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Microbial co-operation in the rhizosphere

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Abstract

Soil microbial populations are immersed in a framework of interactions known to affect plant fitness and soil quality. They are involved in fundamental activities that ensure the stability and productivity of both agricultural systems and natural ecosystems. Strategic and applied research has demonstrated that certain co-operative microbial activities can be exploited, as a low-input biotechnology, to help sustainable, environmentally-friendly, agro-technological practices. Much research is addressed at improving understanding of the diversity, dynamics, and significance of rhizosphere microbial populations and their co-operative activities. An analysis of the co-operative microbial activities known to affect plant development is the general aim of this review. In particular, this article summarizes and discusses significant aspects of this general topic, including (i) the analysis of the key activities carried out by the diverse trophic and functional groups of micro-organisms involved in co-operative rhizosphere interactions; (ii) a critical discussion of the direct microbe–microbe interactions which results in processes benefiting sustainable agro-ecosystem development; and (iii) beneficial microbial interactions involving arbuscular mycorrhiza, the omnipresent fungus–plant beneficial symbiosis. The trends of this thematic area will be outlined, from molecular biology and ecophysiological issues to the biotechnological developments for integrated management, to indicate where research is needed in the future.

Key words: Biological control, microbial inoculates, mycorrhizosphere, nutrient cycling, phytoremediation, rhizobacteria, rhizosphere, soil quality, stress alleviation.

Introduction

The complexity of the soil system is determined by the numerous and diverse interactions among its physical, chemical, and biological components, as modulated by the prevalent environmental conditions (Buscot, 2005). In particular, the varied genetic and functional activities of the extensive microbial populations have a critical impact on soil functions, based on the fact that micro-organisms are driving forces for fundamental metabolic processes involving specific enzyme activities (Nannipieri *et al.*, 2003). Many microbial interactions, which are regulated by specific molecules/signals (Pace, 1997), are responsible for key environmental processes, such as the biogeochemical cycling of nutrients and matter and the maintenance of plant health and soil quality (Barea *et al.*, 2004).

Many studies have demonstrated that soil-borne microbes interact with plant roots and soil constituents at the root–soil interface (Lynch, 1990; Linderman, 1992; Glick, 1995; Kennedy, 1998; Bowen and Rovira, 1999; Barea *et al.*, 2002*b*). The great array of root–microbe interactions results in the development of a dynamic environment known as the *rhizosphere* where microbial communities also interact. The differing physical, chemical, and biological properties of the root-associated soil, compared with those of the root-free bulk soil, are responsible for changes in microbial diversity and for increased numbers and activity of micro-organisms in the rhizosphere micro-environment (Kennedy, 1998). Carbon fluxes are crucial determinants of rhizosphere function (Toal *et al.*, 2000). The release of root exudates and decaying plant material provide sources of carbon compounds for the heterotrophic soil biota as either growth substrates, structural material or signals for the root-associated microbiota (Werner, 1998). Microbial activity in the rhizosphere affects rooting patterns and the supply of available nutrients to plants, thereby modifying the

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quality and quantity of root exudates (Bowen and Rovira, 1999; Gryndler, 2000; Barea, 2000). Two types of interactions in the rhizosphere are recognized, those based on dead plant material (the detritus-based interactions) which affect energy and nutrient flows, and those based on living plant roots. Both types of interactions are relevant to both agronomy and ecology.

Broadly, there are three separate, but interacting, components recognized in the rhizosphere. These are the rhizosphere (soil), the rhizoplane, and the root itself. The rhizosphere is the zone of soil influenced by roots through the release of substrates that affect microbial activity. The rhizoplane is the root surface, including the strongly adhering soil particles. The root itself is a part of the system, because certain micro-organisms, the *endophytes*, are able to colonize root tissues (Kennedy, 1998; Bowen and Rovira, 1999). Microbial colonization of the rhizoplane and/or root tissues is known as *root colonization*, whereas the colonization of the adjacent volume of soil under the influence of the root is known as *rhizosphere colonization* (Kloepper *et al.*, 1991; Kloepper, 1994). The use of molecular techniques to identify micro-organisms (O'Gara *et al.*, 1994) is currently a key tool to study rhizosphere ecology (Puhler *et al.*, 2004).

Because of current public concerns about the side-effects of agrochemicals, there is an increasing interest in improving the understanding of co-operative activities among rhizosphere microbial populations and how these might be applied to agriculture (Kennedy, 1998; Bowen and Rovira, 1999; Barea *et al.*, 2004; Lucy *et al.*, 2004). Certain co-operative microbial activities can be exploited as a low-input biotechnology, and form a basis for a strategy to help sustainable, environmentally-friendly practices fundamental to the stability and productivity of both agricultural systems and natural ecosystems (Kennedy and Smith, 1995). An analysis of the co-operative microbial activities known to affect plant development is the general aim of this review.

The soil micro-biota is often separated into the so-called 'micro-organisms' and the larger 'micro-fauna' (Bowen and Rovira, 1999). Although it is acknowledged that micro-fauna affect plant growth and above-ground food webs (Bonkowski, 2004; Scheu *et al.*, 2005), this review will concentrate on micro-organisms. It will summarize and discuss some key aspects of rhizosphere biology, including (i) analysis of the activities carried out by the diverse trophic and functional groups of micro-organisms involved in co-operative rhizosphere interactions; (ii) direct microbe–microbe interactions which result in processes benefiting sustainable agro-ecosystem development; and (iii) microbial interactions involving arbuscular mycorrhiza. The main conclusions and future trends for research in this area will then be presented.

Diversity of trophic and functional groups of rhizosphere micro-organisms

A variety of microbial forms can be found growing in rhizosphere micro-habitats. It is universally accepted that members of any microbial group can develop important functions in the ecosystem (Giri *et al.*, 2005). However, most studies on rhizosphere microbiology, especially those describing co-operative microbial interactions, have focused their attention on bacteria and fungi (Bowen and Rovira, 1999). Accordingly, this review will focus on these two types of micro-organisms.

The studies involving bacteria discussed here will be restricted to Eubacteria because the interactions of the other bacterial group, the Archaea (or Archaebacteria), with other soil micro-organisms have received very little attention, probably due to their limited success in culture (Pace, 1997). Since molecular approaches are now being used to identify Archaea (Bomberg *et al.*, 2003), their interactions with other soil micro-organisms in the rhizosphere is likely to become the focus of much work in the immediate future.

The prokaryotic bacteria and the eukaryotic fungi have very different trophic/living habits, and a variety of saprophytic and symbiotic relationships, both detrimental (pathogenic) and beneficial (mutualistic), have been described. Barea *et al.* (2004) concluded that detrimental microbes included both the major plant pathogens and the minor parasitic and non-parasitic deleterious rhizosphere bacteria and fungi. Beneficial saprophytes, from a diversity of microbial groups, are able to promote plant growth and health. These include (i) decomposers of organic detritus, (ii) the plant growth promoting rhizobacteria (PGPR), and (iii) fungal and bacterial antagonists of root pathogens. Some of these micro-organisms, the endophytes, colonize the root tissues and promote plant growth and plant protection. Beneficial, plant mutualistic symbionts include the N_2-fixing bacteria and the arbuscular mycorrhizal fungi.

