Interactions between Soil Bacteria and Arbuscular Mycorrhizal Fungi

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Abstract

The extraradical mycelium (ERM) of mycorrhizal fungi constitutes an important pathway for the translocation of energy-rich photoassimilates from plant to soil. Because of the large surface of the mycelium, and its provision of carbon, the ERM potentially constitutes an important site for interactions with other microorganisms in soils. The focus of this thesis is on the ERM of arbuscular mycorrhizal (AM) fungi and its interactions with soil bacteria, and the possible ecological significance of these interactions.

In an *in vitro* study, soil bacteria differed in their ability to attach to AM fungal hyphae. These physical associations between bacteria and fungi were influenced by hyphal vitality and fungal strain. The degree of attachment of bacteria to living or dead hyphae may reflect the ecological niche differentiation of these bacteria, and consequently their trophic relationship (*e.g.* mutualistic or saprotrophic) to the mycorrhizal fungi.

Carbohydrates of mycelial origin, mainly in the form of glucose, acetate and formiate, were identified in exudates from an AM fungus. Mycelial exudates promoted the occurrence of certain bacterial taxa *in vitro*, further supporting the view that specific interactions may occur between some bacteria and AM fungi.

In a field study with long-term applications of different organic and inorganic fertilisers, amendments with sewage sludge or ammonium sulphate changed the bacterial community composition in root-associated soil aggregates. In addition, ammonium sulphate reduced the taxon richness and the occurrence of specific taxa of both AM fungi and bacteria in the mycorrhizosphere. These changes were primarily explained by the low pH of soils amended with sewage sludge or ammonium sulphate. However, taxon richness of both AM fungi and bacteria also appeared to be promoted by high soil organic content.

In summary, interactions between bacteria and the ERM of AM fungi may be specific and physical associations and mycelial exudates may influence these interactions. Microbial interactions are important in determining soil fertility and plant health. In agricultural systems some microorganisms may be either positively or negatively affected by different types of fertilisation, therefore it is possible soil management practices could be customised to promote microorganisms that are beneficial to farming systems.

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Contents

Appendix

Papers I-IV

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

I.   Johansson, J.F., Paul, L.R. & Finlay, R.D. 2004. Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. *FEMS Microbiology Ecology* 48, 1-13.

II.   Toljander, J.F., Artursson, V., Paul, L.R., Jansson, J.K. & Finlay, R.D. 2006. Attachment of different soil bacteria to arbuscular mycorrhizal fungal extraradical hyphae is determined by hyphal vitality and fungal species. *FEMS Microbiology Letters* 254, 34-40.

III.   Toljander, J.F., Lindahl, B.D., Paul, L.R., Elfstrand, M. & Finlay, R.D. Effects of mycelial exudates from an arbuscular mycorrhizal fungus on soil bacterial community composition *in vitro*. *Submitted manuscript*.

IV.   Toljander, J.F., Santos, J., Tehler, A. & Finlay, R.D. Community composition of arbuscular mycorrhizal fungi and bacteria in the maize mycorrhizosphere in a long-term fertilisation trial. *Manuscript*

Papers I & II are reprinted with permission from the respective publisher

Introduction

Background

Microorganisms are key components of soil biodiversity, determining both soil fertility and plant health. Due to high inputs of pesticides and inorganic fertilisers, the roles of these organisms may have become marginalised in conventional agriculture, leading to a loss of biodiversity as well as function. However, increased environmental awareness in many countries, including Sweden, is progressively leading to a development from conventional intensive agriculture to low-input (sustainable) agriculture. Low-input agriculture refers to farming systems where the use of mineral fertilisers and pesticides is restricted. These agroecosystems are consequently more dependent upon biological control of pests and organic fertilisers to maintain crop health and productivity. It is therefore imperative for us to be able to utilise the potential of, or manipulate, naturally occurring microorganisms. A crucial step towards this is to characterise the organisms involved, their interactions with each other and their abiotic environment. The focus of this thesis is on the extraradical mycelium (ERM) of arbuscular mycorrhizal (AM) fungi, its interactions with soil bacteria, and the possible ecological significance of these interactions.

The AM symbiosis and the mycorrhizosphere

Arbuscular mycorrhiza is an ancient (>460 million years BP) and widespread terrestrial symbiotic association formed between fungi of the phylum Glomeromycota and the roots of vascular plants (Schussler, Schwarzott & Walker, 2001). It is estimated that members of 80% of all vascular plant families, or some 250,000 species of plants, are capable of forming the symbiosis worldwide (Smith & Read, 1997). While the number of plant species capable of forming AM is high, relatively few fungal symbionts have so far been identified. Only some 150 species of AM fungi have been described based on spore morphology (Morton & Benny, 1990), however more recent studies using molecular techniques indicate that the true diversity of this fungal phylum has been underestimated (*e.g.* Husband *et al.*, 2002). The ubiquitous distribution of AM in natural and managed (*e.g.* agricultural) ecosystems, makes it an ecologically as well as economically important symbiotic relationship.

During the formation of the symbiosis, fungal hyphae grow between the root cortical cells, penetrate the cell walls, and form highly branched (arbuscules) or coil shaped (coils) hyphal structures that invaginate the plant cell membrane (Fig. 1). This creates a very large surface area between the two symbionts, across which metabolic exchange can take place. When the plant root is colonised, the AM fungus produces runner hyphae, forming the ERM. The ERM is used by the fungus to explore the soil for resources several centimetres from the colonised root (Jakobsen, Abbott & Robson, 1992; Rhodes & Gerdemann, 1975). A gram of soil can contain up to 30 m of AM fungal extraradical hyphae (Smith & Read, 1997). The immediate surrounding of the extraradical hyphae is commonly referred to as

the *hyphosphere* (cf. "rhizosphere", Hiltner, 1904, Fig. 2), and the soil volume influenced by the combined activities of the root and the mycorrhizal fungus is called *mycorrhizosphere* (Rambelli, 1973, Fig. 2). Because of the small diameter of the individual hyphae and the large numbers of hyphae in the soil, it is possible for the fungus to colonise microsites which the roots cannot access. The large surface area of the AM fungal mycelium may significantly increase the nutrient absorptive surface of the roots (Rambelli, 1973; Rhodes & Gerdemann, 1975).
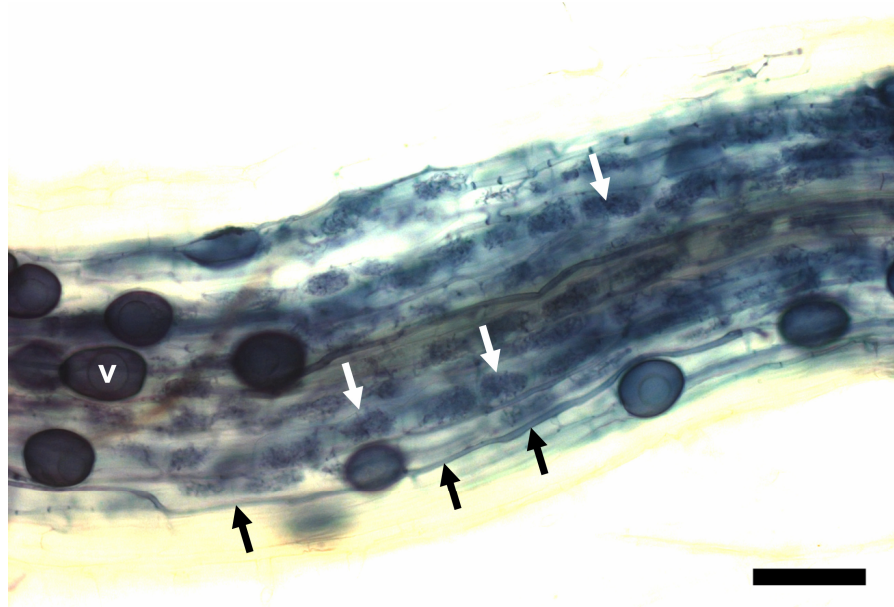

Fig. 1. Structures of an arbuscular mycorrhizal fungus colonising a clover root. Intraradical hyphae growing between the root cortical cells are indicated by black arrows; arbuscules, here seen as dark structures within root cells, are indicated by white arrows; balloon-shaped objects (v) are vesicles, which are fungal storage organs. Bar = 200 μm. (Photograph by Jonas Toljander)

The plant provides the fungus with photosynthetically derived carbohydrates, while the fungus supplies the plant roots with nutrients (Smith & Read, 1997). It is generally believed that the AM fungi primarily are important to their hosts for their superior ability to take up phosphorus which is relatively inaccessible to plants (*e.g.* McGonigle & Miller, 1996; Miller, 2000; Pearson & Jakobsen, 1993). However, the AM association may also increase the phytoavailability of micronutrients, *e.g.* copper and zinc (Smith & Read, 1997). In addition, it has even been suggested that some AM fungi are able to mobilise organically bound nitrogen, inaccessible to plants alone, which is subsequently translocated to the plant host (Hodge, Campbell & Fitter, 2001). This will be discussed in more detail below.

