The Plant – Arbuscular Mycorrhizal Fungi – Bacteria – Pathogen System

Multifunctional Role of AMF Spore-Associated Bacteria

Dharam Parkash Bharadwaj
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Pathology
Uppsala

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Abstract

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The aim of this study was to determine the role of the bacteria associated with arbuscular mycorrhizal (AM) fungi in the interactions between AM fungi, plant hosts and pathogens. Mycorrhizal traits were studied in a potato host using field rhizosphere soils of 12 different plant species as inoculum. High colonisation was found with soil of *Festuca ovina* and *Leucanthemum vulgare*, which contained two dominant AMF species (*Glomus mosseae* and *G. intraradices*). Bacteria associated with spores of AM fungi (AMB) were isolated from these two AM fungal species with either of the two plant species as hosts. Identification based on fatty acid methyl ester profile analysis revealed high diversity and specific occurrence of certain taxa with either of the two AMF. Some AMB were strongly antagonistic against *R. solani* in *in vitro* studies and most of them were spore type-dependent and originated from *G. intraradices* spores. Occurrence of AMB taxa was also plant host-dependent but antagonism was not. The specificity of AMB to AMF could be due to production of specific exudates, since the results showed that exudates collected from either *G. intraradices* or AMB stimulated growth of the other organism in a two-compartment plate system, and that concentrations of certain compounds changed several-fold. Certain AMB (*Pseudomonas* sp. and *Stenotrophomonas* sp.) showed strong multifunctional effects, *i.e*. stimulated AMF colonisation of potato roots in outdoor and greenhouse studies, stimulated potato growth *in vitro* and demonstrated antagonism against several fungal and bacterial plant pathogens. Three AMB from these two genera grown in the presence of exudates of *G. intraradices* resulted in enhanced antagonistic effects. Production of extracellular enzymes and bioactive compounds varied among the AMB species, suggesting different mechanisms for their multifunctional effects. By using *F. ovina* inoculated with *G. intraradices* as a cover crop, it should be possible to enhance the occurrence of strongly pathogen-antagonistic AMB. The association of multifunctional AMB with AMF spores provides evidence that bacteria are involved in the beneficial effects of AM fungi on plants.

Keywords: antagonism, arbuscular mycorrhizal fungi, diversity, *Festuca ovina*, *Glomus, Leucanthemum vulgare*, pathogens, potato, specificity, spore-associated bacteria

Author's address: Dharam Parkash Bharadwaj, Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, P.O. Box 7026, SE-750 07, Uppsala, Sweden. Email: dharam.bharadwaj@mykopat.slu.se

Dedicated to my Mother and Grandfather

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Appendix

Papers I – IV

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Bharadwaj, D.P., Lundquist, P-O. & Alström, S. 2007. Impact of plant species grown as monocultures on sporulation and root colonization by native arbuscular mycorrhizal fungi in potato. *Applied Soil Ecology* 35, 213-225.

II. Bharadwaj, D.P., Lundquist, P-O., Persson, P. & Alström, S. Cultivable bacteria associated with arbuscular mycorrhizal fungal spores: evidence for specificity. (Submitted manuscript)

III. Bharadwaj, D.P., Lundquist, P-O. & Alström, S. Arbuscular mycorrhizal fungal spore-associated bacteria affect mycorrhizal colonization, plant growth and potato pathogens. (Submitted manuscript)

IV. Bharadwaj, D.P., Lundquist, P-O. & Alström, S. Interactions between *Glomus intraradices*, arbuscular mycorrhizal spore-associated bacteria and potato pathogens under *in vitro* conditions. (Manuscript)

Introduction

The rhizosphere is a heterogeneous, continuous and natural habitat in which different types of interactions occur between soil microbes and plants. The beneficial plant-microbe interactions in the rhizosphere are the primary determinants of plant health and soil fertility (Jeffries *et al*., 2003). Arbuscular mycorrhizal (AM) fungi are one of the most important microbial symbionts for the majority of plants. Under phosphate-limited conditions, AM fungi (AMF) can influence plant community development, nutrient uptake, water relations and aboveground productivity. They can also act as bioprotectants against pathogens and toxic stresses (Jeffries *et al*., 2003).

In the rhizosphere AMF also interact with different kinds of bacteria. These interactions can be found at all stages of the AMF life cycle, from spore formation and germination through root colonisation to external hyphae (Bianciotto & Bonfante, 2002; Bianciotto *et al*., 1996, 2003; Roesti *et al*., 2005; Toljander *et al*., 2006). The nature of these interactions may be inhibitory or stimulatory, competitive or mutualistic to each other or for the plant.

Different functional groups of bacteria such as N_2-fixing bacteria (Secilia & Bagyaraj, 1987), plant growth-promoting rhizobacteria (von Alten, Lindermann & Schonbeck, 1993), phosphate-solubilising bacteria (Toro *et al*., 1996) and antagonists of plant pathogens (Citernesi *et al*., 1996; Budi *et al*., 1999) have been reported to be associated with the rhizosphere of different plants colonised by AMF. Some bacteria have also been found to be associated with AM fungal structures such as external hyphae (Toljander *et al*., 2006) and spore or spore walls (Mayo, Davis & Motta, 1986; Xavier & Germida, 2003; Roesti *et al*., 2005). Bacteria have also been reported to live inside the spores of certain AM fungal isolates (Bianciotto *et al*., 1996; 2003).

The ecological importance of bacteria associated with AMF with regard to their interaction with AMF hosts and/or plants is still far from known. There are some reports on the bacteria associated with AMF spores (here denoted AMB) indicating that AMB have the ability to influence spore germination and hyphal growth (Mosse, 1962; Walley & Germida, 1996; Xavier & Germida, 2003). The AMB can degrade biopolymers such as protein, chitin and cellulose (Filippi *et al*., 1998; Roesti *et al*., 2005), inhibit the growth of different plant pathogens (Budi *et al*., 1999) and improve the soil structure (Andrade, Azcon & Bethlenfalvay, 1995). Recently Hildebrandt, Janetta & Bothe (2002) found that AMB have the potential to stimulate the growth of AMF up to the formation of fertile spores in the absence of a host. These reports indicate that AMB might be one important factor involved in AMF development, plant growth and plant protection.

In the rhizosphere, plant species select their bacterial associates (Smith & Goodman, 1999) and strongly influence the composition of the AMF community (Sanders & Fitter, 1992; Bever *et al*., 1996; Eom *et al*., 2000), while AMF species affect the selection of bacteria in the mycorrhizosphere (Roesti *et al*., 2005). In order to understand the various interactions between AMB and AMF, it is

important to know the types of bacteria associated with AMF structures such as spores, whether different types of spores select different bacteria and whether their composition and functional traits are affected by the fungal and plant host. These are some of the questions that still remain to be answered.

Arbuscular mycorrhizal fungi

The term mycorrhiza (*mykes* = fungus, *rhiza* = root) was first coined by Frank (1885) to describe the symbiosis between a soil fungus and plant roots. Based on the type of fungus involved and the resulting structures produced by the root–fungus combination, various mycorrhizal associations have been identified; *e.g.* AMF, ectomycorrhiza, ectendomycorrhiza, ericoid, arbutoid, orchid and monotropoid (Smith & Read, 1997). The AMF are the most common mycorrhiza and it has been estimated that they colonise about 80% of plant families from all terrestrial plants (Schüßler, Schwarzott & Walker, 2001). The AMF have undergone changes to their name in recent years, from endomycorrhiza to vesicular-arbuscular mycorrhiza (VAM) to AM. The name shifted from endomycorrhiza to VAM because VAM do not resemble other types of endomycorrhiza that penetrate the root cells, such as *Rhizoctonia* (mycorrhizal with orchids) and ascomycetes (ectendomycorrhiza). The name also shifted from VAM to AM because not all VAM form vesicles, *e.g.* members of the Gigasporaceae (Morton & Benny, 1990). Hence the term AMF is preferred because of the formation of highly branched intracellular fungal structures or 'arbuscules' by almost all members.

The AMF are recognised on the basis of their specific traits such as obligate biotrophy, asexual reproduction, large and multinucleate spores with layered walls, non-septate hyphae and arbuscule formation in plant roots. Though strictly obligate, *e.g.* AM fungi need living plant roots to survive, some reports show that AM species can grow up to the spore production phase *in vitro* in the absence of plant roots and in the presence of some selected strains of spore-associated bacteria (Hildebrandt, Janetta & Bothe, 2002; Hildebrandt *et al*., 2006).

The AMF reproduce asexually by spore production. There is no evidence that AMF reproduce sexually (Kuhn, Hijri & Sanders, 2001). One study reports the formation of sexual zygospores by *Gigaspora* (Tommerup & Sivasithamparam, 1990) but this has not been confirmed. Only a low level or no genetic recombination has been detected using molecular marker genes (Kuhn, Hijri & Sanders, 2001). Therefore, it is generally assumed that the AMF spores are formed asexually. The spores are relatively large (40-800 μm) with layered walls and lipids in their cytoplasm. Spores are important for identification of AMF. Traditionally AM fungal taxonomy has been based on spore morphology, particularly spore wall layer structure and the method of spore formation on the hypha (Morton, 1988).

Classification

In earlier classifications, the AMF were placed in the order Glomales within the division *Zygomycota*. They have non-septate hyphae, a similar characteristic to that found in hyphae of most *Zygomycota*. However, AMF are distinguished from

the Zygomycotan lineages due to some specific characteristics, *e.g.* mutualistic symbiotic nutritional habit and lack of formation of characteristic zygospores. The rDNA analysis exposed a clear separation of AMF from other fungal groups and the AMF are now placed in a separate new phylum, *Glomeromycota* (Schüßler, Schwarzott & Walker, 2001). The phylum *Glomeromycota* is divided into four orders, eight families and ten genera (Walker & Schüßler, 2004). Recently, two new AMF genera, *Kuklospora* and *Intraspora*, have been described in the phylum *Glomeromycota* (Sieverding & Oehl, 2006). Recent classification is shown in Fig. 1.

