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K. G. Mukerji
C. Manoharachary
J. Singh (Eds.)

Microbial Activity in the Rhizosphere

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Microbial Activity in the Rhizosphere

With 35 Figures

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Preface

The rhizosphere is a very complex environment in which the effects of the plant on soil microorganisms and the effects of the microorganisms on the plant are interacting and are interdependent. Plant root exudates and breakdown products attract microbes and feed them and, in turn, the plants often benefit from the microbes. Interactions among microorganisms and plant roots are essential for nutritional requirements of the plant. Plant growth, development and productivity are largely dependent on the soil environment in the root region rhizosphere. The new techniques of studying the rhizosphere enables us to get a much better understanding of the dynamics of the rhizosphere population, such rhizosphere studies being of interest to agriculturists, soil biologists, chemists, microbiologists and molecular biologists.

The rhizosphere microbes influence the root environment in several ways. They may change the oxidation-reduction potential, influence the availability of moisture and nutrients, produce growth inhibiting or growth promoting substances in the form of exudates, provide competition and possibly induce many other effects. Mycorrhizal associations are beneficial in mineral uptake and in increasing root surface area for effective ion absorption.

Antagonism, competition and synergism in soil and the rhizoplane (rhizosphere) are the most important microbial interactions to consider in the study of rhizosphere biology. With the growing information on the production of growth regulators, competitiveness of the microbes in the rhizosphere, microsymbionts, and other factors, their effect upon plant growth will become more evident. Experiments on the introduction of microbes or their products in the rhizosphere will help to improve our understanding of the biology of the rhizosphere.

Each chapter of the volume has been written by experienced and internationally recognised scientists in the field. The selection of topics and techniques has been assembled in such a way that it will be useful to the beginner as well as to experienced scientists. The need for such a volume was enhanced due to the fact that no book has been published on this aspect for the last two decades. Several chapters included in this volume treat new approaches, which have probably not been reported before.

We are grateful to Professor Ajit Varma, Series Editor and Dr. Jutta Lindenborn, Editor Life Sciences Springer for their help in various ways. Many minds and hands have helped in the preparation of the volume to which we are indebted. We are grateful to all the authors for their contributions to this volume and for accepting suggestions to produce the final shape of the volume. Since the chapters have been independently written by the authors, there may be some slight overlap or repetition, difficult to avoid in these circumstances.

It is our hope that the information presented in this volume will make a valuable contribution to international root (rhizosphere) research. We believe and trust that it will stimulate further discussions and pursuit of new knowledge in this important subject area. We also hope that it will be useful to all students and researchers in microbial biotechnology, microbial ecology, soil microbiology, applied mycology, agriculture and forestry.

Delhi, Hyderabad
and Bedfordshire,
April 2005

K.G. Mukerji,
C. Manoharachary,
and J. Singh

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1 Rhizosphere Biology – an Overview

Chakravarthula Manoharachary, Krishna G. Mukerji

1.1 Introduction

Fungi, bacteria and actinomycetes are known to colonize diverse habitats and substrates and they are known to play substantial role in plant health and productivity besides producing diseases. The specialized ecological niches where the microbial association and their activity amply evidence are soil, rhizosphere, rhizoplane and phylloplane. These microbes may interact with the same plant simultaneously either independently, synergistically and/or antagonistically resulting sometimes in beneficial effect and at other times in harmful consequences. The microbes living in the complex region of rhizosphere influence the crop health and yield. The fungal biotechnologists have forgotten that soil, rhizosphere, rhizoplane and phyllosphere are the natural resources for microbial metabolites, products and other of biotechnological importance.

1.2 Soil Microbes

There is vast microbial flora inheriting the earth and they are found in all types of soils which are virgin or cultivated, sands, deserts, thermal soils or of volcanic origin, in bogs and moors, in snow covered soils, in sediments, semi aquatic ecosystems, on rocks and in rock crevices. The dominating groups of microorganisms are bacteria, actinomycetes, fungi, soil nematodes and protozoans. However, most of these organisms share a common character in being heterotrophic in their nutrition and thereby depend on other organic, dead or living organism and inorganic source of nutrition for their survival and multiplication. Most of the fungi, bacteria and actinomycete are microscopic and show vast variation quantitatively and qualitatively in different sites of collection and at different

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depths. Considerable variation occurs even between soil samples taken a few inches apart. Biotrophic, saprobic and symbiotic microbes are found in soil. The microbial population of soil is, therefore, dependent on composite micro-ecological environments. While most of the microorganisms live as saprophytes, others may be parasitic and symbiotic in relation to living organic substrates. Depending upon their enzymatic activity, they may be cellulolytic, keratinophilic, chitinophilic, pectinolytic, lignicolous or humicolous. The discovery of several parasitic fungi in causing root infection of several economically important crop plants paved way for intensive study of soil microorganisms in different niches-soil, rhizosphere or rhizoplane. Many of the parasitic fungi belong to genera *Pythium, Rhizoctonia* and *Fusarium*. Fusaria are known to cause root rots and wilts in young seedlings and some of them act as saprophytes on some dead plants tissues (Deacon 1996).

The discovery of several isolating techniques by several workers such as dilution plate techniques (Waksman 1911, 1944), soil plate method and modified soil plate methods (Warcup 1950), agar film method (Jones and Mollison 1948), immersion tube method (Chester 1948), immersion plate technique (Thornton 1952), baiting method (Harvey 1925), dilution frequency method (Allen 1957), direct microscopic examination (Conn 1981), uncoated glass slide (Rossi and Riccardo 1927; Cholodny 1930), root maceration (Stover and Waite 1953) and many other techniques for assaying of microbes resulted in their quantitative and qualitative estimations in different niches of the soil. Subsequently specific and nonspecific media were used selectively for isolation of bacteria, fungi and actinomycetes. Utilization of these isolating techniques and culture media contributed immensely in our present day understanding of soil, rhizosphere or rhizoplane microorganisms. The microorganisms in the soil are diverse and they differ quantitatively and qualitatively with the variations in the climatic, edaphic and biotic factors. The population dynamics of microflora of the soil depends on initial substrate and changes in substrate over period of time, nature and changes in other microflora and the genetic ability of a particular microorganisms to utilize or modify the components of the substrate and their interaction with biotic and abiotic components of the soil. Microorganisms in the soil are in constant state of flux and play an active role in recycling of organic wastes, nitrogen, phosphorus cycle and mineralization and other phenomenon and also produce several metabolites useful for humans. Bacteria from the soil were enumerated quantitatively by several workers. *Agrobacterium tumefaciens* was isolated by Schroth and Snyder (1961).

Soil fungi in relation to distinct fungal communities, physico-chemical factors, seasonal variations, biotic and other ecological parameters have been studied by several workers (Behera and Mukerji 1985a,b; Manohara-

chary 1977; Manoharachary and Rama Rao 1975; Mukerji 1966; Rama Rao 1970; Saksena 1967; Warcup 1967). Soil fungi in relation to microhabitat, interaction, physiology, biotic factors, role in decomposition of soil organic matter, antagonism, and role of dilution plate method were some of the facts reviewed extensively at International Symposium on ecology of soil fungi held at Liverpool (Parkinson and Waid 1960). Fungi isolated from different ecological niches including soils have been compiled in several publications by Barron (1968), Gilman (1957), Rai et al. (1961, 1963, 1964a,b,c, 1969), Rai and Mukerji (1962a,b).

The effect of genetically altered *Pseudomonas solanacearum* in soil and rhizosphere of tomato was studied by Hartel et al. (1990) and it was revealed in their studies that the genetically altered *Pseudomonas* slowed down the growth of unaltered *Pseudomonas* as a consequence of recombination. The biological control of soil-borne diseases of crops was achieved by treating soils with VAM fungi (Sharma and Adholeya 2000). It was observed that damping off caused by *Pythium* and *Rhizoctonia* on cucumber seedlings and bacterial wilt caused by *Pseudomonas solanaceraum* was controlled by charcoal compost and VAM combination but control was unsatisfactory with VAM fungi alone. Microorganisms isolated from the soil and the rhizosphere of wheat plants produced growth promoting substances whose effect was similar to those of gibberellins (Voker and Birnstiel 1989).

Pigmented actinomycetes were isolated from Cerrado soils of Brazil using starch agar nutrient medium (Copelhnas and Linhares 1993). The tests revealed that 90% of the pigmented actinomycetes were melanin producers. Melanin production was induced by supplementary inorganic or organic nitrogen source. These strains may have a role in soil organic matter formation. An actinomycetes strain – TB isolated from Korean soils – showed strong antimicrobial activity against Gram-positive bacteria and tumor cell growth inhibition (Jin-Ho et al. 1993).

Survey of actinomycetes from mud and water samples for a period of five years from 12 lakes in China was carried out by Jiang and Ku (1989) and they have found that the number and composition of actinomycetes was related to physical and chemical factors of the water body. Five new species *Micromonospora phaeovivda, Saccharomonospora yumnanensis, Microtetraspora flavorosea, Actinomadura viridoflava* and *Micropholyspora* were reported.

1.3 Rhizosphere Soil

The rhizosphere may be defined as that portion of the soil which is adjacent to the root system of a plant and is influenced by the root exudates. The

area of this zone depends on the soil type and host plant under study and soil environment conditions. The roots exert influences on various type of microorganisms. The stimulatory effect on microorganisms is known as the "Rhizosphere effect" as indicated by the interaction of soil and rhizosphere microbes and their ratio. The chemical and physical nature of the root zone is quite different from the soil away from the root zone and the biology of this complex zone has been studied extensively. The term 'Rhizosphere' was proposed by Hiltner (1904). The phenomenon of accumulation of microorganisms around the root zone was reported by a number of earlier workers (Agnihothrudu 1955; Starkey 1958; Rouatt 1959; Parkinson 1957; Katznelson 1946; Sadasivan 1965; Jackson 1960; Mukerji and Ranga Rao 1968; Ranga Rao and Mukerji 1969, 1971a; Sorensen et al. 1997). Various compounds such as amino acids, vitamins, sugars, tannins etc. are exuded by the roots. These compounds have selective effect on microorganisms with in the root zone. Some root exudates are also known to affect certain microbial species adversely leading to their decrease in the root zone and, in return, microorganisms are known to exert profound influence on the plant itself by decomposition, affecting nutrient uptake, antagonistic effect on other microbes and parasitism.

Rhizosphere is a soil ecological region where soil is subjected to specific influence by plant root due to the exudates from root cells and sloughing of root tissue (Curl and Truelove 1986; Giddens and Todd 1984; Harley and Russell 1968). According to Pinton et al. (2001), rhizosphere represents a poorly defined zone of soil with a microbiological gradient in which maximum changes in the population of microflora in soil is evident adjacent to root and decline with distance away from it (Newman 1978; Bowen 1991; Mukerji 2002). The region of the external surface of plant root together with any closely adhering particles of soil or debris is differentiated from it and has been called rhizoplane. Root exudates stimulate microbial activity selectively in rhizosphere and rhizoplane regions (Bansal and Mukerji 1996). There is an intense competitive activity by the obligate saprobes, unspecialized root parasites and root inhabiting fungi depending on their behaviour towards exudates. In case of root diseases the pathogen has to react with the rhizosphere and rhizoplane fungi before entering the root tissues. These may show antagonism and check its advancement. The various factors and mechanism responsible for fungistasis, although already investigated, still need in-depth investigation. Plant microbe interaction is a regular and continuous feature of the biological world. The beneficial fallouts of such interactions have been extensively exploited for economic gain in recent years.

Once we develop a microbial technology for nif gene transfer and genes of mycorrhizal fungi and chitinase, genes offering resistance to plant pathogens, will definitely boost the crops production and pave the way

for sustainable agriculture, making man's efforts for freedom from hunger a realizable end. This technology will reduce artificial fertilizer application and pesticide use.

Many have emphasized the importance of rhizosphere in plant-microbe interaction (Pinton et al. 2001). It has been documented by several researchers that microorganisms are stimulated in the rhizosphere (Katznelson 1946, 1965). Significant differences in the rhizosphere effect have also been reported. The metabolic state of the plant and the nature of soil appear to influence the extent of the rhizosphere effect (Rovira 1991). There is a need to learn more about the beneficial rhizosphere microorganisms. Ultimately the work on rhizosphere microbes has to aim at augmenting the biomass production (Yang-Ching et al. 2000).

Rhizosphere studies are fascinating and interesting leading to many beneficial consequences though some microbes have harmful effects. Some microbial metabolites like antibiotics and toxins, are crucial factors in determining plant-microbe relations. Microbial enzymes also play an effective role. The interesting relationship between plant root and microbes has attracted the attention of molecular biologists, microbiologists and biochemists throughout the world. Interestingly different types of microbes like fungi, bacteria, nematodes and viruses may interact with the same plant simultaneously either independently, synergistically or antagonistically.

Factors such as soil type, soil moisture, pH, temperature, plant age, relative humidity and several other factors are known to influence the rhizosphere effect. The rhizosphere effect is expressed in terms of R/S ratio (Katznelson 1946; Timonin 1966). The term 'rhizoplane' was proposed by Clark (1949) to refer to the immediate surface of plant roots together with any closely adhering particles of soil or debris. Using different isolation techniques microorganisms were isolated and identified by a number of workers.

In recent studies on rhizosphere, the emphasis was laid on interactions of microflora in the root zone due to effect of systemic or aerial sprays of herbicides or insecticides or fungicides on rhizosphere microflora (Ranga Rao et al. 1972). Data on microflora in Relation to various economically important crop plants or medicinal plants, studies on root diseases (Ranga Rao and Mukerji 1971b), soil environment and various factors of biotic, edaphic, environmental variations on the rhizosphere microflora and Dynamics Of Microorganisms In The Rhizosphere are meagre.

Mineralogical studies of rhizosphere of forest plants from six different sites in north and south of United States (Lynch and Whipps 1991) revealed that mineral grains within these zones are affected mechanically, chemically and mineralogically by invading root microorganisms than compare with non-rhizosphere forest soils. The mineral grains abutting root surfaces

were significantly more fractured. This process of weathering in the root zone micro-environment should have a positive effect on soil complex formation that is biologically active.

Using soil biology methods on rhizosphere soils of six different plant species grown in four different soils and combinations of species were studied (Lynch 1982) to get information on organic metabolites and their origin in the rhizosphere. The amounts of sugar, amino acids and enzyme activity of some metabolites suggested a closer interrelationship with the involved organisms. Sometimes the nature of the soil was the dominating factor while it was host plant species which played a major role.

Recently plant growth promoting rhizobacteria (PGPR) were isolated from a number of economically important plants such as barley, bean, cotton, maize, groundnut, rice, various vegetables, wheat and wood species. In addition to increasing crop yield the strains of PGPR can also affect pathogenic fungi in reducing population densities.

With a higher nitrogen input a higher amount of ^{14}C was released from roots as photosynthates. This indicates that root exudates were significantly used by soil microbes in the rhizosphere. Using different nitrogen regimes, the rhizosphere bacteria of wheat was investigated (Keister and Cregan 1991). The bacterial population in relation to different nitrogen concentrations in the soil application resulted in higher amounts of bacterial populations in the rhizosphere but fluorescent pseudomonas population were low at higher nitrogen rates. The number of species of soil microorganisms, such as bacteria and actinomycetes were significantly correlated with cumulative temperature and rainfall in different agroclimatic zones. Similarly, higher correlations were obtained between rhizosphere microorganisms and pH, K, Ca and Mg. *Acremonium, Alternaria, Cladosporium, Cephalosporium* and *Fusarium* were the dominant fungal species.

Molecular cross talk seems to be the prerequisite mechanism for most root microbial infections. Just as the rhizodeposition can affect the composition of rhizosphere microflora, microbial metabolites can also affect the rhizosphere deposition. Rhizobacteria promote plant growth due to the production of plant growth regulators like auxin derivatives. The most studied molecular cross talk has been between rhizobia and the leguminous and non-leguminous host plant. The root exudates or chemical composition of rhizosphere solution can affect plant growth. It is very much the case that uptake of nutrients may be considerably influenced by the ionic concentration of the rhizosphere solution. However, we do not have the complete picture that takes into account the relative weight of each factor regarding molecular analysis of the interaction between plants, microbes and soil components.

1.3.1 Rhizoplane

Rhizoplane is an ecological niche harbouring a number of bacteria, actinomycetes and fungi. The term rhizoplane was proposed by Clark (1949) to refer to the immediate external surface of plant roots together with any closely adhering particles of soil or debris and microbial communities. It was proposed by Katznelson (1946) that the proportion of microorganisms in the rhizosphere with the population found outside the rhizosphere in numerical terms may be expressed as an R/S ratio. Using serial root washings, Harley and Waid (1955) successfully isolated rhizoplane microorganisms. Stover and Waite (1953) and Singh (1965) using a root maceration technique isolated Fusaria and other root mycoflora. Using baiting technique several pathogenic fungi like *Phytophthora* (Eckert and Tsao 1962) and *Pythium* (Drechsler 1929; Johnson and Curl 1972) have been isolated from infected plant roots and the soil around them.

1.3.2 Root Exudates

Root exudates are the substances released by roots and may affect growth and activity of soil microorganisms in rhizosphere and on rhizoplane (Bansal and Mukerji 1994, 1996; Kapoor 1999; Schottendreier and Falkengren-Greup 1999). Several substances are reported as released by roots in the form of exudates such as amino acids, sugars, vitamins, organic acids, nucleotides, flavones, enzymes, hydrocyanic acid, glycosides, auxins and saponins (Gupta and Mukerji 2002). Root exudates are known to influence the proliferation and survival of root infecting pathogens. Spores or other propagules of many pathogenic fungi such as *Rhizoctonia, Fusarium, Sclerotium, Aphanomyces, Pythium, Colletotrichum, Verticillium* and *Phytophthora* are shown to germinate as a result of stimulation and or food sources provided by root exudates of susceptible cultivars of host plants (Bhuvaneshwari and Subba Rao 1957; Vancura 1964). Root exudates also provide food base for antagonists which in turn suppress the growth of pathogenic microorganisms in soil. The resistant varieties are known to harbour a higher number of *Streptomyces* and *Trichoderma* which in turn may show antagonism against pathogenic fungi, thus rendering host plant resistant (Katznelson and Rouatt 1957; Starkey 1958; Subba Rao and Bailey 1961; Walker 1975).

Root exudates containing toxic substances such as glycosides and hydrocyanic acid may inhibit the growth of pathogens (Rangaswami 1988). It is also possible that the root exudates alter soil pH in rhizosphere and

thus alter the micro-climate or the rhizosphere which possibly reflected in changes and growth of microorganisms.

Age of the plant, temperature and calcium in relation to root exudation and its rhizosphere effect was studied by Vancura (1964). Schroth and Snyder (1961) observed trace amounts of nitrogenous substances in addition to sugar in the root exudates of bean plants.

The compounds released by plant root are diverse and only a few of them have direct effect on the growth of soil-grown plants. Furthermore very few root secretion and root products can be expected to be effective unless the right set of circumstances exist around the root.

1.3.3 Rhizosphere and Compounds Released by Roots

A vast array of compounds gets released into the rhizosphere by plant roots. However few have a direct effect on the growth of soil grown plant root products including organic compounds and these have a direct effect on the growth of the plant. Roots are not only organs for nutrients uptake, but are able to release a wide range of organic and inorganic compounds into the root environment (Giddens and Todd 1984). Soil-chemical changes related to the presence of these compounds and products of their microbial turnover are important factors affecting microbial populations, availability of nutrients, solubility of toxic elements in the rhizosphere and thereby the ability of plants to cope with adverse conditions (organic rhizodepositions – yeast).

In the last two decades due attention was given to functional characterization of plant root exudates. In order to understand the complex interactions at the root-soil interface a multidisciplinary approach is necessary. A number of investigators have suggested that plants treated with humic molecules differ in growth and morphology from control plants. Microbes associated with plant roots are related to plants growth regulators using the precursors released by the roots. Though much work has been done in the stimulation of microbial populations antagonistic to pathogens, manipulation of rhizosphere populations using plant breeding programs has not been seriously pursued. Further, the degradation of pollutants by the rhizosphere microorganisms will open greater opportunities to decontaminate land more effectively. The plant-microbe performance in the rhizosphere is an important aspect. In this regard mineralization and immobilization are the processes which will help in the evaluation. The increasing use of transgenic plants and the release into the environment of genetically modified microorganisms could change the plant-microbe interactions and microbial potential. Four groups of signal organic molecules namely flavonoids,

antibiotics, lipochitooligosaccharides and vitamins have different functions in plant growth promotions, plant defence and plant symbiosis.

Siderophores are iron chelating agents that are secreted by microorganisms in response to iron deficiency. Siderophores have been studied for their importance in plant disease suppression. Recently some studies have been taken up in examining the role of siderophore and phytosiderophores in facilitating heavy metal uptake and food chain transfer of metals. In spite of having wealth of information, there is still considerable debate as to how they function in the rhizosphere and the degree to which they accumulate in soils.

Rhizosphere is considered as a dynamic environment where microbial balance is maintained in spite of having constellation of soil physico-chemical factors. Mycorrhizae, an important component of the rhizosphere, form a mutual plant-fungus association which is an essential feature of biology and ecology of most terrestrial plants, since it influences their growth as well as their water and nutrient absorption and protects them from root diseases. Mycorrhizosphere has still to be understood in detail. Loss of carbon compounds from roots is the driving force for the development of enhanced microbial populations in the rhizosphere in comparison with the bulk soil. There is a need to learn more about carbon flow and microbial communities in natural environments.

The organic substrates released in rhizosphere soil, including plant and animal debris, has the potential to determine the high diversity of the soil microbial community inhabiting the root zone. Physico-chemical factors among rhizosphere soils of different plant species at different developmental stages of plants may dispose rhizosphere microbial communities toward preferential C-metabolite pathways which will impact differently on mineralization or immobilization of nutrients. Productivity of an ecosystem and its performance are dependent on mineralization and immobilization. Estimates of the diversity of DNA-based soil organisms so far have not been able to answer the questions concerning the functions of the soil ecosystem, although they can serve as an efficient tool for explaining DNA diversity in situ. The presence of a certain microbial gene does not assure that the metabolic process for which the gene is responsible is carried out. The increasing utility of transgenic plants and also the release of genetically modified microbes may affect plant-microbe interactions and microbial potential.

Flavonoids are identified as very specific molecules in the rhizobia/legume host plant communication. By activating specific nod genes, a new type of signal molecule, nod factors are produced by the bacteria, triggering host plant reactions such as root hair curling and meristem induction. Antibiotic substances are also released into the rhizosphere ecosystem. Nitrogen fixation is the result of co-evolution of atmosphere and life; one

has to understand the molecular aspects of rhizobia in relation to symbiotic relationship and nitrogen fixation in symbiotic and non-symbiotic plants.

The important function of plant and microbial siderophores in the rhizosphere is for iron acquisition under iron-limiting conditions. Microbes produce siderophores and compete for iron. Plants and microbes also compete for iron. Siderophores also suppress soil-borne and root-borne plant pathogens, besides employing them for phytoremediation.

Mycorrhizae are the rhizosphere soil components and form symbiotic interaction between plant and fungal genomes. Molecular events related to mycorrhization are not understood clearly. Mycorrhizal fungi act as biofertilizer to be used for sustainable agriculture and forestry. One of the interesting fields in microbial ecology is interaction between mycorrhizal fungi and rhizosphere microorganisms.

The rhizosphere microbial community is different from the bulk soil community and differs between plant species. Mathematical models do generate quantitative answers and allow the integration of many individual plants and soil processes to predict a single outcome. There is a requirement for such models to work on rhizosphere ecosystems. Loss of carbon compounds from roots or rhizodeposition is the driving force for the development of enhanced microbial populations in the rhizosphere in comparison with the bulk soil – use of ^{13}C techniques for measuring carbon flow is the best example besides use of biochemical or molecular signatures.

1.4 Decomposition of Organic/Inorganic Compounds in Rhizosphere

Plants and trees interact with soil to produce an area around the roots called the rhizosphere. This is an area of high microbial activity, lower pH, increased organic matter, higher carbon dioxide and lower water and nutrient content. Particularly because of increased microbial activity this area also contributes a unique environment, conducive to the breakdown of organic compounds. Because plants are constantly taking up water the rhizosphere is an area where concentration of metals and organic compounds occurs. Once in the rhizosphere, inorganic and organic compounds, including metals, may be deposited, taken up or broken down by plants.

Active degradation of contaminants involves enzymes, or metabolic pathways, which accommodate the pollutant. The plant or microorganism may use the pollutant as a source of nutrients, carbon, nitrogen, phosphorus etc., as a source of energy or both. Common short-lived insecticides and herbicides are broken down and used in such a manner. Nitrates can be removed from water under anaerobic conditions by a process called

denitrification. During this process nitrate is used as an electron acceptor and thus the organism is using the nitrate in energy production.

Passive removal could be by uptake along with water and release into the atmosphere by transpiration. Gases dissolved in water may be taken up by plants, transported up the plant system and released into the atmosphere during transpiration. On the other hand one might simply wish to have trees remove water from soil to prevent it and associated pollutants from moving into or out of the area.

Passive removal can also take place in the rhizosphere. This could be by concentration or by concentration and precipitation. If a plant can be found which takes up the contaminant, then the plant can be grown and the pollutant "harvested" along with the plant. If the pollutant is a metal the plant material can be burned and the metal recovered. If the pollutant is a heavy metal and is precipitated in the rhizosphere then the plant, roots and surrounding soils may need to be "harvested".

Bioremediation using any type of organism is a valuable remediation tool. It has many advantages including the fact that it is often carried out in situ and thus involves minimal disturbance to the site. In addition microorganisms and plant roots can explore every recess of soil and thus carry out a more complete remediation.

1.5 Root Sensing Signals

Recent advances show that organic compounds present in the rhizosphere can have a specific role in plant-microbe-soil interactions. Signal molecules exchanged between plants and microorganisms have been identified that favour beneficial plant colonization (Giovannetti et al. 1996). Some compounds present in soils, e.g. humic molecules and nutrients, metabolise through stimulation or inhibition of biochemical reactions or processes of root cells and triggering of specific signal transduction pathways. However, molecular information on how the plant senses the environmental conditions of the rhizosphere is still lacking.

1.6 Conclusions

The rhizosphere is the zone of soil adjacent to and influenced by roots. The surface of the root is known as the rhizoplane and the endorhizosphere refers to the root cortex, in the context of its colonisation by bacteria. The rhizosphere is colonised by bacteria, fungi and other microorganisms

and thus constitutes a living interface between roots and the soil. The rhizosphere, rhizoplane and endorhizosphere form a continuum that is difficult to distinguish in terms of microbial ecology. The functional and spatial complexity of the rhizosphere is heightened by mycorrhizal fungi, which in turn support a mycorrhizosphere that is also colonised by bacteria. The rhizosphere generally refers to the zone of soil surrounding the roots with higher microbial activity than in soil remote from roots.

References

Agnihothrudu V (1955) State in which fungi occur in the rhizosphere. Naturwissenschefen 42:515–516
Allen LE (1957) Experiments in soil bacteriology. Burgess, Minneapolis, Minn
Bansal M, Mukerji KG (1994) Positive correlation between VAM induced changes in root exudation and mycorrhizosphere mycoflora. Mycorrhiza 5:39–44
Bansal M, Mukerji KG (1996) Root exudation in rhizosphere biology. In: Mukerji KG, Singh VP, Suvercha (eds) Concepts in applied microbiology and biotechnology. Aditya Books, New Delhi, pp 97–119
Barron GO (1968) The genera of Hyphomycetes from soil. The Williams and Wilkins Company, Baltimore
Behera N, Mukerji KG (1985a) Seasonal variation and distribution of microfungi in forest soils of Delhi. Folia Geobot Phytotaxon 20:291–311
Behera N, Mukerji KG (1985b) Ecology of micromycetes in forest soils of Delhi. Acta Mycol 21:101–108
Bhuvaneshwari K, Subba Rao NS (1957) Root exudates in relation to rhizosphere effect. Proc Indian Acad Sci 45B:299–301
Bowen GD (1991) Microbial dynamis in the rhizosphere: possible strategies in managing rhizosphere population. In: Keister DL, Cregan B (eds) The rhizosphere and plant growth. Kluwer Academic Publishers, Netherlands, pp 25–32
Chester CGC (1948) A contribution to the study of fungi in the soil. Trans Brit Mycol Soc 30:100–117
Cholodny N (1930) Uber eine neue methode zur untersuchung der Boden microflora. Arch Microb 1:620–652
Clark FE (1949) Soil microorganisms and plant roots. Adv Agron 1:241–288
Conn HJ (1981) The microscopic study of bacteria and fungi in the soil. NY Agric Exp St Tech Bull 64
Copelhnas RRR, Linhares LE (1993) Melanogenic actinomycetes (*Streptomyces* sp.) from Brazilion soils. Biol Fertil Soils 15:220–224
Curl EA, Truelove B (1986) The rhizosphere. Springer, Berlin Heidelberg New York
Deacon JA (1996) Ecological implications of recognition events in the pre-infection stages of root pathogens. New Phytol 133:135–145
Drechsler C (1929) The best water mold and several related root parasites. J Agr Res 38: 309–392
Eckert JW, Tsoa PH (1962) A slective antibiotic medium for isolation of *Phytophthora* and *Pythium* from plant roots. Phytopathology 52:771–777
Giddens JE, Todd RL (1984) Rhizosphere microorganisms – overview. In : Todd RL, Giddens JE (eds) Microbial-plant interactions. Proc Soil Sci Soc Am, Madison, pp 51–68
Gilman JC (1957) A manual of soil fungi. Iowa Sate University Press, Ames, Iowa

Giovannetti M, Sbrana C, Citernesi AS, Avio L (1996) Analysis of factors involved in fungal recognition responses to host-desired signals by arbuscular mycorrhizal fungi. New Phytol 133:65–71
Gupta R, Mukerj KG (2002) Root exudate biology. In: Mukerji KG et al. (eds) Techniques in Mycorrhizal studies. Kluwer Academic Publishers, Netherlands, pp 103–131
Harley JL, Russell RS (1968) The soil-root interface. Academic Press, London
Harley HL, Waid JS (1955) A method of studying active mycelia on living roots and other surfaces in the soil. Trans Br Mycol Soc 38:104–118
Hartel PG, Williams JW, Schnell MA (1990) Growth of genetically altered *Pseudomonas solanacearum* in soil and rhizosphere. Soil Sci Soc Am J 54:1021–1025
Harvey TV (1925) A survey of the water molds and Pythiums occurring in the soils of Chapel Hill. J Eisha Mitchell Sci Soc 41:151–164
Hiltner L (1904) Über neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache. Arb Dtsch Landwirt Ges 98:59–78
Jackson TM (1960) Soil fungistasis and rhizosphere. In: Parkinson D, Waid JS (eds) The ecology of soil fungi. Liverpool Univ Press, Liverpool, pp 168–176
Jiang C, Ku L (1989) Studies on actinomycetes flora and resources in the plateau lakes in Yunnan (China). Acta Microbiol Sin 29:7–14
Jin-Ho H, Kim S-C, Chang Y, Konsyeom (1993) Antimicrobial activity and tuner cell growth inhibition of on actinomycetes isolated from Korean soil. Yakhak Hoeji 37:389–396
Johnson LF, Curl EA (1972) Methods for research on the ecology of soil-borne plant pathogens. Burgess Publ, Minneapolis, Minnesota
Jones PCT, Mollison JE (1948) A technique for quantitative estimation of soil microorganisms. J Gen Microbiol 2:54–69
Kapoor R (1999) Root exudation and its implication or rhizospehre mycoflora. In: Tewari JP, Lakhanpal TN, Singh J, Gupta R, Chamola BP (eds) Advances in microbial biotechnology. APH Publ Corp, New Delhi, pp 351–362
Katznelson H (1946) The "Rhizosphere effect" of mangles on certain groups of soil microorganisms. Soil Sci 62:343–354
Katznelson H (1965) Nature and importance of the rhizosphere. In: Baker KF, Snyder WC (eds) Ecology of soil-borne plant pathogens. John Murray Press
Katznelson H , Rouatt JW (1957) Studies on the microflora of the rhizosphere. Trans 6th Int Cong Soil Sci Paris, pp 151–156
Keister DL, Cregan PB (1991) Rhizosphere and plant growth. Kluwer Academic Publishers, Boston
Lynch JM (1982) The rhizosphere. In: Burns RG, Slatter JH (eds) Experimental microbial ecology. Blackwell Sci Publ, Oxford, pp 295–411
Lynch JM, Whipps JM (1991) Substrate flow in the rhizosphere. In: Keister DL, Cregan PB (eds) The rhizosphere and plant growth. Kluwer Academic, Dordrecht, pp 15–24
Manoharachary C (1977) Microbial ecology of scrub jungle and dry wasteland soils from Hyderabad District, Andhra Pradesh (India). Proc Indian Nat Sci Acad 43B:6–18
Manoharachary C, Rama Rao P (1975) Two new additions to the Indian soil fungi. Indian Phytopathol 28:427–428
Mukerji KG (1966) Ecological studies on the microorganic population of usar soils. Mycopath Mycol Appl 29:339–349
Mukerji KG (2002) Rhizosphere biology. In: Mukerji KG et al. (eds) Techniques in Mycorrhizal studies. Kluwer Academic Publishers, Netherlands, pp 87–101
Newman EI (1978) Root microorganisms: their significance in the ecosystem. Biol Rev 53:511–554

Parkinson D (1957) New methods for qualitative and quantitative study of fungi in the rhizosphere. Pedologi Gand 7:146–154

Parkinson D, Waid JS (1960) The ecology of soil fungi. Liverpool Univ Press, Liverpool

Pinton R, Varanini Z, Nannipieri P (2001) The rhizosphere-biochemistry and organic subtances at the soil-plant interface. Marcel Dekker. New York Basel

Rai JN, Mukerji KG (1962a) A new species of *Chaetomium* from Indian soils. Can J Bot 40:857–860

Rai JN, Mukerji KG (1962b) *Sprotrichum carthusioviride* Rai and Mukerji, a new species from Indian soils. Mycopath Mycol Appl 18:122–126

Rai JN, Mukerji KG, Tewari JP (1961) A new *Helicostylum* from Indian soils. Can J Bot 39:1282–1284

Rai JN, Mukerji KG, Tewari JP (1963) *Tripterospora tetraspora* sp. nov., a new cleistothecial Ascomycetes. Can J Bot 41:327–329

Rai JN, Tewari JP, Mukerji KG (1964a) *Achaetomium,* a new genus of Asconycetes. Can J Bot 42:693–697

Rai JN, Tewari JP, Mukerji KG (1964b) A new *Aspergillus* from Indian soils – *A. striatus* sp. nov. Can J Bot 42:1521–1524

Rai JN, Tewari JP, Mukerji KG (1964c) Cultural and texonomic studies on two rare species of *Aspergillus* – *A. paradoxus* and an interesting strain of *A. variecolor* from Indian soils. Mycopath Mycol Appl 26:369–376

Rai JN, Tewari JP, Mukerji KG (1969) Mycoflora of mangrove mud. Mycopath Mycol Appl 38:17–31

Rama Rao P (1970) Studies on soil fungi III. Seasonal variation and distribution of microfungi in some soils of Andhra Pradesh (India). Mycopath Mycol Appl 40:277–298

Ranga Rao V, Mukerji KG (1969) Fungi of Delhi. IX. Additions to our knowledge of soil fungi. J Ind Bot Soc 48:258–261

Ranga Rao V, Mukerji KG (1971a) Fungi in the root zone of four cultivars of wheat. Ann Inst Pasteur 121:533–544

Ranga Rao V, Mukerji KG (1971b) Studies on charcoal rot disease of *Abelmoschus esculentus*. II. Fungal flora of the root zone of healthy and infected plants. Ann Inst Pasteur 122:81–90

Ranga Rao V, Jayakar M, Sharma KR, Mukerji KG (1972) Effect of foliar spray of Morphactin on fungi in the root zone of *Capsicum annum.* Plant Soil 37:179–182

Rangaswami G (1988) Soil-plant microbe interrelationships. Indian Phytopath 41:165–172

Rossi G, Ricardo S (1927) L'seae microscopico ebacteriologico iretto del ferreno agrario. Nuovi Ann Minist Agric 7:457–470

Rouatt JW (1959) Initiation of Rhizosphere effect. Can J Microbiol 5:67–71

Rovira AD (1991) Rhizosphere research – 85 years of progress and frustration. In: Keister DL, Cregan PB (eds) Rhizosphere and plant growth. Kluwer Academic Publishers, Netherlands, pp 3–13

Sadasivan TS (1965) Root and its environment. J Sci Ind Res 24:11–113

Saksena RK (1967) The soil fungi – a biological appraisal. Indian Phytopath 24:13–25

Schottendreier M, Falkengren-Greup U (1999) Plant induced alteration in the rhizosphere and the utilization of soil heterogenicity. Plant Soil 209:297–309

Schroth MN, Snyder WC (1961) Effect of host exudation, chalamydospore germination of the bean root rot fungus *F. solani, F. phaseoli.* Phytopathology 51:389–393

Sharma MP, Adholeya A (2000) Sustainable management of Arbuscular Mycorrhizal fungi in the biocontrol of soil-borne plant disease. In: Upadhyay RK, Mukerji KG, Chamola BP (eds) Biocontrol potential and its exploitation in sustainable agriculture. Kluwer Academic/Plenum Publishers, Dordrect, New York, pp 117–138

Singh KG (1965) Comparison of techniques for the isolation of root infecting fungi. Nature 206:1169–1170

Sorensen J, van Elsas JD, Trevors JT (1997) The rhizosphere as a habitat for soil microorganisms. In: Wellington EMH (ed) Modern soil microbiology. Marcel Dekker, New York, pp 21–45
Starkey RL (1958) Inter relation between microorganisms and plant roots in rhizosphere. Bot Rev 22:154–172
Stover RH, Waite BH (1953) An improved method of isolating *Fusarium* sp. from plant tissues. Phytopathology 43:700–701
Subba Rao NS, Bailey DL (1961) Rhizosphere studies in relation to varietal resistance or susceptibility of tomato to *Verticillium* wilt. Can J Bot 39:1747–1758
Thornton RH (1952) The screened immersion plate. A method of isolating soil microorganisms. Research 5:190–191
Timonin MI (1966) Rhizosphere effect of healthy and diseased lodgepole pine seedlings. Can J Microbiol 12:531–537
Vancura V (1964) Root exudates of plants. I. Analysis of root exudates of barley and wheat in their initial phases of growth. Plant Soil 21:231–248
Voker L, Birnstiel H (1989) Plant growth substances produced by microorganisms of the rhizosphere and the soil. J Basic Microbiol 29:473–476
Waksman SA (1911) Do fungi live and produce mycelium in the soil? Soil Sci NS 44:320–322
Waksman SA (1944) Three decades with soil fungi. Soil Sci 58:89–114
Walker N (1975) Soil microbiology – a critical review. Butterworths, London
Warcup JH (1950) The soil plate method for isolation of soil fungi. Nature, London, 166:117–118
Warcup JH (1967) Fungi in soil. In: Burgess A and Raw F (eds) Soil Biology Academic Press, London, pp 51–110
Yang-Ching H, Crowley DE, Yang CH (2000) Rhizosphere microbial community structure in relation to root location and Plant iron nutritional status. Appl Environ Microbiol 66:345–351

2 Methods and Techniques for Isolation, Enumeration and Characterization of Rhizosphere Microorganisms

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2.1 Introduction

Rhizosphere is a microcosm inhabited by a wide range of soil microorganisms like fungi, bacteria, actinomycetes, algae and nematodes, whose predominance is influenced by the plant root system. The population and functional dynamics of soil microorganisms differ from rhizosphere to non-rhizosphere zone due to the "rhizosphere effect" (Johnson et al. 1959). The complex microbial activities happening around the root zone puts tremendous influence on the plant growth (Fig. 2.1). The symbiotic and associative interactions between rhizosphere microorganisms help in the nutrient influx and acquisition showing beneficial activity on the plant growth (Fig. 2.1).