Apart from influencing the quantity and quality of resources available to the plant host, the ERM also provides an interface for interaction with other soil organisms. A considerable amount (10-20%) of photosynthetically derived carbon may be

invested in the mycorrhizal symbiosis by the plant (Johnson *et al.*, 2002). A proportion of the carbon is built into mycorrhizal fungal biomass, and during the fungus' activities, it could be expected that some of this carbon is actively or passively exuded into the surrounding environment and utilised as a source of energy by other microorganisms in the mycorrhizosphere (**Paper I**). In addition, live or dead mycorrhizal fungal hyphae themselves, or compounds released by hyphae, may serve as a nutrient source or substratum for other organisms (**Papers II and III**).
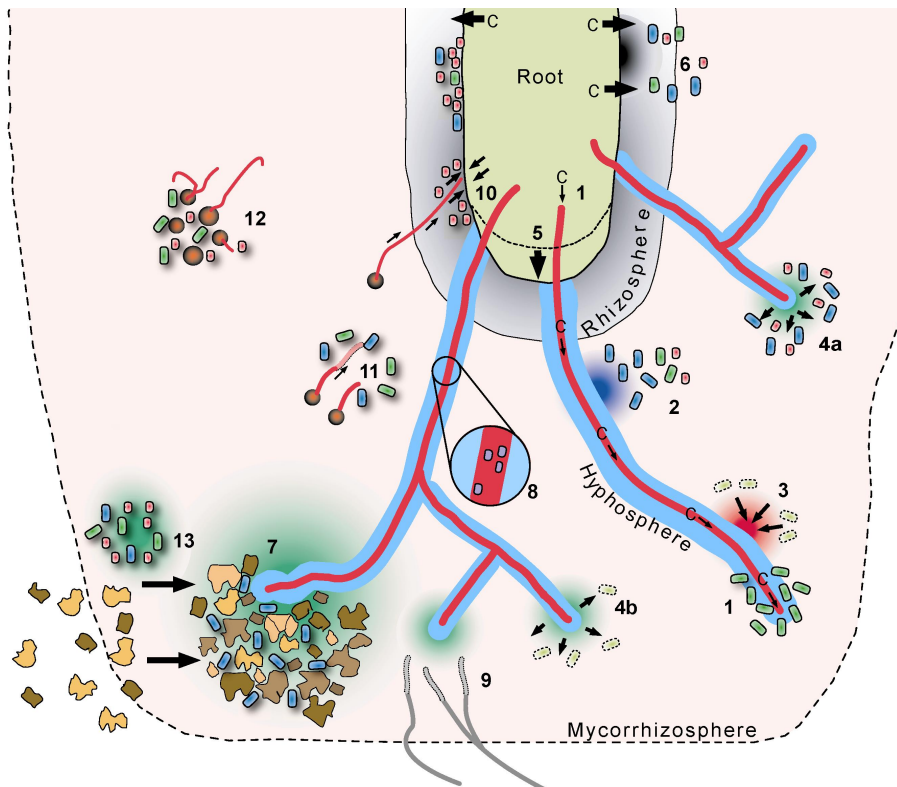
Fig. 2. Schematic view of possible interactions among different components of the mycorrhizosphere. *Indirect and direct influence of AM fungi on other components*: 1. Supply of energy-rich carbohydrates (C) via extraradical hyphae; 2. pH changes; 3. Competition for nutrients; 4. (a) Stimulatory or (b) inhibitory compounds; 5. Stimulated root growth; 6. Changes in root exudation; 7. Changes in soil structure; 8. Endosymbiotic bacteria in AM fungal structures; 9. Interactions with pathogenic fungi. *Effects of bacteria on AM fungi*: 10. Effects on receptivity of roots to AM colonisation; Stimulation or inhibition on (11) growth or (12) germination of AM fungal propagules; 13. Modification of soil chemistry. (Figure is modified from **Paper I**)

Specific associations between AM and bacteria

Arbuscular mycorrhizal colonisation may influence the composition of bacterial communities in the mycorrhizosphere, but little is known about the underlying mechanisms. Proposed general mechanisms include (Fig. 2): supply of energy-rich

compounds via the ERM (Andrade *et al.*, 1997); changes in soil structure due to AM fungal hyphae and mycelial exudates, which may glue soil particles together (Andrade *et al.*, 1998; Tisdall & Oades, 1979); and changes in root exudation patterns induced by AM colonisation (Marschner & Baumann, 2003; Söderberg, Olsson & Bååth, 2002). Changes in bacterial community structure may also be driven by complex interactions between plant species (or genotype) and fungal species involved (Marschner & Baumann, 2003; Marschner & Timonen, 2005). However, bacterial communities in the mycorrhizosphere may in turn influence the mycorrhizal- or plant development in various ways (Fig. 2), further complicating our understanding of bacterial-mycorrhizal interactions. Because of the complex nature of the mycorrhizosphere it has been difficult to couple effects to specific factors/organisms. For example, the changes in microbial communities can be due to direct (*e.g.* belowground carbon allocation) or indirect influence of mycorrhizal colonisation (*e.g.* root exudation) or the combined effects of the mycorrhizal fungi and their biotic and abiotic environment.

An example of an indirect interaction between microorganisms in the mycorrhizosphere is the synergistic interaction between nitrogen fixation in the nodules of legumes and mycorrhizal colonisation of the same plants. Both these processes are symbiotic associations involving the host plant, bacteria belonging to Rhizobiaceae and AM fungi. The increased phosphorus supply provided by the fungus supports the rhizobial production of nitrogenase enzymes, which are important for the nitrogen fixation (Puppi, Azcón & Höflich, 1994). The enhanced nitrogen status of the plant promotes further development of the mycorrhizal symbiosis. An important factor in this interaction may be the small spatial scale, since physical proximity between microorganisms is likely to facilitate interactions between them (see discussions of **Papers I-III**).

Evidence is accumulating that some bacteria live within AM fungi, *i.e.* endosymbiosis. MacDonald *et al.* (1982) observed bacterium-like organisms (BLOs) in the cytoplasm of some AM fungi. The BLOs, however, remained unidentified largely due to difficulties in culturing them. After the advent of PCR based techniques, Bianciotto *et al.* (1996a; 2000) used a combined morphological and molecular approach to study BLOs within the tissues of several species of the AM fungal genera *Gigaspora* and *Scutellospora*. The BLOs were initially identified as *Burkholderia* sp., but were later assigned a new taxonomical identity, *Candidatus* Glomeribacter gigasporarum (Bianciotto *et al.*, 2003). An interesting aspect of these endosymbiotic bacteria is that they are transmitted vertically between fungal generations, suggesting that the bacteria constitute obligate endocellular components of their host fungi (Bianciotto *et al.*, 2004). This deduction is also supported by the fact that total genome size of the endobacterium is estimated to only 1.4 Mb, which is the smallest genome known for a betaproteobacterium (Jargeat *et al.*, 2004). Such small genome sizes are typically found in endocellular bacteria living permanently in their host. The nature of this symbiosis, whether it may be mutualistic, commensalistic or antagonistic, is still unknown, however, the endobacteria cause no apparent harm to the host fungi. *Geosiphon pyriforme* is another example of a fungus in the phylum Glomeromycota that is a host of endosymbiotic organisms. *Ge. pyriforme* is a close

relative of glomalean fungi and it represents the only known example of endocytobiosis between a fungus (macrosymbiont) and a cyanobacterium (Gehrig, Schüssler & Kluge, 1996). Endosymbiotic intracellular bacteria have been identified in ectomycorrhizal (ECM) fungi (Bertaux *et al.*, 2003). These observations indicate that associations between fungi and endobacteria occur within different groups of mycorrhizal fungi, and that mycorrhiza-associated bacteria may be a widespread and ancient phenomenon. It is highly likely that some bacteria and AM fungi have co-evolved over time, and that specialised bacterial communities commonly associate with AM fungi. Consequently, the traditional perception that the mycorrhizal symbiosis is a bipartite association between a plant and a fungus may need to be revised to also include other associated organisms. A first step to understanding the function of these associations is to characterise the organisms involved.

Identifying possible associations between AM and bacteria

Different approaches have been employed in attempts to identify bacteria that are potentially mycorrhiza-associated. Commonly used methods analyse changes in bacterial community composition induced by mycorrhiza. Many earlier studies relied on dilution plate count and various culturing techniques (Ames, Reid & Ingham, 1984; Meyer & Linderman, 1986; Secilia & Bagyaraj, 1987), however more recently, culture independent methods including community fingerprinting methods, such as DGGE or TRFLP have been favoured to characterise bacterial communities in the mycorrhizosphere (Artursson, Finlay & Jansson, 2006a; Marschner & Timonen, 2005; Wamberg *et al.*, 2003).

