

CHAPTER 5.docx

Impacts on Host Plants of Interactions Between AMF and Other Soil Organisms in the Rhizosphere*

Chapter Outline

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The last 30 years has seen a huge increase in the detailed understanding of the microbial environment surrounding plant root systems and of the processes involved in the establishment of the mycorrhiza symbiosis (Martinez-Garcia et al., 2013). Knowledge of the interactions between microbes, particularly how they communicate with each other and with higher plants, has also expanded dramatically (Gobbato, 2015). This development has also allowed a more holistic approach to the investigation of mycorrhiza and the possibility for optimizing the beneficial aspects of the symbiosis.

Arbuscular mycorrhizal fungi (AMF) are an important and ancient component of soil microbial biomass. They inevitably interact with a broad range of soil microbes, not least because there is a concentration of biota in the rhizosphere.

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5.1 INTERACTIONS BETWEEN AMF AND OTHER SOIL MICROBES

ity of bacteria and fungi, including arbuscular mycorrhizal fungi (AMF). Over the 460-million-years of its existence (Sclosse et al., 2015), the symbiosis between two-thirds of the plants on earth and AMF is believed to have developed from the simple exchange of carbon for P and diversified to the extent that

the host plant may receive a number of mineral nutrients, mostly but not exclusively those considered poorly mobile in soil, protection from toxic metals or

metalloids, defense against pathogens, resistance against drought, and enhancement of the structure of the soil in which it grows. In providing these services,

AMF inevitably interact with the millions of microbes also occupying the vol.

ume of soil they and their host plants share. For example, the number of bacteria in soil is typically reported as 10^9 g^{-1} (Table 5.J). However, the distribution of microbes in the soil is not uniform but is several times greater in the *rhizosphere* - the soil volume immediately around the root system or a plant - than in the bulk soil. The ratio can range from 28 to 53 for bacteria, .13 to 12

and bacteria, as these can also be the subject of attack by herbivores and predat-

tors among the protozoa and nematodes (Table 5.1), which also show an increased presence of ⁹¹JO-fold in rhizosphere soil (Bowen and Rovira, 1999). Although the main concentration of microbes is in the topsoil, the upper

(Blume et al., 2002) and between 35% and in excess of 58% of the total microbial biomass can be present below 25 cm (Fierer et al., 2003; Schutz et al., 2010). A significant factor in the *rhizosphere effect* on Ule distribution of microbes in the soil is the release from growing roots of a broad spectrum of carbon compounds that can support their energy requirements. In addition, there are specific compounds released that are important in signaling between microbes and the plants via the root system. But these compounds are also involved in the communication between different groups of microbes. There is increasing evidence that in the rhizosphere and in the soil volume, which is associated with mycorrhiza and the AMF extraradical mycelium (ERM) - the *mycorrhizosphere*.

fungal component (de Boer et al., 2005, 2015; Bonfante and Anca, 2009). All or

these aspects are important in understanding the effects on mycorrhizal host plants of the interactions between AMF and other soil microbes.

TABLE 5.1 The concentration of main groups of microflora and fauna in soil

Units	References
amoA gene copies ^a g ⁻¹ x	Bates et al. (2011); Nicol et al. (2008)
	Habteslassie et al. (2013)
cfu g ⁻¹ × 10 ³	Vieira and Nahas (2003)
10 ³ cells g ⁻¹	Bressan et al. (2015)
10 ³ cells g ⁻¹	Bressan et al. (2015)
cfu g ⁻¹ × 10 ³	Ghorbani-Nasrabadi et al. (2013)
cfu g ⁻¹ × 10 ³	Vieira and Nahas (2005)

A key AMF interaction is with a special group of bacteria (rhizobia) that are capable of taking nitrogen from the atmosphere and converting it to ammonia.

[illegible]

The interaction between microbes, including AMF, is not confined to the bulk soil and the rhizosphere but potentially can also take place within plant

of major significance in productive ecosystems is that of members of the legume family, the Fabaceae and trees of the genus *Parasponia* with rhi-

several symbiotic bacteria genera, including *Azorhizobium*, *Bradyrhizobium*,

Colonization of Roots by Mycorrhizal Fungi

can stimulate the germination of spores, switch on the genes responsible for the signaling system of the AMF as well as affect activity in the

organelles (mitochondria) responsible for generating energy (adenosine tri-

[illegible]

^b Assumes that less than 0.1%–10% of total bacteria are culturable.

*Counts of autotrophic microbes in the soil's crust (1 cm depth samples).

PPA of the epidermal cell, where it grows and branches in the intercellular spaces. These hyphae then induce a PPA-like structure in cells of the inner

cortex, which they enter, branch and form arbuscules, the essential site for nutrient exchange between fungus and host.

There is a great similarity in the processes of colonization by AMF and rhizobia, particularly in the communication between microbe and plant.

Colonization of Roots by Rhizobia

For this symbiosis, development starts with release from the roots of the host plant of signal molecules, mainly flavonoids (Box 5.1) but also included are simple sugars, amino acids, dicarboxylic acid, and hydroxyaromatic acid. These signals not only act to attract rhizobia to the roots of potential hosts but also stimulate changes in the bacteria that activate the rhizobial genes required for initiating the symbiosis and the eventual production of nodules. A critical consequence of the activation of these genes is the production and release into the soil of LCOs, the so-called nodulation factors (Nod Factors). For the legume to participate in the symbiosis, the necessary expression of host genes - the nodulin (nod) genes - have to be activated and this is the role of the Nod Factors. Perception of Nod Factors in the roots of a host initiates numerous changes in the plant, the details of which vary depending on the host. However, these changes can include the curling of root hairs and the formation of subcellular structures and meristems that form the nodules to house the bacteria (Box 5.1). The curling and branching of root hairs entraps rhizobia that may be reached to the hair surface. Rhizobia penetrate the root hair cell wall following localized hydrolysis of the wall (Callaham and Torrey, 1981) and enter an *infection thread*, which is formed by invagination of the plasma membrane (plasmalemma) and generation of new wall material in the form of a tubular lining. The rhizobia therefore remain extracellular as the infection thread extends along the root hair and across the lumen of the cell and then into the root cortex. There, in the mid-cortex, the rhizobia leave the infection thread and enter cells, which have also been undergoing genetically controlled changes induced by the Nod Factors to establish a *nodal primordium*. Within these cells the rhizobia are enveloped by membranes formed by the host, change their shape to become *bacteroids* and within these organelle-like structure, called *symbiosomes*, begin fixing nitrogen. Symbiosomes can be considered as organelles, similar to mitochondria or chloroplasts, being surrounded by a specialized plant membrane that permits metabolite exchange. The Nod Factors form an essential part of the signaling system that controls all stages of the infection process, including the growth of the infection thread, initiation of nodule formation, and the transformation of the bacteria into bacteroids (Ovchinnikova et al., 2011). Mature legume nodules may be *indeterminate*, maintaining the apical meristem and

growing by cell division and expansion. Of the meristem development may be transient, so that nodules are initially only growing by cell enlargement (Box 5.1). In general, temperate legumes, such as pea and alfalfa, develop indeterminate nodules, which are initiated in the pericycle and inner cortex, and grow between the root cells and emerge as club-shaped organs external to the root axis. In contrast the determinate nodules formed by tropical legumes, such as soybean (*Glycine max* L. Merr.), are large drop-shaped structures that are initiated in the outer cortex of the roots (Hirsch, 1992).

BOX 5.1 Cellular and Physiological Changes During Initial Colonization of Roots by Arbuscular Mycorrhizal Fungi (AMF) and Rhizobia

Most members of the Fabaceae form symbiotic relationships with both AMF and nitrogen-fixing bacteria, the latter being commonly referred to as rhizobia. The annual barrel medic (*Medicago truncatula* Gaertn.) has become particularly well studied as a model plant for understanding the physiology of leguminous plants, including the colonization processes by these two groups of microbes (Young et al., 2011). Once an AM fungus establishes signal exchange with the root of the *M. truncatula* host, a hyphopodium forms on the root surface. The epidermal cell below begins to undergo major internal reorganization (Reinhardt, ~2007). It starts with the cell nucleus migrating to a position below the hyphopodium and then moving across the cell lumen toward the inner periclinal wall opposite the SFC (Genre et al., 2005). It leaves behind, in its position under the hyphopodium a collection of microtubules and microfibrils of the cytoskeleton together with cisternae of endoplasmic reticulum (ER). These structures become organized into a finger-like cytoplasmic column, which tracks the route of the nucleus across the cell lumen. A large number of microtubules and bundles of actin microfilaments become aligned parallel to the column and the very dense mass of ER cisternae, which is in reality a hollow tube joining the nucleus to the SFC. The whole arrangement linked to the nucleus is recognized as the PPA. It appears that an invagination of the plasma membrane takes place to line the hollow tube. Once the cytoplasmic column of the PPA has completed the crossing of the cell lumen, the nucleus migrates to the side and a fungal hypha, formed as an outgrowth of the hyphopodium, penetrates the cell wall, possibly by a local degradation caused by the release of enzymes coupled with some mechanical force (Hamson, 1999a). The hypha grows down the hollow tube (Genre et al., 2005) and does not penetrate into the cytoplasm but remains within the apoplast. This general process may be repeated as the fungus penetrates the outer layer of the root cortex or this may take place via the intercellular spaces. Similarly, penetration of the epidermis may be through the wall separating two cells but in this case penetration to the inner layer of the cortex is intercellular. It appears that at least one stage of infection has to be intracellular for colonization of the inner cortex to be successful (Genre et al., 2008).

