2.1 Plants

Tomato seeds (*Lycopersicon esculentum* Mill. cv. 'King Kong II') were surface sterilized by first shaking them in a 75% Ethanol solution for 1 min and then in a 1.5% Sodium hypochloride (NaOCl) solution for 3 min. The seeds were washed with tap water and transferred to sterile sand for germination at greenhouse conditions.

3.2.2 Nematode inoculum

The root-knot nematode *Meloidogyne incognita* (Kofoid & White) Chitwood (race 3) was maintained in the greenhouse at 27°C (±5) in a box filled with sandy loam and permanently cultivated with the susceptible tomato cultivar 'Furore'. Nematode eggs were extracted from heavily galled tomato roots, following a modified extraction technique of Hussey and Barker (1973). Roots were washed free from soil under tap water, cut into 1 cm pieces and macerated in a warring blender at high speed for 20 s and collected in a glass bottle. Sodium hypochloride (NaOCl) was added to obtain a final concentration of 1.5% active Chlorine and the bottle was shaken vigorously for 3 min. The suspension was then thoroughly washed with tap water through a sieve combination 250, 100, 45 and 25 µm mesh to remove the NaOCl. Eggs were collected on the 25 µm sieve and then transferred to a glass bottle.

Nematode eggs were either used directly for inoculation or aerated at 24°C to promote egg development and juvenile hatch. Freshly hatched second stage juveniles (J2) were separated from the remaining unhatched eggs with a modified Baermann technique over 24 h (Oostenbrink, 1960).

3.2.3 Microorganisms and microbial inoculants

Isolation of spore-associated bacteria

The 62 bacterial strains used in the experiments were isolated from AMF spores. First AMF spores were extracted from soil samples by the wet sieving procedure of Gerdemann and Trappe (1963). Chlamydospores were gently surface sterilized with a 2% Chloramine-T solution for 15 min and then washed in sterile water three times. Spore-associated bacteria were isolated by placing the spores on nutrient agar and incubating the Petri dishes for 2-3 days at 28°C. Isolates were purified and stored in liquid Tryptic Soy Broth (TSB) with 15% (v/v) glycerine at -20°C.

Antibiotic resistant mutants

Antibiotic-resistant mutants of *Cellulomonas turbata* SR1 and *Acinetobacter baumannii* SR6 were obtained spontaneously by exposure to concentration gradients of rifampicin. Bacteria taken from the stock culture were grown in TSB media for 24 h at 28°C on a rotary shaker. 100 µl of this cell suspension was transferred to 50 ml of fresh TSB and incubated again at 28°C. After 2 h a rifampicin solution was added to the TSB to achieve a concentration of 20 µl of rifampicin per liter. The medium was subsequently incubated for 2 h intervals and the antibiotic concentration was increased progressively (50/100) to a final concentration of 150 µl per liter. Surviving bacteria were subjected to single colony isolation on Tryptic Soy Agar medium supplemented with 150 µl per l prior to storing as mentioned above.

Inoculum of bacteria and AMF

Inoculum production was carried out by inoculation of liquid TSB medium in Erlenmeyer flasks. The bacteria were incubated on a rotary shaker at 28°C for 48 h. Bacterial suspension was centrifuged for 20 min at 5000 g and the pellet was resuspended in sterile ¼ concentrated Ringer solution (Merck). The optical cell density was measured at 560 nm (OD_{560}) and the cell density was adjusted to 2.0.

Glomus intraradices (isolate no. 510) inoculum in expanded clay, was kindly provided by Dr. H. von Alten, University of Hannover.

3.2.4 Screening of spore-associated bacteria against *Meloidogyne incognita*

Tomato seedlings (see 3.2.1) were transplanted into 500-ml pots containing a sterilized sand-field soil mixture (9:1, v/v). At the time of transplanting a 5 ml bacterial suspension was applied as a soil drench around the stem where as the control was treated with 5 ml sterile ¼ concentrated Ringer solution. The bacterial treatment was repeated one week later. Simultaneously 500 J2 of *M. incognita* were added in 5 ml of tap water in three holes around the stem. Each treatment was replicated six times. Plants were cultivated in the greenhouse at 24°C (± 4) with 16 h of supplemental artificial light (3000 Lux). Plants were fertilized weekly with 0.2% solution of Flory 3 (Euflor, Munich). The experiment was terminated 21 days after nematode inoculation at which time shoot dry weight and root fresh weight were measured. The roots were washed and then boiled in 0.1% lactic acid fuchsin (Ferris, 1985), homogenized with an Ultra-Turrax (IKA-Werk) and the number of penetrated juveniles counted under the stereomicroscope. The experiment was repeated once.

3.2.5 Combination of bacteria and AMF in the biocontrol of *Meloidogyne incognita*

Tomato seedlings (see 3.2.1) were transplanted into 500-ml pots containing a sterilized sand-field soil mixture (9:1, v/v) with or without *G. intraradices* in expanded clay (5% of total substrate volume). At the time of transplanting a 5 ml bacterial suspension was applied as a soil drench around the stem while the control was treated with 5 ml of sterile ¼ concentrated Ringer solution. The treatments were replicated seventeen times. Fertilization with 0.2 % Wuxal Top N solution (Aglukon, Düsseldorf, Germany) was done on a weekly basis and the concentration was increased with plant growth. Four weeks after transplanting the establishment of mycorrhiza in the roots of eight plants was examined by staining the roots according to Vierheilig *et al..* (1998) and estimating the frequency of mycorrhiza (Trouvelot *et al.*, 1986). At the same time roots of the remaining test plants were soaked for three seconds in the same bacterial suspension that was applied initially. The tomatoes were then transplanted into 2-l pots containing a non-sterile sand-field soil mixture (2:1, v/v) infested with 1000 eggs of *M. incognita* per 100 ml of soil. The plants were fertilized with 0.2% Flory 3 solution adjusted to plant growth. Flowers were removed in order to avoid fruit development. Eight weeks after transplanting shoot dry and root

fresh weight were measured. Roots were stained with Phloxine-B (Sikora and Schuster, 2000) and numbers of galls and egg masses counted. The experiment was repeated once.

3.2.6 Persistence of some spore-associated bacteria on mycorrhizal and non-mycorrhizal tomato roots.

This experiment was conducted parallel to the previous experiment (3.2.5) with the same experimental settings. Instead of using the wild strains, the antibiotic resistant bacterial strains were inoculated while no nematodes were applied. Starting in the first week after transplanting, soil samples were taken weekly from each pot with the help of a cork borer. All samples of each treatment were homogenized and subsequently about 5 g were suspended in 50 ml of sterile demineralized water and then mixed for 20 min at 28°C on a rotary shaker. Afterwards the suspension was diluted serially. With the help of a spiral plater (Eddy Jet, Version 1.2, IUL instruments, Königswinter, Germany) 40 µl of each dilution were spread on Petri dishes containing TSA supplemented with rifampicin (150 µg per l). Five replicates of each treatment were incubated at 28°C for 24 to 48 h. Bacterial colonies were then compared with pure cultures on the same medium under the stereomicroscope for colony similarity and finally counted.

3.3 Results

Screening of spore-associated bacteria against Meloidogyne incognita

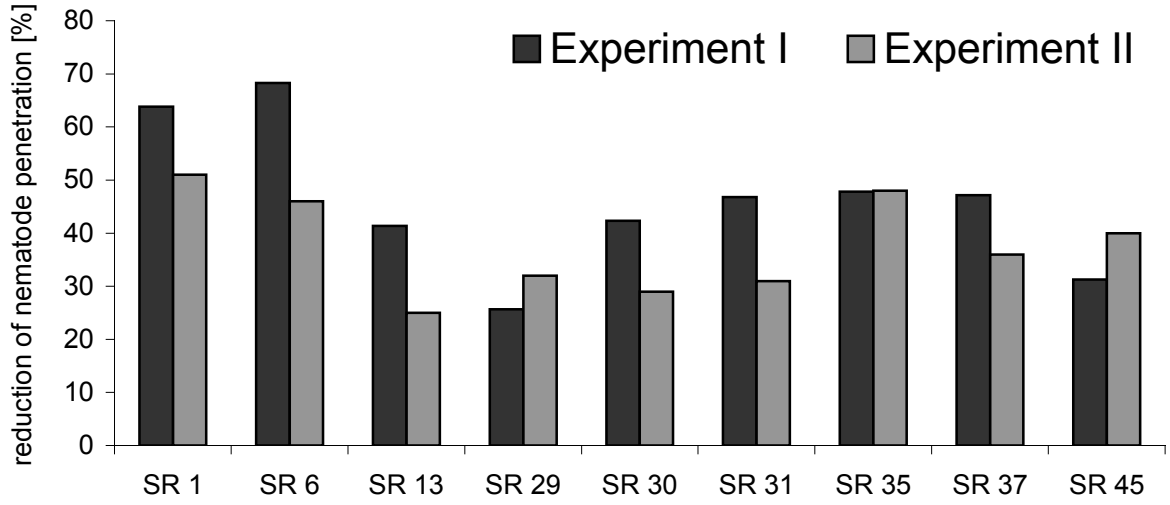

Figure 3.1: Reduction of *M. incognita* penetration on tomato by spore-associated bacteria in two experiments.
Percentage of reduction calculated in comparison to corresponding untreated control.
Results for all bacteria are significant (α = 0,05) according to the Tukey-Test.

Twenty-three of 62 isolates reduced nematode penetration per g root fresh weight significantly in the first screening experiments (see annex). They were tested in a second experiment and again nine bacteria significantly decreased nematode penetration. The reductions of nematode penetration for these nine bacteria in both experiments are summarized in figure 3.1 and the antagonistic bacteria are listed in table 3.1.

Table 3.1: Genus and species of the bacteria reducing nematode penetration in repeated experiments and the average reduction in two experiments.

Strain	Genus and species	Reduction of nematode penetration [%]
SR 1	*Cellulomonas turbata*	57.4
SR 6	*Acinetobacter baumannii*	57.2
SR 13	*Arthrobacter viscosus* *	33.2
SR 26	*Paenibacillus pabuli*	28.9
SR 30	*Bacillus pumilus*	35.7
SR 31	*Bacillus pumilus*	38.9
SR 35	*Arthrobacter viscosus*	47.9
SR 37	*Moraxella osloensis*	41.6
SR 45	*Bacillus pumilus*	35.6

* name not approved, because of low similarity index in FAME identification

Neither root fresh weight nor shoot dry weight were significantly influenced by bacteria inoculation. Only *Arthrobacter viscosus* SR13 increased root fresh weight in both experiments compared with the control (data not shown).

Combination of bacteria and AMF in the biocontrol of Meloidogyne incognita

Precolonization of young plants with AMF and bacteria led to decreased numbers of galls and egg masses. These differences were not statistically significant for single inoculations in experiment I, but in experiment II both single bacteria inoculations resulted in decreased numbers of galls. In the case of *Acinetobacter baumannii* SR6 numbers of egg masses were significantly reduced as well (Figure 3.2).

In both experiments, dual inoculation resulted in reduced galling (38-48%) and egg mass production compared with either single treatment or the untreated control. The combination of SR6 with AMF did not significantly reduce the number of egg masses

in the first experiment, even though the reduction was stronger than the individual inoculation. In general, galling was much lower in the second experiment, but differences between the treatments were more pronounced.

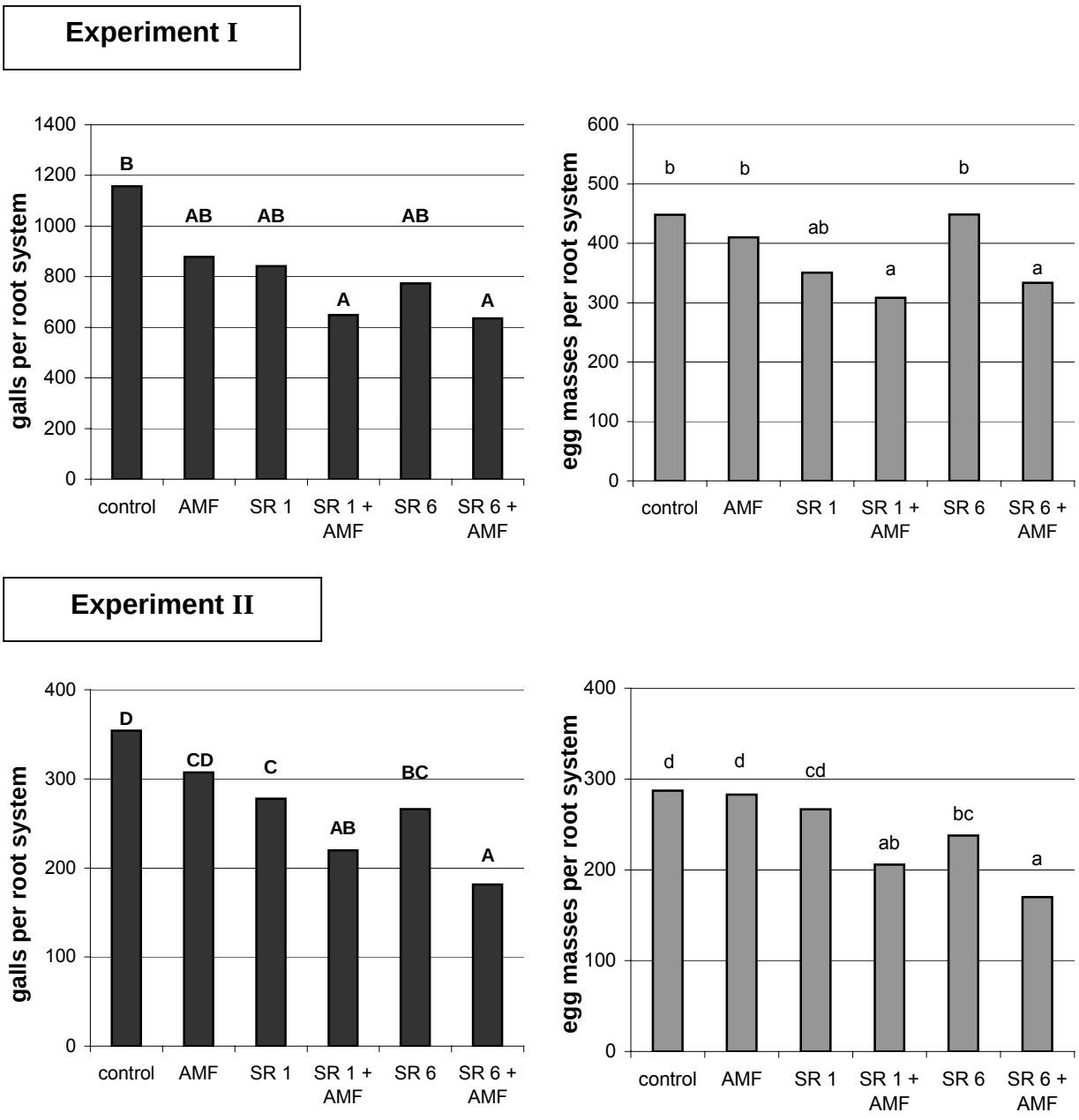

Figure 3.2: Numbers of galls and egg masses formed by *M. incognita* on tomato with or without spore-associated bacteria *Cellulomonas turbata* SR1, *Acinetobacter baumannii* SR6 and *Glomus intraradices* in two experiments.
Means in one graph with the same letters are not significantly different according to the Tukey-Test (α = 0,05).

It is noteworthy that in the control of the first experiment many young, small galls had grown together and were found at the outer part of the root system. Single galls with

egg masses were found in the center of the root. In the dual inoculations, the galls in the outer part of the root system were fewer or completely absent (Fig. 3.3). Staining of the galls from the outer part with lactic acid fuchsin showed that the nematodes inside the roots were in the third or fourth juvenile stage. Up to 40 nematodes were counted in each gall.

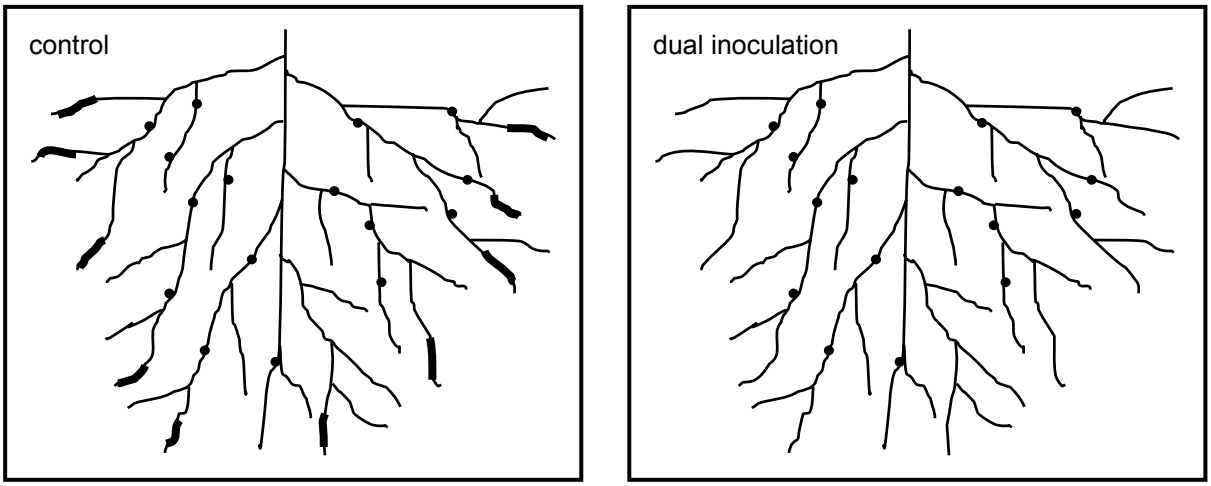

Figure 3.3: Distribution and size of galls on tomato roots of control and double inoculated plants.

Shoot dry weight was not affected by either inoculation, while root fresh weights of mycorrhizal plants were always lower than the corresponding non-mycorrhizal plants. Frequency of mycorrhiza in the roots of four week old plants was 25 to 30% in both experiments. Bacteria inoculation did not affect mycorrhizal establishment (Tab. 3.2).

Table 3.2: Root fresh weight and shoot dry weight [g] of tomato plants inoculated with *M. incognita.*

	Experiment I				Experiment II			
	root		shoot		root		shoot	
control	42,26	b	26,93	a	31,36	c	28,88	a
AMF	35,79	ab	26,92	a	22,53	ab	30,08	a
SR 1	42,81	b	26,89	a	26,19	ab	31,50	a
SR 1 + AMF	37,39	ab	25,84	a	22,38	ab	27,91	a
SR 6	38,80	b	26,30	a	26,60	b	26,71	a
SR 6 + AMF	34,45	a	26,68	a	22,25	a	28,07	a

*values of the same column followed by the same letter are not significantly different according to the Tukey-Test (α=0,05)

Persistence of some spore-associated bacteria on mycorrhizal and non-mycorrhizal tomato roots.

Both bacteria in the combination experiment survived better on mycorrhizal than on non-mycorrhizal roots. The persistence of the two rifampicin resistant strains are shown in figure 3.5.

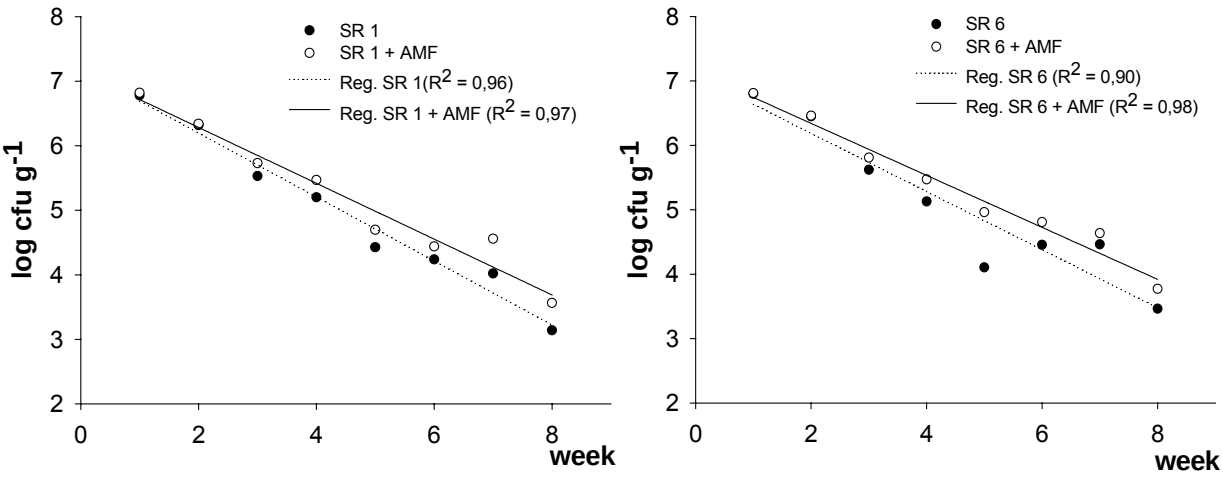

Figure 3.4: Persistence of rifampicin resistant strains of *Cellulomonas turbata* SR 1 and *Acinetobacter baumannii* SR 6 on tomato roots inoculated or not with *Glomus intraradices.*

3.4 Discussion

The high percentage of spore-associated bacteria antagonistic towards the root-knot nematode (14.5% of all isolates) is remarkable. In general, in the rhizosphere less than 10% of all isolated bacteria show antagonistic activity against fungal pathogens (Weller, 1988; Van Bruggen and Semenov, 2000) or nematodes (Sikora, 1992). In earlier studies, conducted with tomato rhizobacteria from Thailand, the number of *M. incognita* per g root fresh weight, galling or egg mass production were reduced by only 5% of the isolates (Terhardt, 1998; Molina-Gayosso, 2004). This leads to the conclusion that spore-associated bacteria have a higher antagonistic potential than rhizobacteria and may be an indication for the involvement of spore-associated bacteria in the antagonistic effects of mycorrhizal fungi against nematodes, as reported in previous studies (Saleh and Sikora, 1984; Rao *et al.*, 1995; Talavera *et al.*, 2001; Diedhiou *et al.*, 2003). In all investigations AMF had to be well established in the root system in order to reduce nematode reproduction or damage. The mycorrhizosphere may have been established at the same time (Linderman, 1994),

and it is most likely that spore-associated bacteria are involved in mycorrhizosphere formation. Therefore these bacteria may have played an important role in the biocontrol activity of AMF.