Another approach is to screen bacterial inoculants for possible affinity to mycorrhizal structures. Physical association between microorganisms may be indicative of a close relationship, since it may facilitate cell-to-cell interactions (**Papers I & II**). Bianciotto *et al*. (1996b) studied the colonisation of spores and hyphae of the AM fungus *Gigaspora margarita* by different strains of fluorescent pseudomonads and rhizobia. Bacteria adhering to fungal tissues typically also adhered to non-living substrata (cotton- and quartz fibres). The authors therefore concluded that specific receptors were not involved in the observed attachment. In another study, a different bacterial strain, *Pseudomonas fluorescens* DF57, did not colonise the hyphae of the AM fungus *Glomus intraradices* (Ravnskov, Nybroe & Jakobsen, 1999). In addition, the fungus had a negative effect on the population size of the introduced bacteria, since both cell counts and culturability were suppressed in hyphosphere soil. The results from these studies suggest that associations between AM fungi and bacteria may be highly specific, and the outcome of the interactions could vary depending on species or strains involved.

A third approach is to screen soil bacteria for possible effects on mycorrhizal fungi. *Paenibacillus* spp. in particular have received much attention as they have been found to interact with AM fungi (Artursson, Finlay & Jansson, 2006a; Bertaux *et al.*, 2003; Mansfeld-Giese, Larsen & Bodker, 2002) or stimulate AM fungal development (Budi *et al.*, 1999; Hildebrandt, Janetta & Bothe, 2002;

Hildebrandt *et al.*, 2006). In addition, further studies have shown that the same *Paenibacillus* spp. also exhibit antagonism against a range of soil borne pathogens (Budi *et al.*, 1999; von der Weid *et al.*, 2003; von der Weid *et al.*, 2005), and could therefore serve as potential inoculants to improve crop yields in low-input agriculture.

Economical and ecological significance of microbial interactions

In low-input cropping systems the natural activities of microorganisms may contribute to the biocontrol of pathogens and improved supply of nutrients, thus maintaining crop health and production. Therefore, the understanding of these interactions and the underlying mechanisms could have implications for the progress of sustainable agriculture. The role of microorganisms in maintaining plant biodiversity and ecosystem functioning was brought into focus by van der Heijden *et al.* (1998). By using macrocosms to simulate old-fields, they showed that plant biodiversity as well as the fundamental ecosystem functions, nutrient capture and plant productivity, increased significantly with increasing species richness of AM fungi, suggesting that these ecosystem functions can be driven by belowground microbial interactions. The results of van der Heijden *et al.* emphasise the need to consider AM fungi, and other microorganims, in management practices. However, only a relatively small number of field studies have investigated the long-term effects of conventional versus sustainable agricultural management practices on microbial diversity in relation to crop production. Helgason *et al.* (1998) showed that the diversity of AM fungal communities was strikingly low in arable sites compared with a woodland. The broad host range of some AM fungi suggests that the change in species composition and low diversity of the fungi in arable fields was probably not the result of plant monoculture, but may reflect other aspects of the agronomic regime such as ploughing, fertilisation or fungicide application. The authors suggested that low AM fungal diversity in arable fields could indicate a smaller functional contribution of AM fungi in these systems. In long term field trials in Switzerland, Mäder *et al.* (2000) found that root length colonised by mycorrhizal fungi in organic farming systems was 30-60% higher than in conventional systems. In addition, there was an overall higher biomass and diversity of soil animals, and a higher microbial activity in organic systems (Mäder *et al.*, 2002). Crop yields were 20% lower in the organic systems, when input of fertiliser and energy (use of fuel, *etc.*) was reduced by 34-53% and pesticide input by 97% (Mäder *et al.*, 2002). They concluded that enhanced soil fertility and higher biodiversity found in organic plots may make these systems less dependent on external inputs. Consequently, the monetary loss in production may, at least to some extent, be compensated for by a reduced need for expensive mineral fertilisers and pesticides.

The high significance of AM associations in the supply of mineral nutrients, in particular phosphorus, to their plant hosts is generally recognised (Caris *et al.*, 1998; Larsen *et al.*, 1996; Miller, 2000; Nielsen *et al.*, 2002; Pearson & Jakobsen, 1993). In addition to this, the possible role of extracellular phosphatase activity of AM fungi, in relation to mineralisation of organic phosphorus pools in soils has

recently attracted attention (Feng *et al.*, 2003; Joner, van Aarle & Vosatka, 2000), although the concept of phosphate solubilising bacteria is probably more well-established. Phosphate solubilising bacteria are widespread in soils and secretion of organic acids and phosphatases are common means for facilitating the conversion of insoluble forms of phosphorus to plant-available forms (Vessey, 2003). The biofertiliser properties of plant growth promoting rhizobacteria are frequently ascribed to their ability to increase the bioavailablity of inorganic and organic phosphorus, and some of these bacteria have documented synergistic effects on *e.g.* nitrogen fixation and formation of mycorrhizal associations. Toro *et al.* (1997) demonstrated that both *Enterobacter* sp. and *Bacillus subtilis* promoted the establishment of the AM fungus *Glomus intraradices* and increased plant biomass as well as tissue nitrogen and phosphorus contents. Kim *et al.* (1998) found that phosphorus content in tomato plants was increased when inoculated with either the AM fungus *G. etunicatum*, or the phosphate solubilising bacterium *Enterobacter agglomerans*. Additionally, the highest nitrogen and phosphorus uptake was observed when tomatoes were inoculated with both organisms, suggesting that bacteria and AM fungi may together increase the nutrient uptake in plants.

It has also been proposed that AM fungi may contribute to the decomposition of organic matter and the subsequent acquisition of organic nitrogen. Hodge *et al.* (2001) grew plants of *Plantago lanceolata* colonised with the AM fungus *Glomus hoi* in compartmentalised soil microcosms. Decomposition of, and nitrogen capture from, dual-labelled ($^{15}N/^{13}C$) plant material was increased by the AM fungus, suggesting that some AM fungi can have saprotrophic capabilities. However, since the microcosms were not monoxenic, it remained a possibility that other microorganisms, possibly in association with the AM fungus, were involved in the decomposition. Using monoxenic cultures of another glomalean fungus, *G. intraradices*, Hawkins *et al.* (2000) showed that the AM fungus alone was indeed able to acquire nitrogen from labelled amino acids, but only at fairly low rates.

The possible significance of synergistic interactions between AM fungi and soil bacteria in relation to general plant nutrition has already been discussed, but in addition to this, microbial interactions may have implications for biological control. A number of studies have indicated that some AM fungi (Caron, 1989; Newsham, Fitter & Watkinson, 1995; Niemira, Hammerschmidt & Safir, 1996), as well as some mycorrhiza associated bacteria (Barea *et al.*, 1998; Budi *et al.*, 1999) exhibit biocontrol properties against root pathogens. Whether AM fungi could be used as biocontrol inoculants practically, or possibly function as co-inoculants with bacteria with biocontrol properties, remains to be explored. A more realistic approach may involve the ability to utilise the potential of indigenous fungi in soils, and promote the activities of beneficial microorganisms already present in the soil.

Objectives

The overall aim of the work described in this thesis was to characterise interactions between AM fungi and soil bacteria and their environment using molecular tools. The specific objectives were:

- To review microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture (**Paper I**). Some aspects of these interactions are investigated in more detail in **Papers II-IV**.

- To examine the ability of different soil bacteria to attach to vital and nonvital extraradical hyphae of different AM fungi *in vitro* (**Paper II**). Physical associations may facilitate metabolic interaction, and therefore the degree of attachment could reflect the trophic status of the bacteria or the ecological function of the association. An increased understanding of these associations has also a direct application when selecting microbial inoculants for biocontrol or biofertilisation

- To characterise the chemical composition of AM fungal extraradical mycelial exudates and their possible influence on soil bacterial community structure *in vitro* (**Paper III**). Very little is known about the range of compounds released by growing AM fungal mycelia, and their effects on other microorganisms. The exudates may have a general stimulatory effect on bacterial growth, but may also specifically influence certain bacteria. Effect of exudates on bacterial communities may reveal synergistic or antagonistic relationships between AM fungi and bacteria.

- To characterise the influence of different long-term fertilisation regimes on the community composition of AM fungi and bacteria in the mycorrhizosphere of maize in a field trial (**Paper IV**). An increasing number of studies report that long term application of mineral fertilisers have detrimental effects on mycorrhizal colonisation, microbial biomass and activity. However, changes in microbial community composition may have consequences for plant productivity. In order to employ management practices that minimise unfavourable effects on the environment and increase the benefits that microorganisms can bring to farming systems (*e.g.* in terms of plant production), there is a need to describe the possible effects of soil management on the composition of microbial communities.

