Mycorrhiza Helper Bacteria

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1 Introduction

Mycorrhizal symbiosis should not be considered merely as a bipartite plant–fungus interaction, but should instead incorporate the associated organisms. These mycorrhiza-associated organisms are known to influence each other mutually, the outcome of which is described as the "mycorrhizosphere" (Foster and Marks 1966; Meyer and Linderman 1986; Frey-Klett and Garbaye 2005). The mycorrhizosphere comprises mycorrhizas, extramatrical mycelium and the associated microorganisms. In the same way the rhizospheres exert a pressure on microbial populations (Barea et al. 2005), the mycorrhizal roots and hyphae of mycorrhizal fungi (MF) shape the bacterial species composition due to root and hyphal exudation and turnover (Bowen 1993; Morgan et al. 2005). This "mycorrhizosphere effect" may lead to improved plant nutrition, growth and disease resistance (Linderman 1988; Frey-Klett et al. 2005). Determining the functional significance of the mycorrhizosphere organisms for plant productivity presents a major challenge for the future (Artursson et al. 2006).

The presence of bacteria that are directly involved in mycorrhiza formation was first indicated by the studies of Bowen and Theodorou (1979) which showed that some bacterial isolates promoted and others inhibited the colonization of *Pinus radiata* roots by *Rhizopogon luteolus*. In subsequent work the presence of bacteria able to promote mycorrhiza formation was confirmed in ectomycorrhiza (ECM) (Garbaye and Bowen 1987; de Oliveira and Garbaye 1989), in arbuscular mycorrhiza (AM) (Meyer and Linderman 1986; Ames 1989) and suggested in orchid mycorrhizal associations (Wilkinson et al. 1989). The bacteria able to promote mycorrhizal development were then collectively named as MHB (mycorrhiza helper bacteria; Duponnois and Garbaye 1991; Garbaye 1994). Their presence in other types of mycorrhizal associations may be expected, but has not been investigated thus far.

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In this review, we will briefly cover the origin and taxonomy of the MHB. An emphasis will be given for the thus far characterized mechanisms that lead to enhanced development of mycorrhizal symbiosis, and on the specificity of this tripartite interaction, which is reflected by the promotion of some and inhibition of other fungal species by individual MHB isolates. Finally, a short overview will be given on the possible applications for MHB in forestry and agriculture. For a deeper insight into bacterium–fungus interactions in mycorrhizas, mycorrhiza–PGPR interactions and other beneficial interactions occurring in the mycorrhizosphere, the reader is advised to take notice of recent reviews (Barea et al. 2005; Morgan et al. 2005; Artursson et al. 2006; Marschner and Timonen 2006; Reddy and Satyanarayana 2006; Frey-Klett et al. 2007; Gamalero et al., this volume), and, for a broader coverage of early literature or ecological aspects regarding the MHB, on the reviews by Garbaye (1991, 1994) and Duponnois (2006).

2 Helper Strains: Origin and Taxonomy

The presence of MHB as an ubiquitous group of micro-organisms and important for mycorrhizal symbiosis is suggested by the following findings: (1) MHB have been found whenever they have been looked for, (2) they are present in very different habitats, (3) many of these bacteria seem to be closely associated with MF, and (4) MHB can be found from taxonomically diverse bacterial groups.

MHB have been isolated from very different habitats. Apart from mycorrhizas and from the mycorrhizospheres, these bacteria have been isolated from ECMF fruiting bodies, AMF spores, galls, termite mounds, and heavy metal-contaminated soils (Garbaye and Bowen 1989; Founoune et al. 2002; Xavier and Germida 2003; Gamalero et al. 2003; Duponnois et al. 2006; Vivas et al. 2003d). Because endocellular bacteria have been reported for a long time in different AM fungi (Bianciotto et al. 1996; Bianciotto and Bonfante 2002) and more recently also in the ectomycorrhizal basidiomycete *Laccaria bicolor* and the ascomycete *Tuber borchii* (Barbieri et al. 2000; Bertaux et al. 2003), one should wonder if these bacteria can harbor mycorrhiza helper activity. Preliminary results have shown that the *Paenibacillus* strain which was suspected to intracellularly colonize *L. bicolor* had a promoting effect on the Douglas fir–*L. bicolor* symbiosis (Frey-Klett, unpublished).

The majority of bacteria from the ectomycorrhizal mantle analyzed by Garbaye and Bowen (1989) had a stimulating effect on the mycelial growth of *R. luteolus* and mycorrhiza formation. Ames (1989) tested 12 actinomycete isolates from AM fungal spores on mycorrhiza formation in onion seedlings, and observed that 7 of these isolates were able to stimulate AM establishment. This indicates that not only single species but microbial communities may have evolved to live in close association with mycorrhizal fungi. Same conclusion was also indicated by the analyses of Frey-Klett et al. (2005), where the authors reported that ectomycorrhizospheres of Douglas fir select for plant-beneficial pseudomonads, while reducing strains that were inhibitory to ECM development.