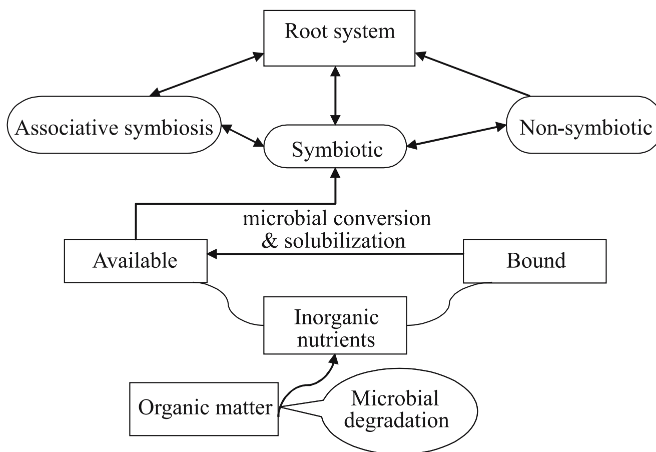

Fig. 2.1. Functional rhizosphere

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In the present review various methods and techniques used in isolation and enumeration of rhizosphere microorganisms are discussed. A focus is also made on the trends in modern molecular techniques involved in their characterization, microbial biomass determination and utilization in the plant productivity.

2.2 Isolation

2.2.1 Sampling

Sampling of rhizosphere soils can be done based on a chosen isolation technique. For direct observation a soil profile is created in the rhizosphere zone and for other techniques like soil plate and soil dilution techniques, soils are collected at different depths by using a soil auger.

The diversity and predominance of rhizosphere microbial populations depends on a number of abiotic and biotic factors of that particular ecological niche (Fig. 2.2).

Methods in microbiological study deal with isolation, estimation of types, numbers, metabolic activities and plant microbial interactions (Paul and Clark 1988) (Fig. 2.3).

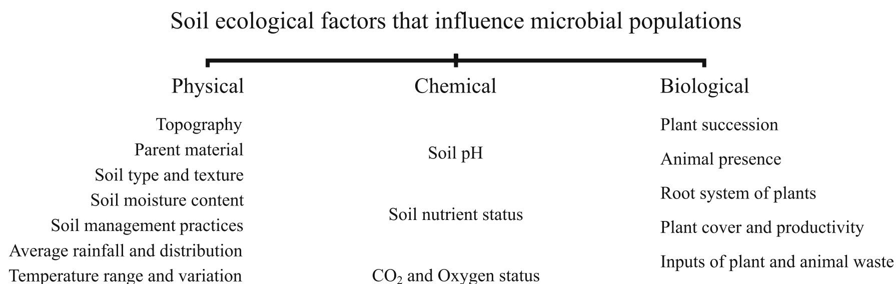

Fig. 2.2. Soil ecological factors

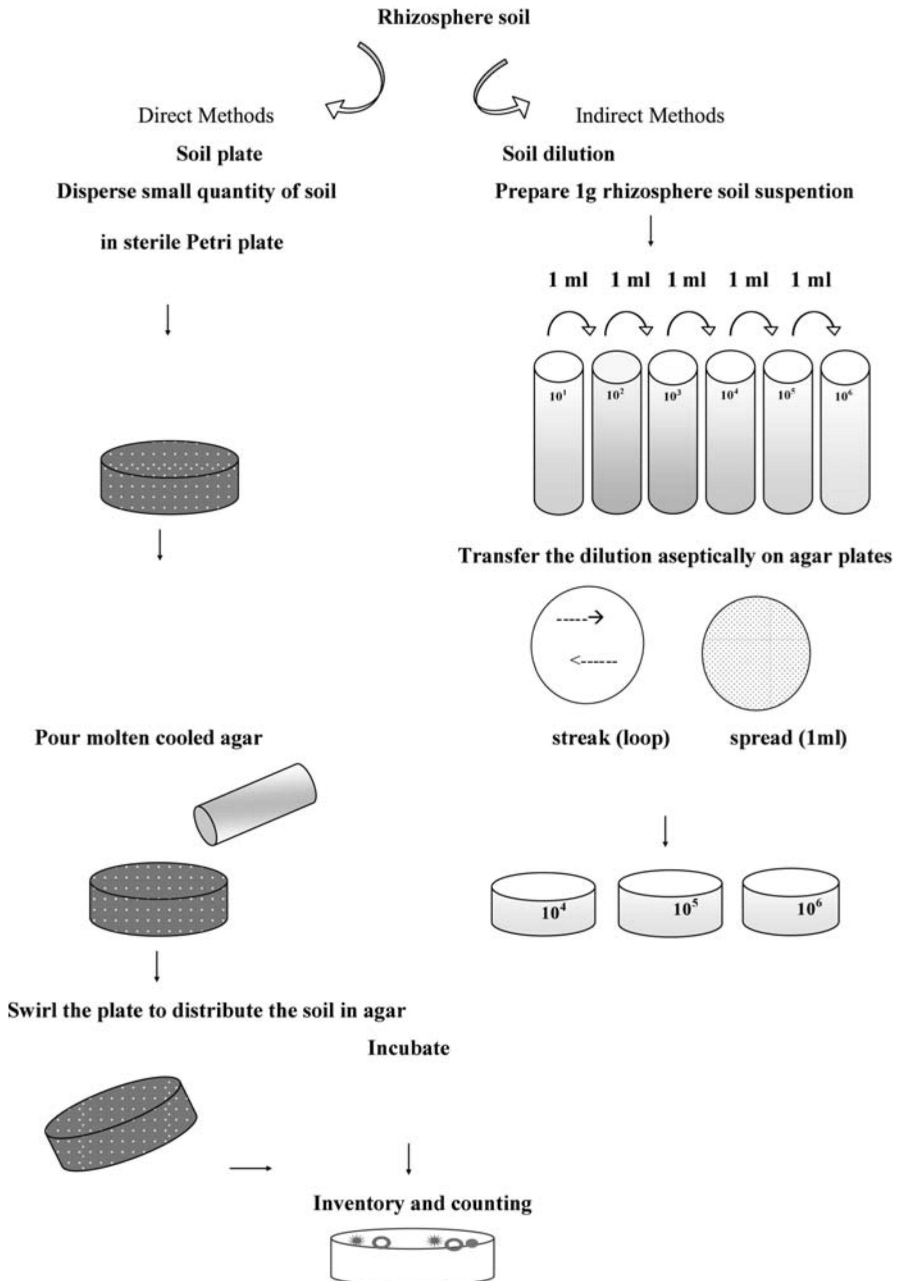

Fig. 2.3. Sample processing and isolation

2.2.2 Fungi

Conventional Methods (Fig. 2.4)

1. Direct methods: these methods are useful for quick scanning of soil fungi and are more appropriate to isolate fungi that do not sporulate and exist as mycelium attached to soil humus. Parkinson (1957) proposed

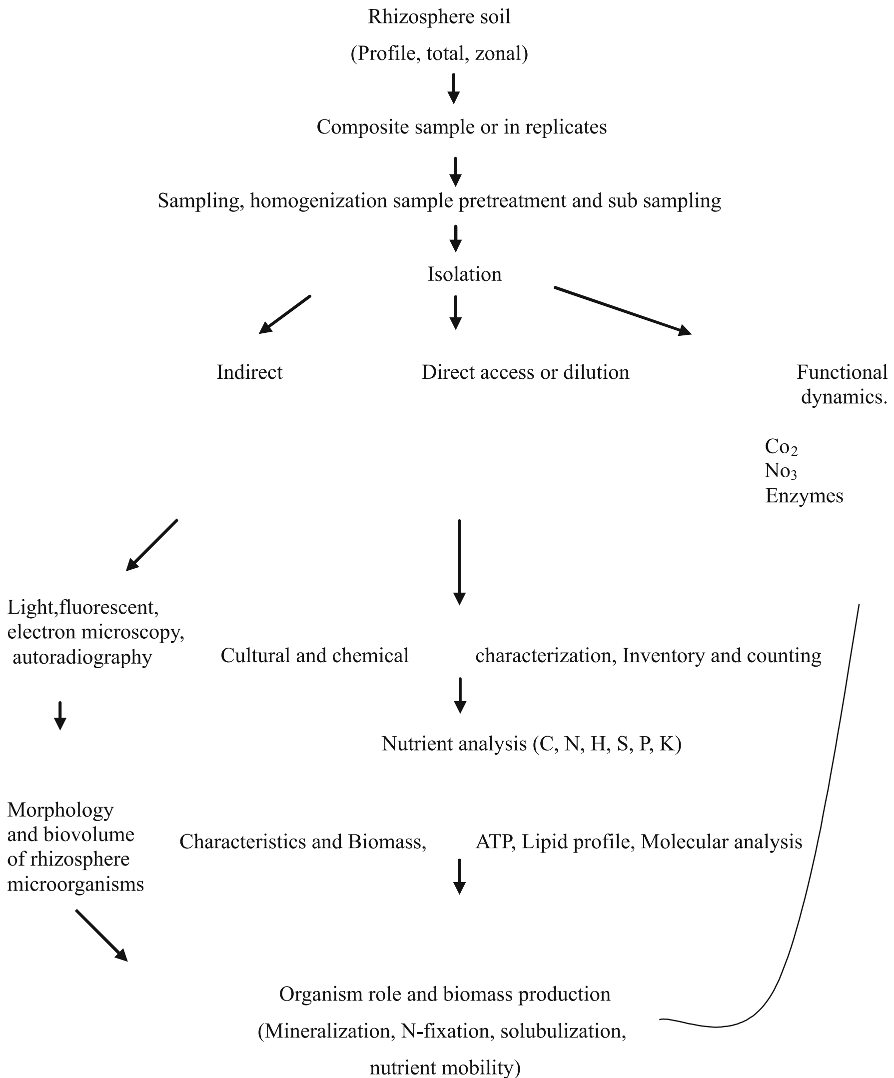

Fig. 2.4. Methods for estimating rhizosphere microbial biomass and function

a method for total and zonal rhizosphere isolations by slightly modifying Warcup's method. In this the plants under study are uprooted carefully and either total rhizosphere soil or soil from different zones of root are collected. Isolations are done by preparing a number of replicate series and after required period of incubation. Other direct observation methods include immersion techniques, soil box and a recent and more appropriate to observe the rhizosphere effect is using a rhizotron (Table 2.1).

2. Indirect methods: sporulating fungi are isolated by these techniques. Soil dilution plate technique (Parkinson 1957) is the most common method; soil suspension of specific dilution can be selected for isolation and to check the fungal population. In serial dilution methods, the spread plate technique is advantageous over pour plating as molten agar used in the latter may kill some heat sensitive fungi or some fungal spores do not germinate if submerged. Serial root washings, sedimentation and sieving, and soil washings are known for good recovery as these techniques involve agitation in soil aggregates and thus the spores can be easily released (Table 2.1).

3. Isolation of specific fungal groups: to isolate specific fungal groups, sample pretreatment and use of selective media are usually considered. Pre-

Table 2.1. Sampling and isolation approaches of rhizosphere fungi

Access	Sampling	Technique	Preferred method
Direct	Total or zonal from the rhizosphere core	Soil plate	Parkinson (1957)
	Profile	Rossi-Cholodny Immersion slide	Parkinson (1957)
	Zonal sampling at desired depth	Soil box with removable microscopic slides	Johnson and Curl (1972)
	Total or zonal from the rhizosphere core	Membrane filter	Hansen et al. (1974)
	Total rhizosphere	Rhizotrons	Heckman and Strick (1996)
Indirect	Total or zonal sample	Soil dilution plate	Timonin (1940); Menzies (1957)
	Total or zonal sample	Sedimentation and sieving	Warcup (1955)
	Total or zonal sample to recover large spores	Flotation in mineral oil emulsion	Ledingham and Chinn (1955)
	Rhizoplane	Serial root washings	Harley and Waid (1955)

Table 2.2. Isolation of specific fungal groups in rhizosphere

Taxonomic group	Sample processing	Technique/ selective medium	Preferred method
Moulds and yeasts		Dilution plate. Malt extract, YM, YNB. Chloremphenicol to inhibit bacteria	Skinner et al. (1980)
Slow growing soil-borne fungi	1. Dilute soil sample with sterile sand	1. Soil plate 2. Dilution plate	Parkinson et al. (1971)
	For slow growing fungi	Rose bengal or oxgall to arrest rapid growth of molds	Domsch et al. (1980)
Ascomycetes	1. Heat shock in water bath 2. Steaming 3. Soak in ethanol followed by heat shock	Soil plate or Dilution plate	Warcup (1951); Johnson and Curl (1972)
Cellulolytic		Enrich culture media with cellulose	Bose (1963)
VAM		1. Wet sieving and decantation 2. Sucrose gradient centrifugation 3. INVAM procedures	Gerdman and Nicolson (1963); Jenkins (1964)

treatment involves heat shock by steaming, immersion in ethanol generally follows. Selective media with enrichment compounds and certain growth retardants like oxagol, rose bengal and streptomycin etc., which inhibit bacteria, actinomycetes and fast growing molds, are used to curtail the interference and to facilitate the slow growing fungi (Table 2.2).

2.2.3 Bacteria

Conventional Methods (Table 2.3)

1. Direct observation: vital staining and fluorescent microscopy with acridine orange for observation of bacteria. Sample pre treatment with 1% Na pyrophosphate enhances the green fluorescence exhibited by living bacterial cells (Strugger 1948). Bacteria can also be detected directly from soil or root surface by IR Photography (Casida 1968). Soil sectioning either by resin (Nicholas et al. 1965) or by gelatin (Minderman 1956) are preferred for quantitative estimation.

Table 2.3. Isolation of soil bacteria

Access	Sampling	Technique	Preferred method
Direct	Total or zonal from the rhizosphere core	Vital staining and fluorescent microscopy	Strugger (1948)
		Soil sectioning	Nicholas et al. (1965); Minderman (1956)
		IR photography	Casida (1968)
		Rhizotrons	Heckman and Strick (1996)
Indirect	Total or zonal sample	Soil dilution plate on soil extract agar	Allen (1957)

2. Indirect isolation: soil dilution technique either by spread plate or by streak plate from the selected soil suspensions. For good recovery of bacteria sample pretreatment Na pyrophosphate, NaCl followed by Na_2CO_3 (Damigi et al. 1961).

2.2.4 Actinomycetes

1. Direct observation: enrichment techniques like baiting, positive chemotaxis and keratin substrates are preferred to isolate actinomycetes by direct access (Table 2.4).
2. Indirect isolation: actinomycetes are generally isolated by soil dilution technique. Actinomycetes are less predominant (tenfold lower) when compared to other soil bacteria. Hence a number of physical and chemical soil processing techniques are suggested for better recovery (Table 2.4). Different selective media used for isolation of actinomycetes is given in Table 2.5.

2.2.5 Algae

Many algal genera inhabit the rhizosphere soil both superficially or in deeper soil layers (Trainor 1983). The soil algae are usually small and unicellular, and include members of Chlorophycophyta, Rhodophycophyta, Euglenophycophyta and Chrysophycophyta. Approximately 10^6 algal cells/g soil are known to occur in surface soils.

Table 2.4. Isolation of actinomycetes

Access	Sampling/processing	Technique	Preferred method
Direct	Profile	1. Baiting with paraffin coated glass rod	Labeda and Shearer (1990)
		2. Positive chemotaxis with KCl	Palleroni (1980)
		3. Keratinophilic substrate	Tribe and Abu Elsound (1979)
		4. Pollen grains	Couch (1954)
		5. Rhizotrons	Heckman and Strick (1996)
Indirect sampling	Total or zonal sample	Soil dilution plate	
	Physical	1. Membrane filter on agar plate	
Pretreatment		2. Alternate drying and wetting	
		3. Centrifugation	Hirsch and Cristensen (1983)
		4. Dry heat (120 °C) or moist heat (45–70 °C)	Makkar and Cross (1982)
		5. Sonication at 30 accou. watts	Baker et al. (1979); Karwowski (1986)
	Chemical	1. Soil slurries with phenol	Lawrence (1956)
		2. Quaternary ammonium compounds	Phillips and Kalpan (1976)
		3. Sodium hypochlorite	Baker (1990)
		4. Osmium tetraoxide	Baker (1990)
	Biological	Actinophages	Williams and Vickers (1988)

Table 2.5. Selective media for isolation of actinomycetes

Medium	Reference
Glucose asparagine agar	Crook et al. (1950)
Mineral medium	Gause et al. (1957)
Starch – caseine agar	Kuster and Williums (1964)
Arginine – glycerol – salts agar	El-Nakeeb and Levchevalier (1963)
Colloidal chitin agar	Hsu and Lockwood (1975)
M_3 agar	Rowbotham and Cross (1977)
Humic acid – salts – vitamin agar (HSV)	Nonomura and Hayakawa (1988)

Isolation Methods

1. Direct technique: soil algae can be isolated directly by immersion technique, moist soil plate, suspending soil in water or growth medium (Dale 1983; Wilson and Forest 1957; Hoshaw and Rosowski 1973).

2. Dilution frequency technique (Allen 1957; Throndsen 1978): soil algal isolation involves certain preliminary techniques to avoid contamination and crowding to make the sample manageable for isolation of specific algal groups. Usually media with specific enrichments and incubation conditions like temperatures and light intensities are considered to select species that grow well under set conditions (Brand 1990). Soil extract or Bristol's sodium nitrate solution are mostly used for isolating algae. For isolation of specific algal groups enrichment or altered ratios of nutrient salts of Na^+, K^+, Ca^+, Mg^+, Cl^-, SO_4^{-2} and nitrogen free media to inhibit bacterial growth and to isolate blue green algae are certain techniques followed.

2.3 Enumeration

Study of microbial populations in the rhizosphere is a challenging task as some organism numbers may be too large or some too small. Hence various approaches have been evolved to enumerate microorganisms (Hurst et al. 1997) which involve dilution, concentration or enrichment of populations to examine the numbers (Fig. 2.5).

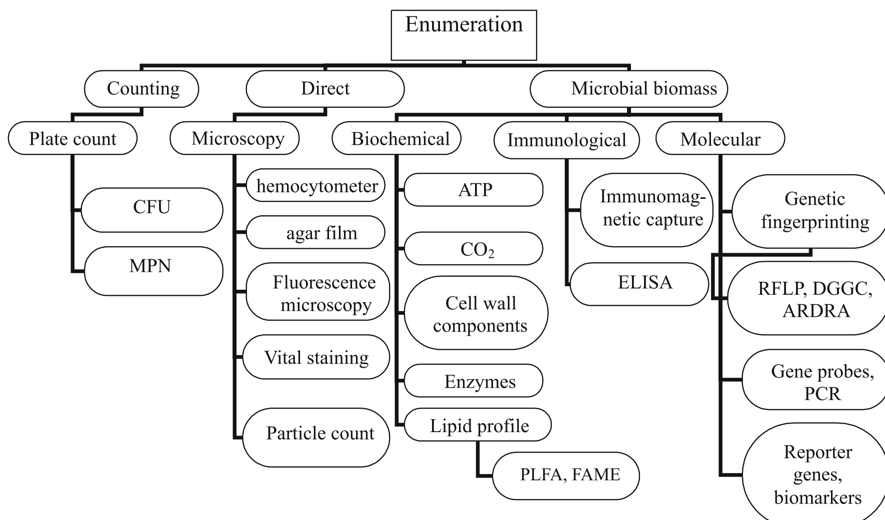

Fig. 2.5. Conventional and modern enumeration methods

2.3.1 Counting

CFUs. Total viable counts are taken in terms of colony forming units (CFU) by a plate counting method. However, this depends on the ability of microbial types to separate and one cannot rely on these for exact estimations as those that are non-culturable cannot be estimated. It has been found that in viable count methods < 1% soil organisms are recovered.

A CFU method would be appropriate when composition of isolating medium and incubation conditions are altered to suit to specific fungal groups. The 'Biolog microtitre' plate assay is a recent method using tetrazolium dye reduction which indicates the utilization of carbon (Lee et al. 1995).

MPN. Most probable number (MPN) gives a realistic estimate of target organisms by noting its presence or absence in the replicates and the highest possible dilution which shows the results is used to calculate the population (Wellington et al. 1997).

2.3.2 Microscopy

Hemocytometer and Agar Film Techniques

Direct counting procedures using microscopy gives the highest numbers and are reliable method when replicate counting is done (Byrd and Colwell 1992). However, in this technique one cannot differentiate between viable and dead organisms and other debris may interfere with accurate counting and further studies cannot be done. Direct enumeration was made easier by using a variety of counting chambers like hemocytometer (Parkinson et al. 1971). Agar film techniques with phenolic aniline blue (Parkinson 1973), or with fluorescein diacetate (FDA) in fluorescence microscopy (Soderstrom 1977) may be used for living fungi as these dyes are adsorbed onto the glycon linkages in fungal cells and thus microscopic imaging is helpful in fungal mycelial estimations.

Vital Staining. Epifluorescence by using vital stains like acridine orange, diamidino phenyl indole and fluorescein isothiocyanate, (FITC) a sulfahydril group adsorber, are used for bacterial counting (Kepner and Pratt 1994). Use of nalidixic acid (Kogure et al. 1984) which inhibit cell division by inactivating DNA gyrase and promotes cell enlargement followed by epifluorescent technique enables easy counting. Epifluorescence microscopy gives twofold higher magnitude numbers when compared to conventional plate counting. Direct counts and image analysis are proportional to biomass hence can

be employed in biomass estimations. FDA gives green fluorescence when hydrolyzed, cyanoditolyl tetrazolium chloride (CTC) gives red fluorescence when biologically reduced and tritiated glucose are used in microautoradiography (Meyer-Reil 1978). A rapid staining procedure for direct enumeration of total and viable counts would be of great help (Boulos et al. 1999).

Electron Microscopy
To get a three-dimensional view on the microbial habitat and micro organisms, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are proved as superior techniques but limited only to naturally dense microbial communities (Atlas and Bartha 1998; Nikitin 1973). Scanning confocal laser microscopy (SCLM) with gene probes can be used to monitor in situ populations (Hartmann et al. 1997).

Particle Count
Coulter counter and flow cytometry (Davey and Kell 1996) along with rRNA targeted gene probes as cell sorters is a very good tool in estimating microbial populations but this is not much use with of bacteria due to size limitations which are close to detection parameters.

2.3.3 Microbial Biomass

Microbial biomass is a parameter to enumerate microorganisms in terms of stored energy in a particular ecological niche. A biochemical approach would be more appropriate but the assay component should be only of biomass. Quantification of ATP, cell wall components, DNA, lipids, proteins, enzymes and C, N, P, S compounds are certain parameters to determine the biomass of microorganisms. The $CHCl_3$ technique (Jenkinson 1976) is used to measure microbial carbon and nitrogen in the form of ^{14}C and ^{15}N incorporated in microorganisms.

Bacterial and fungal biomass in terms of carbon can be calculated by the following equation (Paul and Clark 1988):

$$\text{Bacterial biomass carbon } C_b = N_g V_b \Theta S_c \frac{\%C}{100} \times 10^{-6}$$

where

C_b bacterial biomass carbon ($\mu g\, g^{-1}$ soil)

N_g number of bacteria g^{-1}

V_b average volume μm^{-1} of bacteria (Π r L; r = radius, L = length)

Θ density (1.1×10^{-1} in liquid culture)

%C carbon content (45% dry weight)

S_c solids content (0.2 in liquid culture, 0.3 in soil)

$$\text{Fungal biomass carbon } C_f = \Pi r^2 L\Theta\, S_c\, \%C \times 10^{-10}$$

where

C_f fungal carbon ($\mu g\, g^{-1}$ soil), r = hyphal radius (often 1.13 μm)

L hyphal length ($cm\, g^{-1}$ soil), Θ = density (1.1 in liquid culture, 1.3 in soil)

S_c solids content (0.2 in liquid culture, 0.3 in soil)

%C carbon content (45% dry weight)

ATP

ATP is an ideal coenzyme for determining the microbial biomass (Oades and Jenkinson 1979). ATP content in soil depends on the phosphorus availability in the soil and hence can be employed in soil characterization and to estimate microbial populations in resting state. ATP and cell nutrient ratios are expressed as follows:

ATP	:	C	:	N	:	P	:	S
1	:	250	:	40	:	9	:	2.6

CO_2

Substrate induced respiration (Horwath and Paul 1994) is a physiological method for quantitative estimation of microbial biomass in soils in terms of CO_2. Radiorespiratory methods can also be employed by using isotopes.

Cell Wall Components

Bacterial and fungal cell wall components like muramic acid (MA) (by enzymatic analysis) (King and White 1977), lipopolysaccharide (by *Limulus* amoebocyte lysate (LPS) method) (Watson et al. 1977) and fungal chitin (Sharma et al. 1977) are measured to determine the microbial biomass.

Enzymes

Nutrient cycling in an ecological system involves a series of biological reactions carried out in the vicinity of microorganisms which may be produced at the advent of routine requirement or may be induced. Estimation of certain enzymes like cellulases, dehydrogenases, urease, and phosphotases

can be considered for biomass estimations (Paul and Clark 1988). Enzyme assays specific to microbial strains such as ACC deaminase for plant growth promoting rhizobacteria (PGPR) can also be applied in isolation and characterization procedures (Penrose and Glick 2003).

Lipid Profile

It is a well known fact that all viable cells are enveloped by lipid membrane with polar lipids (White 1995). Total phospholipids content estimation can be applied to determine microbial biomass (Franzmann et al. 1996). Lipid profiles analyses 'serve as signatures to define the community structure' (White 1995) as each microorganism is represented by characteristic lipid composition. Fatty acid methyl ester (FAME) (Eader 1995; Cavigelli et al. 1995) analysis can be used as a rapid method for lipid profile study but most of the fatty acids are common to microorganisms and hence difficult in environmental samples. To avoid such ambiguity, principle component analysis and cluster analysis are useful. Phospholipid linked fatty acid (PLFA) analysis (Petersen and Klug 1994) is employed specifically to determine bacterial biomass. The PLFA profile also provides information about the relative abundance of specific groups and their physiological status.

2.3.4 Immunological Detection Methods

Agglutination and Agar Precipitation Tests

These techniques show visible effects of agglutination in antigen and antibody reactions and a similar detection rate was observed in agar precipitation techniques (Hartmann et al. 1997).

Immunoseparation and Immunomagnetic Capture

Immunoisolation is a serological method, employed in detecting microorganisms (*Bacillus polymyxa* from wheat rhizosphere) (Mavingui et al. 1990) using microtitre plates coated with a specific antibody. In immunomagnetic capture (IMC) antibody coated (Para) magnetic beads (like Dynabeads) (van Elsas et al. 1997) are used to isolate specific microbial community. By this method Alun and Vartdal (1988) isolated *Pseudomonas putida* and Dye (1994) observed 200-fold enrichment in *Rhizobium* recovery.

Immunofluorescence and Immunogold Techniques

Immunofluorescence involves staining of the sample with primary antibody coupled with fluorescence marker (fluorescent isothiocyanate FITC)

or secondary antibody with fluorescent marker and visualized in epifluorescence microscopy (Paul and Clark 1988). Biomass of nitrifying bacteria, *Rhizobium, Aspergillus flavus* were determined by this technique though there are some limitations regarding non-specific reactions discouraging the use of this technique. Immunogold technique is applied in electron microscopy where the primary antibody and secondary antibody labeled with gold particles are employed (Hartmann et al. 1997).

Enzyme-Linked Immunosorbent Assay (ELISA)

This technique is most commonly employed in detection of bacteria depending on the enzyme linked antibody. Linking the enzyme to the secondary antibody is more advantageous as the intrinsic amplification of the signal by the enzyme can even detect low numbers of bound antibodies. The enzymes with high catalytic activity such as alkaline phosphotases, peroxidases and the substrate should become colored due to enzyme action for easy detection (Hartmann et al. 1997).

2.3.5 Molecular Techniques

Molecular methods in microbial ecology are very useful to detect and understand microbial function, as these methods do not require culturing and are thus applicable even to non-culturable microorganisms. Molecular techniques basically depend on the amplification of molecular sequences specific for detection (signature sequences). Phylogenetic relationships can also be drawn by molecular analysis using gene probes. These methods are based on the base sequence and re-association kinetics of denatured DNA and DNA to RNA ratio.

Colony Hybridization

This technique involves a combination of conventional plating followed by transferring the colonies onto a suitable solid support like a nitrocellulose membrane. The viable colony or DNA is lysed and hybridized with gene probes (^{32}P). This method is useful in detection of specific genotypes/phenotypes of microorganisms (Atlas and Bartha 1998).

Genetic Fingerprinting

Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) are helpful in analyzing the genetic divergence in construction of phylogenetic trees. Using *nif* and *nod* probes in this technique separated *Bradyrhizobium japonicum* into four groups (Lunge et al. 1994; Hadrys et al. 1992).

rRNA characterization depends on the nucleic acid recovery and identification of sequences that are highly conserved (16S and 23S) and their rate of change in these sequences which can be used for phylogenetic study (Overmann et al. 1999). 16S rRNA amplified gene fragment separation and fungal-specific PCR by denaturing gradient gel electrophoresis (DGGE) was used to analyze soil fungal dynamics (Muyzer et al. 1993; van Elsas et al. 2000). Amplified ribosomal DNA restriction analysis (ARDRA) was also found to be useful in separation of specific microbial strains (Massol-Deya et al. 1997). Temperature gradient gel electrophoresis was used to encode PCR amplified 18 s RNA in wheat rhizosphere to study the fungal diversity (Smit et al. 1999).

Gene Probes

This is a diagnostic method based on the construction of probe sequences that can bind to homologous DNA sequences for comparable genes in the target organism. Polymerase chain reactions (PCR) involve the amplification of target sequences by annealing the primer to denatured ssDNA and the target gene sequence is hybridized. Arbitrarily primed, AP-PCR is used to differentiate closely related strains. REP-PCR is based on the amplification of repeated and highly conserved sequences with less variability. In REP-PCR individual species can be identified by using species specific probes.

Reporter Genes and Biomarkers

Reporter genes are helpful in determining gene activity in complex communities (Burlage and Kuo 1994). *lacZ* gene is used to cleave X-gal, thus producing blue pigment in the agar medium, and this was used to determine the recombinant and non-recombinant strains of *Pseudomonas. xylE* gene and TOL plasmid-catechol system (Zukowski et al. 1983), *tfdA* (King et al. 1991), and *inaZ* (Lindgren et al. 1989) were also implicated in tracking microbial strains.

Bioluminescence by constructing *lux* genes and production of specific green fluorescent protein (GFP) (King et al. 1990; Chalfie 1995) was successfully used in monitoring the bioavailability of specific indicator components. Bacterial biomass can also be determined by monitoring chitinolytic gene expression and chitin degradation (Baty et al. 2000).

2.4 Mathematical Modeling

Analysis of experimental data in understanding microbial ecology and its function by fitting the observations in a mathematical expression will be

useful in arriving at hypothetical or conceptual conclusions (Robinson 1986). In population dynamics studies a change in the system or a parameter can be expressed in linear or differential equations. In mathematical modeling to define a system, either mechanistic or descriptive models can be applied.

2.4.1 Mechanistic Models

Conceptual simplifications are involved in building such models. This starts with defining the problem in actual and ideal conditions, and its simulation is based on the knowledge gained on the system, assumptions being made leading to hypothetical conclusions and testing of validation (Fig. 2.6) (Hall and Day 1977; Atlas and Bartha 1998).

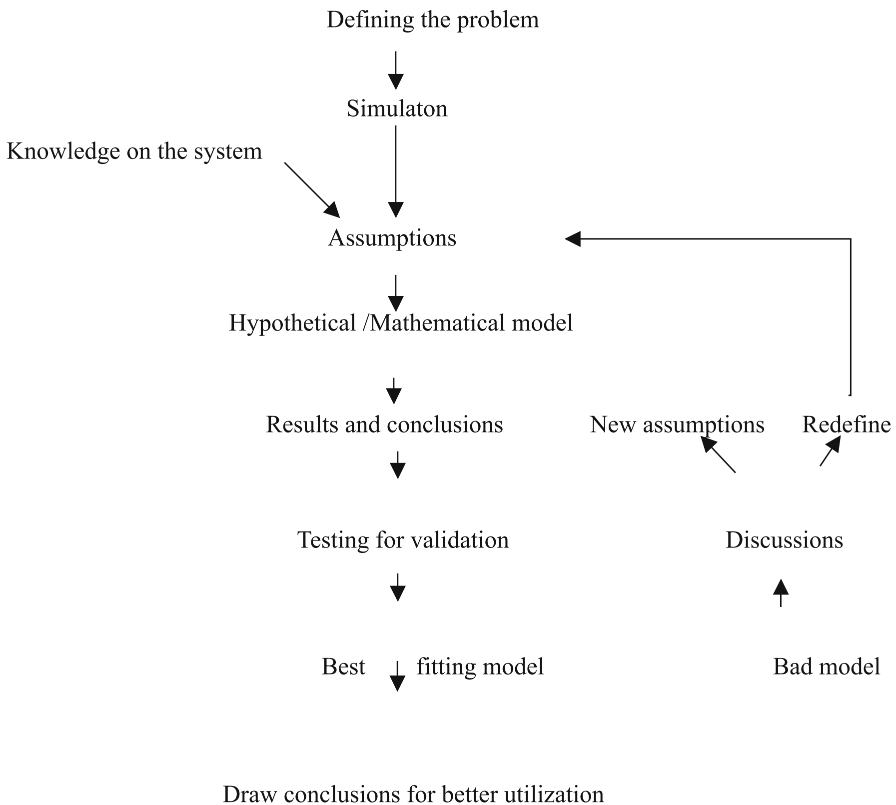

Fig. 2.6. Building a mathematical model

2.4.2 Descriptive Models

These are used to fit the data in simple mathematical equations and the results are tested by fitting in a statistical curve (Robinson 1985). Microbial biomass and interactions can be well understood by employing this tool (Prosser 1997).

2.4.3 Enumeration in Real Time Systems

Mathematical applications in accurate and efficient enumeration of microbial populations can be referred to MPN techniques (Woolmer 1994). Software in real time systems was also in use for microbial enumeration (Bloem et al. 1992).

2.5 Conclusions

A plethora of microorganisms inhabits the rhizosphere whose predominance is influenced by various ecological factors and rhizosphere effects. To monitor the functional rhizosphere and various plant-microbe interactions isolation, enumeration and characterization of microbial populations is very essential. Various conventional methods and techniques are in use for direct and indirect isolation and enumeration. Soil plate soil dilution and immersion methods are mostly followed for preliminary and quick screening. Selective media and various enrichment techniques are also applied in isolation of certain microbial groups for better recovery. Estimation of colony forming units (CFU), most probable number (MPN), hemocytometry, particle counts, vital staining and epifluorescence microscopy are certain approaches used in the enumeration and determination of microbial biomass.

Modern approaches may be through biochemical methods where assays for ATP, CO_2, cell components, enzymes and lipid profiles are used to estimate the relative abundance of specific microbial groups and their physiological states. Immuno-isolation, immuno-magnetic capture and ELISA are significant techniques to detect and estimate the microbial presence. Molecular techniques such as RAPD, DGGE, ARDRA, REP-PCR and reporter genes like *lacZ, X-gal,* TOL plasmid are proved to be highly reliable in detecting specific indicator components and their characterization. Mathematical modeling and real time software packages are applied in

interpreting hypothetical conclusions and their validation in finding the prevalence of microorganisms. Hence isolation, enumeration and characterization are of immense importance in obtaining a microbial continuum with the plant root system for sustainable production.