Methods

In the papers of this thesis a number of techniques were employed to characterise the microorganisms and the interactions between AM fungi and bacteria. Since the details of these methods are presented in the respective papers or in articles referred to in the papers, the purpose of this section is only to outline the principal methods.

Mycorrhizal root organ cultures

Arbuscular mycorrhizal fungi are obligate biotrophs, meaning that they are completely reliant upon the presence of host plant roots to survive and grow. For the purpose of scientific studies these fungi have traditionally been grown in pot cultures in co-culture with whole plants, however increasing numbers of studies have utilised axenic Ri T-DNA-transformed root cultures ("hairy roots" or root organ cultures) (Bécard & Fortin, 1988). Although root cultures represent artificial and less complex system than would be encountered in natural soils, they offer the advantage of being able to study AM fungi in an axenic, and hence more controlled, environment where only the fungus and its host are present. In particular, studies of the AM fungal ERM and its interactions with its abiotic and biotic environment have gained by utilising different modifications of these systems (*e.g.* Filion, St-Arnaud & Fortin, 1999; Hawkins, Johansen & George, 2000; Nielsen *et al.*, 2002; Pfeffer *et al.*, 2004; Rufyikiri, Thiry & Declerck, 2003). By using compartmentalised systems, as described in **Paper III**, the ERM can be studied separately without the direct influence of roots. Only very recently there have been a few reports of certain AM fungal strains being able to complete a life cycle in the absence of plant roots, but in the presence of another, bacterial, symbiont (Hildebrandt, Janetta & Bothe, 2002; Hildebrandt *et al.*, 2006). These studies suggest that the AM association could be more complex than previously held, and that the formation of the symbiosis in soils may involve three symbionts or more.

In the study presented in **Paper II**, carrot (*Daucus carota* L.) root organ cultures were used, which had been Ri T-DNA transformed according to Becard and Fortin (1988). The roots were inoculated with either of the fungal strains *Glomus* sp. MUCL 43205 and *G. intraradices* Schenck & Smith MUCL 43194 and routinely maintained on minimal (M) medium (Bécard & Fortin, 1988) and then transferred to liquid M medium prior to the experiment. For **Paper III**, clover (*Trifolium repens* L.) root organ cultures were developed.

Generation of root organ cultures from clover

Paper III root organ cultures were generated of clover by *Agrobacterium rhizogenes*-mediated root transformation essentially as described by Boisson-Dernier *et al*. (2001). The binary vector p35S GUS INT harboured a *gus* gene and a kanamycin resistance gene under the control of the CaMV 35S promoter and *nos*

promoter respectively (Lindroth *et al.*, 1999). Transformed roots emerged from the inoculated explants on M medium containing kanamycin (50 mg l^{-1}) as selective agent. To establish root cultures, viable roots emerging from the inoculation site after approximately three weeks were isolated and cultured on selective medium. After three subsequent subcultures on M medium with antibiotics, root cultures apparently free of microbial contamination were transferred to M medium without antibiotics. Absence of *A. rhizogenes* was verified by PCR with a v*irD3* specific primer combination (Lindroth *et al.*, 1999). Presence of *gus* was confirmed by PCR using specific primers. One fast-growing culture "LP1" supporting amplification of the *gus* insert, without amplifying the ~800 bp v*irD3* fragment was selected (Fig. 3). To maintain the clover root culture it was subcultured on fresh medium every eight to ten weeks.
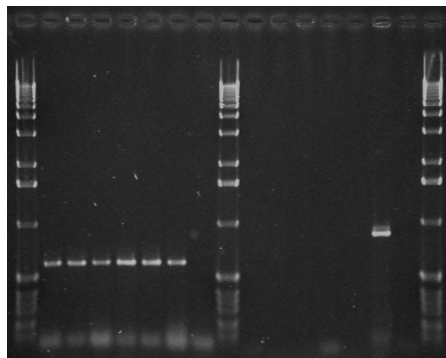

Fig. 3. PCR of crude DNA extracts from five different transgenic *Trifolium repens* L. roots transformed with *Agrobacterium rhizogenes*. From left: Lanes 2-6 amplified *gus*, lane 7 *gus* positive control (p35S GUS INT), lane 8 gus negative control (no DNA template); lanes 10-14 *virD3* (absent), lane 15 *virD3* positive control, lane 16 *virD3* negative control. The *virD3* product has a size of 882 bp and the *gus* product a size of 602 bp. DNA ladder in lanes 1, 9 and 17 represents 1 Kb DNA Ladder (Invitrogen Corporation, Paisley, UK).

The fungal strains

The AM fungal strains used in **Papers II and III** were purchased from GINCO (Louvain-la-Neuve, Belgium), and subcultured in our laboratory on either carrot or clover root organ cultures on M medium. The new clover root organ cultures were successfully colonised with five *Glomus* strains (all affiliated with *Glomus* group A): *Glomus* sp. MUCL 43205, *G. proliferum* Dalpé & Declerck MUCL 41827, *G. intraradices* Schenck & Smith MUCL 43194, *G. intraradices* Schenck & Smith MUCL 43204, and *G. clarum* Nicolson & Schenck MUCL 46238. However, the inoculation of clover roots with the first three of these strains was most successful, in terms of *in vitro* production of spores and ERM. Only *Glomus* sp. MUCL 43205 was used together with clover roots in two-compartment cultures (Fig. 4) in experiments for this thesis (**Paper III**). For **Paper II**, *Glomus* sp. MUCL 43205 and *G. intraradices* MUCL 43194 colonising carrot root organ cultures were used. Both strains were originally isolated from *Fraxinus americana* in Québec, Canada. The latter was the first *G. intraradices* strain to have been propagated on transformed roots (Chabot, Bécard & Piché, 1992), and was selected because it has been used in a range of field- and laboratory studies.
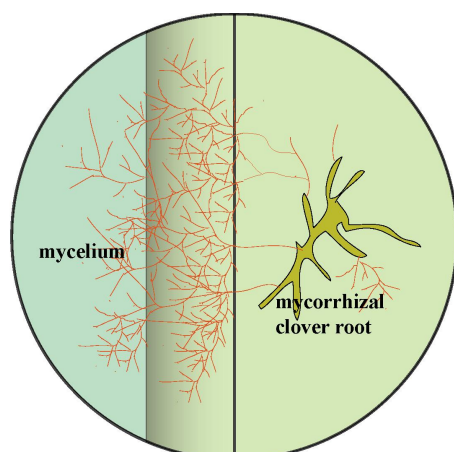
Fig. 4. Schematic view of mycorrhizal root organ culture in the split Petri dish system that was used to produce mycelial exudates in **Paper III**. Arbuscular mycorrhizal extraradical mycelia, but not roots, were allowed to cross the barrier separating the root compartment from the hyphal compartment thus creating an environment influenced only by the fungal mycelium. Mycelial exudates were collected and incubated with soil bacterial communities, to study the specific effect of exudates on different bacterial taxa. (Figure is reproduced from **Paper III**).
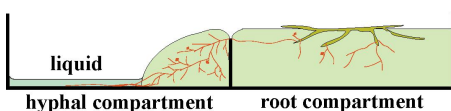
Visualisation of microorganisms

Green fluorescent protein tagging of bacteria

The green fluorescent protein (GFP) is a useful reporter molecule for monitoring *in vivo* gene expression in eukaryotic and prokaryotic cells (Fortineau *et al.*, 2000). The gene encoding the protein, *gfp*, was originally isolated from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992). The protein emits green light when excited with ultraviolet radiation, without the requirement of substrate or cofactor for fluorescence. To visualise and facilitate quantification of bacterial attachment to AM fungal extraradical hyphae in **Paper II,** five different strains of soil bacteria, each tagged with the *gfp* gene, were used. *Paenibacillus brasilensis* PB177 and *Paenibacillus peoriae* NRRL BD62 were both previously transformed (Artursson & Jansson, 2003; von der Weid *et al.*, 2005) with the *pnf8* plasmid containing *gfp* under the control of the strong constitutive promoter from *Listeria* (Fortineau *et al.*, 2000). *Arthrobacter chlorophenolicus* A6G (Elvang *et al.*, 2001) and *Pseudomonas fluorescens* SBW25 *gfp/lux* (Unge *et al.*, 1999) were previously chromosomally tagged with *gfp* or both *gfp* and *luxAB* genes (encoding bacterial luciferase), respectively, using mini-transposon vectors. All of the bacterial strains were routinely grown in Luria–Bertani medium. Erythromycin was added to the medium to select for growth of those bacteria that were tagged with the *pnf8* plasmid to ensure stability of the plasmid and the GFP phenotype. Following short-term incubation experiments with each of the *gfp*-tagged bacterial isolates and vital or nonvital hyphae, fluorescing bacterial cells attached to the surface of AM fungal hyphae were quantified using a fluorescence microscope.