Fluorescent pseudomonads and bacilli have been frequently reported as MHB of ECM symbiosis (Garbaye and Bowen 1989; Garbaye and Duponnois 1992; Founoune et al. 2002; Frey-Klett et al. 2005), but the MHB also include bacterial species from the genera *Burkholderia, Rhodococcus,* and *Streptomyces* (Poole et al. 2001; Schrey et al. 2005). The MHB of AM associations include actinomycetes (Ames 1989; Abdel-Fattah and Mohamedin 2000), pseudomonads (Gryndler and Vosatka 1996; Gamalero et al. 2004), and members of the genus *Alcaligenes* (Will and Sylvia 1990) *Acetobacter* (Paula et al. 1992), *Azospirillum* (Rao et al. 1985), *Bacillus* (Vivas et al. 2003a), *Enterobacter* (Toro et al. 1997), *Klebsiella* (Will and Sylvia 1990; Paula et al. 1992), *Bradyrhizobium* and *Rhizobium* (Xie et al. 1995; Requena et al. 1997). Fluorescent pseudomonads stimulated symbiotic germination of the orchid *Pterostylis vittata* (Wilkinson et al. 1989), suggesting that bacteria may also improve orchid symbiosis.

It is obvious that MHB should be readily cultivable to facilitate their use in controlled mycorrhization assays. However, cultivation-based approaches lead to the isolation of only small proportions of the bacterial species that exist in nature (Torsvik et al. 1990). Mogge et al. (2000) argued that this would suggest that cultivable MHB very likely represent only a small proportion of total mycorrhizosphere bacteria. However, a similarly reasonable suggestion would be that the MHB represent a dominant group of bacteria, an easily cultivable subset of which has been characterized thus far.

3 The Helper Mechanisms

The extent of mycorrhizal colonization depends on the interactions between abiotic and biotic environmental parameters, fungal physiology, and root susceptibility to infection. MHB may promote the mycorrhizal infection rate at different stages of the bacterium–fungus–plant interaction. For instance, pre-infection phases such as spore germination and mycelial growth through soil and on the root surface may be enhanced by MHB, and the root susceptibility to infection may be increased (Bowen 1993). The observation that a similar MHB response could be observed in simple aseptic culture systems as well as in greenhouse experiments (reviewed in Garbaye 1994) has enabled the use of axenic experimental models to address the mechanisms involved in the enhancement of mycorrhiza development. Five major hypotheses explaining the helper effect were presented by Garbaye (1994) (Fig. 1), and some evidence has been presented for each of these putative mechanisms. (For a recent review, refer to Frey-Klett et al. 2007).

3.1 Promoted Germination of Fungal Propagules

The exudates of MHB often stimulate fungal spore germination. Mosse (1962) showed that some rhizosphere bacteria and their culture filtrates were able to stimulate *Glomus mosseae* spore germination; these observations were confirmed

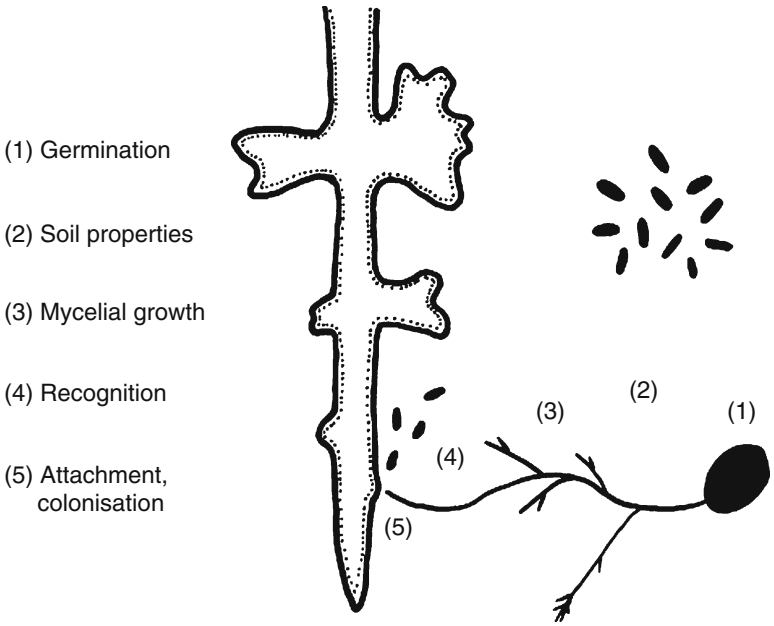


Fig. 1 The sites of action for mycorrhiza helper bacteria. Adapted from Garbaye (1994)