References

Allen ON (1957) Experiments in soil bacteriology, 3rd revised edn. Burgess Publishing, Minn

Alun AH, Vartdal F (1988) Clin Microbiol 26:2572–2575 as cited in van Elsas et al. (1997), pp 279–309

Atlas RM, Bartha R (1998) Microbial ecology–fundamentals and applications. Addison Wesley Logman, Califormia

Baker D (1990) Methods for the isolation, culture and characterization of the *Frankiaciae*: soil actinomycetes and symbionts of actinorrhizal plants. In: Labeda DP (ed) Isolation of biotechnological organisms from nature. MacGraw-Hill, London, pp 213–236

Baker D, Torrey JG, Kidd JH (1979) Isolation by sucrose density fractionation and cultivation in vitro of actinomycetes from nitrogen fixing root nodules. Nature 218:76–78

Baty AM, Eastburn CC, Techkarnjanaruk S, Goodman AE, Geesey GG (2000) Spatial and temporal variations in chitinolytic gene expression and bacterial biomass production during chitin degradation. Appl Environ Microbiol 66:3574–3585

Bloem J, Van Mullem DK, Bolhuis PR (1992) J Microb Methods 16:203 as cited in van Elsas et al. (1997), pp 279–309

Bose RG (1963) A modified cellulose medium for isolation of cellulolytic fungi from infected material and soil. Nature, London 198:505

Boulos L, Prevost M, Barbeau B, Coallier J, Desjardins R (1999) LIVE/DEAD BacLight: application of new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J Microb Methods 37:77–86

Brand LE (1990) The isolation and culture of microalgae for biotechnological applications. In: Labeda DP (ed) Isolation of biotechnological organisms from nature. MacGraw-Hill, London, pp 81–116

Burlage RS, Kuo C-T (1994) Living biosensors for the management and manipulation of microbial consortia. Ann Rev Microbiol 48:291–310

Byrd JJ, Colwell RR (1992) Microscopy analysis of environmental samples. In: Levin MA, Seider RJ, Rogul M (eds) Microbial ecology: principles, methods and applications. McGraw-Hill, New York, pp 93–112

Casida LR Jr (1968) Infrared colour photography. Selective demonstration of bacteria. Science 159:199

Cavigelli MA, Robertson GP, Klug MJ (1995) Fatty acid methyl ester (FAME) profile as a measure of soil microbial community structure. Plant Soil 170:99–113

Chalfie M (1995) Green fluorescent protein. Photochem Photobiol 62:651–656

Couch JN (1954) The genus *Actinoplanes* and its relatives. Trans New York Acad Sci 16: 315–318

Crook P, Carpenter CC, Klens PF (1950) The use of sodium propionate in isolating actinomycetes from soil. Science 112:656

Dale B (1983) Collection, preparation and identification of dinoflagellate resting cysts. In: Taylor DL, Seliger HH (eds) Toxic dinoflagellate blooms. Elsevier/North-Holland, New York, pp 443–452

Damigi SM, Frederick LR, Bremner JM (1961) Effect of soil dispersion techniques on plate counts of fungi, bacteria and actinomycetes. Bacteriol Proc 61:53
Davey HM, Kell DB (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: importance of single-cell analysis. Microbiol Rev 60:641–696
Domsch KH, Gams W, Anderson TH (1980) Compendium of soil fungi. Academic Press, London
Dye M (1994) J Microbiol Methods 19:235 as cited in van Elsas et al. (1977), pp 279–309
Eader K (1995) Gas chromatographic analysis of fatty acid methyl esters. J Chromatogr B Biomed Appl 671:113–131
El-Nakeeb MA, Levchevalier HA (1963) Selective isolation of aerobic actinomycetes. Appl Microbiol 11:75–77
Franzmann PD, Patterson BM, Powers TR, Nichols PD, Davis GB (1996) Microbial biomass in shallow, urban aquifer contaminated with aromatic hydrocarbons: analysis of phospholipids fatty acid content and composition. J Appl Bacteriol 80:617–625
Gause GF, Preobrazhenskaya TP, Kudrina ES, Blinov NO, Ryabova ID, Sveshnikova MA (1957) Problems in the classification of antagonistic Actinomycetes. Medgiz, Moscow
Gerdman JW, Nicolson TH (1963) Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. Trans Br Mycol Soc 46:235–244
Hadrys H, Balik M, Schierwater B (1992) Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol Ecol 1:55–63
Hall CAS, Day JW Jr (1977) Systems and models: terms and basic principles. In: Hall CAS, Day JW Jr (eds) Ecosystems modeling in theory and practice. Wiley, New York, pp 5–36
Hansen JF, Thingstad TF, Goksoyr J (1974) Evaluation of hyphal length and fungal biomass in soil by membrane filter technique. Oikos 25:102
Harley JL, Waid JS (1955) A method for studying active mycelia on living roots and other surfaces in the soil. Trans Br Mycol Soc 38:104–118
Hartmann A, Abmus B, Kirchhof G, Schloter M (1997) Direct approaches for studying soil microbes. In: van Elsas JD et al., pp 279–309
Heckman JR, Strick J (1996) Teaching plant soil relationships with colour images of rhizosphere pH. J Nat Res Life Sci Edu 25:13–17
Hirsch CF, Cristensen DL (1983) Novel method for selective isolation of actinomycetes. App Environ Microbiol 46:925–929
Horwath WR, Paul EA (1994) Microbial biomass. In: Weaver RW, Angle JS, Bottomley PS (eds) Methods of soil analysis. Part 2. Microbiological and biochemical properties. Soil Sci Soc Am, Madison WI, pp 753–773
Hoshaw RW, Rosowski JR (1973) Methods for microscopic algae. In: Stein JR (ed) Handbook of phycological methods. Cambridge University Press, New York, pp 53–68
Hsu SC, Lockwood JL (1975) Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl Microbiol 29:422–426
Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Watter MV (1997) Manual of environmental microbiology. Am Soc Microbiol Press, Washington DC
Jenkins WR (1964) A rapid centrifugal-floatation for separating nematodes from soil. Pl Dis Rep 48:692
Jenkinson DS (1976) The effects of biocidal treatments on metabolism in soil. Part IV. The decomposition of fumigated organisms in soil. Soil Biol Biochem 11:193–199
Johnson LF, Curl EA (1972) Methods for research on ecology of soil-borne plant pathogens. Burgess Publ, Minn
Johnson LF, Curl EA, Bond JH, Fribourg HA (1959) Microorganisms in the plant rhizosphere. In: Johnson LF, Curl EA (eds) Methods for studying soil microflora – plant disease relationships. Burgess Publ, Minn, pp 29–37

Karwowski J (1986) The selective isolation of *Micromonospora* from soil by cesium chloride density gradient ultracentrifugation. J Ind Microbiol 1:181–186

Kepner RL Jr, Pratt JR (1994) Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol Rev 58:603–615

King JD, White DC (1977) Muramic acid as a measure of microbial biomass in estuarine and marine samples. Appl Environ Microbiol 33:777–783

King JMH, DiGrazia PM, Applegate B, Burlage R, Sanseverino J, Dunbar P, Larimer F, Sayler GS (1990) Bioluminescent reporter plasmid for naphthalene exposure and biodegradation. Science 249:778–791

King RJ, Short KA, Seidler RJ (1991) Assay for detection and enumeration of genetically engineered microorganisms which is based on the activity of a deregulated 2,4-dichlorophenoxyacetate monooxygenase. Appl Environ Microbiol 57:1790–1792

Kogure K, Simidu U, Taga N (1984) Arch Hydrobiol 102:117 as cited in van Elsas JD et al. (1997), pp 279–309

Kuster E, Williums ST (1964) Selective media for the isolation of streptomycetes. Nature: 202:928–929

Labeda DP, Shearer MC (1990) Isolation of actinomycetes for biotechnological applications. In: Labeda DP (ed) Isolation of biotechnological organisms from nature. MacGraw-Hill, London, pp 1–20

Lawrence CH (1956) A method of isolating actinomycetes from scabby potato tissue and soil with minimal contamination. Can J Bot 34:44

Ledingham RJ, Chinn SHF (1955) A flotation method for obtaining spores of *Helmithosporium sativum* from soil. Can J Bot 33:298–303

Lee C, Russel NJ, White GF(1995) Rapid screening of bacterial phenotypes capable of biodegrading anionic sulfactants – development and validation of microtitre plate method. Microbiol 141:2801–2810

Lindgren PB, Fredrick R, Govindarajan AG, Panopoulas NJ, Staskawicz BJ, Lindow SE (1989) An ice nucleation reporter gene system: Identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. p*haseolicola.* EMBO J 8:1291–1301

Lunge VR, Ikuta N, Fonseca ASK, Hirigoyen D, Stoll M, Bonatto S (1994) Identification and interrelationship analysis of *Bradyrhizobium japonicum* strains by restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD). World J Microbiol Biotech 10:648–652

Makkar NS, Cross T (1982) Actinoplanets in soil and on plant litter from fresh water habitats. J Appl Bact 52:209–218

Massol-Deya AR, Weller L, Rias-Hernandez J, Zhou Z, Hickey RF, Tiedje JM (1997) Succession and convergence of biofilm communities in fixed-film reactors treating aromatic hydrocarbons in ground water. Appl Environ Microbiol 63: 270–276

Mavingui P, Berge O, Heulin TR (1990) Symbiosis 9:215–221 as cited in van Elsas et al. (1997), pp 279–309

Menzies JD (1957) A dipper technique for serial dilutions of soil microbial analysis. Soil Sci Soc Am Proc 21:660

Meyer-Reil LA (1978) Autoradiography and epifluorescent microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl Environ Microbiol 36:506–512

Minderman G (1956) The preparation of microtome sections unaltered soil for the study of soil microorganisms in situ. Plant Soil 8:42

Muyzer G, deWaal ED, Vitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nicholas DP, Parkinson D, Burges NA (1965) Studies on fungi in podzol. II. Application of the soil-sectioning technique to study of amounts of fungal mycelium in the soil. J Soil Sci 16:258
Nikitin D (1973) Direct electron microscopic techniques for the observation of microorganisms in soil. Bull Ecol Res Com Stockholm 17:85–92
Nonomura H, Hayakawa M (1988) New methods for the selective isolation of soil actinomycetes. In: Okami Y, Beppu T, Ogawara H (eds) Biology of Actinomycetes 88. Japan Sci Soc Press, Tokyo, pp 288–293
Oades JM, Jenkinson DS (1979) Adenosine tri-phosphate content of the soil microbial biomass. Soil Biol Biochem 11:201–204
Overmann J, Coolen MJL, Tuschak C (1999) Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA fragments. Arch Microbiol 173:83–94
Palleroni NJ (1980) A chemotactic method for the isolation of *Actinoplanaceae*. Arch Microbiol 128:53–55
Parkinson D (1957) New methods for the quantitative and qualitative study of fungi in the rhizosphere. Symposium, Methodes d'Etudes Microbiologiques du sol, Louvain, Belgium
Parkinson D (1973) Techniques for the study of soil fungi. Bull Ecol Res Com Stockholm 17:29–36
Parkinson D, Gray TRG, Williams ST (1971) Methods for studying the ecology of soil micro-organisms. IBP Handbook No 19. Blackwell, Oxford
Paul EA, Clark FE (1988) Soil microbiology and biochemistry. Academic Press, San Diego
Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth promoting rhizobacteria. Physiol Plant 118: 10–15
Petersen SO, Klug MJ (1994) Effects of sieving, storage and incubation temperature on the phospholipids fatty acid profile of soil community. Appl Environ Microbiol 60:2421–2430
Phillips BJ, Kalpan W (1976) Effect of cetyl pyrididinium chloride on pathogenic fungi and *Nocordia asteroides*in sputum J Clin Microbiol 3:272–276
Prosser JI (1997) Mathematical modeling and statistical analysis. In: Burlage RS, Atlas R, Stahl D, Geesey G, Sayler G (eds) Techniques in microbial ecology. Oxford University Press, New York, pp 408–437
Robinson JA (1985) Determining microbial kinetic parameters using non-linear regression analysis. Adv Micro Ecol 8:61–114
Robinson JA (1986) Approaches and limits to modeling microbiological processes. In: Meguseu F, Gantar M (eds) Perspective in microbial ecology. Slovene Society of Microbiology, Ljubljana, Yugoslavia, pp 20–29
Rowbotham TJ, Cross T (1977) Ecology of *Rhodocroccus coprophilus* and associated actinomycetes in fresh water and agricultural habitats. J Gen Microbiol 100:231–240
Sharma PD, Fisher PJ, Webster J (1977) Critique of the chitin assay technique for estimation of fungal biomass. Trans Br Mycol Soc 69:479–483
Skinner FA, Passmore FM, Devanport RR (eds) (1980) Media and methods for growing yeasts. In: Biology and activities of yeasts, Academic Press, London, pp 260–301
Smit E, Leeflang P, Glandorph B, Van Elsas JD, Wernars K (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Appl Environ Microbiol 65:2614–2621
Soderstrom DE (1977) Vital staining of fungi in pure cultures and in soil with fluorescein diacetate. Soil Biol Biochem 9:59–63

Strugger S (1948) Fluorescence microscope examination of bacteria in soil. Can J Res 26:188–193

Throndsen J (1978) The dilution-culture method. In: Sournia A (ed) Phytoplankton manual UNESCO. Page Brothers, Paris, pp 218–224

Timonin MI (1940) The interaction of higher plants and soil microorganisms. I. Microbial populations of rhizosphere of seedlings of certain cultivated plants. Can J Res 18:307–317

Trainor FR (1983) Survival of algae in soil after high temperature treatment Phycologia 22:201–202

Tribe HT, Abu-Elsound SM (1979) Colonization of hair in soil-water cultures with special reference to the genera *Pilimelia* and *Spirillospora* (Actinomycetales). Nova Hedwigia 31:789–805

van Elsas JD, Trevoss JT, Wellington EMH (1997) Modern soil microbiology. Marcel Dekker, New York

van Elsas JD, Duarte GF, Keijzer-Wolters A, Smit E (2000) Analysis of dynamics of the fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. J Microbiol Methods 43:133–151

Warcup JH (1951) Soil-steaming: a selective method for the isolation of Ascomycetes from soil. Trans Br Mycol Soc 34:515–518

Warcup JH (1955) On the origin of colonies of fungi on soil dilution plates. Trans Br Mycol Soc 38:298

Watson SW, Novitsky TJ, Quinby HL, Valoris FW (1977) Determination of bacterial numbers and biomass in marine environment. Appl Environ Microbiol 33:940–946

Wellington EMH, Marsh P, Watts JEM, Burdon J (1997) Indirect approaches for studying soil microorganisms based on cell extraction and culturing. In: Van Elsas JD, Trevors JT, Wellington EMH (eds) Modern soil biology. Marcel Dekker

White DC (1995) Chemical ecology: possible link between macro and microbial ecology. Oikos 74:177–184

Williams ST, Vickers JC (1988) Detection of actinomycetes in the natural environment-Problems and perspectives. In: Okami Y, Beppu T, Ogawara H (eds) Biology of actinomycetes 88. Japan Soc Press, Tokyo, pp 265–270

Wilson D, Forest HS (1957) An exploratory study of algae. Ecology 38:309–313

Woolmer PL (1994) Methods of soil analysis. Part 2. In: Weaver RW, Angle S, Bottomley P, Bezdicek D, Smith S, Tabatabai A, Wollum A (eds) Microbiological and biochemical properties. SSSA, Madison, Wisconsin, p 60

Zukowski MM, Gaffney DF, Speck M, Kaffmann M, Findeli A, Wisecup A, Lecocq JP (1983) Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on the expression of a cloned *Pseudomonas* gene. Proc Nat Acad Sci USA 80:1101–1105

3 Root Exudates as Determinant of Rhizospheric Microbial Biodiversity

Geeta Singh, Krishna G. Mukerji

3.1 Introduction

The plant-soil interrelationship is a subject of interest in ecology and agricultural sciences. While it is common knowledge that some plants only grow in special soils or that the yield depends on the condition of the soil, the fact that the plant may strongly influence the soil in which it grows has not very often been taken into account. Many of these effects may be caused by specific ionic uptake or the exudation of specific organic compounds or other rhizosphere effects (Marschner 1984; Peters and Long 1988). The ecological importance of such rhizosphere effects has been emphasized time and again (Mukerji 2002).

The rhizosphere, generally defined as that volume of soil adjacent to and influenced by the plant root, is regarded as a "hot spot" for microbial colonization and activity (Metting 1993). In contrast to bulk soil, where available organic carbon sources are only at low concentrations, rhizospheres are supplied with higher concentrations of nutrient sources generated during plant photosynthesis. (Duineveld et al. 1998, 2001).

Microorganisms in the rhizosphere of plants dominate the cycling of nutrients in soil-plant systems. Rhizosphere microorganisms increase the ability of plants to acquire nutrients from soil by either increasing the extent of the root system (e.g. through fungal hyphae) or solubilizing macronutrients such as phosphorus or sulfur (Smith and Read 1997). In fact, plant growth in nutrient-poor environments is linked to the formation of mutualistic associations with soil microorganisms such as arbuscular mycorrhizal fungi (Smith and Read 1997). Soil microorganisms also influence plant-pathogen interactions. For example, bacteria inoculated onto cucumber root (*Cucumis sativus*) penetrate the root and induce systemic resistance against a range of cucumber diseases (Wei et al. 1996).

The abundance and activities of soil microorganisms are influenced by various environmental (e.g. soil type, nutrients status, pH, texture, organic

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matter content, root zone location, moisture and interactions of these variables etc. (Gorlenko et al. 1997; Kreitz and Anderson 1997; Marschner et al. 1989) as well as plant factors (e.g. species, age). Survival of different introduced strains of fluorescent pseudomonads varies in soils of different textures (Araujo et al. 1994). However, microbial growth in soil is carbon limited and therefore, the presence of organic matter has the greatest influence on microbial populations (Wardle 1992; Mukerji 2002). *Pseudomonas, Flavobacterium, Alcaligenes* and *Agrobacterium* species have been shown to be particularly stimulated in the rhizosphere due to release of exudates and lysates (Curl and Truelove 1986). The variety of organic compounds released by plants has been postulated to be a key factor influencing the diversity of microorganisms in the rhizospheres of different plant species (Bolton et al. 1992; Bansal and Mukerji 1994). However, there is no direct evidence to support this hypothesis.

Rhizosphere is chemically, physically and biologically complex. Plant root release nearly 20% of their photosynthetic assimilates as water soluble sugars, amino acids, hormones, vitamins; root exudates in the form of organic acids, amino compounds and sugar phosphate ester (Uren 2001). The released low molecular weight exudates, lysates, gases, e.g. ethylene and CO_2, mucilage and organic compounds have been postulated to be key factors influencing the diversity and growth rates of micro organisms in the rhizosphere of different plant species. The effect of root exudates depends on the distance that they can diffuse away from the rhizoplane (Gupta and Mukerji 2002).

The identification and quantification of root exudates is of significance as these compounds alter the flow of nutrients in the rhizosphere and stimulate natural transformations in soils (Marschner et al. 1989; Nielsen and Elsas 2001). The diffusion rate into soil is a function of the size and charge of the compound and soil properties such as soil texture, organic matter content, pH, moisture and temperature. Although roots release a variety of organic substances, water-soluble compounds are most readily decomposable substances which serves as a source of nutrients for microbial populations colonizing root surfaces. The microorganism in turn can affect growth and physiology of plants by various microbial metabolites or by modifying soil root environment.

Root exudates may create a niche that influences which microorganisms are to colonize the rhizosphere, thereby altering the composition and diversity of micro organism colonizing the rhizosphere in a plant specific manner (Grayston et al. 1998).

Duineveld et al. (1998) observed that the effect of root exudates on dominant bacterial species can be marginal as most micro organisms are oligotrophic and thus respond slowly to root exudation. However Falchini et al. (2003) showed that Copiotrophs are favoured by the addition of

low molecular substrates passively released by roots. However in reality roots exude a spectra of substrates following a certain dynamic pattern (Kuzyakov and Cheng 2001), thus masking the effect of the individual compound.

3.2 Composition of Root Exudates

Knowledge of the composition and quantity of organic substances released from roots of different plant species gives an insight into chemical and biological processes occurring in the rhizosphere. Root exudates comprises primary metabolites such as sugars, amino acids, sugar alcohols, organic acids and secondary metabolites (Gupta and Mukerji 2002).

The major portion of microbial biodiversity is created by secondary metabolites. Plant roots also accumulate and exude numerous terpenoids, coumarins and flavonoids.

3.3 Factors Affecting Root Exudates

The qualitative and quantitative composition of root exudates are affected by various environmental factors, including pH, soil type, oxygen status, light intensity, soil temperature, nutrient availability and the presence of microorganisms. These factors may have a greater impact upon root exudation than differences due to plant species.

Soil texture has been shown to affect root exudates as it alters the mechanical impedance to the root (Boeuf-Tremblay et al. 1995). Similarly clay content influences the binding of different amino acids and peptides. Microorganisms are known to stimulate amounts and composition of root exudates (Meharg and Killham 1995). Plant species and the age of a plant is known to influence the composition of root exudates (Jaeger et al. 1999).

Soil microorganisms have been shown to respond to plant exudation and plant species can have different root exudation patterns (Brimecombe et al. 2001). Peas and oats exude different amounts of amino acids. Several pines have different exudation patterns. Young maples are known to exude greater and more diverse carbohydrates compared to mature trees, while mature trees exude greater and more diverse amino acids. Chantigny et al. (1996) found a correlation between the water-soluble organic C and microbial biomass C found under various annual and perennial species, suggesting differences in C deposition from the plant species. Martin (1971) showed that clover had a greater exudation rate than wheat, which was higher than

ryegrass. Legumes also have a lower C-to-N ratio, < 20 compared with > 30 for grasses. There is evidence that legumes exude greater concentrations of amino nitrogen than wheat and this could result in the selection of different organisms in these rhizospheres.

Organic acids other than tartaric may prevail in rhizospheres of important crop plants. In a study on the composition of root exudate of maize (*Zea mays*), citric acid was found to be dominating and tartaric acid to be present only at low concentration. In root exudate solutions of the same plant species, Schilling et al. (1998) found citric acid along with succinic acid to be the dominant organic acids. In wheat plants (*Triticum turgidum* var. *durum* L.), oxalic acid and acetic acid were found to be predominant (Cieslinski et al. 1997). Acetate was found to dominate in flax (*Linum usitatissiumum* L.) root exudate (Cieslinski et al. 1997).

3.4 Roles of Root Exudates

Although the mechanism by which plants regulate root exudates are not fully understood, enhanced rate of exudation of organic acids by plants have been found to be in response to toxic levels of aluminium in soil (Kochian 1995) or enhanced acquisition of sparingly soluble plant nutrients, e.g. P, K and Fe. Increased exudation of citrate and malate by plant roots is recorded under P-deficiency and aluminium toxicity (Kochian 1995).

Higher rates of root exudates may be due to zinc deficient conditions. Root exudates in turn affects the availability of inorganic plant nutrients such as Mn, P and Fe (Marschner et al. 1997). Microorganisms present in the rooting media may stimulate the exudation of organic substances by plant roots (Laheurte et al. 1990), in some cases by a factor 2–7, or increase the decomposition of organic root exudates. Microorganisms, such as *Pseudomonas fluorescens*, may themselves exude organic substances (siderophores) into the rhizosphere which may in turn affect the availability or uptake of certain mineral nutrients, especially that of iron.

Root exudates act as chemoattractant to attract bacteria towards roots. Hadacek and Gunther (2002) demonstrated that sugars or sugar alcohols may constitute important signals to soil fungi and determines fungal diversity in natural rhizosphere environments. The sugar alcohol mannitol stimulated the growth of endophytic fungi. However, although non-host carbon sources failed to stimulate the fungi, external sugar concentrations may affect gene expression in ectomycorrhizal basidiomycetes (Nehls et al. 2000). Flavonoids present in root exudates also act as transcriptional signals in the communication between soil bacteria and host plants during nodule formation and BNF by plants and plant-arbuscular mycorrhizae

interactions (Werner 2001). These secondary metabolites act as plant defence mechanism against pathogens and also as a stimulant/behaviour changing signal to the microflora. Legumes release various flavonoids that induce nodulation (*nod*) genes in *Rhizobium* and *Bradyrhizobium* and chalcones (a flavonoid) induce *vir*genes in *Agrobacterium tumefaciens*.

3.4.1 Root Exudates vs AMF

Arbuscular mycorrhizae results from a complex sequence of interactions between fungal hyphae and host cells leading to a functional mutualistic state (Bonfante-Fasolo 1988).

Plant/host factors stimulate AMF hyphal growth in vitro and also during precolonization phase of AMF formation (Becard and Piche 1989). Arbuscular mycorrhizal association in plants increase permeability of roots and/or exudation (Tsai and Phillips 1991). Quality of root exudates as shown by stressed suspension culture legume cells and cultures of Ri-T-DNA transformed roots stimulate AMF colonization. Specific compounds in the root exudates act as signal molecules capable of stimulation/induction of hyphal growth branching differentiation and host penetration (Becard and Piche 1989, 1990). However, Azcon and Ocampo (1984) did not find any relationship between root exudation and AMF infection.

Among the many possible hydrophobic compounds are phenolics in root exudates, which are known to act as signal molecules in plant-microbe interactions (Tsai and Philips 1991). Isoflavone in clover leaves increased under phosphate deficiency, whilst an additional unknown compound was detected in phosphate-deficient parsley (Franken and Gnadinger 1944). Nair et al. (1991) isolated two isoflavonoids, i.e. formononetin (7-hydroxy, 4′ methoxy isoflavone) and biochanin A (5,7-dihydroxy, 4′ methoxy isoflavone) from root exudates of clover roots grown under phosphate stress which act as signal molecules in AM-symbiosis. Hesperitin, naringenin and apigenin have been found to stimulate the hyphal growth of *G. margarita*. Becard and Piche (1990) observed that only flavonoids stimulate fungal growth, whereas, flavones, flavanons and isoflavanons were inhibitary. Organic acids present in root exudates stimulated hyphal growth from germinating spores and amino acids cystine, glycine and thiamine increased hyphal growth from spores of *G. caledonicus*.

Phosphorus nutrition of host plants affects both the quantity and quality of root exudates. Also phosphorus in host plant tissues (Tawaraya et al. 1994) and in the soil solution influences root colonisation by arbuscular mycorrhizal fungi. P-deficiency in plants increases root exudation of amino acids, sugars and organic acids. Root exudates of P-deficient citrus, clover,

onion (Tawaraya et al. 1996a,b) and carrot (Nagahashi et al. 1996) have been shown to increase hyphal growth of arbuscular mycorrhizal fungi and also formation of secondary appressoria by *Gigaspora margarita* on roots of onion (Tawaraya et al. 1994).

Suspension-cultured cell exudates of *Pueraria phaseodes* increased hyphal growth of arbuscular mycorrhizal fungi. In addition, root exudates produced by Ri-T-DNA-transformed *Daucus carota* grown without P gave more hyphal growth from spores of *G. margarita* than exudates produced in the presence of P (Nagahashi et al. 1996). Root exudates from onion of clearly different P status also affected the hyphal growth of *G. margarita* (Tawaraya et al. 1996a) and the formation of secondary appressoria was inhibited by phosphate application to shoots, indicating the inhibitory effect of plant P status (Tawaraya et al. 1994). These results conclusively show that the phosphorus nutrition of the host plant influences the extent of root colonisation by arbuscular mycorrhizal fungi through its effect on root exudates (Tawaraya et al. 1994). Under optimum 'P' levels mycorrhizal infection did not significantly affect amount or composition of root exudate in maize (Azaizeh et al. 1995).

However, factors other than P in root exudates may affect arbuscular mycorrhizal fungi. Hyphal growth of *G. margarita* was stimulated by root exudates of clover and carrot (Becard and Piche 1990) but not of non-host lupin and sugar beet. Host root exudates can elicit hyphal morphogenesis which facilitates root contact and appressorium formation (Giovannetti et al. 1993a,b; Suriyaperruma and Koske 1995). Recent studies on appressorium formation on isolated host cell walls indicate that contact recognition does not require a chemical signal (Nagahashi et al. 1996).

3.5 Host Influence

One of the most important variables in determining the microbial composition (qualitative and quantitative) in the rhizosphere are plant species effects that result in different community structures for various species grown in the same soil (Grayston et al. 1998; Miethling et al. 2000; Nehl et al. 1997; Lemanceau et al. 1995; Wiehe and Hoflich 1995; Berg et al. 2002). The population dynamics of the introduced bioinoculant is also influenced by the plant species. Rhizosphere communities of wheat, ryegrass and clover differ in their ability to utilize a variety of substrates (Grayston et al. 1998).

The plant specific composition of microbial communities in their rhizospheres is governed mainly by the differences in quality and quantity of carbon sources released in the root exudates (Merbach et al. 1999). Even small modifications, as may exist between different cultivars of the same

plant species, can result in the selection of different microbial communities in the rhizosphere (Rengel et al. 1998). In studies with rape (canola; *Brassica napus*), the composition of rhizosphere bacteria of a transgenic cultivars could be distinguished from other non-engineered cultivars. However, in potatoes the composition of microbial communities in the rhizospheres was not significantly affected by genetic engineering (Lukow et al. 2000; Lottmann and Berg 2001).

3.5.1 Diversity of Microbial Populations in the Rhizosphere of Different Plant Species

There are diverse microbial populations in the rhizosphere of different plant species, like wheat (Neal et al. 1970, 1973), clover and ryegrass and grasses. Actinomycetes contributed a high percentage of the microbial community in the rhizospheres of wheat, maize and grass and pseudomonads, though in low percentage were stimulated in the rhizosphere. The selective stimulation of *Pseudomonas* species in the rhizosphere, is also reported by other workers. Cultivars of maize crop harbours genetically different species of *Burkholderia cepacia*. Berg et al. (2002) isolated genotypically and phenotypically diverse antagonistic rhizobacteria from different *Verticillum* host plants. They observed that proportion of isolates with antagonistic activity was highest for strawberry rhizosphere (9.5%) followed by oilseed rape (6.3%) and potato (3.7%) as compared to control soil (3.3%). The abundance and composition of *Verticillium* antagonists was plant species dependent.

3.5.2 Bacterial Population

Each plant species selects its own specific bacterial community from the reservoir of bacteria present in the field soil or on seeds. Studies based on rRNA-gene profiling techniques and community-level physiological profiles demonstrate that plant species are more important in the selection of bacterial communities in rhizospheres than other factors, i.e. soil origin or agricultural treatments (Miethling et al. 2000). Plant age-dependent effects (on bacterial community) are also observed in rhizospheres of crops grown in soil (Gomes et al. 2001; von der Weid et al. 2000). This modification is caused by different compositions or quantities of root exudates during different growth stages.

The rhizosphere community composition is also influenced by plant genotype (Carelli et al. 2000) although there are exceptions. The dominant

operational taxonomic unit (OTU) in rhizosphere of *Trifolium repens* matched the theoretical profile of 16SrRNA gene of *Rhizobium leguminosarum*. This (OTU) was found at substantially lower level in bulk soil. Latour et al. (1996) found that bacterial populations associated with plant roots are selected by plant roots. Populations from the roots of each plant species and from the uncultivated soil differed in their abilities to use different organic compounds and to dissimilate nitrogen. Populations of fluorescent pseudomonads isolated from an uncultivated soil and from roots of two plant species (i.e. flax and tomato) were phenotypically and genotypically different (Lemanceau et al. 1995).

Rengel et al. (1998) showed that wheat genotypes differ in the extent of bacterial colonization of roots because of the influence of soil micronutrient status on the quality and quantity of root exudates released by the wheat genotypes. Root exudates are readily assimilated by the soil microflora. An increased application of N fertilizer increased exudation from wheat roots and increased bacterial counts in the rhizosphere. The selective plant pressure is more pronounced when plant endophytic microbial populations are taxonomically analysed. A greater abundance and diversity of pseudomonads were found in the root interior of *B. napus* cv. Quest (50 isolates) compared to *B. napus* cv. Excel (30 isolates) or *B. rapa* cv. Parkland (15 isolates). The composition of endophytic and rhizosphere bacterial communities is also known to change over the course of the growing season (Mahaffee and Kloepper 1997a). The possible reason for the observed differences in the endophytic population between the cultivars is variation w.r.t. root morphology or root exudates. Endophytic bacterial taxa are a subset of rhizosphere bacterial taxa suggesting a close relationship between the two communities (Lilley et al. 1996; Mahaffee and Kloepper 1997b). Bacteria enters the root interiors by either hydrolyzing wall bound cellulose, entering through auxin induced tumor wounds at lateral root branching (Christiansen-Weniger 1996).

McSpadden et al. (2000) studied genotypic and phenotypic diversity *phlD*-containing *Pseudomonas* strain isolated from the rhizosphere of wheat crops. Based on REP-PCR fingerprints all studies found enormous genetic diversity of *Pseudomonas* sp. at subspecies level. Fromin et al. (2001) reported that the genotypic structure of *Pseudomonas brassicacearum* populations analysed by REP-PCR finger prints are significantly influenced by the *Arabidopsis thaliana* genotype.

Differences exist in root exudates/rhizodeposition patterns between plant species (Bachmann and Kinzel 1992) and among cultivars of the same species (Cieslinski et al. 1997). Activities of rhizosphere bacteria is related to the types and amounts of root exudates/rhizodeposition. Increased associative N_2 fixation by *Azospiriuum brasilence* and roots of three wheat cultivars differed in organic acid exudation consisting of higher amounts

of carboxylic acids such as succinic acid, malic acid, and oxalic acid by host plant (Christensen-Weniger et al. 1992). Tomato mutants with different iron-mobilization properties showed different amounts and kinds of Fe-producing and chelating substances exuded by their roots. They also differed in the amounts of oxalic acid and amino acids exuded. Chickpea exudes large amounts of organic acids especially under 'P' deficient conditions which probably leads to selection of microorganisms tolerant to low pH and capable of growth on these substances and their breakdown products.

3.5.3 Exudation in Genetically Engineered Plants

Genetic manipulation develops crops with improved qualities. The genetically engineered plants might change soil environment and the bacterial consortia qualitatively and quantitatively in the rhizosphere due to the release of gene products or an altered composition of root exudates. Transgenic crops like maize, rape, sugarbeet, are resistant to the herbicidal compounds glufosinate (Syn. L. Phosphino thricin), an L amino acid that inhibits glutamine synthetase. However, a few studies also indicated sensitivity of soil microflora towards this herbicide which may result in diminished soil fertility (Ahmad and Malloch 1995). Similarly transgenics with respect to other traits exists. Microbial populations in the rhizospheres of various transgenic plants such as those producing antibacterial proteins have been analysed (Heuer et al. 2002; Ahrenholtz et al. 2000; Lottmann and Berg 2001).

Gyamfi et al. (2002) evaluated possible shifts/perturbations in eubacterial and *Pseudomonas* rhizosphere community structure due to release of transgenic glufosinate-tolerant *Brassica napus*. DGGE analysis of 16SrRNA gene fragments amplified from rhizospheric eubacterial and *Pseudomonas* communities revealed slightly altered microbial communities in the rhizosphere of transgenic plants due to altered root exudation.

However, evidence from other studies showed that plant species and cultivars strongly influence the composition and diversity of rhizosphere bacterial communities in agricultural soils. Based on FAME analysis of field grown plants fewer *Arthrobacter* and *Bacillus* isolates were recovered in the rhizosphere of a transgenic Canola than the rhizosphere of untransformed Canola cultivar (Dunfield and Germida 2001). The possible reason for observed differences were differences in root exudation pattern. Similarly, rhizosphere bacterial communities of wheat and untransformed canola grown at the same field site differed – *Bacillus* dominated in wheat rhizosphere but not in canola rhizosphere.

Transgenic alfalfa over-expressing a nodule enhanced malate dehydrogenase (ne MDH) (1.6-fold higher MDH) compared to the untransformed alfalfa cultivar differed in the amount and composition of root organic acid produced and exuded into the rhizosphere under field conditions (Tesfaye et al. 2001). Analysis of rhizospheric microbial populations using biolog microtiter plates and nucleotide sequence of PCR based 16S ribosomal DNA (r DNA) showed qualitative changes in the abundance of bacterial phylogenetic groups between rhizospheric soils of transgenic and untransformed alfalfa. Biolog studies also indicated greater microbial functional diversity in rhizosphere of transgenic as compared to untransformed alfalfa. Transgenic alfalfa line with enhanced production (7.1-fold over untransformed) of organic acids induced changes in the distribution and relative abundance of the different phylogentic groups, resulting in qualitative changes in bacterial community structure between the rhizospheres of transgenic and untransformed alfalfa. Utilization of organic acids in root exudates has been shown to be the basis of root colonization by *Pseudomonas* sp. (Lugtenberg et al. 2001).

Potential Applications

By matching rhizobacterium genotypes with crops for which they have extent colonization preference, degree of root colonization could be increased due to the fact that rhizosphere of different plant species differentially supports the added/introduced bioinoculant. The possible role of root exudates in plant-microbe interaction nutrient mobilisation and crop adoption to adverse soil conditions have practical implications for engineering the plants suited to diverse agricultural systems. Transgenic plants differing from wild type plants in composition of root exudates can be valuable tools for assessing the ecological significance of components of root exudates in microbial ecology.

3.6 Conclusions

Root exudation is a important ecological phenomenon which manipulates the plant and (root) microbial succession. Under sterile conditions only 5–10% of the fixed carbon is released by roots compared to 12–18% released from roots in nonsterile soil and this increase is influenced by rhizosphere microflora. The composition and quantity of organic substances released from roots of different plants attract varied types of microbes. Root exudates act as chemoattractants to attract microbes towards roots. Rate of exudation by roots is governed by various factors.

References

Ahmad I, Malloch D (1995) Interaction of soil microflora with the bioherbicide phosphinothricin. Agric Ecosyst Environ 54:165–174

Ahrenholtz I, Harms K, de Vries J, Wackernagel W (2000) Increased killing of *Bacillus subtilis* on hair roots of transgenic T4-lysozyme-producing potatoes. Appl Environ Microbiol 66:1862–1865

Araujo MAV, Mendonca-Hagler LC, Hagler AN, van Elsas JD (1994) Survival of genetically modified *Pseudomonas fluorescens* introduced into subtropical soil microcosms. FEMS Microbiol Ecol 13:205–216

Azaizeh HA, Marschner H, Romheld V, Wittenmayer L (1995) Effects of a vesicular arbuscular mycorrhizal fungus and other soil microorganisms on growth, mineral nutrient acquisition and root exudation of soil grown maize plants. Mycorrhiza 5: 321–327

Azcon R, Ocampo JA (1984) Effect of root exudation on VA mycorrhizal infection at early stages of plant growth. Plant Soil 82:133–138

Bachmann G, Kinzel H (1992) Physiological and ecological aspects of the interactions between plant roots and rhizosphere soil. Soil Biol Biochem 24:543–552

Bansal M, Mukerji KG (1994) Positive correlation between VAM induced changes in root exudation and mycorrhizosphere mycoflora. Mycorrhiza 5:39–44

Becard G, Piche Y (1989) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 112:77–83

Becard G, Piche Y (1990) Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and nonhost Ri T-DNA transformed roots. Can J Bot 68:1260–1264

Berg G, Nicotte R, Anette S, Leo E, Angela Z, Korndia S (2002) Plant dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different verticillium host plants. Appl Environ Microbiol 68:3328–3338

Boeuf-Tremblay V, Plantureux S, Guckert A (1995) Influence of mechanical impedance on root exudation of maize seedlings at two development stages. Plant Soil 172:279–287

Bolton H, Frederickson JK, Elliott LF (1992) Microbial ecology of the rhizosphere. In: Metting FB (ed) Soil microbial ecology, Marcel Dekker, New York, pp 27–36

Bonfante-Fasolo P (1988) The role of the cell wall as a signal mycorrhizal associations, In: Scannerini S, Bonfante-Fasolo P, Pearson G (eds) Cell to signals in plant, animal and microbial symbiosis. Springer, Berlin Heidelberg New York, pp 219–235

Brimecombe MJ, de Leij FA, Lynch JM (2001) The effect of root exudates on rhizosphere microbial communities. In: Pinton R, Varanini Z, Nannipieri P (eds) The rhizosphere. Biochemistry and organic substances at the soil-plant interface. Marcel Dekker, New York, pp 95–140

Carelli M, Gnocchi S, Fancelli S, Mengoni A, Paffetti D, Scotti C, Bazzicalupo M (2000) Genetic diversity and dynamics of *Sinorhizobium meliloti* populations nodulating different alfalfa cultivars in Italian soils. Appl Environ Microbiol 66:4785–4789

Chantigny MH, Prevost D, Angers DA, Vezina LP, Chalifour FP (1996) Microbial biomass and N transformations in two soils cropped with annual and perennial species. Biol Fert Soils 21:239–244

Christiansen-Weniger C (1996) Endophytic establishment of *Azorhizobium caulinodans* through auxin-induced tumors of rice (*Oryza sativa* L.) Biol Fertil Soils 21:293–302

Christensen-Weniger C, Groneman AF, vanVeen JA (1992) Associative N_2 fixation and root exudation of organic acids from wheat cultivars of different aluminum tolerance. Plant Soil 139:167–174

Cieslinski G, van Rees KCJ, Huang PM (1997) Low molecular weight organic acids released from roots of durum wheat and flax into sterile nutrient solutions. J Plant Nutri 20:753–764

Curl EA, Truelove B (1986) The Rhizosphere. Springer-Verlag, New York

Duineveld BM, Rosado A, van Elsas JD, van Veen JA (1998) Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum via denaturing gradient gel electrophoresis and substrate utilization patterns. Appl Environ Microbiol 64:4950–4957

Duineveld BM, Kowalchuk GA, Keijzer A, van Elsas JD, van Veen JA (2001) Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturating gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. Appl Environ Microbiol 67:172–178

Dunfield KE, Germida JJ (2001) Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*. FEMS Microbiol Ecol 38:1–9

Falchini L, Naumova N, Kuikman PJ, Bloem J, Nannipieri P (2003) CO_2 evolution and denaturing gradient gel electrophoresis profiles of bacterial communities in soil following addition of low molecular weight substrates to simulate root exudation. Soil Biol Biochem 36:775–782

Franken P, Gnadinger F (1994) Analysis of parsley arbuscular endomycorrhiza: infection development and mRNA levels of defence-related genes. Mol Plant Microb Interact 7:612–620

Fromin N, Achouak W, Thiery JM, Heulin T (2001) The genotypic diversity of *Pseudomonas brassicacearum* populations isolated from roots of *Arabidopsis thaliana*: influence of plant genotype. FEMS Microbiol Ecol 37:21–29

Giovannetti M, Avio L, Sbrana C, Citernesi AS (1993a) Factors affecting appressorium development in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe. New Phytol 123:115–122

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993b) Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during preinfection stages. New Phytol 125:587–593

Gomes NCM, Heuer H, Schonfeld J, Costa R, Mendonca-Hagler L, Smalla K (2001) Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232:167–180

Gorlenko MV, Majorova TN, Kozhevin PA (1997) Disturbances and their influence on substrate utilization patterns in soil microbial communities. In: Insam H, Rangger A (eds) Microbial communities. Springer, Berlin Heidelberg New York, pp 84–93

Grayston SJ, Wang SQ, Campbell CD, Edwards AC (1998) Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol Biochem 30:369–378

Gupta R, Mukerji KG (2002) Root exudate – biology. In: Mukerji KG et al. (eds) Techniques in mycorrhizal studies. Kluwer Academic, Dordrecht, pp 103–131

Gyamfi S, Ulrike P, Michael S, Angela S (2002) Effect of transgenic glufosinate-tolerant oilseed rape (*Brassica napus*) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere. FEMS Microbiol Ecol 41: 181–190

Hadacek F, Gunther FF (2002) Plant root carbohydrates affect growth behaviour of endophytic microfungi. FEMS Microbiol Ecol 41:161–170

Heuer H, Kroppenstedt RM, Berg G, Smalla K (2002) Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizophere communities are negligible relative to natural factors. Appl Environ Microbiol 68:1325–1335

Jaeger CH, Lindow SE, Miller W, Clark E, Firestone MK (1999) Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. Appl Environ Microbiol 65:2685–2690

Kochian LV (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Ann Rev Plt Physiol Plt Mol Biol 46:237–260

Kreitz S, Anderson TH (1997) Substrate utilization patterns of extractable and non-extractable bacterial fractions in neutral and acidic beech forest soils. In: Insam H, Rangger A (eds). Microbial communities. Springer, Berlin Heidelberg New York, pp 140–160

Kuzyakov Y, Cheng W (2001) Photosynthesis controls of rhizosphere respiration and organic matter decomposition. Soil Biol Biochem 33:1915–1925

Laheurte F, Leyval C, Berthelin J (1990) Root exudates of maize, pine and beech seedlings influenced by mycorrhizal and bacterial inoculation. Symbiosis 9:111–116

Latour X, Corberand T, Laguerre G, Allard F, Lemanceau P (1996) The composition of fluorescent pseudomonad population associated with roots as influenced by plant and soil type. Appl Environ Microbiol 62:2449–2456

Lemanceau P, Corberand T, Gardan L, Labour X, Laguerre G, Boeufgras J, Alabouvette C (1995) Effect of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.) on the diversity of soil-borne populations of fluorescent pseudomonads. Appl Environ Microbiol 61:1004–1012

Lilley AK, Fry JC, Bailey MJ, Day MJ (1996) Comparison of aerobic heterotrophic taxa isolated from four root domains of mature sugar beet (*Beta vulgaris*). FEMS Microbiol Ecol 21:231–242

Lottmann J, Berg G (2001) Phenotypic and genotypic characterization of antagonistic bacteria associated with roots of transgenic and non-transgenic potato plants. Microbiol Res 156:75–82

Lugtenberg BJJ, Ekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. Ann Rev Phytopathol 39:461–490

Lukow T, Dunfield PF, Liesack W (2000) Use of the T-RFLP technique to assess and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol Ecol 32:241–247

Mahaffee WF, Kloepper JW (1997a) Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativa* L.). Microb Ecol 34:210–223

Mahaffee WF, Kloepper JW (1997b) Bacterial communities of the rhizosphere and endorhizosphere associated with field-grown cucumber plants inoculated with a plant growth-promoting rhizobacterium or its genetically modified derivative. Can J Microbiol 43:344–353