Phylum Glomeromycota

Class Glomeromycetes

Orders	**Families**	**Genera**
1. Glomerales	Glomeraceae	*Glomus*
2. Diversisporales	Gigasporaceae	*Gigaspora*, *Scutellospora*
	Acaulosporaceae	*Acaulospora*, *Kuklospora*
	Entrophosporaceae	*Entrophospora*
	Pacisporaceae	*Pacispora*
	Diversisporaceae	*Diversispora*
3. Paraglomerales	Paraglomeraceae	*Paraglomus*
4. Archaeosporales	Geosiphonaceae	*Geosiphon*
	Archaeosporaceae	*Archaeospora*, *Intraspora*

Fig. 1. Recent classification of arbuscular mycorrhizal fungi (Sieverding & Oehl, 2006).

The diversity of AMF depends on the type of ecosystem, agricultural practices and soil conditions. Helgason *et al*. (1998) found higher AMF diversity in a woodland ecosystem in comparison to an arable land ecosystem. Soil disturbance can reduce the density of spores, length of mycelium and species richness of AMF and no-tillage conditions stimulate the mycorrhizal activity (Dodd, 2000; Boddington & Dodd, 2000).

Classical spore morphology and more recently PCR-based molecular approaches are generally used for identification of AMF communities, but there are problems with both these approaches. In the case of spore morphology, it is not always easy to identify all spores when sieved directly from field soil. There is variations in spore development and sometimes AMF colonising the plant roots are not found as spores (Clapp *et al*., 1995; Clapp, Rodriguez & Dodd, 2002). The main problem with molecular approaches is that most are based on rDNA sequences, whereas AMF species have polymorphic rDNA sequences (Sanders, 2002; Redecker, Hijri & Wiemken, 2003). Thus, it is normal to recover multiple sequences by PCR amplification from a single spore because a single spore can contain a thousand or

more nuclei (Antoniolli *et al*., 2000; Pawlowska & Taylor, 2004). At present, there are no individual rDNA primers that permit identification of all major Glomalean lineages and most molecular approaches used to date are not able to detect all rDNA sequences present (Redecker, 2000; Vandenkoornhuyse *et al*., 2002; Redecker, Hijri & Wiemken, 2003; Schüßler, Schwarzott & Walker, 2003). Thus the characterisation of AMF communities based on either spore morphology or molecular identification alone is insufficient to cover the whole spectrum within a community (Landis, Gargas & Givnish, 2004). Hence, in order to assess the total community present at a specific site, use of both methods is recommended because they complement each other (van der Heijden & Scheublin, 2007).

Life cycle

The life cycle of AMF generally starts from the spores present in soil or from adjacent mycorrhizal plant roots. The emerging hyphae (H) from spores or mycorrhizal roots grow towards the plant root. At the root surface, the tip of the hypha swells and forms a specific structure called the appressorium (Ap) (Mandelbaum & Piche, 2000). From these appressoria, infective pegs (Ip) emerge. Hyphae penetrate the adjacent epidermal root cell walls with the help of penetration pegs. The particular point at which hyphae from any propagule first enter the root is called the primary entry point (E). The number of primary entry points formed on a root surface by a fungus is equivalent to its inoculum potential (Garrett, 1956; Bouhot, 1979).

Inside the root, hyphae grow intercellularly to the inner cortical layers and in the inner cortex region hyphae start to grow inside the cells. After that the host cell membrane invaginates and envelopes the fungus and forms a new compartment called the apoplastic space. This space allows the efficient transfer of nutrients between the two symbionts but prevents direct contact between plant and fungal cytoplasm (Sylvia, 2002). The hyphae form different structures such as hyphal coils, arbuscules (Ar) and vesicles (V) inside the cortical cells but outside the cytoplasm. Arbuscules are highly dichotomously branched intracellular structures and could be the site of exchange of phosphorus, carbon, water and other nutrients (Smith & Read, 1997; Wright, 2005). Vesicles are lipid-filled and thought to be carbon storage structures but they can also serve as reproductive propagules (Sylvia, 2002). Not all the AMF form vesicles *e.g*. Gigasporaceae (Morton & Benny, 1990). Formation of the vesicles depends on the fungal symbiont as well as on the environmental conditions (Smith & Read, 1997). Once the infection process has begun, colonisation starts both within a root by intraradical mycelium (InM) and along the root by extraradical mycelium (ExM).

The intraradical mycelium colonises the root in different patterns. Based on its structure, the mycorrhiza is separated into *Arum*, *Paris* and *Intermediate* type (Gallaud, 1905). In the *Arum* type, intercellular hyphae grow in a longitudinal manner along the root and penetrate the cortical cells to form arbuscules. Arbuscules arise from these intercellular hyphae on short side branches, typically at right angles to the main root axis (Smith & Smith, 1997). The *Arum* type morphology is abundant in crop plants (Smith & Smith, 1997; Ahulu, Nakata & Nonaka, 2005). In the *Paris* type, intercellular hyphae are absent and the hyphae

are entirely intracellular and irregularly coiled, some of them forming arbuscules that are not terminal but are localised in definite layers. The arbuscules are formed as intercalary structures and called arbusculate coils (Gallaud, 1905; Yawney & Schultz, 1990; Cavagnaro *et al*., 2001). The *Paris* type morphology is more often seen in plants in natural ecosystems (Brundrett & Kendrick, 1988; Ahulu, Nakata & Nonaka, 2005). Sometimes, both types of structures are formed in the same root system *e.g.* cucumber and tomato (Kubota, McGonigle & Hyakumachi, 2005) and this has been termed the *Intermediate* type (Smith & Smith, 1997).

The extraradical mycelium associated with the root radiates out into the soil. These hyphae are two distinct types, runner and absorbing (Friese & Allen, 1991). The runner hyphae are thicker and grow in the soil to find host roots. The hyphae that penetrate the roots are initiated from the runner hyphae. The absorbing hyphae develop from the running hyphae and form a network of thinner hyphae extending into the soil and absorb the nutrients to transport to the host.

In certain AM fungi, *e.g. Gigaspora* and *Scutellospora* species, typical clustered swellings are formed on extraradical hyphae called auxiliary cells and the function of these structures has yet to be identified. Finally reproductive structures, spores (S) can be formed as hyphal swellings either in the roots or, more commonly, in the soil. Spores may be formed singly or in clusters. Spores function as storage structures, resting stage and propagules. Generally spores are formed when nutrients are remobilised from roots where the AM associations are senescing (Brundrett *et al*., 1996). Several factors such as host dependence, age of host, sporulation ability of AM fungal species, presence of other AM fungal species or composition of indigenous soil micro-flora, spore dormancy and the distribution patterns of AM fungal spores in soils, seasonal influence and other biotic factors can affect AM fungal sporulation in different plant rhizospheres (Walker, Mize & McNabb, 1982; Koske, 1987).

AMF interaction with plants

About 80% of plant families from all phyla of land plants are estimated to be hosts of AMF and AMF usually colonise the host roots by forming intercellular and intracellular hyphae and intracellular arbuscules. The remaining plant species are either non-mycorrhizal or non hosts of AMF. Plant species belonging to the Cruciferae and Chenopodiaceae are not known to form AMF symbiosis (Smith & Read, 1997). Giovannetti & Sbrana (1998) suggested that this is due to the lack of any recognition event leading to the establishment of a functional symbiosis. Glenn, Chew & Williams (1985) reported *Brassica* roots to be colonised by *Glomus mosseae* but only when root cells were dead, *i.e.* when no plasma membrane was present. The AM-crucifer association appears mostly non-functional with regard to nutrient exchange between plant and fungus (Ocampo, Martin & Hayman, 1980).

Association with AMF has generally been assumed to have no, or at least very low, host specificity because many species have been shown to colonise a wide range of hosts and the same plant root can be colonised by a mixture of AMF species (Helgason, Fitter & Young, 1999; Klironomos, 2000). However, van der

Heijden *et al*. (1998) indicated that plants might select the AM fungus and Vandenkoornhuyse *et al*. (2002) demonstrated that distinct AMF communities are associated with different plant hosts. The degree of host specificity could be under the genetic control of the host, the AM fungus, or more likely a complex interaction of both symbionts with the soil environment (Chanway, Turkington & Holl, 1991; Sylvia *et al*., 2003).

AMF interaction with bacteria

The AMF interact with different types of soil bacteria that can influence their development and symbiotic establishment. The interactions between AMF and bacteria can be positive (Bagyaraj & Menge, 1978; Meyer & Linderman, 1986a, b; Gryndler, Hrselova, & Chvatalova, 1996), negative (McAllister *et al*., 1995; Gryndler, Hrselova, & Chvatalova, 1996) or neutral (Edwards, Young & Fitter, 1998). Negative interactions include reduced spore germination and hyphal length in the extramatrical stage, decreased root colonisation and a decline in the metabolic activity of the internal mycelium. Positive interactions include enhanced mycorrhizal development and function. Synergistic positive interactions have been reported between AMF and plant growth promoting bacteria (PGPB) such as nitrogen fixers, fluorescent pseudomonads and sporulating bacilli (Hameeda *et al*., 2007). A PGPB strain of *Pseudomonas putida* was shown to increase AMF root colonisation in subterranean clover (Meyer & Linderman, 1986a). Azcon (1987) reported that the growth of emerging mycelium from *G. mosseae* spores was enhanced in the presence of PGPB. The nodulating bacteria, *e.g. Frankia, Rhizobium* and *Bradyrhizobium*, generally form synergistic interactions with the AM fungi. It is believed that the AM symbiosis reduces phosphate stress for the plant, which is beneficial for the N_2-fixing nitrogenase system of the bacteria, resulting in enhanced fixation and improved N status of the plant. This in turn promotes plant growth and mycorrhizal development (Fraga-Beddiar & Le Tacon, 1990; Bethlenfalvay, 1992). Thus, the type of interactions between AMF and bacteria depend on the soil environment, bacterial species, AMF species and plant species.