The mechanisms responsible for the bacteria mediated reduction of nematode penetration were not determined. Inoculation of *Arthrobacter viscosus* SR13 led to increased root fresh weight, while total numbers of nematodes per root system remained unaffected. Consequently a dilution in the number of nematodes per g root fresh weight may have occurred. Therefore *Arthrobacter viscosus* SR13 might be considered a plant growth promoting rhizobacterium (PGPR) (Weller, 1988). A diluting effect can be excluded for the other eight bacteria and therefore these may be considered plant health promoting rhizobacteria (PHPR) (Sikora and Hoffmann-Hergarten, 1992). Further studies are needed to obtain information as to whether induced resistance, disrupted nematode attraction or nematicidal metabolites are responsible for the reduced penetration rate. Information on mode of action would make it possible to combine bacteria with different mechanisms in order to increase biocontrol activity, as has been previously suggested (Stirling, 1991; Whipps, 2001; Siddiqui and Shaukat, 2002).

The combination of AMF with two strains of spore-associated bacteria resulted in a higher reduction of *M. incognita* infection, as compared with single organism inoculations. Although reduced galling and egg mass production were observed in all treatments, results for single inoculations were only significant in the second experiment. The poor significance in the first experiment may be due to high treatment variation. Combined inoculation led to significant reductions in galling and egg mass production in repeated experiments and these findings were confirmed recently in another laboratory with the same microorganisms (Massadeh, 2005). One explanation for the additive effects could be the combination of different modes of action in nematode control. For example, the spore-associated bacteria reduced nematode penetration in the screening experiments, whereas competition for nutrients and feeding sites is the one possible reason for reduced nematode reproduction after AMF inoculation. (Sikora, 1978; Hussey and Roncadori, 1982; Talavera *et al.*, 2002).

Mycorrhiza frequency at the time of transplanting was between 25-30%, which was lower than the recommended range for biological control of nematodes (Saleh and Sikora, 1984; Diedhiou *et al.*, 2003). Mycorrhiza establishment was not affected by inoculation with either bacteria. Therefore an improved protection of the roots due to increased mycorrhization can be excluded as the mode of action. However, the survival of the two PHPR improved in the rhizosphere of mycorrhizal over that of non-mycorrhizal plants. This higher population density of antagonistic bacteria could be an explanation for the additive effects of dual inoculation.

Changes in phosphate uptake and root morphology are known to affect microbial community composition (Linderman, 1988). Smith *et al.* (2003) have shown that phosphate in mycorrhizal plants can be provided by the AMF while direct phosphate uptake by the plant is downregulated. At the same time changes in root morphology have been reported (Gamalero *et al.*, 2004). Similar changes must have occurred in this experiment, because of the altered shoot/root ratio. Hence, the increased reisolation rates of the rifampicin resistant bacteria strains from roots of mycorrhizal plants could be due to these changes. This observation could be an interesting advantage for future combined applications of spore-associated bacteria and AMF.

Neither of the treatments had an effect on shoot growth, but interestingly all mycorrhizal plants showed decreased root fresh weights. This is in accordance with previous studies reporting that tomato plant growth seems to be independent of endomycorrhizal establishment (Caron *et al.*, 1985; Smith *et al.*, 2003). However, when plants are grown under unsuitable conditions or suffer from pathogen attack, AMF can nullify growth depression (Grandison and Cooper, 1986; Trotta *et al.*, 1996; Cordier *et al.*, 1996). In both experiments galling was not severe, therefore a significant reduction of shoot growth due to nematode attack can be excluded and hence AMF inoculation did not increase shoot growth. Nematode galls act as carbon sinks and therefore can increase root fresh weight until uptake of water and nutrients becomes limiting. This probably explains the decreased root fresh weights of mycorrhizal plants, which showed reduced galling in both experiments, either in comparison with the control or the bacteria treatments.

The differences in gall distribution that appeared in the first experiment (Figure 3.3) may be explained by delayed nematode development. In the control, nematodes

from the first reproduction cycle may have already hatched and reentered the root at the root tips. This would explain the small young galls in the outer part of the root, that had grown together containing many nematodes in the third and fourth juvenile stage. The reduction or absence of these small galls in the treated plants would therefore indicate that delayed nematode growth or reproduction was the mechanism of action, as it was previously proposed for AMF by Elsen *et al..* (2001), Jaizme-Vega *et al..* (1997) and Sikora (1995). The second experiment was conducted in September-October when the overall temperature conditions in the greenhouse were not as good for nematode development as in the first experiment, which was carried-out in August-September. Hence, reproduction was delayed in general and therefore differences in gall distribution were not as obvious as in the first experiment. These results indicate that mechanisms other than delayed nematode development were also involved, such as reduced nematode penetration, as was shown for the two bacteria.

In conclusion, this is the first investigation on biocontrol activity of spore-associated bacteria towards root-knot nematodes and these bacteria show high antagonistic potential against *Meloidogyne incognita*. Spore-associated bacteria could have been involved in the formation of the mycorrhizosphere in previous studies using unsterile AMF inoculum. Therefore these bacteria would have played an important role in the better tolerance/resistance of mycorrhizal plants against nematode attack. In addition, this is also the first investigation combining AMF and spore-associated bacteria in biocontrol. Dual inoculation led to increased plant protection against nematodes than either microorganism alone and these additive effects are important for future nematode management strategies. The improved persistence of these spore-associated bacteria on mycorrhizal roots could be an important advantage for the combination of these microorganisms in the future.

3.5 References

AZCÓN-AGUILAR, C. and BAREA, J.M. (1996) Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - an overview of the mechanisms involved. Mycorrhiza 6, 457-464

BANSAL, M. and MUKERJI, K.G. (1994) Positive correlation between VAM-induced changes in root exudation and mycorrhizosphere mycoflora. Mycorrhiza 5, 39-44

BIANCIOTTO, V. and BONFANTE, P. (2002) Arbuscular mycorrhizal fungi: a specialized niche for rhizosphere and endocellular bacteria. Antonie van Leeuwenhoek 81, 365-371

BUDI, S.W., BLAL, B. and GIANINAZZI, S. (1999) Surface sterilization of sporocarps of *Glomus mosseae* for studying endomycorrhization *in vitro*. Mycorrhiza 9, 65-68

BUDI, S.W., VAN TUINEN, D., MARTINOTTI, G. and GIANINAZZI, S. (1999) Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soil borne fungal pathogens. Appl. Environ. Microbiol. 65, 5148-5150

CARON, M., FORTIN, J.A. and RICHARD, C. (1985) Influence of substrate on the interaction of *Glomus intraradices* and *Fusarium oxysporum* f.sp. *radicis-lycopersici* on tomatoes. Plant Soil 87, 233-239

CORDIER, C., GIANINAZZI, S. and GIANINAZZI-PEARSON, V. (1996) Colonization patterns of root tissues by *Phytophthora nicotinae* var. *parasitica* related to reduced disease in mycorrhizal plants. Plant Soil 185, 223-232

DIEDHIOU, P.M., HALLMANN, J., OERKE, E.C. and H.W. DEHNE (2003) Effects of arbuscular mycorrhizal fungi and a non-pathogenic *Fusarium oxysporum* on *Meloidogyne incognita* infestation of tomato. Mycorrhiza 13, 199-204

ELSEN, A., DECLERCK, S. and DE WAELE, D. (2001) Effects of *Glomus intraradices* on the reproduction of the burrowing nematode (*Radopholus similis*) in dixenic culture. Mycorrhiza 11, 49-51

FERRIS, H. (1985) Crop loss prediction and modeling for management decisions. In: Zuckermann, B.M., Mai, W.F. and Harrison, M.B. (eds.): Plant nematology laboratory manual. Amherst, Massachusetts, USA, 27-33

FRANK, B. (1885) Über die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze. Ber. Dtsch. bot. Ges. 3, 128-145

GAMALERO, E., TROTTA, A., MASSA, N., COPETTA, A., MARTINOTTI, M.G. and BERTA, G. (2004) Impact of two fluorescent pseudomonades and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. Mycorrhiza 14, 185-192

GERDEMANN, J.W. and NICOLSON, T.H. (1963) Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. Trans. Br. Mycol. Soc. 46, 235-244

GRANDISON, G.S. and COOPER, K.M. (1986) Interactions of vesicular-arbuscular mycorrhizae and cultivars of alfalfa susceptible and resistant to *Meloidogyne hapla*. J. Nematol. 18, 141-149

HILTNER, A. (1904) Über neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache (On recent insights and problems in the area of soil bacteriology under special consideration of the use of green manure and fallowing). Arbt. Dtsch. Landwirt. Ges. 98, 59-78

HUSSEY, R.A. and BARKER, K.P. (1973) A comparison of methods for collecting inocula for *Meloidogyne* ssp., including a new technique. Plant Dis. Report. 57, 1025-1028

HUSSEY, R.S. and RONCADORI, R.W. (1982) Vesicular-arbuscular mycorrhizae may limit nematode activity and improve plant growth. Plant Dis. 66, 9-14

JAIZME-VEGA, M.C., TENOURY, P., PINOCHET, J. and JAUMOT, M. (1997) Interactions between the root-knot nematode *Meloidogyne incognita* and *Glomus mosseae* in banana. Plant Soil 196, 37-35

LINDERMAN, R.G. (1988) Mycorrhizal interactions with the rhizosphere microflora: The mycorrhizosphere effect. Phytopathology 78, 366-371

LINDERMAN, R.G. (1994) Role of VAM fungi in biocontrol. In: Pfleger, F.L. and Linderman, R.G. (eds.): Mycorrhizae and Plant Health. St. Paul, Minnesota, USA, 1-25

MARSCHNER, P., CROWLEY, D.E. and LIEBEREI, R. (2001) Arbuscular mycorrhizal infection changes 16 S rDNA community composition in the rhizosphere of maize. Mycorrhiza 11, 297-302

MASSADEH, B. (2005) Bio-Management of *Meloidogyne incognita* (Tylenchida: Meloidogynidae) on tomato using arbuscular mycorrhizal fungi and rhizobacteria. University of Hannover

MAYO, K., DAVIS, R.E. and MOTTA, J. (1986) Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. Mycologia 78, 426-431

MOLINA-GAYOSSO, E. (2004) Investigations on the role of rhizobacteria for control of the root-knot nematode *Meloidogyne incognita* on tomato. University of Bonn, Germany

MOSSE, B. (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J. Gen. Microbiol. 27, 509-520

NAGESH, M., REDDY, P.P., KUMAR, M.V.V. and NAGARAJU, B.M. (1999) Studies on correlation between *Glomus fasciculatum* spore density, root colonization and *Meloidogyne incognita* infection on *Lycopersicon esculentum*. Z. Pflkrankh. Pflschutz. 106, 82-87

OOSTENBRINK, M. (1960) Estimating nematode populations by some selected methods. In: Sasser, J.N. and Jenkins, W.R. (eds.): Nematology. Chapel Hill, North Carolina, USA, 85-102

PENNINSI, E. (2004) The secret life of fungi. Science 304, 1620-1622

RAMBELLI, A. (1973) The rhizosphere of mycorrhizae. In: Marks, G.L. and Koslowski, T.T. (eds.): Ectomycorrhizae. New York, USA, 299-343

RAO, M.S., PARVATHA REDDY, P. and MOHANDAS, S. (1995) Effect of integration of endomycorrhizas (*Glomus mossae*) and neem cake on the control of root-knot nematode on tomato. Z. Pflkrankh. Pflschutz. 102, 526-529

REIMANN, S. and SIKORA, R.A. (2003) Managing the mycorrhizosphere - an approach to sustainable agriculture after the phase out of methyl bromide. Comm. Agric. Appl. Biol. Sci. 68, 129-134

SALEH, H.M. and SIKORA, R.A. (1984) Relationship between *Glomus fasciculatum* root colonization of cotton and its effect on *Meloidogyne incognita*. Nematologica 30, 230-237

SIDDIQUI, I.A. and SHAUKAT, S.S. (2002) Mixtures of plant disease suppressive bacteria enhance biological control of multiple tomato pathogens. Biol. Fertil. Soils. 36, 260-268

SIKORA, R.A. (1978) Einfluss der endotrophen Mykorrhiza (*Glomus mosseae*) auf das Wirt-Parasit-Verhältnis von *Meloidogyne incognita* in Tomaten. Z. Pflkrankh. Pflschutz. 85, 197-202

SIKORA, R.A. (1992) Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. Annu. Rev. Phytopathol. 30, 245-270

SIKORA, R.A. (1995) Vesicular-arbuscular mycorrhizae: their significance for biological control of plant parasitic nematodes. Biocontrol 1, 29-33

SIKORA, R.A. and HOFFMANN-HERGARTEN, S. (1992) Importance of plant health-promoting rhizobacteria for the control of soilborne fungal diseases and plant parasitic nematodes. Arab. J. Plant Protect. 10, 47-53

SIKORA, R.A. and SCHUSTER, R.-P. (2000) Handbuch der Phytonematologie. Shaker Verlag, Aachen, Germany

SMITH, S.E., SMITH, F.A. and JAKOBSEN, I. (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. Plant Physiol. 133, 16-20

STIRLING, G.R. (1991) Biological control of Plant Parasitic Nematodes. CAB International, Wallingford, Great Britain

TALAVERA, M., ITOU, K. and MIZUKUBO, T. (2001) Reduction of nematode damage by root colonization with arbuscular mycorrhiza (*Glomus* spp.) in tomato-*Meloidogyne incognita* (Tylenchida: Meloidogynidae) and carrot-*Pratylenchus penetrans* (Tylenchida: Pratylenchidae) pathosystems. Appl. Entomol. Zool. 36, 387-392

TALAVERA, M., ITOU, K. and MIZUKUBO, T. (2002) Combined application of *Glomus* sp. and *Pasteuria penetrans* for reducing *Meloidogyne incognita* (Tylenchida: Meloidogynae) populations improving tomato growth. Appl. Entomol. Zool. 37, 61-67

TERHARDT, J. (1998) Beeinflussung mikrobieller Gemeinschaften der Rhizosphäre nach Blattbehandlung von Pflanzen und biologische Kontrolle von *Fusarium oxysporum* f.sp. *lycopersici* und *Meloidogyne incognita* mit bakteriellen Antagonisten. University of Bonn, Germany

TROTTA, A., VARESE, G.C., GNAVI, E., FUSCONI, A., SAMPÒ, S. and BERTA, G. (1996) Interactions between the soilborne root pathogen *Phytophthora nicotinae* var. *parasitica* and the arbuscular mycorrhizal fungus *Glomus mosseae* in tomato plants. Plant Soil 185, 199-209

TROUVELOT, A., KOUGH, J.L. and GIANINAZZI-PEARSON, V. (1986) Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. In: Gianinazzi-Pearson, V. and Gianinazzi, S. (eds.): Physiological and genetical aspects of mycorrhizae. Paris, France, 217-221

VAN BRUGGEN, A.H.C. and SEMENOV, A.M. (2000) In search of biological indicators for soil health and disease suppression. Appl. Soil Ecol. 15, 13-24

VIERHEILIG, H., COUGHLAN, A.P., WYSS, U. and PICHÉ, Y. (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Appl. Environ. Microbiol. 64, 5004-5007

WALLEY, F.L. and GERMIDA, J.J. (1996) Failure to decontaminate *Glomus clarum* NT4 spores is due to spore wall-associated bacteria . Mycorrhiza 6, 43-49

WELLER, D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26, 379-407

WHIPPS, J.M. (2001) Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52, 487-511

XAVIER, L.C.J. and GERMIDA, J.L. (2003) Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. Soil Biol. Biochem. 35, 471-478

4 Antagonistic activity of spore-associated bacteria against soilborne plant pathogens

4.1 Introduction

Microbial community composition associated with the plant affects the antagonistic potential against soilborne plant pathogens and this has been intensively studied in the past. Krechel *et al.* (2002) compared the antagonistic potential of different microenvironments on potato in their antagonistic potential against *Rhizoctonia solani* and *Verticillium dahliae* and found out that the majority of antagonistic bacteria can be found in the rhizosphere (10%), followed by endorhiza (9%), phyllosphere (6%) and endosphere (5%). Bacteria isolated from different host plants of *Verticillium dahliae* were tested against the same pathogen and it was demonstrated that the abundance and composition of antagonists is plant species dependent (Berg *et al.*, 2002). Soil microbial community composition is also a major determinant of soil fungistasis, the inhibition of fungal germination and growth, and the antifungal activity can be strongly related to the occurrence of pseudomonades (De Boer *et al.*, 2003).

Among the plant health promoting rhizobacteria (PHPR) *Pseudomonas* spp. and *Bacillus* spp. appear to have the greatest potential as biocontrol agents, because they are dominant in the rhizosphere (Mahaffee and Kloepper, 1997). *Bacillus* spp. are appealing candidates for biocontrol because they produce endospores that are tolerant to heat and desiccation (Weller, 1988). Nevertheless, other bacteria like *Burkholderia* (Mao *et al.*, 1998), *Paenibacillus* (Budi *et al.*, 1999), *Enterobacter* (Chernin *et al.*, 1995) and *Streptomyces* (Singh *et al.*, 1999) have been shown to effectively control diseases caused by soilborne pathogens.

Arbuscular mycorrhizal fungi live in symbiosis with the roots of more than 80% of all terrestrial plants. They have been shown to protect the plant against different soilborne pathogens or decrease the caused damage (Dehne, 1982; Linderman, 1994). Numerous suggestions have been made to explain the enhanced resistance/tolerance of mycorrhizal plants. One possible explanation is the altered microbial composition in the mycorrhizosphere, and there are strong indications that such a mechanism can be involved (Linderman, 1994; Azcón-Aguilar and Barea, 1996; Reimann and Sikora, 2003).

In a first report on the role of the mycorrhizosphere in biocontrol of pathogens extracts of rhizosphere soil from mycorrhizal plants reduced sporangia formation of *Phytophthora cinnamomi* (Meyer and Linderman, 1986). The authors hypothesized that either sporulation-inducing microorganisms were missing or the number of sporulation-inhibiting microorganisms increased. Secilia and Bagyaraj (1987) found more pathogen-antagonistic actinomycetes in the rhizosphere of mycorrhizal plants than in that of the non-mycorrhizal controls. Similarly it was reported by Budy *et al.* (1999) that 12.5% of all bacteria isolated from the mycorrhizosphere had antagonistic potential against various soilborne pathogens *in vitro* and against *Phytophthora parasitica* also *in vivo*. These findings support the hypothesis that the mycorrhizosphere may be rich in PHPR active against soilborne pathogens.

The goal of this investigation was to determine if spore-associated bacteria may be involved in the antagonistic potential of AMF against soilborne pathogens. Therefore these bacteria were tested against fungal pathogens *in vitro* and *in vivo*.

4.2 Materials and methods

4.2.1 Microorganisms and microbial inoculants

Bacteria isolation and inoculum production

62 bacterial strains used in the following experiments were isolated from AMF spores. First, Chlamydospores were extracted from soil samples of tomato fields in Nakhon Ratchasima Province (Thailand) by the wet sieving procedure of Gerdemann and Trappe (1963). These spores were gently surface sterilized with a 2% Chloramine-T solution for 15 min and then washed in sterile water three times. Spore-associated bacteria were isolated by placing the spores on nutrient agar and incubating the Petri dishes for 2-3 days at 28°C. Isolates were purified and stored in liquid Tryptic Soy Broth with 15% (v/v) glycerine at -20°C.