The process of forming an arbuscule within an inner cortical cell is somewhat similar to the initial stage of intracellular penetration by a hypha. Where a

(Continued)

BOX 5.1 (Continued)

hyphal tip makes contact with a cortical cell wall, a localized concentration of ER develops within the cell. This is also associated with the cell nucleus becoming enlarged and moving to the center of the cell (Genre et al., 2008). Invagination of the plasma membrane takes place and the hypha penetrates into the cell, where it undergoes dichotomous branching to form several orders of branches and fill much of the cell. The host cell vacuole may become partly fragmented, or appear so because of distortions of the tonoplast (Pumplin and Harrison, 2009), and ER, large numbers of plastids, and mitochondria congregate around the branches (Hause and Fester, 2005). Actin microfibrils and microtubules form a complex cytoskeleton throughout the cell (Genre et al., 2008). The invaginated plasma membrane of the host cell, often termed the peri-arbuscular membrane, is significantly modified. It contains phosphate transporters and shows intense ATPase activity, especially where it surrounds finer branches of the arbuscule structure (Harrison, 1999b; Hause and Fester, 2005). Between the peri-arbuscular membrane and the plasma membrane of the fungus is cell wall material of the host, but the structure is not consolidated into a secondary wall and the space between the two membranes has an acidic pH, concomitant with the transfer of nutrients between the partners (Rich et al., 2014).

For the establishment of its symbiosis with the rhizobia *Sinorhizobium meliloti*, *M. truncatula*, first has to release the flavone 7,4'-dihydroxyflavone (Ohi) (Zhang et al., 2009). This flavonoid binds directly with receptor proteins, the product of the NodD gene (the only bacterial gene involved in nodulation that is permanently active) within *S. meliloti* and thus activates the bacterial genes required for nodulation of the host plant (Nap and Bisseling, 1990). The various species of legumes release different combinations of flavonoid signals, and it is considered that specificity in the binding with the receptor proteins of the bacteria is one factor in the selectivity of the symbiosis. Mixtures of flavonoids can be more effective in the establishment of root nodules than single compounds in that they encourage the activity of some rhizobia but can be antagonistic to others (Cooper, 2007). The nodulation genes of the rhizobia are required for synthesis of Nod Factors, the compounds that need to be perceived by the host plant to initiate the next steps in the formation of nodules. Typically the exudates from the host roots attract the symbiotic bacteria in the rhizosphere to the root surface, and some attach to the root hairs. This appears to be a two-stage process, with end-on initial attachment to a receptor protein followed by a structural linkage involving either cellulose fibrils (Smil et al., 1987) or proteinaceous fibrillar structures - fimbriae - (Vesper and Bauer, 1986). These structures, formed by the microbe, allow bacterial cells to aggregate at the location following division so that a colony develops at each point of attachment. Collectively these colonies are referred to as infection foci. The host plant detects the bacterial Nod Factor return signals from *S. meliloti*, in the form of LCO molecules (Long, 1996), through receptors, likely lectins, distributed on the plasma membrane localized at the tips of growing root hairs (Dazzo et al., 1978; Law and Strijdom, 1994; Roberts et al., 2013).

(Continued)

BOX 5.1 (Continued)

Nod Factors stimulate a number of different reactions in root hair cells of the host legume, including an initial decrease in osmotic potential, possibly resulting from calcium uptake; the modification of growth; depolarization of their plasma membrane; rapid fluctuations in the levels of intracellular free calcium (called calcium spiking); modifications to the cytoskeleton and stimulate the formation of the preinfection thread in deformed root hairs (van Brussel et al., 1992). They also stimulate cortical cell division at the sites of nodule primordia formation; inhibit the system that generates reactive oxygen; acting together with endogenous flavonoids in the root they perturb auxin flow in roots and induce the activation of regulatory plant genes involved in nodule formation (*nodulin genes*).

In epidermal cells with an emerging root hair, cytoplasm, including the spherical nucleus, is concentrated in the subapical region of the developing protrusion, with a vesicle-rich zone at the tip. The region of dense cytoplasm, the organization of which is maintained by bundles of actin filaments, contains ER, mitochondria, plastids, and Golgi bodies. The main part of the cell contains a large vacuole. Cortical microtubules are oriented obliquely (at varying angles) or transverse to the long axis of the root, especially around the location of the emerging hair, where they are also parallel to one another. The nucleus tracks the polar growth of the extending tip or the hair but remains at a distance from it. As the root hair growth declines, the microtubular cytoskeleton becomes progressively helical and the nucleus changes to ellipsoid. The vacuole progressively extends into the hair and the nucleus finally moves to lie against the cell wall in the lower part of the root hair. Colonized root hairs undergo major changes in the pattern of growth. These changes can result in curling or branching and lead to the entrapment of colonies of the bacteria within an infection pocket, either formed by the curling of the tip back on itself, rather like a shepherd's crook or by a newly established branch growing toward the established part of the hair (Oldroyd and Downie, 2004). From this pocket, preinfection threads then form as invaginations of the cell plasma membrane over which newly synthesized cell wall material is deposited. A network of endoplasmic microtubules, which formed a network around the nucleus, progressively replaces the existing helical arrangement of cortical microtubules. A new network of cortical microtubules forms parallel to the axis of the root hair. Then the nucleus migrates to the tip of the root hair, and during this time the microtubular cytoskeleton gradually concentrates in the region between the nucleus and the root hair tip.

Actual infection by *S. meliloti* starts with a very localized hydrolysis of the root hair cell wall, a process involving the alteration and degradation of cell wall polysaccharides. Microtubules are recruited for the formation of the infection thread and accumulated to form dense parallel arrays extending from the infection pocket. The microtubule cytoskeleton transforms into a dense network surrounding the extending infection thread and connects the nucleus to the infection thread tip. Longitudinal microtubules form parallel to and in close contact with the infection thread.

(Continued)

BOX 5.1 (Continued)

In addition to the Nod Factors, the successful invasion of *M. truncatula* requires the release of an acidic exopolysaccharide, called succinoglycan (Jones and Walker, 2008). This compound acts as a signal to the host plant to permit the entry of *S. meliloti* into the preinfection threads. A second exopolysaccharide, galactoglucan, has also been identified that has similar effects to succinoglycan but its formation seems to be induced when inorganic phosphate levels in the soil are very low (Krol and Becker, 2004; Glenn et al., 2007) and there are sufficient numbers of *S. meliloti* present (Pollock et al., 2002).

Once entry has taken place, rhizobia proliferate in what has become the *infection thread* as it develops along the root hair, so they maintain a position close to the leading end of the tube. The thread grows across the lumen of the cell and then invades cells of the cortex. Similar changes in the arrangement of the cytoskeleton take place as infection threads grow from the activated root hair cells to the first cell layer of the outer cortex. As the infection thread approaches the next inner cell, the latter forms a preinfection thread by establishing a cytoplasmic bridge and its nucleus migrates toward the point of transfer. Localized disruption of the cell wall takes place, microtubules accumulated on both sides of the transfer location, and the nucleus of this cell becomes attached to the infection thread, which follows along the cytoplasmic bridge toward the next cell.

Nodulation requires the coordination of the initial *terminal* infection by rhizobia with cell divisions in the underlying cortex. Even before the infection thread has crossed the epidermis, pericycle, and cortical cells in a zone opposite a protoxylem pole respond in a local manner to the rhizobia. In pericycle cells this is reflected by the rapid induction of a nodulin gene and by rearrangements of the cytoskeleton to one characterized by endoplasmic microtubules (Yang et al., 1993; Timmers et al., 1999). These cells undergo a limited number of anticlinal and periclinal divisions to form a localized bilayer pericycle (Timmers et al., 1999). Next, Nod Factors induce cells of the inner cortex to divide, although rarely those of the endodermis, and form the initial nodule primordium. Prior to division the nucleus swells and moves from the periphery to the center of the cell, remaining linked to parietal cytoplasm by cytoplasmic strands that cross the central vacuole. Mitosis in cells near the middle of the root cortex and next to the initial primordium, results in the generation of the nodule meristem. This begins with each cell undergoing multiple divisions to create groups or meristematic cells that aggregate into a division center. The nodule meristem may continue to produce new cells that can become infected or, once the initial period of cell division is complete, no further divisions occur. In both cases cells expand as they become packed with bacteria. Cells of the outer cortex undergo the same initial structural changes as those of the inner cortex in terms of nucleus size and migration, except that cell division is arrested. The nucleus is located in a central cytoplasmic bridge as indicated previously. Where the bridge is in contact with the parietal cytoplasm, the cell wall becomes modified and it is here that it develops the characteristics of a preinfection thread if the thread from the infection pocket makes contact. After meristem formation, cells

(Continued)

BOX 5.1 (Continued)

steadily rap to show activation by the Nod factors as the nodule grows and eventually emerges through the root surface.