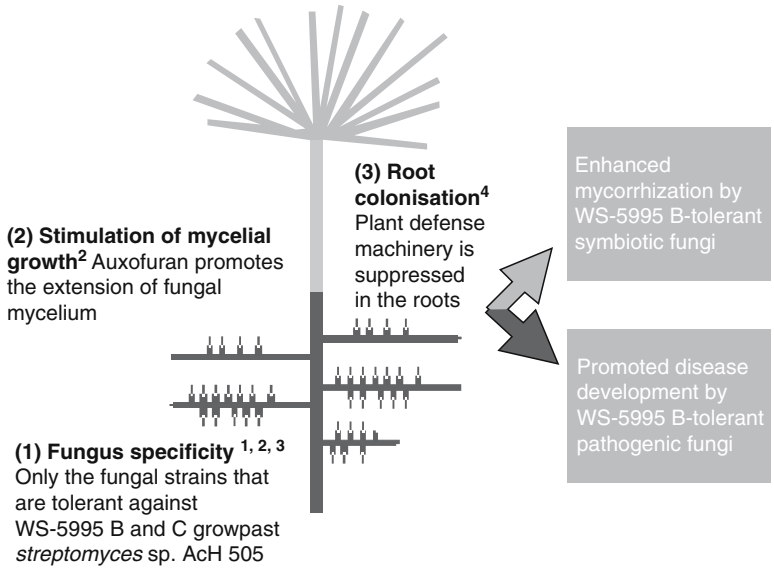
with *G. mosseae* and *G. versiforme* (Mayo et al. 1986; Azcón-Aguilar et al. 1986). The inoculation of sea oats (*Unicola paniculata*) roots with *Klebsiella pneumoniae* led to increased spore germination and faster extension of *G. deserticola* hyphae (Will and Sylvia 1990). Mycorrhiza formation in pot cultures was also increased following bacterial inoculation (Will and Sylvia 1990). Xavier and Germida (2003) observed that a substantial fraction of bacteria from AMF spore cell walls were able to promote *G. clarum* spore germination when a direct contact between the spores and bacteria existed, whereas some bacterial isolates were inhibiting spore germination by producing antagonistic volatiles. Actinomycetes have been observed on the surface of AM fungal spores, and, depending on the species, they either promoted or suppressed AM spore germination. Whereas Krishna et al. (1982) reported an antagonism between a *Streptomyces* sp. and *G. fasciculatus*, Mugnier and Mosse (1987) observed enhanced spore germination rates using a different *Streptomyces* sp. and *G. mosseae*. ECM fungal spore germination has received surprisingly little attention with respect to helper bacteria, whereas it is well known that, depending on the ECM fungi, the bacterial communities inhabiting the sporocarp can be very numerous and diverse (e.g., *Cantharellus:* Danell et al. 1993; *Tuber*: Bedini et al. 1999; Gazzanelli et al. 1999; Barbieri et al. 2005). Fries (1987) reported that basidiospore germination is stimulated by both soil yeasts and bacteria, and Ali and Jackson (1989) showed that *Corynebacterium* and several *Pseudomonas* isolates were able to stimulate basidiospore germination.

3.2 Promoted Mycelial Growth

Fungus–bacterium co-cultures are easily implemented and thus were often used as first indicators for the screening of MHB strains promoting hyphal growth. If MHB inoculation leads to increased mycelial biomass in the soil, the occurrence of root–fungus encounters should increase too, resulting in faster mycorrhization (Brulé et al. 2001). In line with this hypothesis, a significant correlation has been shown to exist between improved mycelial extension and promoted mycorrhiza establishment (Garbaye and Bowen 1989; Garbaye and Duponnois 1992; Gryndler and Vosatka 1996; Founoune et al. 2002; Schrey et al. 2005; Riedlinger et al. 2006). For the ECM fungus *Amanita muscaria*, however, Maier (2003) observed that the fungal mycelial density decreases in co-culture with the MHB *Streptomyces* sp. AcH 505. This suggested that the MHB effect of AcH 505 is caused by faster spread of the mycelial front but not by higher mycelial density.

The relative ease to perform bacterium–fungus co-cultures in a reproducible manner has enabled Deveau et al. (2007) and Schrey et al. (2005) to perform an analysis on ectomycorrhizal fungal gene expression levels during the interaction with helper bacterial strains. Using a microarray approach, Deveau et al. (2007) identified early stage-responsive genes presumably involved in the priming effect of the helper bacterial strain *P. fluorescens* BBc6R8 on the growth and morphology of its ectomycorrhizal fungal associate *L. bicolor* A238N. In the case of the interaction between *Streptomyces* sp. AcH 505 and the ECM fungus *A. muscaria*. Schrey et al. (2005) and Tarkka et al. (2006) demonstrated that the fungal genes upregulated in co-culture with AcH 505 included members of signal transduction pathways and genes related to cell stress and cell growth, metabolism and cell structure. One of the analyzed genes, the cyclophilin gene *AmCyp40*, was similarly upregulated by the cell-free culture supernatants of AcH 505 and *Streptomyces setonii* AcH 1003, but not by those of *S. argenteolus* AcH 504. Since AcH 505 and AcH 1003 promote the growth of *A. muscaria* and enhance mycorrhization, but AcH 504 does not, this suggested that *AmCyp40* could respond to MHB in general (Schrey et al. 2005). The fact that *AmCyp40* responds to cell stress in general (S. Schrey, unpublished) indicates that AcH 505 and AcH 1003 produce stress-inducing substances, later confirmed with AcH 505 (Riedlinger et al. 2006). AcH 505 also posses an effect on hyphal architecture, since the *A. muscaria*-AcH 505 dual culture hyphae are thinner than the non-inoculated hyphae (Maier 2003).

To screen for bacterial compounds responsible for growth promotion by *Streptomyces* sp. AcH 505, the suspension cultures of the bacterium were analyzed for dominant secondary metabolites. Several chromatographic steps were used to isolate a fungal growth-promoting factor in pure form, and its structure was elucidated by nuclear magnetic resonance spectroscopy (Riedlinger et al. 2006; Keller et al., unpublished). The fungal growth promoter was a novel compound, classified due to its auxin related structure as auxofuran (Fig. 2b). The production rate of auxofuran into the culture medium ranged from 10 nM to 10 µM, ideal for growth promotion of fungi, since all tested homobasidiomycete fungi respond to 1 nM to 1 µM auxofuran (Riedlinger et al. 2006; M. Tarkka, unpublished). In the presence of *A. muscaria,* AcH 505 produces 4-fold more auxofuran than in single culture, due to the acidification of the culture