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Fungal hyphal vitality

In **Paper II** vital and nonvital extraradical hyphae of the two AM fungal isolates *Glomus* sp. MUCL 43205 and *G. intraradices* MUCL 43194 were used in attachment assays involving single bacterial isolates. To produce nonvital extraradical hyphae, hyphae were excised from axenic mycorrhizal root cultures (*Daucus carota* L.) grown in liquid media ca. 7 days prior to the experiment. At the time of performing the experiment, excised hyphae, as well as hyphae from intact mycorrhizal cultures, were randomly collected and subjected to staining using fluorescein diacetate (FDA) (Söderström, 1979). Fluorescein diacetate is a non-fluorescent compound which can pass through the cell membrane. When taken up by the hyphae, intracellular esterases cleave off the diacetate upon which a highly fluorescent product (fluorescein) is produced and can be detected using a fluorescence microscope. Because cells with compromised membrane integrity rapidly leak out the product, green-fluorescing hyphae can be assumed to be vital. In addition to the visual vitality assay of hyphae, other observations were made: Nonvital hyphae were generally more brittle than vital hyphae, and vital hyphae tended to be more flexible. However, being more subjective than the FDA staining, these observations were not used to determine hyphal vitality.

Bacterial cell vitality

In **Paper III** bacterial numbers and vitality were quantified to monitor bacterial growth over time in the mycorrhizal and non-mycorrhizal treatment. To rapidly distinguish live bacteria from dead the LIVE/DEAD® *Bac*Light™ Bacterial Vitality kit (Molecular Probes, Leiden, The Netherlands) was used. With this method two nucleic acid stains, SYTO 9 dye (cell-permeant) and propidium iodide (cell-impermeant) are added to a bacterial suspension. The dyes cause live bacteria to fluoresce green and dead bacteria to fluoresce red under a fluorescence microscope. Live and dead cells are quantified using fluorescence microscopy and thereby the proportion of live to dead, or live to total cells, can then be calculated and used to estimate total bacterial vitality or population growth.

Characterisation of microbial communities

Terminal restriction fragment length polymorphism of bacterial communities

In order to characterise the composition of bacterial communities in samples of **Papers III and IV** the PCR based method terminal restriction fragment length polymorphism (TRFLP) was employed. With this method DNA template is amplified using fluorescently labelled primers which are homologous to a conserved sequence of the desired gene (usually the 16S rRNA gene for bacteria). In both **Papers III and IV**, the universal eubacterial forward primer 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 534r (5'-ATT ACC GCG GCT GCT GG-3') were used in combination for TRFLP PCR. Each primer was labelled with fluorescent labels in the 5'-end. This set of primers amplifies a ~500 base pair (bp) region of the bacterial 16S rDNA sequence. Following PCR, the

amplified products were digested with restriction endonucleases to produce restriction fragments of varying sizes. For **Paper III** the restriction endonucleases *Cfo*I, *Hae*III and *Msp*I were used, and for **Paper IV** only *Cfo*I and *Hae*III were used. The sizes of the obtained fragments depend on the presence and location of restriction sites recognised by the endonucleases, which is sequence specific and therefore potentially taxon specific. An internal fluorescent DNA size standard is added to each of the samples, which are then analysed using an automated fluorescence-based DNA sequencing instrument. In this process the DNA fragments are separated by capillary electrophoresis. Terminal restriction fragments (TRFs) with the fluorescent 5'-label can thereby be detected and assigned a fragment size measured in base pairs.

The method has several advantages compared with its predecessor RFLP. TRFLP is a high-throughput method employing high-resolution separation of fragments by capillary electrophoresis, rather than low-resolution agarose gel separation. In addition, automated fluorescence-based detection enables sensitive detection of fragments at very low concentrations (Felske & Osborn, 2005). Consequently, TRFs that would not be distinguished with RFLP can be detected. Typically, in an environmental sample many fragments of different sizes, each potentially representing a taxon, will be detected. The fragments, or peaks, can be assigned putative identities in several ways: by comparing fragments against fragments produced from clones or isolates with a known identity; by comparison against a public TRFLP database; by comparing the obtained fragment size with the predicted size, by studying the location of specific restriction sites in the DNA sequences of identified clones/isolates.

Although TRFLP and other community profiling methods are sensitive methods to detect species in environmental samples, all PCR based methods have inherent problems that need to be considered when interpreting community profiles. PCR primers may have a higher affinity to the DNA belonging to certain taxa, resulting in a skewed representation of species abundance (Allmér, 2005). Furthermore, bias in the PCR reaction caused by impurities in the sample, the relative proportions of reaction constituents (chemicals, and target and non-target DNA) may also be introduced. To test these assumptions, the effect of template dilution prior to PCR and TRFLP on relative abundance of restriction fragments was investigated. Undiluted and diluted samples were all subjected to TRFLP PCR, and amplified products were subsequently digested using *Cfo*I. Following TRFLP the relative abundance of TRFs within each sample was calculated by dividing the area of each peak (TRF) by the total peak area in the sample. This simple test showed that in three out of four dilution series, the ratio between individual peaks in a sample was altered by dilution. In the most extreme case, the relationship between peaks that were high or low in abundance was reversed, even though samples had only been diluted 1:2 (Fig. 5). This indicated that the relative abundance of peaks should be interpreted with great caution when translating the TRFLP profiles into relative abundance of taxa in environmental samples. The occurrence of bacterial taxa identified by TRFLP in **Papers III and IV** was therefore only recorded in terms of presence, and not their relative abundance abundance, in samples.
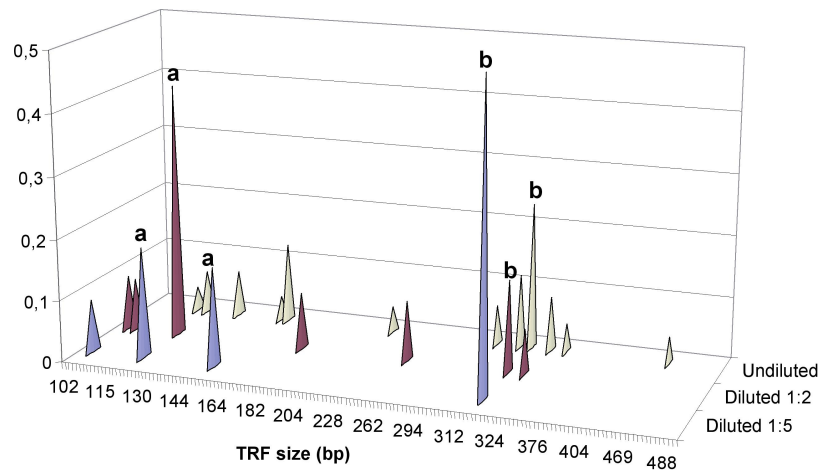
Fig. 5. Example of the effect of dilution of DNA template prior to PCR and TRFLP on the the relative abundance of terminal restriction fragments (TRFs) in an environmental sample (unpublished data). Relative abundance of TRFs (peaks) has been calculated by dividing the area of each peak by the total peak area in the sample. Each TRF represents a putative bacterial taxon. The figure shows the TRF profile of an undiluted sample, and the same sample diluted two and five times, respectively. For the undiluted sample, the ratio of a:b = 0.25; for the sample diluted two times a:b = 2.67; for the sample diluted five times a:b = 0.36. The test demonstrated that even a small dilution of template before PCR may cause the relative abundance of peaks to be reversed.

AM fungal community composition

For **Paper IV**, AM fungal community composition was analysed by PCR and cloning of fungal 18S rDNA in maize roots, and subsequent sequencing of clone inserts. For PCR the universal eukaryotic primer NS31 (Simon, Lalonde & Bruns, 1992) was used as forward primer. As reverse primers a mixture of three different primers was used: the AM1 primer (Helgason *et al.*, 1998), the AM2 (5'-GTT TCC CGT AAG GTG CCA AA-3') and AM3 (5'-GTT TCC CGT AAG GTG CCG AA-3') (Santos, Finlay & Tehler, In Press). The AM1 primer was originally designed as AM fungal specific, but results in important mismatches with sequences of certain glomalean groups. The AM2 and the AM3 are designed to match the same priming site as AM1 in taxa belonging to *Glomus* group B and group C, respectively, as defined by Schüssler *et al.* (Schüssler *et al.*, 2001). This primer combination amplifies a 550 bp long sequence in the SSU 18 rRNA gene. Following cloning of PCR products, clones were collected and reamplified using the same primers as above. Purified, reamplified fragments were subsequently sequenced using the NS31 primer as sequencing primer.

Analysis of chemical composition of hyphal exudates

In **Paper III**, analysis of the composition of hyphal exudates of *Glomus* sp. MUCL 43205 was performed by proton nuclear magnetic spectrometry (HNMR). A portion of the liquid from each culture was vacuum-centrifuged at 40ºC until dry. The dry residues were washed with deuterium oxide (D_2O) and then vacuum-centrifuged again. The washing procedure was repeated three times. Washed and dried samples were subjected to analysis performed at the Department of Chemistry, SLU, Uppsala, Sweden.