AMF-associated bacteria

The mycorrhizosphere is the soil surrounding and influenced by the mycorrhizal fungi (Rambelli, 1973), where the fungus colonises the roots and modifies the root soil aggregation and water distribution in the soil through its extramatrical hyphae (Andrade *et al*., 1998). Several types of bacteria have been found to be closely associated with the mycorrhizosphere, ranging from apparently simple to more intimate and obligatory symbiotic types (Perotto & Bonfante, 1997; Johansson, Paul & Finlay, 2004). The composition of bacterial populations in the mycorrhizosphere of AM plants can affect the interaction between plant and AM fungi (Andrade *et al*., 1997), or alternatively the AM fungi can influence a shift in specific groups of bacteria in the rhizosphere of mycorrhizal plants towards more facultative anaerobic bacteria and fewer fluorescent pseudomonads (Meyer & Linderman, 1986b). The change in bacterial populations can take place through several modes; *e.g.* competition for nutrients, changes in soil structure, changes in plant root exudate patterns and energy-rich compounds provided by the extra-radical mycelium of AM fungi (Tisdall & Oades, 1979; Mayo, Davis & Motta,

1986; Andrade *et al*., 1997; Ravnskov, Nybroe & Jakobsen, 1999; Söderberg, Olsson & Bååth, 2002).

Some bacteria also associate with AMF structures. Mansfeld-Giese, Larsen & Bodker (2002) reported the bacterial genus *Paenibacillus* to be intimately associated with the mycelium of the AMF *G. intraradices*. Artursson & Jansson (2003) found that *Bacillus cereus* isolated from soil showed higher levels of attachment to the hyphae of *G. dussii* in comparison with other bacterial strains. It seems that some bacteria are more specific to a particular type of AMF, which might be due to the secretion of specific exudates by specific AMF species (Artursson & Jansson, 2003). Mosse (1962) first showed that bacteria colonise the spores of AM fungi. Different studies since then have shown that the spore-associated bacteria can influence the germination of AMF spores, the growth of AMF (Walley & Germida, 1996; Bianciotto & Bonfante, 2002; Hildebrandt, Janetta & Bothe, 2002; Xavier & Germida, 2003) and the formation of the mycorrhizosphere (Budi *et al*., 1999). Budi *et al*. (1999) found that some AMB have antagonistic potential against several soil-borne plant pathogens. The antagonistic potential of spore-associated bacteria against pathogens needs to be fully explored in order to obtain information on the plant health promoting effect of the mycorrhizae. Interest in research on spore-associated bacteria has increased because these have shown the potential to support AMF to complete spore production *in vitro* in the absence of a host (Hildebrandt, Janetta & Bothe, 2002).

The AMF also harbour bacteria-like organisms (BLO) in their cytoplasm. Bianciotto *et al*. (1996) reported that these BLO are actually of true bacterial origin and have endobacterial properties, *i.e.* they complete their life cycle within fungal cells. The BLO are gram-negative, rod-shaped and present in several AM fungal species such as *Acaulospora laevis, Gigaspora margarita* and *Glomus versiforme*. These bacteria were initially identified as belonging to *Burkholderia* on the basis of their 16S ribosomal RNA sequence, but have recently been assigned to new taxon called *Candidatus* Glomeribacter gigasporarum (Bianciotto *et al*., 2003). They have never been cultured on cell-free medium (Bianciotto *et al*., 2004; Jargeat *et al*., 2004). The physiological role of these bacteria is still unclear but results from the genomic library developed from *G. margarita* spores indicate the presence of interesting genes such as a putative phosphate transporter gene, pst (Ruiz-Lozano & Bonfante, 1999) in these organisms.

Mechanisms controlling associations of bacteria with AMF and plant roots in the mycorrhizosphere (Artursson, Finlay & Jansson, 2005) are not fully elucidated. However, a deeper understanding of interactions between the AM fungi and their associated bacteria can partly be gained by characterising the bacterial spectrum of different habitats in the plant rhizosphere.

Characteristics of AMF spore-associated bacteria

The AMF spore-associated bacteria (AMB) have different types of characteristics. Some have the ability to hydrolyse biopolymers such as protein, chitin and cellulose (Filippi *et al*., 1998; Roesti *et al*., 2005). They can degrade the plant material and fungal cell walls around them, resulting in improved soil structure

(Andrade, Azcon & Bethlenfalvay, 1995). Some AMB such as *Bacillus pabuli* have the ability to enhance AMF root colonization (Xavier & Germida, 2003) and could also improve plant growth (Artursson, Finlay & Jansson, 2006). Budi *et al*. (1999) reported that *Paenibacillus* sp. isolated from surface-sterilised *G. mosseae* spores significantly stimulated mycorrhizal colonisation in *Sorghum bicolor*. Thus, AMB from spores can have potential both as mycorrhiza helper bacteria (MHB) and PGPB. It seems that the multifunctional traits could confer an advantage to the AMB in colonising the spore surface and spore walls and ensure their survival in specific microhabitats in competition for nutrients and space with other soil microbes.

Important potato pathogens

Potato (*Solanum tuberosum* L.) is one of the most important crops in terms of its food and economic value. There are several pathogens that cause problems in potato production worldwide. The model pathogens used for interactions in this study were the bacterial pathogen *Erwinia carotovora* var *carotovora* and the fungal pathogens *Phytophthora infestans*, *Rhizoctonia solani* and *Verticillium dahliae*, which affect potato production in terms of both quality and quantity.

The pathogen *E. carotovora var. carotovora* (*Ecc*) causes blackleg and tuber soft rot of potato (Perombelon & Kelman, 1980). The pathogen survives in plant residues and is transmitted through water. It penetrates through cracks in the tubers and lenticels. The disease in the daughter tubers continues to develop in the soil under conditions of high humidity, and in poorly ventilated storage areas. The symptoms start at the base of the stem with hollowing above the blackened area and then stunting and yellowing of the foliage with upward curling of leaflets starts. As the disease progresses, the plant wilts and dies. In the tubers, soft black rot begins usually from the stolon and develops until the tuber disintegrates (Perombelon, 2002). There is a characteristic foul odour due to rotting. Rot may also develop on the sides of the tuber lenticels and wounds.

The pathogen *P. infestans* causes late blight disease in potato (Agrios 2005). It is worldwide in distribution and causes great yield losses every year. Symptoms appear on the stems and leaves. Grey-green patches form at the edges of the leaves at first and turn brown later. A white fungal coating forms on the underside of the leaves, which then dry up or rot. *Phytophthora* spreads via spores, which use a germ tube to penetrate the plant tissue. It also infects the tubers which results in inedible potatoes with blue-grey patches, whose flesh eventually turns brown and rots. Late blight becomes an epidemic during cool, wet weather when the fungus can produce spores and infect leaves nearby.

The pathogen *R. solani*, a member of the anastomosis group 3 (AG-3 group), is an important pathogen of potato and commonly occurs in most potato-producing areas throughout the world. Its importance seems to be increasing in Swedish potato cultivation. The fungus can infect most parts of the potato plant in favourable environmental conditions. It causes black scurf and stem and stolon canker in potato. Canker is economically significant due to malformation of developing daughter tubers and reduction in yield in some early cultivars. In the

case of black scurf, the surface of the tubers becomes covered with irregularly shaped black sclerotia. There are occasionally cracks, depressions and malformations of the tubers. Black scurf has no effect on the yield but affects the market value of the tubers (Jeger *et al*., 1996; Grosch *et al*., 2005). Transmission of this pathogen takes place through sclerotia on the tubers and within the soil or plant residues. Development of sclerotia on tubers is pronounced in poorly drained soils.

The pathogen *V. dahliae* causes early dying wilt of potato. It is also an important pathogen on many other plants, including oilseed rape. It infects through roots and invades the plant's water-conducting tissues. The vascular bundles of the stem and tubers become brown and dark. It can cause a yield losses up to 30-50%. In some cases tuber quality is reduced (Johnson, 1988). The pathogen has been found to occur in diseased potato plants in Sweden but its economic importance is not yet known (S. Alström, pers. comm.).

Disease control

Many practices, such as crop rotation, seed certification, resistant cultivars, chemical fungicides, soil fumigation *etc.*, are used to control pathogens, especially soil-borne pathogens. However there are many problems associated with controlling pathogens with long-term persistent survival structures due to difficulties in reducing pathogen inoculum and lack of good sources of plant resistance. Soil fumigants are not allowed in Sweden but in countries where they are used the most commonly used compound is methyl bromide, which is highly toxic and also depletes the stratospheric ozone layer (Gan *et al*., 1997). Therefore many researchers are trying to find alternate approaches based on either adding or manipulating microorganisms to enhance plant protection against pathogens (Grosch *et al*., 2005). Both seeds and soil can be treated. The beneficial microorganisms (antagonistic bacteria) (*e.g. Pseudomonas fluorescens*, *Bacillus subtilis, etc*.) and fungi (*e.g*. AMF, *Trichoderma, etc.*) compete with plant pathogens for nutrients and space, by producing antibiotics, by parasitising pathogens, or by inducing resistance in the host plants. These have been used for biocontrol of pathogens (Berg, Grosch, & Scherwinski, 2007).

AMF in disease control

The AM fungi play an important function in the reduction of plant pathogens (St-Arnaud *et al*., 1995; Azcón-Aguilar & Barea, 1996; Whipps, 2004). Many workers have observed an antagonistic effect of AMF against some fungal pathogens such as *Fusarium oxysporum* (Dehne & Schönbeck, 1979; Caron, Richard & Fortin, 1986; St-Arnaud *et al*., 1997; Filion, St-Arnaud & Fortin, 1999), different *Phytophthora* species (Davis & Menge, 1980; Cordier, Gianinazzi & Gianinazzi-Pearson, 1996; Trotta *et al*., 1996), *Rhizoctonia solani* (Yao, Tweddell & Desilets, 2002) and *Pythium ultimum* (Calvet, Pera & Barea, 1993) in different crops. The AMF have also been shown to reduce bacterial diseases (Dehne, 1982). For example, *G. intraradices* suppresses *Fusarium sambucinum,* causal organism of potato dry rot (Niemira, Hammerschmidt, & Safir, 1996) and *R. solani* (Yao, Tweddell & Desilets, 2002), while *G. etunictum* suppresses *R. solani* in potato (Yao, Tweddell & Desilets, 2002). The mode of action of AMF

biocontrol activity is assumed to be the direct interactions between AMF and pathogens, but mycorrhiza-mediated triggering of plant defence reactions have also been proposed (Azcón-Aguilar & Barea, 1996; Whipps, 2004). In addition, antagonism from bacteria inhibiting the mycorrhizosphere has also been suggested as a possible mechanism (Budi *et al*., 1999).