Non-symbiotic beneficial rhizosphere bacteria and fungi

The term *rhizobacteria* is used to describe a subset of rhizosphere bacteria able to colonize the root environment (Kloepper *et al.*, 1991; Kloepper, 1994). Beneficial, root-colonizing, rhizosphere bacteria, the PGPR, are defined by three intrinsic characteristics: (i) they must be able to colonize the root, (ii) they must survive and multiply in microhabitats associated with the root surface, in competition with other microbiota, at least for the time needed to express their plant promotion/protection activities, and (iii) they must promote plant growth. Novel techniques to identify and characterize PGPR, and to study the colonization pattern and molecular determinants of root colonization have been discussed recently (Lugtenberg *et al.*, 1991, 2001; Rothballer *et al.*, 2003; Espinosa-Urgel, 2004; Gamalero *et al.*, 2004).

The PGPR are known to participate in many important ecosystem processes, such as the biological control of plant pathogens, nutrient cycling, and/or seedling growth (Persello-Cartieaux *et al.*, 2003; Barea *et al.*, 2004; Zahir *et al.*, 2004). *Pseudomonas* and *Bacillus* are the genera most commonly described as having PGPR, but many other taxa also contain PGPR. Selected strains of PGPR are being used as seed inoculates (Dobbelaere *et al.*, 2001; Vessey, 2003; Lucy *et al.*, 2004; Sahin *et al.*, 2004; Zahir *et al.*, 2004). Some of these are based on ecologically-tested, genetically-modified bacteria (Morrissey *et al.*, 2002), in accordance with European Union regulations (Nuti, 1994).

The PGPR have been divided into two groups: those involved in nutrient cycling and phytostimulation, and those involved in the biocontrol of plant pathogens (Bashan and Holguin, 1998). The PGPR-mediated processes involved in nutrient cycling include those related to non-symbiotic nitrogen-fixation, and those responsible for increasing the availability of phosphate and other nutrients in the soil. Many asymbiotic diazotrophic bacteria have been described and tested as biofertilizers (Kennedy *et al.*, 2004). Many results are inconclusive, but encouraging enough to improve selection procedures and the production of quality inocula for practical application. The selection of effective PGPR diazotrophs is critical for further development of this technology.

Many rhizobacteria (and rhizofungi) are able to solubilize sparingly soluble phosphates, usually by releasing chelating organic acids (Kucey *et al.*, 1989; Whitelaw, 2000; Richardson, 2001; Vessey *et al.*, 2004). Phosphate-solubilizing bacteria (PSB) have been identified, but their effectiveness in the soil–plant system is still unclear (Barea *et al.*, 2002*a*). Firstly, the inoculated PSB must become established in the root-associated soil habitats. Hence it is recommended that the inoculate PSB is selected from existing PGPR populations to take advantage of their ability to colonize the rhizosphere micro-environment. Secondly, the ability of an inoculated PSB to supply P to a plant may be limited, either because the compounds released by PSB to solubilize phosphate are rapidly degraded or because the solubilized phosphate is re-fixed before it reaches the root surface. However, if the phosphate released by PSB is taken up by a mycorrhizal mycelium, the result would be a co-operative synergistic microbial interaction that improved P acquisition by the plant, as will be discussed later in this review.

In a similar context, bacteria colonizing the rhizoplane of rock-weathering desert plants were found to release a significant amount of minerals (P, K, Mg, Mn, Cu, Zn) from the rocks, and were also thermo-tolerant and/or halo-tolerant (Puente *et al.*, 2004). The role of soil fungi has also been studied in these situations (Hoffland *et al.*, 2004), and there are likely to be synergistic interactions here too.

Azospirillum species are also considered to be PGPR (Okon, 1994; Bashan, 1999; Lucy *et al.*, 2004; Zahir *et al.*, 2004). A significant activity of these bacteria is the production of auxin-type phytohormones that affect root morphology and, thereby, improve nutrient uptake from soil. This may be more important than their N_2-fixing activity (Dobbelaere *et al.*, 1999). *Azospirillum* species are being used as seed inoculates under field conditions (Dobbelaere *et al.*, 2001; Lucy *et al.*, 2004; Zahir *et al.*, 2004). Despite many studies reporting the benefits of *Azospirillum* inoculates, some studies present inconsistent results. However, it can be assumed that, upon establishing appropriate management practices, the use of these inoculates will have a beneficial effect on plant nutrition.

Specific PGPR have been screened as biocontrol agents of microbial plant pathogens (Lugtenberg *et al.*, 1991; Alabouvette *et al.*, 1997; Chin-A-Woeng *et al.*, 2003; de Boer *et al.*, 2003; Persello-Cartieaux *et al.*, 2003). Biological control of soil-borne diseases is known to result from (i) the reduction of the saprophytic growth of the pathogens and then of the frequency of root infections through microbial antagonism, and/or (ii) the stimulation of 'induced systemic resistance (ISR)' in the host-plants (van Loon *et al.*, 1998). The former may be achieved through the release of antibiotics by the PGPR. Among the different antifungal factors produced by PGPR, acetylphloroglucinols (Landa *et al.*, 2003; Picard *et al.*, 2004) and phenacines (Chin-A-Woeng *et al.*, 2003; Ownley *et al.*, 2003) are the products receiving most attention. Good examples of recent advances in our knowledge of PGPR-elicited ISR are provided by Kloepper *et al.* (2004) and Zhang *et al.* (2004). Some micro-organisms can benefit plants in several ways. For example, *Trichoderma* species control fungal pathogens by acting both as a microbial antagonist and by inducing localized and systemic plant defence responses (Harman *et al.*, 2004). Endophytic bacteria and fungi (Sturz and Novak, 2000; Surette *et al.*, 2003; Landa *et al.*, 2004; Sessitsch *et al.*, 2004) act both as growth promoters and as biocontrol agents.

It has recently being postulated that an additional mechanism for plant growth promotion by PGPR could be their altering of microbial rhizosphere communities (Ramos *et al.*, 2003). Agreeing with such an indirect mechanism, it would be interesting to evaluate the actual impact of this activity in rhizosphere biology.

The mutualistic symbionts: N_2-fixing bacteria and arbuscular mycorrhizal fungi

Symbiotic N_2-fixation is a well-known process exclusively driven by bacteria, the only organisms possessing the key enzyme nitrogenase, which specifically reduces atmospheric N_2 to ammonia in the symbiotic root nodules (Postgate, 1998; Leigh, 2002). N_2-fixation is the first step for cycling N to the biosphere from the atmosphere, a key

input of N for plant productivity (Vance, 2001). The bacteria responsible belong to the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Azorhizobium*, collectively termed rhizobia. These bacteria interact with legume roots leading to the formation of N_2-fixing nodules (Spaink *et al.*, 1998; Sprent, 2002). The signalling processes (Lindström *et al.*, 2002), the evolutionary history (Henson *et al.*, 2004) and, particularly, the molecular aspects determining host specificity in the rhizobial–legume symbiosis (Young *et al.*, 2002) have been reviewed recently. Other bacteria (actinomycetes) of the genus *Frankia* form nodules on the root of 'actinorrhizal' plant species, which are of great ecological importance (Vessey *et al.*, 2004). The genetics and genomics of their root symbiosis is a matter of current attention (Stougaard, 2001; Riely *et al.*, 2004).