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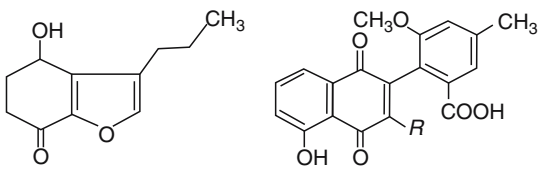

Auxofuran

WS-5995 B: *R* = H

WS-5995 C: *R* = OH

b

Fig. 2 The secondary metabolites produced by *Streptomyces* sp. AcH 505 and the MHB effect. **a** Model explaining the interaction between AcH 505, fungi and plants. References: [1]Maier et al. (2004); [2]Schrey et al. (2005); [3]Riedlinger et al. (2006); [4]Lehr et al.(upublished). **b** Dominant secondary metabolites of AcH 505, auxofuran, WS-5995 B and WS-5995 C

medium by fungal exudates. The expression levels of *A. muscaria* gene acetoacyl co A synthetase (*AmAacs*) were upregulated by auxofuran treatment, indicating an activation of sterol biosynthesis by this substance (Riedlinger et al. 2006).

The fungal influence on the production of a bacterial growth-promoting factor was also indirectly suggested in a recent study by Duponnois and Kisa (2006). These authors showed that the MHB *Pseudomonas monteilii* produces currently unknown gaseous compounds that increase the growth rate of *Pisolithus albu,* when the fungus is grown on tryptic soy broth agar or on a minimal medium with trehalose, a carbohydrate that it is frequently accumulated in fungal mycelium. The stimulatory volatiles are not produced when the bacteria are grown on a minimal medium with simple organic acids, chitin, or starch as carbohydrate sources. With *Streptomyces*

sp. AcH 505, we have observed that the fungal growth stimulator auxofuran is not produced at pH values lower than 5 or higher than 7.5 (Riedlinger 2006). As the organic acids lower and chitin increases the pH significantly, one should expect that medium pH might also have an influence on stimulatory volatile production by *P. monteilii*. The volatiles produced by MHB may also have growth inhibitory effects against some fungal species. With the Douglas fir–*L. laccata* system, Garbaye and Duponnois (1992) observed that at least some of the growth inhibitory factors produced by the MHB were volatiles. The nature of these compounds is still unknown, but could perhaps be unraveled by gas chromatography/mass spectrometry.

AM infection rate of the roots with *Glomus fistulosum* and the growth rate of soil substrate hyphae were significantly higher when the fungus was co-inoculated with *Pseudomonas putida* or with the culture supernatant of the bacterium (Gryndler and Vosatka 1996). The application of a low molecular weight fraction from the *P. putida* culture increased mycorrhiza formation and the extension of extraradical hyphae (Vosatka and Gryndler 1999), indicating that the effective substances were in this fraction. Although not yet tested for mycorrhization, the investigations on *Paenibacillus validus–Glomus intraradices* interaction have already given insight into the mechanisms that enhance AM mycelial development. Hildebrandt et al. (2002) showed that this otherwise obligately symbiotic fungus could grow and sporulate in fungus–bacterium co-cultures. A specific carbon source, raffinose, was detected in bacterial cultures, and mycelial growth was apparently supported by this sugar (Hildebrandt et al. 2006). The production of fertile spores did not take place after raffinose applications, indicating that in addition to raffinose other bioactive substances are involved in the *Paenibacillus validus–G. intraradices* interaction.

3.3 Modification of the Mycorrhizosphere Soil

In a long-term survey, Brulé et al. (2001) trapped fungal mycelium with Douglas fir seedlings, with and without the influence of *P. fluorescens* BBc6. The authors suggested that BBc6 promotes the survival of the fungal inoculum in the soil, since they observed a significant positive bacterial influence on fungal biomass only after autoclaving the nursery soil prior to adding bacteria and fungal inoculum, e.g., under adverse conditions for fungal development (Brulé et al. 2001). The data from Brulé et al. (2001) suggest that, with certain fungus–plant–substrate combinations, the MHB effect may only be observable when fungal growth is inhibited. Many of the soil microbes, including mycorrhizal fungi, produce toxic metabolites to suppress the growth of competitors. Duponnois and Garbaye (1990) analyzed how the MHB influenced the concentrations of antagonistic substances produced by mycorrhizal fungi. They could show that the bacteria were able to detoxify the liquid media from the inhibitory fungal metabolites. Helper bacteria could perhaps also suppress the production of toxic substances by soil microbes. We have found that antibiotic production by *Streptomyces* sp. AcH 505 can be suppressed by acidic substance production by *Amanita muscaria* (Riedlinger et al. 2006). The

presence of organic acid-producing bacteria in the soil suggests that some MHB may possess a similar activity.

Environmental parameters, e.g., drought or pollution stress, show a strong influence on mycorrhizal symbiosis and on the extent of mycorrhiza helper effect. By subjecting plants to polyethylene glycol- (PEG) induced drought stress conditions, Vivas et al. (2003a) addressed the question if the influence of a *Bacillus* sp. on the colonization or on physiological activities of arbuscular mycorrhizal fungi depends on the water supply. Bacterial inoculation with *Bacillus* sp. had a stronger positive influence on the colonization intensity and arbuscule abundance in the mycorrhizal roots when the plants were subjected to drought stress. Moreover, succinate dehydrogenase staining, indicative of active intraradical AMF mycelium, was also much stronger in the co-inoculated lettuce roots subjected to drought. In experiments using Cd- or Zn-contaminated substrates, the AM colonization and the development of extraradical mycelium in plants colonized by *G. mosseae* was observed to increase in the presence of *Brevibacillus brevis* (Vivas et al. 2003b, 2003c). These effects could be related to an increased carbohydrate transport from the host plant to the fungus. In a subsequent study, Vivas et al. (2005) were able to show that the bacteria had a strong positive impact on spore germination and on presymbiotic fungal growth in heavy metal-contaminated solutions. Bacterial inoculation not only reduced damage to *G. mossae* hyphae but even resulted in increased hyphal growth from 195% (without Cd) to 254% (with Cd solution). The effect was similarly strong under Zn treatment where mycelial growth ranged from 125% (without Zn) to 232% (with Zn solution).