Marschner H (1984) Nährstoffdynamik in der *Rhizosphare*. Vortrag Botaniker-Tagung, Wien

Marschner H, Treeby M, Romheld V (1989) Role of root-induced changes in the rhizosphere for iron acquisition in higher plants. Z Pflanzenernahr Bodenk 152:197–204

Marschner P, Crowley DE, Higashi RM (1997) Root exudation and physiological status of a root-colonizing fluorescent pseudomonad in mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum* L.) Plant Soil 189:11–20

Martin JK (1971) ^{14}C-labelled material leached from the rhizosphere of plants supplied with ^{14}CO. Aust J Biol Sci 24:1131–1142

McSpadden-Gardener BB, Schroeder KL, Kalloger SE, Raajmakers JM, Thomashow LS, Weller DM (2000) Genotypic and phenotypic diversity of *phlD*-containing *Pseudomonas* strain isolated from the rhizosphere of wheat. Appl Environ Microbiol 66:1939–1946

Meharg AA, Killham K (1995) Loss of exudates from the roots of perennial ryegrass inoculated with a range of micro-organisms. Plant Soil 170:345–349

Merbach W, Mirus E, Knof G, Remus R, Ruppel S, Russow R, Gransee A, Schulze J (1999) Release of carbon and nitrogen compounds by plant roots and their possible ecological importance. J Plant Nutr Soil Sci 162:373–383
Metting BF (1993) Soil microbial ecology. Marcel Dekker, New York
Miethling R, Wieland G, Backhaus H, Tebbe CC (2000) Variation of microbial rhizosphere communities in response to crop species, soil origin, and inoculation with *Sinorhizobium meliloti* L33. Microb Ecol 40:43–56
Mukerji KG (2002) Rhizosphare biology. In: Mukerji KG et al. (eds) Techniques in mycorrhizal studies. Kluwer Academic, Dordrecht, pp 87–101
Nagahashi G, Douds DD Jr, Abney GD (1996) Phosphorus amendment inhibits hyphal branching of the VAM fungus *Gigaspora margarita* directly and indirectly through its effect on root exudation. Mycorrhiza 6:403–408
Nair MG, Safir GR, Siqueira JO (1991) Isolation and identification of vesicular-arbuscular mycorrhiza-stimulatory compounds from clover roots. Appl Environ Microbiol 57: 434–439
Neal JL, Atkinson TG, Larson RI (1970) Changes in the rhizosphere microflora of spring wheat induced by disomic substitution of a chromosome. Can J Microbiol 16:153–158
Neal JL, Larson RJ, Atkinson TG (1973) Changes in rhizosphere populations of selected groups of physiological groups of bacteria related to substitution of specific pairs of chromosomes in spring wheat. Plant Soil 39:209–212
Nehl DB, Allen SJ, Brown JF (1997) Deleterious rhizosphere bacteria: an integrating perspective. Appl Soil Ecol 5:1–20
Nehls U, Wiese J, Hampp R (2000) External sugar concentration as a signal controlling ectomycorrhizal fungal gene expression. In: Poldila G, Douds DD Jr (eds) Current advances in mycorrhizal research. APS Press, St Paul, Minn, pp 1–26
Nielsen KM, Elsas JD (2001) Stimulatory effects of compounds present in the rhizosphere on natural transformation of Acinetobacter sp. BD413 in soil. Soil Biol Biochem 33:345–357
Peters NK, Long SR (1988) Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plt Physiol 88:396–400
Rengel Z, Ross G, Hirsch P (1998) Plant genotype micro-nutrient influence colonization of wheat roots by soil bacteria. J Plant Nutr 21:99–113
Schilling G, Gransee A, Deubel A, Lezovic G, Ruppel S (1998) Phosphorus availability, root exudates, and microbial activity in the rhizosphere. Zcits Pflanzenernahrung Boden 161:465–478
Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, San Diego, Calif
Suriyaperruma SP, Koske RE (1995) Attraction of germ tube and germination of spores of the arbuscular mycorrhizal gus *Gigaspora gigantea* in the presence of roots of maize posed to different concentrations of phosphorus. Mycology 87:772–778
Tawaraya K, Sasai K, Wagatsuma T (1994) Effect of phosphate application on the contents of amino acids and reducing sugars in the rhizosphere and VA mycorrhizal infection of wheat clover. Soil Sci Plant Nutr 40:539–543
Tawaraya K, Watanabe S, Yoshida E, Wagatsuma T (1996a) Effect of onion (*Allium cepa*) root exudates on the hyphal growth of *Gigaspora margarita*. Mycorrhiza 6:57–59
Tawaraya K, Saito M, Morioka M, Wagatsuma T (1996b) Effect of the concentration of phosphate on spore germination and hyphal growth of the arbuscular mycorrhizal fungus, *Gigaspora margarita* Becker & Hall. Soil Sci Plant Nutr 42:667–667
Tesfaye M, Temple SJ, Allan DL, Vance CP, Samac DA (2001) Over-expression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. Plant Physiol 127:1836–1844
Tsai SM, Phillips AD (1991) Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. Appl Environ Microbiol 57:1485–1488

Uren NC (2001) Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants. In: Pinton R, Varanini Z, Nannipieri P (eds) The rhizosphere. Marcel Dekker, New York, pp 19–40

von der Weid I, Paiva E, Nobrega A, van Elsas JD, Seldin L (2000) Diversity of *Paenibacillus polymyxa* strains isolated from the rhizosphere of maize planted in Cerrado soil. Res Microbiol 151:369–381

Wardle DA (1992) A comparative assessment of factors which influence microbial biomass carbon and nitrogen levels in soil. Biol Rev 67:321–358

Wei G, Kloepper JW, Tuzun S (1996) Induced systemic resistance to cucumber diseases and increased plant growth by plant-promoting rhizobacteria under field conditions. Phytopathol 86:221–224

Werner D (2001) Organic signals between plants and microorganisms. In: Pinton R, Varanini Z, Nannipieri P (eds) Rhizosphere, biochemistry and organic substances at the soil-plant interface, Marcel Dekker, New York, pp 197–222

Wiehe W, Hoflich G (1995) Survival of plant growth promoting rhizosphere bacteria in the rhizosphere of different crops and migration to non-inoculated plants under field conditions in north-east Germany. Microb Res 150:201–206

4 Rhizosphere Microbial Community Dynamics

Roopam Kapoor, Krishna G. Mukerji

4.1 Introduction

The rhizosphere is the rooting zone of plants and includes the root, soil attached to the root, and adjacent soil that is influenced by the root (Wallace 2001). It is a densely populated area in which the roots must compete with the invading root systems of neighbouring plant species for space, water and mineral nutrients, and with soil inhabiting microorganisms, including bacteria, fungi and insects feeding on an abundant source of organic materials (Ryan and Delhaize 2001). Thus, root–root, root–microbe, and root–insect communications are likely continuous occurrences in this biologically active soil zone (Bachman and Kinzel 1992). Root–root and root–microbe communication can either be positive (symbiotic) to the plant, such as the associations of epiphytes, microrhizal fungi, and nitrogen fixing bacteria with roots; or negative to the plant including interactions with parasitic plants and pathogens (bacteria, fungi, insects etc.).

The term "rhizosphere" in recent times has evolved to include the endophytic microbes that have proven capable of penetrating and colonizing root tissue (Sturz and Nowak 2000). In this model the root cortex becomes part of the soil, root microbial environment, resulting in a continuous apoplastic pathway from the root epidermis to the shoot, sufficient for movement of microorganisms into the xylem. This creates a continuum of root associated microorganism that are able to inhabit the rhizosphere, the root cortex and other plant organs.

4.2 Root-Microbe Communication

Considering the complexity and biodiversity of the underground world, roots are clearly not passive targets for soil organisms. In addition to providing mechanical support and allowing water/nutrient uptake, roots

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also perform certain specialized roles, including the ability to synthesize, accumulate and secrete a diverse array of compounds (Clegg and Murray 2002; Flores et al. 1999).

4.2.1 Root Exudates

Nearly 5 to 23% of all photosynthetically fixed carbon is transferred to the rhizosphere through root exudates (Marschner 1995; Walker et al. 2003). The compounds secreted by plant roots serve important roles as chemical attractants and repellants in the rhizosphere (Estabrook and Yoder 1998; Bais et al. 2001). Through the exudation of a wide variety of soluble and easily diffusible components (e.g. cellulose, lignin and proteins), plants influence the community structure and diversity of microorganisms in the rhizosphere soil (see Chap. 3).

The microbial relationships in the rhizosphere are dynamic in nature. The length of root survival may have a bearing on the efficiency of these microbial relationships and seems to indicate that long-lived perennials would be most beneficial. For instance, the specific rhizobial relationships that establish with legume roots exist only as long as that root is alive and then need to be re-established with newly developing roots (Atkinson and Watson 2000).

The nature and quality of the carbon supply from root to the rhizosphere is a major factor that influences its colonization by soil organisms. To reach the root tip, organisms must either migrate forward with, or towards the root tip or interfere with the growth of the root tip in some way. A potentially crucial part of this system is the sheath of up to 10,000 living border cells that are produced by the growing root cap each day, and released into the surrounding rhizosphere. It has been suggested that these border cells act as decoys to pathogenic organisms in soil around the root tips, thus decreasing the probability of pathogenic attack at the root tip (Hawes et al. 2000). If the pathogen attack does occur in the border cells at the root tip, these cells may become detached before the root tip proper is infected.

4.2.2 Microbial Interactions

Equally, soil microorganisms can influence which compounds the roots exude, thus creating a more favourable environment for themselves (Lynch and Brown 2001). Bacteria and fungi live around roots and feed on root exudates and dead root cells. Competition between microbial species in this

area is stiff. In the battle for the carbon compounds (organic matter) bacteria use several strategies including production of antibiotics and poisonous chemicals that remove the competition. Bacteria also produce substances that stimulate the plant to increase root growth (Sturz and Nowak 2000), increase root area for colonization and increase root exudates (Wallace 2001).

Total biological community rather than just immediate root microflora plays a role in rhizosphere interactions with plant roots and that relationships between plant roots and microorganisms are all regulated by complex molecular signalling. There has been a co-evolution between plants and soil microbes resulting in microbial responses to plant exudation and plant exudation responses to the presence of microbes (Atkinson and Watson 2000; Sturz and Nowak 2000).

Root secretions play symbiotic or defensive roles as a plant ultimately engages in positive or negative communications, depending on the other elements in the rhizosphere. Flavonoids present in the root exudates of legumes activate *Rhizobium meliloti* genes responsible for the nodulation process (Peters et al. 1986). In contrast, survival of the delicate and physically unprotected root cells under continued attack by pathogenic microorganisms depends on a continuous "underground chemical warfare" mediated by secretion of phytoalexins, defence proteins, and other as yet unknown chemicals (Flores et al. 1999).

The unexplored chemodiversity of root exudates is an obvious place to search for novel biologically active compounds, including antimicrobials. Bais et al. (2002) identified rosmarinic acid (RA) in the root exudates of sweet basil (*Ocimum basilicum*) elicited by fungal cell wall extracts from *Phytopohthora cinnamomi*. The dynamics of rhizosphere microbial communities has been related to root exudation in P-deficient *Lupinus albus* (Horst et al. 2001). Bacterial population on cluster roots were affected by the alternation of root exudation during cluster root development that are responsible for dramatic changes in the composition of the rhizosphere soil solution. Bacterial population on cluster roots was influenced by the concentrations of citrate, malate and *cis*-aconitate in the rhizosphere soil solution but not by fumerate. In contrast, fungal populations were influenced by citrate exudation in cluster roots but not in other root zones. Cluster roots of white lupin have been functionally linked with a very efficient chemical mobilization of sparingly soluble soil P-sources by organic chelators (citrate, malate, phenolics) and ectoenzymes (acid phosphatases), released into the rhizosphere in extraordinary amounts. Drastic alterations in rhizosphere pH, redox potential, release of carboxylates and phenolics, and a high expression of chitinase suggest marked influence on microbial communities during cluster root development. This may in turn have important consequences for microbial turnover of root exudates and

on production of microbial metabolites involved in nutrient mobilization. Plant age also has a determinant effect on the numbers of microorganisms (Gomez-de-Guinan and Nageswara 1996).

4.2.3 Signalling Factors

Cell–cell communication between bacteria are mediated by small diffusible signalling molecules as acylated homo-serlactones (AHLs) for Gram-negative bacteria and peptide-signalling molecules for Gram-positive bacteria. Upon reaching a threshold concentration at high population densities, an auto-inducer activates transcriptional activator proteins that induce specific genes. Thus, intercellular signals enable a bacterial population to control the expression of genes in response to cell density (quorum sensing). It was also reported that crude aqueous extracts from several plant species exhibited AHL inhibitory activity. Root exudates from pea (*Pisum sativum*) seedlings were found to contain several bioactive compounds that mimicked AHL signals in well-characterized bacterial reporter strains, stimulating AHL-regulated behaviours in others (Knee at al. 2001).

Thus, it is possible that roots may have developed defence strategies by secreting compounds into the rhizosphere that interfere with bacterial quorum sensing responses such as signal mimics, signal blockers, and/or signal-degrading enzymes. However, the chemical nature of such active mimic secondary metabolites is not fully identified (Tepelitski et al. 2000; Knee et al. 2001).

4.3 Microbial Dynamics in Terrestrial Ecosystem

Soil microorganisms constitute the world's largest reservoir of biological diversity and are crucial to the functioning of terrestrial ecosystems. Soil provides a spatially and temporally heterogeneous environment, especially at the scale encountered by microbes (de Brito-Alvarez et al. 1995).

Heterotrophic microbial communities inhabiting soil mediate key processes that control ecosystem carbon (C) and nitrogen (N) cycling, and they potentially represent a mechanistic link between plant diversity and ecosystem function (Warenbourg and Estelrich 2000). It is expected that plant diversity could affect microbial communities and the ecosystem processes they mediate (Bargett and Shine 1999; Wardle et al. 1999; Stephan et al. 2000). The availability of growth limiting resources shapes the composition of biotic communities (Tilman 1982, 1987) and resource availability for

soil microbial communities is constrained by organic compounds in dead leaves and roots (i.e. detritus) that can be used to generate cellular energy (Smith and Paul 1990). Plant species differ in their biochemical composition, and therefore changes in plant diversity could alter production as well as the range, of organic compounds in detritus that limit, and thus control, the composition and function of heterotrophic microbial communities.

4.4 Soil Microbial Community in Relation to Plant Diversity

The plant diversity significantly enhances rates of microbial processes that mediate ecosystem C and N cycling; this effect is more strongly dependent on plant production than on species richness (Zak et al. 2003). During organic matter decomposition, microbial respiration returns photosynthetically fixed C to the atmosphere.

Plants in species rich communities use greater amounts of soil water than those at the lower levels of diversity. Such a response would reduce soil matric potential as plant diversity increase, which would alter microbial community composition and function by favouring fungal dominance (Paul and Clark 1996).

The pattern of gross N mineralization matches the increase in microbial respiration. Although the exact mechanisms causing greater rates of N mineralization remains to be determined, plant diversity alters microbial community composition and function which in turn increase the supply of soil N to plants and contribute to greater productivity in the most species with experimental plant communities. However, these associations may not hold for all microbial groups. In a study of grasslands taken out of production, it was found that genetic diversity of ammonia-oxidising bacteria, which are responsible for the first step of nitrification, did not respond to changes in the diversity of the above ground vegetation. Plants mainly affect microbial communities on the root surface rather than in the rhizosphere soil. Outside this zone the dominant microbial populations appear to be very stable in time and space. On the root surface, plants appear to select consistently a distinct microbial community that comprises a broad taxonomic range of microorganisms. Grayston et al. (1996, 1998) showed selective influence of four plant species (wheat, ryegrass, bentgrass and clover) on microbial diversity in the rhizosphere. There was clear discrimination between the carbon sources utilised by the microbial community from the different plant rhizospheres. Carbohydrates, carboxylic acids and amino acids are the substrates mainly responsible for this discrimination, suggesting plants may differ in the exudation of these compounds. Grayston et al. (1996, 1998) confirmed the stimulatory effect of the rhizosphere on microbial growth

and in particular pseudomonad proliferation. The bacterial genera most commonly detected in the rhizosphere consist of a number of well characterized groups such as *Bacillus, Pseudomonas, Stenotrophomonas* and *Comamonas*, as well as non-culturable *Verrucomicrobium* and *Acidobacterium*. This selection, i.e. 'rhizosphere effect', appears to be at strain level rather than at higher taxonomic levels (see Chap. 1).

4.5 Microbial Community and Plant Genotype

Plant genotype affects the composition of the microbial community in the rhizosphere. Chaudhury (1999) studied the microbial population of wheat rhizosphere as influenced by wheat genotypes. The rhizosphere of wild diploid and tetraploid wheat contained larger populations of bacteria and actinomycetes than did cultivated hexaploid wheat. These two groups of microorganisms were mostly stimulated under the influence of diploid DD and VV chromosomes. It has been observed that secondary metabolites reduce the development of mycelium of several fungi isolated from the rhizosphere. Genotypes that differ in the production of these compounds have different effects on the composition of the microbial community under field conditions.

4.5.1 Transgenics Expressing T_4 Lysozyme

A transgenic plant expressing T_4 lysozyme is successful in management of a variety of diseases of economically important crop plants. T_4 lysozyme affects Gram positive and Gram negative bacteria either by its muramidase activity against bacterial cell wall component murein (Tsugita et al. 1968) or by a nonenzymatic mechanism which may involve disruption of membranes. This genetic modification of the plant results in reduced susceptibility of transgenic potato plant to infection by *Erwinia carotovora* (During 1993). Also a detectable amount of T_4 lysozyme is released from the roots causing bactericidal activity on the root surface (Ahrenholtz et al. 2000). Many plant associated bacterial species were susceptible in vitro (During 1993; Lottmann and Berg 2001). As the effect on different species varied significantly, the T_4 lysozyme expression has the potential to change the rhizosphere bacterial community and thereby affect the quality or the function of the soil. Rhizospheric bacterial communities of two transgenic potato lines which produce T_4 lysozyme for protection against bacterial infections when analysed in comparison to rhizospheric communities of

wild type plants and transgenic controls not harbouring the lysozyme gene showed no detectable differences (Heuer et al. 2002). They found that environmental factors and not the T_4 lysozyme expression of the transgenic plant influenced the rhizospheric community as revealed by DGGE profiles, the compositions of bacterial isolates and Biolog profiles of different samplings.

Lukow et al. (2000) biomonitored the highly diverse bacterial communities of three distinct soil plots located within the same agricultural field and planted with different plant variants, i.e. (i) transgenic Barnase/Barstar (tBB), (ii) transgenic GUS (tGUS) and (iii) non-transgenic (WT-wild-type) potato plants. The transgenic Barnase/Barstar potato plants carried two modified gene constructs. One gene construct consisted of a bacterial ribonuclease from *Bacillus amyloliquefaciens*, termed *barnase*, coupled with the *gstl* promoter. Selective induction of the promoter after infection with various types of pathogenics, e.g. *Phytophthora infestans*, or symbiotic organisms should lead to suicide of the infected cells and thus prevent spreading of the pathogen. To minimise the detrimental effects of the potential background activity of the *barnase* gene in non-infected tissue, the gene encoding *barstar* coupled with the constitutively expressed viral *CaMV 35S* promoter was also inserted into the genome of the transgenic plants. The *barstar* gene inhibits *barnase* synthesis and is also derived from *B. amyloliquefaciens.* Instead of the *barnasel/barstar* gene construct the transgenic GUS potato plants carried a *GUS* gene (*uidA* gene) coding for b-glucuronidase. They showed that the T-RFLP finger printing technique enables the detection of both spatial heterogeneities and change over time in the structural composition of highly diverse bacterial communities. The effect of transgenic factor was negligible but seasonal shifts were more prominent under field grown plants (Gomes et al. 2001; Smalla et al. 2001; Heuer et al. 2002).

Schmalenberger and Tebbe (2002) compared the bacterial communities in the rhizosphere of transgenic maize (having *pat* genes conferring resistance to the herbicide glufosinate) to its isogenic non-transgenic cultivar. No detectable difference was observed between SSCP profiles of the two rhizospheric communities. In contrast PCR-SSCP profiles of bacterial communities from rhizosphere of sugar beet grown under similar field conditions were different.

Other reports showed different microbial communities associated with the roots of field-grown transgenic and non-transgenic glyphosate-tolerant oilseed rape. However, in these studies three different cultivars were tested and one of them was genetically engineered. Therefore, the authors could not exclude the possibility of additional genotypic differences besides the genetic insertion conferring herbicide resistance. Becker et al. (2001) studied the diversity of *Rhizobium leguninosarum* bv. *viciae* in fields cultivated

with transgenic, Basta-tolerant and wild-type rapeseed cultivars. Various strains were found in soils around the transgenic lines that were not detected in control treatments. In addition, fields of some transgenic lines possessed a higher *Rhizobium* diversity. However, no significant effects on rhizobial numbers or soil basal respiration were observed. Transient but significant effects on microbial communities due to unintentionally altered plant characteristics by genetic engineering were also reported by Donegan et al. (1995, 1996). In that study, total bacterial numbers, bacterial species and DNA fingerprints of micro-organisms associated with cotton expressing the *Bacillus thuringiensis* endotoxin were compared to those of the wild-type plant.

4.6 Atmospheric Carbon Dioxide

The concentration of atmospheric CO_2 has risen from 280 to 362 ppm in the last nine decades and is predicted to continue to rise by an average of 1.5 ppm per year. Plants respond to elevated CO_2 by fixing more carbon, leading to increased plant biomass and below ground carbon allocation. Quantitative and qualitative changes in carbon entering the soil as litter or rhizodeposition, under elevated CO_2 will have important consequences for nutrient cycling and acquisition, through effects on both the composition and size of soil microbial community. In particular the C:N ratio of plant litter influences the decomposition rate. It has been hypothesized that this increase in C:N ratio will slow the rate of litter decomposition, reducing N mineralization and N availability to the plant.

Microbial community of the rhizosphere changes when the plants are grown at elevated CO_2 with preferential stimulation of fungal growth (Panikov 1994). These changes were due to the gross exuding compounds with higher C:N ratio. This was reflected in a significantly greater utilization of all C sources, except those with a low C:N ratio (neutral and acidic amino acids, amides, N-heterocycles, long chain aliphatics) by microbial communities from the rhizosphere of elevated CO_2 grown plants. The preferential stimulation of fungi under elevated CO_2 could be due to an increase in quantity, but decrease in quality of litter, because fungi grow on more complex substrates and they are more efficient at converting substrate into biomass than bacteria.

Lussenhop et al. (1998) observed that the strongest response to increased atmospheric CO_2 in the rhizosphere where unchanged microbial biomass and greater numbers of protozoa suggested faster bacterial turnover, where arbuscular mycorrhizal root length increased and where the number of microanthropods observed on fine roots increased.

4.7 Microbial Community and Rhizodeposition

The microbial community is the main agent responsible for litter decomposition and nutrient cycling in the forest soils. The rhizosphere is continually supplying readily available forms of carbon and nitrogen to the soil system (Smith and Paul 1990). Thereby, the growth of the microbial biomass is promoted.

The quantity and the composition of soil microbial biomass are particularly sensitive to changes in soil environment. Microbial biomass parameters have frequently been used as indicators of ecosystem stress and disturbance (Anderson and Domsch 1993). Across the forest stand, values of microbial biomass varied widely (Hack et al. 2000). Among forest types different limitations were found acting upon the growth of the microbial biomass. In the more acidic soils, microbial biomass was significantly correlated to soil pH. Positive correlations between microbial biomass and soil moisture and total soil N were found in soils of all forest types (Hack et al. 2000).

Low molecular weight compounds enter the soil through plant litter decomposition, rhizodeposition and microbial metabolism as well as from decaying soil organisms. Once in soil, these substances may be used up very rapidly, with typical half-lives for amino acids being in the region of 1–12 h (Jones 1999). Therefore the concentration of sugars and amino acids not only depend on the amount, quality and decomposition rate of litter and on rhizodeposition but also on turnover times of these substances. High concentrations of sugars and amino acids may indicate high substrate abundance as well as low turnout rate.

During a growth season the soil environment may undergo significant changes as a result of both local climate conditions and cultivation practices, which could impact soil microbial communities and the associated nutrient dynamics. It was observed that the size of the microbial community fluctuated only moderately, and there was always higher concentration of microbes in the top layer. Carbon utilization ability of the bacterial community in the soil changed over time, colour formation patterns suggested a decrease in microbial activity level.

4.8 Agronomical Regimes and Microbial Diversity

Microorganisms in agricultural soils are known to exert profound influence on the soil's fertility status in particular with respect to the availability of plant nutrients (Kennedy and Smith 1995). These key life sup-

port functions of soil are likely to be dependent on crucial constituents of the soil microbial community. The health of the soil can be defined in terms of its microbiological capacity to counteract (suppress) the activity of plant-pathogenic or plant-deleterious microorganisms (van Bruggen and Semenov 2000). The extent and degree of microbial activity in soils is clearly affected by agronomical regimes. The covercrops affect the soil microbial communities and thereby potentially affect the suppresiveness to plant diseases (Mazzola1999; Raaijmakers and Weller 1998: Smalla et al. 2001). A major driving force in these effects is the influx of sources of carbon and energy into the grossly oligotrophic soil system (Yang and Crowely 2000). Differences in these inputs by different crop plants induce different microbial community structures (Smalla et al. 2001). Nussleien and Tiedje (1999) showed that a change in soil use from forest to pasture caused a shift in the G+C content profile of soil DNA, resulting in an estimated 49% change of microbial community. These changes may be due to direct (vegetation-related) as well as indirect (soil abiotics like pH, organic matter content) effects. A clear example of the cover crop directly affecting the below ground microbiota can be visualised by a link between suppression of take-all pathogen *Geaumannomyces graminis* var. *tritici* and the prevalence of 2,4-diacetyl phloroglucinol-producing pseudomonads (Raaijmakers and Weller 1998). Similarly, Mazzola (1999) found that apple orchards established in wheat soils suppressed *Rhizoctonia solani* to a greater extent than those planted in non-wheat areas. The analysis of the total microbial community level based on the sequencing of 16S and 18S rDNA genes and on PCR-DGGE of soil DNA with different primer systems used showed rather consistent differences in the microbial communities between arable and the grass land plots (van Elsas et al. 2002). In spite of the clear-cut differences in the microbial communities in the two habitats at the phylogenetic and functional levels, it is not clear what these differences really indicate with respect to soil functioning in terms of the mechanisms of disease suppression. It is supposed that key abiotic determinants such as soil organic matter and temperature can exert strong controls over microbial activities in the soil (van Veen et al. 1997). Certain soil-borne pathogens such as *R. solani*, actually can thrive in soil given appropriate conditions (van Elsas et al. 2002). Predictions with respect to the degree of suppression of soil-borne diseases on the basis of an overview of microbial community will, therefore, always have to be related to the soil abiotics. Schonfeld et al. (2002) found that different sources of organic matter such as piggery manure vs compost resulted in different longevity of disease suppression.

4.8.1 Crop Sequence

Crop sequence can favour the build up of advantageous associations of bacterial endophyte populations leading to the development and maintenance of beneficial host-endophyte allelopathies. Rumberger and Marschner (2003) have demonstrated that canola crops inhibit soil-borne pathogens in following crops. Although this effect was mainly attributed to the release of low molecular S-containing compounds, such as isothiocyanates, during microbial degradation of crop residues they also concluded that canola may affect the soil microbial community structure such as active living plants. Forage crop such as alfalfa, clovers and oats contribute greatly to organic matter of soils through their extensive root systems and by their dense leafy canopies (Scott and Cooper 2002). Their deep tap root expand the functional rhizosphere and therefore increase the infiltration of air, water and uptake of nutrient for the crop following. By maintaining high organic matter, these plants increase the microbial activity that contribute to good soil physical structure.

Hoflich et al. (2000) found that mineral fertilizers inhibit the microbial activity in the root zone compared to organic fertilization with green manures especially for the bacterial species *Pseudomonas, Agrobacterium* and *Xanthomonas*. The leghaemoglobin content of pea nodules, which are indicator of nitrogen fixing activity, was reduced by nitrogen application. Compost amendments caused small variations in the total numbers of bacteria, actinomycetes and fungi in the rhizosphere (de Brito-Alvarez et al. 1995). Compost, depending on the degree of maturity provides rich medium supporting high microbial activity (Chen et al. 1988).

The importance of microbial diversity in soil is still not fully understood. It has been suggested that those soil harbouring a greater diversity of microorganisms are more likely to be resilient to stress such as heavy metal, hydrocarbon contamination, or long term water logging. Human land use and agricultural practices have been identified as the most important factors affecting biodiversity. Biodiversity studies have revealed that an increased management of grassland results in a decrease in plant and animal diversity and also microbial diversity. Population profiles have been generated for general bacterial population (eubacteria), actinomycetes, ammonia-oxidising bacteria and the pseudomonads in plots under differing drainage and N-fertilizer status. The banding pattern obtained revealed distinct clustering for each of the bacterial grouping where grassland management was discriminating factor. These results provide the basic information regarding microbial diversity and evidence that N-fertilizer and drainage are factors responsible for the development of distinct bacterial communities.

4.9 Conclusions

Microorganisms are fundamentally important component of the soil habitat where they play key roles in ecosystem functioning through controlling nutrient cycling reactions. Within soil there exist many microbial interactions all regulated by complex molecular signalling. Each individual member of the microbial community plays its specific role in its specific niche and this role may not be easily replaceable by the other organisms present in the system. Diversity of the microbial communities inhabiting soil is influenced by plant diversity and environmental fluctuations due to shifts in composition of root exudates. Disturbance of a soil microbial system may not lead to great effects on soil functioning as a result of functional redundancy present in the system. The ecology of the key microbial interactions that take place in the soil, needs scrutiny. We need to understand the root and soil factors controlling the microbial dynamics.

References

Ahrenholtz I, Harms K, de Vries J, Wackernagel W (2000) Increased killing of *Bacillus subtilis* on hair roots of transgenic T4-lysozyme-producing potatoes. Appl Environ Microbiol 66:1862–1865

Anderson TH, Domsch KH (1993) The metabolic quotient for CO_2 (qCO_2) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soil. Soil Biol Biochem 25:393–395

Atkinson D, Watson CA (2000) The beneficial rhizosphere: a dynamic entity. Appl Soil Ecol 15:99–104

Bachman G, Kinzel H (1992) Physiological and ecological aspects of the interaction between plant roots and rhizosphere soil. Soil Biol Biochem 24:543–552

Bais HP, Loyola VM, Flores HE, Vivanco JM (2001) Root specific metabolism: the biology and biochemistry of underground organs. In Vitro Cell Dev Biol P l 37:730–741

Bais HP, Walker TS, Schweizer HP, Vivanco JM (2002) Root specific elicitation and antimicrobial activity of rosamarinic acid is hairy root cultures of sweet basil (*Ocimum basilicum* L.). Plant Physiol Biochem 40:983–995

Bargett RD, Shine A (1999) Linkages between plant litter diversity, soil microbial biomass and ecosystem function in temperate grasslands. Soil Biol Biochem 31:317–321

Becker R, Ulrich A, Hedtke C, Hornermeier B (2001) Einfluss des Anbaus von transgenem herbizidresistentem Raps auf das Agrar-Ökosystem. Bundesgesundheitsbl. Gesundheitsforsch-Gesundheitsschutz 44:159–167

Chaudhury A (1999) Microbial population of wheat rhizosphere as influenced by wheat genotypes. J Interacademia 3:230–231

Chen W, Hoilink HAJ, Schmitthenner AF, Taovinen OH (1988) The role of microbial activity on the suppression of damping-off caused by *Pythium ultimum*. Phytopathol 78:314–322

Clegg C, Murray P (2002) Soil microbial ecology and plant root interactions. In: Gordon A J (ed) IGER Innovations No 6, pp 36–39

de Brito-Alvarez MA, Gaugne S, Antoun H (1995) Effect of compost on rhizosphere microflora of the tomato and on the incidence of plant growth-promoting rhizobacteria. Appl Environ Microbiol 61:194–199

Donegan KK, Palm CJ, Fieland VJ, Porteous LA, Ganio LM, Schaller DL, Bucao LQ, Seidler RJ (1995) Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin. Appl Soil Ecol 2:111–124

Donegan KK, Schaller DL, Stone JK, Ganio LM, Reed G, Hamm PB, Seidler RJ (1996) Microbial populations, fungal species diversity and plant pathogen levels in field plots of potato plants expressing the *Bacillus thuringiensis* var. *tenebrionis* endotoxin. Transgenic Res 5:25–35

During K, Porsch P, Fladung M, Lorz H (1993) Transgenic plants resistant to the phytopathogenic bacterium *Ervinia carotovans* 3:587–598

Estabrook EM, Yoder JI (1998) Plant-plant communications: rhizosphere signating between parasitic angiosperms and their hosts. Pl Physiol 116:1–7

Flores HE, Vivanco JM, Loyola-Vargas VM (1999) "Radicle" biochemistry: the biology of root-specific metabolism. Trend Plant Sci 4:220–226

Gomes NCM, Heuer H, Schonfeld J, Costa R, Mendonca-Hagler L, Smalla K (2001) Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232:167–180

Gomez-de-Guinan YG, Nageswara J (1996) Effect of the rhizosphere of peanut (Papilloniaceae) on the fungal flora and the activity of phosphatases. Caribbean J Sci 32: 214–220

Grayston SJ, Vaughan D, Jones D (1996) Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. Appl Soil Ecol 5:29–56

Grayston SJ, Wang SQ, Campbell CD, Edwards AC (1998) Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol Biochem 30:369–378

Hack E, Bachmann G, Zechmeister-Boltenstern S (2000) Soil microbial biomass and rhizosphere effects in natural forest stands. Phyton 40:83–90

Hawes MC, Gunawardena U, Mijasaka S, Zhao X (2000) The role of root border cells in plant defense. Trends Plant Sci 5:128–133

Heuer H, Kroppenstedt RM, Berg G, Smalla K (2002) Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizophere communities are negligible relative to natural factors. Appl Environ Microbiol 68:1325–1335

Hoflich G, Tauchler M, Kuhn G, Rogasik J (2000) Influence of agricultural crops and fertilization on microbial activity and microoorganisms in the rhizosphere. J Agron Crop Sci 184:49–59

Horst WJ, Kanh M, Jilarin JM, Chude VO (2001) Agronomic measures for increasing availability to crops. Plant Soil 237:211–223

Jones DC (1999) Amino acid biodegradation and its potential effects on organic nitrogen capture by plants. Soil Biol Biochem 31:613–622

Kennedy AC, Smith KL (1995) Soil microbial diversity and sustainability of agricultural soils. Plant Soil 170:75–86

Knee EM, Gong FC, Gao M, Teplitski M, Jones AR, Foxworthy A, Mort AJ, Baur WD (2001) Root mucilage from pea and its utilization by rhizosphere bacteria as a sole carbon source. Mol Plant Microbe Interact 14:775–784

Lottmann J, Berg G (2001) Phenotypic and genotypic characterization of antagonistic bacteria associated with roots of transgenic and non-transgenic potato plants. Microbiol Res 156:75–82

Lukow T, Dunfield PF, Liesack W (2000) Use of the T-RFLP technique to assess and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol Ecol 32:241–247
Lussenhop J, Treonis A, Curtes PS, Teeri JA, Vogel CS (1998) Response of soil biota to elevated atmospheric CO_2 in poplar model systems. Oecologia 113:247–251
Lynch JP, Brown J (2001) Topsoil foraging: an architectural adaptations of plant to low phosphorus availability. Plant Soil 37:225–237
Marschner H (1995) Mineral nutrition of higher plants. Academic Press, London
Mazzola M (1999) Transformation of soil microbial community structure and *Rhizoctonia*-suppressive potential in response to apple roots. Phytopathol 89:920–927
Nusslein K, Tiedje JM (1999) Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. Appl Environ Microbiol 65:3622–3626
Panikov NS (1994) Response of soil microbial community to global warming: simulation of seasonal dynamics and long term succession in typical tundra. Microbiol 63:289–404
Paul EA, Clark FE (1996) Soil microbiology and biochemistry. Academic Press, New York
Peters NK, Frost JW, Long SR (1986) A plant flavone, luteolin, induces expression if *Rhizobium meliloti* nodulation genes. Science 233:977–980
Raaijmakers JM, Weller DM (1998) Natural plant protection by 2,4-diacetyl phloroglucinol-producing *Pseudomonas* spp. in take-all decline soil. Mol Plant-Microbe Interact 11:144–152
Rumberger A, Marschner P (2003) 2 Phenyl ethyl iso thiocyanate concentration and microbial community composition in the rhizosphere of canola. Soil Biol Biochem 35:445–452
Ryan PR, Delhaize E (2001) Function and mechanism of organic anion exudation from plant roots. Ann Rev Plant Physiol Mol Biol 52:527–560
Schmalenberger A, Tebbe CC (2002) Bacterial community composition in the rhizosphere of a transgenic, herbicide-resistant maize (*Zea mays*) and the comparison to its non-transgenic cultivar *Bosphore*. FEMS Microbiol Ecol 40:29–37
Schonfield J, Gelsomino A, van overbeek LS, Smalla K, van Elsas JD (2002) Effects of comport addition and simulated solarisation on the fate of *Rastonia solanacearum* biovar. 2 and indigenous bacteria in soil. FEMS Microbiol Ecol 40:87–105
Scott J, Cooper J (2002) GPI Agricultural Accounts Part II: Resource capacity and use soilqualtiy and productivity. GPI Atlantic, Atlanta Canada
Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heure H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis plant-dependent enrichment and seasonal shifts revealed. Appl Environ Microbial 67:4742–4751
Smith JL, Paul EA (1990) The significance of soil biomass estimates. In: Bollag JM, Stotzky G (eds) Soil biochemistry. Marcel Dekker, New York, pp 357–396
Stephan A, Meyer AH, Schmid B (2000) Plant diversity affects culturable soil bacteria in experimental grassland communities. J Ecol 88:988–999
Sturz AV, Nowak J (2000) Endophytic communities of rhizobacter and the strategies to create yield enhancing associations with crops. Appl Soil Ecol 15:183–190
Teplitski M, Robinson JB, Bauer WD (2000). Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviours in associated bacteria. Mol Plant Microbe Interact 13:637–648
Tilman D (1982) Resource competition and community structure. Princeton University Press, New Jersey, USA
Tilman D (1987) Secondary succession and the pattern of plant dominance along experimental nitrogen gradients. Ecol Monographs 57:189–214
Tsugita A, Inouye M, Terzaghi E, Streinger G (1968) Purification of bacteriophage T4 lysozyme. J Biol Chem 243:391–397

van Bruggen AHC, Semenov AM (2000) In search of biological indicators for soil health and disease suppression. Appl Soil Ecol 15:13–24

van Elsas JD, Garbeva P, Salles J (2002) Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soil-borne plant pathogens. Biodegradation 13:29–40

van Veen JA, Van Overbeck LS, van Elsas JD (1997) Fate and activity of microorganisms following release into soil. Microb Mol Biol Rev 61:121–135

Walker TS, Bais HP, Grotewold E, Vivanco JM (2003) Root exudation and rhizosphere biology. Plant Physiol 132:49–51

Wallace J (2001) Organic field crop handbook, 2nd edn. Canadian Organic Growers. Mothersill Printing, Canada

Wardle DA, Bonner KI, Barker GM, Yeates GW, Nicholson KS, Bardgett RD, Watson KN, Ghani A (1999) Plant removals in perennial grassland: vegetation dynamics, decomposers, soil biodiversity and ecosystem properties. Ecol Monographs 69:535–568

Warenborag FR, Estelrich HD (2000) Towards a better understanding of carbon flow in he rhizosphere: a time dependent approach using C^{14}. Biol Fert Soil 30:528–534

Yang CH, Crowley DE (2000) Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. Appl Environ Microbiol 66:345–351

Zak DR, Holmes WE, White DC, Peacock AD, Tilman D (2003) Plant diversity, soil microbial communities and ecosystem function; are there any links? Ecology 84:2042–2050

5 The Role of Rhizotrons and Minirhizotrons in Evaluating the Dynamics of Rhizoplane-Rhizosphere Microflora

Bobbie L. McMichael, John C. Zak

5.1 Introduction

Methods for observing the dynamic growth and decline of plant root systems and in-situ evaluation of associated saprophytic and symbiotic root fungi and microfauna have changed considerably over the last 20 years (Lussenhop et al. 1991). The efforts to provide for a direct and less intrusive evaluation of fine root turnover and rhizosphere/root region biotic interactions have come about in part in response to the realization that plant root systems regulate energy flow and biogeochemical cycles in all terrestrial ecosystems. Schimell and Bennett (2004) have recently proposed a new paradigm for interpretation of the abiotic and biotic components of nitrogen mineralization and cycling that more directly involves root region dynamics, microsite microbial processes, and plant uptake than has been previously postulated or realized. A critical evaluation of the new paradigm for the role of roots and soil microsites in nitrogen dynamics will demand a greater understanding of the abiotic and biotic factors that control root production, root turnover, and rhizoplane and rhizosphere interactions. Fahey et al. (1999) have argued that the use of standardized techniques to follow root dynamics among multiple landscapes and through time should provide insights into the subsequent mechanism that regulate the structure and function of terrestrial ecosystems.