AMB in disease control

Meyer & Linderman (1986b) first reported the role of the mycorrhizosphere in biocontrol of pathogens. They found that extracts of rhizosphere soil from mycorrhizal plants reduced sporangia formation of *Phytophthora cinnamomi* in comparison with extracts of rhizosphere soil from non-mycorrhizal plants. These authors postulated that either the sporulation-inducing microorganisms were missing or that the number of sporulation-inhibiting microorganisms increased. The bacteria associated with AMF may also have antagonistic properties. Budi *et al*. (1999) reported that a *Paenibacillus* strain isolated from surface-sterilised *G. mosseae* spores inhibited a number of different plant pathogens, *viz. Aphanomyces euteiches*, *Chalara elegans*, *Pythium* sp., *Fusarium culmorum*, *F. oxysporum*, *Phytophthora parasitica* and *R. solani.*

Some studies indicate that AMF and AMF-associated bacteria have the potential to control plant pathogens (Secilia & Bagyaraj, 1987; Mao *et al*., 1998; Budi *et al*., 1999; Filion, St-Arnaud & Fortin, 1999). Reports on the direct interactions between AMB and potato pathogens are still limited. The PGPB and AMF are co-inoculated to control the growth of potato pathogens (Akköpru & Demir, 2005; Akhtar & Siddiqui, 2007) but there are no reports on the effect of dual inoculation of AMF and AMB on potato pathogens.

Aims of the study

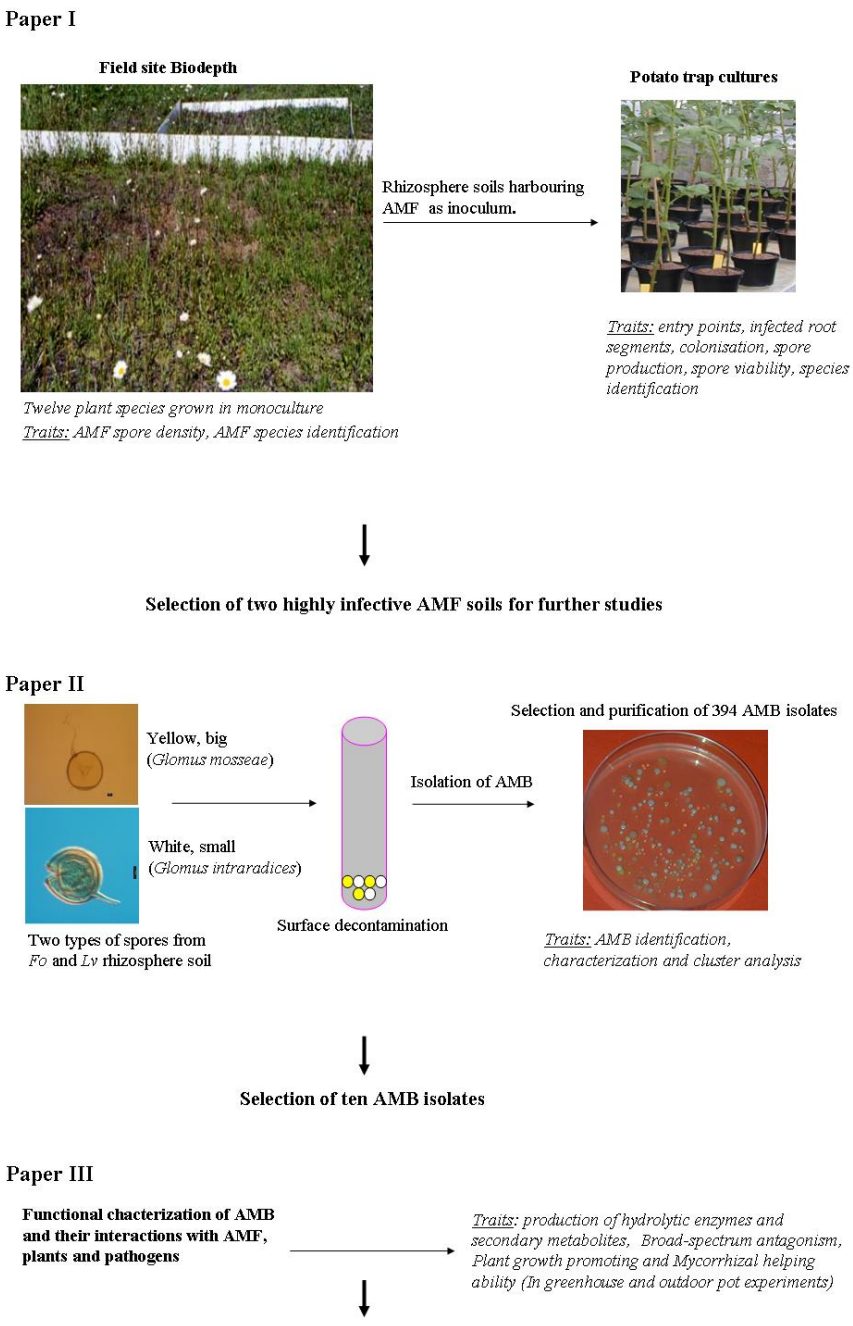
The overall aim of this study was to determine the role played by bacteria associated with AMF in the interaction of AMF with its plant hosts. Plant species play an important role in regulation of the composition and diversity of AM fungal and bacterial communities due to different spectra of their root exudates (Grayston *et al*., 1998; Eom, Hartnett & Wilson, 2000; Marschner *et al*., 2001; Johnson *et al*., 2003). On the other hand, both AMF and bacteria play an important role in the development of the plant community (Eom, Hartnett & Wilson, 2000). Knowledge about the specific effects of individual plant species on the composition of AMF and AMB and also about the ecological function of these AMB in the development of AMF and plants is still very limited.

This study focused on the bacteria associated with the AM fungal spore. This fungal structure was chosen since it is an important long-term reproductive structure. It could act as a habitat for bacteria that play a potential role during the formation of the AM-plant symbiosis. In order to achieve the research aim, the specific objectives of this study were to:

- Examine the composition and efficacy of AM fungal communities as affected by plant species grown as monocultures.
- Investigate the composition of cultivable bacteria associated with AM fungal spores (AMB) and characterise them.
- Study AMF interactions with AMB in host and non-host plant species.
- Study the interactions between AMF, AMB and pathogens *in vitro*.

Materials and methods

The work strategy used in this study is summarised in Fig. 2.



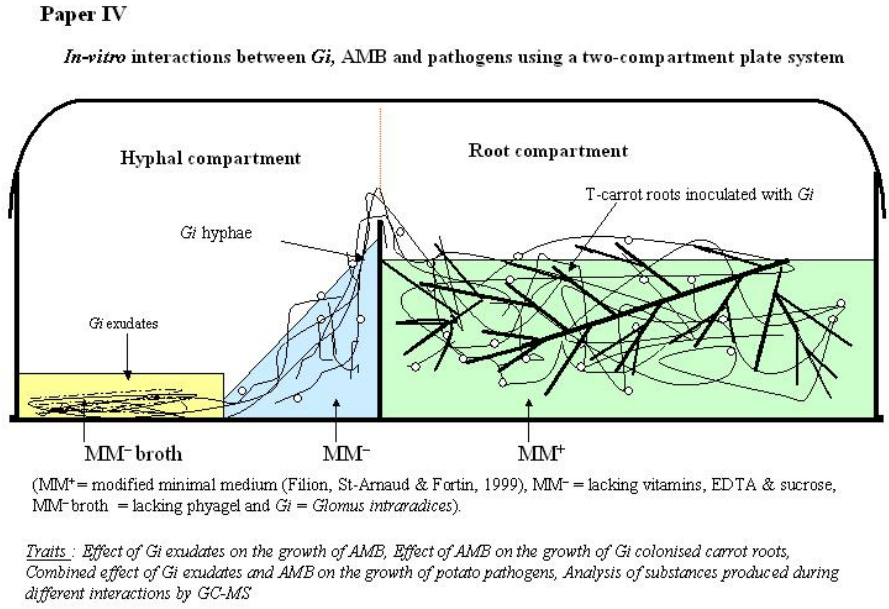


Fig. 2. Schematic view of plan of work used in this thesis.

Table 1. *Biological material and organisms used in this study*

Organisms/materials	**Origin/ source**	**Paper**
Plants		
12 plant species in monoculture	BIODEPTH soil	I
Festuca ovina (Fo)	” ”	I
Leucanthemum vulgare (Lv)	” ”	I
Potato (*Solanum tuberosum*)		
Tubers	Svenskt Potatisutsäde AB, Umeå, Sweden	I & III
Plantlets		III
Seeds		III
Rape seeds		III
Transformed carrot roots	GINCO, Belgium	IV
Arbuscular mycorrhizal fungi		
AMF	BIODEPTH soil, Ultuna soil	I & III
AMF spores	*Festuca ovina*	II
	Leucanthemum vulgare	II
Glomus intraradices	GINCO, Belgium	II & III
Bacteria		
AMB	AMF spores	II, III& IV
Pathogens		
Erwinia carotovora var *carotovora*	Own collection at the Dept	III &IV
Phytophthora infestans	” ” ”	III
Rhizoctonia solani	” ” ”	II, III &IV
Verticillium dahliae		III & IV

Soil sampling

All samples were taken from the field site BIODEPTH, Umeå, northern Sweden (63°45′N, 20°17′E, 12 m above sea level) (Hector *et al*., 1999; Mulder *et al*., 2002). The rhizosphere soil samples with roots were collected from 12 different plant species belonging to three different functional groups:
a) The grasses *Dactylis glomerata* L. (*Dg*), *Festuca ovina* L. (*Fo*), *Phalaris arundinacea* L. (*Pa*), *Phleum pratense* L. (*Pp*).
b) The legumes *Lotus corniculatus* L. (*Lc*), *Trifolium hybridum* L. (*Th*), *Trifolium pratense* L. (*Tp*), *Trifolium repens* L. (*Tr*).
c) The non-leguminous forbs *Achillea millefolium* L. (*Am*), *Leucanthemum vulgare* Lam. (*Lv*), *Ranunculus acris* L. (*Ra*), *Rumex acetosa* L. (*Ru*).