3.4 Host Recognition and Changes in Root System Architecture

The recognition process between the host plant and the mycorrhizal fungus includes the reception of plant signals by the fungal mycelium, chemotrophic hyphal extension growth to the prospective infection site, and characteristic changes in mycelial and hyphal morphology. Xie et al. (1995) showed that MHB may be able to enhance the production of stimulatory signals that direct mycelial growth towards the root. *Bradyrhizobium japonicum* stimulated AM colonization by inducing changes in the host plant's flavonoid spectrum. Mycorrhization of soybeans was not only enhanced in the presence of Nod factor-producing rhizobia but also by exogenous application of specific Nod factors and flavonoids, suggesting that the Nod factor-induced stimulation of mycorrhizal colonization in soybean roots is mediated by plant flavonoids. As specific flavonoids produced by the roots of host plants also serve as signaling molecules promoting ECM fungi (Lagrange et al. 2001), the helper mechanism involving modulation of plant–fungus signaling should be further investigated, as has not been the case so far.

Lateral root production can be positively influenced by MHB (Garbaye 1994; Poole et al. 2001; Vivas et al. 2003d; Schrey et al. 2005), probably due to the production of auxins or auxin-related substances by the bacteria. The formation of novel root tips may lead to the establishment of more mycorrhizas, as the density of colonization sites per soil volume increases. However, the mycorrhizal rate (mycorrhizas / total

fine roots) may decrease if the lateral roots form in areas poor in fungal hyphae. In some cases, MHB exhibited differential effects on the development of the root system. The *Bacillus* strain isolated by Bending et al. (2002) increased the formation only of first order ECM roots, but *Burkholderia* and *Rhodococcus* strains isolated by Poole et al. (2001) increased the formation only of secondary order ECM roots in Scots pine. Localization of the bacteria did not reveal how the bacteria induced these specific root branching patterns, and the authors suggested that the bacteria possessed differential hormonal effects on the Scots pine roots (Poole et al. 2001). During the development of ECM in pine short roots, dichotomous (root tip) branching leads to the formation of coralloid mycorrhizal roots, producing as many as 40 root tips at their maturity. *Paenibacillus* sp. EJP73 and *Burkholderia* sp. EJP67, two strains isolated from *L. rufus* mycorrhiza, were found to promote dichotomous root branching in Scots pine (*Pinus sylvestris*) seedlings. Aspray et al. (2006a) suggested that the number of individual root tips rather than absolute number of mycorrhizal roots may be an important previously overlooked parameter for defining MHB effects.

Hormonal effects may be responsible for the MHB effect exhibited by some bacteria towards symbiotic germination of orchids (Wilkinson et al. 1989, 1994). From seven tested bacterial strains isolated from inside the underground parts of the orchid *P. vittata*, three were able to significantly promote symbiotic seed germination, one showed no difference to the uninoculated control, and three significantly suppressed seedling development. Bacterial auxin production may be a key factor behind enhanced symbiotic germination, as auxin treatments also enhanced symbiotic germination (Wilkinson et al. 1994).

The morphology of fungal mycelia upon mycorrhization in the presence of MHB has not received much attention. We have observed thinning of the hyphae of the ECM fungus *Amanita muscaria* and changed cytoskeletal architecture owing to the influence of *Streptomyces* sp. AcH 505. These changes in hyphal cell structure can be induced by the application of cell-free AcH 505 culture filtrates into the culture medium (S. Schrey et al., unpublished). Similarly, in in vitro co-cultures of *L. bicolor* S238N with MHB bacterial strains, Deveau et al. (2007) observed significant morphological modifications of the hyphal apex density and branching angles, which depended on the bacterial strains.

3.5 Receptivity of the Roots

According to the fifth hypothesis, the bacterium facilitates the colonization of the root system while growing in the rhizosphere prior to the contact between the mycorrhizal fungus and the host plant. This could occur through controlled production by the MHB of cell wall digesting enzymes, permitting the enhanced penetration of the roots by the fungal hyphae and easing their spread inside the root tissues. The suppression of plant defense response prior to fungal colonization could also potentially lead to enhanced mycorrhization.

The recent work of Aspray et al. (2006b) showed, that *Paenibacillus* sp. EJP73 only promoted mycorrhiza establishment in Scots pine when the bacterium was in direct contact with the short roots. The application of EJP73 culture filtrate showed

no positive effect on mycorrhiza development, suggesting that three non-exclusive hypotheses: (1) the bacterium exudes the effector molecules only when in contact with the roots, (2) the effectors are attached to the bacterial cell wall, and/or (3) the substances are short-lived and produced by live bacteria. Softening of root cell walls by the bacteria could also render the plants more susceptible to fungal colonization. The early work by Mosse (1962) showed that some microorganisms belonging to the genus *Pseudomonas* produce cell wall degrading enzymes and promote the establishment of AM on clover roots. Bacterial culture filtrates and enzyme preparations were similarly efficient in promoting AM development, an indication of a role for these enzymes in the MHB effect.