The methodologies that have been employed to study root region dynamics have ranged from using trenching techniques where roots are excavated using heavy equipment, to extractions of soil cores where roots are washed from a specific soil volume and counted, to more sophisticated methods utilizing transparent wall techniques (slant tubes, root boxes, rhizotrons and minirhizotrons). Many of the early techniques for evaluation of static root parameters are described by Bohm (1979) along with some of the advantages or disadvantages of each method. A more recent discussion of techniques for measurement of static root parameters can be found in

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Bledsoe et al. (1999). The use of rhizotrons, root windows, root walls and the more versatile minirhizotron techniques have been utilized in many studies ranging from measuring root dynamics in forest ecosystems (e.g. Hendrick and Pregitzer 1992, 1996) to root observations in annual crop plants.

The minirhizotron method, as described in Taylor (1987), has gained wide acceptance as a useful tool in root research since the use of the method is not restricted to a given site and has certain advantages over the rhizotron approach. After a discussion of the use of both rhizotrons and minirhizotrons, the type of data that can be collected and advantages and disadvantages of both methods, this chapter will address the use of rhizotrons and minirhizotrons in rhizosphere research specifically focusing on the potential of the approaches to address key aspects of the dynamics of mycorrhizae, fungal root pathogen activity, and rhizosphere microflora and microfauna.

5.2 Use of Rhizotrons and Minirhizotrons in Root Research

Rhizotrons are some of the earliest non-destructive techniques for observing root growth. Early root "laboratories" or so-called "root cellars" were used in Germany in the early 1900s by Noll and Kromeer (Bohm 1979). Other rhizotrons were built in the USSR in 1915 (Kolesnikov 1971). In the 1960s rhizotrons were constructed in England with covered walkways and glass sides for viewing roots of plants growing in the excavated trenches (Rogers 1969). The facility first called a rhizotron was constructed by Lyford and Wilson (1966). Other facilities (e.g. University of Georgia, Auburn University) have been constructed which used the rhizotron in England as a prototype with some refinements to meet specific objectives at the new research sites. These refinements included individual observation chambers with improved viewing panels, some of which include a grid system imbedded in the panel to facilitate measurements of changes in root length within a given area of the chamber (Figs. 5.1 and 5.2). The above-ground area of the rhizotrons may have the roof close to the ground to prevent changes in the microclimate of the plants growing in the chambers. The roof of the rhizotron at Auburn University was also covered with soil (Taylor 1969). A long-term rhizotron was also constructed at the Michigan Biological Station, with smaller rhizotron trenches constructed at several National Sciences Foundation funded Long-Term Ecological Research sites (e.g. Hubbard Brook, and Konza) across the United States to examine site specific aspects of root-growth dynamics over the last several decades.

Some of the rhizotrons listed above include facilities for measuring water use by the plants growing in an individual chamber either by water balance methods or by actually weighing the soil volume in the chamber by using load cells and the appropriate electronic equipment. Several of the rhizotrons have been constructed to study nutrient uptake (Long and Huck 1980) while others were designed to study the effects of soil temperature on the dynamics of root development (Bhar et al. 1970). Van de Geijn et al. (1994) described a rhizotron installation that included a rain shelter that automatically closed at the onset of rain to prevent rain from entering the experimental area.

5.2.1 Minirhizotrons

The concept and design for the minirhizotron was first described by Bates (1937). In the experiments he conducted, he used sections of lamp glass that were inserted into the soil to observe the roots that intersected the glass. He accomplished this by inserting a long pole with a mirror attached to the pole to observe the roots. Waddington (1971) later refined the technique by using acrylic tubes and a fiber optic system. The term 'minirhizotron'

Fig. 5.1. Top view of a rhizotron field installation. Each *circular section* represents an individual compartment for observing root development in the underground portion of the installation

Fig. 5.2. View of rhizotron field installation showing observer measuring root development on the glass face of each plant growth compartment. The compartments are separated by a concrete wall

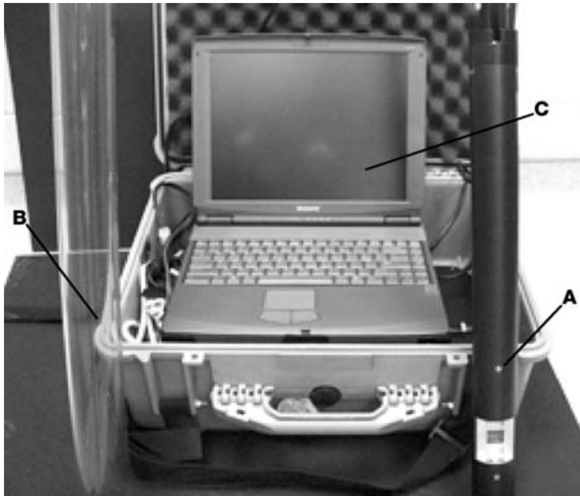

Fig. 5.3. View of minirhizotron system for imaging roots in a field. (**A**) Camera system; (**B**) Polycarbonate minirhizotron tube for insertion into soil; (**C**) Computer system for acquiring images (Bartz Technology Corp., Santa Barbara, CA)

was first used by Bohm (1974) when he used glass tubes inserted in the soil to observe root growth. Bohm (1974) further modified the mirror attached to a rod technique of Bates (1937) by adding a small battery operated light source to improve viewing the roots at lower depths (Fig. 5.3).

The basic overall concept of the minirhizotron has not changed over the years, but the equipment for viewing has evolved considerably. The mirror on a pole has been replaced with devices such as endoscopes (Vos and Groenwold 1983), duodenoscopes (Sanders and Brown 1978), and rigid borescopes with a television camera attached (Upchurch and Ritchie 1983), to specialized camera systems and accompanying computer and software (Fig. 5.3) for collecting analyzing information (Bartz Technology Corp., 116-A East Yanonali St, Santa Barbara, CA 93101, USA). The design of the observation tubes has changed likewise with most observation tubes now usually 50 mm in diameter and constructed of acrylic or glass with etchings on the tubes for depth marks and orientation for camera equipment. Initially, minirhizotron tubes were installed vertically in the field but in later studies the tubes were installed at some angle, usually 30–45 ° from the vertical to reduce the possibility of the roots following the soil tube interface once the roots intersect the tubes.

5.2.2 Data Collection from Rhizotrons and Minirhizotrons

The types of data that can be collected and analyzed are similar in many respects between studies with rhizotrons and minirhizotrons since both are essentially transparent wall techniques. To summarize briefly, both systems can be used to measure changes in root length and view fungal hyphae developing along the walls of the chamber by direct methods such as tracing the positions of roots and hyphae on the face of the glass and using methods developed by Newman (1966) to estimate root or hyphal lengths. Indirect methods can involve counting the intersections of roots on a grid system (Huck and Taylor 1982; Taylor et al. 1970) and using equations developed again by Newman (1966) to estimate changes in root length. In the case of minirhizotrons, the Newman (1966) grid system can also be applied to images derived from the camera observations and recorded on videotape. The field of view however is considerably less than what is observed using a rhizotron. Since root length can be estimated using both systems it is possible to estimate changes in rooting density or root length density (RLD) from either technique. These measurements can provide an assessment of cm root/cubic cm of soil volume. The soil volume is calculated as the surface area of the field of view of the minirhizotron times the assumed depth of observation, which is generally within 2 to 3 mm behind the wall (Sanders and Brown 1978; Taylor et al. 1970). This approach has been used by a number of researchers (Moore 1981; Taylor and Klepper 1973). Upchurch and Ritchie (1983) have used a somewhat different concept in estimating RLD with minirhizotrons. They made the assumption that the root growth angle and root morphology would essentially be the same if the minirhizotron was not present. Upchurch (1985) later validated this assumption to deal with potential biases caused by the presence of the minirhizotron tube.

Root dynamics, or rates of root growth or death can also be measured in a rhizotron or by using a series of minirhizotrons. Huck and Taylor (1982) described the physical approach to make these types of measurements while Head (1965) used a time-lapse photography approach to obtain similar data. Dyer and Brown (1980) used a fluorescence technique to determine changes in root age and root dieback in soybean roots observed with a minirhizotron system. Other dynamic functions, such as water and nutrient uptake have been measured in a rhizotron system (Taylor and Klepper 1973). Karnok and Kucharski (1982) measured above-ground photosynthesis and transpiration rates and related these physiological parameters to patterns of root growth using a rhizotron.

Other measurements such as the leaching of chemicals, changes in soil temperature, changes in soil oxygen, effects of liming, and high aluminum

soils have all been studied in relation to root growth and dynamics with rhizotron systems (Long and Huck 1980; Bhar et al. 1970; Pearson et al. 1973). A recent review of minirhizotron research by Johnson et al. (2001) discussed the advantages and limitation when using this method in field research to address questions of root growth and dynamics.

5.3 Advantages and Disadvantages of Rhizotrons and Minirhizotrons

There are several aspects of rhizotrons and minirhizotron systems that should be considered concerning their use in a particular research project to answer specific questions regarding rhizosphere and root region research. Foremost is that the incorporation of these techniques into a research program can constitute a major commitment of financial and time resources to accomplish the project objectives.

Rhizotrons are more suitable for long-term research due to the immobility of the facility once it is constructed and in place. They are particularly suitable for measuring root dynamics and root turnover (root dieback) since the same root system can be observed over the life cycle of the plants in question. Rhizotrons can also be equipped with more permanent measuring equipment, such as collection systems for measuring nutrient leaching and nutrient and water uptake dynamics. Moreover, since the rhizotron can consist of several different chambers or soil compartments, several different experiments can be run simultaneously within the same growing season.

Probably the main disadvantage of the using the rhizotron if one is not available within a reasonable distance is the cost of installing and maintaining a facility (Upchurch and Taylor 1990). The price for a state-of-the-art facility could easily be within the six to seven figure range depending on the size and sophistication of the facility. There are also problems with filling the compartments with soil for specific experiments. These problems include the formation of air gaps in the glass-soil interface, which could lead to unrealistic root distributions. Moreover, the aerial environment surrounding the rhizotron may be different from the environment adjacent to the facility which may lead to differences in the growth of the plants within the rhizotron (Huck and Taylor 1982). Problems due the lack of sufficient replication of experiments may also arise and mask the ability to determine differences in root growth in response to some treatment variable (Bohm 1979).

One of the main advantages of using minirhizotrons for rhizosphere and root region research is the portability of the system. Minirhizotron

tubes can be installed in many locations throughout a field site and in various configurations depending on the experiments to be conducted and the data to be collected. The cost of the tubes is relatively inexpensive and since they are a small (50 mm) diameter they are less disruptive to root systems in field plots. The primary cost of a minirhizotron system is the camera or viewing and recording equipment which can be in the neighborhood of $20,000–$25,000 depending on the recording devices. Importantly, the minirhizotron system provides for repeated measurements in the same location and since the number of observation tubes that can be established is dependent upon the questions asked and inherent variability at the field location, sufficient replications can be established that allows for statistical rigor (Johnson et al. 2001). In addition to quantifying root growth, depending upon the depth of the access tube, changes in rooting depth can be readily observed using the minirhizotron system.

Some of the disadvantages that have been mentioned for the rhizotron system also plague the minirhizotron system as well. One of the major problems concerns the formation of air gaps at the soil-tube interface. Compaction of the soil immediately adjacent to the tube can also occur leading to lower root densities near the tube interface (Kosola 1999; McMichael personal observation). Also, root densities may also be lower in the near surface presumably due to light effects (Levan et al. 1987). Some statistical problems may also arise with the use of minirhizotrons since a large number of tubes may be required to successfully characterize differences in root dynamics between treatments. These issues have been addressed by Upchurch (1985) and more recently by Johnson et al. (2001). Additional issues that are not well evaluated concern the impact of access tube composition (i.e. glass, acrylic and butyrate) on root growth and development. In a recent investigation Withington et al. (2003) determined that while the tube material had little impact on root production of apple and six forest tree species (*Acer pseudoplantanus*, *Fagus sylvatica*, *Quercus robur*, *Picea abies*, *Pinus nigra*, and *Pinus sylvestris*), the composition of the tube did impact root survivorship. Withington et al. (2003) found that cumulative apple root production was greater adjacent to glass and least for butyrate. For the forest tree species, differences in root production between acrylic and butyrate were not significant. The composition of the access tubes did influence the rate of root pigmentation and survivorship. Roots adjacent to butyrate access tubes had a greater risk of becoming pigmented. In addition, root life span was usually greater by the acrylic tubes than for butyrate. There were species specific differences in root life span in response to access tube composition (Withington et al. 2003).

5.4 Use of Rhizotrons and Minirhizotrons in Rhizoplane and Rhizosphere Research

In this chapter we will examine and briefly discuss the use of rhizotrons and minirhizotrons in three main research areas that are focused towards understanding the dynamics of rhizoplane (root surface) and rhizosphere microbes. These root observation systems have their greatest benefit in allowing a direct examination of: i) the production, longevity and survivorship of mycorrhizal root tips and associated fungal structures, ii) the interactions of root growth dynamics with root pathogen activity and iii) changes in root surface phenomena (suberization, etc.) as root systems develop. Each of these research venues will be discussed with the acknowledgement that some portion of the results being discussed were obtained using either rhizotrons or minirhizotrons to show the ability or inability of these systems to collect specific types of data.

5.5 Mycorrrhizae

The potential for using rhizotrons and minirhizotrons to assess patterns of mycorrhizal colonization of grasses, shrubs or tree roots lies with the ability of these techniques to capture the dynamic nature of these fungal-plant interactions. Of the two approaches, the minirhizotron system is the most versatile for field observations and manipulation studies. Unfortunately most of the efforts expended to understand the dynamics of arbuscular and ecto-mycorrhizal colonization have been obtained from the collection of roots from static soil cores. The standard techniques are designed to evaluate the percentage of the root length colonized by arbuscular mycorrhizal fungi or the numbers of root tips that are mycorrhizal per soil volume (Sollins et al. 1999 for standard methods for assessment of arbuscular and ecto-mycorrhizae).

One of the best designed studies to follow the dynamic nature of arbuscular mycorrhizal fungal colonization that employed a modified minirhizotron was presented by Friese and Allen (1991) using thin sections of soil sandwiched between two glass plates. Though the design was not a minirhizotron in the strictest sense the study employed the concept of continuous observation of root growth along a glass surface and a dissecting microscope instead of a video camera to record observations. The technique employed by Friese and Allen (1991) was similar to one used by Finlay and Read (1986) to evaluate the translocation of ^{14}C labeled carbon between tree seedlings interconnected by a common ectomycorrhizal

fungal mycelium. The glass-plate system allowed Friese and Allen (1991) to describe in detail the architectural structure of arbuscular mycorrhizal mycelium in a heterogeneous soil environment. The glass-plate/soil system (two 12 × 30 cm plates) were attached to one another by a layer of silicon adhesive on three sides. The silicon was beaded such that there was a 5-mm gap between the plates. Each plate system was planted with a shrub seedlings (*Artemisia tridentata*) and *Oryzopsis hymenoides*, a grass. The glass plates were examined daily under a compound microscope and the morphology of the AM fungi that developed described for 25 days. Arbuscular mycorrhizal activity inside of the roots was evaluated using autofluorescence (Ames et al. 1982; Allen et al. 1989). By employing this approach, Friese and Allen (1991) were able to describe how AM mycelium changes from exploratory to absorptive mycelium as the fungus encountered various substrates.

One of the technical challenges with using minirhizotron systems in the field to evaluate arbuscular mycorrhizal dynamics is in determining if hyphae that is associated with the roots of grasses or other herbaceous plants near the access tube is indeed that from an arbuscular mycorrhizal fungus. Certain morphological features of AM fungal hyphae are known (Friese and Allen 1991) and with practice, an investigator could ascertain if the hyphae is from an AM fungus or not. However, careful training and verification will be necessary to determine if the accuracy of the observations and assignment of the hyphae as arbuscular mycorrhizal mycelium is correct. The production of chlamydospores along the mycelium by some AM fungi can be used to verify the accuracy of the observation. To be sure, minirhizotron systems can be employed in greenhouse or growth chamber studies, with greater accuracy to examine growth rates, or turnover rates of AM fungal mycelium in response to changes in soil-root conditions and plant response to increases in CO_2 levels if the incidence of saprophytic fungi are minimized.

Rillig and Allen (1999) and Rillig et al. (1999) previously established the direct impact of arbuscular mycorrhizal fungi on soils in response to rising levels of atmospheric CO_2. Minirhizotrons and minirhizotron like approaches could be used to evaluate further the interactions between atmospheric levels of CO_2 and arbuscular mycorrhizal dynamics in the detail necessary for these data and the consequences of their interactions to be used in global carbon models. For example, Fitter et al. (1997) observed an increase in root turnover in a grassland ecosystem in response to elevated CO_2 and used a minirhizotron system to estimate rates of root production and mortality from captured images to determine root turnover estimates. Changes in root longevity, as measured using minirhizotrons could have a direct impact on AM inoculum levels and spore production. The minirhizotron system, under controlled conditions and possibly in the

field, has the potential to examine the impacts of accelerated root turnover on AM colonization dynamics and inoculum production and the contribution of this microbial biomass to soil nutrient pools and global carbon cycles.

The application of minirhizotrons to examine mycorrhizal dynamics in response to changes in soil conditions, climate change, or atmospheric deposition has focused primarily on the impacts of these parameters on ectomycorrhizae in forest systems. This bias is due in part to our ability to easily evaluate with either rhizotrons or minirhizotrons the occurrence and dynamics of ectomycorrhizal root tips in response to some abiotic or biotic manipulation (Lussenhop and Fogel 1999; Curtis et al. 2000). Furthermore, a great deal of attention has recently been given towards examining ecotomycorrhizal associations in temperate forests in response to their ability to contribute large amounts of carbon to the soil system.

Changes in root dynamics in response to global climate change, atmospheric levels of CO_2, and increases in anthropogenic nitrogen deposition will undoubtedly impact the number, composition and lifespan of mycorrhizal root tips. Although ectomycorrhizae and their associated mycelial network and morphological structures can account for up to 75% of the photosynthetic production of a tree (Vogt et al. 1980; Fogel and Hunt 1983) there is only minimal understanding of how ectomycorrhizae respond to changes in plant carbon dynamics as a consequence of increasing atmospheric CO_2 levels. Hungate et al. (1997) and Langley et al. (2003) have stated, that depending upon the longevity and decomposability of ecotomycorrhizal root tips, ecotomycorrhizal biomass could represent a mechanism for net carbon storage or a pathway by which carbon is rapidly returned to the atmosphere. Treseder and Allen (2000) have stated that shifts in carbon allocation to ecotomycorrhizal root tips vs non-mycorrhizal fine roots could drastically alter the quality of the organic matter entering the soil carbon pools. However, using minirhizotron imaging, Dilustro et al. (2001) reported no change in root longevity with increasing levels of atmospheric CO_2 at a scrub-oak ecosystem in Florida. Langley et al. (2003) reported that while elevated CO_2 levels did increase the biomass of ectomycorrhizal root tips, non-mycorrhizal fine roots also increased thereby resulting in an unaltered ratio between the two types of roots under ambient and elevated CO_2. Given the variability in responses to CO_2 by ectomycorrhizal tree species, the development of a multisite comparison of CO_2 impacts on ectomycorrhizae employing a minirhizotron system could make a significant contribution toward understanding the impacts of CO_2 on ectomycorrhizal root tip dynamics.

Several studies have used minirhizotrons to examine the impacts of atmospheric nitrogen deposition on ectomycorrhizae. Using a growth

chamber design, Rygiewicz et al. (1997) reported that ectomycorrhizal root tips of *Pinus ponderosa* had a medium turnover time (lifespan + decomposition) of 139 days with no impact of additional nitrogen on turnover. The opposite was observed by Majdi and Nylund (1996), who reported a reduction in the lifespan of ecotomycorrhizal root tips following nitrogen addition to a Norway spruce stand in Sweden. The ability to address fundamental questions of ecotomycorrhizal turnover and longevity in response to climate change or nitrogen deposition rates is directly linked to the ability of the minirhizotron system to provide real time data on root growth and lifespan.

Extramatrical mycelium and rhizomorph production by ecotomycorrhizal fungi can represent an important component of the carbon and nutrient demand of the fungus and is usually overlooked or underappreciated in the examination of global climate change or anthropogenic impacts on ecotomycorrhizal dynamics. Carbon, nitrogen and phosphorus pools in fungal tissue can make significant contributions to overall nutrient budgets of forested ecosystems (Boddy and Watkinson 1995; Schmidt et al. 1999; and Zogg et al. 2000). For ectomycorrhizal fungi and other basidiomycetes, rhizomorphs are large, differentiated bundles of fungal hyphae that translocate nutrients over large distances and which can also be employed to establish new colonization sites of neighboring plants (Cairney 1991, 1992). In a recent investigation of the impacts of nitrogen addition on the life span of ectomycorrhizal rhizomorphs in three North American coniferous forests, Treseder et al. (2004) employed minirhizotrons to follow the impacts of nitrogen addition on abundance, lifespan, and decomposition rates on ectomycorrhizal rhizomorphs in a pinyon-juniper (*Pinus edulis* and *Juniperous monosperma*) woodland at the Sevilleta LTER site in New Mexico, a white spruce (*Picea glauca*) forest at the Bonanza Creek LTER in Alaska, and in a red pine (*Pinus resinosa*) forest in Michigan. In this study minirhizotron tube angles varied among sites due to soil conditions and were implanted at a shallow angle (20°) at the Sevilleta, and at a steeper angle (45°) at Bonanza Creek and in Michigan. After almost one year following the initial application of nitrogen at the rate of 10 gN/m^2/year, the additional nitrogen did not change the number of rhizomorphs observed at any site, though the minirhizotron data indicated a seasonal shift in rhizomorphs abundance at each forest location. Treseder et al. (2004) also reported a decrease in rhizomorphs lifespan with nitrogen fertilization in the white spruce site and under juniper, but not under pinyon pine. The study just described by Treseder et al. (2004) underscores the versatility of minirhizotron systems for addressing questions related to the production and lifespan of ectomycorrhizal structures.

5.6 Soil Borne Pathogens

Pathogen activity has traditionally been documented by destructive sampling of the plant roots for the particular pathogen along with observing the above ground symptoms, such as leaf chlorosis. With the development of the glass wall techniques for observing plant root development, it then became possible to obtain at least qualitative data of the occurrence of soil borne pathogens and relate the observations with above ground symptoms. One of the first studies using this approach was conducted by Rush et al. (1984) using the minirhizotron techniques with a borescope observation system equipped with a 35-mm camera to determine the occurrence of *Phymatotrichum omnivorum* on cotton root systems in the field and sorghum root systems under greenhouse conditions. Rush et al. (1984) were able to observe mycelial strands of *P. omnivorum* on the cotton roots in the field and the germination of sclerotia on sorghum roots growing in pots in the greenhouse. They also alluded to the fact that pathogens that produce necrotic lesions should be readily observable using the minirhizotron system. In later studies, Waipara et al. (1997) used a similar minirhizotron-borescope system to measure the effects of two fungal pathogens, *Cylindrocladium scoparium* and *Fusarium crookwellense*, on root growth of white clover and perennial ryegrass. They were able to measure differences in root length in both species as a function of the pathogen activity. As with mycorrhizal activity and dynamics, the minirhizotron system allows one the opportunity to evaluate directly abiotic or biotic factors that influence the spread and establishment of root pathogens without having to destructively sample the root systems.

5.7 Root Surface or Rhizoplane Phenomena

The use of both rhizotrons and minirhizotrons for observing and quantifying root growth parameters such as changes in root length, root turnover, and root diameters is, as mentioned earlier, well documented (Dyer and Brown 1980; Sanders and Brown 1978; Upchurch and Ritchie 1983). Changes in root surface characteristics such as surface area, suberization, etc. have been studied but to a lesser degree. However, these methods do provide the means for evaluating these phenomena that destructive techniques do not afford.

Ludovici and Morris (1995) studied the changes in root surface area in loblolly pine, sweetgum and crab grass roots using rhizotron cells. They observed a reduction in root surface area when loblolly pine seedlings were

grown with crab grass and also when the pine seedlings were grown along with sweetgum. They were also able to observe changes in root surface areas due to changes in water availability.

In many instances changes in root surface area are calculated from measurements of root length and root diameter in both rhizotron and minirhizotron systems (Huck and Taylor 1982). They also point out that in some cases this calculation may be underestimated since root hairs are not included in the measurements. Johnson et al. (2001) explained that not only may root areas be determined using minirhizotrons, but that areas of fine roots per area of minirhizotron image may also be determined. Box (1996) also indicates that both rhizotrons and minirhizotrons can be utilized for root surface determinations. Majdi (1996) in his discussion of the applications and limitations of minirhizotrons indicates that this system can be used to determine root diameters, which if the root length is known, root surface areas can be calculated.

Majdi (1996) also mentions that minirhizotrons can also be used to determine changes in root color as manifested on the root surface. These changes in root color may be due to changes in degree of suberization of the roots or due to root pathogen infection. McMichael and Taylor (1987) also mention that the degree of suberization may be possible to document particularly with color camera equipment. Dyer and Brown (1980) use fluorescence techniques to determine changes in root age in soybeans. Smit and Zuin (1996) determined that over time the UV fluorescence of Brussels sprouts roots decreased but increased in leek roots using minirhizotrons. They concluded that UV fluorescence could not be used as a universal indicator of root age or activity but might be used in some species to separate transparent roots from the background using image analysis techniques. Comas et al. (2000) used specially constructed root boxes with acetate windows to monitor changes in root age and root metabolic activity using tetrazolium chloride in root systems of grape plants. They concluded from their findings that even though some cells of brown colored roots were still alive, they were no longer functional. They also used a minirhizotron system to observe differences in root appearance (color changes) with age. Lopez et al. (2001) used minirhizotrons to determine the longevity of holm oak roots and to also determine when white roots became brown as a result of ageing. Wells and Eissenstat (2001) used minirhizotrons to determine the longevity of apple roots. They also used the tetrazolium assays to determine the relationship between changes in root color as shown in the minirhizotron images and root activity during the year. They observed that the majority of the roots that survived from October until May of each year were brown but produced new white roots during the spring.

5.8 Conclusions

A key benefit from an investment in a minirhizotron system is that the approach allows for the continuous evaluation of root dynamics and associated microbial growth and development in time and space. The acquisition of continuous data provides the opportunity to determine with greater accuracy and precision the impacts of such factors as soil moisture and nutrients, above-ground growth and microbial response to precipitation patterns or elevated CO_2 on root dynamics, rather than through inferential statistical evaluation of growth patterns obtained from static point measurements. For example, root turnover values are a crucial component of local and global carbon balance models. The precision of these data is increased through the use of minirhizotron systems. Likewise, questions regarding impacts of climate change or anthropogenic nitrogen deposition on mycelial growth of mycorrhizal and saprophytic fungi along root regions in natural ecosystems, as their growth and turnover impacts nutrient dynamics, can only be determined using the minirhizotron system under field conditions. The added advantage of using a minirhizotron in the field is that one can examine root region dynamics of various aged trees growing in a heterogeneous environment, rather than using only seedling or juvenile sized plants as what occurs in most greenhouse studies.

References

Allen MF, Allen EB, Friese CF (1989) Responses of the non-mycotrophic plant *Salsola kali* to invasion by vesicular-arbuscular mycorrhizal fungi. New Phytol 111:45–49

Ames RN, Ingham ER, Reid CPP (1982) Ultraviolet-induced autofluorescence of arbuscular mycorrhizal root infections: an alternative to clearing and staining methods for assessing infection. Can J Microbiol 28:351–355

Bates GH (1937) A device for the observation of root growth in the soil. Nature 139:966–967

Bhar DS, Mason GF, Hilton RJ (1970) In situ observations on plum root growth. J Am Soc Hortic Sci 95:237–239

Bledsoe CS, Fahey TJ, Day FP, Ruess RW (1999) Measurements of static root parameters: Biomass, length and distribution in the soil profile. In: Robertson GP, Coleman DC, Bledsoe CS, Sollins P (eds) Standard methods for long-term ecological research. Long-Term Ecological Research Network Series 2, Oxford University Press, Oxford, pp 413–436

Boddy L, Watkinson SC (1995) Wood decomposition, higher fungi, and their role in nutrient redistribution. Can J Bot 73:S1377–S1383

Bohm W (1974) Mini-rhizotrons for root observations under field conditions. Z Acker Pflanzenbau 140:282–287

Bohm W (1979) Methods of studying root systems. Springer, Berlin Heidelberg New York

Box JE Jr (1996) Modern methods for roit Investigations. In: Waisel Y, Eshel A, Kafkafi U (eds) Plant roots: the hidden half. Marcel Dekker, New York, NY, pp 193–237

Cairney JWG (1991) Structural and ontogenic study of ectomycorrhizal rhizomorphs. Methods Micro 23:331–340

Cairney JWG (1992) Translocation of solutes in ectomycorrhizal and saprotrophic rhizomorphs. Mycol Res 96:135–141

Comas LH, Eissenstat DM, Lakso AN (2000) Assessing root death and root system dynamics in a study of grape canopy pruning. New Phytol 147:171–178

Curtis PS, Vogel CS, Wang X, Pregitzer KS, Zak DR, Lussenhop J, Kubiske M, Terri JA (2000) Gas exchange, leaf nitrogen, and growth efficiency of *Populas tremuloides* in a CO_2-enriched atmosphere. Ecol Appl 10:3–17

Dilustro JJ, Day FP, Drake BG (2001) Effects of elevated atmospheric CO2 on root decomposition in s scrub-oak ecosystem. Global Change Biol 7:581–590

Dyer D, Brown DA (1980) In situ root observation using fiber optic video and fluorescence. Agron Abstr American Society of Agronomy. Madison, WI, p 80

Fahey TJ, Bledsoe CS, Ruess RW, Smucker AJM (1999) Fine root production and demography. In: Robertson GP, Coleman DC, Bledsoe CS, Sollins P (eds) Standard methods for long-term ecological research. Long-Term Ecological Research Network Series 2, Oxford University Press, Oxford, pp 437–455

Finlay RD, Read DJ (1986) The structure and function of the vegetative mycelium of ectomycorrhizal plants. I. Translocation of 14C-labelled carbon between plants interconnected by a common mycelium. New Phytol 103:143–156

Fitter AH, Graves JD, Wolfenden J, Self GK, Brown TK, Bogie D, Mansfield TA (1997) Root production and turnover and carbon budgets of two contrasting grasslands under ambient and elevated atmospheric carbon dioxide concentrations. New Phytol 137: 247–255

Fogel R, Hunt G (1983) Contribution of mycorrhizae and soil fungi to nutrient cycling in a Douglas Fir ecosystem. Can J Forest Res 13:219–232

Friese CF, Allen MF (1991) The spread of VA mycorrhizal fungal hyphae in the soil: Inoculum types and external hyphal architecture. Mycologia 83:409–418

Head GC (1965) Studies of diurnal changes in cherry root growth and nutritional movements of apple root tips by time-lapse cinematography. Ann Bot NS 29:219–224

Hendrick RI, Pregitzer KS (1992) The demography of fine roots in a northern hardwood forest. Ecology 73:1094–1104

Hendrick RI, Pregitzer KS (1996) Temporal and depth-related patterns of fine root dynamics in northern hardwood forests. J Ecol 84:167–176

Huck MG, Taylor HM (1982) The rhizotron as a tool for root research. Agronomy 35:1–35

Hungate BA, Holland EA, Jackson RB, Chapin FSI, Mooney HA, Field CB (1997) The fate of carbon in grasslands under carbon dioxide enrichment. Nature 388:576–579

Johnson MG, Tingey DT, Phillips DL, Storm MJ (2001) Advancing fine root research with minirhizotrons. Environ Exp Bot 45:263–289

Karnok KJ, Kucharski RT (1982) Design and construction of a rhizotron-lysimeter facility at the Ohio State University. Agron J 74:152–156

Kolesnikov VA (1971) The root system of fruit plants. Mir Publications, Moscow, USSR

Kosola KR (1999) Laparascopic sampling of roots of known age from an expandable-wall minirhizotron system. Agron J 91:876–879

Langley JA, Dijkstra P, Drake BG, Hungate BA (2003) Ectomycorrhizal colonization, biomass, and production in a regenerating scrub oak forest in response to elevated CO_2. Ecosystems 6:424–430

Levan MA, Ycas JW, Hummel JW (1987) Light leak effects on near surface soybean rooting observed in minirhizotrons. In: Taylor HM (ed) Minirhizotron observation tubes: methods and applications for measuring rhizosphere dynamics. Spec Publ 50, American Society of Agronomy, Madison, WI, pp 89–98

Long FL, Huck MG (1980) Nitrate movement under corn and fallow conditions. Soil Sci Soc Am Proc 44:787–792

Lopez B, Sabate S, Gracia CA (2001) Fine-root longevity of *Quercus ilex*. New Phytol 151: 437–441
Ludovici KH, Morris LA (1995) Responses of loblolly pine, sweetgum and crab grass roots to localized increases in two watering regimes. Tree Physiol 16:933–939
Lussenhop J, Fogel R (1999) Seasonal changes in phosphorous content of *Pinus strobes – Cenococcum geophilium* ectomycorrhizae. Mycologia 91:742–746
Lussenhop J, Fogel R, Pregitzer K (1991) A new dawn for soil biology: video analysis of root-soil-microbial-faunal interactions. In: Crossley DA Jr, Coleman DC, Hendrix PF, Cheng W, Wright DH, Beare MH, Edwards CA (eds) Modern techniques in soil. Elsevier, Amsterdam, pp 235–250
Lyford W, Wilson BF (1966) Controlled growth of forest tree roots: technique and application. Harvard For Pap no 16. Harvard University. Petersham, MA
Majdi H (1996) Root sampling methods – applications and limitations of the minirhizotron technique. Plant Soil 185:255–258
Majdi H, Nylund JE (1996) Does liquid fertilization affect fine root dynamics and lifespan of mycorrhizal short roots? Plant Soil 185:305–309
McMichael BL, Taylor HM (1987) Applications and limitations of rhizotrons and minirhizotrons. In: Minirhizotron observation tubes: methods and applications for measuring rhizosphere dynamics. American Society of Agronomy, Madison, WI, pp 1–13
Moore TJ (1981) Rooting behavior and soil water extraction of several grain sorghum genotypes. MS thesis. Texas Tech Univ, Plant and Soil Science Department, Lubbock, Texas
Newman EI (1966) A method of estimating the total length of root in a sample. J Appl Ecol 3:139–145
Pearson RW, Childs J, Lund ZF (1973) Uniformity of limestone mixing in acid subsoil as a factor in cotton root penetration. Proc Soil Sci Soc Am 37:727–732
Rillig MC, Allen MF (1999) What is the role of arbuscular mycorrhizal fungi in plant-to-ecosystem responses in elevated atmospheric CO_2? Mycorrhiza 9:1–8
Rillig MC, Wright SF, Allen MF, Field CF (1999) Rise in carbon dioxide changes soil structure. Nature 400:628
Rogers WS (1969) The East Malling root-observation laboratories. In: Whittington WJ (ed) Root growth. Butterworth, London, pp 361–376
Rush CM, Upchurch DR, Gerik TJ (1984) In situ observations of Phymatotrichum omnivorum with a borescope mini-rhizotron system. Phytopathology 74:104–105
Rygiewicz PT, Johnson MG, Ganio LM, Tingey DT, Storm MJ (1997) Lifetime and temporal occurrence of ectomycorrhizae on Ponderosa pine (Pinus–Ponderosa Laws) seedlings grown under varied atmospheric CO2 and nitrogen levels. Plant Soil 189:275–287
Sanders JL, Brown DA (1978) A new fiber optic technique for measuring root growth of soybeans under field conditions. Agron J 70:1073–1077
Schimell JP, Bennett J (2004) Nitrogen mineralization: challenges of a changing paradigm. Ecology 3:591–602
Schmidt IK, Jonasson S, Michelsen A (1999) Mineralization and microbial immobilization of N and P in Arctic soils in relation to season, temperature, and nutrient amendment. Appl Soil Ecol 11:147–160
Smit AL, Zuin A (1996) Root growth dynamics of Brussels sprouts (*Brassica olearacea* var. *gemmifera*) and leeks (*Allium porrum* L.) as reflected by root length, root colour and UV fluorescence. Plant Soil 185:271–280
Sollins P, Glassman C, Paul E, Lajtha K, Heil J, Elliot ET (1999) Soil carbon and nitrogen: pools and fractions. In: Robertson GP, Bledsoe CS, Coleman DC, Sollins C (eds) Standard soil methods for long term ecological research. Oxford University Press, New York, pp 89–105

Taylor HM (1969) The rhizotron at Auburn, Alabama – a root observation laboratory. Auburn Univ Exp Stn Circ no 171
Taylor HM (1987) Minirhizotron observation tubes: methods and applications for measuring rhizosphere dynamics. American Society of Agronomy, Madison, WI
Taylor HM, Huck MG, Klepper B, Lund ZF (1970) Measurement of soil-grown roots in a rhizotron. Agron J 62:807–809
Taylor HM, Klepper B (1973) Rooting density and water extraction patterns for corn (Zea mays L). Agron J 65:965–968
Treseder KK, Allen MF (2000) Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO2 and nitrogen deposition. New Phytol 174:189–201
Treseder KK, Allen MF, Ruess R, Pregitzer K, Hendricks R (2004) Turnover of mycorrhizal rhizomorphs under N fertilization in three diverse ecosystems. Plant Soil 270:249–255
Upchurch DR (1985) Relationship between observations in mini-rhizotrons and true root length density. PhD dissertation. Texas Tech University, Lubbock, Texas
Upchurch DR, Ritchie JT (1983) Root observations using a video recording system in mini-rhizotrons. Agron J 75:1009–1015
Upchurch DR, Taylor HM (1990) Tools for studying rhizosphere dynamics. In: Box JE Jr, Hammond LH (eds) Rhizosphere dynamics. Westview Press, Boulder, CO, pp 83–115
van de Geijn SC, Vos J, Groenwold J, Goudrian J, Leffelaar PA (1994) The Wageningen Rhizolab – a facility to study soil-root-shoot-atmosphere interactions in crops. Plant Soil 161:275–287
Vogt KA, Edmonds RL, Grier CC, Piper SR (1980) Seasonal changes in mycorrhizal and fibrous-textured root biomass in 23-and 180-year-old Pacific silver fir stands in western Washington. Can J Forest Res 10:523–529
Vos J, Groenwold J (1983) Estimation of root densities by observation tubes and endoscope. Plant Soil 74:295–300
Waddington J (1971) Observation of plant roots *in situ*. Can J Bot 49:1850–1852
Waipara NW, Di Menna ME, Skipp RA, Cole ALJ (1997) In situ examination of white clover and perennial ryegrass roots inoculated with fungal pathogens. Proc 50th NZ Plant Protection Conf., New Zealand, pp 78–83
Wells CE, Eissenstat DM (2001) Marked differences in survivorship among apple roots of different diameters. Ecology 82(3):882–892
Withington JM, Elkin AD, Bulaj B, Olesinski J, Tracy KN, Bouma TJ, Oleksyn J, Anderson LJ, Modrzynski J, Reich PB, Eissenstat DM (2003) The impact of material used for minirhizotron tubes for root research. New Phytol 10:1–12
Zogg GP, Zak DR, Pregitzer KS, Burton AJ (2000) Microbial immobilization and the retention of anthropogenic nitrate in a northern hardwood forest. Ecology 81:1858–1866

6 Significance of Bacteria in the Rhizosphere

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6.1 Introduction

Bacteria are organisms of paradox. They are the simplest organisms, yet the most diverse. They are single-celled, yet during their 3.5 billion years of existence they have developed the functional capacity to permeate and exploit every conceivable location on the planet. They are 'unseen', yet they are integral drivers of biogeochemical pathways in every ecosystem. Bacteria play key roles in cycling the organic and inorganic compounds that sustain life and the physical environment, capturing and releasing molecules from air, water, soil and other organisms. They are the smallest organisms, yet they are 'giants' in the global ecosystem.