These plants were maintained as monocultures in different plots at BIODEPTH. The soil samples from different plots for each plant species were mixed with care to give one pooled sample per plant species. These pooled samples were used as inocula containing native AMF communities of each plant.

Mycorrhizal traits in potato

The efficacy of native AM fungal communities associated with each plant species was tested in a greenhouse experiment using the above rhizosphere soils. Potato (*cv* King Edward) was used as the host plant. Different mycorrhizal traits were investigated. Based on spore density and percentage root colonisation (Biermann & Linderman, 1981), soils from two highly infective plant species (*Fo* and *Lv*) and two poorly infective plant species (*Pa* and *Tp*) were used for further studies (**Paper I**).

Isolation and identification of AMF

The AMF spores were extracted by wet sieving and a sucrose gradient centrifugation method (Daniels & Skipper, 1982) both from rhizospheres of the *Fo*, *Pa*, *Tp* and *Lv* and from corresponding potato trap cultures. Healthy spores were used for identification based on colour, size and surface ornamentation in collaboration with Prof. S. Rosendahl, Department of Microbiology, University of Copenhagen, Denmark, and in accordance with the descriptions available at the INVAM (International culture collection of Vesicular Arbuscular Mycorrhizal Fungi) site, http://invam.caf.wvu.edu. The AM fungal spores isolated as above and the potato roots treated with *Fo*, *Pa*, *Tp* and *Lv* soils were used for molecular identification of AM fungi by 16S rDNA sequencing (Kjøller & Rosendahl, 2000) (**Paper I**).

Isolation and identification of AMB

Rhizosphere soils from *F. ovina* (*Fo*) and *L. vulgare* (*Lv*) plants were selected for further studies because they caused the highest colonisation of potato roots when used as an inoculum. For isolation of AMB, dominant AMF spores from the *Fo* and *Lv* rhizosphere soils were selected and divided into four groups.

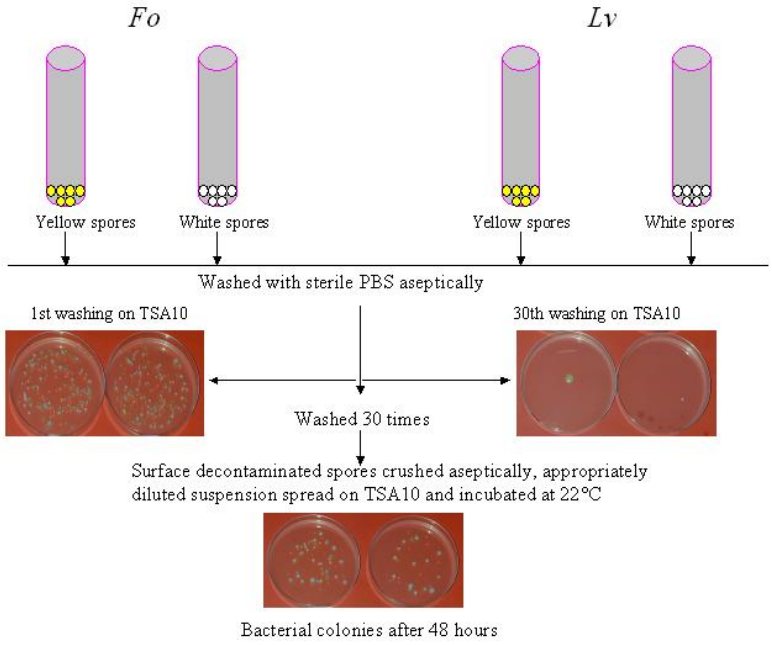


Fig. 3. Isolation procedure for bacteria associated with arbuscular mycorrhizal spores.

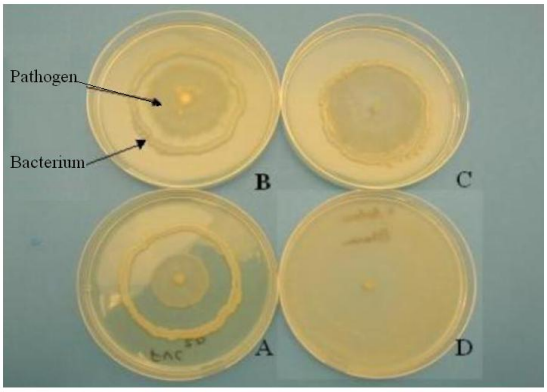


Fig. 4. Effect of three different AMB isolates inhibiting the radial growth of *Rhizoctonia solani*. A=Strong inhibition, B= Moderate inhibition, C= Weak or no inhibition, and D= control (*R. solani* only).

These groups were labelled LY (yellow large spores from *L. vulgare*), LW (white small spores from *L. vulgare*), FY (yellow large spores from *F. ovina*) and FW (white small spores from *F. ovina*) (**Paper II**).

Spores were surface-decontaminated by multiple washings with sterile phosphate buffer saline (PBS) and AMB were isolated from the crushed surface-

decontaminated spores (Fig. 3). All the AMB isolates were identified based on their fatty acid methyl ester (FAME) profile (Sasser, 1990). Of these, 25 isolates were further identified based on 16S rDNA and sequencing. These isolates were selected as representatives of above four groups FW, FY, LW and LY.

Antagonism and fluorescence

Antagonistic activity of all AMB isolates against *R. solani* was assessed in a dual culture assay *in vitro* (Fig. 4, Montealegre *et al*., 2003). The ability of all AMB to produce fluorescence was tested on Kings B medium (KBA) under UV light.

Effects of AMB on plant, AMF and pathogens

a) On plant growth

The effects of ten selected AMB isolates (FWC14, FWC16, FWC30, FWC42, FWC70, FWC94, FWC101, FWC110, LWC2 and LYC39) were studied on AMF host (potato) and non-host (oilseed rape) plants. They were selected on the basis of their antagonistic activity against *R. solani* and *P. infestans* (Hedenskog *et al*., 2005; **Paper II**). For this purpose, the surface-sterilised seeds of potato and oilseed rape were inoculated with each AMB isolate after sowing on water agar (WA). Number of seeds germinated and their radicle length were recorded. The direct effect of the isolates was also tested on the growth of potato shootlets grown on modified minimal (MM) medium (Filion, St-Arnaud & Fortin, 1999).

b) On AMF growth and colonisation

Five isolates (FWC14, FWC16, FWC30, FWC42 and FWC70) that showed strongest *in vitro* inhibition of *R. solani* were selected (**Paper II**). Their effect on AMF colonisation was assessed as percentage root colonisation by *G. intraradices* in potato and oilseed rape in greenhouse experiments. On the basis of the significant response of FWC70 on percentage root colonisation in greenhouse conditions, this bacterium with five other AMB isolates (FWC94, FWC101, FWC110, LYC39 and LWC2) were further tested for their effect on percentage potato root colonisation by native AMF in an outdoor pot experiment.

c) Biocontrol activity against potato pathogens

The ten AMB isolates listed above were further tested *in vitro* for their activity against *V. dahliae* and *Erwinia carotovora* var *carotovora* (*Ecc*). The antagonistic activity against *V. dahliae* was tested on a dual culture assay. Any inhibition due to AMB was recorded as strong, moderate or less and no effect. In the case of *Ecc*, slices from surface-sterilised potato tubers were used. Antagonism was measured as number of slices showing rotting and/or reduction in weight of rotten tissue compared with that in diseased controls.

Interactions between AMF, AMB and pathogens

The interactions between AMF, AMB and pathogens were examined using a two-compartment (St-Arnaud *et al*., 1996, Filion, St-Arnaud & Fortin, 1999; Toljander *et al*., 2007) and multiwell plate system.

Collection of exudates from AMF

This study was limited to *in vitro* cultured *G. intraradices* (*Gi*) as the AMF. The interaction of *Gi* with AMB and potato pathogens was investigated through its exudates. The exudates were collected by culturing the fungus with transformed (T) carrot roots in the two-compartment system (Fig. 2).

The composition of exudates produced by *Gi* and AMB during their growth in different interaction studies (Fig. 2) was analysed using GC-MS (Gullberg *et al*., 2004) to identify any substance(s) that could possible explain the effect mediated by exudates on growth of AMB and pathogens (**Paper IV**).

Effect of AMF on AMB

The effect of *Gi* exudates on the growth of AMB was studied in multiwell and two -compartment plate system. In the multiwell system, Gi exudates were inoculated as a component of the growth substrates at one point in time, while in the two-compartment system there was direct continuous secretion of *Gi* exudates only. In the multiwell plate system, growth of each AMB was measured at different time intervals up to 48 hrs by measuring absorbance at 560 nm wavelength. In the two-compartment plate, average number of colony-forming units (cfu) per mL was determined and any change in pH was also recorded for each combination.

Effect of AMB on AMF

The ten AMB isolates listed above were analysed in terms of their effect on the growth of *G. intraradices*-colonised T-carrot roots (Fig. 2). Average number and length of newly formed T-carrot roots were recorded. Of the ten AMB tested, three isolates (FWC30, FWC70 and LWC2) were selected on the basis of their strong antagonistic activity against potato pathogens (**Paper III**) for further study. The effect of these three selected isolates on *Gi* spore production was also recorded (**Paper IV**)

Effect of AMF and AMB on potato pathogens

The combined effect of *Gi* and AMB (FWC30, FWC70 and LWC2) on the growth of potato pathogens was studied in sterile 24-multiwell plates. To investigate whether there was any increase in antagonistic activity of AMB in the presence of AMF, the pathogens *R. solani*, *V. dahliae* and *Ecc* were added to the interaction products of *Gi* exudates +AMB isolates. Effect on fungal pathogens (*R. solani* and *V. dahliae*) was measured as mycelial dry weight and on *Ecc* as change in cfu mL^{-1} of the two co-inoculants using specific media and comparing the results with their individual growth.

Functional characteristics of AMB

The ten selected AMB isolates (**Papers III** and **IV**) were functionally characterised in order to get some information about their ecological importance and possible clues to mechanisms behind their effects on plant growth, AMF and pathogens. They were characterised in terms of production of various enzymes and some secondary metabolites that are suggested to be important for their role in nutrient acquisition and biocontrol (Paulsen *et al*., 2005). Production of chitinases, proteases, cellulases and phosphatases was assessed qualitatively according to Arora *et al*. (2005). Production of siderophores and hydrogen cyanide (HCN) was detected according to methods described in Schwyn & Neilands (1987) and Alström & Burns (1989), while production of the plant growth hormone indole-acetic-acid (IAA) was determined according to Sarwar & Kremer (1995).