The other major group of microbial plant mutualistic symbionts are the fungi which establish a (mycorrhizal) symbiosis with the roots of most plant species. The soil-borne mycorrhizal fungi colonize the root cortex biotrophycally, then develop an external mycelium which is a bridge connecting the root with the surrounding soil microhabitats. Mycorrhizal symbioses can be found in almost all ecosystems worldwide to improve plant fitness and soil quality through key ecological processes. Most of the major plant families form arbuscular mycorrhiza (AM) associations, the most common mycorrhizal type (Smith and Read, 1997). The AM fungi responsible are obligate microbial symbionts, unable to complete their life cycle without colonizing a host plant. They are ubiquitous soil-borne microbial fungi, whose origin and divergence have been dated back to more than 450 million years ago (Redecker *et al.*, 2000). The AM fungi were formerly included in the order Glomales in the Zygomycota (Redecker *et al.*, 2000), but they have recently been moved to a new phylum Glomeromycota (Schüßler *et al.*, 2001). As this is the most widespread mycorrhizal symbiosis, this review will focus only on the AM fungal symbiosis with plants. However, the importance of microbial interactions involving ectomycorrhizal associations, particularly in forest ecosystems (Frey-Klett *et al.*, 2005), must be recognized. There is a great analogy between these two types of mycorrhizal symbioses, as will be commented on later in this review.

Studies on the diversity of AM fungi in natural environments have been hampered by difficulties in their identification, a process traditionally based on the ontogeny and morphological characters of their large multinucleate spores. However, recent reports indicate that ribosomal DNA sequence analysis is a suitable tool with which to infer the phylogenetic relationships of AM fungi and to analyse the diversity of natural AM populations (Rodríguez *et al.*, 2004; Ferrol *et al.*, 2004*b*). Fingerprinting techniques, using gel electrophoresis of PCR-amplified rDNA fragments, are being applied (Cornejo *et al.*, 2004). In particular, temporal temperature gradient gel electrophoresis (TTGE) was found useful for identifying AM fungal species colonizing the rhizosphere soil and/or the root itself (Cornejo *et al.*, 2004). Recent advances in the genetic and genomics of the AM fungi have been reviewed (Ferrol *et al.*, 2004*a*; Gianinazzi-Person *et al.*, 2004; Parniske, 2004).

The obligate character of the AM fungi has meant that analysis of the processes involved in the formation of AM symbioses has required careful methodological approaches (Giovannetti *et al.*, 2002).

The AM symbiosis influences nutrient cycling in soil–plant systems, and improves plant health through increased protection against biotic and abiotic stresses, and soil structure through aggregate formation (Bethlenfalvay and Linderman, 1992; Gianinazzi and Schüepp, 1994; Smith and Read, 1997; Kapulnik and Douds, 2000; Gianinazzi *et al.*, 2002; Turnau and Haselwandter, 2002; van der Heijden and Sanders, 2002; Jeffries *et al.*, 2003; Barea *et al.*, 2005*a*; Turnau *et al.*, 2005). Briefly, the AM symbiosis increases the supply of mineral nutrients to the plant, particularly those whose ionic forms have poor mobility or those present in low concentrations in the soil solution. This mainly applies to phosphate, ammonium, zinc, and copper. The AM association also improves plant health through increased protection against biotic and abiotic stresses, with possible applications in *biocontrol* of plant soil-borne microbial pathogens, and in *bioremediation* of polluted soils.

Since the AM symbiosis can benefit plant growth and health, there is an increasing interest in ascertaining its effectiveness in particular plant production systems and, consequently, in manipulating them, when feasible, so that they can be incorporated into production practices. Evidence is accumulating to show that indigenous and/or introduced AM fungi can benefit annual crops, such as cereals and legumes, vegetable crops, temperate fruit trees or shrubs, tropical plantation crops, ornamentals, and spices (Azcón-Aguilar and Barea, 1997; Vestberg *et al.*, 2002). Selection of the appropriate AM fungi (Estaún *et al.*, 2002), the production of quality inocula (von Alten *et al.*, 2002), and the analysis of the ecology of AM inoculation (Vosatka and Dodd, 2002; Feldmann and Grotkass, 2002) are critical issues for the application of AM technology in agriculture. A Federation of European Mycorrhizal Inoculum Producers has been created.

Some AM fungi have established a particular type of symbiosis with endosymbiotic bacteria, previously assigned to the genus *Burkholderia* (Bianciotto *et al.*, 2002; Bianciotto and Bonfante, 2002) and recently reassigned to a new taxon named '*Candidatus* glomeribacter gigasporarum' (Jargeat *et al.*, 2004). These bacteria have interesting metabolic genes that may influence AM functions, and current investigations are aimed at exploting this cooperative relationship.

Microbe–microbe interactions benefiting sustainable agro-ecosystem development

Direct interactions occurring between members of different microbial types often result in the promotion of key processes benefiting plant growth and health. It is obvious that all interactions taking place in the rhizosphere are, at least indirectly, plant-mediated. However, this section will deal with direct microbe–microbe interactions themselves, with the plant as a 'supporting actor' in the rhizosphere. Three types of interactions have been selected for discussion here because of their relevance to the development of sustainable agro-ecosystems. These are: (i) the co-operation between PGPR and *Rhizobium* for improving N_2-fixation; (ii) microbial antagonism for the biocontrol of plant pathogens; and (iii) interactions between rhizosphere microbes and AM fungi to establish a functional mycorrhizosphere.

PGPR-Rhizobium *co-operation to improve N_2-fixation*

As they share common microhabitats in the root–soil interface, rhizobia and PGPR must interact during their processes of root colonization. Some PGPRs can improve nodulation and N_2-fixation in legume plants (Polenko *et al.*, 1987; Fuhrmann and Wollum, 1989; Zhang *et al.*, 1996; Andrade *et al.*, 1998; Lucas-Garcia *et al.*, 2004). Studies carried out under field conditions (Dashti *et al.*, 1998; Bai *et al.*, 2002, 2003), particularly those using ^{15}N-based techniques (Dashti *et al.*, 1998) reinforce such beneficial co-operative effects between microbes.

Research on the mechanisms by which PGPR enhance nodule formation implicates their production of plant hormones among the co-inoculation benefits. For example, Chebotar *et al.* (2001) demonstrated that some *Pseudomonas* strains, but not all, increased nodule number and acetylene reduction in soybean plants inoculated with *B. japonicum*. The use of *gus-A* marked rhizobacteria allowed the authors to demonstrate that the bacteria colonized the root. Azcón-Aguilar and Barea (1978), using both cell-free supernatants of PGPR cultures and pure chemicals, first demonstrated that plant-growth-regulating substances produced by PGPR affected nodulation and nitrogen fixation. Recently, Mañero *et al.* (2003) extended these observations. The possibility that metabolites other than phytohormones, such as siderophores, phytoalexins, and flavonoids, might enhance nodule formation has also been proposed (Lucas-Garcia *et al.*, 2004), but this hypothesis has not been verified.