Water constitutes roughly two-thirds of the planet's surface but, despite the ubiquity of bacteria, their diversity in water is relatively low and is dominated by a few groups. Curtis et al. (2002) estimated that 1 ml of seawater may contain around 160 bacterial taxa. In contrast, the diversity of bacteria in soil was estimated to range between 6000 and 38,000 taxa in a single gram, with possibly as many as 4×10^6 taxa in a tonne of soil (Torsvik et al. 1996; Curtis et al. 2002). Furthermore, the abundance of culturable cells in a gram of soil may be $> 10^9$, which probably only represents 1–10% of the actual total (Alexander 1987). Soil is clearly the major habitat for bacteria.

Soil is also the substrate that provides physical support and mineral elements to plants and, therefore, is a fundamental component of terrestrial ecosystems. The rhizosphere is the zone of soil adjacent to, and influenced by, roots. The surface of the root is known as the rhizoplane and the endorhizosphere refers to the root cortex, in the context of its colonisation by bacteria (Bolton et al. 1993). The rhizosphere is colonised by bacteria (hereafter *rhizobacteria*) and other microorganisms and thus constitutes

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a living interface between roots and the soil. The rhizosphere, rhizoplane and endorhizosphere form a continuum that is difficult to distinguish in terms of microbial ecology (Bolton et al. 1993). The functional and spatial complexity of the rhizosphere is heightened by mycorrhizal fungi which, in turn, support a mycorrhizosphere that is also colonised by bacteria (Linderman 1988).

The importance of specific functions of rhizobacteria, such as nitrogen fixation, has been recognised for many decades. However, a broader understanding and appreciation of their significance in soil ecosystems has increased rapidly in recent years. The objective of this review is to evaluate the significance of bacteria in the rhizosphere, from both a functional and evolutionary perspective. The potential for specific functions of rhizobacteria to impact upon the soil, plants and other organisms is outlined and the implications for ecosystem function are discussed. Evidence for co-evolution of plants and rhizobacteria is appraised, including speculation that rhizobacteria may have been an enabling force in the adaptation of plants to the terrestrial environment.

6.2 Functional Diversity of Rhizobacteria

Although the 'smallness' of bacteria renders them vulnerable to desiccation and predation by higher organisms, smallness has other advantages. Being single-celled, bacteria are affected by, and respond to, changes in their environment more rapidly than do multicellular organisms. Bacterial ribosomes attach themselves to mRNA before transcription from DNA is completed (Lewin 1990), enabling rapid metabolic responses to their environment. The diversity of metabolic capacity of rhizobacteria is vast. The functions that have been studied the most are those that impact upon plants and soil properties, especially functions associated with nutrient cycling, symbioses, pathogens and plant growth promotion.

Bacteria are much more abundant and metabolically active in the rhizosphere than in non-rhizosphere soil. Plant roots are effectively a conduit for photosynthate, from the leaves to the soil. Root exudates and the contents of sloughed cortical cells are labile and readily metabolised by bacteria (Bolton et al. 1993). In contrast, the organic matter in non-rhizosphere soil is metabolised slowly by microbes and, without external organic inputs, will not maintain constant microbial biomass (De Nobili et al. 2001). The abundance of bacteria and their associated metabolic activity are key differentials between the rhizosphere and bulk soil.

The earliest examination of specific functions of bacteria in the rhizosphere began more than a century ago with the discovery that legumes were

able to derive their entire nitrogen requirements through symbiosis with the rhizobial bacteria *Bradyrhizobium, Mesorhizobium*, *Sinorhizobium* and the recently-described *Methylobacterium nodulans* (Sy et al. 2001). Since then, research and discovery has concentrated on rhizosphere processes with relevance to human activity, particularly agricultural productivity. However, the functional processes mediated by rhizobacteria are not restricted to agricultural ecosystems alone. To highlight the diversity and importance of microbial functions affecting plant-soil interactions, a few of the more critical functions of rhizobacteria are now discussed.

6.2.1 Soil Structure

Soil structure and stability depends upon the formation of aggregates of sand, silt and clay. Aggregates are held together by organic matter and soil organisms. Aggregation affects several properties of soil that are critical for plant and microbial growth, including porosity, aeration and water infiltration. Microaggregates (2–250 μm in diameter) are stabilised by humic substances, polysaccharides, fungal hyphae and bacteria. Macroaggregate formation is more complex with polysaccharide, plant root and the soil flora and fauna involved in their stabilisation (Carter 2004). Plant roots physically bind and stabilise soil. While the physical contribution of roots to aggregate stability is not well understood (Degens 1997), root exudates and their breakdown products contribute to the bonding of microaggregates. In addition to stimulating microbial growth, root exudates establish contact between the root and the soil, providing lubrication and protection from desiccation (Hawes et al. 2000).

Microorganisms in the rhizosphere contribute to aggregate stability through the synthesis of new compounds from root exudates and decomposition of soil organic matter (Lynch 1995; Degens 1997). Furthermore, fungal hyphae, particularly those of arbuscular mycorrhizal fungi, contribute to soil stability by enmeshment of aggregates or cross-linking between them (Tisdall 1991; Degens 1997). Arbusuclar mycorrhizal fungi also secrete large quantities of a glycoprotein known as glomalin (Rillig and Steinberg 2002). Glomalin potentially plays a very important role in aggregate stability (Wright and Upadhyaya 1998). Changes in protein production levels in response to different environmental stimuli suggest that aggregate formation is an active and regulated process of the soil microbiota rather than a by-product of their activity (Rillig and Steinberg 2002). The relative importance of organic compounds derived from bacteria and fungi to aggregate stability under field conditions is presently not well understood (Degens 1997). However, rhizobacteria are key players in soil stability and

are intimately linked to interactions between roots and mycorrhizal fungi (as discussed below).

6.2.2 Phosphate Solubilisation

Phosphate solubilising bacteria produce organic acids that solubilise mineral phosphates, including calcium phosphates in high-pH soils and rock phosphate fertilisers. These bacteria occur in most soils and potentially represent 40% of the culturable population (Richardson 2001). The extent to which P-solubilising rhizobacteria contribute to plant P-uptake in natural systems has not been clearly elucidated (Goldstein 1995; Richardson 2001). The organic acids released by plant roots probably have a greater impact on the solubilisation of P than do the acids produced by rhizobacteria (Jones 1998). However, rhizobacteria consume the organic acids in root exudates and, therefore, indirectly may moderate solubilisation of P and other immobile elements, such as Fe and Mn. Organic acids are metabolised two to three times faster in the rhizosphere than in bulk soil, typically with 60% being mineralised and the remainder incorporated into microbial biomass (Jones 1998).

A range of organic acids with P-solubilising activity are produced by rhizobacteria but gluconic acid and 2-ketogluconic acid appear to be the most active and important (Moghimi and Tate 1978; Goldstein et al. 1999; Hwangbo et al. 2003). Some Gram-negative rhizobacteria have membrane-bound enzymes that enable the extracellular conversion of glucose to gluconic acid (glucose dehydrogenase) and thence to 2-ketogluconic acid. Moghimi et al. (1978) observed that 2-ketogluconic acid represented as much as 20% of the soluble products produced in the rhizosphere of wheat.

The possibility of enhancing P uptake of crops by artificial inoculation with P-solubilising strains of rhizobacteria has been an attractive proposition for research. Plant responses to inoculation in the field have been widely reported but are variable (Richardson 2001). Early attempts at inoculation failed due to the use of non-indigenous strains (Lynch 1983). Solubilisation of phosphate and enhancement of plant growth has been demonstrated in non-soil potting media (Piccini and Azcon 1987) but inappropriate laboratory screening procedures and a lack of understanding of plant-bacterium-soil interactions are impediments to successful deployment of P-solubilising inoculants (Richardson 2001). For example, the potential for intimate interaction, equivalent to mutualism, between P-solubilising rhizobacteria and plants was recently identified by Hwangbo et al. (2003). They found that the population of rhizobacteria on *Helianthus annus jaegeri*, growing in a highly alkaline soil with very low available

phosphate, was dominated by *Enterobacter cloacae*. In culture, this bacterium only produced glucose dehydrogenase in response to a bioactive component contained in the root exudates of the host plant, suggesting regulation of gluconic acid production through plant-bacterium signalling.

6.2.3 Nitrogen Cycling

The importance of bacteria as key drivers of the nitrogen cycle is exemplified in both bulk soil and the rhizosphere (Delwiche 1970; Rosswall 1983). Carbon substrates in the rhizosphere provide a large proportion of the energy required for nitrogen cycling in soil as a whole. Microbial-driven nitrogen fixation, ammonification, nitrification and denitrification all impact upon assimilation of ammonium and nitrate by plants and other organisms in the soil ecosystem. The bulk of plant-assimilated nitrogen comes from nitrate added as either fertilisers or nitrate produced from ammonification and nitrification in the rhizosphere (Rosswall 1976). Bacterial ammonification is important for returning large quantities of ammonia to the soil, through the mineralisation of nitrogen-based organic compounds in animal waste products (Berks et al. 1995). Fertilisers such as urea, ammonium sulphate or metal nitrates, can have a significant role in affecting and determining microbial community structure in the rhizosphere. The application of ammonium sulphate [$(NH_4)_2SO_4$] acidifies soil, which in turn influences antibiotic production by rhizobacteria (Cooper and Chilton 1950) and their community dynamics. Similarly, artificial introduction of nitrate to nitrogen-limited populations of bacteria in a carbon-rich environment will favour rapid bacterial growth, with competition for nitrate (Lockwood 1988).

The ability to carry out denitrification allows many bacteria to use nitrate as an alternative to oxygen as a respiratory electron acceptor (Firestone 1982; Smith 1990). Oxygen becomes limited in soils when as soil pores are filled with water, which acts as a barrier to oxygen diffusion (Patrick 1982; Smith 1990). However, respiration by rhizobacteria, driven by catabolism of root exudates, and respiration by the plant combine to create reduced oxygen availability in the rhizosphere (Woldendorp 1962, 1963; Smith and Tiedje 1979; Stefanson 1972; Firestone 1982). This rhizosphere respiration establishes anoxic and anaerobic zones in which oxygen demand outweighs supply. Denitrifying rhizobacteria can maintain metabolic activity under anoxic or anaerobic conditions and so remain influential within the rhizosphere community. Almost all denitrifiers are aerobic organisms capable of facultative anaerobic growth in the presence of nitrogenous oxides.

Despite the discovery of high numbers of Gram-negative bacteria capable of carrying out aerobic nitrate respiration (Payne 1973), the full extent and

importance of denitrification in the rhizosphere has not yet been realised (Carter et al. 1995). However, the amount of nitrogen lost to the atmosphere at the end point of denitrification is relatively small in comparison to that being cycled through soil microbes, plants and animals (Bolin et al. 1983). In unfertilised soils, losses of nitrogen to the atmosphere must be balanced by natural inputs. A large portion of this nitrogen input is derived from nitrogen-fixing (diazotrophic) bacteria. Symbiotic bacteria in the rhizosphere contribute substantially to these inputs, with direct assimilation of fixed nitrogen by their plant hosts.

6.2.4 Symbioses Between Plants and Diazotrophic Rhizobacteria

Symbiotic nitrogen fixation by rhizobia in legumes has a profound impact upon agriculture and human endeavour. Global fixation of nitrogen arising from intensive cultivation of legumes is estimated to be up to 40 million tonnes annually, providing around 20% of the available nitrogen in agricultural soils (Crews and Peoples 2004). This symbiosis involves intimate mutual interaction between both partners, with altered morphology of both the bacteria and the plant. The evolution, biology and genetics of the legume symbiosis with rhizobia has been extensively reviewed (for recent examples see Gualtieri and Bisseling 2000; Colebatch et al. 2002; Simms and Taylor 2002; Lodwig and Poole 2003).

In natural ecosystems, the symbiosis between legumes (Fabaceae) and rhizobia is important but other symbioses between plants and diazotrophic rhizobacteria can be equally prominent. The contribution of fixed nitrogen to the soil pool by the actinomycete *Frankia* is comparable to that of legumes, on both a global and individual scale (Dawson 1986; Myrold and Huss-Danell 2003). *Frankia* forms actinorrhizal symbiosis with at least 200 species of woody plants from 24 genera among eight Angiosperm families (Schwencke and Carú 2001). Cyanobacteria form morphologically diverse symbioses with a range of higher and lower plants. The symbiosis between cyanobacteria and cycads is analogous to that formed by the rhizobia and *Frankia*. The cyanobacteria, especially *Nostoc*, colonise apogeotropic roots (coralloid roots) of approximately 30 species of cycads (Smith and Douglas 1987; Rai et al. 2000). Cycads are rarely dominant in plant ecosystems but levels of nitrogen fixation approaching 20 kg of N ha^{-1} $year^{-1}$ have been observed (Rai et al. 2000).

The symbioses that plants develop with cyanobacteria, *Frankia* and the rhizobia are clearly defined by the trophic interdependence of the host and symbiont, and the physiological and morphological changes to both that arise from the interaction. The interactions between plants and free-living

diazotrophic rhizobacteria lack structural definition but nonetheless play an important role in rhizosphere ecology.

6.2.5 Free-Living Diazotrophic Rhizobacteria

The association of diazotrophic rhizobacteria with grasses is well documented (Baldani et al. 1997) and includes several bacterial genera and many important agricultural plants (Table 6.1). Free-living diazotrophs are frequently the predominant culturable bacteria in the rhizosphere of wheat (Ruppel 1988; Heulin et al. 1994). Such associations are not restricted to the grasses and also occur in roots of *Atriplex* (Bilal et al. 1990), conifers (Chanway and Holl 1991), many horticultural trees and crops (Hill et al. 1982; Subba Rao 1983; Ghai and Thomas 1989), mangroves (Zuberer and Silver 1978), fresh and salt water marsh plants (Patriquin and Keddy 1978; Dierberg and Brezonik 1983) and some marine angiosperms (Smith and Douglas 1987).

Under certain circumstances, free-living diazotrophic bacteria that associate with roots of non-leguminous plants can increase the growth and yield of crops (Boddey et al. 1991; Kennedy and Tchan 1992; Peoples and Crasswell 1992; Sarig and Okon 1992; Abbass and Okon 1993; Fulchier and Fioni 1994). However, nitrogen fixation by free-living rhizobacteria is thought to contribute only a small proportion of the nitrogen assimilated directly by plants (Michiels et al. 1989; Wood et al. 2001) with the observed growth responses being attributed to secretion of plant growth-promoting substances (Dobbelaere et al. 2001). The associations between plants and diazotrophic bacteria have not been considered to be symbioses due to the absence of morphological changes comparable to those of nodulation (Michiels et al. 1989). However, direct transfer of substantial amounts of fixed nitrogen to the plant has been demonstrated, particularly in tropical grasses (Boddey and Victoria 1986; Lima et al. 1987; Reis et al. 2001) and salt marshes dominated by the grass *Spartina* (Bagwell and Lovell 2000). Such associations demonstrate a high degree of adaptation between plants and diazotrophic rhizobacteria (Dobereiner and Pedrosa 1987; Bagwell and Lovell 2000; Piceno and Lovell 2000; Reinhold-Hurek and Hurek 2000). Many diazotrophic rhizobacteria are facultative or even obligate endophytes of roots (Baldani et al. 1997). These factors all suggest that mutualism between plants and diazotrophic rhizobacteria extends well beyond the morphologically defined symbioses.

Processes analogous to mutualism may also encompass the indirect effects on plants of nitrogen fixation in the rhizosphere. Much of the nitrogen that free living diazotrophic rhizobacteria fix may be retained within their

Table 6.1. Examples of the diversity of associations between nitrogen-fixing (diazotrophic) rhizobacteria and grasses

Bacterium	Principal association	Reference
Azospirillum amazonense	Many grasses in Amazonia, *Brachiaria* spp.	Boddey and Dobereiner 1988; Reis et al. 2001
Azospirillum lipoferum	Many grasses and cereals	Boddey and Dobereiner 1988
Azospirillum halopraeferans	Kallar grass	Boddey and Dobereiner 1988
Azospirillum irakense	Rice	Baldani et al. 1997
Azospirillum spp.	*Atriplex* spp.; Guinea grass, sugarcane	Bilal et al. 1990; Ghai and Thomas 1989
Acaligenes denitrificans	Sea oats	Will and Sylvia 1990
Azospira oryzae	Kallar grass and rice	Reinhold-Hurek and Hurek 2000
Azotobacter chroococcum	Maize	Martinez-Toledo et al. 1988
Azotobacter paspali	*Paspalum*	Dobereiner and Pedrosa 1987
Azotobacter vinelandii	*Paspalum*, grasses	Dobereiner and Pedrosa 1987
Azovibrio restrictus	Kallar grass and rice	Reinhold-Hurek and Hurek 2000
Gluconacetobacter diazotrophicus	Sugarcane	Michiels et al. 1989
Bacillus azotofixans	Wheat, sugarcane, grasses	Boddey and Dobereiner 1988
Bacillus circulans	Maize	Berge et al. 1991
Bacillus macerans	Wheat, prairie grasses	Nelson et al. 1976; Dobereiner and Pedrosa 1987
Bacillus polymixa	Prairie grasses, xeric grasses, wheat	Nelson et al. 1976; Dobereiner and Pedrosa 1987; Heulin et al. 1994
Beijerinkia spp.	Rice, sugarcane, grasses	Dobereiner and Pedrosa 1987
Campylobacter nitrofigilis	*Spartina alterniflora* (saltmarsh grass)	Boddey and Dobereiner 1988
Derxia spp.	Tropical grasses	Dobereiner and Pedrosa 1987
Enterobacter cloacae	Wheat, sorghum, maize, grasses	Raju et al. 1972; Nelson et al. 1976; Pedersen et al. 1978
Enterobacter aglomerans	Temperate and tropical, grasses *Atriplex* spp.	Haahtela and Korhonen 1985; Bilal et al. 1990
Erwinia herbicola	Wheat, sorghum	Pedersen et al. 1978
Herbaspirillum seropedicae	*Brachiaria* sp., maize, grasses, cereals, elephant grass	Boddey and Dobereiner 1988; Reis et al. 2001
Herbaspirilluim frisinguense	Elephant grass	Reis et al. 2001
Klebsiella pneumoniae	Wheat, sorghum, Kallar grass, *Poa pratensis*	Pedersen et al. 1978; Haahtela and Kari 1986; Qureshi et al. 1988
Klebsiella oxytoca	Rice	Dobereiner and Pedrosa 1987
Klebsiella terrigena	Grasses	Haatela and Korhonen 1985
Pseudomonas sp.	Wetland rice	Boddey and Dobereiner 1988
Rahnella aquatilis	Wheat	Heulin et al. 1994
Saccharobacter nitrocaptan	Sugarcane	Graham 1988

cells in the form of ammonia, thus preventing direct transfer to the plant (Wood et al. 2001). Ultimately, however, most of the nitrogen fixed by free-living rhizobacteria must enter the soil nitrogen pool. Kuikman and Van Veen (1989) observed that inoculation of wheat with diazotrophic bacteria and protozoa increased mineralisation of fixed nitrogen and its uptake by 65%, through predation of the bacteria by the protozoa, resulting in a 20% increase in nitrogen uptake by the wheat. Bacteriophageous nematodes can mineralise up to six times more nitrogen than the same biomass of protozoa grazing on bacteria (Griffiths 1990). While the direct transfer of fixed nitrogen to plants appears to be a variable phenomenon, the widespread occurrence of diazotrophic bacteria in the rhizosphere suggests that trophic interactions within the soil food web may play a greater role in nitrogen assimilation by plants than previously recognised.

6.2.6 The Soil Food Web

Plants are thought to release 12–40% of the carbohydrates produced by photosynthesis into the soil surrounding their roots (Lynch 1995). Deposition of this vast carbon source into the rhizosphere makes plants the major driving force of the soil food web. However, the fate of this carbon in the soil is poorly understood. Basic information about soil trophic interactions, including the energy involved and environmental effects, and techniques to measure biodiversity are all lacking (Scheu 2002).

Molecular techniques have been used to attempt to answer ecological questions about how plant species interact with the below ground microbial communities (Colebatch et al. 2002; Kent and Triplett 2002). Ecological studies often hypothesise that the most productive ecosystems are those with the greatest diversity and this holds true at the continental level. However, on a smaller scale this hypothesis fails, with some of the most productive areas being those with low plant diversity, such as a field under monoculture (Moore 2003). Observations of this nature have lead many plant ecologists to examine the role of roots in establishing plant diversity (Rajaniemi et al. 2003). Investigation of the implications of plant diversity for soil microbial communities has limitations, the majority of which are due to poor understanding of the complex interactions that occur between plants, soil fauna, and soil abiotic conditions (Gastine et al. 2003). Much of the work presented to date is conflicting and the hypothesis that diverse plant communities support a greater microbial diversity than monocultures, and monocultures a greater diversity than bare soil does not always hold true (Johnson et al. 2003). Reasoning for this revolves around concepts that plants are not interacting solely with microbes, but have to contend with

temporal and environmental variation, the abiotic properties of soil, as well as other micro-organisms, plants and soil micro fauna (Gastine et al. 2003).

Buffering effects between the different trophic levels are often used to explain why above ground diversity does not appear to affect that below ground (Gastine et al. 2003). The degree of buffering is hard to discern due to the lack of understanding of interactions between trophic levels within the soil (Scheu 2002). For example, while earthworms and nematodes are important in the soil ecosystem and can significantly increase the size of microbial populations in the rhizosphere (Knox et al. 2003; Stephens et al. 1993), the diet of these soil animals is only just being investigated (Scheu 2002; Wolter and Scheu 1999). Until recently it was assumed that earthworms digest soil microbes, including rhizobacteria, as a major part of their diet. Evidence now exists to the contrary (Fischer et al. 1997). In contrast, protozoa are known to digest bacteria. However, factors such as selective grazing complicate our attempts to understand their role within the soil food web. Grazing on rhizobacteria varies among protozoans species. Particular populations of protozoa can select for cell size and/or in favour of Gram-positive bacteria (Ronn et al. 2002). Selective grazing by protozoa has also been demonstrated to increase plant growth (Bonkowski et al. 2000; Jentschke et al. 1995). This phenomenon is not necessarily a result of increased nitrogen turnover (Griffiths and Robinson 1992) and it is now believed that the observations were a result of protozoa feeding on bacteria that were incapable of producing the plant hormone indole-3-acetic acid (IAA) (Bonkowski and Brandt 2002). Rhizobacteria form an important link between plants and the soil food web, with complex interactions that affect plants and other microorganisms in the rhizosphere.

6.2.7 Interactions Among Rhizosphere Microorganisms

Mycorrhizal symbiosis is ubiquitous in plant ecosystems. Mycorrhizal fungi colonise roots and the surrounding soil, and transfer P and other relatively immobile elements to the plant in exchange for plant carbohydrate. The biology of the arbuscular mycorrhizas, ectomycorrhizas and other types has been comprehensively reviewed elsewhere (Smith and Read 1997). Mycorrhizal fungi are fundamental players in the rhizosphere and have potential to both influence and be influenced by rhizobacteria. Mycorrhizal fungi alter the root exudation of plants and also produce their own exudates, which has prompted development of the mycorrhizosphere concept (Linderman 1992).

The potential impacts of rhizobacteria on mycorrhizal fungi include changes in root-fungus signalling, recognition and receptivity, as well as

effects on fungal growth and germination (Johansson et al. 2004). Rhizobacteria can either increase or inhibit plant growth, indirectly, through effects on the competency of arbuscular mycorrhizas (Hetrick et al. 1988; Linderman 1992; Meyer and Linderman 1986) and ectomycorrhizas (Garbaye 1994). Some of the interaction between rhizobacteria and mycorrhizal fungi probably occurs by default, through joint occupation of the rhizosphere, but specialised associations are also evident. Fastidious endophytic bacteria are now known to colonise the cytoplasm of arbuscular mycorrhizal fungi, with vertical transmission from generation to generation of fungi (Bonfante 2003).

Free-living diazotrophic bacteria have the potential to act in synergism with arbuscular mycorrhizal fungi to increase plant growth (Subba Rao et al. 1985; Paula et al. 1992; Singh 1992). The same applies for interaction between mycorrhizas and the 'symbiotic' diazotrophs, such as the rhizobia and *Frankia*. Biological nitrogen fixation expends large amounts of energy, requiring the synthesis of around 16 molecules of adenosine 5′ triphosphate (ATP) for every molecule of N_2 converted to ammonia (Gottschalk 1986). Phosphorus supply is thus a limiting factor to symbiotic nitrogen fixation and legumes restrict nodulation accordingly (Simms and Taylor 2002). The capacity for mycorrhizas to enhance P uptake by plants enables synergistic interaction between *Frankia* and both endo- and ectomycorrhizal fungi (Chatarpaul et al. 1989; Rojas et al. 2002; Tian et al. 2002). Similarly, synergistic interactions have been observed between arbuscular mycorrhizal fungi and rhizobia (Galleguillos et al. 2000; Vázquez et al. 2001). Rhizobial symbioses, in turn, can also be influenced by rhizobacteria. Improvement in the efficiency of nitrogen fixation has been reported when plants are co-inoculated with rhizobia and strains of *Pseudomonas* and *Bacillus* (Parmar and Dardarwal 1999). The complexity of all these interactions is further complicated by the potential for dynamic changes in the functions expressed by rhizobacteria.

6.2.8 Functional Flux in the Rhizosphere

Rhizobacteria that have a positive or negative influence on plant growth are classified, respectively, as plant growth promoting rhizobacteria (PGPR) or deleterious rhizobacteria (DRB). PGPR can increase plant growth directly through various mechanisms, including production of phytohormones (Okon and Kapulnik 1986; Zimmer and Bothe 1988), fixing nitrogen in the rhizosphere (Lima et al. 1987; Reis et al. 2001), solubilising P (Piccini and Azcon 1987), promoting mycorrhizal function (Linderman 1992) and regulating ethylene production in roots (Glick 1995). PGPR can increase

plant growth indirectly by suppressing plant pathogens through a range of mechanisms, including carbon substrate competition, sequestration of Fe^{3+} in siderophores that the pathogen cannot access, increasing the availability of Mn in the soil, production of antibiotic substances and induction of systemic induced resistance in the plant (Weller 1988; Jacobsen and Backman 1993; Elmer 1995; Glick 1995; Leeman et al. 1996; Wei et al. 1996). The potential to deploy PGPR for biological control of disease has attracted much research, especially with pseudomonads, as they are highly rhizosphere-competent. Induction of systemic resistance in the plant and the production of antibiotic substances, especially phenazines and phloroglucinols, are key mechanisms in biological control by pseudomonads (Chin-A-Wong et al. 2003).

Rhizosphere communities generally contain bacteria that function across the spectrum from PGPR to DRB. Deleterious effects of rhizobacteria have been observed in a broad range of crops as well as pasture and weed species and DRB have been implicated in yield declines associated with continuous monoculture (Nehl et al. 1997). Mechanisms by which DRB may inhibit plant growth include: production of phytotoxins, including HCN, phytotoxic metabolites and phytohormones, especially IAA; inhibition of the function of mycorrhizal fungi, diazotrophs and PGPR; and competition with the plant for nutrients such as P and Fe (Nehl et al. 1997; Barazani and Friedman 1999).

The classification of rhizobacteria as either PGPR or DRB is equivocal (Nehl et al. 1997). The effect of individual isolates of rhizobacteria on plants can fluctuate from growth inhibition to growth promotion, according to environmental conditions, the host genotype and mycorrhizal status (Nehl et al. 1997; Sturz and Christie 2003). Rhizobacterial communities have a major impact on the suppressiveness or conduciveness of soil to fungal pathogens (Garbeva et al. 2004). Given the existence of subtle species-specific bacterium-host interactions in the rhizosphere, the potential for rhizobacteria to have deleterious effects on plant growth while simultaneously suppressing fungal plant pathogens is evident (Nehl et al. 1997).

6.2.9 Rhizobacteria are Moderators at the Plant-Soil Interface

The discussion above has highlighted the capacity for rhizobacteria to moderate numerous physical and chemical processes and biological interactions that are important for the function of soil ecosystems. Increasing knowledge of these processes underpins efforts to utilise rhizobacteria to enhance the productivity and sustainability of plant production. However, the incredible biodiversity and complexity of community structures

and interactions within the rhizosphere remains a hurdle to manipulation (Sturz and Christie 2003). Genomic studies have been used to investigate interactions between legumes and rhizobia (Colebatch et al. 2002) and should help unravel the complexity of rhizosphere communities and their functional dynamics.

Strategies for manipulation of rhizobacteria include inoculation of seed or tissue cultures, manipulation of crop production systems, crop cultivar selection and genetic modification of plants or rhizobacteria (Sturz and Nowak 2000). In the case of the latter, modification of plants and PGPR strains with genes for production and catabolism of opines offers the exciting possibility of engineering rhizosphere competitiveness (Savka et al. 2002). Competitiveness and other determinants of rhizosphere community structure are now discussed.

6.3 Selective Enrichment and Diversity of Bacteria in the Rhizosphere

An enriched microflora is one of the major characteristics that distinguish the rhizosphere from bulk soil. The rhizosphere contains abundant substrates that can be exploited by bacteria. Roots release a diverse range of compounds, including sugars, amino acids, organic acids, fatty acids, sterols, growth factors, nucleotides, enzymes and many others (Bolton et al. 1993). As stated eloquently by Hawes et al. (1998), “in the rhizosphere ... the key problem facing microorganisms is the need to obtain water and nutrients, and the key problem facing plants is that they are water and nutrients”.

To date, the most-studied rhizobacteria are those that are easily culturable. Molecular techniques are now revealing the presence of substantial numbers of non-culturable bacteria in the rhizosphere. Using ribosomal RNA sequences and in situ hybridisation, Simon et al. (2000) found that bacteria from the Crenarchaeota represented respectively 3 and 16% of the total population of bacteria on young and old roots of tomato. Growth rate appeared not to be a factor limiting colonisation by the Crenarchaeota, suggesting other mechanisms for niche specialisation. The functions of these apparently competitive rhizobacteria, including their role in the rhizobacterial community, is yet to be determined (Simon et al. 2000).

The diversity and community structure of bacteria in the rhizosphere varies dramatically according to plant genotype, soil type, agricultural practices, and root morphology (Dalmastri et al. 1999; Reis et al. 2001; Marschner et al. 2004). Different populations of bacteria will be associated with root tips, free-living border cells, the root hair zone and older parts

of the root (Hawes et al. 1998; Simon et al. 2000; Marschner et al. 2004). Root border cells and eukaryotic microbes, including saprophytic, parasitic and mycorrhizal fungi, bridge these zones to varying degrees and create additional microbial biospheres. Indeed, free-living root border cells may contribute the majority of the carbon rich 'exudates' released from roots (Hawes et al. 1998) and thus have an important role in rhizosphere community dynamics. The variation in quality and quantity of root exudates among plant genotypes also exerts a major selective influence on rhizosphere community structure. Accordingly, the competitive adaptations of individual strains or species of rhizobacteria will affect their success or failure with any given set of substrates. Recent research is revealing the extent and complexity of adaptation by bacteria for colonisation of the rhizosphere, including bacterial competitiveness and selective plant factors.

6.3.1
Competitiveness of Rhizobacteria

Rhizobacteria are a subset of the whole population of bacteria in soil but are better adapted to colonisation of roots. In the absence of root exudates or other organic inputs, soil microbial populations are metabolically inactive. However, irrespective of the availability of labile substrates, it appears that the ATP content of soil may generally be maintained at relatively constant levels in proportion to microbial biomass (Contin et al. 2000). These ATP levels are commensurate with that of exponential microbial growth in vitro, even when the microbial community is inactive. An explanation was hypothesised by de Nobili et al. (2001). They observed that the addition of trace amounts of soluble sugars, amino acids or root extracts to soil triggered rapid increases in microbial metabolic activity – activity over and above that which could be supported by the amount of substrate provided by the 'trigger' solution alone. Less labile substrates, such as cellulose, did not act as triggers but their presence did help sustain metabolic activity at a higher level following trigger events. They hypothesised that maintaining high ATP levels enables microbes to be primed for rapid exploitation of substrates. Importantly, the greatest trigger-response was to root extracts (de Nobili et al. 2001), suggesting that rhizobacteria have a genetic predisposition that primes them for rapid colonisation and exploitation of the rhizosphere.

Given the relative abundance of labile substrates in the rhizosphere, the efficiency of nutrient uptake and catabolism by bacteria is a key factor in competitiveness. Rapid uptake of nutrients depends on tactical responses to nutrient supply and, therefore, chemotaxis and motility are also key factors (Chin-A-Woeng et al. 2003). The capacity for rapid growth when substrates

are encountered is not the only factor affecting rhizosphere competence, as rhizobacteria deploy many other metabolic strategies. For example, the capacity for extracellular conversion of glucose to gluconic acid and 2-ketogluconic acid enables some bacteria, including several species of *Pseudomonas* to sequester glucose effectively. When glucose is in plentiful supply, external conversion to gluconic acid and 2-ketogluconic acid gives pseudomonads a competitive advantage over microorganisms that lack the ability to utilise these compounds (Whiting et al. 1976; Gottschalk 1986).

An even more specialised strategy is found in the association of *Agrobacterium tumefaciens* with plant roots. DNA from the tumour inducing (Ti) plasmid in the bacterium is transferred to cells in the root and where it integrates with the plant's genome. The transformed cells proliferate as gall tissue and subsequently produce amino acid-like compounds, known as opines, that are specific to the infecting strain (Zupan et al. 2000) and may represent as much as 7% of the dry mass of the gall tissue (Savka et al. 2002). The genes for opine production (in planta) and catabolism in the bacterium are both located on the Ti plasmid and are linked to virulence. *A. tumefaciens* is thus able to genetically engineer plants to create an ecological niche in which it is the only rhizobacterium that can utilise the most abundant substrate excreted by the plant (Zupan et al. 2000). Very few rhizobacteria can utilise opines; a feature that could be exploited to genetically engineer highly specific plant-rhizobacterium associations based on opine production and catabolism (Oger et al. 2000; Savka et al. 2002).

Some strains of rhizobia produce opine-like molecules, known as rhizopines, inside legume root nodules. Rhizopines released into the rhizosphere can act as a selective substrate for free-living rhizobia that have the ability to catabolise them (Murphy et al. 1995). The genes for rhizopine production and catabolism are located within the same plasmid, suggesting that the rhizobial bacterioids within nodules are able to confer a competitive advantage to free living bacteria of the same strain. Since rhizobial bacterioids cannot reproduce and die within senescing nodules, rhizopines provide a mechanism for the free-living cells and, hence, the species as a whole to benefit from its symbiosis with plants (Simms and Bever 1998).

Antagonism is another adaptation for competitiveness of rhizobacteria, especially against fungi. Production of secondary metabolites, such as antibiotics, has been determinant in studies of the competitiveness of some combinations of rhizobacteria but not others (Chin-A-Woeng et al. 2003). Production of HCN has been suggested as a mechanism by which PGPR may inhibit DRB (Kloepper 1993). However, more subtle mechanisms of refined control of rhizosphere community dynamics are now recognised. A range of Gram-negative rhizobacteria can secrete and respond to furanones that regulate growth patterns in proportion to bacterial population density

(Newton and Fray 2004). This autoregulation of reproduction is known as quorum sensing and acyl homoserine lactones (AHL) are the major known group of furanones involved. In contrast to non-rhizosphere soil, which is an oligotrophic environment, the rhizosphere is an enriched copiotrophic environment that supports populations large enough for quorum sensing.

Among culturable bacteria, AHL production occurs in a greater proportion of those from the rhizosphere than from bulk soil (Cha et al. 1998; Elasri et al. 2001). Work on individual Gram-negative rhizosphere isolates has also demonstrated these bacteria produce a number of chemically distinct AHL molecules (Cha et al. 1998), each of which has the potential to influence expression of a different gene array. In addition AHL produced in vitro by individual species or strains can switch on expression of AHL-regulated genes in other species or strains (Pierson et al. 1998). Quorum sensing is presumed to increased rhizosphere competence (Newton and Fray 2004) and the potential for one strain to regulate growth in another supports this hypothesis. Such inter-specific bacterial communication may, in future, offer opportunities to manipulate bacterial community structure in the rhizosphere for the benefit of agriculture. However, selective enrichment of the rhizosphere is not merely the result of competitive substrate utilisation by opportunistic bacteria. A complex array of plant factors is also involved.

6.3.2 Selective Plant Factors

Small differences in host genotype can regulate bacterial colonisation of the rhizosphere in a very specific manner (Nehl et al. 1997; Garbeva et al. 2004). For example, Larson and Neal (1978) observed preferential colonisation of a genetically defined line of wheat by diazotrophic *Bacillus* spp. The total numbers of bacteria on roots of that cultivar were less than or equal to numbers on other wheat lines indicating that the quality of the root exudates led to this host specific effect. In spring wheat the occurrence of a rhizoplane microflora that was antagonistic to *Cochliobolis sativus* was determined by genes on a single chromosome (Cook and Baker 1983; Bruehl 1987).

In most plant species, living cells at the periphery of the root cap, known as border cells, become detached as individuals or small groups of cells (Hawes et al. 2003). Border cell production does not occur in a number of aquatic and parasitic plants whose roots are devoid of a root cap structure (Bowers et al. 2003). Members of the Brassicaceae, including the model plant *Arabidopsis thaliana*, do not produce viable border cells and border cells in solanaceous plants tend to have low viability (Wang et al. 1996;

Hawes et al. 2003). The cross-phyla phenomenon of border cell production in angiosperms, gymnosperms and the pteridophytes (Bowers et al. 2003) suggests that they have been part of the rhizosphere throughout the evolution of roots.

Border cells were originally thought to act as a protective barrier, reducing friction between the soil and the root surface, and their production in sufficient numbers to perform this role has been demonstrated (Iijima et al. 2003). However, once border cells are differentiated, they undergo a change in structure, size and gene expression. The altered gene expression results in production of several border cell-specific proteins that are predominantly exported to the environment (Brigham et al. 1995). Once in the environment, these excreted proteins have the potential to select and impact upon microbes in the rhizosphere (Brigham et al. 1998; Hawes et al. 2000). The root cap is a major site of root exudation, thus influencing the early establishment of microorganisms (Hawes et al. 2000; Hawes et al. 2003).

Hawes and Smith (1989) demonstrated the potential for selective mediation of rhizobacterial community structure by border cells. The border cells excreted extracellular galactosidase which released galactose from their mucilage, variously stimulating or inhibiting colonisation by different bacterial species. Furthermore, border cells can also produce specific extracellular signal molecules that directly influence microbial growth and gene expression, and the attraction of pathogenic fungi or nematodes (Hawes et al. 2000; Rodger et al. 2003).

Molecular compounds similar to bacterial AHL have been isolated from the root exudates of several higher plants (Bauer and Teplitski 2001). These plant compounds have the ability to modify AHL signalling in microbial populations by either promoting or inhibiting AHL mediated quorum sensing (Bauer and Teplitski 2001). One of the best studied plant AHL analogues is furanone production by red alga *Delisea pulcra*. *D. pulcra* produces over 30 halogenated furanone compounds that are capable of inhibiting AHL signalling between Gram-negative bacteria. These furanone compounds, similar to bacterial AHL in structure, prevent Gram-negative bacteria from colonising the algal surfaces whilst allowing less abundant Gram-positive species to do so (Bauer and Teplitski 2001). Extrapolation of this algal system of selective colonisation and the detection of AHL analogues in the root exudates of higher plants could be considered as the basis of a plant mediated mechanism for selective root colonisation by either stimulation or inhibition of specific bacterial species (Bauer and Teplitski 2001).

Root exudate-mediated effects on rhizobacteria are not simply a one-way interaction. Jones and Darrah (1994, 1996) reported that roots of maize plants can actively re-sorb sugars and amino acids lost by passive leakage. Re-sorption occurred against concentration gradients between roots and

the rhizosphere. It is conceivable, therefore, that plants might exert a degree of regulation on both the quantity and the quality of carbon substrate available in the rhizosphere. Rhizobacteria can also induce highly specific root exudation responses (Nehl et al. 1997) and alter metabolic process of roots, such as the production of plant flavonoids (Parmar and Dadarwal 1999). The existence and diversity of so many multifaceted, complex interactions between plants and rhizobacteria reflects a continuum of co-evolutionary events in the rhizosphere.