Results and Discussion

In the rhizosphere, microorganisms are influenced by plant roots and plants have the ability to change or modify rhizosphere microbes during their growth. The effect of rhizosphere microorganisms can be directly detrimental *e.g.* in the case of pathogens, directly beneficial *e.g.* AMF and plant growth promoting bacteria (PGPR), or indirectly beneficial. The different interactions studied here are shown in Fig. 5.

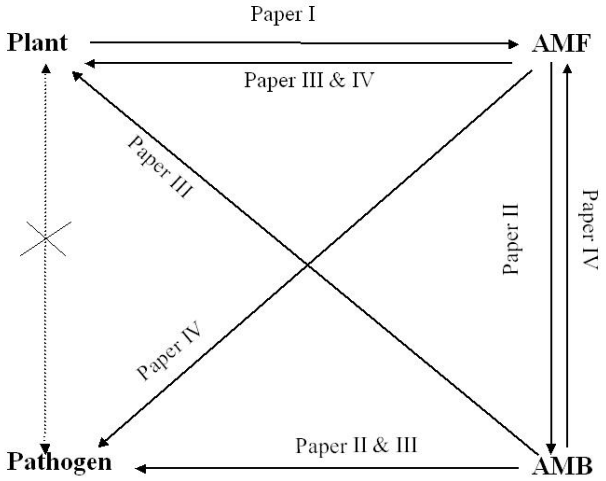


Fig. 5. Outline of different interactions studied in this thesis (X = not investigated).

Impact of plant species in monoculture on AMF

Generally, AM fungi are non-host specific but some degree of host specificity has been reported. For example *Acaulospora colossica* has been shown to sporulate more profusely in association with *Allium vineale* (field garlic) than with *Plantago lanceolata* but the reverse is true with *Scutellospora calospora* (Bever *et al*., 1996; Schultz, 1996). The composition of an AMF community may be strongly affected by individual plant species through differential effects on hyphal growth and sporulation (Sanders & Fitter, 1992; Bever *et al*., 1996). When a host plant grows in monoculture, it is conceivable that it gradually selects the AMF assemblage that can optimise its growth. Bever *et al*. (1996) reported that different AMF species sporulated differentially with different plant species with which they were associated.

At the study site BIODEPTH in Umeå, Sweden (Hector *et al.* 1999; Mulder *et al.* 2002), 12 plant species had been grown as monocultures for seven years (**Paper I**). Thus, host plants of AMF had been able to affect the soil microbiota for a long time. We postulated that a lower number of AMF species would be associated with each plant species than would be present with mixed cultures. Plots with mixed plant species were not investigated in this study.

We assumed that the AM fungal communities developing in the rhizosphere of the 12 plant species would be specific for, or at least more efficient with, each of the 12 plant species. The monocultures were assumed to have functioned as a kind of trap culture in nature. The density of AMF spores estimated in the soils of these 12 plant species was shown to vary depending on plant species. The highest spore density occurred in *Dg* (70 spores g^{-1} soil) and the lowest in *Tr* (20 spores g^{-1} soil). This variation might be due to AMF-host combinations and may also depend on seasonal influences (Borg *et al*., 2003; Panwar & Tarafdar, 2006).

Potato, a major crop in temperate regions, was the most important plant studied as a host of AMF. To investigate the compatibility between potato and the AMF in each of the 12 soils, potatoes were grown in a greenhouse and the soil from each of the 12 plant species was used as an inoculum of AMF communities.

The AMF from all 12 plant soils were able to colonise the potato roots and different types of typical AMF structures of their development stages were observed in colonised potato roots (Fig. 6). The results showed that the response of potato roots differed depending on the plant species, although all the plant species were grown in the same field under the same environmental conditions. This difference was observed in terms of spore density, percentage root colonization and AMF species composition. The potato rhizosphere harboured the highest spore density when it was cultivated in *Fo* and *Tp* soils but the highest colonisation was observed when potato was cultivated in *Fo* and *Lv* soils. This difference can be explained by the presence of different AMF species and their different degree of sporulation (Land & Schönbeck, 1991) and colonisation in the potato rhizosphere. An additional explanation can be difference in amounts of viable AMF propagules. Type of propagules are spores and infected host roots present in the soil inoculum. These may interact with substances in potato root

exudates that may delay or enhance AMF colonisation or may initiate or inhibit the sporulation. Alternatively, different AMF species composition in the soil inoculum of monoculture plants may have different preferences for the potato roots.

Characterisation of AMF communities in soils from four selected plant species and from potato plant roots grown in presence of these four soils was based on spore morphology (Abbott & Robson, 1979; Merryweather & Fitter, 1998; Landis, Gargas & Givnish, 2004) and molecular sequencing (van der Heijden & Scheublin, 2007). Using only one method is suggested to be insufficient to cover the whole spectrum, hence we used both methods because they give more complete information (van der Heijden & Scheublin, 2007). Two highly 'infective' soils (*Lv* and *Fo*) and two poorly 'infective' soils (*Tp* and *Pa*) were used for AMF identification and a total of seven AMF species were identified. These belonged to *Acaulospora* sp, *Glomus caledonium*, *G. intraradices, G. geosporum, G. microaggregatum, G. mosseae*, and *G. sinuosum*, of which *G. intraradices* and *G. mosseae* were the most common. Only two species (*G. intraradices* and *G. geosporum*) were found in *Tp* soil, whereas seven species were found in *Fo* and *Lv* soils. It seems that some AMF species from *Fo* and *Lv* are more compatible with growing potato roots than other AMF species.

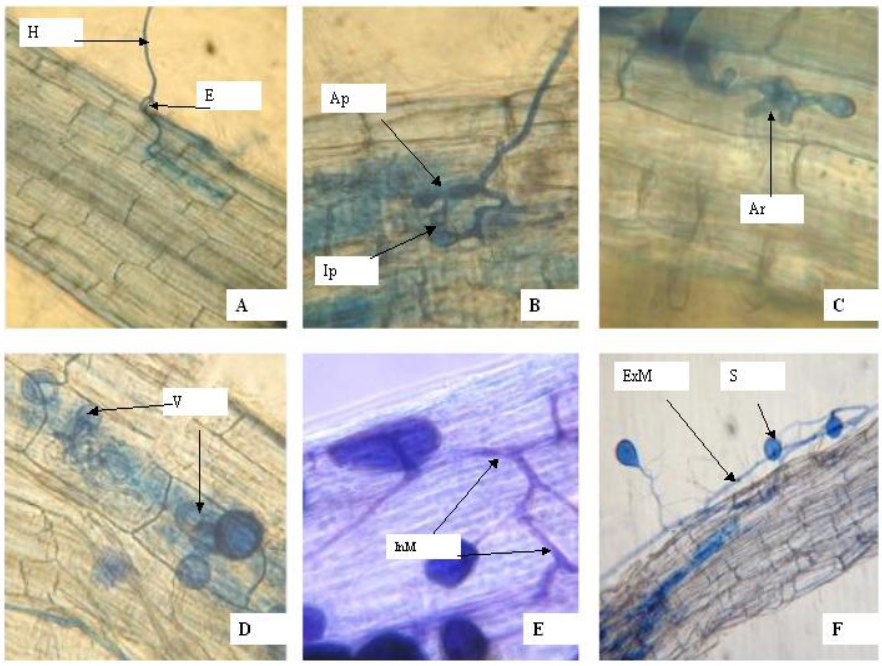


Fig. 6. Different structures reported to be involved in the AMF life cycle and observed in potato roots during this study: hyphae (H), entry point (E), appresorium (Ap), infection peg (Ip), arbuscule (Ar), vesicle (V), intraradical mycelium (InM), extraradical mycelium (ExM) and spore (S).

The species *G. mosseae* was present in both *Fo* and *Lv* soils but absent in *Tp* soil and we found that potato roots were mainly colonised by *G. mosseae*. It can be concluded that *G. mosseae* is a more active AMF in *Fo* and *Lv* soils than in soil under other plant species grown at the BIODEPTH site. The results suggest that the efficacy of naturally occurring AMF inoculum might depend on different abilities of individual AMF species to interact with the host plant, regardless of differences in spore numbers and colonisation levels (van der Heijden *et al*., 1998). The spore production and colonisation ability depends on genetic control of the host, the AM fungus, or more likely a complex interaction of both symbiotic partners with soil environmental factors (Sylvia *et al*., 2003).

Bacterial communities associated with AMF spores

The AM fungi provide specific niches on their spores, extraradical hyphae and intraradical mycelia for a large population of bacteria (Scannerini & Bonfante 1991). There are a few reports on the interactions between AM fungi and bacteria associated with their spores (Mayo, Davis & Motta, 1986; Budi *et al*., 1999; Xavier & Germida, 2003). The main focus in this thesis is on AM spore-associated bacteria (AMB) because of their potential role in spore germination and root colonisation. In this study, a total of 394 AMB were isolated from two dominant spore types, *i.e.* large yellow and small white spores belonging mainly to *G. mosseae* and *G. intraradices* respectively. These two types of AMF spores were extracted from the two highly 'infective' *Fo* and *Lv* soils (**Paper I**). It proved possible to identify half the AMB based on their fatty acid methyl ester (FAME) profiles at species level. They belonged to 16 genera and 36 species. *Arthrobacter, Pseudomonas* and a cluster of mostly unidentified isolates related to *Stenotrophomonas* dominated. The genera *Pseudomonas* and *Arthrobacter*, found to be dominant in this study, have also been reported as dominant genera in other studies on AMF-associated bacteria (Mayo, Davis & Motta, 1986; Mansfeld-Giese, Larsen & Bodker, 2002). The identity of selected AMB was confirmed using 16S rDNA sequence analysis.