The infection thread traverses several cells in the root cortex to reach the newly dividing cells below the nodule meristem. As infection threads enter this region, the bacteria are released into cells from wall-less branches of the infection threads into the plant cytoplasm and enveloped by a plant membrane, the peribacteroid membrane, derived from the host plasma membrane. The bacteria then enlarge and differentiate into nitrogen-fixing forms that are known as bacteroids. These bacteroids, with the surrounding membrane, are known as symbiosomes. It is in these structures that the symbiotic nitrogen fixation takes place. The mature nodule also incorporates two or more peripheral vascular bundles that converge toward the nodule apex and provide the means for exchange of nutrients between plant and nodules (Guan et al., 2013).

From these brief accounts of the formation of these two symbioses, it is evident that there is considerable similarity in the development of the symbiosis between the contrasting microbial symbionts - fungi and bacteria - and the host legume plant (Gianinazzi-Pearson and Denarie, 1997). For example, although the signal compounds from the host plant are specific, strigolactones for AMF and flavonoids for rhizobia, the response signal from both microbial symbionts is a LCO and there appears to be some commonality in the nature of the receptors used by the host plant. Furthermore, both require the development of an infection thread-like structure for the symbionts to enter or pass through cells without penetrating the host plasma membrane (Kistner and Fritzsche, 2002). Some of the plant molecules associated with early events of rhizobia and legumes interactions have been located in AM symbiotic structures. For example, in pea, plant proteins and glycoproteins in the matrix surrounding bacteria in nodule infection threads are present in the host wall material around arbuscule hyphae. Oligosaccharides or glycoconjugates of the plant-derived membrane or interfacial matrix around the bacteroids in nodule cells are common to the peri-arbuscular membrane and arbuscule interface (Gianinazzi-Pearson et al., 1991b, 1996; Perotto et al., 1994). Both symbioses are inhibited by the phytohormone ethylene (Guinel and Geil, 2002) and there is evidence for the involvement of several phytohormones in the development and maintenance of the symbiotic structures, both pre- and postinfection (Hirsch et al., 1997; Downie, 2010). In addition, naturally occurring and chemically induced single gene mutants of pea (*Pisum sativum* L.) and faba bean (*Vicia faba* L.) are not able to form either functional root nodules with appropriate rhizobia or mycorrhizas with AM fungi (Due et al., 1989). However, one major difference is the high level of specificity for the rhizobia partner shown by a host, whereas that is less obvious for AMF. Nevertheless, it is commonly considered that the

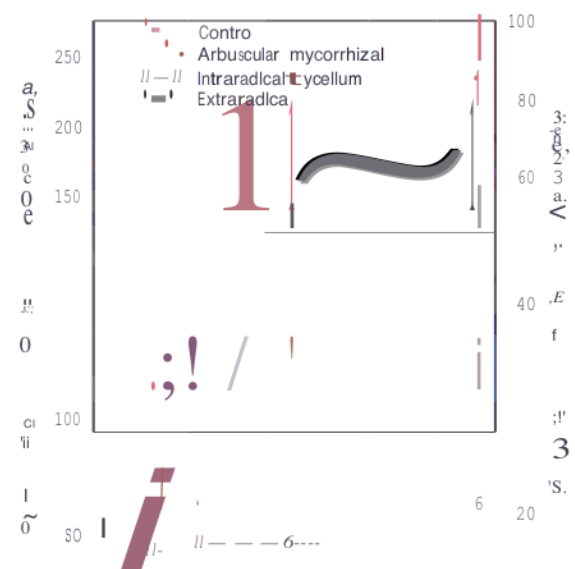
development of the symbiosis with rhizobia involved the exploitation of the preexisting signaling system for mycorrhiza formation (Roberts et al., 2013). One consequence is the possibility that the two microbial symbionts could be competitive over the formation of a symbiosis, either for sites of infection or for plant resources, or, alternatively, that a tripartite symbiosis involving both microbes could be synergistic.

Interactions Between AMF and Rhizobia Affecting the Growth

of the Legume Host and N Fixation

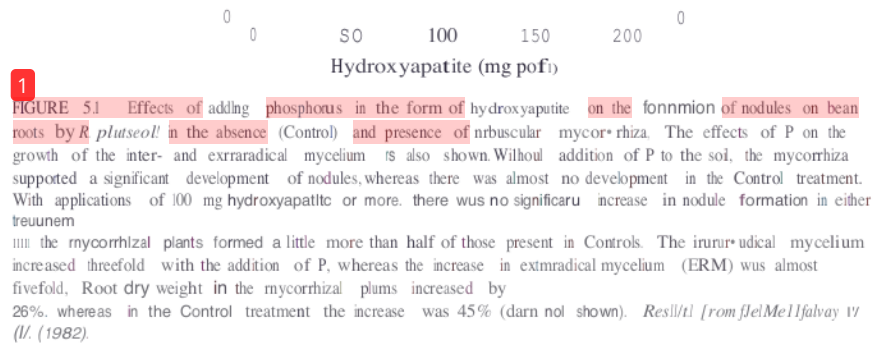
Smith and Bowen (1979) concluded from their study on the effect of temperature on the colonization of *M. truncatula* that there was no competition for infection sites between native AM fungi and *S. meliloti*. From a metaanalysis of results from 20 papers published before 1983, Cluett and Boucher (1983) reported that the presence of mycorrhizal infection significantly increased

nodulation in a range of legumes relative to those grown in the absence of AM fungi. However, the growth of the host legumes was greater when mycorrhiza were formed but differences were not significant if calculated as a function of plant dry weight. In one case (data from Bethlenfalvai et al., 1982) considered by Cluett and Boucher (1983), nodulation was significantly reduced by mycorrhiza formation. The legume was bean (*Phaseolus vulgaris* L. cv. Dwarf) and the two microbial symbionts were *Glomus fasciculatum* Gerd. and Trappe for the AM fungus and the rhizobia was *Rhizobium pliazeoli*. The experiment considered the effect of the addition of P in the form of hydroxyapatite to the rooting medium, a mixture of perlite and sand, on the formation of nodules and mycorrhiza. Bethlenfalvai et al. (1982) concluded that when soil P greatly limits the overall growth of the tripartite symbiosis, there was competition between the two microbes through limitations in the supply of P. At levels of P that allow extensive hyphal development and in the absence of sources of N other than the symbiosomes, it is competition for carbohydrates between the two microbes that negatively impacts nodule development (Fig. 5.1). Importantly, at intermediate levels of P supply from the soil, the tripartite symbiosis is very effective for each participant.



The relative timing of colonization by AMF and rhizobia is important in the formation of the tripartite symbiosis but interaction between the symbionts does not take place within functioning root nodules.

If, in another experiment this time using a 2:1 mixture of silt loam soil and sand, Bethlenfalvai et al. (1985) investigated the tripartite symbiosis between soybean (*G. max* [L.] Merr.), the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, and the rhizobium *Bradyrhizabium japonicum*.



The microbial symbionts were either applied singly (Treatments F₁ or R₁) or simultaneously (Treatment FR₁) to the roots of soybean plants at the start of the experiment. For those plants receiving only AM fungus, N as ammonium nitrate was added to the soil after 10 days. Plants receiving *only* rhizobia had P as potassium phosphate added to the soil, also after 10 days; others had no additional P (Treatment R₂). Other plants received both minerals N and P (after 10 days) but no symbiotic microbe (Treatment NS). After 20 days the soil was leached to remove minerals N and P before the "missing" microbe was applied to some of the plants that had previously received only one (Treatments F₁R₂₀ and F₂₀R₁), some plants that had not received a microbe now received both the AM fungus and the rhizobia (Treatment F₂₀R₂₀) or just rhizobia (Treatment R₂₀). Some plants continued all Treatments P₁, R₁, R₂, and others as Treatment NS. At harvest after 50 days growth, plants that received minerals N and P or either N or

Pin conjunction with the complementary microbe (Treatments NS, Fi, and Ri), all had the same total dry weight and equal to that of plants that were inoculated with both microbes 20 days after the start of the experiment (Treatment F20R20). Similarly total dry weight of plants inoculated with one or both microbes at the start of the experiment (Treatments FiR20, F20Ri, and FiRi) was similar but smaller than the previous group or treatments. Plants inoculated with *B. japonicum* at the start of the experiment but received no N or P (Treatment R?) had the smallest dry weight. Nodule dry weight in Treatment FiRi was greater than that in Treatment FiR20 but similar to that in treatments F20Ri and F20R20. Apparently nodulation was adversely affected if colonization by AMF occurred an extended period before that of rhizobia. Similarly if colonization by AMF took place well after nodulation (Treatment F20Ri), then the dry weight of fungal material produced was significantly reduced compared with when the two inoculums were applied at the same time (Treatment FiRi). Delaying AMF colonization, even if it took place at the same time as the introduction of rhizobia (Treatment F20R20), reduced AMF colonization compared with early colonization (Treatment FiRi). These results were consistent with the previous study and strongly suggest that there is some level of competition between the two microbial symbionts. This is further supported by the fact that the greatest nodulation or AM fungus colonization occurred in the absence of the other symbiont and suggests that resource availability is important.