Results and discussion

Interactions between extraradical mycelia and soil bacteria

The continuous provision of energy-rich compounds, coupled with the large surface area of the mycelium that interacts with the surrounding soil environment, suggests that the AM fungal ERM constitutes an important niche for bacterial colonisation and growth. In **Paper II** *Paenibacillus brasilensis* PB177 showed a higher attachment to vital hyphae of both *Glomus* sp. MUCL 43205 (Fig. 6) and *G. intraradices* MUCL 43194 *in vitro*. This bacterial strain, along with several other described *Paenibacillus* spp., has documented antagonistic effects on plant pathogenic soil fungi (von der Weid *et al.*, 2003; von der Weid *et al.*, 2005). Many *Paenibacillus* spp. are also nitrogen fixers, exhibiting stimulatory effects on AM development (Budi *et al.*, 1999; Hildebrandt, Janetta & Bothe, 2002; Hildebrandt *et al.*, 2006). Synergistic interactions between microorganisms may have implications for biocontrol or biofertilisation. The degree of dependence on mycorrhizal fungi exhibited by the bacteria may determine the fate of these bacteria in soils, and consequently their usefulness as inoculants. For example, bacterial strains closely associated with AM fungi may utilise fungal hyphae as a substratum, and hyphal exudates for sustenance. Arbuscular mycorrhizal fungi may thereby aid dispersal and persistence of some bacteria in the soil environment.

Other bacteria associating with mycorrhizal fungal hyphae may act as saprotrophs and derive carbon and other nutrients through degradation of fungal hyphae. In **Paper II**, *Bacillus cereus* VA1 showed higher attachment to nonvital hyphae of both glomalean fungi in the assay (Fig. 6). In another study (Artursson & Jansson, 2003) the same *B. cereus* strain was frequently associated with damaged parts of hyphae, indicating that these bacteria were utilising the hyphae, or cell contents released from the hyphae, as a substrate. Staddon *et al*. (2003) demonstrated a rapid turnover of AM fungal hyphae in soil. Their results suggested that AM fungal hyphae in soils live on average only 5–6 days, contributing to a rapid pathway for carbon in soil ecosystems. The results of **Paper II** suggest that *B. cereus* may behave as a saprotroph or mycoparasite. Saprotrophic activity contributing to the degradation of hyphae and utilisation of compounds released by damaged hyphae may have contributed to the rapid hyphal turnover observed by Staddon *et al*.
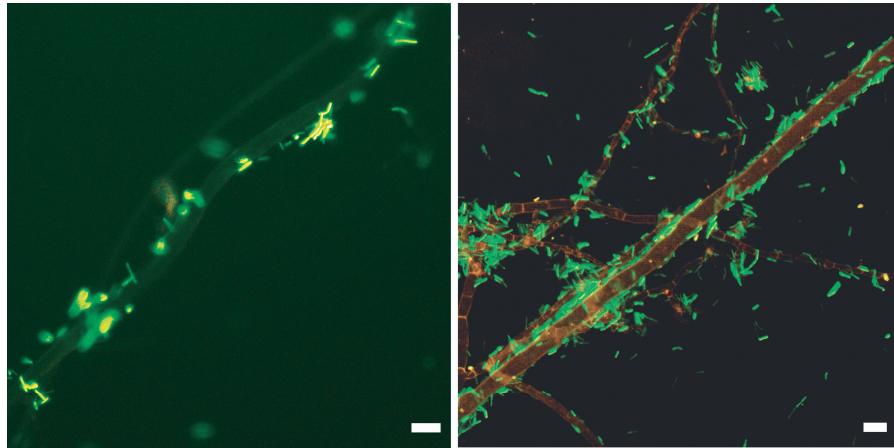
Fig. 6. Examples of attachment of *gfp*-tagged bacteria to extraradical hyphae of an arbuscular mycorrhizal fungus (*Glomus* sp. MUCL 43205): Attachment of *Paenibacillus brasilensis* PB177 to vital hyphae (left); attachment of *Bacillus cereus* VA1 to nonvital hyphae (right). Bars = 10 μm (Both photographs are reproduced from **Paper II**)

In **Paper II**, individual bacterial strains exhibited differences in their degree of attachment to vital hyphae of the two tested fungi. *Pseudomonas fluorescens* SBW25 showed a greater attachment to vital than to nonvital hyphae of *Glomus* sp. MUCL 43205, whereas this trend was opposite for the attachment to *G. intraradices* hyphae. *Paenibacillus peoriae* BD62 did not attach to *Glomus* sp. MUCL 43205 at all, but showed a high attachment to nonvital hyphae of *G. intraradices*. These observations indicate that in addition to hyphal vitality, other factors may influence bacterial attachment to hyphae. Differential attachment may also be caused by differences in hyphal surfaces at the cellular level. Another possibility is that the quantity or quality of compounds of mycelial origin may differ between fungal strains. The growth and attachment of certain bacteria may therefore be promoted or inhibited accordingly.

It is unclear to which extent mycelia, compared with roots, contribute to the total quantity of carbon available for consumption by soil organisms. There may however be qualitative differences between fungal mycelia and plant roots in the composition of exudates. In addition, root deposition is localised in the vicinity of the root systems, whereas fungal mycelia can create micro-niches or access fractions of the soil which roots alone never access. Consequently, the carbon released by active mycelia, or by hyphae being decomposed, may create hotspots in the soil, and could therefore significantly influence the activity of other organisms where mycelia are present.

Fungal metabolites actively or passively exuded by AM fungal hyphae may result in general attraction and growth stimulation of some bacteria that utilise exuded compounds for growth. However, it is also possible that some bacteria are suppressed by inhibitory compounds produced by AM fungi. Antagonism of single bacterial or fungal isolates by AM fungi has previously been reported (Filion, St-

Arnaud & Fortin, 1999; Ravnskov, Nybroe & Jakobsen, 1999). It seems likely that such antagonistic interactions are mediated by the AM fungal exudation of compounds with inhibitory or antimicrobial properties. While there is information on the metabolism and translocation of carbon within AM fungal tissues (Bago *et al.*, 2002), the nature of compounds that are actively or passively exuded by mycelia, and their possible effects on their biotic and abiotic environments are not well-known. Some ectomycorrhizal (ECM) fungi, however, have been shown to exude acetic and oxalic acid into their environment (Sun *et al.*, 1999), and ECM fungal production of siderophores and their possible relevance in mineral weathering and nutrient acquisition has also been discussed (Holmström *et al.*, 2004; van Hees *et al.*, 2006). Allen *et al.* (1996) used scanning electron microscopy to examine production of oxalate crystals on the surfaces of ecto- and arbuscular mycorrhizal fungal hyphae. Only hyphae of some mat-forming ectomycorrhizal fungal species had crystal structures associated with them. Their data suggested that there are fundamental differences in chemical exudation between mycorrhizal fungi, and that this could have implications for uptake and cycling of phosphorus in soils. Organic acids may in addition increase the bioavailability of inorganic phosphorus and metallic ions. Consequently, mycelial exudation and nutrient acquisition by fungi could indirectly influence the composition of bacterial communities in the soil, through competition with other microorganisms for nutrients.