Inoculation of phosphate-solubilizing bacteria (PSB) enhanced nodulation and N_2-fixation (^{15}N) by alfalfa plants, in parallel with an increase in the P content of plant tissues (Toro *et al.*, 1998). It is therefore thought that an improvement in P nutrition of the plant resulting from the presence of PSB was responsible for increased nodulation and N_2-fixation, as it is well-known that these processes are P-dependent (Barea *et al.*, 2005*b*).

In a recent study it was demonstrated that PGPR isolated from a Cd-contaminated soil increased the nodulation of clover plants growing in this soil (Vivas *et al.*, 2005). One explanation for this effect may be that the PGPR accumulated Cd, and therefore reduced solution Cd concentrations and Cd uptake by plants and rhizobia, thereby preventing Cd toxicity and enabling nodulation. In addition, an increase in soil enzymatic activities (phosphatase, β-glucosidase, dehydrogenase) and of auxin production around PGPR-inoculated roots could also be involved in the PGPR effect on nodulation.

Microbial antagonism in the biological control of plant pathogens

In the early 1970s several researchers identified microbial populations in the rhizosphere as constituting the first barrier to pathogen infection. Nowadays, it is well known that some soils are naturally suppressive to some soil-borne plant pathogens including *Fusarium*, *Gaeumannomyces*, *Rhizoctonia*, *Pythium*, and *Phytophthora*. Although this suppression relates to both physicochemical and microbiological features of the soil, in most systems the biological elements are the primary factors in disease suppression and the topic of 'biological control of plant pathogens' gained feasibility in the context of sustainable issues (Weller *et al.*, 2002). The groups of micro-organisms with antagonistic properties towards plant pathogens are diverse, including plant-associated prokaryotes and eukaryotes. A detailed overview of mechanisms involved in microbial antagonism, and a compilation of organisms with demonstrated antagonistic properties used in the biocontrol of pathogens, appears in Whipps (1997, 2001). Among the prokaryotes, a wide range of bacteria such as *Agrobacterium*, *Bacillus* spp. (e.g. *B. cereus*, *B. pumilis*, and *B. subtilis*), *Streptomyces*, and *Burkholderia* have been shown to be effective antagonists of soil-borne pathogens. The most widely studied bacteria by far in relation to biocontrol are *Pseudomonas* spp., such as *P. aeruginosa* and *P. fluorescens*, which are probably amongst the most effective root-colonizing bacteria. Among the eukaryotes, there are a variety of fungal species and isolates that display antagonistic properties and have been applied in biocontrol, but the ubiquitous *Trichoderma* species clearly dominate. In addition, non-pathogenic species of fungi such as *Pythium* and *Fusarium* are receiving increasing interest as antagonists.

Pathogen suppression by antagonistic micro-organisms can result from one or more mechanisms depending on the antagonist involved. Direct effects on the pathogen include competition for colonization or infection sites, competition for carbon and nitrogen sources as nutrients and signals, competition for iron through the production of iron-chelating compounds or siderophores, inhibition of the pathogen by antimicrobial compounds such as antibiotics and HCN,

degradation of pathogen germination factors or pathogenicity factors, and parasitism. These effects can be accompanied by indirect mechanisms, including improvement of plant nutrition and damage compensation, changes in root system anatomy, microbial changes in the rhizosphere, and activation of plant defence mechanisms, leading to enhanced plant resistance. An effective biocontrol agent often acts through the combination of several different mechanisms (Whipps, 2001).

Rhizobacteria from the genus *Pseudomonas* provide an excellent example of a combination of multiple mechanisms for effective biocontrol including direct antagonism and induction of plant resistance. *Pseudomonas* spp. produce several metabolites with antimicrobial activity towards other bacteria and fungi (Haas and Keel, 2003). Indeed, the first clear-cut experimental demonstration that a bacteria-produced antibiotic could suppress plant disease in an ecosystem was made by Tomashow and Weller (1988). Using an elegant genetic approach, they demonstrated the direct correlation between the production of a phenazine antibiotic by a fluorescent *Pseudomonas* sp. and its biocontrol activity against take-all disease of wheat. Competition is another key factor in the antagonistic properties of *Pseudomonas* spp. In addition to competition for substrates (Couteaudier and Alaboubette, 1990), research on the siderophores produced by *Pseudomonas* species (pyoverdine, pyochelin) has shown the involvement of siderophore-mediated competition for iron in the control of *Fusarium* and *Pythium* in soils (Duijff *et al.*, 1994; Raaijmakers *et al.*, 1995).

Another well-studied example illustrating a combination of mechanisms for successful antagonism of plant pathogens is provided by the filamentous fungus *Trichoderma* spp. These ubiquitous soil fungi are well-known for their effectiveness in controlling a broad range of phytopathogenic fungi such as *Rhizoctonia solani*, *Pythium ultimum*, and *Botrytis cinerea*. The direct mechanisms involved in this protective effect include competition, antibiosis (Howell, 1998), and mycoparasitism (Jeffries, 1997). *Trichoderma* grows towards the fungal pathogen and releases toxic compounds (e.g. the antibiotics gliotoxin, gliovirin, and peptabiols) and a battery of lytic enzymes, mainly chitinases, glucanases, and proteases. These enzymes facilitate penetration into the host by *Trichoderma* and the utilization of the host for nutrition (Lorito *et al.*, 1996). Direct evidence for the role of cell-wall degrading enzymes in biocontrol *in vivo* comes from studies utilizing mutant strains over-expressing or lacking a particular enzyme, or transgenic plants expressing these enzymes (Baek *et al.*, 1999; Lorito *et al.*, 1998; Mendoza-Mendoza *et al.*, 2003; Pozo *et al.*, 2004). In addition, recent studies indicated the importance of the induction of plant defence mechanisms in biocontrol by *Trichoderma* (Harman *et al.*, 2004).

Several reports show the potential of combining different biocontrol agents with different disease-suppressive mechanisms in the field (de Boer *et al.*, 1999, 2003). The development of appropriate combinations should provide a higher level of plant protection, a wider range of effectiveness and a reduction of variability in the results. Thus, the optimal use of the antagonistic properties of the microbiota will result in a more effective and more reliable biocontrol of plant pathogens, and constitutes a very promising research area.

Interactions between rhizosphere microbes and AM fungi to establish a functional mycorrhizosphere

Microbial populations in the rhizosphere are known either to interfere with or to benefit the establishment of mycorrhizal symbioses (Gryndler, 2000). A typical beneficial effect is that exerted by the 'mycorrhiza-helper-bacteria' (MHB), a term that was coined by Garbaye (1994) for those bacteria known to stimulate mycelial growth of mycorrhizal fungi and/or enhance mycorrhizal formation. This applies both to Ectomycorriza (Garbaye, 1994; Founoune *et al.*, 2002; Frey-Klett *et al.*, 2005) and to AM associations (Azcón-Aguilar and Barea, 1995; Gryndler, 2000; Barea *et al.*, 2004; Johansson *et al.*, 2004). Soil micro-organisms are known to produce compounds that increase the rates of root exudation. This, in turn, stimulates AM fungal mycelia in the rhizosphere or facilitates root penetration by the fungus. Plant hormones, as produced by soil micro-organisms, are known to affect AM establishment (Azcón-Aguilar and Barea, 1992). Rhizosphere micro-organisms are also known to affect the presymbiotic stages of AM development, such as spore germination rate and mycelial growth (Azcón-Aguilar and Barea, 1992, 1995).