Smith et al. (1979) investigated the tripartite symbiosis in subterranean clover (*Trifolium subterraneum* L.) inoculated with *Rhizobium trifolii*, the indigenous AMF. In soil with a similar supply of available nutrients, the growth of mycorrhizal plants was greater than nonmycorrhizal plants grown in autoclaved soil to which a soil filtrate was added. The purpose of the filtrate was to rebuild the bacterial population. In a 9:1 mixture of autoclaved and fresh soil, shoot weight of *T. subterraneum* was intermediate between that of mycorrhizal and nonmycorrhizal plants. In this case, mycorrhiza formation was much slower than in the fresh soil, which was attributed to a much smaller level of inoculum. Nodule development of mycorrhizal plants in fresh soil was greater, with more and larger nodules than on nonmycorrhizal plants. In the soil mixture nodules tended to be smaller than those in the fresh soil but after 8 weeks the number of nodules was greater and they were widespread on lateral roots as well as on the taproot of *T. subterraneum*. In contrast, in an experiment using soil with limited nutrient availability, Smith et al. (1979) observed that growth of nonmycorrhizal plants was better than that of mycorrhizal plants but the reverse was true for nodulation based on volume and activity of nodules (nonmycorrhizal plants formed slightly more but much smaller nodules). Here poorer growth in mycorrhizal plants most likely resulted from increased carbon demand to support the mycorrhiza and nodules. Other potentially limiting factors can include the supply of trace elements (Smith and Dalt, 1977) or photosynthate (Bethlenfalvay et al., 1982).

All these early experiments investigating the interaction of mycorrhiza and rhizobia in the tripartite interaction with legumes indicated the importance of rapid mycorrhizal infection for enhancing nodulation by rhizobia. Smith et al. (1979) pointed out that a delay in colonization could occur if the mycorrhizal inoculum applied consisted of spores or infected root segments. We will consider this further later in this section. Importantly the research was consistent in that irrespective of the outcome in terms of shoot growth, mycorrhiza development resulted in enhanced P inflow to the host plant and the concentration of P in tissue tended to be least in the shoot, greatest in nodules and intermediate in colonized roots. The issue of competition between the symbionts for carbon from the host plant is also worthy of further consideration, not least because it contributes to the assessment of the role of the AMF component in the tripartite symbiosis. Another area that has received relatively little attention is whether the two symbionts interact directly within nodules. Early reports indicated that AMF hyphae are not found in nodules but that has been challenged (e.g., Scheublin et al., 2004, and references therein) but in a subsequent paper Scheublin and van der Heijden (2006) concluded that only nonfunctional nodules were colonized. The presence of spores in colonized nodules suggested that the AMF were making use of the resources in the nodule material rather than contributing to nodule functioning (Scheublin and van der Heijden, 2006).

1) key feature of the AMF symbiosis is the exchange of carbon, in the form of sugars from the host, for phosphorus from the fungus. The AMF component of the tripartite symbiosis results in greater photosynthesis by the host, which may result from a larger leaf area or greater photosynthetic efficiency.

Direct effects of the two microbial symbionts on the supply of photosynthate in the tripartite symbiosis between legume, AMF, and rhizobia were investigated by Kucey and Paul (1981, 1982). They found that the rate of carbon fixation per unit leaf area by *Yicia faba* increased by 13.9% in the tripartite symbiosis than in the absence of the microbial symbionts. The AM fungus (*G. mosseae*) utilized 4% of the C fixed by the host and the rhizobia (*R. leguminosarum*) used 12% of the fixed carbon in the tripartite symbiosis (6% in the absence of the fungal symbiont). Harris et al. (1985) reported a similar value for the proportion of host-fixed carbon used by the rhizobial symbiont *B. japonicum*, symbiotic with soybean and *G. fasciculatum* (Thaxter sensu Gerd). Kucey and Paul (1981, 1982) found that although nodule biomass was 18.6% greater in the tripartite symbiosis than in the absence of the AM fungus, the rate of nitrogen fixation per unit nodule weight remained the same. Brown and Bethlenfalvay (1987, 1988) compared the plant carbon exchange rate of leaves from soybean involved in a tripartite

symbiosis with *G. mosseae* and *B. japonicum*, in a simple symbiosis with two microbial symbionts separately, or with no microbial symbiont. In the absence of *G. mosseae* plants were provided with minerals P and N was supplied if the rhizobia was not present. In the tripartite symbiosis, the carbon exchange rate per unit area of leaf in the two studies increased by 19.4% and 30.6% relative to controls with no microbial symbionts. The increase in carbon fixation in *M. truncatula* symbiotic with the AM fungus *Rhizophagus irregularis* BEGL41 was ascribed by Adolfsson et al. (2015) to increased branching and leaf canopy rather than carbon fixation per unit leaf area. The results from these various experiments suggest that the AMF and rhizobia symbionts act as additional carbon sinks, which result in increases in carbon fixation by the host plant. When AM fungi are investigated separately, whether in legumes or nonleguminous plants, the increase in CUE sink size ranges from 4% (Kucey and Paul, 1981, 1982) to 20% (Jakobsen and Rosendahl, 1990; Peng et al., 1993) of the total photosynthate produced by the host. The evidence for plants, including legumes, suggests that the enhanced carbon fixation by the host is the result of a combination of increased photosynthetic area and photosynthetic rate per unit area (e.g., Miller et al., 2002).

In a metaanalysis, Kaschuk et al. (2009) concluded that both symbionts provided an additional carbon sink, which were additive and synergistic, and this was important in enhancing photosynthesis in the host plant. Larimer et al. (2010) also reported additive effects of AM fungi and rhizobia following a metaanalysis of published material. In their investigation of the growth of the prairie legume *Amorpha canescens*, Larimer et al. (2014) reported that the AM fungi *G. mosseae* and *G. claroideum* increased the number and mass of nodules, even in soils where inorganic N adversely affected nodulation. However, the presence of rhizobia decreased colonization by AM fungi. Depending on the soil nutrient environment, the growth of the legume was enhanced by a particular combination of AM fungus and rhizobial strain. However, a contrasting combination could be more beneficial to the host plant in a different environment. For example, plants mycorrhizal with *G. mosseae* alone in combination with rhizobial strain 2 produced the best growth when P was added to the soil but when the mycorrhizal inoculum was a mixture of ~38% *G. mosseae* and 62% *G. claroideum*, the best growth for the combination with rhizobial 2 was in soil with no additional P. Overall both inoculation with AMF or rhizobia increased biomass production in *A. canescens* compared with controls provided with P or N as mineral nutrients, respectively. However, the effect of developing a tripartite symbiosis was synergistic and not simply additive. To explain the contrast between these results and the conclusions of Kaschuk et al. (2009) and Larimer et al. (2010), Larimer et al. (2014) suggested that many previous experiments had focused on annual plants important in agriculture, where insufficient time was available for the tripartite symbiosis to become synergistic.

The tripartite interaction is greatly enhanced if AMF colonization is initiated from an intact mycorrhizal mycelium network. The ERM network can be a transport highway for nitrogen between a legume and a nonlegume host. Protection of the symbiosis against abiotic stress includes the defense of the rhizobial bacteroid.