6.4 Evolution of the Rhizosphere

There is no doubt that plants evolved in intimate contact with soil microorganisms. In some cases, highly specific mutualisms have evolved, such as the diazotrophic symbioses. Bolton et al. (1993) hypothesised that root exudation evolved in plants as a means of stimulating an active rhizosphere microflora. Nehl et al. (1997) considered that this hypothesis was feasible because of the selectivity for rhizobacteria among plant genotypes and the potential for some rhizobacteria to improve plant health, and hence fecundity. The potential for either an exudation response to bacteria or a response by bacteria to exudation suggests a degree of coevolution between plants and rhizobacteria. Homology of genetic themes associated with plant-microbe associations (Sanchez et al. 2004) and cross-species (horizontal) gene flow provide further evidence that selective interaction between plants and rhizobacteria has occurred on a broad evolutionary scale.

6.4.1 Gene Flow in the Rhizosphere

Foreign DNA in the form of transposable elements has been causing phenotypically detectable mutations and changes to plant genomes for millions of years (Casacuberta and Santiago 2003). Transposable elements fall broadly into two classes; class I elements that transpose through a RNA-intermediate and are found in eukaryotes, and class II elements, which transpose via a DNA-intermediate and are found in both eukaryotic and prokaryotic genomes (Hamer et al. 2001). Retrotransposons, which belong to class I transposable elements, are both ubiquitous and highly heterogeneous within plant genomes, which has lead to suggestions that they were probably present in the first plants to evolve (Flavell et al. 1992; Voytas et al. 1992). However, it remains conceivable that retrotransposons originated after the evolution of eukaryotes and subsequently spread by vertical and horizontal transmission (Kumar and Bennetzen 1999).

In rhizobacteria, genetic determinants for genes involved in chemotaxis, competition (particularly antibiotic production) and nutrition are often located on non-chromosomal DNA, such as plasmids (Lynch 1995). The existence of these functional genes, implicated in rhizosphere colonisation, in what are potentially mobile elements in transformation and conjugation is potentially of immense importance. The transfer of plasmids, transposable elements or the uptake of foreign DNA can potentially alter the phenotype of recipient plants or bacteria, and in some cases this might be dramatic enough to assign the transformed organism to a new species (De La Cruz and Davies 2000; Richter and Ronald 2000; Sy et al. 2001).

Such dramatic phenotypic change occurs with transfer of the Ti plasmid from *A. tumefaciens* to plant cells, with the subsequent formation of gall tissue. The Ti plasmid and its derivatives are complex, usually carrying several genes involved in signal recognition (Ashby et al. 1988), virulence genes associated with plasmid insertion (Kerr 1969; Hoekema et al. 1983), IAA synthesis and cytokinin regulation that cause gall formation. The Ti plasmid can also carry genes for production of opines, which are involved in chemotaxis and metabolite production. This is a clear example of where a bacterial mobile element (the Ti plasmid) can enter plant cells, integrate into the chromosome, utilise the plant's transcription and translational mechanisms, produce active cell functional signals altering its morphogenic programme, and produce and secrete novel plant cell compounds.

The Ti plasmid is so efficient at transferring DNA to plants that it was developed as a tool for genetic engineering of plants (Hoekema et al. 1983). Transfer of DNA in the *Agrobacterium*-plant association is not unique. Mechanisms for cross-species DNA transfer, integration and expression occur across all the kingdoms with transformations occurring in more than one direction (Binns 2002). The rhizosphere is focal point for movement of mobile genetic elements among bacteria. Furthermore, the horizontal transfer of plant-derived DNA to rhizobacteria (other than *Agrobacterium*) has also been observed (van Elsas et al. 2003). Since horizontal gene transfer has been occurring for millions of years, it is conceivable that the transfer of genetic material from plants to bacteria and vice versa has contributed to their speciation, altering the selective forces that shaped the plant-bacterium interactions that exist today.

6.4.2 Selection Processes in the Rhizosphere

The rhizosphere is a highly variable environment in which bacterial community structure is subject to selective enrichment, determined by the interaction among a multitude of plant and microbial parameters. These

interactions occur at intra-specific, inter-specific and inter-kingdom levels. The rhizosphere is a substrate-rich habitat that clearly selects for competitiveness in rhizobacteria. Consequently, rhizobacteria have developed a diverse pallet of adaptations that enhance their own fitness (discussed above).

The extent to which rhizobacteria select for the fitness of plants encompasses a continuum, ranging from mutualism to pathogenism (Hall 1974). Selection mechanisms are well defined at the extremities; pathogens and symbionts can both influence the fecundity of plants. However, the vast majority of rhizobacteria function neither as symbionts or pathogens. Furthermore, the expression of deleterious or growth-promoting functions by rhizobacteria can fluctuate according to plant genotype, microbial community structure and environmental variables (Nehl et al. 1997). Even when bacterial taxa can be identified as specialist pathogens or mutualists, the effectiveness of strains is variable within their populations. Strains of rhizobia with poor nitrogen fixation efficiency are effectively cheating the plant, yet they occur widely in nature (Simms and Taylor 2002). A 'market choice' model has been proposed to explain how plants might constrain cheating and thus enable mutualism to persist.

The market choice model assumes that, given a range of potential symbionts, plants can constrain cheating through selective enrichment of efficient taxa or strains, and that the cost of selective enrichment does not exceed the benefits of it (Simms and Taylor 2002). Many rhizobacteria provide no direct reciprocal benefit to the plant in return for its investment in root exudates. However, the market choice model seems to be a useful analogy because rhizobacteria can moderate the rhizosphere to benefit plants indirectly (e.g. nutrient cycling, disease suppression) and plants appear to exert a degree of control on rhizobacterial community structure. Mechanisms for controlling cheating are not clearly characterised in the rhizobial symbiosis (Simms and Taylor 2002), let alone other rhizobacterium associations with plants. However, mechanisms to control pathogens, which represent the extreme of cheating, are ubiquitous in plants. Given the vast diversity of bacteria and fungi in the soil (Hawksworth and Rossman 1997; Curtis et al. 2002) an extremely small number of microbes manage to be major pathogens of any given host plant. This is because plants have evolved complex suites of defence responses, based on signal recognition and polygenic expression of a suite of antimicrobial molecules (Richter and Ronald 2000; Pietiese et al. 2002). Plants actively provided substrates for microbial growth in their rhizospheres but most plants are able to prevent most microbes from becoming pathogenic, implying that plant defences buffer against pathogenicity in the rhizosphere.

Persistent and highly specific associations between rhizobacteria provide further evidence that plants have evolved root exudation as a means of supporting specific rhizosphere communities. For example, the abundance of

diazotrophic free-living rhizobacteria, which supply large amounts of nitrogen to salt marsh grasses, remains resilient, even with application of luxury amounts of nitrogen fertiliser (Bagwell and Lovell 2000). Hence, the association of diazotrophs with these grasses appears to be obligate even in the face of functional redundancy, reflecting a highly specific association (Bergholz et al. 2001). Similarly, *Azotobacter paspali* has almost absolute specificity for a single ecotype of *Paspalum notatum* (Dobereiner and Pedrosa 1987). The Kallar grass association (Table 6.1) entails a high degree of adaptation between both the host and the associative diazotrophs (Reinhold-Hurek and Hurek 2000). Highly specific associations between plants and free-living rhizobacteria are not restricted to diazotrophs (Goldstein et al. 1999).

Our current understanding of rhizosphere dynamics is modest but already demonstrates that interaction between plants and rhizobacteria has been pivotal in the evolution of a rhizosphere that selects for bacterial community structure. Less obvious is the extent to which rhizobacteria select for plant community structure.

6.4.3 Rhizobacteria as Determinants of Plant Diversity

Recent research is indicating that mycorrhizal fungi play a key role in determining the diversity of plant communities (Bever 2003). In reviewing the significance of rhizosphere microorganisms to ecosystems, Newman (1978) speculated that rhizobacteria might influence plant diversity but supporting data was lacking at that time. The potential for DRB to selectively influence competitive interaction between plants has been demonstrated (Nehl et al. 1997) and such interactions may be of evolutionary importance.

Newman et al. (1977) found that inoculation of a mixture of perennial ryegrass and garden sorrel in sterilised soil with a soil filtrate containing bacteria increased the growth of the ryegrass at the expense of the sorrel. Thus inoculation with a plant growth promoter had a deleterious effect on the sorrel. There is increasing evidence that soilborne plant pathogens play a definitive role in modulating the abundance of plants and increasing the diversity of plant species (Klironomos 2002; Packer and Clay 2004). The full extent to which plant community structures are mediated by rhizobacterial communities remains to be determined.

6.5 Conclusions

It is evident from the few examples we have presented that rhizobacteria engage in a diverse range of metabolic processes and interactions with

numerous functional consequences that influence the dynamics among microbial communities, plants and the soil. Rhizobacteria are a major component of the global biosphere, whether regarded from a plant or microbe or soil perspective. The rhizosphere is one of the oldest features of terrestrial ecosystems, having first established during the Silurian period (Retallack 1997). Yet, we still know relatively little about the full extent of the diversity, functional capabilities, adaptations, resilience and subtle, intricate signalling interactions of rhizobacteria.

Research on rhizobacteria has largely focussed on their critical functions in plant production systems: a 'top down' approach. We are now beginning to realise their ecological importance. The ancient existence of rhizospheres underpins the role that rhizobacteria have undoubtedly played in coevolution with plants and other organisms. It has been hypothesised that the evolution of terrestrial plants was made possible by symbiosis between plants and fungi (Pirozynski and Malloch 1975) and supporting evidence is accumulating (Brundrette 2002). It is conceivable that rhizobacteria have played an equally enabling role in the evolution of plants, through their impact on the development of soils that are physically, chemically and biologically conducive for higher plants. Rhizobacteria clearly are intimately involved in all three disciplines. Future research will strengthen our understanding of the many roles of rhizobacteria, providing positive economic and environmental outcomes. There is no doubt that we are living in an age where, even after 3.5 billion years, the microbe is still king.

References

Abbass Z, Okon Y (1993) Plant growth promotion by *Azotobacter paspali* in the rhizosphere. Soil Biol Bioch 25:1075–1083

Alexander M (1987) Introduction to soil microbiology. Krieger Publishing, Malabar

Ashby AM, Watson MD, Loake GJ, Shaw CH (1988) Ti plasmid specified chemotaxis of *Agrobacterium tumefaciens* C58c1 toward vir-inducing phenolic-compounds and soluble factors from monocotyledonous and dicotyledonous plants. J Bacteriol 170:4181–4187

Bagwell CE, Lovell CR (2000) Microdiversity of culturable diazotrophs from the rhizoplanes of the salt marsh grasses *Spartina alterniflora* and *Juncus roemerianus*. Microb Ecol 39:128–136

Baldani JI, Caruso L, Baldani VLD, Goi SR, Dobereiner J (1997) Recent advances in BNF with non-legume plants. Soil Biol Bioch 29:911–922

Barazani O, Friedman J (1999) Is IAA the major root growth factor secreted from plant-growth-mediating bacteria ? J Chem Ecol 25:2397–2406

Bauer WD, Teplitski M (2001) Can plants manipulate bacterial quorum sensing? Aust J Plant Physiol 28:913–921

Berge O, Heulin T, Balandreau J (1991) Diversity of diazotroph populations in the rhizosphere of maize (*Zea mays* L.) growing on different French soils. Biol Fert Soil 11:210–215

Bergholz PW, Bagwell CE, Lovell CR (2001) Physiological diversity of rhizoplane diazotrophs of the saltmeadow cordgrass, *Spartina patens:* implications for host specific ecotypes. Microbial Ecol 42:466–473

Berks BC, Ferguson SJ, Moir JWB, Richardson DJ (1995) Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. Biochem Biophys Acta 1232:97–173

Bever JD (2003) Soil community feedback and the coexistence of comptetitors: conceptual frameworks and empirical tests. New Phytol 157:465–473

Bilal R, Rasul G, Mahmood K, Malik KA (1990) Nitrogenase activity and nitrogen-fixing bacteria associated with the roots of Atriplex spp. growing in saline soils of Pakistan. Biol Fert Soil 9:315–320

Binns AN (2002) T-DNA of *Agrobacterium tumefaciens*: 25 years and counting. Trend Plant Sci 7:231–233

Boddey RM, Dobereiner J (1988) Nitrogen fixation associated with grasses and cereals: recent results and perspectives for future research. Plant Soil 108:53–65

Boddey RM, Victorial RL (1986) Estimation of biological nitrogen fixation associated with *Brachiaria* and *Paspalum* grasses using 15 N labelled organic matter and fertilizer. Plant Soil 90:265–292

Boddey RM, Urquiaga S, Reis V, Dobereiner J (1991) Biological nitrogen fixation associated with sugar cane. Plant Soil 137:111–117

Bolin B, Crutzen PJ, Vitouesk PM, Woodmansee RG, Goldberg ED, Cook RB (1983) Interactions in biogeochemical cycles. In: Bolin B, Cook RB (eds) The major biogeochemical cycles and their interactions. Wiley, New York, pp 1–39

Bolton HJ, Fredrickson JK, Elliott LF (1993) Microbial ecology of the rhizosphere. In: Metting FBJ (ed) Soil microbial ecology. Marcel Dekker, New York, pp 27–63

Bonfante P (2003) Plants, mycorrhizal fungi and endobacteria: a dialog among cells and genomes. Biologic Bull 204:215–220

Bonkowski M, Brandt F (2002) Do soil protozoa enhance plant growth by hormonal effects? Soil Biol Biochem 34:1709–1715

Bonkowski M, Griffiths B, Scrimgeour C (2000) Substrate heterogeneity and microfauna in soil organic 'hotspots' as determinants of nitrogen capture and growth of ryegrass. Appl Soil Ecol 14:37–53

Bowers JE, Parke JL (1993) Colonization of pea (*Pisum sativum* L.) taproots by *Pseudomonas fluorescens*: effect of soil temperature and bacterial motility. Soil Biol Bioch 25:1693–1701

Bowers JE, Chapman BA, Rong JK, Paterson AH (2003) Unraveling angiosperm genome evolution by phylogenic analysis of chromosomal duplication events. Nature 422:433–438

Brigham LA, Woo HH, Nicoll SM, Hawes MC (1995) Differential expression of proteins and mRNAs from border cells and root tips of pea. Plant Physiol 109:457–463

Brigham LA, Woo HH, Wen F, Hawes MC (1998) Meristem-specific suppression of mitosis and a global switch in gene expression in the root cap of pea by endogenous signals. Plant Physiol 118:1223–1231

Bruehl GW (1987) Soilborne plant pathogens. Macmillan, New York

Brundrett MC (2002) Coevolution of roots and mycorrhizas of land plants. New Phytol 154:275–304

Carter JP, Hsiao YH, Spiro S, Richardson, DJ (1995) Soil and sediment bacteria capable of aerobic nitrate respiration. App Environ Microbiol 61:2852–2858

Carter MR (2004) Researching structural complexity in agricultural soils. Soil Till Res 79:1–6

Casacuberta JM, Santiago N (2003) Plant LTR-retrotransposons and MITEs: control of transposition and impact on the evolution of plant genes and genomes. Gene 311:1–11

Cha C, Gao P, Chen YC, Shaw PD, Farrand SK (1998) Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria. Mol Plant Microbe Interact 11:1119–1129

Chanway CP, Holl FB (1991) Biomass increase and associative nitrogen fixation of mycorrhizal *Pinus contorta* seedlings inoculated with a plant growth promoting *Bacillus* strain. Can J Bot 69:507–511

Chatarpaul L, Chakravarty P, Subramaniam P (1989) Studies in tetrapartite symbioses I. Role of ecto- and endomycorrhizal fungi and *Frankia* on the growth performance of *Alnus incana*. Plant Soil 118:145–150

Chin-A-Woeng TFC, Bloemberg GV, Lugtenberg BJ (2003) Phenazines and their role in biocontrol by *Pseudomonas* bacteria. New Phytol 157:503–523

Colebatch G, Trevaskis B, Udvardi M (2002) Symbiotic nitrogen fixation research in the postgenomics era. New Phytol 153:37–42

Contin M, Corcimaru S, De Nobili M, Brookes PC (2000) Temperature changes and the ATP concentration of the soil microbial biomass. Soil Biol Bioch 32:1219–1225

Cook RJ, Baker KF (1983) The nature and practice of biological control of plant pathogens. APS Press, St. Paul

Cooper WE, Chilton SJP (1950) Studies on antibiotic soil organisms I. Actinomycetes antibiotic to *Pythium arrhenomanes* in sugarcane soils of Louisiana. Phytopathol 40:544–552

Crews TE, Peoples MB (2004) Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. Agric Ecosys Environ 102:279–297

Curtis TP, Sloan WT, Scannell JW (2002) Estimating prokaryotic diversity and its limits. Proc Nat Acad Sci 99:10494–10499

Dalmastri C, Chiarini L, Cantale C, Bevivino A, Tabacchioni S (1999) Soil type and maize cultivar affect the genetic diversity of maize root-associated *Burkholderia cepacia* populations. Microbial Ecol 38:273–284

Dawson JO (1986) Actinorhizal plants: their use in forestry and agriculture. Outlook Agric 15:202–208

De La Cruz F, Davies J (2000) Horizontal gene transfer and the origin of species: lessons from bacteria. Trends Microbiol 8:128–133

De Nobili M, Contin M, Mondini C, Brookes PC (2001) Soil microbial biomass is triggered into activity by trace amounts of substrate. Soil Biol Bioch 33:1163–1170

Degens BP (1997) Macro-aggregation of soils by biological bonding and binding mechanisms and the factors affecting these: a review. Aust J Soil Res 35:431–460

Delwiche CC (1970) The nitrogen cycle. Sci Am 223:137–146

Dierberg FE, Brezonik PL (1983) Nitrogen and phosphorus mass balances in natural and sewage-enriched cypress domes. J Appl Ecol 20:323–327

Dobbelaere S, Croonenborghs A, Thys A, Ptacek D, Vanderleyden J, Dutto P, Labandera-Gonzalez C, Caballero-Mellado J, Aguirre JF, Kapulnik Y, Brener S, Burdman S, Kadouri D, Sarig S, Okon Y (2001) Responses of agronomically important crops to inoculation with *Azospirillum*. Aust J Plant Physiol 28:871–879

Dobereiner J, Pedrosa FA (1987) Nitrogen fixing bacteria in nonleguminous Plants. Science Tech Publishers, Madison

Elasri M, Delorme S, Lemanceau P, Stewart G, Laue B, Glickmann E, Oger PM, Dessaux Y (2001) Acyl-homoserine lactone production is more common among plant-associated *Pseudomonas* spp. than among soilborne *Pseudomonas* spp. Appl Environ Microbiol 67:1198–1209

Elmer WH (1995) Association between Mn-reducing root bacteria and NaCl applications in suppression of Fusarium crown and root rot of asparagus. Phytopathol 85:1461–1467

Firestone MK (1982) Biological denitrification. In: Stevenson FJ (ed) Nitrogen in agricultural soils. American Society of Agronomy, Madison, pp 289–326
Fischer K, Hahn D, Hönerlage W, Zeyer J (1997) Effect of passage through the gut of the earthworm *Lumbricus terrestris* L. on *Bacillus megaterium* studied by whole cell hybridization. Soil Biol Biochem 29:1149–1152
Flavell AJ, Dunbar E, Anderson R, Pearce SR, Hartley R, Kumar A (1992) Ty1-copia group retrotransposons are ubiquitous and heterogeneous in higher-plants. Nucleic Acids Res 20:3639–3644
Fulchier M, Fioni L (1994) *Azospirillum* inoculation on maize (*Zea mays*): effect on yield in a field experiment in central Argentina. Soil Biol Biochem 26:921–923
Galleguillos C, Aguirre C, Barea JM, Azcón R (2000) Growth promoting effect of two Sinorhizobium meliloti strains (a wild type and its genetically modified derivative) on a non-legume plant species in specific interaction with two arbuscular mycorrhizal fungi. Plant Sci 159:57–63
Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210
Garbeva P, van Veen JA, van Elsas JD (2004) Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Ann Rev Phytopath 42:243–270
Gastine A, Scherer-Lorenzen M, Leadley PW (2003) No consistent effects of plant diversity on root biomass, soil biota and soil abiotic conditions in temperate grassland communities. App Soil Ecol 24:101–111
Ghai SK, Thomas GV (1989) Occurrence of *Azospirillum* spp. in coconut-based farming systsems. Plant Soil 114:235–241
Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117
Goldstein AH (1995) Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by Gram negative bacteria. Biol Agric Hort 12:185–193
Goldstein AH, Braverman K, Osorio N (1999) Evidence for mutualism between a plant growing in a phosphate-limited desert environment and a mineral phosphate solubilizing (MPS) rhizobacterium. FEMS Microbiol Ecol 30:295–300
Gottschalk G (1986) Bacterial metabolism, 2nd edn. Springer, Berlin Heidelberg New York
Graham JH (1988) Interactions of mycorrhizal fungi with soilborne plant pathogens and other organisms: an introduction. Phytopathol 78:365–366
Griffiths B, Robinson D (1992) Root-induced nitrogen mineralization – a nitrogen-balance model. Plant Soil 139:253–263
Griffiths BS (1990) A comparison of microbial-feeding nematodes and protozoa in the rhizosphere of different plants. Biol Fert Soil 9:83–88
Gualtieri B, Bisseling T (2000) The evolution of nodulation. Plant Mol Biol 42:181–194
Haahtela K, Kari K (1986) The role of root-associated *Klebsiella pneumoniae* in the nitrogen nutrition of *Poa pratensis* and *Triticum aestivum* as estimated by the method of ^{15}N isotope dilution. Plant Soil 90:245–254
Haahtela K, Korhonen TK (1985) In vitro adhesion of N_2-fixing enteric bacteria to roots of grasses and cereals. Appl Environ Microbiol 49:1186–1190
Hall R (1974) Pathogenism and parasitism as concepts of symbiotic relationships. Phytopathol 64:576–577
Hamer L, Dezwaan TM, Montenegro-Chamorro MV, Frank SA, Hamer JE (2001) Recent advances in large-scale transposon mutagenesis. Curr Opin Chem Biol 5:67–73
Hawes MC, Smith LY (1989) Requirement for chemotaxis in pathogenicity of *Agrobacterium tumefaciens* on roots of soil-grown pea-plants. J Bacteriol 171:5668–5671

Hawes MC, Brigham LA, Wen F, Woo HH, Zhu Y (1998) Function of root border cells in plant health: pioneers in the rhizosphere. Annu Rev Phytopath 36: 311–327
Hawes MC, Gunawardena U, Miyasaka S, Zhao X (2000) The role of root border cells in plant defense. Trends Plant Sci 5:128–133
Hawes MC, Bengough G, Cassab G, Ponce G (2003) Root caps and rhizosphere. J Plant Growth Reg 21:352–367
Hawksworth DL, Rossman AY (1997) Where are all the undescribed fungi? Phytopathol 87:888–891
Hetrick BAD, Wilson GT, Kitt DG, Schwab AP (1988) Effects of soil microorganisms on mycorrhizal contribution to growth of big bluestem grass in non-sterile soil. Soil Biol Bioch 20:501–507
Heulin T, Berge O, Mavingui P, Gouzou L, Hebbar KP, Balandreau J (1994) *Bacillus polymyxa* and *Rahnella aquatilis* the dominant N_2-fixing bacteria associated with wheat rhizosphere in French soils. Europ J Soil Biol 30:35–42
Hill WA, Bacon-Hill P, Crossman SM, Stevens C (1982) Characterization of N_2-fixing bacteria associated with sweet potato roots. Can J Bot 29:860–862
Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary vector strategy based on separation of the *vir* and T-region of the *Agrobacterium tumefaciens* Ti plasmid. Nature 303:179–180
Hwangbo H, Park RD, Kim YW, Rim YS, Park KH, Kim TH, Suh JS, Kim KY (2003) 2-Ketogluconic acid production and phosphate solubilization by *Enterobacter intermedium*. Curr Microbiol 47:87–92
Iijima M, Barlow PW, Bengough AG (2003) Root cap structure and cell production rates of maize (*Zea mays*) roots in compacted sand. New Phytol 160:127–134
Jacobsen BJ, Backman A (1993) Biological and cultural plant disease controls: alternatives and supplements to chemicals in IPM systems. Plant Dis 77:311–315
Jentschke G, Bonkowski M, Godbold DL, Scheu S (1995) Soil protozoa and plant growth: non-nutritional effects and interactions with mycorrhizas. Biol Fert Soil 20:263–269
Johansson JF, Paul LR, Finaly RD (2004) Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiol Ecol 48:1–13
Johnson D, Booth RE, Whiteley AS, Bailey MJ, Read DJ, Grime JP, Leake JR (2003) Plant community composition affects the biomass, activity and diversity of microorganisms in limestone grassland soil. Eur J Soil Sci 54:671–678
Jones DL (1998) Organic acids in the rhizosphere – a critical review. Plant Soil 205:25–44
Jones DL, Darrah PR (1994) Amino-acid influx at the soil-root interface of *Zea mays* L. and its implications in the rhizosphere. Plant Soil 163:1–12
Jones DL, Darrah PR (1996) Re-sorption of organic compounds by roots of *Zea mays* L. and its consequences in the rhizosphere. III. Characteristics of sugar influx and efflux. Plant Soil 178:153–160
Kennedy IR, Tchan YT (1992) Biological nitrogen fixation in non-leguminous field crops: recent advances. Plant Soil 141:93–118
Kent AD, Triplett EW (2002) Microbial communities and their interactions in soil and rhizosphere ecosystems. Ann Rev Microbiol 56:211–236
Kerr A (1969) Transfer of virulence between isolates of *Agrobacterium tumefaciens*. Nature 223:1175–1176
Klironomos JN (2002) Feedback with soil biota contributes to plant rarity and invasiveness in communities. Nature 417:67–70
Kloepper JW (1993) Plant growth-promoting rhizobacteria as biological control agents. In: Metting FBJ (eds) Soil microbial ecology. Marcel Dekker, New York, pp 255–274
Knox OGG, Killham K, Mullins CE, Wilson MJ (2003) Nematode-enhanced microbial colonization of the wheat rhizosphere. FEMS Microbiol Lett 225:227–233

Kuikman PJ, Van Veen JA (1989) The impact of protozoa on the availability of bacterial nitrogen to plants. Biol Fert Soil 8:13–18
Kumar A, Bennetzen JL (1999) Plant retrotransposons. Annu Rev Genet 33:479–532
Larson RI, Neal JLJ (1978) Selective colonization of the rhizosphere of wheat by nitrogen-fixing bacteria. Ecologic Bull 26:331–342
Leeman M, den Ouden FM, van Pelt JA, Dirkx H, Steijl H, Bakker PAHM, Schippers B (1996) Iron availability affects induction of systemic resistance to fusarium wilt of radish by *Pseudomonas fluorescens*. Phytopathology 86:149–155
Lewin B (1990) Genes IV. Oxford University Press, Oxford
Lima E, Boddey RM, Dobereiner J (1987) Quantification of biological nitrogen fixation associated with sugarcane using a ^{15}N aided nitrogen balance. Soil Biol Biochem 19:165–170
Linderman RG (1988) Mycorrhizal interactions with the rhizosphere microflora: the mycorrhizosphere effect. Phytopathology 78:366–371
Linderman RG (1992) Vesicular-arbuscular mycorrhizae and soil microbial interactions. In: Bethlenfalvay GJ, Linderman RG (eds) Mycorrhizae in sustainable agriculture. American Society of Agronomy, Madison, pp 45–70
Lockwood JL (1988) Evolution of concepts associated with soilborne plant pathogens. Ann Rev Phytopathol 26:93–121
Lodwig E, Poole P (2003) Metabolism of *Rhizobium* bacteroids. Crit Rev Plant Sci 22:37–78
Lynch JM (1983) Soil biotechnology. Blackwell Scientific, Oxford
Lynch MR (1995) The rhizosphere. Wiley, Chichester
Marschner P, Crowley D, Yang CH (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant Soil 261:199–208
Martinez-Toledo MV, Gonzalez-Lopez J, de la Rubia T, Moreno J, Ramos-Cormenzana A (1988) Effect of inoculation with *Azotobacter chroococcum* on nitrogenase activity of *Zea mays* roots grown in agricultural soils under aseptic and non-sterile conditions. Biol Fert Soil 6:170–173
Meyer JR, Linderman RG (1986) Response of subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth promoting bacterium, *Psuedomonas putida*. Soil Biol Bioch 18:191–196
Michiels K, Venderleyden J, Van Gool A (1989) *Azospirillium*-plant root associations: a review. Biol Fert Soil 8:356–368
Moghimi A, Tate ME (1978) Does 2-ketogluconate chelate calcium in the pH range 2.4 to 6.4? Soil Biol Bioch 10:289–292
Moghimi A, Tate ME, Oades JM (1978) Characterization of rhizosphere products, especially 2-ketogluconic acid. Soil Biol Bioch 10:283–287
Moore PD (2003) Ecology – roots of diversity. Nature 424:26–27
Murphy PJ, Wexler W, Grzemski W, Rao JP, Gordon D (1995) Rhizopines – their role in symbiosis and competition. Soil Biol Bioch 27:525–529
Myrold DD, Huss-Danell K (2003) Alder and lupine enhance nitrogen cycling in a degraded forest soil in Northern Sweden. Plant Soil 254:47–56
Nehl DB, Allen SJ, Brown JF (1997) Deleterious rhizosphere bacteria: an integrating perspective. Appl Soil Ecol 5:1–20
Nelson AD, Barber LE, Tjepkema J, Russell SA, Powelson R, Evans HJ, Seidler RJ (1976) Nitrogen fixation associated with grasses in Oregon. Can J Bot 22:523–530
Newman EI (1978) Root microorganisms: their significance in the ecosystem. Biol Rev 53:511–54
Newman EI, Campbell R, Rovira AD (1977) Experimental alteration of soil microbial populations for studying effects on higher plant interactions. New Phytol 79:107–118
Newton JA, Fray RG (2004) Integration of environmental and host-derived signals with quorum sensing during plant-microbe interactions. Cell Microbiol 6:213–224

Oger P, Mansouri H, Dessaux Y (2000) Effect of crop rotation and soil cover on alteration of the soil microflora generated by the culture of transgenic plants producing opines. Mol Ecol 9:881–890

Okon Y, Kapulnik Y (1986) Development and function of *Azospirillum*-inoculated roots. Plant Soil 90:3–16

Packer A, Clay K (2004) Development of negative feedback during successive growth cycles of black cherry. Proc R Soc London Biol Sci 271:317–324

Parmar N, Dadarwal KR (1999) Stimulation of nitrogen fixation and induction of flavonoid-like compounds by rhizobacteria. J Appl Microbiol 86:36–44

Patrick WH (1982) Nitrogen transformations in submerged soils. In: Stevenson FJ (ed) Nitrogen in agricultural soils. American Society of Agronomy, Madison, pp 449–465

Patriquin DG, Keddy C (1978) Nitrogenase activity (acetylene reduction) in a Nova Scotian salt marsh: its association with angiosperms and the influence of some edaphic factors. Aquat Bot 4:227–244

Paula MA, Urquiaga S, Siquiera JO, Dobereiner J (1992) Synergistic effects of vesicular-arbuscular mycorrhizal fungi and diazotrophic bacteria on nutrition and growth of sweet potato (*Ipomoea batatas*). Biol Fert Soil 14:61–66

Payne WJ (1973) Reduction of nitrogenous oxides by microorganisms. Bacteriol Rev 37:409–452

Pedersen WL, Chakrabarty K, Klucas RV, Vidaver AK (1978) Nitrogen fixation (acetylene reduction) associated with roots of winter wheat and sorghum in Nebraska. Appl Environ Microbiol 35:129–135

Peoples MB, Craswell ET (1992) Biological nitrogen fixation: Investments, expectations and actual contributions to agriculture. Plant Soil 141:13–39

Piccini D, Azcon R (1987) Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizal fungi on the utilization of Bayobvar rock phosphate by alfalfa plants using a sand-vermiculite medium. Plant Soil 101:45–50

Piceno YM, Lovell CR (2000) Stability in natural bacterial communities: I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. Microbial Ecol 39:32–40

Pierson LS, Wood DW, Pierson EA (1998) Homoserine lactone-mediated gene regulation in plant-associated bacteria. Annu Rev Phtytopath 36:207–225

Pieterse CMJ, van-Wees SCM, Ton J, van-Pelt JA, van-Loon LC (2002) Signalling in rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. Plant Biol 4:535–544

Pirozynski KA, Malloch DW (1975) The origin of land plants: a matter of mycotrophism. BioSystems 6:153–164

Qureshi JA, Zafar Y, Malik KA (1988) *Klebsiella* sp. *NIAB-I*: a new diazotroph, associated with roots of Kallar grass from saline sodic soils. Plant Soil 110:219–224

Rai AN, Soderback E, Bergman B (2000) Cyanobacterium-plant symbioses. New Phytol 147:449–481

Rajaniemi TK, Allison VJ, Goldberg DE (2003) Root competition can cause a decline in diversity with increased productivity. J Ecol 91:407–416

Raju PN, Evans HJ, Seidler RJ (1972) An asymbiotic nitrogen-fixing bacterium from the root environment of corn. Proc Nat Acad Sci 69:3474–3478

Reinhold-Hurek B, Hurek T (2000) Reassessment of the taxonomic structure of the diazotrophic genus *Azoarcus sensu lato* and description of three new genera and new species, *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov. and *Azonexus fungiphilus* gen. nov., sp. nov. Int J Sys Evol Microbiol 50:649–659

Reis VJ, dos Reis FBJ, Quesada DM, de Oliveira OCA, Alves BJR, Urquiaga S, Boddey RM (2001) Bilogical nitrogen fixation associated with tropical pasture grasses. Aust J Plant Physiol 28:837–844

Retallack A (1997) Early forest soils and their role in Devonian global change. Sci 276:583–585
Richardson AE (2001) Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. Aust J Plant Physiol 28:897–906
Richter TE, Ronald PC (2000) The evolution of disease resistance genes. Plant Mol Biol 42:195–204
Rillig MC, Steinberg PD (2002) Glomalin production by an arbuscular mycorrhizal fungus: a mechanism of habitat modification? Soil Biol Biochem 34:1371–1374
Rodger S, Bengough AG, Griffiths BS, Stubbs V, Young IM (2003) Does the presence of detached root border cells of *Zea mays* alter the activity of the pathogenic nematode *Meloidogyne incognita*? Phytopathol 93:1111–1114
Rojas NS, Perry DA, Li CY, Ganio LM (2002) Interactions among soil biology, nutrition, and performance of actinorhizal plant species in the H.J. Andrews Experimental Forest of Oregon. Appl Soil Ecol 19:13–26
Ronn R, McCaig AE, Griffiths BS, Prosser JI (2002) Impact of protozoan grazing on bacterial community structure in soil microcosms. Appl Environ Microbiol 68:6094–6105
Rosswall T (1976) The internal nitrogen cycle between micro-organisms, vegetation and soil. In: Svensson BH, Soderlund R (eds) Nitrogen, phosphorus and sulphu – global cycles. SCOPE report No 7. Ecol Bull 22:157–167
Rosswall T (1983) The nitrogen cycle. In: Bolin B, Cook RB (eds) The major biogeochemical cycles and their interactions, Wiley, Chichester, pp 46–50
Ruppel S (1988) Isolation of diazotrophic bacteria from the endorhizosphere and rhizoplane of winter wheat in a temperate region. Zent Mikrobiol 143:621–629
Sanchez L, Weidmann S, Brechenmacher L, Batoux M, van Tuinen D, Lemanceau P, Gianinazzi S, Gianinazzi-Pearson V (2004) Common gene expression in *Medicago truncatula* roots in response to *Pseudomonas fluorescens* colonization, mycorrhiza development and nodulation. New Phytol 161:855–863
Sarig S, Okon Y (1992) Effect of *Azospirillum brasilense* inoculation in growth dynamics and hydraulic conductivity of *Sorghum bicolor* roots. J Plant Nutr 15:805–819
Savka MA, Dessaux Y, Oger P, Rossbach S (2002) Engineering bacterial competitiveness and persistence in the phytosphere. Mol Plant Microbe Interact 15:866–874
Scheu S (2002) The soil food web: structure and perspectives. Eur J Soil Biol 38:11–20
Schwencke J, Carú M (2001) Advances in actinorhizal symbiosis: host plant-Frankia interactions, biology, and applications in arid land reclamation. A review. Arid Land Res Manag 15:285–327
Simms EL, Bever JD (1998) Evolutionary dynamics of rhizopine within spatially structured rhizobium populations. Proc R Soc London Biol Sci 265:1713–1719
Simms EL, Taylor DL (2002) Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. Integ Compar Biol 42:369–380
Simon HM, Dodsworth JA, Goodman RM (2000) Crenarchaeota colonize terrestrial plant roots. Environ Microbiol 2:495–505
Singh CS (1992) Mass inoculum production of vesicular-arbuscular (VA) mycorrhizae: I. Selection of host in the presence of *Azospirillum brasilense*. Zent Mikrobiol 147:447–453
Smith DC, Douglas AE (1987) The biology of symbiosis. Edward Arnold, London
Smith KA (1990) Anaerobic zones and denitrification in soil: modelling and measurement. In: Revsbech NR, Sorensen J (eds) Denitrification in soil and sediment. Plenum Press, New York, pp 229–244
Smith MS, Teidje JM (1979) The effect of roots on soil denitrification. J Am Soil Sci Soc 43:951–955

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic Press, London
Stefanson RC (1972) Soil denitrification in sealed soil-plant systems I. Effect of plants, soil water content and soil organic matter content. Plant Soil 37:113–127
Stephens PM, Davoren CW, Ryder MH, Doube BM (1993) Influence of the lumbricid earthworm *Aporrectodea trapezoides* on the colonization of wheat roots by *Pseudomonas corrugata* strain 2140r in soil. Soil Biol Bioch 25:1719–1724
Sturz AV, Christie BR (2003) Beneficial microbial allelopathies in the root zone: the management of soil quality and plant disease with rhizobacteria. Soil Till Res 72:107–123
Sturz AV, Nowak J (2000) Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. Appl Soil Ecol 15:183–190
Subba Rao NS (1983) Nitrogen-fixing bacteria associated with plantation and orchard plants. Can J Microbiol 29:863–866
Subba Rao NSS, Tilak KVBR, Singh CS (1985) Synergistic effect of vesicular-arbuscular mycorrhizas and *Azospirillum brasilense* on the growth of barley in pots. Biol Fert Soil 17:119–121
Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis M, Boivan-Masson C, Dreyfus B (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J Bacteriol 183:214–220
Tian CJ, He XY, Zhong Y, Chen JK (2002) Effects of VA mycorrhizae and *Frankia* dual inoculation on growth and nitrogen fixation of *Hippophae tibetana*. For Ecol Manag 170:307–312
Tisdall JM (1991) Fungal hyphae and structural stability of soil. Aust J Soil Res 29:729–743
Torsvik V, Sorheim R, Goksoyr J (1996) Total bacterial diversity in soil and sediment communities – a review. J Ind Microbiol 17:170–178
van Elsas JD, Turner S, Bailey MJ (2003) Horizontal gene transfer in the phytosphere. New Phytol 157:525–537
Vázquez M, Barea J, Azcón R (2001) Impact of soil nitrogen concentration on *Glomus* spp.-*Sinorhizobium* interactions as affecting growth, nitrate reductase activity and protein content of *Medicago sativa*. Biol Fert Soil 34:57–63
Voytas DF, Cummings MP, Konieczny A, Ausubel FM, Rodermel SR (1992) Copia-like retrotransposons are ubiquitous among plants. Proc Natl Acad Sci 89:7124–7128
Wang H, Li J, Bostock RM, Gilchrist DG (1996) Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. Plant Cell 8:375–391
Wei G, Kloepper JW, Tuzun S (1996) Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. Phytopathol 86:221–224
Weller DM (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Ann Rev Phytopath 26:379–407
Whiting PH, Midgley M, Dawes EA (1976) The role of glucose limitation in the regulation of the transport of glucose, gluconate and 2-oxogluconate, and of glucose metabolism in *Pseudomonas aeruginosa*. J Gen Microbiol 92:304–310
Will ME, Sylvia DM (1990) Interaction of rhizosphere bacteria, fertilizer, and vesicular-arbuscular mycorrhizal fungi with sea oats. Appl Environ Microbiol 56:2073–2079
Woldendorp JP (1962) The quantitative influence of the rhizosphere on denitrification. Plant Soil 17:267–270
Woldendorp JP (1963) The influence of living plants on denitrification. Med Land Wagen 63:1–100
Wolter C, Scheu S (1999) Changes in bacterial numbers and hyphal lengths during the gut passage through *Lumbricus terrestris* (Lumbricidae, Oligochaeta). Pedobiologia 43:891–900

Wood CC, Islam N, Ritchie RJ, Kennedy IR (2001) A simplified model for assessing critical parameters during associative $^{15}N_2$ fixation between *Azospirillum* and wheat. Aust J Plant Physiol 28:969–974

Wright SF, Upadhyaya A (1998) A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant Soil 198:97–107

Zimmer W, Bothe H (1988) The phytohormonal interactions between *Azospirillum* and wheat. Plant Soil 110:239–247

Zuberer DA, Silver WS (1978) Biological dinitrogen fixation (acetylene reduction) associated with Florida mangroves. Appl Environ Microbiol 35:567–575

Zupan J, Muth TR, Draper O, Zambryski P (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. Plant J 23:11–28

7 Interactions Among Beneficial Microorganisms

Anil Kumar Saxena, Rasika Shende, Minakshi Grover

7.1 Introduction

The plant rhizosphere is a unique environment characterized by the continuous supply of low molecular weight compounds exuded from the roots. The rhizosphere supports a large and metabolically active microbial population that can be several orders of magnitude higher than in non-rhizosphere soil (Schloter et al. 1997). Interactions between the plant and microorganisms and among rhizosphere microorganisms are largely unknown and recent works show that these interactions are complex and are dependent on multiple traits (Lugtenberg and Dekkers 1999). Although the rhizosphere appears to be too complex to allow its manipulation, specific bacteria can be applied to seed or roots, which cause an alteration in the composition of the rhizosphere. In addition to the manipulation of the microorganisms to discourage disease causing organisms, it should be possible to promote the activity of beneficial ones, such as arbuscular mycorrhizal (AM) fungi, plant growth promoting rhizobacteria (PGPR) and *Rhizobium* sp. Thus the focus of attention has now shifted from plant-microbe interactions to plant-microbe-microbe interactions. Attempts have been made not only to highlight increase in biological activities in two or three membered associations of organisms (Barea et al. 2004) but also to decipher the mechanisms involved in such interactions (Ma et al. 2003). Such syntropic associations are of ecological importance with implied agricultural significance. In the present review, an attempt has been made to discuss interactions among certain groups of beneficial microorganisms like AM fungi, *Rhizobium* sp. and PGPR.