The establishment of the AM fungus in the root cortex is known to change many key aspects of plant physiology. These include the mineral nutrient composition of plant tissues, the hormonal balance, and the patterns of C allocation. Therefore, the AM symbiotic status changes the chemical composition of root exudates, while the development of an AM soil mycelium, which can act as a carbon source for microbial communities, introduces physical modifications into the environment surrounding the roots.

AM-induced changes in plant physiology affect the microbial populations, both quantitatively and qualitatively, in either the rhizosphere and/or the rhizoplane. Therefore, the rhizosphere of a mycorrhizal plant can have features that differ from those of a non-mycorrhizal plant (Barea *et al.*, 2002*a*, *b*; Johansson *et al.*, 2004). However, there are specific modifications in the environment surrounding the AM mycelium itself, the *mycorrhizosphere* (Linderman, 1988; Gryndler, 2000). In addition to this term, the soil space affected by extraradical hyphae is also called the *mycosphere* (Linderman, 1988) or *hyphosphere* as an analogy with the term rhizosphere (Gryndler, 2000). Large numbers of bacteria (including actinomycetes) and

fungi can be associated with both AM fungal structures (Budi *et al.*, 1999) and ectomycorrhizal structures (Bedini *et al.*, 1999; Frey-Klett *et al.*, 2005). Since the AM mycelium releases energy-rich organic compounds, an increased growth and activity of microbial saprophytes can be expected to occur in the mycorrhizosphere. However, the enrichment of this particular environment by organic compounds is much lower than that of the rhizosphere, corresponding to lower counts of bacteria in mycorrhizosphere soil, compared with those in the rhizosphere (Andrade *et al.*, 1997).

The establishment of PGPR inoculates in the rhizosphere can be affected by AM fungal co-inoculation (Ravnskov *et al.*, 1999; Bianciotto *et al.*, 2002; Bianciotto and Bonfante, 2002). In particular, AM inoculation improves the establishment of both inoculated and indigenous phosphate-solubilizing rhizobacteria acting as MHB (Toro *et al.*, 1997; Barea *et al.*, 2002*c*).

Interactions between arbuscular mycorrhiza and rhizosphere micro-organisms

The AM symbiosis occupies a central position in rhizosphere development and many types of interactions involving this microbial symbiosis and significant microbial groups have been reported (Barea *et al.*, 2004). The main conclusions from key information will be critically summarized here by considering interactions related to: (i) symbiotic N_2-fixation; (ii) phosphate solubilization; (iii) phytoremediation of heavy metal contaminated soils; (iv) biological control of root pathogens; and (v) improvement of soil quality.

Interactions with symbiotic N_2-fixing bacteria

The widespread presence of the AM symbiosis in nodulated legumes and the role of AM fungi in improving nodulation and rhizobial activity within the nodules, are both universally recognized processes (Barea *et al.*, 2005*b*). In the last 50 years much work has been carried out on the tripartite symbiosis of legume–AM fungi–rhizobia. Particularly interesting were the findings demonstrating that the evolution and interaction patterns of both the N_2-fixing and mycorrhizal symbioses are similar (Parniske, 2000). A common ancestral plant–fungal interaction has been proposed, and because the rhizobia–legume symbiosis evolved much later than AM associations (Provorov *et al.*, 2002), it has been hypothesized that the cellular and molecular events occurring during legume nodulation may have evolved from those already established in the AM symbiosis (Gianinazzi-Pearson, 1997). In fact, the legume–rhizobia symbiosis seems to have evolved from a set of pre-adaptations during co-evolution with AM fungi (Provorov *et al.*, 2002). However, the possibility that some plant genes can modulate both types of legume symbiosis has been challenged (Ruiz-Lozano *et al.*, 1999; Novero *et al.*, 2002; Stracke *et al.*, 2002; Lum and Hirsch, 2003; Demchenko *et al.*, 2004). Most studies take advantage of the mycorrhiza-defective mutants (Myc^-), which have allowed the common cellular and genetic programmes responsible for the legume symbioses to be dissected. These mutants have also allowed an insight into the common signal-transduction pathways shared by both microbe–plant symbioses (Gollotte *et al.*, 2002).

From the trophic point of view, AM establishment has been shown to improve nodulation and N_2 fixation, and the use of the isotope ^{15}N has made it possible to ascertain and quantify the amount of N that is fixed in a particular situation, as well as the contribution of the AM symbiosis to N_2 fixation (Barea *et al.*, 1987, 1989, 1992, 2002*c*). The physiological and biochemical basis of AM fungal×*Rhizobium* interactions in improving legume productivity indicated that the main effect of AM in enhancing *Rhizobium* activity is through a generalized stimulation of host nutrition, but some localized effects may also occur at the root or nodule level (Barea *et al.*, 1992).

Multi-microbial interactions, including not only AM fungi and *Rhizobium* spp. but also PGPR, have also been tested (Requena *et al.*, 1997). In general, the results support the importance of physiological and genetic adaptation of microbes to the environment. Thus, local isolates are recommended for biotechnological applications. Several microbial combinations are effective in improving plant development, nutrient uptake, N_2-fixation (^{15}N) or root system quality, and these show selective and specific functional compatibility relationships among the microbial inoculates.

Since AM colonization can help plants to cope with drought and salinity stresses (Augé, 2001, Ruiz-Lozano, 2003), the role of this symbiosis in legumes is particularly interesting. AM inoculation improved nodulation and N_2 fixation at low levels of water potential (Azcón *et al.*, 1988; Goicoechea *et al.*, 1997, 1998) and compensated for the negative effects of salinity on nodulation and N_2 fixation (Ruiz-Lozano and Azcón, 1993).

More recent experiments have corroborated a positive effect of the interactions between AM fungi and rhizobia under drought conditions (Ruiz-Lozano *et al.*, 2001). For example, it was found that inoculation with AM fungi protected soybean plants against the detrimental effects of drought and helped them cope with the premature nodule senescence induced by drought stress (Porcel *et al.*, 2003).

Interactions with phosphate-solubilizing bacteria (PSB)

The primary effect of AM establishment is the improvement of phosphate uptake by plants due to the ability of the external mycelium of AM fungi to act as a bridge between roots and the surrounding soil microhabitats. This gives access to the phosphate ions from the soil solution beyond the phosphate-depletion zone surrounding the roots (Smith

and Read, 1997). The AM fungi can contribute to P capture and supply, by linking the biotic and geochemical portions of the soil ecosystem, therefore affecting P cycling rates and patterns in both agricultural and natural ecosystems (Jeffries and Barea, 2001). Because the phosphate made available by PSB acting on sparingly soluble P sources may not reach the root surface due to limited diffusion, it was proposed that if the solubilized phosphate were taken up by an AM mycelium, this synergistic microbial interaction should improve P supply to the plant (Barea *et al.*, 1983). This was investigated in studies that included the application of poorly reactive rock phosphate to a non-acidic soil and the use of ^{32}P-labelling methodologies (Toro *et al.*, 1997). Upon adding a small amount of ^{32}P to label the exchangeable soil P pool, the isotopic composition, or 'specific activity' (SA=^{32}P/^{31}P), is determined in plant tissues (Zapata and Axmann, 1995). It was found that dual inoculation reduced the SA of the host plants, indicating that they accumulated more ^{31}P solubilized from P sources not directly available to control plants.