Goss and colleagues considered the importance of the speed of establishing the tripartite symbiosis to its efficacy (see Section 6.1 for a general account). Building on the work of Miller on the establishment of effective AM mycorrhiza in maize (Miller, 2000), detailed consideration was given to the potential of a preformed ERM as a primary inoculum (see Section 6.2.3) instead of spores or colonized root fragments, which can be slow to colonize (Smith et al., 1979). This was achieved, either by sieving the Canadian silt loam soil or leaving it undisturbed after growing a mycorrhizal ERM development plant. Instead of using sterilized soil and inoculating a laboratory strain or strains, Goss and coworkers followed Smith et al. (1979) in using the indigenous AMF population in the soil and generated two levels of inoculum potential: one level having spores, colonized root and an intact ERM, the other comprising spores and infected root fragments. They used a commercially available, peat-based inoculum of *B. japonicum* strain 532 C in their work with soybean. In a greenhouse experiment, Goss and de Varennes (2002) showed that the presence of the ERM in the inoculum resulted in a faster colonization by both AMF and rhizobia compared with the presence of spores and root fragments. For example, 10 days after emergence arbuscules were present in 56% of root length and 14 nodules had been produced per plant when ERM was present compared to 14% of root length and 8 nodules when it was not. Importantly, AMF colonization increased in both treatments to podfill, but was always greater in plants with ERM in the inoculum. However, the number of nodules was unchanged after 23 days and similar in both treatments but the dry weight was consistently greater where ERM was in the inoculum and this was reflected in a threefold difference in N fixation at podfill. Plant dry weight was similar in the two treatments at 10 and 23 days after emergence but by podfill (49 days after emergence) dry weight of plants having ERM present at sowing was 42% greater than where it was not. In contrast the number of trifoliate leaves was greater 10 and 23 days after emergence, when ERM had been present, but inoculum potential made no difference to leaf number by podfill. The content of P in plants declined similarly in both treatments until 10 days after emergence, after which uptake started in the plants with ERM in the inoculum. However, uptake of P was delayed until 23 days after emergence in plants where there was no ERM in the inoculum. Total N acquisition by soybean at podfill was greater in plants with ERM in the inoculum but the concentration in the shoot was

less than in plants where there had been no ERM. The faster AMF colonization from an intact ERM resulting in earlier nodulation of soybean was confirmed in a similar soil type but with a greater P content (Antunes et al., 2006b). However, in this field experiment, where rotary tillage to 10 cm was

used to disrupt ERM or the soil was left undisturbed before soybean was planted with a no-till seeder, the effects did not result in any measurable dif-

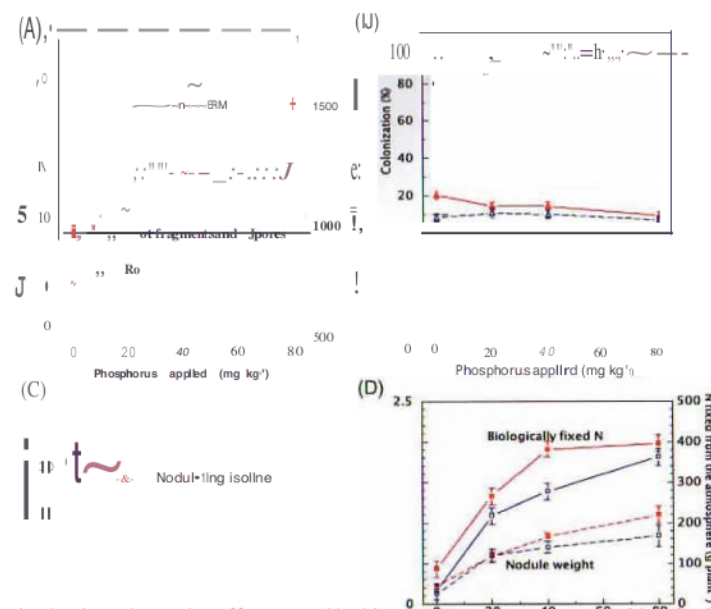
ferences in the soybean plants at podfill.

In a greenhouse experiment similar to that of Goss and de Varennes (2002) but using the annual medic, *M. truncatula*, instead of the grain legume, soybean, with *S. meliloti* as the rhizobia, de Varennes and Goss (2007) also found a more rapid colonization by indigenous AMF from a

Portuguese clay soil when the AMF inoculum included ERM as well as spores and infected root fragments. However, by podfill, no differences in colonization remained. At 14 days and 29 days after emergence (flowering) there were no differences in shoot weight or nodule numbers between treat-

ments but by podfill shoot weight and nodule size were greater where ERM was present in the AMF inoculum. Both the concentration and content of P in plants was greater throughout the experiment in the treatment with the ERM in the AMF inoculum. A greater proportion of N in the plants at podfill had been derived from the atmosphere where earlier AMF colonization had taken place.

Earlier, Kadir (1994) investigated the tripartite symbiosis in soybean under greenhouse conditions following different applications of P to the soil. The mycorrhiza were formed by indigenous AMF and either free-living rhizobia or the *B. japonicum* strain 532 C. The main treatment comparison was between an AMF inoculum that did or did not contain intact ERM. In soil containing intact ERM, plants grew faster and had a greater trifoliolate leaf area after 4 weeks than those infected by inoculum containing only colonized root fragments and spores. The difference in leaf area persisted to the time the plants were harvested at the end of podfill and was reflected in a difference in plant dry weight (Fig. S.2A). By that time there were no significant differences between the main treatments in root colonization by AMF hyphae or arbuscules but the proportion of root length containing vesicles was significantly greater when an intact ERM was present in the inoculum (Fig. S.2B). The difference in vesicle colonization was more consistent in the nodulating isolate than in the nonnodulating isolate (Fig. S.2C). In contrast to the small effects on AMF colonization when ERM was the key propagule



in the inoculum, the effects on rhizobia were much greater resulting in 38% more nodules than when only colonized root fragments and spores were present. The addition of phosphate to the soil produced a reduction of 5%–15% in the intensity of colonization by AMF (Fig. S.2B) but increased the colonization by rhizobia, as assessed by the total weight of nodules (Fig. S.2D). However, the negative effects on the proportion of root length containing arbuscules were large, but only at a concentration of 80 mg P kg⁻¹; when the

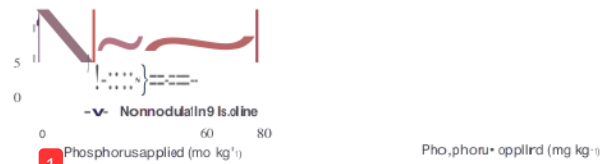


FIGURE 5.2 The effects of applying phosphate fertilizer on the development and effectiveness of the tripartite symbiosis between soybean, indigenous AM17, and *Bradyrhizobium japonicum*. (A) Variation in shoot dry weight (red markers) and leaf area (blue markers) at podfill in plants colonized using inoculum with extraradical mycelium (ERM) kept intact (solid markers) or made up mainly of root fragments, spores, and disrupted ERM (open markers). The application of phosphorus in excess of 20 mg kg⁻¹ to disturbed soil without an intact ERM was not beneficial to growth but plants colonized from intact ERM showed a significant response up to 40 mg kg⁻¹. (B) At podfill there was no effect of inoculum type on colonization, except for the concentration of vesicles within the roots, where the effect of the presence of an intact ERM was significant at $P < 0.001$. Red markers, ERM Intact; blue markers, ERM disrupted. Negative effects on colonization or applying phosphorus were small. (C) The negative impact of phosphorus on colonization was greater in soybeans that were genetically incapable of establishing a viable symbiosis with Rhizobia and could not form nodules (blue markers) than in the nodulating isolate (red markers). The benefit to colonization from the inoculum containing intact ERM (closed markers) was also less consistent in the nonnodulating isolate. (D) Both colonization by Rhizobia, as indicated by nodule weight (dashed lines), and biological nitrogen fixation (solid lines) were enhanced by the presence of intact ERM (closed markers) when soybeans were planted compared with those colonized from spores, root fragments and disrupted ERM (open markers). Source: Data from Kadir (1994).

value with till in excess of 70%. By podfill the main treatments had no significant effect on the concentration of P or N in the shoots but the proportion of N in the plant resulting from biological fixation in the nodules was greater where AMF colonization took place in the presence of intact ERM and was also enhanced by the application of P (Fig. S.2D). The relationship between nodule weight and N acquired by biological fixation was enhanced if the ERM was kept intact prior to planting the soybean and free-living wild-type

rhizobia were available to establish functional nodules. Inoculating with the more effective 532 C strain further increased Ure N derived from biological fixation (Fig. 5.3).

The variation in the sensitivity of the tripartite symbiosis to added P in the soil, seen across the experiments discussed in this section, may also reflect the normal phosphate environment experienced by the AMF before the imposition of experimental treatments (Jasper et al., 1979).

One other important aspect of colonization by an existing ERM is that the potential exists for the new mycorrhizal plant to be linked to other plants. Enhanced transport between soybean and maize via a common ERM (van Kessel et al., 1985) indicated the potential for AMF to facilitate the transfer of N between legumes and grasses, and this was demonstrated by Haystead et al. (1988) with the transfer of N from white clover (*Trifolium repens* L.) to ryegrass (*Lolium perenne* L.). However, at least in laboratory microcosms, if more than one potential recipient host plant is linked to the same ERM network, there can be considerable competition between them (Walder et al., 2012).