According to the current model for mycorrhizal symbiosis, mycorrhizal fungi evoke a temporary defense response in their host plants which is subsequently attenuated. We have recently obtained evidence that the MHB inoculation may lead to the attenuation of plant defense response in Norway spruce prior to fungal colonisation (N. Lehr et al., unpublished). The inoculation of spruce roots with *Streptomyces* sp. AcH 505 led to decreased peroxidase activities and gene expression levels in roots, markers for a defense response in spruce seedlings (Asiegbu et al. 1993; Fossdal et al. 2001). Simultaneously the colonization of roots by *Heterobasidion abietinum* 331 was promoted by AcH 505, although mycelial extension of this fungal strain was not affected by AcH 505. This suggests that AcH 505 promotes plant root colonization by fungi. Whether this indeed results from the production of unknown bacterial factors that suppress plant defense response remains to be investigated.

3.6 Fungus Specificity

Specificity in MHB-mycorrhizal fungus interactions was already indicated in early studies, which described bacterial species that promote and others that were either neutral or inhibitory to mycorrhiza formation (Garbaye and Bowen 1987, 1989). Frey-Klett et al. (2005) demonstrated that the Douglas fir–*L. bicolor* mycorrhizas and ectomycorrhizosphere selected *P. fluorescens* isolates that inhibited the mycelial growth of a larger range of phytopathogens in vitro than bulk soil isolates. In regard to ECM formation, they could demonstrate that there was a significantly higher proportion of ECM formation-inhibiting bacteria in the bulk soil zone in comparison with the symbiotic area. Fungus specificity has been addressed in several studies by Garbaye and co-workers. Garbaye and Duponnois (1992) showed that mycorrhiza formation of Douglas fir with *L. laccata* and some related *Laccaria* species was enhanced in the presence of MHB from *L. laccata* fruitbodies or Douglas fir–*L. bicolor* ECM whereas the establishment of the symbiosis with other fungi was inhibited. Two bacteria, *Pseudomonas fluorescens* BBc6 and *Pseudomonas* sp. SBc5, had a positive effect on *L. laccata* (from which they were isolated) and *L. bicolor,* and a negative effect on *Hebeloma cylindrosporum* and *Paxillus involutus*. Duponnois et al. (1993) reasoned that because of their selectivity,

MHBs might be an interesting, cheaper and safer alternative to soil fumigation. In a nursery experiment, the selectivity of two of these bacteria was confirmed; in a methyl bromide fumigated nursery soil, the MHB strains *Pseudomonas* sp. SBc5 and *P. fluorescens* BBc6 markedly improved the efficiency of the inoculation by *L. laccata* and closely-related species, but suppressed the mycorrhization of *P. menziensii* with *H. cylindrosporum* (Duponnois et al. 1993). Seven Western Australian bacterial isolates from *Laccaria fraterna* sporocarps or ectomycorrhizas, as well as *P. fluorescens* BBc6 and *Bacillus subtilis* MB3, were tested for their influence on ECM development of *Eucalyptus diversicolor* seedlings with three *Laccaria* spp. (Dunstan et al. 1998). Mycorrhiza formation by *L. fraterna* increased significantly with the indigenous isolates *Bacillus* sp. Elf28 and *Pseudomonas* sp. Elf29 and with the strains BBc6 and MB3. However, co-inoculation with the Australian *L. laccata* strain and the MHB isolate *P. fluorescens* BBc6 resulted in significantly inhibited ECM development. This was in stark contrast to the data from Duponnois and Garbaye (1991), who observed a significant promotion of ECM formation between a French *L. laccata* isolate and Douglas fir. Dunstan et al. (1998) suggested that this "fungal isolate specificity" reflects the genetic distance between the French and Australian *L. laccata* isolates.

Clear evidence for a fungus specificity factor came up recently, when the two antibiotics WS-5995 B and C were isolated from the culture supernatant of *Streptomyces* sp. AcH 505 (Riedlinger et al. 2006; Fig. 2b). The growth of *A. muscaria* was inhibited by micromolar concentrations of these substances, WS-5995 B being more effective than WS-5995 C. The production rate of the antibiotics by the bacterium ranged from 10 nM to 1 µM, and in co-culture with *A. muscaria*, down-regulation of WS-5995 B and C production to low nM levels was observed, due to the acidification of the culture medium (Riedlinger et al. 2006). The ECM fungus *Hebeloma cylindrosporum* which was suppressed in its growth in co-culture with *Streptomyces* AcH 505 was more sensitive to WS-5995 B than *A. muscaria*, promoted through this streptomycete. This indicates that the resistance towards WS-5995 B/C serves as a determining factor for fungus specificity by AcH 505 (Fig. 2a). Three genes in *A. muscaria* were observed to be upregulated after a treatment with WS-5995 B: a cell growth related aceto-acyl Coenzyme A synthetase (*AmAacs)*, a cell growth and cell stress related cyclophilin (*AmCyp40*), and a gamma-amino butyric acid/polyamine transporter (*Uga4)*. Riedlinger et al. (2006) speculated that *AmAacs* may be upregulated as a result of membrane damage, and *AmCyp40* and *Uga4* due to cell stress posed by the treatment with the antibiotic. The latter hypothesis was supported by increased gamma-amino butyric acid (GABA) levels in WS-5995 B treated hyphae, since GABA catabolism is involved in scavenging reactive oxygen species (Coleman et al. 2001). This suggests that WS-5995 B could cause oxidative stress and membrane damage in fungal hyphae, and that *A. muscaria* is either able to deal with these adverse effects on fungal physiology or to prevent the transfer of WS-5995 B into the fungal hyphae, to detoxify the substance, to export it or to transfer it into the vacuole. If any of these parameters were more pronounced in *A. muscaria* than in *H. cylindrosporum*, they could explain the fungus specificity of AcH 505.