A model experiment involving the use of isotopic techniques and field trials to validate results from greenhouse assays (Barea *et al.*, 2002*c*), is summarized here to illustrate the effect of PSB×AM interactions on P capture, cycling, and supply. This experiment involved a factorial combination of four microbial and two chemical treatments. The microbial treatments were: (i) AM inoculation; (ii) PSB inoculation; (iii) AM plus PSB dual inoculation; and (iv) non-inoculated controls, exposed to the naturally existing AM fungi and PSB. The two chemical treatments were: (i) non-amended control without P application, and (ii) rock phosphate application. For the greenhouse experiment, the exchangeable soil P pool was labelled with ^{32}P. The ^{32}P activity in the plant material was measured and the SA was calculated. Both rock phosphate addition and microbial inoculation improved biomass production and P accumulation in the test plants, with dual microbial inoculation being the most effective treatment. Independently of rock phosphate addition, AM-inoculated plants showed a lower SA (^{32}P/^{31}P) than their comparable non-AM inoculated controls, particularly when they were inoculated with PSB. This means that AM-inoculated plants were taking soil P which was labelled differentially from that taken up by control plants. Possibly, the PSB were effective in releasing ^{31}P from sparingly soluble sources, either from the soil components or from the added rock phosphate. This release of P would constitute a part of the total ^{31}P pool from which the AM mycelium tapped phosphate and transferred it to the plants. Such microbial activities could result in the lower SA in dually-inoculated plants. Results from the field trial corroborated the interactions between AM fungi and PSB in a co-operative fundamental role for P-cycling, stimulating considerable interest in their application to sustainable agro-ecosystems.

Multi-microbial interactions, including those between locally isolated AM fungi, PSB, and *Azospirillum*, have also been reported, which indicate clearly that micro-organisms act synergistically when inoculated simultaneously (Muthukumar *et al.*, 2001).

Interactions involved in phytoremediation of soil contaminated with heavy metals

The use of living organisms for the remediation of soils contaminated with heavy metals, radionuclide or polycyclic aromatic hydrocarbon is known as 'bioremediation' (Kumar *et al.*, 1995; Brooks and Robinson, 1998; Salt *et al.*, 1998; Baker *et al.*, 2000). AM fungi are involved in bioremediation through phytoremediation, the technique based on the use of plants for soil remediation (Leyval *et al.*, 1997; Turnau *et al.*, 2005). Depending on the type of pollutant, different strategies for phytoremediation, such as phytostabilization, phytodegradation, and phytoextraction, can be used. Only examples involving heavy metals (HMs) will be discussed here, which illustrate microbial co-operation in the rhizosphere. For phytoremediation of soil polluted with HMs, the phytostabilization strategy involves the immobilization of HMs in the soil by establishing plants. This reduces both soil erosion and transfer of the HMs to aquifers, thus avoiding their dispersion by the wind. Alternatively, phytoextraction takes advantage of the ability of plants to hyperaccumulate metals (Turnau *et al.*, 2005).

AM can help phytoremediation activities, particularly in phytostabilization (Gonçalves *et al.*, 1997; Leyval *et al.*, 1997, 2002; Orlowska *et al.*, 2002; Regvar *et al.*, 2003; Turnau *et al.*, 2005). Among the possible mechanisms by which AM fungi improve the resistance of plants to HMs is the ability of the AM fungi to sequester HMs through the production of chelates or by absorption. AM plants typically translocate less HM to their shoots than the corresponding non-AM controls. The role of AM fungi in phytoextraction is thought to be less significant. However, the involvement of AM is being investigated now because of the recent interest in plants able to hyper-accumulate HMs (Turnau *et al.*, 2005). Hyperaccumulating plants are usually non-mycorrhizal and produce little biomass, but there are several reports of the presence of AM in hypeaccumulating plants such as *Berkheya coddii*. This plant is capable of accumulating high concentrations of HMs under natural conditions and produces a biomass that exceeds most other hyperaccumulators. Although AM fungi do not necessarily stimulate phytoextraction, the potential to increase the biomass of the plants, to enhance nutrient and water uptake and to improve soil conditions are important reasons to include AM fungi in further research (Turnau *et al.*, 2005).

Among the diverse types of mycorrhizosphere interactions known to benefit plant growth and health, those

related to phytoremediation processes merit special attention. As rhizobacteria and AM fungi interact synergistically to the benefit of phytoremediation, the selection of target rhizobacteria is necessary (Takács *et al.*, 2001). Selection procedures must achieve: (i) isolation of adapted bacteria from HM contaminated soils; (ii) ecological compatibility with AM fungi also adapted to HM-contamination; and (iii) functional compatibility of both types of micro-organisms in terms of promoting phytoextraction and/or phytostabilization of metals from the polluted soil.

A key point in phytoremediation is the use of HM-adapted microbes. Soil microbial diversity and activity are both negatively affected by excessive concentration of HMs. Indigenous bacterial populations (Giller *et al.*, 1998) and AM fungi (del Val *et al.*, 1999) must be adapted to metal toxicity and have evolved abilities to enable them to survive in polluted soils.

Long-term experiments using soils supplemented with specific HMs can demonstrate the individual toxic effects of each HM on the beneficial microbes, and hence indicate which can be used in phytoremediation studies (Biró *et al.*, 1998). To achieve this, an agricultural soil from Nagyhörcsök Experimental Station (Hungary) was contaminated in 1991 with suspensions of 13 microelement salts applied separately (Biró *et al.*, 1998). Using this soil, the role of a tailored mycorrhizosphere in phytoremediation was investigated for the first time in a series of studies (Vivas *et al.*, 2003*a*, *b*, *c*, *d*, 2005). These studies consisted of: (i) isolation and characterization of micro-organisms from a target HM contaminated site; (ii) development of several phytoremediation experiments; and (iii) analysis of the mechanisms accounting for the demonstrated phytoextraction and/or phytostabilization activities found. Micro-organisms isolated from the HM-contaminated soils ('autochthonous metal-adapted AM fungi and/or bacteria') were compared with micro-organisms in the same taxa from culture collections, which were non-adapted to the HM-contaminated sites. The microbial isolates were tested for their influence on plant growth, nutrient acquisition, and metal accumulation by plants in soils containing Zn, Cd, Pb, or Ni. The main achievements from these experiments are summarized below.

The most efficient bacterial isolates were identified by 16S rDNA sequence analysis as *Brevibacillus* spp., with *B. brevis* being most common (Vivas *et al.*, 2003*a*, *c*). The test bacteria accumulated large amounts of metals in *in vitro* assays. The AM fungus *Glomus mosseae* was present in all the HM-polluted soil samples, and was the target AM fungus used for phytoremediation inoculation experiments. *Trifolium repens* L., the commonest plant species found in the target contaminated areas, was used as the test plant and was inoculated with a HM-tolerant strain of *Rhizobium leguminosarum* bv. *trifoli*.