Our results (**Paper III**) show that AM fungal mycelia may exude simple carbohydrates, in the form of glucose, as well as low molecular weight aliphatic acids. Organic acids exuded by roots or mycorrhizal fungal mycelia may be utilised as sources of carbon and nutrients or as chemoattractants for some microorganisms (Grayston, Vaughan & Jones, 1997; Sood, 2003). The presence of mycelial exudates from *Glomus* sp. MUCL 43205 had a general stimulatory effect on total bacterial growth (Fig. 7) and changed the composition of the bacterial community, with certain bacterial taxa either decreasing or increasing in occurrence. The occurrence of several taxa, in particular three taxa related to *Buttiauxella gaviniae* (gammaproteobacteria), *Legionella wadsworthii* (gammaproteobacteria), *Flavobacterium* sp. (flavobacteria), was significantly increased in response to the mycelial exudates. Several of the bacterial taxa with an increased occurrence in response to mycelial exudates were gammaproteobacteria. In contrast, none of the taxa with a higher frequency of occurrence in samples without AM fungal mycelial exudates was affiliated to this class of bacteria. This is in agreement with many studies reporting synergistic interactions between AM and gammaproteobacteria, leading to increased mycorrhizal colonisation and nutrient uptake in plants (see Artursson, Finlay & Jansson, 2006b and references therein). As indicated in the introduction, bacterial strains belonging to Enterobacteriaceae have previously been shown to promote mycorrhizal establishment (Toro, Azcon & Barea, 1997) and plant nitrogen and phosphorus uptake (Kim, Jordan & McDonald, 1998; Toro, Azcon & Barea, 1997), which may suggest that these strains are interacting synergistically with AM fungi. Indeed, the gammaproteobacterium identified as *Buttiauxella gaviniae* (Enterobacteriaceae) in **Paper III** increased in frequency of occurrence in response mycelial exudates from an AM fungus.
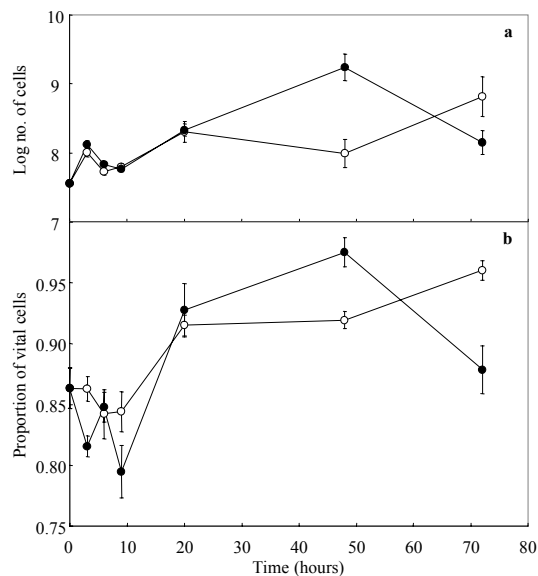
Fig. 7. Bacterial population growth and vitality at different times post incubation in the presence and absence of mycelial exudates from an arbuscular mycorrhizal fungus (*Glomus* sp. MUCL 43205). (a) Log total number of cells counted at each sampling occasion. (b) Proportion of vital cells, calculated as the number of vital cells divided by the number vital and nonvital cells. Closed and open circles symbolise mean values of samples with or without mycelial exudates, respectively. Error bars represent standard error of the means. Y axes are broken and do not begin at zero. (Figure is reproduced from **Paper III**)

The results from **Papers II and III** support the view that interactions between certain bacteria and AM fungi are commonplace, and that there is a degree of specificity in these interactions. These interactions may be facilitated by the physical proximity between the symbiotic organisms. The nature of these interactions, whether mutualistic or parasitic, is highly dependent on which interacting organisms are involved, and may be determined on the species-, or strain level. However, there are also opportunistic bacteria that at times rely on mycelial products released by AM fungi, or bacteria that may utilise the hyphae themselves as a nutrient source or substratum. The understanding of interactions occurring between the AM fungal ERM and its biotic and abiotic environment is still in its infancy. The characterisation of the composition of AM fungal exudates and the effects of these compounds on soil microbial communities may have wide implications: from understanding the specific interactions between the microorganisms in the mycorrhizosphere, to more applied areas of research, such as the study of nutrient cycling and contribution of soil microbial interactions to soil fertility and crop production.

Influence of different nitrogen fertilisers on soil microbial community composition

In **Paper I** the significance of interactions occurring between AM fungi and other microorganisms to sustainable agriculture is outlined. Clearly microorganisms play crucial roles in soil processes of both natural and managed ecosystems, however it is also evident from a large number of studies that intensive management practices (*i.e.* tillage, mono-cropping, landscape homogeneity, and application of mineral fertilisers) can have detrimental effects on the activity and occurrence of a wide range of organism groups (Eriksson, 1999; Gosling *et al.*, 2006; Mäder *et al.*, 2002; Nyberg, 2006; Salles, 2005; Weibull, 2002). Many studies reporting

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negative effects of intensive management practices on soil microorganisms have mainly considered total microbial activity or microbial biomass. As indicated by van der Heijden *et al.* (1998) it may also be important to consider the species composition of these microbial communities, since microbial diversity alone may be an important factor determining ecosystem functions. Removal of crops at harvest, or crop rotation using non-mycorrhizal plants, each represent disturbances, which are also likely to have profound effects on the composition of microbial communities belowground. In order to employ management practices to increase the benefits that microorganisms can bring to sustainable farming systems, we need to describe the possible effects of soil management on the composition of microbial communities.

We therefore aimed to describe the changes in community composition of AM fungi and bacteria in the mycorrhizosphere of maize (*Zea mays* L.) in response to long-term application of different organic and mineral nitrogen fertilisers (**Paper IV**). An additional aim of this study was to see how removal of crops influenced the community composition of bacteria. At crop harvest the allocation of photosynthates to the mycorrhizal roots is interrupted, resulting in a decline in the mycorrhizal fungal population and, presumably, also in bacteria that associate with the living mycorrhizal roots. A pulsed input of carbon in the form of senescing roots and mycorrhizal fungal mycelia in the soil may favour certain other groups of microorganisms that are saprotrophic or mycotrophic.

The main finding presented in **Paper IV** was that both numbers of taxa and the occurrence of specific AM fungal and bacterial taxa were influenced by the fertilisation treatments. Overall, fewer AM fungal and bacterial taxa were detected in plots amended with $(NH_4)_2SO_4$, whereas the number of taxa in the different treatments tended to increase with soil pH and organic amendments. *Glomus intraradices* was detected in all treatments except in the one amended with $(NH_4)_2SO_4$, and there was a significant reduction in the occurrence of six different bacterial taxa in plots fertilised with $(NH_4)_2SO_4$. In addition, most of the bacterial taxa that occurred in all treatments were recorded in a smaller number of samples in that treatment. This indicates that there may have been an overall decrease in bacterial and AM fungal population density in plots amended with $(NH_4)_2SO_4$, causing these taxa to be excluded from detection. This is supported by previous studies at this experimental site, demonstrating that long-term fertilisation with $(NH_4)_2SO_4$ can result in a reduction in the ratio of microbial biomass-C to soil-C (Witter & Dahlin, 1995; Witter, Mårtensson & Garcia, 1993) and basal respiration (Nyberg, 2006).

In **Paper IV**, the bacterial community composition of the two treatments, $(NH_4)_2SO_4$ and sewage sludge, were markedly different from the other treatments, and the two treatments were, in addition, different from each other (Fig. 8). The differences in bacterial community composition in plots amended with $(NH_4)_2SO_4$ and sewage sludge, compared with the other treatments, was primarily related to the lower pH of soils amended with $(NH_4)_2SO_4$ and sewage sludge. The bacterial community composition was also related to the soil carbon content, which may explain some of the differences in bacterial community composition between

$(NH_4)_2SO_4$ and sewage sludge amended plots. It is also possible that the high metal concentrations of the sewage sludge, or bacterial communities brought with that fertiliser, were influential in determining the bacterial community composition.
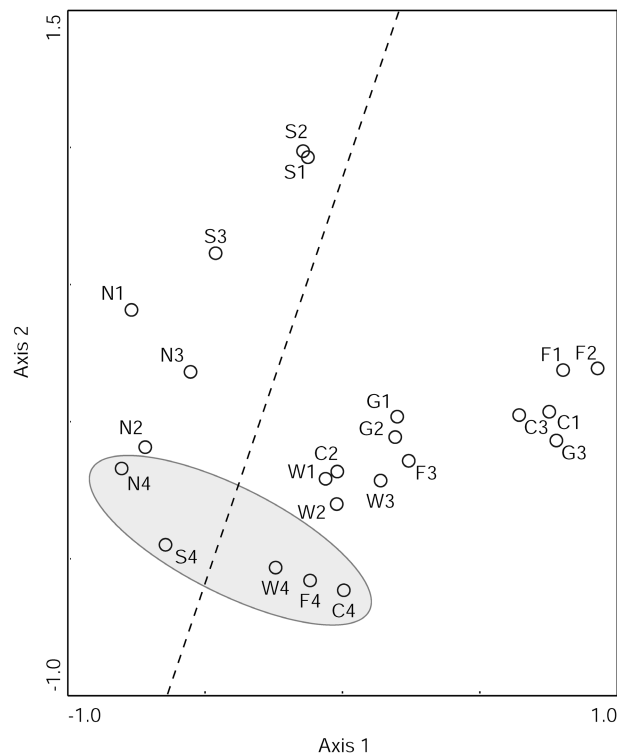

Fig. 8. Scatter plot derived from Principal component analysis (PCA) of bacterial TRFLP profiles in root-associated soil aggregates from a Swedish long-term field experiment with amendments of different organic and inorganic nitrogen fertilisers. The cumulative variance in the species data explained by the two first axes in the PCA is 43.8%. The dashed line separates samples from (N) and (S) from the other treatments (W; C; G; F). The shaded area highlights samples taken three weeks after crop harvest. Treatment abbreviations: W without organic material or mineral fertiliser; C $Ca(NO_3)_2$; N $(NH_4)_2SO_4$; G green manure; F farmyard manure; S sewage sludge. Numbers (1-4) after treatment abbreviations in the plot denote time of sampling: 1 July; 2 August; 3 September; 4 October – post harvest. Data for green manure (G) at the October sampling are not available. (Figure is reproduced from **Paper IV**)