Garbaye et al. (1992) demonstrated a mycorrhiza helper effect which was not specific to the host plant. Indeed, he showed that the helper effect, observed with the conifer Douglas fir, may be reproduced using deciduous tree species. When oak seedlings *(Quercus robur)* were inoculated with *L. laccata* and two helper pseudomonad isolates from Douglas fir–*L. laccata* mycorrhizas, both helper strains significantly increased the mycorrhiza formation in/on? the oak seedlings. The significant promotion of the formation of eucalyptus–*L. laccata* mycorrhizas by a MHB isolate from Douglas fir–*L. laccata* mycorrhizas (Dunstan et al. 1998) also supports this conclusion.

4 Potential for Use of Mycorrhiza Helper Bacteria in Agri- and Silviculture

Apart from the positive influence of MHB on mycorrhiza formation, stimulation of plant nutrition, growth, and suppression of phytopathogens by the helper bacteria have been observed. In the following, potential applications are discussed in the light of MHB research.

4.1 Plant Growth Promoting Helper Bacteria

Plant growth promoting bacteria (PGPB) exert their functions through mineral weathering (Calvaruso et al. 2006) mineralisation, plant hormone production and biological control (Barea et al. 2005). Several reports state that combined inoculation with PGPB and mycorrhizal fungi may yield synergistic positive effects on plant growth. The inoculation with *Azospirillum brasilense* and the AM fungi *Gigaspora margarita* or *Glomus fasciculatum* led to increased shoot and root biomass in pearl millet (*Pennisetum americanum*), due to improved phosphorus uptake (Rao et al. 1983). The MHB and PGPB *Pseudomonas fluorescens* 92 stimulates the growth of cucumber plants, and Gamalero et al. (2003) suggested that the reason for this was the strong rate of IAA production by the bacterium. Illustrating the complexity of the interactions within the mycorrhizosphere, Gamalero et al. (2004) showed that the use of two bacterial strains together with an AMF strongly improves tomato growth. The MHB strain *P. fluorescens* 92 was used together with the PGPB strain *P. fluorescens* P190r, and the combination of these two bacteria with *G. mossaeae* BEG12 led to strongly increased plant growth (Gamalero et al. 2004).

It is well known that mycorrhizosphere bacteria, including the MHB, may improve plant nutrition, and that P and N content of the soil affects the magnitude of plant responses to any microbial inoculation (Barea et al. 2005; Morgan et al. 2005). Barea and co-workers have found rhizobacteria that promote the establishment of AM and the solubilization of P from rock phosphate (Toro et al. 1997). The inoculation of onion with *Enterobacter* sp., *Bacillus subtilis* and AMF had a

positive influence on plant growth, P and N status. *B. subtilis* was especially effective. The inoculation of this bacterial isolate significantly increased AM symbiosis, shoot dry weight, shoot P content and shoot N content. These effects were at their greatest in dual inoculation experiments with the AM fungus *G. intraradices*, indicating an important role for the MHB effect in plant nutrition and growth. From a collection of *Rhizobium* strains native to the legume *Anthyllis cytisoides,* Requena et al. (1997) found *Rhizobium* strains that improve both AM establishment and the N status of *A. cytisoides*. The mycelium of the investigated AM fungi interacted differentially with the *Rhizobium* strains, whereas *G. intraradices* appeared to be more effective in P and N uptake with the *Rhizobium* strain NR 4, *G. coronatum* was more effective with the *Rhizobium* strain NR 9. Interestingly when these AM fungi were simultaneously co-inoculated with other *Rhizobium* and other rhizobacteria, the *Rhizobium* strain preferences of *G. intraradices* and *G. coronatum* were modulated. Therefore, these results indicate that a collection of native microbial isolates could be a good starting point for the selection of multifunctional microbe inocula for commercial purposes, but also underline the fact that it is difficult to predict the outcome of the interactions between plant beneficial microbes.

4.2 Phytoremediation or Increased Plant Survival in Polluted Soils

Microbial interactions to improve soil quality, remediation of polluted soils, have been addressed in several studies. In petroleum contaminated soils, Sarand et al. (1998) observed that the hyphal patches of the ECM fungus *Suillus bovinus* supported bacterial growth. These mycorrhizosphere bacteria were able to grow on media with toluate and xylene as sole sources, and to cleave catechol (Sarand et al. 1998). It should be tested if these bacteria, capable of mycorrhizo-degradation, could be mixed with MHB inocula to promote plant fitness in polluted soils. MHB increasing plant tolerance to Cd were characterized by Vivas et al. (2003a), and these were used as co-inoculants in remediation experiments. In a report on lead contaminated soils, Vivas et al. (2003d) showed that a *Brevibacillus* isolate was able to promote mycorrhization and nodulation, to decrease the amount of Pb absorbed by plants, and to improve shoot biomass, and N and P accumulation in *Trifolium pratense*. Due to the marked changes in root architecture, Vivas et al. (2003d) suggested that IAA production by the *Brevibacillus* isolate could be significant for the observed plant-beneficial capabilities.