In the Cd-contaminated soil (Vivas *et al.*, 2003*c*, *d*), co-inoculation with a Cd-adapted autochthonous *Brevibacillus* sp. and *G. mosseae* increased biomass, N and P content as compared to non-inoculated plants, and also enhanced the establishment of symbiotic structures (nodule number and AM colonization), which were negatively affected as the level of Cd in soil increased. Dual inoculation lowered Cd concentrations in *Trifolium* plants, inferring a phytostabilization-based activity. However, the total Cd content in plant shoots was higher in dually-inoculated plants due to the effect on biomass accumulation indicating a possible phytoextraction activity. Further studies (Vivas *et al.*, 2005) demonstrated that the inoculated Cd-adapted bacteria increased dehydrogenase, phosphatase, and β-gluconase activities in the mycorrhizosphere, indicating an enhancement of microbial activities related to plant development. Similar conclusions were obtained in experiments on Pb- or Ni-spiked soil (Vivas *et al.*, 2003*a*), where auxin production by the test bacteria could account for the beneficial role of these bacteria on AM-plant development (Barea *et al.*, 2002*b*). Both phytostabilization and phytoextraction activities were also evident.

The mechanisms by which the tested bacterial isolates enhanced phytoremediation activity in AM plants can therefore be summarized as follows: (i) improved rooting, and AM formation and functioning; (ii) enhanced microbial activity in the mycorrhizosphere; and (iii) accumulation of metals in the root–soil environment, thus avoiding their transfer to the trophic chain, or to aquifers. In conclusion, a clear effect of mycorrhizosphere co-operative interactions was demonstrated on 'phytostabilization', but a significant effect on 'phytoextraction' was also shown. Therefore, whatever the mechanisms involved, a selected HM-adapted mycorrhizosphere can apparently be tailored to improve plant tolerance to HMs and to benefit bioremediation of HM-contaminated soils.

Interactions that influence the biological control of root pathogens

The establishment of AM fungi in plant roots has been shown to reduce damage caused by soil-borne plant pathogens with an enhancement of plant resistance/tolerance in mycorrhizal plants. In any case, the effectiveness of AM in biocontrol is dependent on the AM fungus involved, as well as the substrate and the host plant (Azcón-Aguilar and Barea, 1996; Linderman, 2000; Whipps, 2004). Different mechanisms have been suggested to account for this effect of AM fungi (Azcón-Aguilar and Barea, 1992, 1996; Linderman, 1994, 2000; Elmer, 2002; Azcón-Aguilar *et al.*, 2002). One mechanism is via the changes in microbial communities that are produced as the mycorrhizosphere develops. There is strong evidence that shifts in microbial community structure and the resulting microbial equilibria can influence the growth and health of plants (Azcón-Aguilar and Barea, 1992, 1996; Linderman, 1994, 2000). Activation of plant defence mechanisms, including

the development of systemic resistance have also been proposed (Cordier *et al.*, 1998; Pozo *et al.*, 2002), but the occurrence of this mechanism, and its impact in biological control, needs further research. All in all, the use of AM fungi in biocontrol is a promising practice, and current research is trying to determine its potential (Whipps, 2004).

Since specific PGPR antagonistic to root pathogens are being used as biological control agents (Alabouvette *et al.*, 1997), an aim is to exploit the prophylactic ability of AM fungi in association with these antagonists (Linderman, 1994, 2000; Azcón-Aguilar and Barea, 1996). Experimental evidence is accumulating for this activity, but information is too scarce for general conclusions. However, Vestberg *et al.* (2004) conducted a model and comprehensive set of experiments that merits further discussion. Seven nursery experiments were carried out to test different conditions and/or inoculation patterns on the effect of five diverse rhizosphere micro-organisms involved in the biological control of two strawberry diseases, crown rot (caused by *Phytophothora cactorum*) and red stele (caused by *P. fragari*). The micro-organisms tested were registered strains of the AM fungus *Glomus mosseae*, the biocontrol bacteria *Bacillus subtilis* and *Pseudomonas fluorescens*, and the biocontrol fungi *Trichoderma harzianum* and *Gliocladium catenulatum*. Inocula from these microbes were applied singly or in dual mixtures. In most experiments, all the inoculated micro-organims except *T. harzianum* and *G. mosseae* established in the rhizosphere. The growth-promoting effects were not consistent and dual inoculation did not increase growth to any greater extent than a single inoculation. *B. subtilis* was the most promising PGPR. In some treatments a decrease in crown rot symptoms in the shoot was found, with the mixture *T. harzianum*+*G. catenulatum* being most effective. The general conclusion was that, 'the great variation between experiments indicates that more studies are needed for optimization of the whole plant–substrate–micro-organism system'. It can thus be concluded that the use of mycorrhizosphere interactions for the enhancement of root resistence/tolerance to pathogen attack is a promising biotechnological tool. However, because the prophylactic effect is not exerted with the same effectiveness by all microbial combinations, it is not applicable to all pathogens, all substrates, or all environmental conditions. More research is needed for the successful application of microbial consortia in sustainable agricultural practices.

A key point is to ascertain whether an antifungal biocontrol agent will negatively affect beneficial fungi, such as AM fungi. Several studies have demonstrated that microbial antagonists of fungal pathogens, either fungi or PGPR, do not exert any anti-microbial effect against AM fungi (Calvet *et al.*, 1993; Barea *et al.*, 1998; Edwards *et al.*, 1998; Vazquez *et al.*, 2000). This is the key to exploiting the possibilities of dual (AM fungi and PGPR) inoculation to aid plant defence against root pathogens. Barea *et al.* (1998) carried out a series of experiments to test the effect of *Pseudomonas* strains producing 2,4-diacetylphloroglucinol (DAPG) on AM formation and functioning. Three *Pseudomonas* strains were tested for their effects on AM fungi: a wild type (F113) producing the antifungal compound DAPG; the genetically-modified strain (F113G22), a DAPG-negative mutant of F113; and another genetically-modified strain [F113 (pCU203)], a DAPG-over-producer. The results from *in vitro* and *in situ* experiments under controlled conditions demonstrated no negative effects of these *Pseudomonas* strains on spore germination. There was, however, a stimulation of hyphal growth of *G. mosseae*. A field experiment was designed to validate these results. None of these *Pseudomonas* strains affected: (i) the numbers or diversity of the native AM fungal population; (ii) the percentage of root length that became mycorrhizal; or (iii) AM performance. Furthermore, the antifungal *Pseudomonas* improved plant growth and nutrient (N and P) acquisition by the mycorrhizal plants (Barea *et al.*, 1998).

Interactions for improving soil quality

Physico-chemical soil properties are fundamental for soil quality, with soil structure being one of the most influential factors (Buscot, 2005). Soil particles are bound together into aggregates and these influence the precise pore structure of the soil (Tisdall, 1996). When the soil is exposed to environmental stresses, maintaining its structural stability is critical in the prevention of soil erosion (Oades, 1993). A well-aggregated soil structure ensures appropriate soil tilth, soil–plant water relations, water infiltration rates, soil aeration, root penetrability and organic matter accumulation, which all contribute to soil quality (Miller and Jastrow, 2000).