Long-term application of mineral nitrogen in the form of $(NH_4)_2SO_4$ had a negative impact on AM fungal and bacterial diversity. In comparison, more AM fungal species and bacterial taxa were detected in plots amended with $Ca(NO_3)_2$, and there was in addition a positive effect on *Glomus intraradices*. This species has previously been reported as being 'nitrophilic' (Jumpponen *et al.*, 2005; Scheublin *et al*., 2004). The occurrence of this AM fungus in all the fertiliser treatments (except $(NH_4)_2SO_4$) may reflect its wide ecological adaptation, or possibly its tolerance to fertilisation and other soil management. Helgason *et al.* (1998) showed that diversity of AM fungal communities of arable fields was lower compared with a woodland. In all the arable fields, regardless of host plant or location, the

dominant AM fungal type was a putative *G. mosseae* not found in the woodland. This species is known to sporulate abundantly and colonises readily from spores, which may be more important in a field that is ploughed annually than in woodland. Several studies have reported decreases in levels of root colonisation (Jensen & Jakobsen, 1980; Mäder *et al.*, 2000), sporulation (Jensen & Jakobsen, 1980) or mycelial development (Gryndler *et al.*, 2001) of AM fungi in response to mineral fertilisers. These studies indicate that nitrogen fertilisation can have a negative impact on the functional structures of AM fungi. The effects of mineral nitrogen on AM fungal community composition are, however, not well-documented. In a previous study a negative correlation was observed between soil mineral nitrogen and the number of AM fungal sequence groups in the roots of *Festuca pratensis* and *Achillea millefolium* along a nitrogen gradient of a Swedish pasture (Santos, Finlay & Tehler, In Press), suggesting that N fertilisation may negatively affect species richness of AM fungi colonising plant roots. In **Paper IV,** the highest number of glomalean species was detected in two treatments (without nitrogen fertilisation; farmyard manure) exhibiting high soil pH, and intermediate-to high levels of total nitrogen.

Time of sampling or harvest did not affect taxon richness of bacteria, but did influence the composition of the bacterial community in the mycorrhizosphere, as indicated by the ordination graphs and the record of taxa found in samples at different sampling times (**Paper IV**). A number of taxa either increased or decreased in frequency of occurrence over time, however only a small number of taxa did so significantly in response to crop harvest (after third sampling), which may be surprising considering the apparently strong correlation between community composition and time of sampling shown in the Redundancy analysis (see **Paper IV**). This is, however, explained by the stringent analysis of species data employed in **Paper IV**, which only considered highly significant ($P < 0.01$) effects of treatment or time of sampling on the bacterial frequency of occurrence.

The results from **Paper IV** suggest that changes of AM fungal and bacterial community composition in the field trial were primarily linked to differences in soil pH induced by fertilisation. Due to the fact that taxon richness of bacteria was also high in plots with sewage sludge amendment, which exhibited a high soil carbon content and low pH, the low pH alone may not explain all changes in microbial community composition. The soil organic matter itself represents a source of carbon and nutrients, which may favour some microorganisms. In addition, stunted plant growth due to long-term $(NH_4)_2SO_4$ application may have resulted in plants with small root systems, unable to support a large microbial population belowground, *e.g.* leading to roots with an overall lower level of mycorrhizal colonisation. Low pH in combination with the high phosphorus availability in the sewage sludge treatment may have inhibited AM fungal development and favoured the growth of bacteria. There was also a clear influence of time of sampling, in particular of the last sampling occasion, on the community composition of bacteria. This showed that in addition to the seasonal effect, coupled to climatic conditions and crop growth, there was an effect of crop harvest. This suggests that there was a shift in the bacterial community composition, from bacteria that are dependent on living mycorrhizal roots, to a community dominated

by saprotrophic (and mycotrophic) bacteria. However, due to the limited amount of data for the AM fungal community, no relationship between AM fungal and bacterial community composition could be demonstrated. **Paper IV,** and other studies cited above, show that the structure of microbial communities can be modified according to different agricultural practices. Certain groups of microrganisms may be more sensitive than others in agroecosystems. For example, tillage represents a major disturbance for symbiotic fungi, because the mycelial network in the soil is disrupted (Jansa *et al.*, 2003). In addition, organisms that interact with living roots are dependent on the presence of host plants and, potentially, plant species composition. A relationship between the aboveground plant diversity and belowground microbial diversity has been implied in a number of studies (*e.g.* Johnson *et al.*, 2004; O'Connor, Smith & Smith, 2002; van der Heijden *et al.*, 1998), however the influence of plant species composition on belowground microbial community composition is poorly understood. Kowalchuk *et al.* (2002) investigated bacterial community structure in bulk soil and in the rhizosphere of soils with different compositions of plant species. They showed that there was a plant induced-effect on the bacterial community structure. Rhizosphere soil from experimental plots with lower plant diversity showed lower bacterial diversity. In addition, bacterial diversity was generally lower in the rhizosphere than in bulk soil. It was hypothesised that certain microbial functional groups (*e.g.* mycorrhizal fungi or obligate root pathogens) which are dependent on direct associations with plant roots are likely to be affected more by plant diversity. Therefore, the type and level of interaction between microorganisms and plants, rather than the plant species composition alone, may significantly influence the composition of soil microbial communities. In summary, the combined effects of monoculture, tillage and other management practices have profound effects on soil microbial communities. In a historical perspective it appears possible that millennia of agriculture have favoured certain microbial groups, leading to a general decrease in the diversity of mycorrhizal fungi and bacteria.

General conclusions

- The mycorrhizosphere constitutes an important niche for microbial activity. Microbial interactions in the mycorrhizosphere are determinants of soil fertility and plant health. Our understanding of these interactions therefore has implications for sustainable soil management (**Paper I**).

- Soil bacteria differ in their ability to attach to AM fungal hyphae. These physical associations between bacteria and fungi can be influenced by hyphal vitality and the fungal strain concerned. The degree of attachment of bacteria to living or dead hyphae may reflect the ecological niche differentiation of these bacteria, and consequently their trophic relationship (*e.g.* biotrophic or saprotrophic) to the mycorrhizal fungi (**Paper II**).

- In addition to the physical proximity of the interacting organisms, low molecular weight carbohydrates of mycelial origin may also mediate

interactions between AM fungi and bacteria. Mycelial exudates can promote the occurrence of certain bacterial taxa, further supporting the view that specific interactions may occur between some bacteria and AM fungi (**Paper III**).

- Intensive soil management can be detrimental to the diversity and activity of AM fungi and mycorrhizosphere bacteria (**Papers I and IV**). In general, taxon richness and the occurrence of specific taxa of both AM fungi and bacteria in the mycorrhizosphere were reduced in soils with the lowest pH. In addition a higher taxon richness of both AM fungi and bacteria tended to be associated with soil with high organic content (**Paper IV**). Crop harvest changed the soil bacterial community composition, probably leading to an increase of saprotrophic bacteria. Agricultural soil management practices can be customised to promote beneficial microorganisms in order to increase the benefit these organisms could bring to farming systems, *e.g.* in terms of increased productivity and a reduced need for application of mineral fertilisers or pesticides.

Outlook

Many studies of bacterial-AM interactions have only utilised culturable strains of bacteria. However, since most bacteria in soils are unculturable, potentially important interactions between these organisms may have been overlooked. Alternative culturing methods to isolate previously uncultured, slow-growing, bacteria are now available (Zengler *et al.*, 2002). The utilisation of these techniques, possibly in combination with culture independent methods, may further increase our knowledge of microbial communities and the interactions occurring in the mycorrhizosphere. These methods are also likely to improve taxonomical descriptions of bacterial taxa that are currently only described as uncultured or unknown sequences in public databases. This thesis provides a preliminary description of mycelial exudates produced by an AM fungal strain. The continued characterisation of the mycelial exudates of different AM fungi and their involvement in interactions with their biotic and abiotic environment *in situ* will also improve our understanding of the ecological roles of AM fungi.

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List of abbreviations used in this thesis

AM   Arbuscular mycorrhiza(l)
BLO   Bacterium-like organism
CaMV 35S   Cauliflower Mosaic Virus 35S
DGGE   Denaturing gradient gel electrophoresis
ECM   Ectomycorrhiza(l)
ERM   Extraradical mycelium/mycelia
FDA   Fluorescein diacetate
GFP   Green fluorescent protein
GUS   β-glucosidase
HNMR   Proton nuclear magnetic resonance
luxAB   Luciferase A and B genes
PCA   Principal component analysis
rDNA   Ribosomal DNA
RFLP   Restriction fragment length polymorphism
Ri T-DNA   Root inducing transfer-DNA
rRNA   Ribosomal RNA
TRF   Terminal restriction fragment
TRFLP   Terminal restriction fragment length polymorphism
virD3   Virulence D3 gene

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