Bacteria from the stock culture were plated out on Tryptic Soy Agar and incubated for 48 h at 28°C. They were either used directly or transferred to Erlenmeyer flasks containing Tryptic Soy Broth. The flasks were incubated for 48 h at 28°C on a rotary shaker. Afterwards the bacterial suspension was centrifuged for 20 min at 5000 g and the pellet was resuspended in sterile ¼ concentrated Ringer solution (Merck). Finally the optical cell density was adjusted to $OD_{560\ nm}$= 2.0.

Plant pathogens and inoculum production

The four soilborne plant pathogens used in the experiments were

1) *Fusarium oxysporum* f.sp. *lycopersici* (DSMZ no. 62059)
2) *Pythium aphanidermatum* (AIT, Bangkok, Thailand)
3) *Pythium ultimum* (Bayer Crop Science AG)
4) *Rhizoctonia solani* (Bayer Crop Science AG)

All fungal isolates were maintained on Petri dishes with Potato Dextrose Agar and frequently renewed. Three pieces of infested agar were transferred into Erlenmeyer flasks containing liquid Potato Dextrose Broth medium. The flasks were incubated at 24°C for 7 days on a rotary shaker in the dark. Afterwards the spore suspension was filtered and then centrifuged at 10000 g and 20°C for 10 min. The pellet was resuspended in sterile, ¼ concentrated Ringer (Merck) solution and adjusted to a concentration of 10^5 conidia ml^{-1} for inoculation.

4.2.2 Antagonistic activity of spore-associated bacteria towards soilborne pathogens *in vitro*

Spore-associated bacteria were streaked in two parallel lines on Sabouroud Dextrose Agar, which proved to be a good medium for the growth of both microorganisms in a pretest, and incubated for 24 h at 28°C in the dark. Subsequently one 3-mm piece of agar containing living mycelia of the pathogen was placed in the center of the plate and the Petri dish was incubated at 24°C in the dark for 2 to 6 days, depending on the growth of the fungus (see figure 4.1). Experiments were performed with five replicates for each bacterium-pathogen combination and were repeated at least twice. Antibiosis of the bacteria against the pathogen was evaluated by inhibition of radial mycelia growth in comparison to the control.

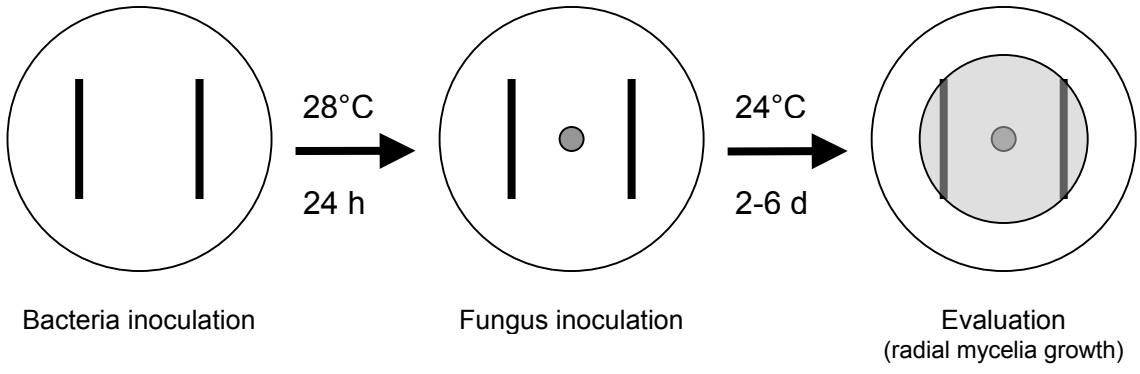

Figure 4.1: Course diagram of the *in vitro* testing system for antagonistic activity against fungi.

4.2.3 Antagonistic activity of spore-associated bacteria towards *Fusarium oxysporum in vivo*

Seven bacteria that inhibited the growth of *Fusarium oxysporum* f.sp. *lycopersici* in the dual culture test were tested again *in vivo* against this pathogen (Tab. 4.2). Tomato seeds (*Lycopersicon esculentum* Mill. cv. 'King Kong II') were surface sterilized in a 75% Ethanol solution for 1 min and then in a 1.5% NaOCl solution for 3 min. The seeds were then washed with tap water and transferred to sterile sand for germination under greenhouse conditions for one week. Eighteen seedlings per treatment were transplanted into 900 ml pots containing Klassmann plant growth substrate. The pots were inoculated separately with 5 ml of bacteria suspension (OD_{560}=2.0) by drenching the soil surface around the stem. The bacteria were inoculated again one week later and afterwards 5 ml of *Fusarium* spore suspension (5 x 10^5 conidia) were applied per pot on the soil surface. The plants were fertilized weekly with 0.2% Wuxal Top N (Aglukon, Düsseldorf, Germany).

Four and six weeks after *Fusarium* inoculation, nine plants of each treatment were evaluated. First wilting due to *Fusarium* infection was assessed on a scale of 1 to 5 (Tab. 4.1) as described by Mwangi (2002). Afterwards *Fusarium oxysporum* was reisolated from the tomato shoots by the method described by Terhardt (1998). After removing the leaves, 3 cm long pieces were cut out from the lower, middle and upper part of the shoot. These segments were surface sterilized by immersion in 75% alcohol for one minute followed by three minutes in 0.5% sodium hypochloride (NaOCl). The shoot segments were then washed in sterile water three times to remove the chloride. After sterilization the two ends were cut off with a sterile scalpel and the remaining 1 cm piece was split lengthwise. The two sections were placed on rifampicin supplemented Potato Dextrose Agar and incubated at 24°C for 3 to 6 days depending on the extent of fungal growth. The pathogen was easily identified by its characteristic pink stained mycelium.

Table 4.1: Tomato wilt index for the evaluation of damage caused by *Fusarium oxysporum*.

scale	symptoms
1	0-24% - healthy plants with green leaves or first yellow leaf
2	25-59% - lower leaves yellow
3	50-74% - Some lower leaves dead and some upper leaves wilted
4	75-99% - lower leaves dead and most upper leaves wilted
5	100% - dead plants

4.3 Results

Antagonistic activity of bacteria towards soilborne pathogens in vitro

Twelve out of the 62 bacteria isolated from AMF spores inhibited at least one of the tested pathogens in repeated dual cultures tests. All bacteria that inhibited *P. ultimum* also negatively affected mycelia growth of *P. aphanidermatum*. Seven bacteria were able to decrease mycelia growth of the slow growing *Fusarium oxysporum* f.sp. *lycopersici*. Only six bacteria reduced radial mycelia growth of all four soilborne pathogens. Six out of nine bacilli isolated had an effect on the development of at least one pathogen *in vitro*. The results are summarized in table 4.2 and pictures of the *in vitro* study are shown in figure 4.2.

Table 4.2: Antagonistic activity of spore-associated bacteria against soilborne pathogens *in vitro.*

	Bacillus pumilus SR 8	*Bacillus cereus* SR 11	*Bacillus cereus* SR 12	*Bacillus cereus* SR 16	*Arthrobacter viscosus* SR 24	*Bacillus pumilus* SR 30	*Bacillus pumilus* SR 31	*Sphingobacterium heparinum* SR 43	*Pseudomonas putida* SR 52	*Pseudomonas putida* SR 57	*Enterobacter cloaceae* SR 58	*Paenibacillus macerans* SR 60
Fusarium oxysporum		+		+	+				+	+	+	+
Pythium aphanidermatum	+	+	+	+	+	+	+	+		+	+	+
Pythium ultimum	+	+	+	+	+	+	+	+		+	+	+
Rhizoctonia solani	+	+		+	+	+	+	+	+	+	+	+

+ = inhibition of mycelia growth

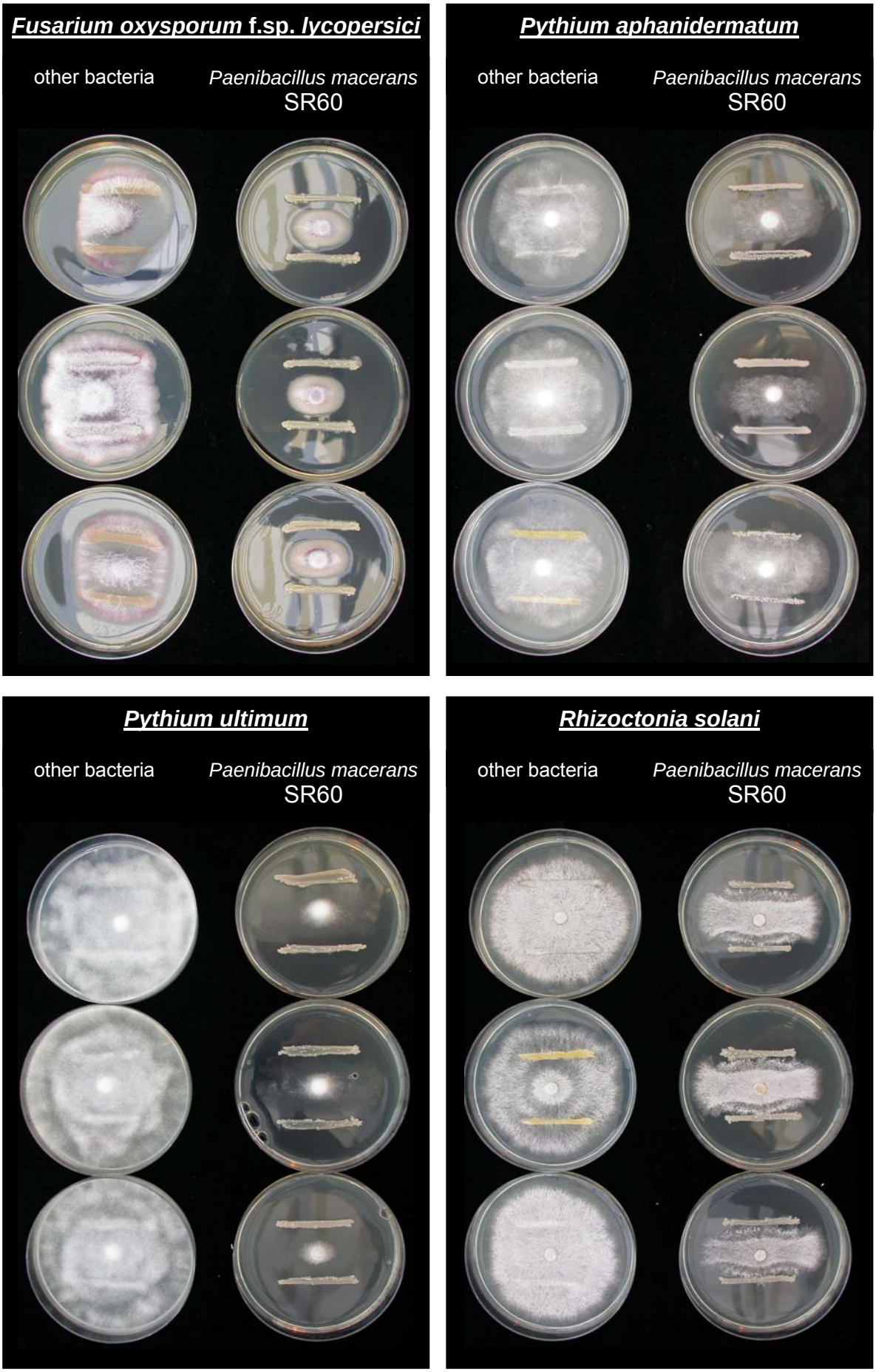

Figure 4.2: Antagonistic activity of *Paenibacillus macerans* SR60 *in vitro* in comparison to other non-effective strains of spore-associated bacteria.

Antagonistic activity of bacteria towards Fusarium oxysporum in vivo

The seven bacteria that inhibited mycelia growth of *Fusarium oxysporum* f.sp. *lycopersici in vitro* were tested *in vivo* against this pathogen. The tomato plants did not develop severe wilting symptoms and therefore the results of the wilt index were not statistically different (Fig. 4.3).

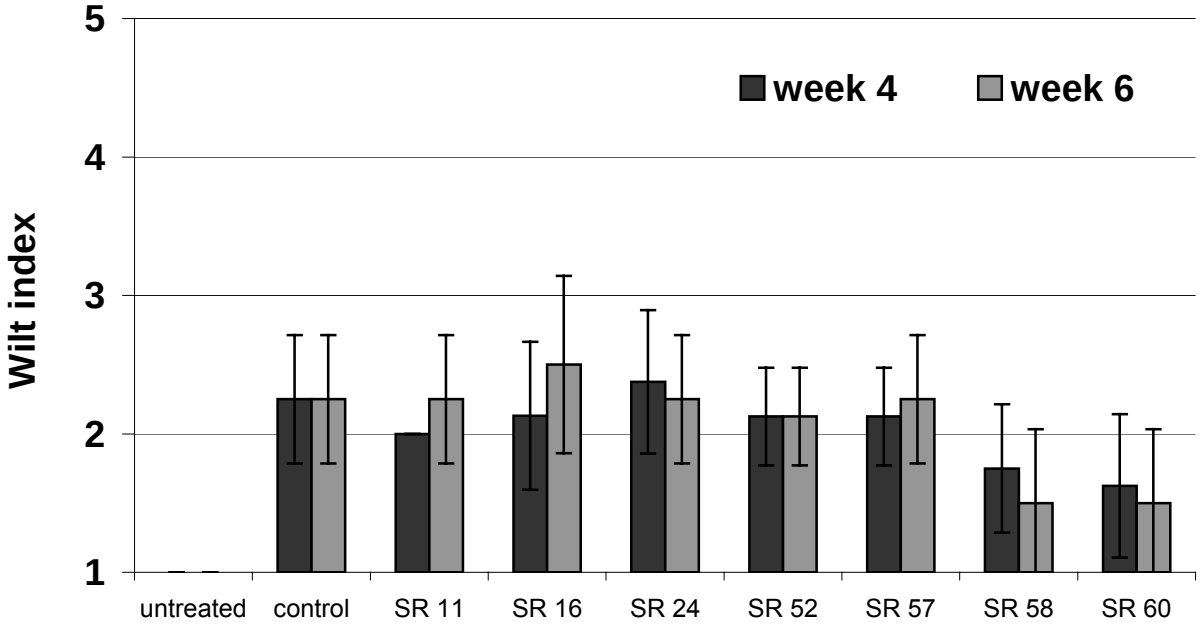

Figure 4.3: Wilt index of tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* with and without spore-associated bacteria.
(Wilting scale: 1 = healthy; 2 = 25-49%, lower leaves yellow; 3 = 50-74%, lower leaves dead & first wilting, 4 = 75-99%, wilting of whole plant, 5 = dead plant)

Reisolation frequency, representing the percentage of shoot segments infested with mycelium of *F. oxysporum*, is shown in figure 4.4. In general the fungal frequency decreased from the base to the top of the shoot as was expected. However no differences between the two evaluation dates were observed. Four of the seven bacteria reduced the spread of the fungus in the plant tissue. *Paenibacillus macerans* SR60 inoculation led to the greatest decline in the frequency of reisolation. Shoot segments from plants treated with *Bacillus cereus* SR16, *Pseudomonas putida* SR52 and *Enterobacter cloaceae* SR58 also showed lower levels of fungal colonization than the control.

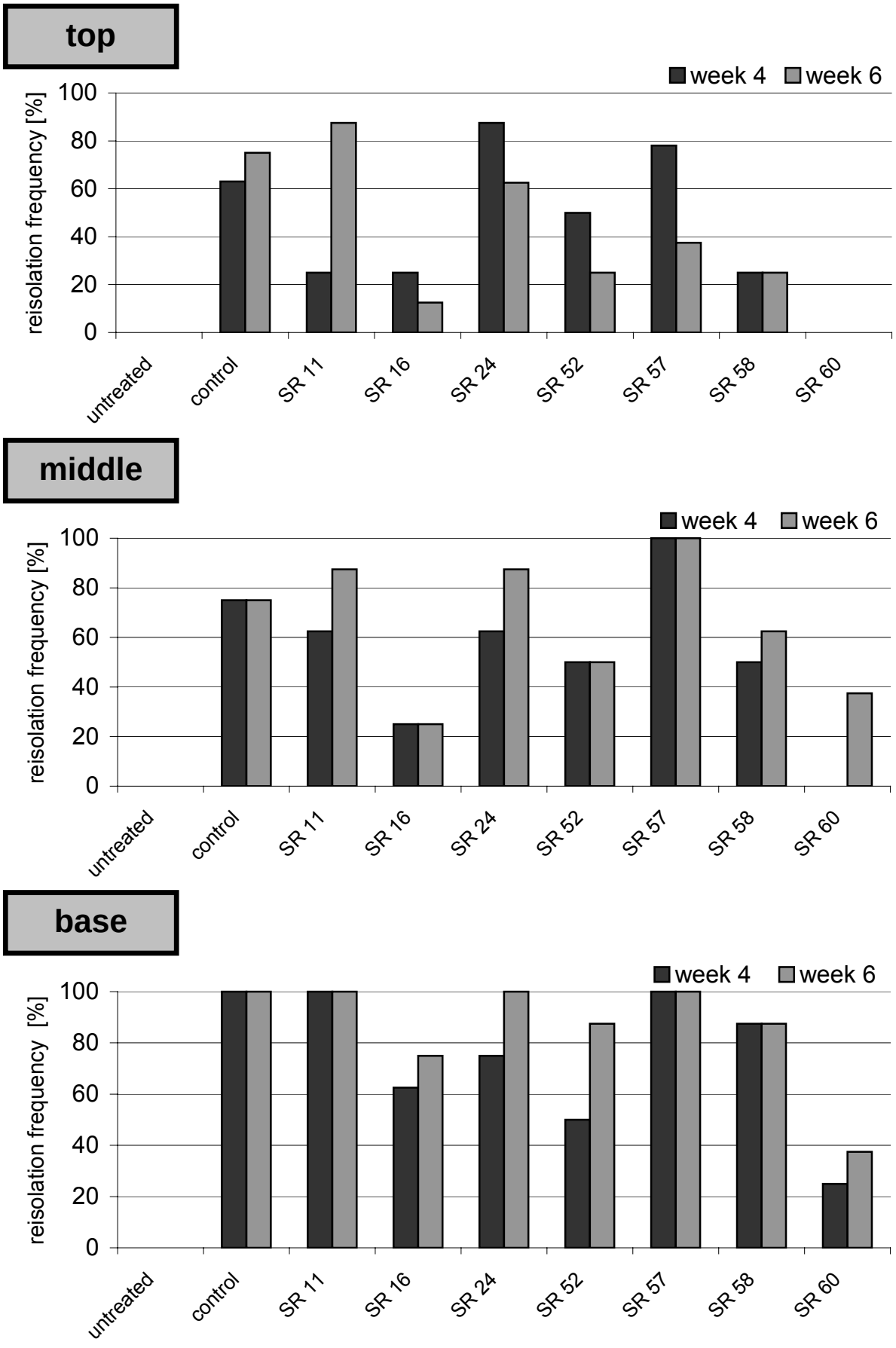

Figure 4.4: Effect of spore associated bacteria on frequency of *Fusarium oxysporum* reisolation from different tomato shoot segments in %.

4.4 Discussion

Twelve out of 62 bacteria (19.4%) inhibited growth of four fungal pathogens *in vitro* to different degrees. Eleven bacteria decreased fungal growth of *Rhizoctonia solani*, *P. aphanidermatum* and *P. ultimum,* while only *s*even isolates were able to reduce mycelia growth of *Fusarium oxysporum in vitro*. Six isolates (9,8%) were able to inhibit all fungal pathogens *in vitro*.

Isolates from different habitats of potato inhibited growth of *Rhizoctonia solani* and *Verticillium dahliae* in dual culture tests to a comparable degree (Krechel *et al.*, 2002). The authors observed that 10% of all isolates from the rhizosphere inhibited fungal growth of these two pathogens. It was reported that 4 to 10% of all bacteria from rhizosphere of different plant species showed antagonistic potential against *Verticillium dahliae* (Berg *et al.*, 2002). In another study conducted with *Phytophthora sojae*, 6% of 133 rhizosphere isolates showed biocontrol traits (Sharifi Tehrani *et al.*, 2002).

The antagonistic potential of spore-associated bacteria observed in the present study is relatively high compared with the other studies. Therefore the 19.4% level of activity against individual pathogens and 9.8% against all pathogens tested suggests that the spore-associated bacteria are an important and productive source for detection of bacterial based antagonism. Budi *et al.* (1999) reported similar results. They isolated eight bacteria from the mycorrhizosphere and tested them against different soilborne pathogens *in vitro*. The *Paenibacillus* strain B2 suppressed mycelia growth of *Phytophthora parasitica*, *Pythium ultimum*, *Fusarium oxysporum*, *Rhizoctonia solani in vitro*. Studies *ad planta* confirmed the antagonistic potential of this bacterial strain against *Phytophthora parasitica* on tomato.