4.3 Biocontrol and Controlled Mycorrhization

Root pathogens are a major concern in agriculture and forestry, and suppression of seedling death in nurseries is often not effective by conventional practices. Methods of biological control have, therefore, received increasing interest, including the use

of ECM and AM fungi against forest diseases (see reviews by Barea et al. 2005). The fungus specificity among the MHB indicates that the MHB could be used for a simultaneous promotion of certain symbiotic fungi and for the inhibition of plant pathogenic fungi. In vitro antagonism against phytopathogens has been frequently observed by MHB (Schelkle and Pererson 1996; Barea et al. 1998; Becker et al. 1999; Budi et al. 1999; Maier et al. 2004). With Norway spruce–*Heterobasidion annosum* pathosystem, we recently observed that *Streptomyces* sp. AcH 505 is antagonistic to against 11 of the 12 tested *Heterobasidion annosum* isolates. The antagonism led to a suppression of fungal colonization of Norway spruce roots and of agar covered wood disks. In contrast, mycelial growth rate of the 12th strain tested, *Heterobasidion abietinum* 331, was not affected by AcH 505, and the colonization of roots by this fungal strain was promoted by AcH 505. Bacterial inoculation led to decreased plant peroxidase activities and gene expression levels in roots. With these results it may be predicted that AcH 505 generally promotes plant root colonisation by fungi, restricted to fungal strains that are tolerant to antifungal metabolites produced by the bacterium (Fig. 2a). This indicates that fungus specificity and stimulated root receptivity by a MHB should be regarded as a potential risks in regard to biocontrol efficacy.

4.4 Persistence in the Soil and Dose-Dependent Effect on Mycorrhization

For the applications of MHB in nurseries and in the field it would be desirable that the bacteria would have a strong short-term influence on mycorrhization, but a minimal effect on native microbial populations. Frey-Klett et al. (1997) measured the development of MHB *P. fluorescens* BBc6 populations in nursery soil under greenhouse conditions. They showed a positive effect of bacterial inoculation on the Douglas fir- *L. bicolor* symbiosis in spite of the apparent survival of the bacterium of only 19 weeks in nursery soil. After a 4-year experiment in a forest plantation, no further effect on the MHB on mycorrhization was observable (Heinonsalo et al. 2004). Toro et al. (1997) inoculated phosphate solubilising rhizobacteria with AM fungi to onion seedlings and detected a similar drop in bacterial numbers as did Frey-Klett et al. (1997). After 60 days, the density of bacteria was dropped from 10^7 cfu g^{-1} of dry rhizosphere soil to 10^3 cfu g^{-1}. Still, highly significant positive effects on symbiosis development, seedling growth, N, and P contents were observed (Toro et al. 1997).

The effective dose of MHB that has to be used for increased mycorrhization varies between bacteria. Aspray et al. (2006) used *Pinus sylvestris–Lactarius rufus* symbiosis to test dose-effects with two MHB strains, *Paenibacillus* sp. EJP73 and *Burkholderia* sp. EJP67. Whereas EJP73 promoted mycorrhization at all doses tested, EJP67 only stimulated mycorrhiza formation at a narrow range of inoculum densities. Frey-Klett et al. (1999) showed that the mycorrhiza helper *P. fluorescens* BBc6 promotes mycorrhization only at low population doses (10 cfu/cm^3 soil), and

suggested that this helper strain could have some detrimental effects toward the plant or the fungus at higher population densities.

5 Perspectives

The complexity of the interactions within the mycorrhizosphere (Frey-Klett et al. 2005) can be exploited to the benefit of plants especially by using combinations of PGPR, MHB and mycorrhizal fungi in horticulture and tree nurseries (Gamalero et al. 2004). Recent data from polluted soils (Vivas et al. 2003d) indicates that MHB, possibly in combination with soil detoxifying bacteria, can also be used to improve plant fitness in toxic soils and for phytoremediation.

Novel and specific targeting and visualizing techniques for the microorganisms have to be developed in order to obtain a better understanding of pre-symbiotic fungal growth and root colonization processes in relation with the density of viable MHB. This is especially important since the speed of bacterial spread on the root surfaces as well as the morphology of the bacterial colony depend on the bacterial isolate (Poole et al. 2001; Aspray et al. 2006). Specific localization techniques have already been developed for certain bacteria (Artursson et al. 2005; Rincon et al. 2005; Aspray et al. 2006), and are under development for mycorrhizal fungi (Grimaldi et al. 2005; Müller et al. 2006).

A recent analysis of fungal gene expression during interaction with a MHB (Schrey et al. 2005; Deveau et al. 2007) will soon be followed by detailed microarray studies, as the sequencing of mycorrhizal fungal (*Laccaria bicolor*, *Tuber borchii*) and plant (*Lotus japonicus*, *Medicago truncatula, Oryza sativa, Populus trichocarpa*) genomes is finished. We may in the near future be able to identify gene clusters that are linked to the MHB effect and fungus specificity. The next step would then be to target the genome of selected MHB.

The development of molecular methods should, however, not distract from the continuing importance of physiological and biochemical studies. Physiological data will not only be essential for the interpretation of data from future microarray analyses, but also for addressing the functional diversity of MHB. Perhaps one of the most challenging fields ahead is the isolation of effector molecules from the MHB (Xie et al. 1995; Riedlinger et al. 2006).

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