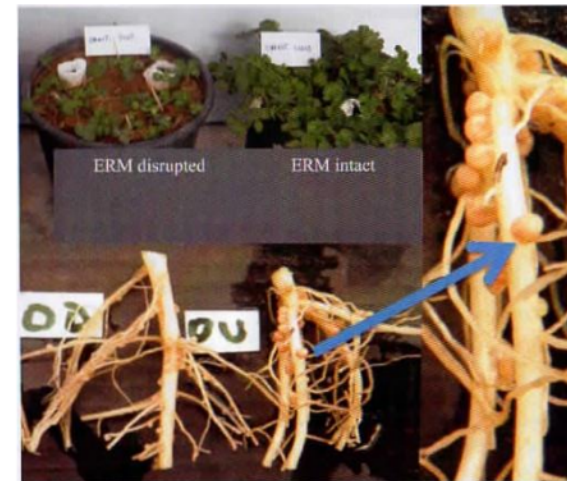
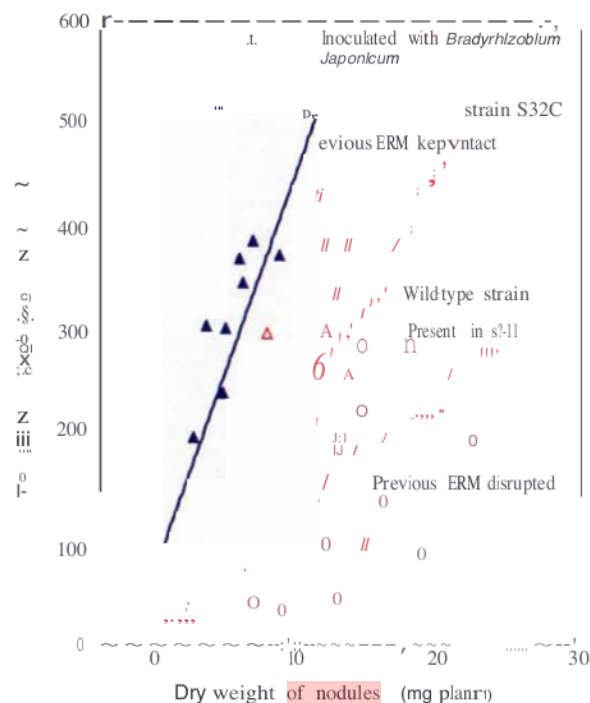


FIGURE 5.4 Pots of *Trifolium subterraneum* L. 6 weeks after planting in soil containing 22.6 mg Mn kg⁻¹. Left, soil was sieved after the growth of the previous plants (*Oenothera compressa* L.) and the roots were cut into sections and mixed back into the soil before subterranean clover was sown. Roots were colonized by indigenous AMF from spores, colonized root fragments, and short pieces of disrupted extraradical mycelium (ERM). Note the sparse formation of

nodules. Right, prolific growth of *T. subterraneum* in undisturbed soil, colonized by indigenous AMF from an intact ERM (associated with *O. compressa* and spores). More large nodules were formed on the main axis (arrow points to nodule on enlargement of main axis).

Resilience to Stress in the Tripartite Symbiosis

It is well established that abiotic stress, such as from toxic ions, can directly affect N-fixation. The impact of toxic levels of Mn can greatly inhibit plant growth but a number of researchers (e.g., Dobereiner, 1966; Evans et al.,

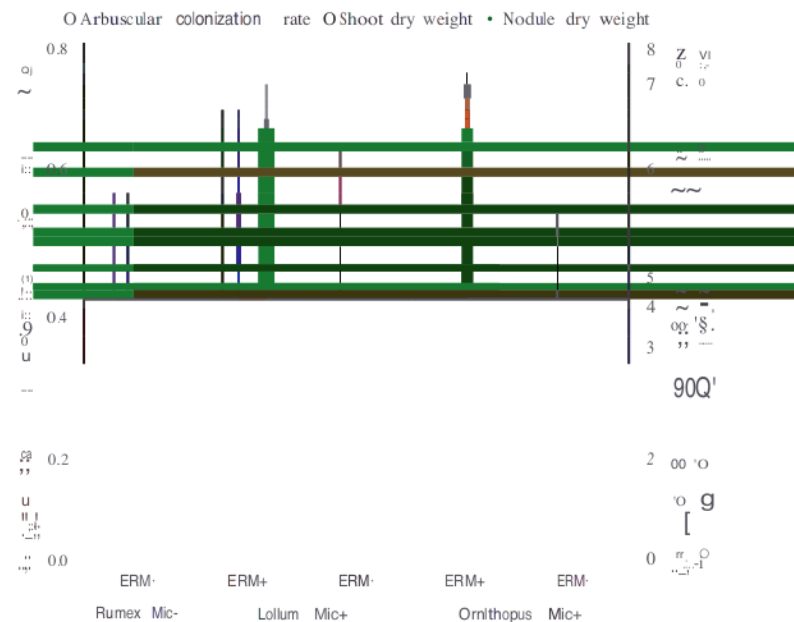
1987; de Varennes et al., 2001) found that this was greater, when legume plants were dependent on symbiotic N-fixation rather than on mineral N.

The effects of Mn can reduce Ure formation of root nodules in terms of numbers and size as well as the rate of symbiotic N-fixation (Evans et al., 1987; DeHaan et al., 2002). Alho et al. (2015) showed that, in *T. subterraneum* L., colonization by AMF from inoculum containing intact ERM was

FIGURE 5.3 Effect of keeping the extraradical mycelium intact (—) or disrupted (---) prior to sowing soybeans on the effectiveness of nodules colonized by free-living wild type rhizobium and the

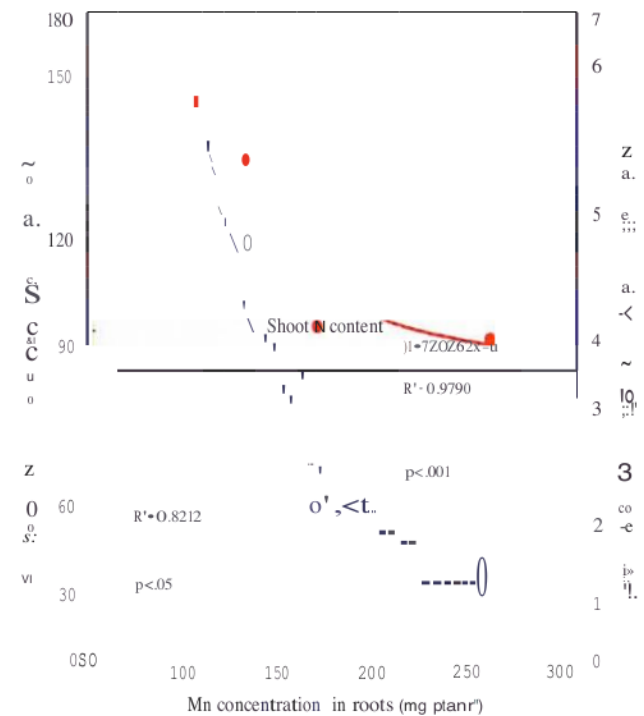
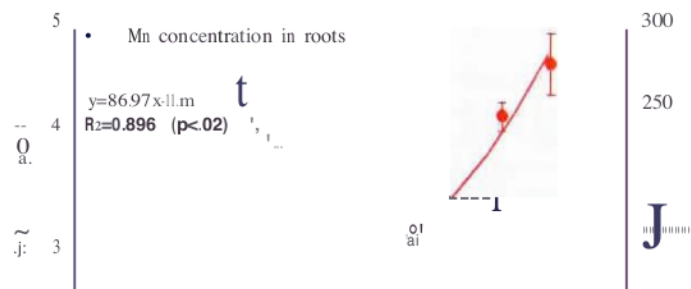
added benefit from inoculation (— —) with the more effective strain 532C. Source: Data from Kadir (1994).

very effective in protecting plants from Mn (Figs. 5.4, 5.5). Shoot dry weight was up to 3.3 times larger after 21 days and, as indicated in Fig. 5.4, a maximum of 16.2 times greater after 42 days relative to colonization from spores or infected root fragments. The protection seemed to be associated with a smaller concentration of Mn in the plant roots (Fig. 5.6). As Mn in the roots increased, nodule dry weight decreased and so did the N content of shoots (Fig. 5.7). The decrease in Mn in the roots as a result of enhanced AMF



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Developer genus, mycotrophy, and ERM integrity
FIGURE 5.5 Effects of developer ERM, presence and integrity, on colonization rate by indigenes