The bacterial antagonistic activity in the dual culture tests could be due to a number of mechanisms of action, such as direct effects through the production of antifungal compounds (Thomashow and Weller, 1988; Levy *et al.*, 1992; Raaijmakers and Weller, 1998; Ligon *et al.*, 2000). Siderophores that transport iron into bacterial cells are able to sequester iron (III) thus reducing the iron availability for the pathogen (Kloepper *et al.*, 1980; Thomashow and Weller, 1996; Duffy and Défago, 1999; Yang and Crowley, 2000). Some bacteria are able to produce lytic enzymes that can lead to cell wall degradation of the pathogen (Chet *et al.*, 1990; Pleban *et al.*, 1997; Singh

et al., 1999). The induction of systemic resistance is another possible mode of action observed in many plant-bacteria interactions (Van Loon *et al.*, 1998). Additional investigations are needed to study the mechanisms for the isolates in detail, because a mixture of different biocontrol agents and therefore a combination of different mechanisms can improve biological control (Raupach and Kloepper, 1998; Siddiqui and Shaukat, 2002).

Wilting of tomato plants inoculated with *Fusarium oxysporum* was not significantly reduced by any of the seven bacteria isolates that had shown antagonistic activity *in vitro*. However, wilting was very low even in the control and therefore it was difficult to detect any differences between the treatments. The frequency of *F. oxysporum* reisolation, representing fungal spread inside the shoot, gave interesting results. *Paenibacillus macerans* SR60 reduced the frequency in all shoot segments and the segments from the top of the shoot were free of fungal mycelium. Three other bacteria, namely *Bacillus cereus* SR16, *Pseudomonas putida* SR52 and *Enterobacter cloaceae* SR58, also reduced fungal spread in the shoot.

Terhardt (1998) isolated 1900 bacteria strains from the rhizosphere of different crops in Thailand and used the same techniques to study their antagonistic potential against the same *F. oxysporum* strain. In his study 62 isolates, 4% of all bacteria strains, showed antagonistic activity against the pathogen *in vitro* but only three of the isolates reduced the frequency of reisolation of the pathogen from the stem. Interestingly the three antagonistic bacteria were in the same species or at least the same genus as in this study: *Paenibacillus macerans* B60, *Pseudomonas putida* B53 and *Bacillus megaterium* B4. The antagonistic potential of these three genera against different fungal pathogens is well known (Weller, 1988; Whipps, 2001).

It can be concluded that the antagonistic potential of spore-associated bacteria was high, when compared with other investigations. These findings are in accordance with other studies (Meyer and Linderman, 1986; Budi *et al.*, 1999; Avio *et al.*, 2000). Therefore an influence of mycorrhiza on the enrichment of antagonistic bacteria (Linderman, 1988; Barea *et al.*, 2002) is clearly demonstrated by the findings presented here and in other studies. Further investigations combining AMF and spore-associated bacteria in the biocontrol of soilborne pathogens are needed and

could give similar additive effects as observed in the biocontrol of *Meloidogyne incognita* in Chapter 3.

4.5 References

AVIO, L., SBRANA, C., FILLIPPI, C., BAGNOLI, G. and GIOVANETTI, M. (2000) Antagonistic bacteria tightly associated to spores of three arbuscular mycorrhizal fungi species. In: Elad, Y., Freeman, S. and Monte, E. (eds.): Biocontrol agents: Mode of action and interaction with means of control. Sevilla, Spain, 279-284

AZCÓN-AGUILAR, C. and BAREA, J.M. (1996) Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - an overview of the mechanisms involved. Mycorrhiza 6, 457-464

BAREA, J.M., AZCÓN, R. and AZCÓN-AGUILAR, C. (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie van Leeuwenhoek 81, 343-51

BERG, G., ROSKOT, N., STEIDLE, A., EBERL, L., ZOCK, A. and SMALLA, K. (2002) Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Appl. Environ. Microbiol. 68, 3328-3338

BUDI, S.W., BLAL, B. and GIANINAZZI, S. (1999) Surface sterilization of sporocarps of *Glomus mosseae* for studying endomycorrhization *in vitro*. Mycorrhiza 9, 65-68

BUDI, S.W., VAN TUINEN, D., MARTINOTTI, G. and GIANINAZZI, S. (1999) Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soil borne fungal pathogens. Appl. Environ. Microbiol. 65, 5148-5150

CHERNIN, L., ISMAILOV, Z., HARAN, S. and CHET, I. (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens . Appl. Environ. Microbiol. 61, 1720-1726

CHET, I., ORDENTLICH, A., SHAPIRA, R. and OPPENHEIM, A. (1990) Mechanisms of biocontrol of soil-borne pathogens by rhizobacteria. Plant Soil 129, 85-92

DE BOER, W., VERHEGGEN, P., KLEIN GUNNEWIEK, P.J.A., KOWALCHUK, G.A. and VAN 'VEEN, J.A. (2003) Microbial community composition affects soil fungistasis. Appl. Environ. Microbiol. 69, 835-844

DEHNE, H.W. (1982) Interaction between vesicular-arbuscular mycorrhizal fungi and plant pathogens. Phytopathology 72, 1115-1119

DUFFY, B.K. and DÉFAGO, G. (1999) Environmental factors modulating antibiotic and siderophore production by *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. 65, 2429-2438

GERDEMANN, J.W. and NICOLSON, T.H. (1963) Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. Trans. Br. Mycol. Soc. 46, 235-244

KLOEPPER, J.W., LEONG, J., TEINTZE, M. and SCHROTH, M.N. (1980) *Pseudomonas* siderophores: a mechanism explaining disease suppressive soils . Curr. Microbiol. 4, 317-320

KRECHEL, A., FAUPEL, A., HALLMANN, J., ULRICH, A. and BERG, G. (2002) Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode *Meloidogyne incognita* (Kofoid & White) Chitwood. Can. J. Microbiol. 48, 772-786

LEVY, E., GOUGH, F.J., BERLIN, K.D., GUIANA, P.W. and SMITH, J.T. (1992) Inhibition of *Septoria tritici* and other phytopathogenic fungi and bacteria by *Pseudomonas fluorescens* and its antibiotics. Plant Pathol. 41, 335-341

LIGON, J.M., HILL, D.S., HAMMER, P.E., TORKEWITZ, N.R., HOFMANN, D., KEMPF, H.J. and VAN PEE, K.H. (2000) Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. Pest Manage. Sci. 56, 688-695

LINDERMAN, R.G. (1988) Mycorrhizal interactions with the rhizosphere microflora: The mycorrhizosphere effect. Phytopathology 78, 366-371

LINDERMAN, R.G. (1994) Role of VAM fungi in biocontrol. In: Pfleger, F.L. and Linderman, R.G. (eds.): Mycorrhizae and Plant Health. St. Paul, Minnesota, USA, 1-25

MAHAFFEE, W.F. and KLOEPPER, J.W. (1997) Temporal changes in the bacterial communities of soil, rhizosphere and endorhiza associated with field grown cucumber (*Cucumis sativus*, L.). Can. J. Microbiol. 34, 210-223

MAO, W., LEWIS, J.A., LUMSDEN, D. and HEBBAR, K.P. (1998) Biocontrol of selected soil-borne diseases of tomato and pepper plants. Crop Protection 6, 535-542

MEYER, J.R. and LINDERMAN, R.G. (1986) Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. Soil Biol. Biochem. 18, 191-196

MWANGI, M. (2002) Mechanisms of action in biological control of *Fusarium oxysporum* f.sp. *lycopersici* in tomato using rhizobacteria. University of Bonn, Germany

PLEBAN, S., CHERNIN, L. and CHET, I. (1997) Chitinolytic activity of an endophytic strain of *Bacillus cereus*. Lett. Appl. Microbiol. 25, 284-285

RAAIJMAKERS, J.M. and WELLER, D.M. (1998) Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. Mol. Plant-Microbe Interact. 11, 144-152

RAUPACH, G.S. and KLOEPPER, J.W. (1998) Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88, 1158-1164

REIMANN, S. and SIKORA, R.A. (2003) Managing the mycorrhizosphere - an approach to sustainable agriculture after the phase-out of methyl bromide. Comm. Agric. Appl. Biol. Sci. 68, 129-134

SECILIA, J. and BAGYARAJ, D.J. (1987) Bacteria and actinomycetes associated with pot cultures of vesicular-arbuscular mycorrhizas. Can. J. Microbiol. 33, 1069-1073

SHARIFI TEHRANI, A., ZEBARJAD, A., HEDJAROUD, G.A. and MOHAMMADI, M. (2002) Biological control of soybean damping-off by antagonistic rhizobacteria. Comm. Agric. Appl. Biol. Sci. 67, 377-380

SIDDIQUI, I.A. and SHAUKAT, S.S. (2002) Mixtures of plant disease suppressive bacteria enhance biological control of multiple tomato pathogens. Biol. Fertil. Soils. 36, 260-268

SINGH, P.P., SHIN, Y.C., PARK, C.S. and CHUNG, Y.R. (1999) Biological control of *Fusarium* wilt of cucumber by chitinolytic bacteria. Phytopathology 89, 92-99

TERHARDT, J. (1998) Beeinflussung mikrobieller Gemeinschaften der Rhizosphäre nach Blattbehandlung von Pflanzen und biologische Kontrolle von *Fusarium oxysporum* f.sp. *lycopersici* und *Meloidogyne incognita* mit bakteriellen Antagonisten. University of Bonn, Germany

THOMASHOW, L.S. and WELLER, D.M. (1988) Role of phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J. Bacteriol. 170, 3499-3508

THOMASHOW, L.S. and WELLER, D.M. (1996) Molecular basis of pathogen suppression by antibiosis in the rhizosphere. In: Hall, R. (ed.): Principles and Practice of Managing Soilborne Plant Pathogens. St. Paul, Minnesota, USA, 80-103

VAN LOON, L.C., BAKKER, P.A.H.M. and PIETERSE, C.M.J. (1998) Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36,

WELLER, D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26, 379-407

WHIPPS, J.M. (2001) Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52, 487-511

YANG, C.H. and CROWLEY, D.E. (2000) Rhizosphere microbial community structure in relation to root location and plant iron status. Appl. Environ. Microbiol. 66, 345-351

5 Influence of *Rhizobium etli* G12 on *Glomus intraradices* and their combination in the biocontrol of *Meloidogyne incognita*

5.1 Introduction

Interest in alternative pathogen control strategies for crop production increased in response to environmental and health concerns about the use of pesticides. For instance, many microorganisms have been tested against plant pathogens in the last decades to develop biological control strategies (Buchenauer, 1998; Whipps, 2001). Plant health promoting rhizobacteria (PHPR) show single or multiple antagonistic activity against different soilborne pathogens and are promising organisms for commercial use (Weller, 1988; Sikora and Hoffmann-Hergarten, 1993; Whipps, 1997). However, biocontrol agents show inconsistent performance, because they are normally based on a single microorganism that is not likely to be active under all environmental conditions. Therefore the combination of different strains and organisms has been recommended, in order to enhance biological control activity (Raupach and Kloepper, 1998; Sikora and Reimann, 2004).

AMF are important components in agroecosystem (Barea and Jeffries, 1995) and therefore biocontrol agents introduced into the soil must be compatible with the formation and functioning of this symbiosis (Barea *et al.*, 1998). Different strains of *Trichoderma harzianum* inhibited AMF development and colonization (Wyss *et al.*, 1992; Burla *et al.*, 1996), while mycorrhizal establishment was increased by another strain of the same fungus (Filion *et al.*, 1999). DAPG-producing *Pseudomonas* strains have been shown to either have no effect on mycorrhizal development or even improve AMF establishment (Paulitz and Linderman, 1989; Barea *et al.*, 1998). On the other hand, AMF are also known to inhibit pathogen attack or decrease the damage that is caused by the deleterious organisms (Linderman, 1994; Sikora, 1995).

The PHPR *Rhizobium etli* G12, initially identified as *Agrobacterium radiobacter*, was originally isolated from the rhizosphere of potatoes (Racke and Sikora, 1992). It has been repeatedly demonstrated that this bacterium is able to suppress early infection by the potato cyst nematode *Globodera pallida* (Hasky-Günther *et al.*, 1998) and the

root-knot nematode *Meloidogyne incognita* (Hallmann *et al.*, 2001). Lipopolysaccharides were identified as an inducing agent of the systemic resistance (Reitz *et al.*, 2000) and the mechanism involved in resistance development seems to be directly related to nematode recognition and penetration of the root (Reitz *et al.*, 2001). *Rhizobium etli* was recently reported to promote establishment of the mycorrhizal fungus *Glomus intraradices* on tomato roots (Reimann and Sikora, 2003).

The objectives of this investigation were to study (i) the effects of *Rhizobium etli* on AMF *in vitro* and *in vivo* in order to obtain information about the mechanisms involved and (ii) the combined application of AMF and G12 in the biocontrol of the root-knot nematode *Meloidogyne incognita*.

5.2 Materials and methods

5.2.1 Plants

Tomato seeds (*Lycopersicon esculentum* Mill.) were surface sterilized in a 75% Ethanol solution for 1 min and then in a 1.5% NaOCl solution for 3 min. Seeds were then washed with tap water and transferred to sterile sand in a seedling box for germination at greenhouse conditions.

The cultivar 'King Kong II' (AVRDC, Taiwan) was the standard variety for all experiments, while 'Hildares' (Juliwa-Enza, Heidelberg, Germany) and 'Seedatip' (AIT, Bangkok, Thailand) were only used for one experiment.

The culture substrate was a mixture of sterile sand and field soil (9:1, v/v). Inoculum of *Glomus intraradices* in expanded clay was mixed into this substrate at 5% (v/v). One g of rock phosphate per liter substrate was incorporated as an initial fertilizer.

The temperature in the greenhouse was kept at 22°C (± 4°C) and the plants were supplied with artificial light (3000 Lux).

5.2.2 Nematode inoculum

The root-knot nematode *Meloidogyne incognita* (Kofoid & White) Chitwood (race 3) was maintained in the greenhouse at 27°C in a large container filled with sandy loam and permanently cultivated with tomato (cv. 'Furore'). Nematode eggs were extracted

from heavily galled tomato roots according to Hussey and Barker (1973). Galled roots were washed free from soil under tap water, cut into 1 cm pieces and macerated in a warring blender at high speed for 20 s and collected in a glass bottle. Sodium hypochloride (NaOCl) was added to a final concentration of 1.5% active Chlorine and the bottle was shaken vigorously for 3 min. The suspension was thoroughly washed with tap water through a sieve combination (250, 100, 45 and 25 µm mesh) to remove the NaOCl. Eggs were collected on the 25 µm sieve and washed with tap water into a glass bottle. The Number of eggs was counted, the concentration adjusted to 1000 eggs ml^{-1} with tap water and then mixed into the substrate, where needed as inoculum.

5.2.3 Microorganisms and microbial inoculants

Rhizobium etli G12 was maintained for long-term storage at –80°C in cryo vials (Pro Lab Diagnostic). Bacteria were cultured in liquid King's medium B (King *et al.*, 1954) on a rotary shaker or on solid agar of the same medium and incubated for 36 h at 28°C. The bacterial suspension was centrifuged at 10°C for 20 min at 5000 g (Heraeus Varifuge RF). The resulting pellet was resuspended in sterile ¼-concentrated Ringer-solution (Merck) and the concentration was adjusted to an optical cell density of 2 at 560 nm (OD_{560}). The concentration of this bacteria suspension corresponded to cell numbers of approximately 1.4 x 10^{10} cfu ml^{-1}. Control plants were always inoculated with same volume of sterile Ringer solution.

Glomus intraradices (isolate 510) inoculum in expanded clay was kindly provided by Dr. H. von Alten (University of Hannover).

Spores of *Glomus intraradices* (Sy 167) for the *in vitro* experiments were extracted from monoxenic culture with Ri T-DNA transformed carrot roots (Hildebrandt *et al.*, 2002).

5.2.4 Evaluation of mycorrhizal establishment

Establishment of mycorrhiza on the roots was examined by staining the roots according to Vierheilig *et al.* (1998). Twenty root pieces of 1 cm length were placed on a slide and than examined for mycorrhizal features under the microscope in order to estimate the frequency and intensity of mycorrhiza establishment (Trouvelot *et al.*, 1986).

5.2.5 Influence of *Rhizobium etli* on mycorrhiza establishment on different cultivars

One week old seedlings of the three tomato cultivars were transplanted into 900-ml pots containing the culture substrate supplemented with 5% (v/v) *Glomus intraradices* inoculum. At the time of transplanting 5 ml of *Rhizobium etli* was applied as a soil drench around the stem of nine replicates. Plants were cultivated under greenhouse conditions. Fertilization was carried out weekly with 0.2% Wuxal Top N (Aglukon, Düsseldorf, Germany) solution as needed. Five weeks after bacteria application mycorrhiza establishment was estimated and shoot dry weight and root fresh weight were measured.

5.2.6 Influence of *Rhizobium etli* on mycorrhiza establishment over time

In this experiment the cultivar 'King Kong II' was used. All the other experimental parameters were the same as described in 1.2.5. Each week six plants, treated with *Rhizobium etli* or not, were taken for the evaluation of mycorrhiza establishment until mycorrhiza establishment reached its maximum after 9 weeks.

5.2.7 Interaction of *Rhizobium etli* and *Glomus intraradices in vitro*

All studies were conducted on a modified gellangum (GelGro, ICN) M-Medium (Bécard and Fortin, 1988) as described previously by Hildebrandt *et al.* (2002). Petri dishes subdivided into three compartments were used for the first experiments. About 1 mg of spores and hyphae of *Glomus intraradices* Sy167, coming from monoxenic culture, was placed on the medium in the middle of each compartment. A small portion of bacteria was scratched from a *Rhizobium etli* culture growing on King's medium B agar and was placed approximately 15 mm away from the fungus. The Petri dish was incubated at 27°C in the dark and growth of the AMF was examined daily.

Square Petri dishes were used for the second experiment. AMF spores and hyphae (~1 mg) were placed in the center of the plate and covered with a dialysis membrane with a molecular weight cut-off of 12 kDa. One drop of bacterial cells, precultured on King's medium B agar, was placed on the membrane directly over the AMF spores. Plates were incubated for 6 weeks at 27°C in the dark and examined daily. Finally newly formed spores were counted and spore size and hyphal diameter were

measured under the microscope applying the diskus program (Technisches Büro Hilgers, Königswinter, Germany). Hyphal branching was estimated by counting the branches of 100 segments of primary hyphae with a length of 1.4 µm. Mother spores were removed and the agar was collected in an Erlenmeyer flask. The agar was then dissolved in a 10 mM sodium citrate solution (pH 6.0) by shaking on a rotary shaker at 28°C. The hyphae were collected by filtering the solution, then dried on a filter paper and finally weighed.

5.2.8 Combination of *Glomus intraradices* and *Rhizobium etli* G12 in the biocontrol of *Meloidogyne incognita* on tomato

One week old tomato seedlings were transplanted in 500-ml pots containing the culture substrate supplemented with 5% (v/v) *Glomus intraradices* inoculum. At the time of transplanting a 5 ml suspension of *Rhizobium etli* or sterile Ringer-solution was applied as a soil drench around the stem of 16 replicates. Plants were cultivated under greenhouse conditions and fertilized weekly with 0.2% Wuxal Top N solution as needed.

Four weeks after bacterial treatment the establishment of mycorrhiza on the roots of 8 plants was examined. The pots of the other 8 plants were soaked in a beaker containing the *R. etli* suspension for three seconds. These tomato plants were transplanted again into 2-l pots containing non-sterile sand-field soil mixture (2:1, v/v) infested with 1000 eggs of *M. incognita* per 100 ml of soil. The plants were fertilized with increasing amounts of 0,2% Flory 3 solution (Euflor, Munich, Germany). Flowers were removed in order to avoid fruit development. Eight weeks after transplanting shoot dry weight and root fresh weight were measured. The roots were stained with Phloxin-B (Merck) and subsequently galls and egg masses were counted (Sikora and Schuster, 2000).

5.3 Results

Influence of Rhizobium etli on mycorrhiza establishment on different cultivars

Dual inoculation of AMF with *Rhizobium etli* G12 led to a significant increase in mycorrhizal establishment on all three tomato cultivars (Fig. 5.1). Levels of mycorrhiza frequency were similar for ‘Hildares’ and ‘King Kong II’ and both were increased by about 40% with *Rhizobium etli* application. Root colonization of the

cultivar ‘Seedatip’ by *Glomus intraradices* was much lower and the inoculation with bacteria led to a 100% increase of AMF establishment over the control.