Hierarchical Cluster analysis of FAME data of the majority of isolates (385 isolates) revealed a distribution of most isolates into two major clusters, with sub-clusters dominated by the genera *Pseudomonas, Acidovorax, Agrobacterium*, *Arthrobacter*, *Cellulomonas*, *Micrococcus* and *Bacillus*. Most unidentified isolates were found in a cluster with *Stenotrophomonas* sp. The genera *Acidovorax*, *Agrobacterium*, *Arthrobacter*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Cellulomonas*, *Clavibacter*, *Corynebacterium*, *Micrococcus*, *Paenibacillus* and *Pseudomonas* have also been previously reported from either AM fungal spores or mycelia (Mayo, Davis & Motta, 1986; Bianciotto *et al*., 1996; Mansfeld-Giese, Larsen & Bodken, 2002; Xavier & Germida, 2003; Roesti *et al*., 2005; Toljander *et al*., 2006). In our study we found all of these to be associated with spores. In addition, we found the genera *Aureobacterium*, *Curtobacterium*, *Hydrogenophaga*, *Janthinobacterium* and *Stenotrophomonas* associated with spores (**Paper II**).

Our results demonstrate that the species composition of cultivable AMB in AM fungal spores is affected by their source environment, *i.e*. AMF species, plant species and the AMF-plant species combination. Support for this was found in the

differences in distribution among the two major AMB clusters, the differences in distribution among origins within clusters at the genus level and the exclusive occurrence of certain AMB species with specific AMF or plant species and their combinations (**Paper II**). The possible explanation behind the species-specificity of AMB might be the production of plant species-specific exudates and AMF species-specific exudates (Bais *et al*., 2006; Artursson, Finlay & Jansson, 2006). The plant root and AMF exudates can contain substances that support the growth of certain groups of bacteria or induce production of antimicrobial compounds that inhibit the growth of competing microbes and allow the growth of only such microbes that can adapt to the particular niche of spore wall. These microhabitats can vary depending on spore morphology and the environment where they are produced. The AMF spores developed in monoculture in our study might have selected bacteria that can multiply in a specific environment provided by the continuous growth of the same host and its associated AMF flora. Plant root exudates were not analysed in this study. Andrade *et al*. (1997) found that the composition of bacterial populations in the rhizosphere and hyphosphere of AMF plants was affected by the presence of plant host species and isolates of AM fungi.

All AMB isolates tested for inhibition of *R. solani* exhibited different degrees of inhibition (Fig. 4 and 8). A total of 14% of the isolates were strongly antagonistic, of which a quarter were fluorescent *Pseudomonas*. The AMB showing strong inhibition belonged to *P. putida*, *Agrobacterium radiobacter*, *Cellulomonas flavigena*, *Arthrobacter oxydans*, *B. subtilis*, *Micrococcus kristinae*, *P. pseudoalcaligenes* , *S. maltophilia* and unidentified isolates. Occurrence of most antagonistic isolates was spore type-dependent and not plant host-dependent and they originated from *G. intraradices* spores.

The methods used for selecting the colonies representing a particular source environment were shown to have some influence on the AMB distribution pattern. Use of more than one method to select bacterial colonies provided better information about the bacterial diversity associated with spores. We conclude that there was high AMB diversity with AMF spores in this system and this depended on AMF species and plant host. Our results on AMB provide a mechanism for the often found positive effect of AMF against plant pathogens.

Effects of AMB on plant and AMF

Ten AMB isolates belonging to the genera *Arthrobacter*, *Bacillus*, *Pseudomonas* and *Stenotrophomonas* were selected to investigate the importance of AMB in the development of AMF colonisation, plant growth and inhibition of pathogens. The basis of selection was the ability to inhibit growth of fungal pathogens. Root colonisation of host potato by *Glomus mosseae* and by native AMF community was stimulated by a *Pseudomonas putida* isolate (FWC70), both in greenhouse and outdoor pot experiments. Such stimulation also recorded for two isolates of *Stenotrophomonas* and *Arthrobacter* in outdoor pot experiments. Meyer & Linderman (1986b) found that *P. putida* enhanced mycorrhizal colonisation in subterranean clover but inhibited the germination of *G. clarum* NT4 spores, probably due to a non-volatile substance being produced by the pseudomonad (Walley & Germida, 1996). This variation in effects on AMF may depend on the

amount and type of isolates of the bacteria used (Requena *et al*., 1997), the time of inoculation (Krishna, Balakrishna & Bagyaraj, 1982) and the time of harvest (Staley, Lawrence & Nance, 1992).

In non-host oilseed rape, the growth of *G. mosseae* was very low, as expected. The low growth was probably due to the lack of production of a diffusible growth stimulus, which is present near the roots of compatible hosts (Glenn, Chew & Williams, 1988). In the presence of three of the five AMB isolates tested, a negative effect on the growth of *G. mosseae* mycelium along the oilseed rape root was observed. A possible reason for this could be that presence of AMB increased the production or toxicity of exudates from the rapeseed root, leading to lower *G. mosseae* growth and possible inhibition of AMF spore germination. The two AMB that did not have a negative effect were the *Pseudomonas* isolates FWC70 and FWC30.

Potato seeds inoculated with each of the ten isolates germinated in all cases and there was no deleterious effect on their seedling growth. In a separate experiment in which potato shootlets grown on a minimal medium in the absence of AMF, both root induction and growth were stimulated by the same *Pseudomonas putida* isolate, FWC70 (Fig. 7, **Paper III**) that improved the AMF colonisation discussed above. The same response was also observed to some extent for another *Stenotrophomonas* isolate. Significant increases in some parameters of potato shootlets were also observed in the presence of two more AMB isolates, FWC14 and FWC30. A tendency was also observed for the shoot and root dry weight of potato plants to increased (by 7-15% respectively) in the presence of FWC70 in the greenhouse experiment. This increase was not confirmed statistically (data not shown). The duration of this experiment was short and further studies of longer duration are needed to verify these trends of AMB on plant growth.

The *Pseudomonas* and *Stenotrophomonas* isolates that stimulated potato growth also stimulated oilseed rape radicle length on water agar medium. None of ten isolates studied exhibited deleterious effects on emergence of seeds of oilseed rape. The radicle length of rapeseed was instead found to increase in their presence.

The PGP effect of both FWC30 and FWC70 can be explained by their ability to produce the plant growth hormone IAA, siderophores and phosphate-solubilising enzyme(s). These were shown to be produced *in vitro*. Whether they are produced in natural conditions has not yet been confirmed. However, their ability to produce these substances *in vitro* is indicative of their plant-growth-promoting potential. Production of phytohormones, siderophores and phosphatases by the rhizosphere bacteria has been reported to be involved in enhancement of growth and yield of several plant species including crucifers (*e.g.* canola), solanaceous crops (*e.g.* tomato and potato) and graminaceous crops (*e.g.* wheat) (Bakker *et al*., 1986; Abbass & Okon, 1993; de Fritas, Banerjee & Germida, 1997).

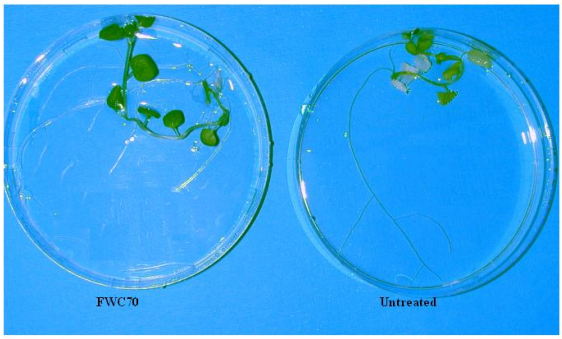


Fig. 7. *In vitro* response in growth of potato plantlets after inoculation by AMB isolate FWC70. Control to the right.

Fig. 8. Changes in hyphal morphology of *R. solani* after inoculation with AMB isolate FWC70. Control to the right.

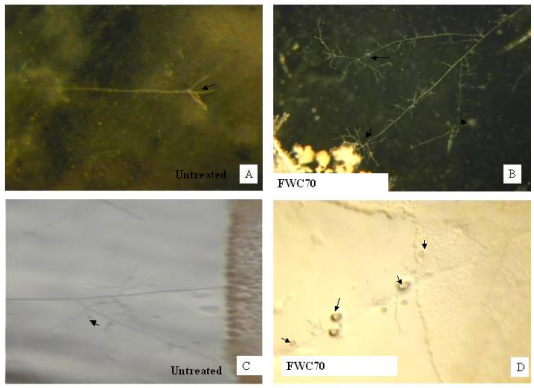


Fig. 9. The AMB isolate FWC70 increased the number of hyphal branches (B) and spores (D) of *Glomus intraradices*. Control A, C to the left. Arrow shows hyphal branches and spores.

Ayyadurai *et al*. (2006) suggest a potential plant growth-promoting ability of *Pseudomonas aeruginosa*, which produces IAA, siderophores and phosphate-solubilising enzyme. In this study, the production of IAA by bacteria could be one of the mechanisms behind increased number of roots and thus increased absorptive surface of plant roots. Siderophores and phosphatases are known to be involved in effective uptake of nutrients, *e.g.* iron and phosphorus respectively. Thus, improved nutrient availability by AMB could be another mechanism behind the enhanced plant growth in potato and oilseed rape plants.

Effects of AMB on pathogens

Antagonism against the two potato pathogens *Verticillium dahliae* and *Erwinia carotovora* was found for all three *Pseudomonas* isolates. The four different *Stenotrophomonas* isolates were variable with regards to inhibition of pathogens. Extracellular activities of protease(s) and production of siderophores and the plant growth hormone indole acetic acid (IAA) were found for all isolates. Chitinase(s) was produced mostly by *Stenotrophomonas* and not by *Pseudomonas* isolates. Extracellular phosphatase was detected in all *Pseudomonas* isolates, one *Stenotrophomonas* and one *Arthrobacter* isolate. We concluded that some of the AMB were multifunctional, *i.e*. the same AMB enhanced mycorrhizal colonisation and plant growth and was antagonistic against pathogens. The multifunctional effects varied with AMB isolate and more than one mechanism seems to be used by the AMB studied. Our results show that some AMB are likely to contribute to the often described ability of AMF to inhibit pathogens, acquire nutrients and modify plant root growth.