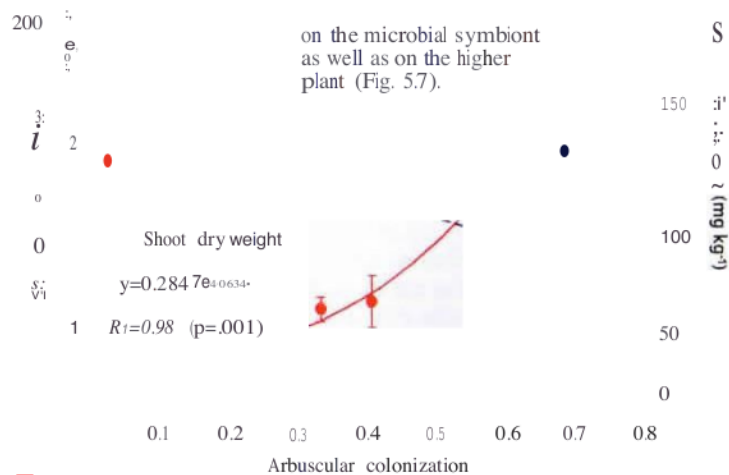
nous AMF (based on arbuscule formation) and dry weight of shoots and root nodules of *Trifolium subterraneum* L. 21 days after sowing. Mycorrhizae were initially formed in association with roots of two common Mediterranean weed species *Ornithopus compressus* L. or *Lolium rigidum* Gaudin, both being mycotrophic (Mic +). A third plant, *Rumex bucephalophorus* L. is considered not to form mycorrhiza (Mic-) and hence provided a control for soil disturbance and the contribution of disrupted ERM. ERM intact - ERM+, ERM disrupted - ERM-. Source: Based on Illmer (2015).



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Relationship between Mn concentration in the roots, shoot N content (—) and Nodule dry weight (---) in *Trifolium subterraneum* L. 42 days after sowing.

colonization would explain the greater nodule dry weight, shoot N content, and dry matter production. Although at 21 days after planting, the proportion of root length containing arbuscules was up to 2.8 times that following colonization from colonized root fragments and spores, after 42 days the maximum difference between treatments was only 18% better when intact ERM was present. The protection granted by an enhanced AMF root colonization resulted in nodule weight ranging between 6.4 and 4 times greater when intact ERM was

present in the soil than when it was not. As nodule weight is a good indicator of the effectiveness of the symbiosis between the legume and rhizobia, this suggests that the benefit of AMF to the tripartite symbiosis was through an effect



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FIGURE 5.6 Relationship between colonization rate, based on arbuscule formation 21 days after sowing, and shoot dry weight (---), and Mn concentration in the roots (—) of *Trifolium subterraneum* L. 42 days after sowing.

Bacteria, both free-living and endophytic within the hyphae, can enhance the

effectiveness of AM mycorrhiza, both by aiding colonization and increasing the

effectiveness of protection of the plant host against biotic and abiotic stresses.

5.1.2 Other Interactions With Bacteria

The ERM is commonly associated with bacteria that are attached to the surface of Ute hyphae (Scheublin et al., 2010) or even living as endocellular organisms within the cytoplasm of hyphae (Bonfante and Anca, 2009). With modern molecular techniques, the different taxonomic groups of bacteria found within the mycorrhizosphere have begun to be identified (Fig. 5.8).

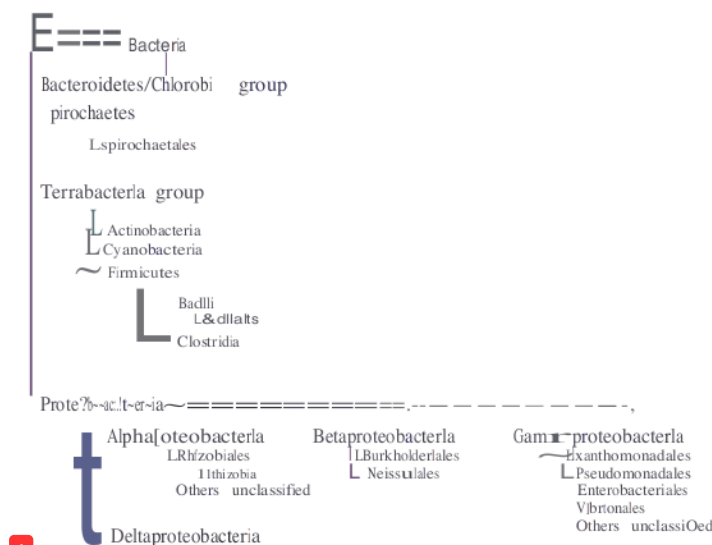


FIGURE 5.8 Main bacterial groups considered to participate in activities in the mycorrhizosphere (based on Bonfante and Anca, 2009). Although rhizobacteria include the rhizobia that form the tripartite symbiosis with legumes, some members of the rhizobacteria can also fix nitrogen from the atmosphere and have been identified as endobiotrophs (Cage, 2004; Leveau and Preston, 2008).

Toljander et al. (2006) observed differences in the mode of attachment to living and dead hyphae between taxa of bacteria, consistent with some being saprophytic and others, whose functioning depended on being more intimately involved with the living fungus. Some of the bacterial species that are found in close association with AM fungi have been shown to enhance the formation of mycorrhiza on receptive hosts (Garbaye, 1994). The specificity shown by individual bacterial strains for increasing AMF colonization in specific soils has resulted in the concept of *mycorrhiza helper bacteria* - MHB - (Garbaye, 1994). Some bacteria that apparently enhance the benefit to the host plant from the formation of a mycorrhiza are also known to be directly beneficial to host plants, being identified as plant growth-promoting rhizobacteria (PGPR). Possible mechanisms by which mycorrhiza formation can be improved have been distilled into four hypotheses:

1. MHB improve soil properties that are conducive to improved fungus colonization. Such properties include the creation of a more appropriate soil pH and the complexing of ions, especially those that can be toxic to growth, by siderophores.

2. MHB promote the germination of spores together with the growth and survival of mycelium, both gaseous and small molecular weight compounds as well as sugars have been shown to affect germination or hyphal growth and branching (Artursson et al., 2006). MHB may also act through antagonism or competition with other bacteria or fungi that are inhibitory of AMF activity and hence links to the first hypothesis (Anursson et al., 2006).
3. The presence of MHB improves the receptiveness of roots to initial interaction with the mycorrhizal fungi. Root branching stimulated by MHB will increase the intensity of root development and the likelihood of interaction with mycorrhizal fungi. The formation of compounds, such as indole-3-acetic acid (IAA), by bacteria in the rhizosphere could not only modify root branching (though this still has to be established in MHB) but reinforce the impacts of the local inhibition of phytohormone transport during the initial responses to the recognition in the plant of the "myc factors" released by the fungus. One example is provided by the isolate UW4 of *Pseudomonas putida*. This MHB promotes mycorrhiza formation (increased root colonization and arbuscule formation) in cucumber by the AMF *Gigaspora rosea* by producing ACC deaminase that reduced the formation of the phytohormone ethylene, which is known to inhibit the colonization of roots and formation of fully developed arbuscules (Herrera-Medina et al., 2007). A mutant of *P. putida* UW4, lacking the deaminase, had no beneficial effect on mycorrhizal development (Garnalero et al., 2008).
4. MHB enhance the early stages of signal recognition between the host plant and the mycorrhizal fungus (Garbaye, 1994; Frey-Kjeller et al., 2007). Sanchez et al. (2004) compared the colonization of *M. truncatula* roots by the AMF *G. mosseae*, *S. meliloti*, and the MHB *Pseudomonas fluorescens* strain C7R12. *Glomus mosseae* increased the activity of 12 genes of *M. truncatula*, whereas *S. meliloti* upregulated only three of the same set of genes and down regulated five of them. *P. fluorescens* strain C7R12, which colonized the surface of root tips and grew between cells within the root cap and also entered some cells of the root cortex, increased the activity of seven of the gene set, consistent with the MHB causing a number of the same host responses as the AMF during colonization.

In contrast to MHB, some bacteria as well as a number of fungi can be antagonistic to AMF and are capable of parasitizing them (de Boer et al., 2005; Lee and Keske, 1994). Other bacteria use the exudates from AMF hyphae and this has been proposed as one mechanism that can affect the relationship or the fungus with specific bacteria associations (Andrade et al., 1997). The range of bacterial mycophagy, the active feeding of fungal material, covers extracellular necrotrophy, extracellular biotrophy, and endocellular biotrophy (Leveau and Preston, 2008). Necrotrophic actions involve the

secretion by the mycophagous bacteria of proteins or toxins with a relatively small molecular weight, which increase the permeability of fungal hyphae and lyse them and inhibit fungal metabolism, leading to hyphal death and the release of metabolites that are used in bacterial growth. Extracellular biotrophy does not kill fungal hyphae, but the bacteria live in close proximity and may colonize the hyphal surfaces. The colonization can involve exopolysaccharides, surfactants, and fibrillae, reminiscent of the attachment or rhizobia. The bacteria may be able to modify the metabolism of the host, thereby increasing the exudation of nutrients from living hyphae or other fungal cells. Such biotrophs can tolerate or actively suppress production of antibacterial metabolites by the fungus. Endocellular biotrophy involves the absorption of nutrients from the fungal cytoplasm by bacteria located within living fungal cells, where they grow and multiply. AMF are likely to be subject to all three forms of bacterial mycophagy (Bonfante and Anca, 2009).