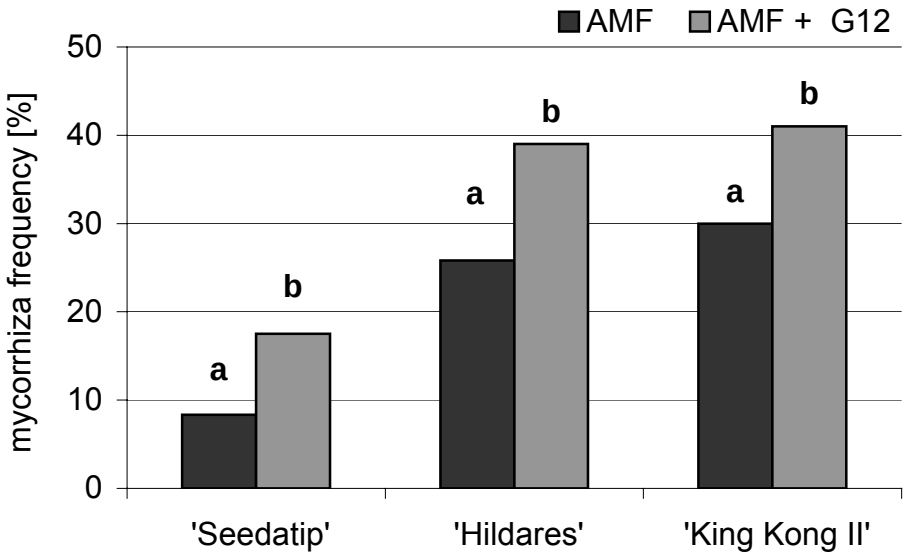

Figure 5.1: Root colonization by *Glomus intraradices* on three tomato cultivars with and without *Rhizobium etli* G12.
Different letters indicate statistical significant differences (α = 0,05; Student’s T-Test)

Influence of Rhizobium etli on mycorrhiza establishment over time

Mycorrhiza establishment was accelerated in plants simultaneously inoculated with *R. etli*. After 3 weeks, more than 50% of all examined root pieces showed the presence of arbuscules, vesicles or hyphae. In the uninoculated control, mycorrhiza

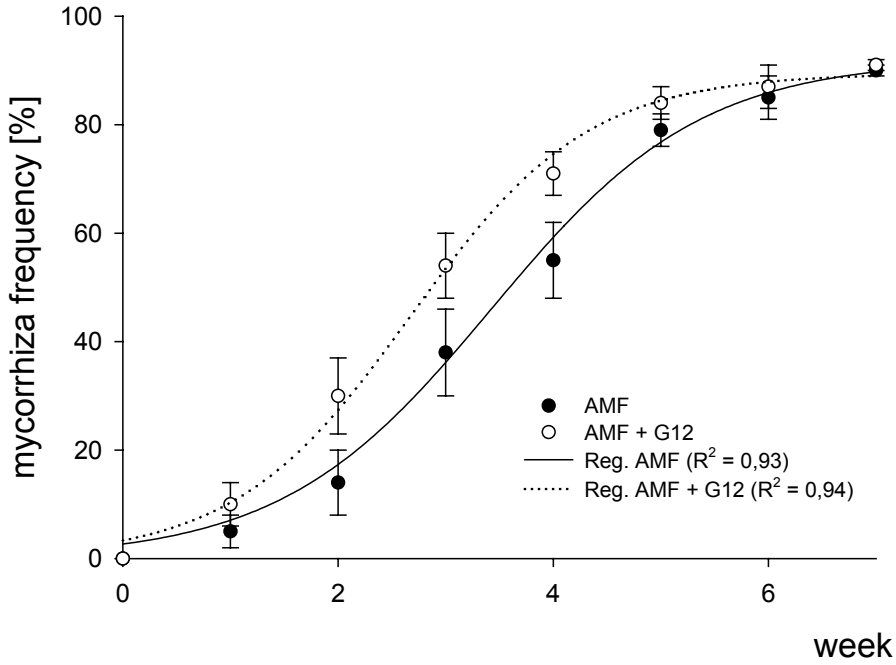

Figure 5.2: Establishment of *Glomus intraradices* on tomato plants cv. ‘King Kong II’ with or without *Rhizobium etli* G12.

structures were observed on only 38% of all root pieces at the same time. From week seven on no differences were observed because mycorrhizal colonization reached its maximum (~ 85%) in both treatments (Fig 5.2).

Interaction of Rhizobium etli and Glomus intraradices in vitro

Two weeks after inoculation with *Rhizobium etli, g*erminating spores of *Glomus intraradices* started to build so-called branched absorbing structures (Bago *et al.*, 1998) and new spores were formed. In contrast only long "runner hyphae" and few spores were formed in the control. Densely packed coils (DPC) as described for the interaction between *Glomus intraradices* and *Paenibacillus validus* (Hildebrandt *et al.*, 2002) were not found. When *Rhizobium etli* came in contact with the mycorrhizal hyphae, the bacterium moved along the mycelium. Finally the neighboring control compartments were contaminated with bacteria via hyphae that grew over the separation barriers (Fig.5.3).

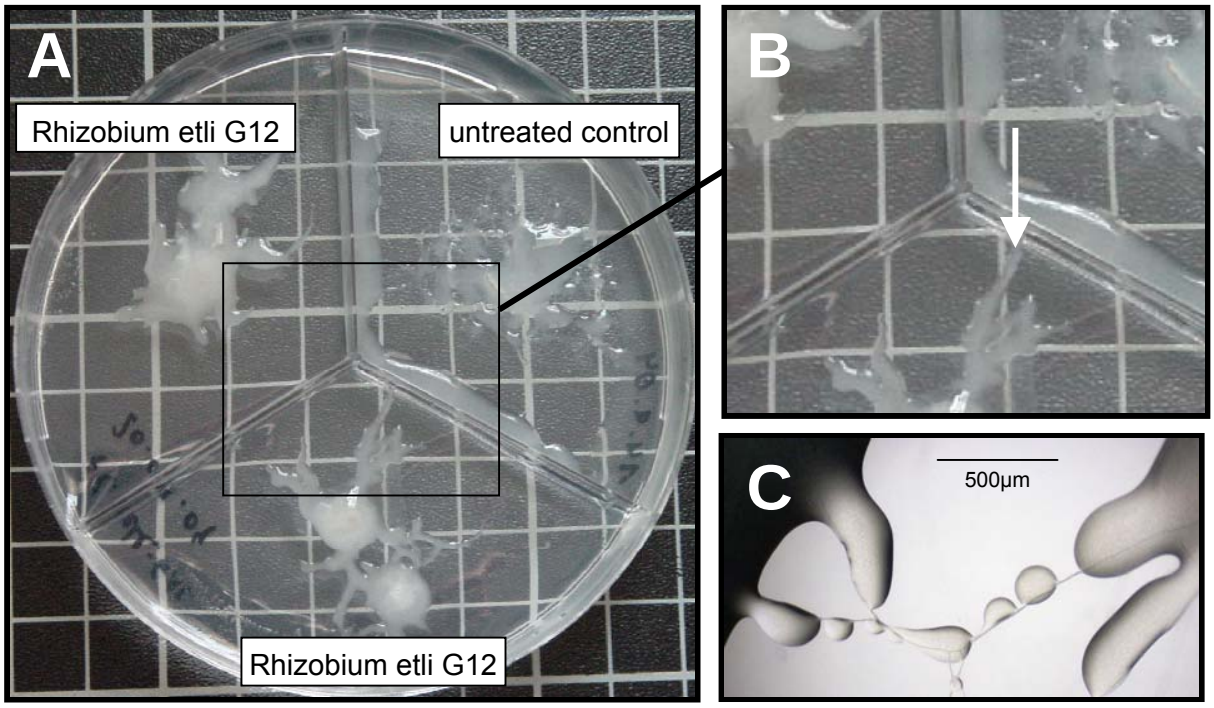

Figure 5.3: Coculture of *Glomus intraradices* (Sy167) with *Rhizobium etli* G12 (A), latter contaminating the untreated control via hyphae that grew over the separation barriers of the AMF (B, arrow) and attachment of the bacteria to AMF hyphae (C).

When a dialysis membrane separated *Rhizobium etli* from *Glomus intraradices*, the fungus again started to sporulate two weeks after inoculation. Sporulation was mainly observed where the hyphae were covered by colonies of growing bacteria on the other side of the membrane. The fungus seemed to have stopped hyphal and spore

growth in the Petri dishes. Upon observing this the newly formed fungal biomass was harvested and the fresh weight determined. The ratio of newly formed fungal biomass to biomass of the initial inoculum was inhibited by inoculation with G12, whereas hyphal diameter and hyphal branching increased at the same time (Tab. 5.1). In the control septation of the hyphae was visible, whereas this did not occur in the AMF exposed to *Rhizobium etli*.

Table 5.3: Influence of *Rhizobium etli* G12 on development of *Glomus intraradices* (Sy167) *in vitro*.

	AMF	AMF + G12
Biomass AMF inoculum (old)	1.40 mg	1.11 mg
Biomass mycelium (new)	0.32 mg	0.07 mg
Biomass ratio (new/old)	0.23	0.06
Hyphae diameter	2.91 ± 0.70 µm	4.78 ± 1,01 µm
First degree ramifications	0.95 ± 0.51	2.07 ± 0.52
Newly formed spores *	< 1	59.6
Size of mother spores	74.07 ± 4.89 µm	74.07 ± 4.89 µm
Size of daughter spores	n.d.	32.81 ± 4.82 µm

n.d. = not determined, * per 100 mother spores

Numerous newly formed spores were observed in the Petri dishes co-inoculated with *R. etli*, whereas spores were only occasionally found in the control (Tab 5.1). Sporulation most frequently occurred on branched hyphae directly below bacteria colonies, growing on the other side of the membrane. All daughter spores remained much smaller than the mother spores, but they were filled with lipid droplets or globules in the bacteria treated Petri dishes while the few spores in the control seemed to be empty (Fig. 5.4).

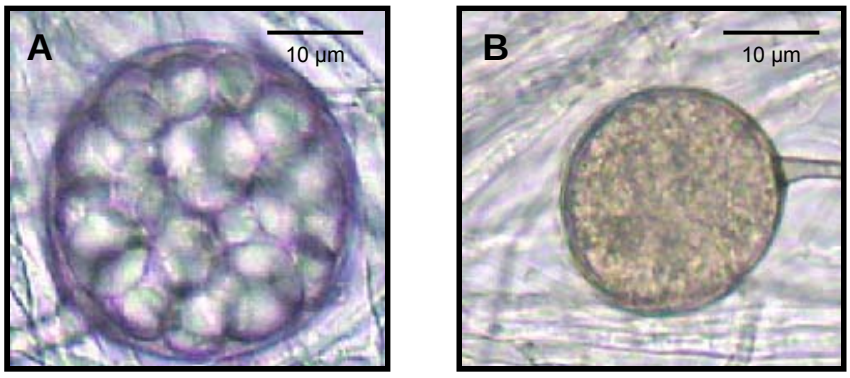

Figure 5.4: Spores of *Glomus intraradices* (Sy167) formed either in co-culture with *Rhizobium etli* G12 (A) or on M-Medium alone (B).

Combination of Glomus intraradices and Rhizobium etli G12 in the biocontrol of Meloidogyne incognita on tomato

The number of galls formed by *Meloidogyne incognita* in the AMF treatment were reduced in two experiments by 24 and 13% respectively, but results were not significant in the second experiment (Fig. 5.5).

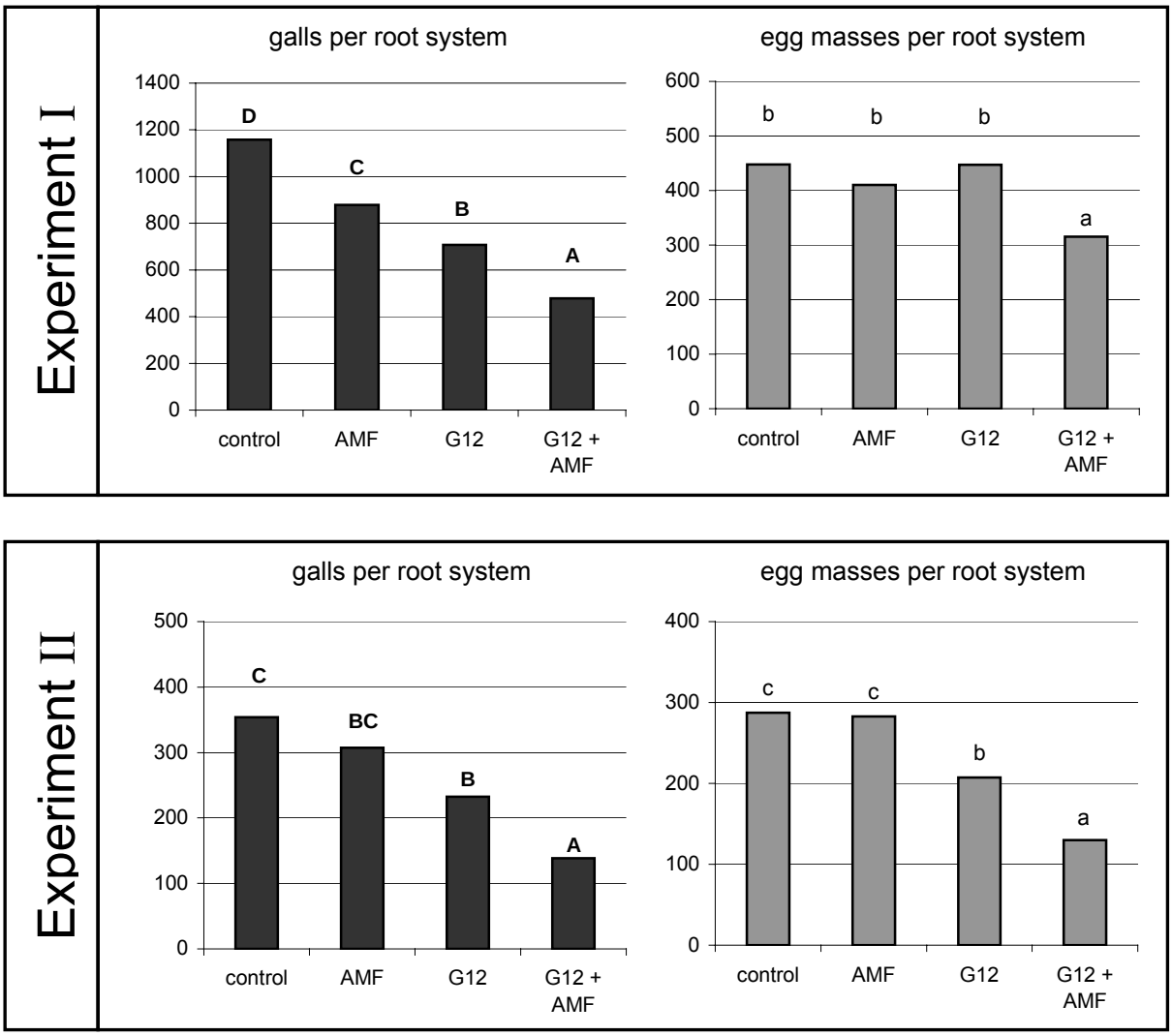

Figure 5.5: Effects of combined and single inoculation of *Glomus intraradices* and *Rhizobium etli* G12 on gall and egg mass formation by *Meloidogyne incognita* on tomato in two experiments.

Egg mass production was not affected by inoculation with *Glomus intraradices*. Application of *Rhizobium etli* G12 alone resulted in significant reduction of galling in both experiments (39/34%) and the number of egg masses in the second experiment. Combining the two microorganisms led to significant reduction in the

numbers of galls (59/61%) and egg masses (54/30%) over the other treatments in both experiments.

In the first experiment, the number of egg masses for all treatments were generally very similar, while differences in galling were more pronounced. In the second experiment, both galling and egg mass production were strongly reduced, even if the numbers for galls and egg masses were much lower for all treatments. It is noteworthy that the first experiment was conducted in August-September and the second in September-October. Hence development and hatching of the nematode was most probably delayed in the second experiment due to lower temperatures in the greenhouse and lower irradiation intensity.

Table 5.4: Effects of *Glomus intraradices* and *Rhizobium etli* in the biocontrol of *Meloidogyne incognita* on mycorrhiza establishment after four weeks and plant growth parameters at the end of the experiment.

Experiment I	**control**	**AMF**	**G12**	**AMF + G12**
Mycorrhiza frequency [%] *	n.d.	25.10 ± 6.07	n.d.	42.30 ± 7.80
Shoot dry weight [g]	26.93 ± 0.69	26.92 ± 1.04	25.84 ± 0.85	27.12 ± 0.80
Root fresh weight [g]	42.26 ± 6.38	35.79 ± 5.94	37.25 ± 6.63	31.45 ±6.04
Experiment II	**control**	**AMF**	**G12**	**AMF + G12**
Mycorrhiza frequency [%] *	n.d.	25.30 ± 4.80	n.d.	38.20 ± 7.04
Shoot dry weight [g]	28.88 ± 1.15	30.08 ± 4.15	27.72 ± 0.50	30.45 ± 5.49
Root fresh weight [g]	31.36 ± 3.28	21.53 ± 3.60	25.55 ± 3.30	21.30 ± 1.71

n.d.= not determined, * four weeks after inoculation at the time of transplanting

Shoot dry weight was not affected by either treatment, whereas root fresh weight of all mycorrhizal plants decreased. Mycorrhiza establishment in the roots of four week old plants prior to transplantation increased to 42 and 38% respectively in the *Rhizobium etli* treatment compared to 25% in both experiments when AMF was inoculated alone (Fig. 5.2).

5.4 Discussion

The results presented here confirm earlier findings (Reimann and Sikora, 2003). *Rhizobium etli* G12 was able to enhance establishment of *Glomus intraradices* on three tomato cultivars, differing in their compatibility with the chosen AMF isolate. Over time the bacterium accelerated AMF colonization. Consequently *Rhizobium etli* G12 should be regarded as a mycorrhiza helper bacterium (MHB) as described for other bacteria (Garbaye, 1994).

Azcón-Aguilar *et al.* (1978) reported better AMF root colonization of *Medicago sativa* after inoculation with *Rhizobium meliloti*. The same group was able to demonstrate that plant hormones are involved in this AMF promotion (Azcón *et al.*, 1978). Therefore it was hypothesized that plants were stimulated by the bacterium to form the mycorrhiza symbiosis, while a direct effect on the fungus was not excluded. In general AMF and rhizobia seem to have many things in common. They are both endosymbionts, and flavanoids have been shown to be involved in the formation of their symbioses (Bauer and Caetano-Anolles, 1990; Siqueira *et al.*, 1991; Koide and Schreiner, 1992). Gianinazzi-Pearson *et al.* (Gianinazzi-Pearson *et al.*, 1989) demonstrated that flavonoids which activate *nod* gene expression in *Rhizobium* elicited AMF mycelia growth. Furthermore both microorganisms have to suppress the host-defence response in order to successfully establish in the root and it was shown that some *nod*$^-$ mutants are also incompatible to AMF (*myc*$^-$) (Hirsch and Kapulnik, 1998).

Due to these similarities it has been hypothesized that the two symbioses share a common genetic basis (Sagan *et al.*, 1995). Bonanomi *et al.* (2001) studied the induction of genes in *Medicago sativa* after AMF inoculation. The authors observed that the same genes were upregulated after *Sinorhizobium meliloti* application and concluded that they are general symbiosis-related genes. Frühling *et al.* (1997) reported the activation of the late nodulin gene encoding for the bean leghemoglobin *VfLb*29 in nodule-free roots colonized by an arbuscular mycorrhizal fungus. There are other reports on the expression of early nodulin genes in mycorrhizal root tissues of legumes (Wyss *et al.*, 1990; Van Rhijn *et al.*, 1997; Journet *et al.*, 2001). All these results were achieved with legumes and therefore a common genetical basis in the formation of both symbioses seems to be evident. However, experiments with non-

legume plants have shown that these findings may be transferred to a certain degree. Vieweg *et al.* (2004) observed that the activation of the *VfLb*29 promoter by AMF establishment is not limited to legume plants but also occurred in *Nicotinia tabacum*. The authors concluded that a common trigger exists for this promoter in arbuscule-containing root cells of different plant species. In another study the inoculation of barley and wheat with *Rhizobium leguminosarum* resulted in enhanced accumulation of secondary compounds such as blumenin, which were found to play an important role in AMF establishment (Fester *et al.*, 1999). Additionally the bacterium was able to enhance the establishment of *Glomus intraradices* on the roots of these non-legume plants. In this context, it seems noteworthy that nonlegumes, e.g. tomato, responses to *nod* factors (Staehelin *et al.*, 1994; Bono *et al.*, 1995). Consequently the enhanced establishment of *Glomus intraradices* in the present study following inoculation with *Rhizobium etli* G12 could be plant mediated and further studies are needed to test this hypothesis.