The contribution of microbial co-operation in the rhizosphere to the formation and stabilization of soil aggregates has been demonstrated frequently (Miller and Jastrow, 2000). Firstly, soil particles are bound together by bacterial products and by hyphae of saprophytic and AM fungi, into stable microaggregates (2–20 μm in diameter). These are bound by microbial products into larger microaggregates (20–250 μm in diameter), with bacterial polysaccharides acting as binding agents. Microaggregates are then bound into macroaggregates (>250 μm in diameter), with bacterial polysaccharides acting as binding agents and AM mycelia increasing the size of macroaggregates. The role of AM is accounted for by the size, branching habits and three-dimensional structure of the external mycelium colonizing the soil surrounding the roots, an activity that can persist up to 22 weeks after the plant has died (Miller and Jastrow, 2000).

The effect of the AM fungi in co-operation with other microbes in the formation of water-stable soil aggregates is evident in different ecological situations (Andrade *et al.*, 1995, 1998; Bethlenfalvay and Schüepp, 1994; Bethlenfalvay

et al., 1999; Requena *et al.*, 2001), and the involvement of glomalin, a glycoprotein produced by the external hyphae of AM fungi, has been demonstrated (Wright and Upadhyaya, 1998). Because of its glue-like hydrophobic nature, glomalin participates in the initiation and stabilization of soil aggregates (Miller and Jastrow, 2000).

As a result of degradation/desertification processes, disturbance of natural plant communities is often accompanied, or preceded by, loss of physicochemical and biological properties of the soil, such as soil structure, plant nutrient availability, organic matter content, and microbial activity (Jeffries and Barea, 2001). Management of AM fungi, together with rhizosphere bacteria, aimed at restoring these soil traits has been investigated (Requena *et al.*, 2001).

A representative area within a desertified semi-arid ecosystem in southeast Spain, was chosen for field studies on this topic. The existing natural vegetation was a degraded shrubland where *Anthyllis cytisoides*, a drought-tolerant legume able to form symbioses with both rhizobial and AM microsymbionts, was the dominant species (Requena *et al.*, 1997). *Anthyllis* seedlings inoculated with an indigenous rhizobial+AM fungal inoculum, were transplanted to field plots for a 5-year trial. The experimental variables tested were seedling survival rates, growth, N-fixation, and N-transfer from N-fixing to associated non-fixing species in the natural succession, while those in the rhizosphere soil were N content, levels of organic matter, and hydrostable soil aggregates. A long-term improvement in these physicochemical properties was evident in the soil around the *Anthyllis* plants. The increase in N content in the rhizosphere of the legume can be accounted for by an improvement in nodulation and N-fixing capacity resulting from inoculation with both symbionts (Barea *et al.*, 2005*a*, *b*). Inoculation with native co-operative microbial symbionts also benefited plant growth, N fixation, and N-transfer. Improved N status of non-leguminous plants grown in association with legumes has previously been described for agricultural crops (Azcón-Aguilar *et al.*, 1979), but this was the first demonstration of this phenomenon for natural plant communities in a semi-arid ecosystem. The dually-inoculated shrub legumes were a source of AM fungal inoculum for the surrounding area and for improving the N nutrition of non-N-fixing vegetation. The general conclusion was that the co-operation of microbial symbionts inoculated in the rhizosphere of target indigenous species of plants is a successful biotechnological tool to aid the recovery of desertified ecosystems. This can be used as an initial step in the restoration of a self-sustaining ecosystem.

Conclusions and future trends

There is considerable experimental evidence that certain bacteria and fungi are able to colonize the root–soil environments where they carry out a variety of interactive activities known to benefit plant growth and health, and also soil quality. Since it was realized that the appropriate management of target co-operative microbial activities can reduce the use of chemicals and energy, there has been an increasing interest in applying selected microbial consortia, as plant inoculates, to benefit plant production systems. Molecular techniques are being used in microbial ecology to understand the soil ecosystem, for the production of microbial inoculates, and for monitoring these inoculates after field release. These inoculates may or may not be genetically modified strains. Thus, future research in rhizosphere biology will rely on the development of molecular and biotechnological approaches to increase our knowledge of rhizosphere biology and to achieve an integrated management of soil microbial populations.

From the agricultural and ecological viewpoints, the aims will be to increase food quality, and to improve sustainable plant productivity, while maintaining environmental quality. However, to achieve this, basic and strategic studies must be undertaken to improve our understanding of microbial interactions in the rhizosphere. Only then can the corresponding agro-biotechnology be applied successfully. Hence, future investigation in the field of microbial co-operation in the rhizosphere will include: (i) advances in visualization technology; (ii) analysis of the molecular basis of root colonization; (iii) signalling in the rhizosphere; (iv) functional genomics; (v) mechanisms involved in beneficial co-operative microbial activities; (vi) engineering of micro-organisms for beneficial purposes; and (vii) biotechnological developments for integrated management.

Non-disruptive *in situ* visualization techniques are already being used for detailed studies on the interactions of micro-organisms within the rhizosphere, both between themselves and with the root. Improving these techniques, based on the use of confocal laser scanning microscopy and fluorescent proteins, will not only allow the simultaneous imaging of different populations of microbes in the rhizosphere, but also the temporal–spatial visualization of gene expression. Novel research is needed to improve immunofluorescence techniques to assess gene transfer in rhizosphere environments without the need to cultivate micro-organisms.

Many traits of root colonization by rhizo-microbes have already been identified, but novel molecular approaches are being used to screen for new traits. These are important to decipher the genes encoding proteins involved in transport or signal transduction pathways involved in colonization. An increase in current knowledge on quorum sensing systems, such as those based on *N*-acyl-homoserine lactones, will be important for understanding the ecodynamics of microbial populations in the rhizosphere, and the cellular and molecular aspects of signalling processes in microbe–microbe interactions.

Future developments in functional genomics (including proteomics and metabolomics) will be useful to identify the

genes expressed in the rhizosphere, while the use of promoters to drive gene expression specifically at the root–soil interfaces will allow the engineering of micro-organisms for beneficial purposes.

The specific management of mycorrhiza/bacteria interactions, through the manipulation of appropriate mycorrhizospheres, should be one of the main objectives of applied research in the future. The use of microbial inoculates must take into account the importance of retaining microbial diversity in rhizosphere ecology, and in achieving realistic and effective biotechnological applications ('rhizosphere technology'). The improvement of molecular biology-based approaches will be fundamental for analysing microbial diversity and community structure, and to predict responses to microbial inoculation/processes in the environment ('ecological engineering'). Further studies must address the consequences of the co-operation between microbes in the rhizosphere under field conditions to assess their ecological impacts and biotechnological potential.

Despite the difficulty in selecting effective multifunctional microbial inoculates, appropriate combinations can already be recommended. New environmentally-friendly, genetically-modified, microbial inoculates are being produced commercially and used to protect plants from disease and to promote plant growth. These new products are expected to lead to a reduction in the use of biocides and chemical fertilizers. Nevertheless, biological safety issues must be considered prior to the release of these transgenics into the environment.

All in all, the availability of new and powerful technologies for studying co-operative microbial interactions in the rhizosphere guarantees a greater understanding of these processes, which will facilitate their successful applications in biotechnology.

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