Endocellular bacterial biotrophs in AMF were first identified in microscopy studies, where they were reported as bacteria-like objects (BLO) (MacDonald and Chandler, 1981). Combining morphological and molecular techniques have allowed BLO to be identified (e.g., Bianciouo et al., 1996). The evidence indicates that the bacteria can be passed from one generation of AMF to the next as part of vegetative spore formation (Bianciouo et al., 2004). Much of our understanding of endotrophic bacteria in AMF has resulted from the study of isolate BEG34 of *Gigaspora margarita* and its endobacterium "*Candidatus Glomeribacter Gigasporarum*," which has been used as a model system (Bonfante and Anca, 2009). The inability of the bacterium to be grown in culture outside of its host results in its designation of *Candidatus*. The endobacterium, which is a rod-shaped, gram-negative organism $0.8\text{--}1.2\text{ }\mu\text{m}$ in diameter \times $1.5\text{--}2.0\text{ }\mu\text{m}$ in length, is found singly or grouped and often in protein-filled vacuoles within cells of the AMF, both in spores and hyphae. However, this organism is confined to the Gigasporaceae but a coccoid endobacterium (MacDonald et al., 1982) is more widely distributed across different groups of AMF and, although having a gram-positive cell wall, its ribosomal DNA indicates that it is related to Mollicutes (Naumann et al., 2010), which are common endophytic pathogens but do not produce a cell wall (Dybvig and Voelker, 1996). The two bacteria are able to coexist in the same fungal cell, although there is evidence that *Ca. G. gigasporarum* can be surrounded by a membrane of fungal origin, whereas the Mollicutes-related coccoid is free within the fungal cytoplasm (Desiro et al., 2014).

The transcription of the marker gene for cell division in *Ca. G. gigasporarum* is most active during the symbiotic phase of the AMF, particularly in the ERM (Bonfante and Anca, 2009). Treatment of *Gi. margarita* spores with strigolactone also stimulated the division of the bacterium. When the bacterium was selectively lost from the AMF hyphae, the elongation and branching of hyphae, following the application of root exudate, was greatly

impaired, suggesting that the endobacterium had an important role in the preparation of the fungus for the formation of a symbiosis with a host plant (Bonfante and Anca, 2009). This would be consistent with the endobacterium being a MHB.

AMF appear to interact with other fungi as well as soil fauna, with positive and negative impacts on the effectiveness of the mycorrhiza as well as some evidence of predation on fine hyphae.

5.2 INTERACTIONS BETWEEN AMF AND OTHER FUNGI

There has been considerable testing of the hypothesis that AMF provide some protection to host plants against fungal pathogens. Filler and Garbaye (1994) reported French research, which involved both ecto- and endomycorrhiza, that found significant reductions in disease were achieved in 76% of cases studied. In many natural environments, particularly in sandy soils, AMF hyphae within roots have been associated with dark colored septate hyphae or fungi that are known to be or may be affiliated to the ascomycetes (Mandryk and Jumpponen, 2005). Despite many reports on the fungi there appears to be no indication of any interaction with AMF in the same plant. Yeasts, which produce vitamin B₁₂, have also been shown to act as mycorrhizal helpers, increasing root colonization and spore production, and also increasing the beneficial effects of the AMF on the host plant (Boby et al., 2008).

5.3 INTERACTIONS BETWEEN AMF AND SOIL FAUNA

Much of the interest has been focused on whether grazing by arthropods, such as mites and Collembola, have a serious impact on AMF, including reducing the size of the spore bank in the soil. The other area that has received considerable attention is the interaction with burrowing organisms, such as earthworms.

5.3.1 Interactions With Arthropods

In the herb *Geranium robertianum* L., growth was enhanced by the formation of a mycorrhiza but the introduction of the collembolan *Folsomia candida* into the soil caused a reduction in growth that was not directly related to grazing pressure (Harris and Boemer, 1990). McGonigle and Fitter (1988) reported broadly similar results. Such results suggested that grazing of the fine hyphae of the ERM was detrimental to the effectiveness of arbuscular mycorrhiza (Fitter and Garbaye, 1994; Hodge, 2000). Klironomos and Ursic (1998) found that in their study with *F. candida* the impact on the efficiency of the symbiosis was a function of grazing pressure. However, Larsen and Jakobsen

(1996) concluded that because the AMF hyphae were the preferred diet of the Collembola, the real interaction between the two organisms was quite limited. Consequently in the specially developed experimental microcosm, which allowed *F. candida* to graze the extraradical hyphae of the AMF *Glomus caledonium* (Nicol. & Gerd.) Trappe and Gerdemann without roots being present, there was no effect of grazing on the growth of *T. subterraneum* over 6 weeks. However, thereafter the growth of mycorrhizal plants was slower than nonmycorrhizal plants. Adding yeast cells as an alternative food source for the Collembola increased hyphal length whether Collembola were present or not but a small apparent effect of grazing at 4 weeks was not detected thereafter (Larsen and Jakobsen, 1996). Gange (2000) concluded that on the balance of evidence available, there was no consistent indication that Collembola adversely impacted the beneficial effects of AMF on plants. In a microcosm experiment using maize (*Zea mays* L.) as host plant, Ngosonga et al. (2014) investigated the grazing of the collembolan *Protaphorura fimata* on the AMF *G. mosseae*. The growth of maize shoots was greatest where *P. fimata* was present in the compartment containing mycorrhizal roots or where it was also present in the compartment containing only AMF hyphae. The P content of roots was greatest where the Collembola were present in the root compartment of mycorrhizal roots but there was no effect of *P. fimata* on N acquisition of N. Grazing of mycorrhizal roots increased the dry matter invested by the AMF in the hyphal compartment, particularly if grazing was restricted to that area. Rather than developing spores or storage structures, the grazing of mycorrhizal roots encouraged greater hyphal exploration of the root compartment. The evidence from fatty acid profiles was that Collembola preferentially fed on soil bacteria but consumed more fungal material in compartments containing mycorrhizal roots. Overall the Collembola were beneficial to the mycorrhiza.

In a field microcosm study, Bakonyi et al. (2002) found that Collembola reduced the number of AMF spores in the soil under maize and *Festuca rubra*. At grazing densities between 0.2 and 0.4 adult Collembola g⁻¹ soil, the number of spores declined rapidly but at greater densities the decline was much less. At small grazing densities the colonization of roots by AMF increased, which was assumed to result from spores being transported by the Collembola to root surfaces. However, at grazing densities slightly greater than those that reduced spore numbers, there was a decline in the percentage of root length colonized by AMF.

5.3.2 Interactions With Earthworms

The presence of the earthworm *Lumbricus rubellus* in the soil increased the dry matter production of mycorrhizal plants of *Plantago lanceolata* but the presence of the animals did not affect the level of root colonization by indigenous AMF (Gormsen et al., 2004). The content of ERM in the soil, based

on fatty acid-specific analysis for fungi was greatly enhanced by the presence of earthworms. Eisenhuenger et al. (2009) also found that earthworms did not affect root colonization by AMF (*Glomus intraradices*) but also found no interaction between AMF and earthworms on the growth of representative grass, herb, and legume hosts. In contrast, Li et al. (2013) inoculated maize with AMF, which significantly improved shoot growth and maize yield; the addition of earthworms resulted in a further yield enhancement. There was a significant increase in the activity of alkaline phosphomonoesterase, in the soil and C and N in microbial biomass were also enhanced by the presence of both AMF and earthworms, whereas AMF reduced the availability of P.

5.4 CONCLUSIONS

Far from being a large diversity of competing organisms inhabiting the rhizosphere of plants, there is considerable evidence of both cooperation and synergism between groups concentrated around mycorrhiza. It seems that where the interaction between microbes and plants is of particular interest to the development of a sustainable agriculture, the relationship is carefully choreographed through complex signaling systems. Much of our detailed knowledge of the interaction between AMF, bacteria, and plants comes from legumes involved in a tripartite interaction that may also involve additional endophytic partners, which are only now being identified and their possible roles elucidated. The benefits of mycorrhiza in the tripartite interaction are best achieved when the host plant is colonized early, especially from an inoculum based on intact ERM. The outcome ultimately depends on the subsequent growing conditions. Very large improvements in growth can result, at least in the presence of abiotic stresses. The interactions between AMF and other organisms is less well understood but most can have some benefit to the development of the mycorrhizal host plant.

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