Conversely the results of the *in vitro* study demonstrate that a direct influence of *Rhizobium etli* on *Glomus intraradices* may be involved in the enhanced mycorrhiza establishment. Inoculation of *R. etli* resulted in increased hyphal ramification and the formation of branched absorbing structures. Mosse (1962) reported similar findings after inoculation with different bacteria and she was able to show that increased branching led to better AMF colonization of the root. In another study, mycorrhizal hyphae were found to branch and form a fan-like structure in the presence of plant roots, whereas AMF alone did not change hyphal development (Powell, 1976). The author concluded that these morphological changes can be regarded as a preinfection stage of AMF establishment.

Mosse (1962) reported that hyphae of sterile spores had a strong apical dominance and that septation became visible when the spore reserves were exhausted. In the untreated controls in the present study, the hyphae of *G. intraradices* showed the same characteristics. *Rhizobium etli* stimulated spore production of the AMF soon after germination, while the fungus without bacteria inoculation produced more hyphae. However, fungal development was not prolonged by *Rhizobium etli* as was described for a *Paenibacillus validus* under the same conditions (Hildebrandt *et al.*, 2002). *Glomus intraradices* treated with *R. etli* did not form the densely packed coils that were observed after *P. validus* inoculation. These arbuscule-like structures may

be an important prerequisite for the nutrition of the fungus and therefore the saprophytic growth in monoxenic culture (Azcón-Aguilar and Barea, 1995).

Extracellular polysaccharides play a mayor role in biofilm formation and these are involved in the attachment of bacteria to different surfaces (Costerton *et al.*, 1995). Bianciotto *et al.* (2001) reported that extracellular polysaccharides (EPS) of *Rhizobium leguminosarum* are involved in the attachment of the bacteria to extraradical hyphae of AMF. In this study *Rhizobium etli* G12 moved along the hyphae and Hasky-Günther *et al.* (1998) demonstrated that this isolate produces EPS. Therefore it seems likely that these compounds are involved in the attachment of the bacterium to fungal hyphae. Bacteria attachment to a surface seems to be important for the interaction between bacteria and other organisms (Nurmiaho-Lassila *et al.*, 1997), e.g. mycorrhizal fungi in this study. However, the separation of the *Rhizobium etli* from the AMF *in vitro* led to similar effects on fungus development. Therefore direct contact of the bacterium is not necessary for the changes in fungal development. *Rhizobium etli* grew along the hyphae on the other side of the dialysis membrane indicating that the two microorganisms “communicate” through the membrane.

Dual inoculation of the PHPR and AMF led to additive effects in the biological control of *M. incognita* and decreased the numbers of galls by more than 50% in two experiments. Shoot dry weight was not affected by either treatment. This is in accordance with earlier studies that described growth of tomato plants as independent of endomycorrhizal establishment (Caron *et al.*, 1985). However, Smith *et al.* (2003) reported that mycorrhizal fungi can control phosphate uptake of tomato plants irrespective of growth responses. Roots with less galls in general are expected to provide better nutrient uptake and, since fruit quality of tomatoes suffers significantly from nutrient deficiency, increased tomato quality should be expected. On the other hand decreased galling reduces attack of secondary pathogens (Jones *et al.* 1991; Sikora and Fernández, 2005). Hence an increased plant fitness can be expected, even if shoot dry weight was not affected.

One explanation for the increased plant protection could be the combination of different modes of action. *Rhizobium etli* G12 reduces nematode penetration on crops in different pest-host combinations (Hasky-Günther *et al.*, 1998; Hallmann *et*

al., 2001). The mechanisms by which AMF reduces nematode damage has not been determined, but the hypothesis range from depression of nematode development by competition for nutrient and space (Smith *et al.*, 1986; Smith, 1988; Elsen *et al.*, 2001) to microbial changes in the mycorrhizosphere that disturb nematode chemotaxis (Linderman, 1988). Mycorrhizal fungi have to be established in the root before the nematodes attack the roots in order to provide biological control (Grandison and Cooper, 1986; Diedhiou *et al.*, 2003). Saleh and Sikora (1984) demonstrated that at least 38% of the root should be mycorrhizal in order to suppress nematode development. In the present study the inoculation with *Rhizobium etli* resulted in increased mycorrhiza establishment in the roots of four week old transplants and this may have enhanced the biocontrol potential of *Glomus intraradices*.

In conclusion, Rhizobium etli G12 can be regarded to be a mycorrhiza helper bacterium. Morphological and developmental changes of *Glomus intraradices* after *Rhizobium etli* inoculation demonstrated that a direct interaction between the two microorganisms occurs. This interaction may be the reason for the increased mycorrhizal establishment. However, a plant mediated effect of the bacterium on AMF establishment cannot be excluded and further investigation are necessary.

The combination of *Glomus intraradices* and *Rhizobium etli* led to increased biocontrol activity compared with single inoculations. Further studies are needed to test different forms of bacteria application and different AMF isolates in order to obtain the best combination. In this context, the attachment of the bacterium to the mycorrhizal hyphae may be important for the development of an effective biocontrol agent based on AMF and this bacteria strain.

5.5 References

AZCÓN-AGUILAR, C. and BAREA, J.M. (1978) Effects of interactions between different culture fractions of 'phosphobacteria' and *Rhizobium* on mycorrhizal infection, growth and nodulation of *Medicago sativa*. Can. J. Microbiol. 24, 520-524

AZCÓN-AGUILAR, C. and BAREA, J.M. (1995) Saprophytic growth of arbuscular mycorrhizal fungi. In: Varma, A. and Hock, B. (eds.): Mycorrhiza. Structure, Function, Molecular Biology and Biotechnology. Berlin, 391-407

AZCÓN, R., AZCÓN-AGUILAR, C. and BAREA, J.M. (1978) Effects of plant hormones present in bacterial cultures on the formation and responses to VA mycorrhizas. New Phytol. 80, 359-364

BAGO, B.C., AZCÓN-AGUILAR, C., GOULET, A. and PICHÉ, Y. (1998) Branched absorbing structures (BAS): a feature of the extraradical hyphae of symbiotic arbuscular mycorrhizal fungi. New Phytol. 139, 375-388

BAREA, J.M., ANDRADE, G., BIANCIOTTO, V., DOWLING, D., LOHRKE, S., BONFANTE, P., O'GARA, F. and AZCÓN-AGUILAR, C. (1998) Impact on arbuscular mycorrhizal formation of *Pseudomonas* strains used as inoculants for biocontrol of soilborne plant pathogens. Appl. Environ. Microbiol. 64, 2304-2307

BAREA, J.M. and JEFFRIES, P. (1995) Arbuscular mycorrhizas in sustainable soil-plant systems. In: Hock, B. and Varma, A. (eds.): Mycorrhiza structure, function, molecular biology and biotechnology. Berlin, Germany, 521-559

BAUER, W.D. and CAETANO-ANOLLES, G. (1990) Chemotaxis, induced gene expression and competitiveness in the rhizosphere. Plant Soil 129, 45-52

BÉCARD, G. and FORTIN, J.A. (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol. 108, 211-218

BIANCIOTTO, V., ANDREOTTI, S., BALESTRINI, R., BONFANTE, P. and PEROTTO, S. (2001) Extracellular polysaccharides are involved in the attachment of *Azosporillum brasilense* and *Rhizobium leguminosarum* to arbuscular mycorrhizal structures. Eur. J. Histochem. 45, 39-49

BONANOMI, A., OETIKER, J.H., BOLLER, T., WIEMKEN, A. and VÖGELI-LANGE, R. (2001) Symbiosis-related genes of *Medicago truncatula* induced in the early phase of its interaction with *Glomus intraradices*. In: Sikora, R.A. (ed.): Integrated Control of Soil Pests. Gent, Belgium, 19-24

BONO, J.-J., RIOND, J., NICOLAOU, K.C., BOCKOVICH, N.J., ESTEVEZ, V.A., CULLIMORE, J.V. and RANJEVA, R. (1995) Characterization of a binding site for chemically synthesized lipo-oligosaccharides NodRm factors in particulate fraction prepared from roots. Plant J. 7, 253-270

BUCHENAUER, H. (1998) Biological control of soil-borne diseases by rhizobacteria. Z. Pflkrankh. Pflschutz. 105, 329-348

BURLA, M., GOVERDE, M., SCHWINN, F.J. and WIEMKEN, A. (1996) Influence of biocontrol organisms on root pathogenic fungi and on the plant symbiotic micro-organisms *Rhizobium phaseoli* and *Glomus mosseae*. Z. Pflkrankh. Pflschutz. 103, 156-163

CARON, M., FORTIN, J.A. and RICHARD, C. (1985) Influence of substrate on the interaction of *Glomus intraradices* and *Fusarium oxysporum* f.sp. *radicis-lycopersici* on tomatoes. Plant Soil 87, 233-239

COSTERTON, J.W., LEWANDOWSKI, Z., CALDWELL, D.E., KORBER, D.R. and LAPPIN-SCOTT, H.M. (1995) Microbial biofilms. Annu. Rev. Microbiol. 49, 711-745

DIEDHIOU, P.M., HALLMANN, J., OERKE, E.C. and H.W. DEHNE (2003) Effects of arbuscular mycorrhizal fungi and a non-pathogenic *Fusarium oxysporum* on *Meloidogyne incognita* infestation of tomato. Mycorrhiza 13, 199-204

ELSEN, A., DECLERCK, S. and DE WAELE, D. (2001) Effects of *Glomus intraradices* on the reproduction of the burrowing nematode (*Radopholus similis*) in dixenic culture. Mycorrhiza 11, 49-51

FESTER, T., MAIER, W. and STRACK, D. (1999) Accumulation of secondary compounds in barley and wheat roots in response to inoculation with an arbuscular mycorrhizal fungus and co-inoculation with rhizosphere bacteria. Mycorrhiza 8, 241-246

FILION, M., ST-ARNAUD, M. and FORTIN, J.A. (1999) Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere micro-organisms. New Phytol. 141, 525-533

FRÜHLING, M., ROUSSEL, H., GIANINAZZI-PEARSON, V., PÜHLER, A. and PERLICK, A.M. (1997) The *Vicia faba* leghemoglobin gene VfLb29 is induced in root nodules and in roots colonized by the arbuscular mycorrhizal fungus *Glomus fasciculatum*. Mol. Plant-Microbe Interact. 10, 124-131

GARBAYE, J. (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol. 128, 197-210

GIANINAZZI-PEARSON, V., BRANZANTI, B. and GIANINAZZI, S. (1989) *In vitro* enhancement of spore germination and early hyphal growth of a vesicular arbuscular mycorrhizal fungus by host root exudates and plant flavanoids. Symbiosis 7, 243-255

GRANDISON, G.S. and COOPER, K.M. (1986) Interactions of vesicular-arbuscular mycorrhizae and cultivars of alfalfa susceptible and resistant to *Meloidogyne hapla*. J. Nematol. 18, 141-149

HALLMANN, J., QUADT-HALLMANN, A., MILLER, W.G., SIKORA, R.A. and LINDOW, S.E. (2001) Endophytic colonization of plants by the biocontrol agent *Rhizobium etli* G12 in relation to *Meloidogyne incognita* infection. Biol. Control 91, 415-422

HASKY-GÜNTHER, K., HOFFMANN-HERGARTEN, S. and SIKORA, R.A. (1998) Resistance against the potato cyst nematode *Globodera pallida* systemically induced by the rhizobacteria *Agrobacterium radiobacter* (G12) and *Bacillus sphaericus* (B43). Fundam. Appl. Nematol. 21, 511-517

HILDEBRANDT, U., JANETTA, K. and BOTHE, H. (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl. Environ. Microbiol. 68, 1919-1924

HIRSCH, A.M. and KAPULNIK, Y. (1998) Signal transduction pathways in mycorrhizal associations: comparisons with the *Rhizobium*-legume symbiosis. Fungal Genet. Biol. 23, 205-212

HUSSEY, R.A. and BARKER, K.P. (1973) A comparison of methods for collecting inocula for *Meloidogyne* ssp., including a new technique. Plant Dis. Report. 57, 1025-1028

JOURNET, E.P., EL-GACHTOULI, N., VERNOUD, V., DE BILLY, F., PICHON, M., DEDIEU, A., ARNOULD, C., MORANDI, D., BARKER, D.G. and GIANINAZZI-PEARSON, V. (2001) Medicago truncatula ENOD11: a novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. Mol. Plant-Microbe Interact. 14, 737-748

JONES, J.P., STALL, R.E. and ZITTER, T.A. (1991) Compendium of Tomato Diseases. APS Press, St. Paul, Minnesota, USA

KING, E.O., WARTH, M. and RANEY, E.D. (1954) Two simple media for the demonstration of procyanin and fluorescin. J. Lab. Clin. Med. 44, 301-307

KOIDE, R.T. and SCHREINER, R.P. (1992) Regulation of the vesicular-arbuscular mycorrhizal symbiosis. Annu. Rev. Plant Physiol. 43, 557-581

LINDERMAN, R.G. (1988) Mycorrhizal interactions with the rhizosphere microflora: The mycorrhizosphere effect. Phytopathology 78, 366-371

LINDERMAN, R.G. (1994) Role of VAM fungi in biocontrol. In: Pfleger, F.L. and Linderman, R.G. (eds.): Mycorrhizae and Plant Health. St. Paul, USA, 1-25

MOSSE, B. (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J. Gen. Microbiol. 27, 509-520

NURMIAHO-LASSILA, E.L., TIMONEN, S., HAAHTELA, K. and SEN, R. (1997) Bacterial colonization patterns of intact *Pinus sylvestris* mycorrhizospheres in dry pine forest soil: an electron microscopy study. Can. J. Microbiol. 43, 1017-1035

PAULITZ, T.C. and LINDERMAN, R.G. (1989) Interactions between fluorescent pseudomonades and VA mycorrhizal fungi. New Phytol. 113, 37-45

POWELL, C.L.L. (1976) Development of mycorrhizal infections from *Endogone* spores and infected root segments. Trans. Br. Myc. Soc. 66, 439-445

RACKE, J. and SIKORA, R.A. (1992) Isolation, formulation and antagonistic activity of rhizobacteria toward the potato cyst nematode *Globodera pallida*. Soil Biol. Biochem. 24, 521-526

RAUPACH, G.S. and KLOEPPER, J.W. (1998) Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88, 1158-1164

REIMANN, S. and SIKORA, R.A. (2003) Managing the mycorrhizosphere - an approach to sustainable agriculture after the phase-out of methyl bromide. Comm. Agric. Appl. Biol. Sci. 68, 129-134

REITZ, M., HOFFMANN-HERGARTEN, S., HALLMANN, J. and SIKORA, R.A. (2001) Induction of systemic resistance in potato by rhizobacterium *Rhizobium etli* strain G12 is not associated with accumulation of pathogenesis-related proteins and enhanced lignin biosynthesis. Z. Pflkrankh. Pflschutz. 108, 11-20

REITZ, M., RUDOLPH, K., SCHRÖDER, I., HOFFMANN-HERGARTEN, S., HALLMANN, J. and SIKORA, R.A. (2000) Lipopolysaccharides of *Rhizobium etli* strain G12 act in potato roots as inducing agent of systemic resistance to infection by the cyst nematode *Globodera pallida*. Appl. Environ. Microbiol. 66, 3515-3518

SAGAN, M., MORANDI, D., TARENGHI, E. and DUC, G. (1995) Selection of nodulation and mycorrhizal mutants in the model plant *Medicago truncatula* (Gaertn.) after γ-ray mutagenesis. Plant Sci. 111, 63-71

SALEH, H.M. and SIKORA, R.A. (1984) Relationship between *Glomus fasciculatum* root colonization of cotton and its effect on *Meloidogyne incognita*. Nematologica 30, 230-237

SIKORA, R.A. (1995) Vesicular-arbuscular mycorrhizae: their significance for biological control of plant parasitic nematodes. Biocontrol 1, 29-33

SIKORA, R.A. and FERNÁNDEZ, E. (2005) Nematode Parasites of Vegetables. In: Luc, M., Sikora, R.A. and Bridge, J. (eds.): Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. Melksham, Great Britain,

SIKORA, R.A. and HOFFMANN-HERGARTEN, S. (1993) Biological control of plant parasitic nematodes with plant-health-promoting rhizobacteria. In: Lumsden, R.D. and Vaughn, J.L. (eds.): Pest Management: Biologically based technologies. Proceedings of Beltsville Symposium XVIII. Washington, DC,

SIKORA, R.A. and REIMANN, S. (2004) Suppressive soils, the edge of chaos and multitrophic strategies for biocontrol of pests and diseases in soil ecosystems. In: Sikora, R.A., Gowen, S., Hausschild, R. and Kiewnick, S. (eds.): Multitrophic interactions in soil. Gent, Belgium, 251-258

SIKORA, R.A. and SCHUSTER, R.-P. (2000) Handbuch der Phytonematologie. Shaker Verlag, Aachen, Germany

SIQUEIRA, J.O., SAFIR, G.R. and NAIR, M.G. (1991) Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavanoid compounds. New Phytol. 118, 87-93

SMITH, G.S. (1988) The Role of phosphorus nutrition in interactions of vesicular-arbuscular mycorrhizal fungi with soilborne nematodes and fungi. Phytopathology 78, 371-374

SMITH, G.S., HUSSEY, R.S. and RONCADORI, R.W. (1986) Penetration and postinfection development of *Meloidogyne incognita* on cotton as affected by *Glomus intraradices* and Phosphorus. J. Nematol. 18, 429-435

SMITH, S.E., SMITH, F.A. and JAKOBSEN, I. (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. Plant Physiol. 133, 16-20

STAEHELIN, C., GRANADO, J., MULLER, J. and WIEMKEN, A. (1994) Perception of *Rhizobium* nodulation factors by tomato cells and inactivation by root chitinases. Proc. Natl. Acad. Sci. USA 91, 2196-2200

TROUVELOT, A., KOUGH, J.L. and GIANINAZZI-PEARSON, V. (1986) Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. In: Gianinazzi-Pearson, V. and Gianinazzi, S. (eds.): Physiological and genetical aspects of mycorrhizae. Paris, France, 217-221

VAN RHIJN, P., FANG, Y., GALILI, S., SHAUL, O., ATZMON, N., WININGER, S., ESHED, Y., LUM, M., LI, Y., TO, V., FUJISHIGE, N., KAPULNIK, Y. and HIRSCH, A.M. (1997) Expression of early nodulin genes in alfalfa mycorrhizae indicates that signal transduction pathways used in forming arbuscular mycorrhizae and rhizobium-induced nodules may be conserved. Proc. Natl. Acad. Sci. USA 94, 5467-5472

VIERHEILIG, H., COUGHLAN, A.P., WYSS, U. and PICHÉ, Y. (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Appl. Environ. Microbiol. 64, 5004-5007

VIEWEG, M.F., FRÜHLING, M., QUANDT, H.-J., HEIM, U., BÄUMLEIN, H., PÜHLER, A., KÜSTER, H. and PERLICK, A.M. (2004) The promoter of the *Vicia faba* L. leghemoglobin gene *VfLb29* is specifically activated in the infected cells of root nodules and in the arbuscule containing cells of mycorrhizal roots from different legume and nonlegume plants. Mol. Plant-Microbe Interact. 17, 62-69

WELLER, D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26, 379-407

WHIPPS, J.M. (1997) Developments in the biological control of soilborne pathogens. Adv. Bot. Res. 26, 1-133

WHIPPS, J.M. (2001) Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52, 487-511

WYSS, P., BOLLER, T.H. and WIEMKEN, A. (1992) Testing the effect of biological control agents on the formation of vesicular arbuscular mycorrhiza. Plant Soil 147, 159-162

WYSS, P., MELLOR, R.B. and WEIMKEN, A. (1990) Vesicular-arbuscular mycorrhizas of wild-type soybean and non-nodulating mutants with *Glomus mosseae* contain symbiosis-specific polypeptides (mycorrhizins), immunologically cross-reactive with nodulins. Planta 182, 22-26

6 General conclusions

Rambelli (1973) created the term mycorrhizosphere, describing the altered microbial composition in the rhizosphere after mycorrhizal establishment on the root. Meyer and Linderman (1986) reported that this habitat is rich in *Actinomycetes*, which showed antagonistic potential against *Phytophthora cinnamomi*. The authors stated that the shift in microbial composition might be one explanation for increased plant health after AMF inoculation and in 1988 Linderman called this the "mycorrhizosphere effect". The mycorrhizosphere is now an accepted concept and cited in every article about the effects of AMF on plant health. In contrast the majority of the studies concerning this specific niche are based on:

i) description of microbial diversity (Vancura *et al.*, 1990; Andrade *et al.*, 1998; Artursson and Jansson, 2003)

ii) effects of biocontrol agents on mycorrhiza formation (Calvet *et al.*, 1993; Barea *et al.*, 1998; Vázquez *et al.*, 2000)

iii) interactions between fungus and bacteria involved in nutrient cycling (Toro *et al.*, 1998; Tsimilli-Michael *et al.*, 2000; Hodge and Campbell, 2001; André *et al.*, 2003)

iv) interactions to improve soil quality (Andrade *et al.*, 1998; Requena *et al.*, 2001; Barea *et al.*, 2002)

Plant health aspects in mycorrhizosphere literature are scarce, even if results are promising. Secilia and Bayaraj (1987) observed more pathogen antagonistic actinomycetes in the rhizosphere of mycorrhizal plants than in that of non-mycorrhizal plants. AMF root colonization of grapevine reduced the population density of fluorescent pseudomonades in the soil that are responsible for grapevine replant disease (Waschkies *et al.*, 1994). Budi *et al.* (Budi *et al.*, 1999) isolated eight bacteria from the mycorrhizosphere of *Sorghum bicolor* and one *Paenibacillus* was able to suppress fungal development of different soilborne pathogens *in vitro* and of *Phytophthora cinnamomi* also *in vivo*. Together with the report of Linderman this is the complete literature about microbial interactions in the mycorrhizosphere with emphasis on plant pathological aspects.