The AMF can inhibit growth of plant pathogens (**Paper II**) but there are a few reports on the involvement of AMB in the inhibition of growth of pathogens (Budi *et al*., 1999; Li *et al*., 2007). Budi *et al*. (1999) reported that the *Paenibacillus* ssp. isolated from the AMF spores suppressed *in vitro* mycelium growth of several plant pathogens**.** Li *et al*. (2007) found that AM-associated bacteria from the genus *Paenibacillus* have biocontrol ability against *Pythium*-caused damping-off of cucumber. The results from ten AMB isolates tested for antagonistic activity against four different potato pathogens in this study revealed that AMB have strong antagonistic potential both *in vitro* and *in vivo* (**Papers II, III**). The broad spectrum activity of AMB depends upon the pathogen and the bacterial isolate involved. It can be concluded from our observation that among ten isolates, FWC70 is one of most promising candidates that can be considered both as single or co-inoculant with AMF, provided that its consistency in field conditions is confirmed. This study reported the possible mechanisms behind the inhibition of pathogens by AMB, *i.e.* competition for nutrients such as Fe and P and production of pathogen cell wall-degrading enzymes. The other possible mechanism could be the production of antibiotics, but further studies are necessary. However, in the studies of interactions *in vitro*, it was shown that *Gi* exudates alone inhibited the growth of two pathogens (*Ecc* and *R. solani*). In the presence of three AMB isolates tested this inhibition was enhanced and also extended to *V. dahliae* (**Paper IV**). This seems to indicate that the strong antagonistic response is the result of interactions between AMF and AMB.

Effects of AMB on AMF development *in vitro*

In further *in vitro* studies conducted in compartmentalised plates, no negative impact of the AMB tested was observed on the growth of carrot roots colonised with *G. intraradices*. Co-inoculation with FWC70 led to a significant increase in the number and length of newly formed carrot roots (**Paper IV**). The effects of FWC30, FWC70 and LWC2 were examined *in vitro* on spore production by *G. intraradices* (Fig. 9). No negative impact on spore production by any of the three isolates tested was recorded. On the other hand, the maximum number of spores was produced in the presence of FWC70. Hildebrandt, Janetta & Bothe (2002) also isolated a bacterium *Paenibacillus validus* from spores of *G. intraradices*. Their *Paenibacillus* isolate stimulated the growth of *G. intraradices* up to the formation of newly colonising spores (Hildebrandt *et al*., 2006)**.** All these results indicate clearly that some AMF spore-associated isolates, *e.g.* FWC70, have an additional role to play as *e.g.* MHB (Fig. 9). The mycorrhizal helper effect might be due to increased absorptive surface and improved availability of nutrients, *e.g.* Fe-mediated through their siderophores. however, evidence in support of this statement is needed.

Effects of AMF on AMB growth

The AMF fungi produce different compounds during their growth that might improve the growth of bacteria or might allow growth of only selected bacteria (Toljander *et al*., 2007). The analysis of *Gi* exudates by GC-MS detected high amounts of different compounds, *e.g.* sucrose, fructose, glucose, trehalose, raffinose (carbohydrates), succinic acid, citric acid (organic acids), asparagine, glutamic acid, pyroglutamic acid, glutamine, leucine, proline (amino acids) and several other unidentified compounds (**Paper IV**). These compounds are important source of nutrients for AMB.

We found that three of six unidentified compounds that were present in high amounts in *Gi* exudates were reduced in the presence of the AMB isolates FWC30, FWC70 and LWC2. It seems that these compounds were consumed during AMB growth. There were also certain compounds that were detected in very low amounts in *Gi* exudates. Example of these were lactose, lysine and uric acid. These compounds increased during AMB growth.

Some unidentified compounds were also detected as a result of AMB interaction with *Gi*, as they were not detected in *Gi* exudates. Our results prove that *Gi* produce different compounds that can be involved in improved growth of AMB and allow them to compete and survive in their environment.

Mechanisms for the roles of AMB in the plant – AM fungi – bacteria – pathogen system

The AMB studied in detail here seem to have functions both as MHB, PGPB and/or antagonists. The mechanisms behind their observed effects (*e.g.* FWC70) can be multifold. They can produce an array of several enzymes, *e.g.* chitinases, proteases, phosphatases and cellulases, and several extracellular metabolites, *e.g.* siderophores and IAA. All of these are considered to be involved in *e.g.* nutrient acquisition, colonisation competence and biocontrol ability (**Papers II, IV**).

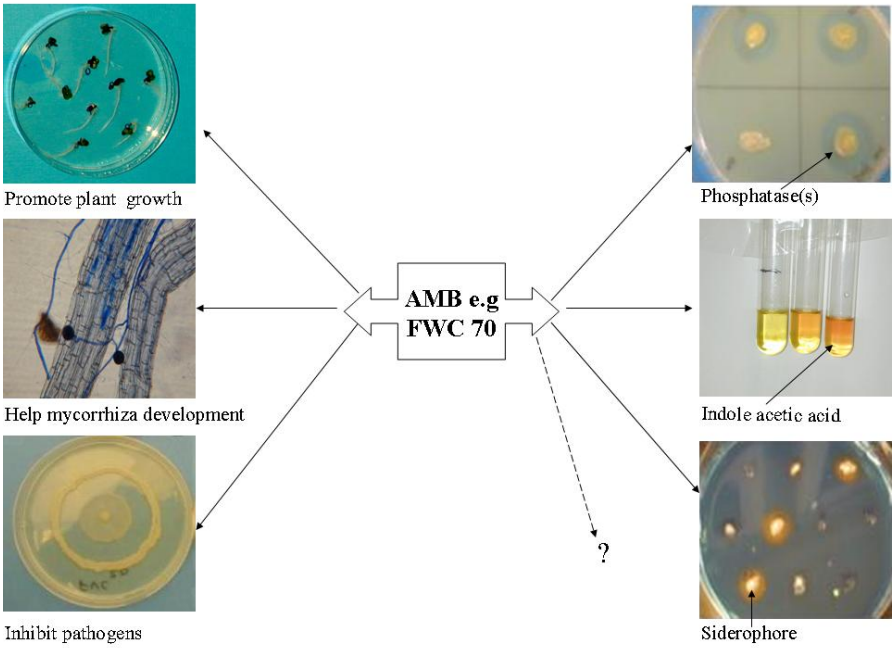


Fig. 10. Multifunctional characteristics of AMF spore-associated bacteria (AMB) *e.g.* isolate FWC70 in this study.

Conclusions

- Soil from the rhizosphere of the plants *Festuca ovina* and *Leucanthemum vulgare* contained AMF species highly infective for potato roots. Based on spore morphology, more AMF species were present in these two soils than in two other soils giving low colonisation of potato. *Glomus intraradices* and *G. mosseae* were fungal dominant species in the *F. ovina* and *L. vulgare* soils.
- The AMB associated with *G. intraradices* and *G. mosseae* spores from *F. ovina* and *L. vulgare* soils showed a broad range of diversity. Certain AMB genera were specific to particular AMF species, plant species and combinations of these.
- The combination of AMF and plant species was important for isolates in the genus *Arthrobacter*, where certain isolates mostly co-occurred with spores from the combination *G. intraradices* - *F. ovina* and the combination *G. mosseae* - *L. vulgare*.
- The specificity of AMB to certain AMF might be due to production of specific exudates by plant and/or AMF species and due to different morphologies of the AMF spore surface. Exudates collected from *G. intraradices* stimulated growth of AMB and *vice versa*.
- Certain AMB showed strong multifunctional effects, *i.e.* stimulated AMF colonisation of plant roots, plant growth and antagonism against several fungal and bacterial plant pathogens. The effects varied with AMB isolate and pathogen tested.
- The AMB showing strong inhibition against *R. solani* growth mostly belonged to the genera *Pseudomonas*, *Agrobacterium* and several unidentified isolates related to the genus *Stenotrophomonas*.
- Some AMB species showed strong inhibition of the growth *in vitro* of several potato pathogens (*Erwinia carotovora*, *Phytophthora infestans*, *Rhizoctonia solani* and *Verticillium dahliae*). These AMB were identified as *Arthrobacter ilicis*, *Bacillus subtilis*, *Pseudomonas putida*, *P. fluorescens* and *Stenotrophomonas maltophilia*.
- Occurrence of most strongly antagonistic AMB isolates was spore type-dependent and not plant host-dependent and they originated from the spores of the AM fungal species *G. intraradices*.
- Production of extracellular enzymes and bioactive compounds by AMB varied among the AMB species, suggesting that different mechanisms were used in antagonism against pathogens.
- AMB in the presence of *G. intraradices* exudates resulted in enhanced antagonistic effects against the pathogens *E. carotovora*, *R. solani* and *V. dahliae*.
- Analysis of exudates collected from *G. intraradices* grown in the absence or presence of AMB by gas chromatography/mass spectrometry showed many compounds that were several-fold increased or decreased in concentration depending on the *G. intraradices* – AMB isolate interaction.

- Plants of *F. ovina* and *L. vulgare* can be used as alternate hosts to maintain or enhance the AM fungal inoculum for potato cultivation. By using *F. ovina* inoculated with *G. intraradices* as a cover crop, it should be possible to enhance the occurrence of strongly antagonistic AMB.
- The association of multifunctional AMB with AMF spores provides evidence for the mechanism by which bacteria might be involved in the often reported positive effect of AM fungi against plant pathogens.

Future perspectives

Many new questions arise from this study. Some of the issues that need immediate attention are listed below.

- It is evident that bacteria associated with AMF and their multifunctional properties will attract increasing attention as an unexploited resource in biological control. Their performance in the real world should be given increased attention through field testing and may provide an environmentally safe alternative to chemical pesticides.

- In monoculture cropping systems, population build-up of plant pathogens poses one of the major problems. Suitable AMB in combination with AMF should be investigated for minimising the harmful effects due to monoculture.

- Further research on metabolic interactions of the different AMB isolates *e.g. Pseudomonas, Bacillus etc*. with different AM fungal species is required to understand the ecological roles of metabolites produced by AMB and AMF species alone or in interaction, for the organisms in their surroundings and for disease suppression and development of AMF symbiosis and plant growth promotion.

- The extent to which the production of enzymes and bioactive compounds, *e.g*. IAA, produced by AMB is actually involved in AMF development and in the inhibition of plant pathogens needs to be unravelled. For this purpose AMB mutants will be required.

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