The bacteria from the mycorrhizosphere can be isolated in two ways. The first method is to inoculate plants with mycorrhiza and later bacteria are isolated from rhizosphere soil remaining on the mycorrhizal roots (Meyer and Linderman, 1986; Vancura *et al.*, 1990; Waschkies *et al.*, 1994). With this isolation procedure bacteria coming from the bulk soil, the plant, the irrigation water or the AMF inoculum that successfully established in the mycorrhizosphere can be extracted. The second method is to extract AMF spores from an open field or a greenhouse culture and either isolate the bacteria directly from the spores (Mayo *et al.*, 1986; Avio *et al.*, 2000; Xavier and Germida, 2003) or inoculate plants under “sterile” conditions (Budi *et al.*, 1999). Following this procedure it can be guaranteed that the bacteria isolated are living in association with the fungus and the contamination from other microbial resources can be excluded. The spore-associated bacteria seem to be more interesting for the examination of interrelationships between AMF and bacteria and were therefore chosen for the investigations presented here.

In this study many different species of bacteria were isolated from AMF spores extracted from tomato fields in Thailand. The gram-negative bacteria from the main genus *Neisseria* were dominant. These bacteria have been isolated from AMF spores before (Gazanelli *et al.*, 1999; Xavier and Germida, 2003) but were never found to be dominant. This may be due to the fact that the majority of the former isolations were conducted with AMF spores from the greenhouse and not from an agricultural field soil. Since bacteria from this main genus are known bio-degraders it could be hypothesized that these bacteria are involved in the degradation of organic matter by AMF (Hodge and Campbell, 2001).

The other genera of spore-associated bacteria in the present study are *Bacillus* and *Arthrobacter* that are generally found on AMF spores. Only three isolated strains belong to the genus *Pseudomonas* and were all classified as *P. putida*. These results are in agreement with previous reports (Waschkies *et al.*, 1994; Marschner *et al.*, 1997; Andrade *et al.*, 1997; Vázquez *et al.*, 2000) who observed less pseudomonades in the presence of arbuscular mycorrhizal fungi.

The antagonistic potential of spore-associated bacteria was remarkably high. Nine out of 62 isolates (14.5%) reduced the penetration rate of second stage juveniles of *Meloidogyne incognita* significantly in repeated experiments. Compared with the

rhizosphere, where less than 10% of all bacteria have been shown to reduce nematode attack, this should be considered a high percentage. This is the first report on the antagonistic potential of spore-associated bacteria towards root-knot nematodes. Six isolates (10%) of the spore-associated bacteria *in vitro* affected mycelia growth of the plant pathogens *Fusarium oxysporum, Pythium aphanidermatum, Pythium ultimum* and *Rhizoctonia solani,* and four bacterial isolates were able to inhibit the spread of *Fusarium oxysporum* in the stem of tomato plants, while wilting was not affected. In a similar study with bacteria from the rhizosphere of different plants (Terhardt, 1998), the percentage of antagonistic bacteria was much lower (3%). Interestingly, the antagonistic bacteria in both studies belonged to the same genera (*Bacillus*, *Paenibacillus*, *Pseudomonas*). These findings strongly support the hypothesis that AMF establishment enriches the antagonistic potential of soil microbial communities (Linderman, 1994) and spore-associated bacteria seem to be an interesting source for antagonists against soilborne diseases.

The combination of spore-associated bacteria and *Glomus intraradices* for the biocontrol of *M. incognita* resulted in additive effects in the reduction of damage caused by the nematode. Roots treated with each microorganism alone showed less galling than the control but the reductions were not significant. In contrast disease suppression following dual inoculation of both antagonists was significant. Mycorrhizal establishment was not improved but the persistence of the antagonistic bacteria was slightly increased by AMF establishment, which may have been responsible for increased biocontrol activity and durability. This is the first attempt to combine spore-associated bacteria with AMF in biocontrol. The results obtained here are promising and have been confirmed recently with the same AMF and bacterial isolates under different conditions (Massadeh, 2005).

Since the combination of AMF and bacteria was more successful than single inoculations in nematode control, the combined inoculation should also be tested for biocontrol of other soilborne pathogens. In addition, the bacteria should be combined with different AMF isolates and plant species to determine optimum combinations. The mechanisms of action of the antagonistic bacteria should be studied and, according to their mechanisms, a biocontrol strategy with multiple antagonistic organisms developed. This has been suggested previously in order to overcome inconsistent biocontrol activity (Raupach and Kloepper, 1998; Siddiqui and Shaukat,

2002). Elsen *et al.* (2001) working with *Radopholus similis* found effects of sterile AMF spores in dixenic cultures and therefore concluded that spore-associated bacteria played no role in this interaction. It would be interesting to test the spore-associated bacteria in combination with AMF under the same conditions in order to see if the nematode control can be improved by dual inoculation.

Arbuscular mycorrhizal fungi are part of the agro-ecosystem and respond to a range of cultural practices. Consequently biocontrol agents such as PHPR, that are introduced into the soil have to be tested for their compatibility with these important plant symbionts. *Rhizobium etli*, an antagonist of root-knot and cyst nematodes, was able to improve mycorrhizal establishment in repeated greenhouse studies. Mycorrhiza promotion varied between different tomato cultivars (40-100%) and was highest in a cultivar that was not well colonized by the AMF isolate. The bacterium should therefore be considered a mycorrhiza helper bacterium (Garbaye, 1994). A possible application of these properties could be AMF promotion on poor hosts in order to ensure good nutrition or biocontrol and adaptation of the plant to adverse environmental conditions (Requena *et al.*, 2001).

The *in vitro* studies suggested that one explanation for the increased mycorrhization could be the altered morphology of the AMF after *R. etli* inoculation. Hyphal branching was significantly increased after bacteria application in comparison to the untreated control. Powell (1976) and Mosse (1962) reported that AMF hyphal branching can be interpreted as the preinfection stage according to their results. However, a plant mediated effect cannot be excluded and since AMF and rhizobia have many things in common it would be very interesting to study these interactions in detail. For example it would be interesting to determine if *Rhizobium etli* is able to activate genes that are involved in the formation of the mycorrhizal symbiosis (Frühling *et al.*, 1997; Vieweg *et al.*, 2004). These experiments could provide more information about the possible similarities between the mycorrhizal and rhizobial symbiosis in general or at least about the interactions between the plant, *Rhizobium etli* and AMF in particular.

Dual inoculation of the *R. etli* with AMF led to increased biocontrol activity against the root-knot nematode *M. incognita*. The number of galls and egg masses was reduced by more than 50% in repeated experiments. Single inoculation especially of AMF

gave inconsistent results. One explanation for the increased biocontrol of root-knot nematodes is the improved mycorrhizal establishment. Saleh and Sikora (1984) reported that at least 38% of cotton roots had to be mycorrhizal at the time of nematode attack in order to obtain biocontrol of *M. incognita* through *G. fasciculatum*. In another study conducted with *Glomus coronatum* and tomato the threshold level was 30% (Diedhiou *et al.*, 2003). The establishment of *Glomus intraradices* on four week old transplants was enhanced by the bacterium from 25% in the control up to 39% and therefore *R. etli* increased mycorrhization over the threshold level. Another explanation for the increased nematode control could be the combination of different modes of action. *Rhizobium etli* induces systemic resistance against root-knot nematode and reduces penetration (Hasky-Günther *et al.*, 1998), while AMF competes with the nematode for nutrients and space after penetration (Sikora, 1995).

The separation of AMF and *Rhizobium etli* by a dialysis membrane did not prevent the induction of morphological changes in the mycorrhizal fungus seen when in direct contact. Consequently, the two microorganisms communicate through production of diffusible substances such as hormones or metabolites (Azcón *et al.*, 1978; Azcón-Aguilar and Barea, 1978; Kothari *et al.*, 1991). Direct contact between the bacterium and the mycorrhizal hyphae is not necessary for interactions between these microorganisms to occur. However, the attachment of the bacterium to the mycorrhizal hyphae and movement along the hyphae observed in this study is extremely important for the development of effective biocontrol agents using combinations of AMF spores with bacteria.

In degraded nutrient-poor and arid soils, it has been proposed that plants must be mycorrhizal to thrive the adverse conditions (Barea, 1991) and it was demonstrated that these mycorrhizal effects can be improved by co-inoculation with mycorrhiza-helper bacteria (Requena *et al.*, 1997) or other free-living rhizobacteria (Requena *et al.*, 2001; Vivas *et al.*, 2003). The results obtained in this study offer further evidence for the importance of combining plant health promoting rhizobacteria and AMF in biocontrol of soilborne pathogens. Inconsistent performance of PHPR (Weller and Thomashow, 1994) is the rule rather than the exception and a combined use of biocontrol agents with AMF is regarded as a possible approach for achieving more consistent results (Stirling, 1991; Budi *et al.*, 1999; Bianciotto *et al.*, 2001). In the past different authors reported positive effects of biocontrol agents on mycorrhiza

establishment and *vice versa*, influencing the success of biocontrol (Meyer and Linderman, 1986; Barea *et al.*, 1998).

Based on the literature and the findings presented here a hypothetical IPM strategy to control root-knot nematodes on tomato can be further optimized:

1) Tomato seeds are sown into nematode free substrate inoculated with AMF and a mycorrhiza helper bacterium. In the following four or five weeks the plants then have enough time for successful establishment of the symbiosis which is important for biocontrol.

2) At the time of transplanting plant roots are inoculated with PHPR which inhibit nematode movement or penetration in the early stages of plant development in the field. These PHPR could be spore-associated bacteria that are able to survive longer in the mycorrhizosphere and therefore provide plant protection for a longer period of time

The microorganisms inoculated at transplanting provide the initial protection of the young plant. Numbers of these antagonists will probably decrease over time and therefore protection level will be reduced as well. Mycorrhizal establishment supported by the mycorrhiza helper bacterium will take over long term biocontrol in the field. This is schematically shown in figure 6.1. In addition the application of other biocontrol agents is possible:

3) Fungal endophytes, e.g. non-pathogenic *Fusarium oxysporum* strains (Hallmann and Sikora, 1994), enhance plant growth and reduce damage caused by the nematode. They also could be added to the seedlings in order to successfully establish in the plant before nematode attack. Possible positive interactions with AMF and PHPR might occur in the seedling stage and increase the level of overall plant protection after transplanting.

4) Before transplanting the field soil could be treated with egg parasites such as *Pochonia chlamydosporia* (Rao *et al.*, 1997), *Paecilomyces* lilacinus (Al-Raddad, 1995) or toxin producing *Trichoderma* spp. (Sharon *et al.*, 2001) in order to reduce initial nematode population densities and to establish an antagonistic potential in the field soil over time.

5) The nematode parasite *Pasteuria penetrans* (Stirling, 1991) could be inoculated to the plant root balls prior to transplanting. Nematodes penetrating the root would be parasitized by the bacterium and even if the nematodes enter the root they are not able to multiply. Therefore nematode population densities would be further reduced and *Pasteuria* populations would be established in the soil, leading to high levels of suppressiveness.

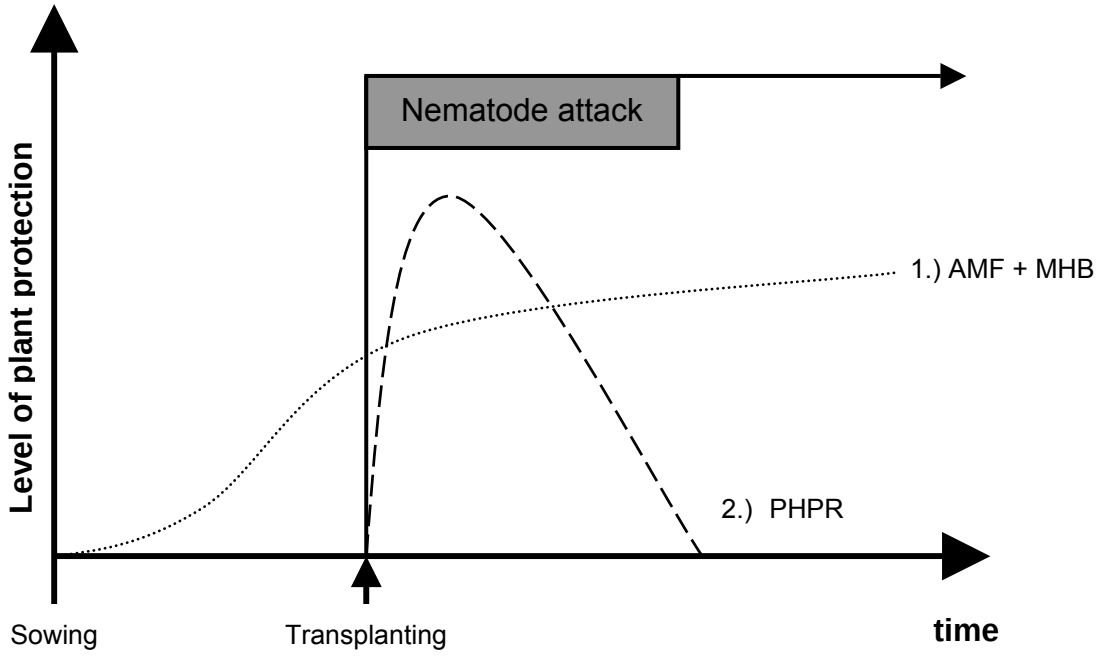

Figure 6.1: Scheme of an IPM strategy based on combined use of arbuscular mycorrhizal fungi (AMF), mycorrhiza helper bacteria (MHB) and plant health promoting rhizobacteria (PHPR) that provide short term nematode control.

All these applications are cost intensive and the farmers have to choose the combination that fit best to their needs. The IPM strategy would be useful mainly for horticultural crops which are first grown under protected conditions and later transplanted to the field. In these systems the producer can probably afford additional costs of one or more pre-treatments, because of the higher profit margin.

6.1 References

AL-RADDAD, A.M. (1995) Interaction of *Glomus mossae* and *Paecilomyces lilacinus* on *Meloidogyne javanica* of tomato. Mycorrhiza 5, 233-236

ANDRADE, G., LINDERMAN, R.G. and BETHLENFALVAY, G.J. (1998) Bacterial associations with the mycorrhizosphere and hyphosphere of the arbuscular mycorrhizal fungus *Glomus mosseae*. Plant Soil 202, 79-87

ANDRADE, G., MIHARA, K.L., LINDERMAN, R.G. and BETHLENFALVAY, G.J. (1997) Bacteria from rhizosphere and hyphosphere soils of different arbuscular mycorrhizal fungi. Plant Soil 192, 71-79

ANDRADE, G., MIHARA, K.L., LINDERMAN, R.G. and BETHLENFALVAY, G.J. (1998) Soil aggregation status and rhizobacteria in the mycorrhizosphere. Plant Soil 202, 89-96

ANDRÉ, S., NEYRA, M. and DUPONNOIS, R. (2003) Arbuscular mycorrhizal symbiosis changes the colonization pattern of *Accacia tortilis* spp. *Raddiana* Rhizosphere by two strains of Rhizobia. Microb. Ecol. 45, 137-144

ARTURSSON, V. and JANSSON, J.K. (2003) Use of Bromodeoxyuridine immunocapture to identify active bacteria associated with arbuscular mycorrhizal hyphae. Appl. Environ. Microbiol. 69, 6208-6215

AVIO, L., SBRANA, C., FILLIPPI, C., BAGNOLI, G. and GIOVANETTI, M. (2000) Antagonistic bacteria tightly associated to spores of three arbuscular mycorrhizal fungi species. In: Elad, Y., Freeman, S. and Monte, E. (eds.): Biocontrol agents: Mode of action and interaction with other means of control. Sevilla, Spain, 279-

AZCÓN-AGUILAR, C. and BAREA, J.M. (1978) Effects of interactions between different culture fractions of 'phosphobacteria' and *Rhizobium* on mycorrhizal infection, growth and nodulation of *Medicago sativa*. Can. J. Microbiol. 24, 520-524

AZCÓN, R., AZCÓN-AGUILAR, C. and BAREA, J.M. (1978) Effects of plant hormones present in bacterial cultures on the formation and responses to VA mycorrhizas. New Phytol. 80, 359-364

BAREA, J.M., ANDRADE, G., BIANCIOTTO, V., DOWLING, D., LOHRKE, S., BONFANTE, P., O'GARA, F. and AZCÓN-AGUILAR, C. (1998) Impact on arbuscular mycorrhizal formation of *Pseudomonas* strains used as inoculants for biocontrol of soilborne fungal plant pathogens. Appl. Environ. Microbiol. 64, 2304-2307

BAREA, J.M. (1991) Vesicular-arbuscular mycorrhizae as modifiers of soil fertility . In: Stewart, B.A. (ed.): Advances in soil science, vol. 15. New York Berlin Heidelberg, 1-39

BAREA, J.M., AZCÓN, R. and AZCÓN-AGUILAR, C. (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie van Leeuwenhoek 81, 343-51

BIANCIOTTO, V., ANDREOTTI, S., BALESTRINI, R., BONFANTE, P. and PEROTTO, S. (2001) Extracellular polysaccharides are involved in the attachment of *Azosporillum brasilense* and *Rhizobium leguminosarum* to arbuscular mycorrhizal structures. Eur. J. Histochem. 45, 39-49

BUDI, S.W., VAN TUINEN, D., MARTINOTTI, G. and GIANINAZZI, S. (1999) Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soil borne fungal pathogens. Appl. Environ. Microbiol. 65, 5148-5150

CALVET, C., PERA, J. and BAREA, J.M. (1993) Growth response of mariegold (*Tagetes erecta*) to inoculation with *Glomus mosseae*, *Trichoderma aureoviride* and *Pythium ultimum* in a peat-perlite mixture. Plant Soil 148, 1-6

DIEDHIOU, P.M., HALLMANN, J., OERKE, E.C. and H.W. DEHNE (2003) Effects of arbuscular mycorrhizal fungi and a non-pathogenic *Fusarium oxysporum* on *Meloidogyne incognita* infestation of tomato. Mycorrhiza 13, 199-204

ELSEN, A., DECLERCK, S. and DE WAELE, D. (2001) Effects of *Glomus intraradices* on the reproduction of the burrowing nematode (*Radopholus similis*) in dixenic culture. Mycorrhiza 11, 49-51

FRÜHLING, M., ROUSSEL, H., GIANINAZZI-PEARSON, V., PÜHLER, A. and PERLICK, A.M. (1997) The *Vicia faba* leghemoglobin gene VfLb29 is induced in root nodules and in roots colonized by the arbuscular mycorrhizal fungus *Glomus fasciculatum*. Mol. Plant-Microbe Interact. 10, 124-131

GARBAYE, J. (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol. 128, 197-210

GAZANELLI, G., MALATESTA, M., PIANETTI, A., BAFFONE, W., STOCCHI, V. and CITTERIO, B. (1999) Bacteria associated to fruit bodies of the ectomycorrhizal fungus *Tuber borchii* Vittad. Symbiosis 26, 211-222

HALLMANN, J. and SIKORA, R.A. (1994) Occurrence of plant pathogenic nematodes and non-pathogenic species of *Fusarium* in tomato plants in Kenya and their role as mutualistic synergists for biological control of root-knot nematodes. Int. J. Pest Manag. 40, 321-325

HASKY-GÜNTHER, K., HOFFMANN-HERGARTEN, S. and SIKORA, R.A. (1998) Resistance against the potato cyst nematode *Globodera pallida* systemically induced by the rhizobacteria *Agrobacterium radiobacter* (G12) and *Bacillus sphaericus* (B43). Fundam. Appl. Nematol. 21, 511-517

HODGE, A. and CAMPBELL, C.D.F.A.H. (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413, 297-299

KOTHARI, S.K., MARSCHNER, H. and RÖMHELD, V. (1991) Effect of vesicular-arbuscular mycorrhizal fungus and rhizosphere micro-organisms on manganese reduction in the rhizosphere and manganese concentrations in maize (*Zea mays* L.). New Phytol. 117, 649-655

LINDERMAN, R.G. (1988) Mycorrhizal interactions with the rhizosphere microflora: The mycorrhizosphere effect. Phytopathology 78, 366-371

LINDERMAN, R.G. (1994) Role of VAM fungi in biocontrol. In: Pfleger, F.L. and Linderman, R.G. (eds.): Mycorrhizae and Plant Health. St. Paul, USA, 1-25

MARSCHNER, P., CROWLEY, D.E. and HIGASHI, R.M. (1997) Root exudation and physiological status of a root-colonizing fluorescent pseudomonad in mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum* L.). Plant Soil 189, 11-20

MASSADEH, B. (2005) Bio-Management of *Meloidogyne incognita* (Tylenchida: Meloidogynidae) on tomato using arbuscular mycorrhizal fungi and rhizobacteria. University of Hannover

MAYO, K., DAVIS, R.E. and MOTTA, J. (1986) Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. Mycologia 78, 426-431

MEYER, J.R. and LINDERMAN, R.G. (1986) Response of subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth-promoting bacterium, *Pseudomonas putida*. Soil Biol. Biochem. 18, 191-196

MEYER, J.R. and LINDERMAN, R.G. (1986) Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. Soil Biol. Biochem. 18, 191-196

MOSSE, B. (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J. Gen. Microbiol. 27, 509-520

POWELL, C.L.L. (1976) Development of mycorrhizal infections from *Endogone* spores and infected root segments. Trans. Br. Myc. Soc. 66, 439-445

RAMBELLI, A. (1973) The rhizosphere of mycorrhizae. In: Marks, G.L. and Koslowski, T.T. (eds.): Ectomycorrhizae. New York, USA, 299-343

RAO, M.S., KERRY, B.R., GOWEN, S.R., BOURNE, J.M. and REDDY, P.P. (1997) Management of *Meloidogyne incognita* in tomato nurseries by integration of *Glomus deserticola* with *Verticilium chlamydosporum*. Z. Pflkrankh. Pflschutz. 104, 419-422

RAUPACH, G.S. and KLOEPPER, J.W. (1998) Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88, 1158-1164

REQUENA, N., JIMINEZ, I., TORO, M. and BAREA, J.M. (1997) Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in mediterranean semi-arid ecosystems. New Phytol. 136, 667-677

REQUENA, N., PEREZ-SOLIS, E., AZCÓN-AGUILAR, C., JEFFRIES, P. and BAREA, J.M. (2001) Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Appl. Environ. Microbiol. 67, 495-498

SALEH, H.M. and SIKORA, R.A. (1984) Relationship between *Glomus fasciculatum* root colonization of cotton and its effect on *Meloidogyne incognita*. Nematologica 30, 230-237

SECILIA, J. and BAGYARAJ, D.J. (1987) Bacteria and actinomycetes associated with pot cultures of vesicular-arbuscular mycorrhizas. Can. J. Microbiol. 33, 1069-1073

SHARON, E., BAR-EYAL, M., CHET, I., HERRERA-ESTRELLA, A., KLEIFELD, O. and SPIEGEL, Y. (2001) Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology 91, 687-693

SIDDIQUI, I.A. and SHAUKAT, S.S. (2002) Mixtures of plant disease suppressive bacteria enhance biological control of multiple tomato pathogens. Biol. Fertil. Soils. 36, 260-268

SIKORA, R.A. (1995) Vesicular-arbuscular mycorrhizae: their significance for biological control of plant parasitic nematodes. Biocontrol 1, 29-33

STIRLING, G.R. (1991) Biological control of Plant Parasitic Nematodes. CAB International, Wallingford, Great Britain

TERHARDT, J. (1998) Beeinflussung mikrobieller Gemeinschaften der Rhizosphäre nach Blattbehandlung von Pflanzen und biologische Kontrolle von *Fusarium oxysporum* f.sp. *lycopersici* und *Meloidogyne incognita* mit bakteriellen Antagonisten. University of Bonn, Germany

TORO, M., AZCÓN, R. and BAREA, J.M. (1998) The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotype, mycorrhizal fungi, phosphate solubilizing rhizobacteria and rock phosphate on nitrogen and phosphorus acquisition by *Medicago sativa*. New Phytol. 138, 265-273

TSIMILLI-MICHAEL, M., EGGENBERG, P., BIRO, B., KOVES-PECHY, K., VOROS, I. and STRASSER, R.J. (2000) Additive and antagonistic effects of arbuscular mycorrhizal fungi and *Azospirillum* and *Rhizobium* nitrogen-fixers on the photosynthetic activity of alfalfa, probed by the polyphasic chlorophyll a fluorescence transient O-J-I-P. Appl. Soil Ecol. 15, 169-182

VANCURA, V., OROZCO, M.O., GRAUOYÁ, O. and PRIKRYL, Z. (1990) Properties of bacteria in the hyphosphere of a vesicular-arbuscular mycorrhizal fungus. Agric. Ecosys. Environ. 29, 421-427

VÁZQUEZ, M.M., CÉSAR, S., AZCÓN, R. and BAREA, J.M. (2000) Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azosporillum*, *Pseudomonas*, *Trichoderma*) and their effects on mycorrhizal population and enzyme activities in the rhizosphere of maize plants. Appl. Soil Ecol. 15, 261-272

VIEWEG, M.F., FRÜHLING, M., QUANDT, H.-J., HEIM, U., BÄUMLEIN, H., PÜHLER, A., KÜSTER, H. and PERLICK, A.M. (2004) The promoter of the *Vicia faba* L. leghemoglobin gene *VfLb29* is specifically activated in the infected cells of root nodules and in the arbuscule containing cells of mycorrhizal roots from different legume and nonlegume plants. Mol. Plant-Microbe Interact. 17, 62-69

VIVAS, A., MARULANDA, A., RUIZ-LOZANO, J.M., BAREA, J.M. and AZCÓN, R. (2003) Influence of a *Bacillus* sp. on physological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress. Mycorrhiza 13, 249-256

WASCHKIES, C., SCHROPP, A. and MARSCHNER, H. (1994) Relations between grapevine replant disease and root colonization of grapevine (*Vitis* sp.) by fluorescent pseudomonades and endomycorrhizal fungi. Plant Soil 162, 219-227

WELLER, D.M. and THOMASHOW, L.S. (1994) Current challenges in introducing beneficial microorganisms into the rhizosphere. In: O'Gara, F., Dowling, D.N. and Boesten, B. (eds.): Molecular Ecology of Rhizosphere Microorganism Biotechnology and the Release of GMOs. Weinheim, Germany, 1-18

XAVIER, L.C.J. and GERMIDA, J.L. (2003) Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. Soil Biol. Biochem. 35, 471-478

7 Annex

Table 7.1: Spore-associated bacteria identified or not by FAME-GC (SR1 to SR 31)

soil sample	name	genus	species	Similarty index
1	**SR 1**	*Cellulomonas*	*turbata*	0,5
1	**SR 2**	*Acinetobacter*	*baumannii*	0,7
1	**SR 3**	*Acinetobacter*	*baumannii*	0,6
1	**SR 4**	*Acinetobacter*	*baumannii*	0,8
1	**SR 5**	*Acinetobacter*	*baumannii*	0,4
1	**SR 6**	*Acinetobacter*	*baumannii*	0,8
1	**SR 7**	*Acinetobacter*	*baumannii*	0,9
1	**SR 8**	*Bacillus*	*pumilus*	0,9
1	**SR 9**	*Acinetobacter*	*baumannii*	0,7
2	**SR 10**	*Bacillus*	*sphaericus*	0,2
2	**SR 11**	*Bacillus*	*cereus*	0,4
2	**SR 12**	*Bacillus*	*cereus*	0,6
2	**SR 13**	*Arthrobacter*	*viscosus*	< 0,2
2	**SR 14**	*Bacillus*	*sphaericus*	< 0,2
2	**SR 15**	*Acinetobacter*	*johnsonnii*	0,9
2	**SR 16**	*Bacillus*	*cereus*	0,6
3	**SR 17**	*no match*		0
3	**SR 18**	*Moraxella*	*catarrhalis*	0,2
3	**SR 19**	*Brevundimonas*	*vesicularis*	0,6
3	**SR 20**	*Enterococcus*	*gallinarum*	1,0
3	**SR 21**	*Arthrobacter*	*ilicis*	0,7
3	**SR 22**	*Burkholderia*	*cepacia*	0,5
3	**SR 23**	*Pseudomonas*	*putida*	0,9
3	**SR 24**	*Arthrobacter*	*viscosus*	0,6
3	**SR 25**	*Micrococcus*	*luteus*	0,6
3	**SR 26**	*Paenibacillus*	*pabuli*	0,6
3	**SR 27**	*Flavobacterium*	*resinovorum*	< 0,2
3	**SR 28**	*Rhodococcus*	*equi*	0,2
3	**SR 29**	*Moraxella*	*osloensis*	0,4
4	**SR 30**	*Bacillus*	*pumilus*	0,8
4	**SR 31**	*Bacillus*	*pumilus*	0,9

Table 7.2: Spore-associated bacteria identified or not by FAME-GC (SR32 to SR 62)

soil sample	name	genus	species	Similarity index
4	**SR 32**	*Moraxella*	*osloensis*	0,5
4	**SR 33**	*Enterococcus*	*faecium*	< 0,2
4	**SR 34**	*Moraxella*	*catarrhalis*	0,4
4	**SR 35**	*Arthrobacter*	*viscosus*	0,5
4	**SR 36**	*Rathayibacter*	*tritici*	0,4
4	**SR 37**	*Moraxella*	*osloensis*	0,8
4	**SR 38**	*Acinetobacter*	*baumannii*	0,7
4	**SR 39**	*Moraxella*	*catarrhalis*	0,4
5	**SR 40**	*Moraxella*	*catarrhalis*	< 0,2
5	**SR 41**	*Acinetobacter*	*haemolyticus*	0,7
5	**SR 42**	*Paenibacillus*	*macerans*	0,2
5	**SR 43**	*Sphingobacterium*	*heparinum*	0,4
5	**SR 44**	*Arthrobacter*	*ilicis*	0,8
5	**SR 45**	*Bacillus*	*pumilus*	0,2
5	**SR 46**	*Acinetobacter*	*haemolyticus*	0,6
5	**SR 47**	*Streptoverticillium*	*reticulum*	< 0,2
5	**SR 48**	*Micrococcus*	*halobius*	0,2
5	**SR 49**	*Moraxella*	*osloensis*	0,4
5	**SR 50**	*Moraxella*	*osloensis*	0,6
5	**SR 51**	*Moraxella*	*catarrhalis*	0,4
5	**SR 52**	*Pseudomonas*	*putida*	0,6
5	**SR 53**	*Moraxella*	*catarrhalis*	0,4
5	**SR 54**	*Moraxella*	*osloensis*	0,4
5	**SR 55**	*Acinetobacter*	*haemolyticus*	0,7
5	**SR 56**	*Moraxella*	*catarrhalis*	0,4
5	**SR 57**	*Pseudomonas*	*putida*	0,3
6	**SR 58**	*Enterobacter*	*cloacae*	< 0,2
6	**SR 59**	*Arthrobacter*	*ilicis*	0,8
6	**SR 60**	*Paenibacillus*	*macerans*	0,8
6	**SR 61**	*Corynebacterium*	*aquaticum*	0,4
6	**SR 62**	*Bacillus*	*circulans*	< 0,2

Table 7.3: Results of screening spore-associated bacteria against *Meloidogyne incognita* on tomato (set 1 to 5)

n = 6	Treatment	Root fresh weight (g)	Shoot dry weight (g)	Nematodes per root system	Nematodes per g root fresh weight	Significant reduction
SET 1	**Control**	0,96	0,43	115	129,23	
	SR 1	1,22	0,51	55	46,73	X
	SR 2	1,30	0,46	62	51,40	
	SR 3	1,18	0,56	68	59,76	
	SR 4	1,24	0,55	78	64,26	
	SR 5	1,20	0,66	83	70,47	
	SR 6	1,28	0,71	45	40,98	X
	SR 7	1,28	0,60	98	76,57	
	SR 8	1,12	0,59	116	111,96	
	SR 9	1,12	0,50	207	215,19	
SET 2	**Control**	1,57	0,33	124	79,91	
	SR 30	1,80	0,42	79	46,07	X
	SR 31	1,60	0,41	68	42,51	X
	SR 11	1,51	0,38	64	46,48	X
	SR 12	1,73	0,42	102	67,40	
	SR 15	1,59	0,37	105	68,40	
	SR 16	1,73	0,39	101	58,44	
SET 3	**Control**	1,37	0,30	134	99,91	
	SR 10	1,51	0,23	127	84,98	
	SR 13	1,81	0,36	106	58,58	X
	SR 14	1,74	0,35	128	73,64	
	SR 32	1,61	0,32	124	79,68	
	SR 33	1,80	0,43	94	53,49	X
	SR 35	1,72	0,35	89	52,11	X
	SR 36	1,72	0,32	129	78,54	
	SR 38	1,88	0,38	119	63,38	X
SET 4	**Control**	1,35	0,33	147	113,58	
	SR 34	1,52	0,37	157	105,07	
	SR 17	1,30	0,34	156	129,84	
	SR 18	1,31	0,34	168	128,94	
	SR 19	1,48	0,35	133	90,10	
	SR 20	1,47	0,37	87	60,17	X
	SR 21	1,26	0,32	198	158,99	
	SR 22	1,56	0,41	133	86,46	X
SET 5	**Control**	1,00		102	104,72	
	SR 23	1,39		89	72,10	X
	SR 24	1,18		120	110,95	
	SR 25	1,69		134	80,93	X
	SR 26	1,74		127	75,30	X
	SR 27	1,63		108	66,44	X
	SR 28	1,32		127	97,71	
	SR 37	1,55		83	55,35	X

Table 7.4: Results of screening spore-associated bacteria against *Meloidogyne incognita* on tomato (set 6 to 8)

n = 6	Treatment	Root fresh weight (g)	Shoot dry weight (g)	Nematodes per root system	Nematodes per g root fresh weight	Significant reduction
SET 6	**Control**	2,41	0,86	134	57,15	
	SR 29	2,67	0,87	116	44,18	X
	SR 58	2,59	0,88	120	47,05	X
	SR 59	2,65	0,90	134	50,69	
	SR 60	2,56	0,86	135	52,93	
SET 7	**control**	1,93	0,65	200	104,28	
	SR 61	2,22	0,74	200	90,38	
	SR 62	2,04	0,70	223	109,59	
	SR 40	1,94	0,68	217	113,28	
	SR 41	2,03	0,66	218	108,78	
	SR 42	1,95	0,63	216	111,99	
	SR 43	2,15	0,73	199	94,77	
	SR 44	1,84	0,62	216	118,68	
	SR 45	2,09	0,66	173	71,20	X
	SR 46	2,17	0,69	170	79,92	X
	SR 48	2,12	0,69	209	100,89	
	SR 49	2,03	0,68	231	117,15	
	SR 50	1,87	0,63	215	116,29	
	SR 51	1,88	0,61	194	104,24	
	SR 52	1,70	0,53	225	132,81	
	SR 53	1,98	0,68	215	108,56	
SET 8	**control**	1,66	0,32	230	142,35	
	SR 47	1,70	0,41	192	117,55	X
	SR 54	1,69	0,38	216	134,26	
	SR 55	1,64	0,40	255	156,85	
	SR 56	1,77	0,40	161	93,59	X
	SR 57	1,74	0,37	202	117,37	X

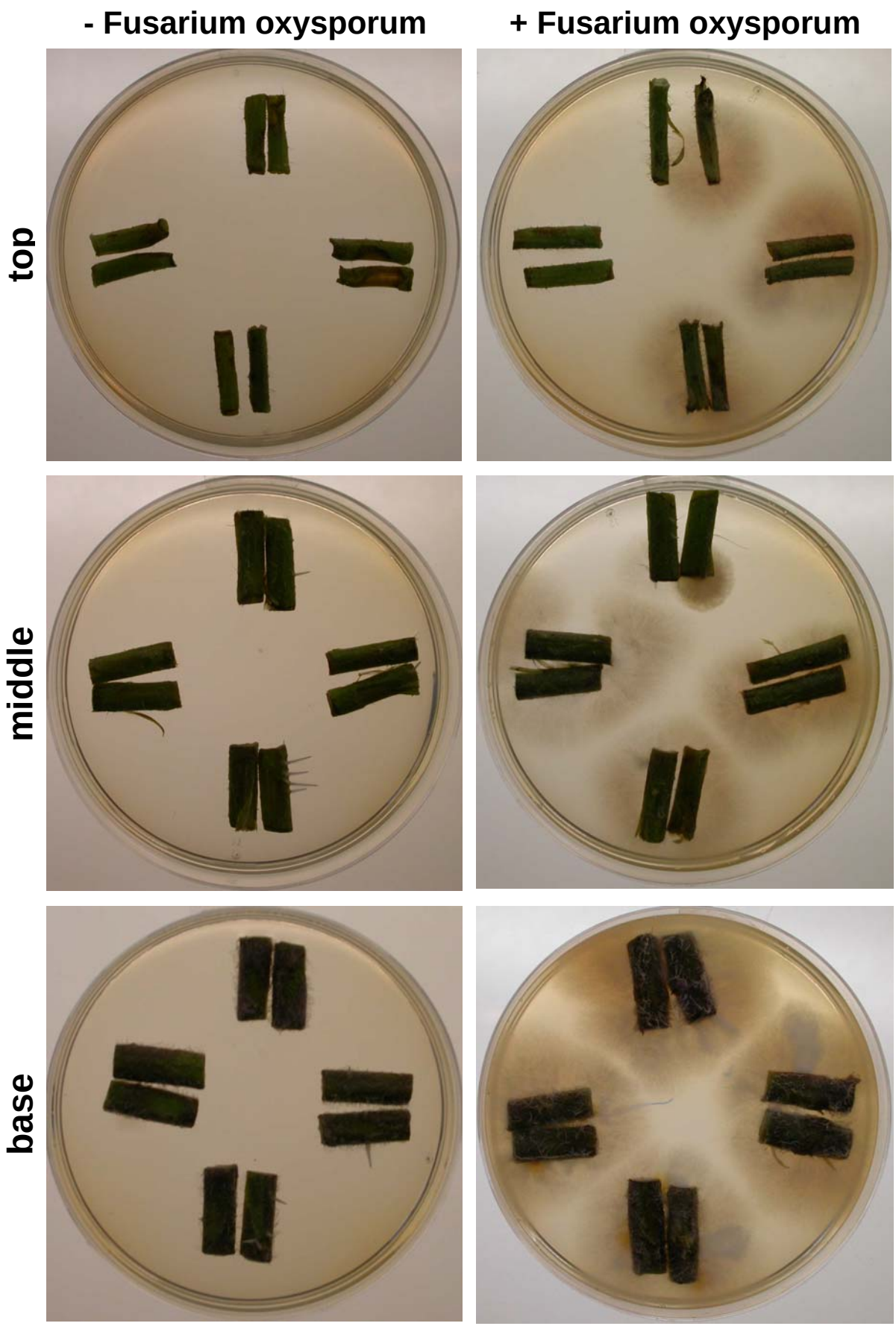

Figure 7.1: Reisolation of *Fusarium oxysporum* from the three tomato shoot segments. Segments of four shoots are placed on one plate.

ACKNOWLEDGEMENTS

First of all I would like to express my thanks to my supervisor Prof. Dr. Richard A. Sikora who gave me this exciting topic. His interest in my work, his helpful criticism and ever present support were an important prerequisite for the successful completion of this thesis.

I would like to thank Dr. Rüdiger Hauschild for his helpful advice in all laboratory questions related to my work. At the same time, I want to thank him and Dr. Jon Padgham for critical review of the manuscript.

I want to thank all the colleagues in the “BÖS” team and the Institute for Plant Diseases at the University of Bonn for fruitful discussions and all the encouraging meetings that we had in these three years. Special thanks to Angelika Nilgen, for her never ending willingness to help in all organisational problems.

I would like to thank Prof Poonpilai Suwanarit, Salisa Suwannapak and Yeerapan Upanasikorn for their hospitality and help during my stay at Kasetsart University in Bangkok. This was a great experience.

Thanks to Dr. Henning von Alten and Dr. Ban Massadeh from the University of Hannover for providing me with AMF inoculum and teaching me how to work with this fungus. I want to thank Dr. Ulrich Hildebrandt for his willingness to conduct some of the experiments at the University of Cologne and his suggestions for the experimental design.

I would like to thank my family and friends who always believed in me, and made sure that I never felt alone.

I am grateful for the financial support that I received from Deutsche Forschungsgemeinschaft (DFG) and Landesgraduiertenkolleg NRW.