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7.2
Interaction Between *Rhizobium* and PGPR

Rhizospheric bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Azorhizobium* can interact with roots of legumes to form nodules, which function as sites for atmospheric nitrogen fixation (Relic et al. 1994). Root nodule formation is a complex developmental process involving sequential exchange of chemical signals between the bacterial microsymbiont and the host plant (Long 1996). Both plant and bacterial signals have been identified and shown to play a major role in the specificity of the legume-*Rhizobium* interaction (Downie 1994).

7.2.1
Rhizobium and Nodulation Promoting Rhizobacteria

Specific microorganisms have considerable potential to alter the composition and activity of rhizosphere microflora such as *Rhizobium* (Schroth and Ole Becker 1990). Bacteria that promote nodulation of legumes by rhizobia are referred to as nodulation promoting rhizobacteria (NPR) (Kloepper et al. 1988). These NPR belong to diverse group of microorganisms and include *Azospirillum*, *Azotobacter*, *Pseudomonas* (Villacieros et al. 2003), *Bacillus*, *Streptomyces* (Samac et al. 2003), *Serratia* and *Aeromonas*.

Coinoculation studies with PGPR and *Rhizobium* have been shown to increase root and shoot dry weight, plant vigour, nitrogen fixation and nodule number in various legumes such as alfalfa (Knight and Langston-Unkefer 1988), common bean (Grimes and Mount 1984), green gram (Gupta et al. 1998), pigeon pea (Podile 1995), chickpea (Sindhu et al. 2002), pea (Bolton et al. 1990), soybean (Dashti et al. 1998), cowpea (Agarwal and Tilak 1989), clovers (Burns et al. 1981) and Sesbania (El-Gamai 1992). The beneficial effects of these bacteria have been variously attributed to their ability to produce different compounds including phytohormones (Molla et al. 2001), toxins (Knight and Langston-Unkefer 1988) and antibiotics (Li and Alexander 1990) to suppress deleterious rhizobacteria (Turner and Beckman 1991) or through some other unidentified mechanism (Halverson and Handelsman 1991). Recently it has been shown that the presence of 1-aminocyclopopane-1-carboxylated deaminase (ACC deaminase) enzyme in *Rhizobium leguminosarum* bv. *viceae* enhances the nodulation of *Pisum sativum*, likely by modulating ethylene levels in the plant roots during the early stages of nodule development (Ma et al. 2003). Insertion mutants, with mutations in the rhizobial ACC deaminase gene (acdS) and its regulatory gene, that could not synthesize ACC deaminase showed decreased nodulation efficiency compared to that of the parental strain. In bean

plants, nodulation was increased when *Rhizobium* sp. were coinoculated with *Bacillus polymyxa* (Peterson et al. 1996) or *Azospirillum brasilense* (Burdman et al. 1996). Burdman et al. (1996) related *A. brasilense* mediated stimulation in nodulation of common bean to an increased production of flavonoids by the legume host. Presence of rhizobacteria, belonging to fluorescent *Pseudomonas* and *Bacillus*, in the rhizosphere of chickpea enhanced the level of flavonoid-like compounds in the roots, suggesting that the rhizobacteria have a direct influence on root flavonoids which might be an additional factor in nodule promotion by these bacteria (Parmar and Dadarwal 1999). It was shown that coinoculation of *Rhizobium etli* TAL 182 with *Bacillus* sp. induced root hair proliferation in *Phaseolus vulgaris* and enhanced nodulation of *P. vulgaris* by *Rhizobium* (Srinivasan et al. 1996). Coinoculation with both bacterial species also facilitated heterologous nodulation of *Rhizobium* Tal 182 on *Phaseolus acutifolius* (Srinivasan et al. 1996). *Rhizobium etli* is a narrow host range microsymbiont specific for nodulation on *P. vulgaris* whereas *P. acutifolius* belongs to the cowpea cross inoculation group and has a very specialized bradyrhizobial requirement for nodulation (Somasegaran et al. 1991). Heterologous nodulation of *P. acutifolius* by *R. etli* TAL 82 coinoculated with *B. megaterium* S49 implicates the *Bacillus* more directly in the nodulation process. Similar heterologous nodulation on hairy roots of clover plants by a non-specific symbiont *Rhizobium leguminosarum* bv. *viceae* has been reported (Diaz et al. 1995). In contrast, *Bacillus* sp. CECT 450 that increased nodulation on bean when co inoculated with *Bradyrhizobium tropici* CIAT 899 reduced nodulation on soybean when coinoculated with *B. japonicum* USDA 110 (Camacho et al. 2001). Likewise, *Bacillus cereus* UW85 increased nodulation in soybean indirectly by increasing root growth and not stimulating the nodulation process. However the bacterium had little to no positive effects on pea and common bean symbiosis (Vessey and Buss 2002).

Inoculation of soybean crops with effective *B. japonicum* strains singly or in combination with PGPR strains was found to be important for improving and maximizing the plant growth and nitrogen fixation potential of the crop either in soil which lacks indigenous population of *Bradyrhizobium* sp. or in those soils high in indigenous population, but less effective than the introduced bacteria (Kucey 1988). Dual inoculation with a mixture of *B. japonicum* and *Azospirillum brasilense* was superior over single inoculation with *B. japonicum* with regards to nitrogen fixation and dry biomass of soybean (Galal 1997). Molla et al. (2001) found *A. brasilense* to perform better in root growth and nodule development of soybean compared to *A. lipoferum*. Coinoculation of *Serratia proteamaculans* 1–102 and *S. liquefaciens* 2–68 with *B. japonicum* on soybean not only improved nodule number, plant dry weight and fixed nitrogen but also shortened the time for nodule initiation and increased the nodulation rate (Bai et al. 2002).

The influence of rhizospheric bacteria on the competitive ability of the introduced strain is another interesting area where definite conclusions are yet to be made. Singh and Gaur (1995) reported two strains of rhizospheric bacteria that further improves the nodulation as well as competitiveness of an effective strain of chickpea-*Rhizobium*. It was demonstrated that the rhizospheric isolates produce flavonoid like substances which in turn induced nod gene expression. *Enterobacter* isolates EG-ER-2 and KG-ER-1 improved the nodule occupancy of *Bradyrhizobium* strain S 24 and COG 15 respectively (Gupta et al. 1998). Both isolates produced antibiotics and siderophores that might have inhibited other rhizospheric rhizobia enabling the inoculant bradyrhizobial strains to occupy successfully the nodulation sites.

7.2.2 *Rhizobium* and Biocontrol Agents

Most rhizobacteria used as biocontrol agents have been shown to stimulate legume-*Rhizobium* symbiosis. In contrast several reports suggest inhibition of rhizobia by rhizospheric bacteria on agar plates. Smith and Miller (1974) showed that eight out of nine rhizospheric bacteria inhibited *B. japonicum* on agar plates. Their study also revealed that, as a group, rhizosphere organisms were more inhibitory to *B. japonicum* than non-rhizospheric organisms. In another study, of the 115 rhizospheric rizobacteria tested, 23 inhibited one or more strains of *B. japonicum*. All of the rhizospheric bacteria that inhibited bradyrhizobia produced fluorescent, yellow green, diffusible pigments and were classified as fluorescent *Pseudomonas*. Most of the inhibitory effects of *Pseudomonas* sp. on bradyrhizobia were caused by siderophore-induced iron deprivation (Fuhrmann and Wollum 1989). Three *P. fluorescens* strains UP61, UP143 and UP 148 did not modify the shoot dry weight and rate of nodulation of birdsfoot trefoil, despite antagonistic activity against rhizobia in vitro (De la Fuente et al. 2002). Cattelan et al. (1999) found several rhizospheric isolates which stimulated aspects of soybean-bradyrhizobia symbiosis and which had β-glucanase or cyanide production. These attributes are required for biocontrol of pathogenic fungi but their exact role in stimulation of symbiosis is not clear. In another study inoculation of HCN producing strain of *P. fluorescens* F113rif alone had a deleterious effect on alfalfa plants grown in gnotobiotic systems. However this effect was reversed due to coinoculation with *Sinorhizobium meliloti* EFB1 (Villacieros et al. 2003). Different strains of antibiotic producing *Streptomyces* used to control leaf spot in alfalfa could inhibit in vitro growth of *S. meliloti* but did not significantly reduce the number of nodules. However there was an adverse affect on the growth of plant. It

was postulated that the number of *S. meliloti* per nodule or their metabolic activity might have been reduced leading to diminished nitrogen fixation. It is also possible that the *Streptomyces* strains produced plant growth-inhibiting compounds (Samac et al. 2003). However, there is a report on the increase in nodulation frequency, possibly at the level of infection by *Rhizobium* sp. on inoculation of root colonizing actinomycetes *Streptomyces lydicus*. It was shown that *S. lydicus* colonizes and then sporulates within the surface layers of the nodules. Colonization leads to an increase in the average size of the nodules that form and improves the vigour of bacteroids within the nodules by enhancing nodular assimilation of iron and possibly other soil nutrients (Tokala et al. 2002). Another interesting mechanism of enhancement of growth, nodulation and nitrogen fixation of alfalfa has been reported for toxin releasing *Pseudomonas* pv. *tabaci*, a tobacco leaf pathogen. The toxin, tabtoxinine-β-lactam, inactivates selectively one form of glutamine synthetase in the nodules. Thus, normal glutamine synthetase-catalysed ammonia assimilation is significantly impaired, yet these plants assimilated about twice the amount of nitrogen (Knight and Langston-Unkefer 1988). These increases as well as concomitant decrease in glutamine synthetase activity are observed only in plants infested with pv. *tabaci*. Higher assimilation of nitrogen even in the presence of low amounts of glutamine synthetase suggests the operation of alternative routes of ammonia assimilation like through glutamate dehydrogenase and asparagine synthetase. It was further demonstrated that the changes in glutamine synthetase activity in nodules and roots of pv. *tabaci*-infested plants resulted in altered glutamate and glutamine pools; these changes may collectively influence nitrogen fixation and assimilation as well as nodulation in these infested plants. In another study the impact of biological control strain *Pseudomonas fluorescens* CHAO and its genetically modified, antibiotic over-producing derivative CHAO/pME3424 on a reconstructed population of *Sinorhizobium meliloti* bacteria was assessed in gnotobiotic systems. In sterile soil there was growth inhibition and the population of *S. meliloti* declined due to production of pyoluteorin. In plant tests, inoculation of over producing derivative did not affect nodulation, however, lack of plant growth promotion was observed. In contrast, addition of wild type strain CHAO significantly improved shoot dry weight of alfalfa plants (Nieman et al. 1997). Stimulation of plant growth by coinoculation of alfalfa with *S. meliloti* and *P. fluorescens* has also been reported previously (Liste 1993). *Pseudomonas* species isolated from the rhizosphere of green gram could inhibit the growth of several pathogenic fungi and enhanced the nodule number and growth of green gram and chickpea on coinoculation with specific rhizobia (Sindhu et al. 1999, 2002).

7.3 Interaction Between AM Fungi and *Rhizobium*

Associative effects of AMF with rhizobia have been reviewed earlier (Saxena et al. 2002) and reported for crop legumes like soybean (Zhao et al. 1997); fababeans (Ishac et al. 1994); Medicago (Azcon et al. 1991); green gram (Saxena et al. 1997) and tree legumes like *Anthyllis cystisoides* (Requena et al. 1997); *Leucaena leucocephala* (Dixon et al. 1993); *Sesbania* (Sengupta and Choudhuri 1995) and *Prosopis* (Dixon et al. 1993).

7.3.1 Influence on Nodulation and Nitrogen Fixation

Nodule number and biomass has been shown to increase significantly in several studies due to coinoculation of both microsymbionts (Saxena et al. 1997; Zhao et al. 1997). In *Acacia laeta* dual inoculation of *Rhizobium* and *Glomus mosseae* resulted in 176 and 305% increase in nodule biomass and number of nodules respectively (Badji et al. 1989). In black locust *(Robinia pseudoacacia)* mycorrhizal colonization increased nodule biomass, nodule number and N content by 78, 48 and 300% respectively (Olesniewicz and Thomas 1999). Sreenivasa et al. (1995) reported that AM fungi assist nodulation by *Bradyrhizobium japonicum* in soybean whereas *Astragalus sinicum* has been shown to be completely dependent on AM formation for nodulation (Zhao et al. 1997). However, *Glomus etunicatum* colonized soybean plants with the highest nodule numbers have been shown to have the shortest colonized root lengths (44%) while high levels of AM root colonization (76%) in *Glomus mosseae* and *Gigaspora rosea* treated plants were accompanied by low nodule counts indicating antagonism between the two symbionts (Schreiner et al. 1997). Such negative relationships between rhizobia and AM fungi have been related to competition for nutrients (Bethlenfalvay 1992) and to selective compatibilities between the microsymbionts of the legume association (Azcon et al. 1991).

7.3.2 Specific Compatibility and Yield

Like all symbiotic parameters, yield of legumes coinoculated with AM and rhizobia has been reported to increase significantly when compared to uninoculated or inoculated with either microsymbiont (Corbera and Hernandez 1997). However neutral or negative response to inoculation has also been reported (Saxena et al. 1997). Several studies have shown

that there is competition for carbon between plants, mycorrhiza and bacteria in symbiotic nitrogen fixing systems (Michelsen and Sprent 1994). For example, in soybean seedlings, development of both microsymbionts is reduced in the tripartite association when compared with plants colonized by only one symbiont (Brown and Bethlenfalvay 1986, 1987). It is believed that mycorrhizal roots constitutes a stronger sink for assimilates than non-mycorrhizal roots (Smith and Gianinazzi-Pearson 1988). Such negative interaction could be due to specific compatibilities between the two microsymbionts (Saxena et al. 1997). Pacovsky (1986) also claimed that by changing *Bradyrhizobium* strain, mycorrhizal plants have shown improvement in the yield of soybean.

Selective interactions between the two endosymbionts can enhance the yield of crop plants. Combinations of *Bradyrhizobium japonicum* ICA-8001 gave best results for soybean in combination with *G. fasciculatum* (Corbera and Hernandez 1997). In a recent study, pea plants were inoculated with the AMF species *Glomus clarum* NT4 or *G. mosseae* NY6 and/or ten *Rhizobium* strains. The growth and yield response of pea to coinoculation with AMF and *Rhizobium* strains depended on the particular AMF-*Rhizobium* combination. Careful co selection of AMF species and *Rhizobium* strains enhanced pea yield and nutrition (Xavier and Germida 2003). Specific compatibilities have also been reported for other hosts like *Anthyllis cystisoides* (Requena et al. 1997); *Medicago arborea* (Valdenegro et al. 2001); kidney bean (Daniels-Hylton and Ahmad 1994) green gram (Saxena et al. 1997) and *Acacia tortilis* (Andre et al. 2003).

Plant or genotype dependent functional specificity between the microsymbionts has also been reported (Chang et al. 1992). The symbiotic parameters and yield of soybean cv. Ludous improved with a combination of *Rhizobium* 2048 and *Glomus versiforme* whereas that of cultivar zhongdous 14 improved with *Rhizobium* 61A76 and *Glomus mosseae* (Chang et al. 1992). Such plant mediated interactions could be dependent on differences in the metabolic impact of the microsymbionts on plant metabolism (Brown et al. 1988).

Specific compatibility between microsymbionts can exist both in terms of competitive ability of introduced rhizobia and percent root colonization by introduced AM fungi (Saxena et al. 1997). Thiagarajan and Ahmad (1993) reported that in the presence of AM fungus (*Glomus pallidum*), introduced strains of *Bradyrhizobium* become more competitive than native rhizobia to nodulate cowpea. Nodulation competitiveness of introduced *Bradyrhizobium* sp. (*Vigna*) strain S24 was significantly higher (60–65%) in the presence of *Glomus mosseae, G. fasiculatum* and *Scutellospora calospora* when compared to treatment with single inoculation of S24 (50%) (Saxena et al. 1997). They further reported that percentage AM colonization was higher in treatments having higher nodule occupancy of introduced strain.

7.3.3
Interaction Under Stress

The microbial-based approach, particularly use of AM fungi and *Rhizobium* for legumes has been evaluated and recommended for reclamation or for rehabilitation of desertified ecosystems (Requena et al. 1997), wastelands (Bhatia et al. 1998), saline soils (Azcon and Atrash 1997) and drought prone soils (Goicoechea et al. 1998). *Rhizobium leguminosarum* bv. *viceae* strain 102 F84 and AM inoculation in desert soils of Egypt (calcareous soils) improved growth of fababeans to a much greater extent than that can be attributed to either inoculum on a singular basis (Ishac et al. 1994). Requena et al. (1997) showed that compatible combination of AM fungi, *Rhizobium* and PGPR strain can improve plant performance in nutrient-deficient and degraded habitats. The results of a four-year trial showed that inoculation with selected rhizobia and mycorrhizal fungi improved outplanting performance, plant survival and biomass development of woody legumes in a desertified Mediterranean ecosystems (Herrera et al. 1993). Goicoechea et al. (1998) reported that symbiotic alfalfa plants are better adapted than non-symbiotic ones to cope with water deficit. It was found that symbiotic plants maintained higher polyamine (spermidine and spermine) concentrations than non-symbiotic ones under water stress.

In saline soils, the high concentration of salts is detrimental to the growth of both plant and microorganisms. Dual inoculation with AM fungi and rhizobia may help *Leucaena* and *Prosopis* species mitigate the adverse effects of NaCl on juvenile growth and development (Dixon et al. 1993; Ahmad 1996). Under salt stress, nodules formed in mycorrhizal root system were generally more abundant than the P-fertilized plants (Azcon and Atrash 1997). In stressed soils, the symbiosis with both endophytes enhances the ability of the plant to become established and cope with stress situations (nutrient deficiency, drought, trace element imbalance, soil disturbance).

7.4
Interaction of AM Fungi with PGPR

7.4.1
Mycorrhiza Development and Population Density of PGPR

AM fungi are obligate symbionts and, to date, all attempts to culture it in artificial media have failed. They survive, multiply and colonize in and around the root system. In other words, they share the common habitat that is the root surface with the plant growth promoting rhizobacteria. In the process of sharing a niche, they are bound to interact with each other and these

interactions can range from beneficial to neutral to harmful. These rhizobacteria can influence the AM formation and function, and, conversely, mycorrhizas can affect the microbial population. Various spore associated bacteria including *Pseudomonas* and *Corynebacterium* have been reported to stimulate the spore germination in case of *Glomus versiforme* (Mayo et al. 1986). Cell free extracts of free living nitrogen fixing microorganisms like *Azospirillum* and *Azotobacter* significantly enhanced the spore germination of *Glomus fasciculatum*. However, non-nitrogen fixing organisms except *Pseudomonas putida* and *P. fluorescens* did not show significant increase in spore germination (Tilak et al. 1990). In contrast strains of *Pseudomonas cepacia* R85 and *P. putida* were shown to inhibit the germination of spores of *Glomus clarum* NT4 (Walley and Germida 1997). Mycorrhiza-helper bacteria are known to stimulate mycelial growth and enhance mycorrhizal formation (Gryndler et al. 2000). Soil microorganisms can produce compounds that increase root cell permeability, increase the rates of root exudation and in turn stimulate mycorrhizal fungal mycelia in the rhizosphere or facilitate root penetration by the fungus (Barea 2000). *Glomus fasciculatum* in association with *Zea mays* or *Trifolium subterraneum* reduced the viable counts of fluorescent pseudomonads (FP) but increased total bacterial numbers compared to non AM plants (Meyer and Linderman 1986), while the total viable counts of bacteria in the rhizoplane of guinea grass was reduced by *Acaulospora laevis* but increased by *G. fasciculatum* (Secilia and Bagyaraj 1987). Since microbial activity in soil is stimulated by root exudates, root colonization by AM fungi may alter bacterial growth by changing exudation patterns (Azaizeh et al. 1995). It was reported that the mycorrhizal status of soils might selectively influence persistence of bacterial inoculants as well as affect the number of other native bacteria (Andrade et al. 1998). The population density of *Pseudomonas fluorescens* 2–79RL declined by 50% and its physiological status by one order of magnitude due to root system mycorrhizal with *Glomus deserticola*. In contrast, for *Glomus intraradices*, both the population density and the physiological status of *P. fluorescens* decreased on the mycorrhizal and non-mycorrhizal side by one order of magnitude. Thus some mycorrhizal fungi can reduce both the population density and the physiological status of certain bacterial groups in the rhizosphere (Marschner and Crowley 1996).

7.4.2 Interaction of AMF and PGPR in Plant Growth Promotion

Crop productivity is usually limited by nitrogen and phosphorus availability, particularly in the tropical soils. Recent research in association between AM fungi and free living nitrogen fixers suggests that these endophytes may

provide enough phosphorus and nitrogen to enhance the growth and the yield of cereals in marginal environments. Probiotic influence of associative nitrogen fixers and those of mycorrhizal fungi in major agricultural crops have been reviewed (Barea et al. 2004). In sorghum, pearlmillet and wheat coinoculation of *Azospirillum* and AMF significantly increased the growth and chlorophyll content and mycorrhizal infection of the roots (Walley and Germida 1997). However no significant effect of coinoculation was reported on nitrogen nutrition of C_3 and C_4 plants (Barea and Azcon-Aguilar 1983). Synergism of *Azotobacter* and AM fungi have also been cited in the literature (Toro et al. 1997, 1998). Dual inoculation of *A. chroococcum* and *G. fasciculatum* enhanced root infection of AM fungi, stimulated the plant growth, and increased shoot N, Ca, Mg and K in luxor tomatoes (El-Shanshoury et al. 1989). Improved growth and yield of lettuce plants in both sterile and non-sterile soils due to simultaneous inoculation with AMF and *A. chroococcum* has also been reported (Brown and Carr 1984). The synergistic host response could be mainly due to the production of phytohormones or growth regulators by these microbes rather than the small increases in N and P availability (Barea et al. 1975).

The phosphate solubilizing microorganisms (PSM) interacts well with the AMF in P deficient soils or soils amended with rock phosphate (Poi et al. 1989). The PSM can release some phosphate from otherwise sparingly soluble phosphate sources and it was postulated that AM fungal hyphae can tap these ions and translocate them to the plant. Nevertheless, Azcon-Aguilar et al. (1986) did not find that PSM improved the utilization by mycorrhizal plants of a labelled source (^{32}P–^{45}Ca-tricalcium phosphate) of insoluble phosphate added to the soil. Inoculation of Neem (*Azadirachta indica*) seedlings with *Azospirillum brasilense* and phosphate-solubilizing bacteria (PSB) in presence of AMF *Glomus intraradices* and *G. geosporum* resulted in increased mycorrhizal colonization, greater plant height, leaf area and number, root collar diameter, biomass, phosphorus, nitrogen and potassium content, and seedling quality. Inoculated seedlings also had low root/shoot ratios and low nutrient utilization efficiencies. Populations of PSB declined with seedling growth; contrarily populations of *A. brasilense* increased. *A. brasilense* and PSB populations were related to each other and influenced root colonization by AM fungi. Microbial inoculation effects were greatest when seedlings were inoculated with a combination of microbes rather than individually. This clearly indicates that these microorganisms act synergistically when inoculated simultaneously, with maximum response being when both AM fungi were coinoculated with *A. brasilense* and PSB. The results emphasize the importance of microbial inoculations for the production of robust, rapidly growing seedlings in nurseries and illustrate the advantage of inoculating soils of a low microbial population with indigenous microbes (Muthukumar et al. 2001).

Even under adverse environmental conditions or in a polluted environment, coinoculation of PGPR and AMF can be beneficial. Of the two bacterial strains isolated from lead (Pb) polluted soil, strain A (*Brevibacillus*) alone or when coinoculated with AMF enhanced plant growth of *Trifolium pratense* L., nitrogen and P accumulation, nodule formation and mycorrhizal infection. It also decreases the amount of Pb absorbed by the plants (Vivas et al. 2003). Interaction of *Rhizobium* with AMF has been discussed in an earlier section. However, there are reports on plant growth promoting activity of *Rhizobium* in the rhizosphere of non-leguminous crops (Noel et al. 1996). There is also evidence that *Rhizobium* strains were able to colonize the rhizosphere of non-legume hosts where they establish positive interactions with AM fungi and behave as PGPR (Galleguillos et al. 2000).

In fruit crops like papaya, dual inoculation of *Azospirillum* and AMF enhanced the total dry matter and leaf area than non-colonized plants (Alarcon et al. 2002). Coinoculation effects were significant even in field-established papaya and mulberry. *Bacillus coagulans* when co-inoculated with a mixture of *Glomus fasciculatum* and *G. caledonium* increase the leaf yield in ten-year-old mulberry and fruit yield in 1.5-year-old papaya (Mamatha et al. 2002).

7.5 Conclusions

The rhizosphere is the site of intense and complex microbial activity. Microbes interact with each other and contribute to soil fertility and crop productivity. In recent years emphasis on the use of two or more microorganisms has been made with the aim of maximizing beneficial plant growth responses. It is important, therefore, to identify the best strains of beneficial microbes for the planting situation, verify their compatibility and combined efficacy, both in vitro and in vivo, and employ this combination inoculum to real agricultural situation as part of the management and production practices. Compatible combinations have been tested in field, yield increases have been attained but with limited success and consistency. Inconsistency in field experimentation can be due to poor understanding of edaphic factors, composition of native microflora and even inoculum dosage. In addition the level of interaction between the beneficial microorganisms is still an unsolved mystery. The interactions can be at genetical, molecular or metabolic level. One of the most exciting discoveries in the area of symbiotic plant-microbe interactions has been the identification of common genetic determinants underlying AM and *Rhizobium* symbioses (Duc et al. 1989). Similar studies on other systems will throw light on specific interaction among microorganisms. Recently it has been reported

that there is common gene expression in *Medicago truncatula* roots in response to *Pseudomonas fluorescens* colonization, mycorrhiza development and nodulation by *Sinorhizobium meliloti*. This report supports the hypothesis that some plant cell programmes may be shared during root colonization by these beneficial microorganisms. Thus research should be directed towards deciphering the intricacies of plant-microbe-microbe interaction at the genetic level.

References

Agarwal R, Tilak KVBR (1989) *Azospirillum brasilense* and *Rhizobium* sp. (cowpea misc) inoculum effect on yield and nitrogen uptake of various minor millets. Indian J Microbiol 29:229–232

Ahmad AE (1996) Effect of salinity and vesicular arbuscular mycorrhizal fungi on growth and symbiotic properties of faba beans (*V. faba*). Khartoum, Sudan, p 110

Alarcon A, Davies FT Jr, Egilla JN, Fox TC, Estrada-Luna AA, Ferrera-Cerrato R (2002) Short term effects of *Glomus claroideum* and *Azospirillum brasilense* on growth and root acid phosphatase activity of *Carica papaya* L. under phosphorus stress. Microbiologia 44:31–37

Andrade G, De Leij FAAM, Lynch JM (1998) Plant mediated interactions between *Pseudomonas fluorescens, Rhizobium leguminosarum* and arbuscular mycorrhizae on pea. Lett Appl Microbiol 26:311–316

Andre S, Neyra M, Duponnois R (2003) Arbuscular mycorrhizal symbiosis changes the colonization pattern of *Acacia tortilis* bv. *radiana* rhizosphere by two strains of rhizobia. Microb Ecol 45:137–144

Azaizeh HA, Marschner A, Romheld V, Wittenmayer L (1995) Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth, mineral nutrient acqusition and root exudation of soil grown maize plants. Mycorrhiza 5:321–327

Azcon R, El-Atrash E (1997) Influence of arbuscular mycorrhizae and phosphorus fertilization on growth, nodulation and N_2 fixation (^{15}N) in *Medicago sativa* at four salinity levels. Biol Fertil Soils 24:81–86

Azcon R, Rubio R, Barea JM (1991) Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N_2 fixation (^{15}N) and nutrition of *Medicago sativa* L. New Phytol 117:399–404

Azcon-Aguilar C, Gianinazzi-Pearson V, Fardeau JC, Gianinazzi S (1986) Effect of vesicular-arbuscular mycorrhizal fungi and phosphote-solubilizing bacteria on growth and nutrition os soybean in a neutral calcareous soil amended with ^{32}P–^{45}Ca-tricalcium phosphate. Plant Soil 96:3–15

Badji S, Ducousso M, Thoen D, Colonna JP (1989) Effects of double inoculation with *Rhizobium-Glomus mosseae* on the nodulation and growth of young *Acacia laetea*. In: Tress for development in sub-Saharan Africa. Proceedings of a regional seminar held by the International Foundation of Science (IFS), ICRAF House, Nairobi, Kenya, Feb 20–25, pp 323–329

Bai Y, Pan B, Charles TC, Smith DL (2002) Co-inoculation dose and root zone temperature for plant growth promoting rhizobacteria on soybean [*Glycine max* (L.) Merr] grown in soil-less media. Soil Biol Biochem 34:1953–1957

Barea JM (2000) Rhizosphere and mycorrhiza of field crops. In: Toutant JP, Balazs E, Galante E, Lynch JM, Schepers JS, Werner D, Werry PA (eds) Biological resource management

connecting science and policy. (OECD) INRA Editions and Springer, Berlin Heidelberg New York, pp 110–125
Barea JM, Azcon-Aguilar C (1983) Myocrrhizas and their significance in nodulating, nitrogen fixing plants. Agronomy Adv 36:1–54
Barea JM, Azcon R, Hayman DS (1975) Possible synergistic interactions between *Endogyne* and phosphate-solubilizing bacteria in low phosphate soils. In: Sanders FE, Mose B, Tinker PB (eds) Endomycorrhizas. Academic Press, London, pp 409–417
Barea JM, Azcon R, Azcon-Aguilar C (2004) Mycorrhizal fungi and PGPR. In: Kamp RM, Calvete JJ, Choli-Papadopoulou T (eds). Principles and practice: methods in proteome and protein analysis. Springer, Berlin Heidelberg New York
Bethlenfalvay GJ (1992) Vesicular-arbuscular mycorrhizal fungi in nitrogen-fixing legumes: problems and prospects. Methods Microbiol 24:375–389
Bhatia NP, Adholeya A, Sharma A (1998) Biomass production and changes in soil productivity during long term cultivation of *Prosopis juliflora (Swartz)* DC inoculated with VA mycorrhiza and *Rhizobium* spp. in semi-arid wasteland. Biol Fertil Soils 26:208–214
Bolton H Jr, Elliott LF, Turco RF, Kennedy AC (1990) Rhizoplane colonizing of pea seedlings by *Rhizobium leguminosarum* and a deleterious root colonizing *Pseudomonas* sp. and effects on plant growth. Plant Soil 123:121–124
Brown ME, Carr GR (1984) Interactions between *Azotobacter chroococcum* and VAM and their effect on plant growth. J Appl Bacteriol 56:429–437
Brown MS, Bethenfalvay GJ (1986) The *Glycine-Glomus-Rhizobium* symbiosis II. Endophyte effects on leaf carbon, nitrogen and phosphorus nutrition. J Plant Nutr 9:1199–1212
Brown MS, Bethenfalvay GJ (1987) The *Glycine-Glomus-Rhizobium* symbiosis. VI. Photosynthesis in nodulated, mycorrhizal, or N- and P-fertilized soybean plants. Plant Physiol 85:120–123
Brown MS, Thamasurakul S, Bethlenfalvay GJ (1988) The *Glycine-Glomus-Bradyrhizobium* symbiosis VIII. Phosphorus-use efficiency of CO_2 and N_2 fixation in mycorrhizal soybean. Physiol Plant 74:159–163
Burdman S, Volpin H, Kigel J, Kapulnik Y, Okon Y (1996) Promotion of nod gene inducers and nodulation in common bean (*Phaseolus vulgaris*) roots inoculated with *Azospirillum brasilense* Cd. Appl Environ Microbiol 62:3030–3033
Burns TA, Bishop PE, Israd DW (1981) Enhanced nodulation of leguminous plant root by mixed cultures *Azotobacter vinelandii* and *Rhizobium*. Plant Soil 62:399–412
Camacho M, Santamaria C, Temprano F, Rodriguez-Navarro DN, Daza A (2001) Co-inoculation with *Bacillus* sp. CECT 450 improves nodulation in *Phaseolus vulgaris* L. Can J Micobol 47:1058–1062
Cattelan AJ, Hartel PG, Fuhrmann JJ (1999) Screening for plant growth promoting Rhizobacteria to promote earley soybean growth. Soil Sci Soc Am J 63:1670–1680
Chang CY, Lu ZH, Dai SJ, Zhao CJ, Guo DX (1992) Studies on selection of the best symbiotic system of high yielding and high protein content soyabean, high efficient N fixing *Rhizobium* and mycorrhiza fungus. Soybean Sci 11:226–233
Corbera J, Hernandez A (1997) Assessment of the effects of the rhizobia-vesicular arbuscular mycorrhiza association on growth and development of soybeans (*Glycine max. L.* Merrill). Cultivos-Tropicales 18:10–12
Daniels-Hylton KDM, Ahmad MH (1994) Inoculation response in kidney beans (*Phaseolus vulgaris* L.) to vesicular-arbuscular mycorrhiza fungi and rhizobia in non-sterilized. Soil Biol Fertil Soils 18:95–98
Dashti N, Zhang F, Hynes RK, Smith DL (1998) Plant growth promoting rhizobacteria accelerate nodulation and increase nitrogen fixation activity by field grown soybean [*Glycine max* (L.) Merr.] under short season conditions. Plant Soil 200:205–213

De la Fuente L, Quagliotto L, Bajsa N, Fabiano E, Altier N, Arias A (2002) Inoculation with *Pseudomonas* biocontrol strains does not affect symbiosis between rhizobia and forage leumes. Soil Biol Biochem 34:545–548

Diaz CL, Spaink HP, Wijffelman CA, Kijne JW (1995) Genomic requirements of *Rhizobium* for nodulation of white clover hairy roots transformed with the pea lectin gene. Mol Plant-Microbe Interact 8: 348–356

Dixon RK, Garg VK, Rao MV (1993) Inoculation of *Leucaena* and *Prosopis* seedlings with *Glomus* and *Rhizobium* species in saline soils: rhizosphere relations and seedling growth. Arid Soil Res Rehabilit 7:133–140

Downie JA (1994) Signalling strategies for nodulation of legumes by rhizobia. Trends Microbiol 2:318–324

Duc G, Trouvelot A, Gianinazzi-Pearson V, Gianinazzi S (1989) First report of non-mycorrhizal, plant mutants (myc^{--}) obtained in pea (*Pisum sativum* L.) and fababean (*Vicia faba* L.). Plant Sci 60:215–222

El-Gamai MS (1992) Interactions between *Azotobacter* spp. and *Rhizobium sesbani* in the rhizosphere of *Sesbania sesban* (L.) Merril plants and its efficiency on growth and symbiotic nitrogen fixation. Zbl Mikrobiol 147:112–118

El-Shanshoury AR, Hasan MA, Abdel-Ghaffar BA (1989) Synergistic effects of VAM and *Azotobacter chroococcum* on the growth and the nutrient contents of tomato plants. Phyton (Horn) 29:203–212

Fuhrmann J, Wollum AG II (1989) In vitro growth responses of *Bradyrhizobium japonicum* to soybean rhizosphere bacteria. Soil Biol Biochem 21:131–135

Galal YGM (1997) Dual inoculation with strains of *Bradyrhizobium japonicum* and *Azospirillum brasilense* to improve growth and biological nitrogen fixation of soybean (*Glycine max* L.). Biol Fertil Soil 24:317–322

Galleguillos C, Aguirre C, Barea JM, Azcon R (2000) Growth promoting effect of two *Sinorhizobium meliloti* strains (a wild and its genetically modified derivative) on a non-legume plant species in specific interaction with two arbuscular mycorrhizal fungi. Plant Sci 159:57–63

Goicoechea N, Szalai G, Antolin MC, Sanchez-Diaz M, Diaz M, Paldi E (1998) Influence of arbuscular mycorrhizae and *Rhizobium* on free polyamines and proline level in water stressed alfalfa. J Plant Physiol 153:706–711

Grimes HD, Mount MS (1984) Influence of *Pseudomonas putida* on nodulation of *Phaseolus vulgaris*. Soil Biol Biochem 16:27–30

Gryndler M, Hrselova H, Striteska D (2000) Effect of soil bacteria on growth of hyphae of the arbuscular mycorrhizal (AM) fungus *Glomus claroideum*. Folia Microbiol 45:545–551

Gupta A, Saxena AK, Gopal M, Tilak KVBR (1998) Enhanced nodulation of green gram by introduced *Bradyrhizobium* when co-inoculated with plant growth promoting rhizobacteria. J Sci Indust Res 57:720–725

Halverson LJ, Handelsman J (1991) Enhancement of soybean-nodulation by *Bacillus cereus* UW85 in the field and in growth chamber. Appl Environ Microbiol 57:2767–2770

Herrera MA, Salamanca CP, Barea JM (1993) Inoculation of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified mediterranean ecosystems. Appl Environ Microbiol 59:129–133

Ishac YZ, Angle JS, El-Borollosy MA, El-Demerdash ME, Mostafa MI, Fares CN (1994) Growth of *Vicia faba* as affected by inoculation with vesicular arbuscular mycorrhizae and *Rhizobium leguminosarum* bv. *viceae* in two soils. Biol Fertil Soils 17:27–31

Kloepper JW, Hume DT, Scher FM, Singleton C, Tipping B, Laliberte M, Franley K, Kutchaw T, Simonson C, Lifshitz R, Zaleska I, Lee L (1988) Plant growth promoting rhizobacteria on canola (rapeseed). Plant Dis 72:42–46

Knight TJ, Langston-Unkefer PJ (1988) Enhancement of symbiotic dinitrogen fixation by a toxin releasing plant pathogen. Science 241:951–954

Kucey RMN (1988) Alteration of size of wheat root systems and nitrogen fixation by associative nitrogen-fixing bacteria measured under field conditions. Can J Microbiol 34:735–739

Li De M, Alelxander M (1990) Factors affecting co-inoculation with antibiotic producing bacteria to enhance rhizobial colonization and nodulation. Plant Soil 129:195–201

Liste HH (1993) Stimulation of symbiosis and growth of lucerne by combined inoculation with *Rhizobium meliloti* and *Pseudomonas fluorescens*. Zentralbl Mikrobiol 148:163–176

Long SR (1996) *Rhizobium* symbiosis: nod factors in perspective. Plant Cell 8:1885–1898

Lugtenberg BJJ, Dekkers LC (1999) What makes *Pseudomonas* bacteria rhizosphere competent? Environ Microbiol 1:9–13

Ma W, Guinei FC, Glick BR (2003) *Rhizobium leguminosarum* biovar *viciae* 1-aminocyclopropane-1-carboxylate deaminase promotes nodulation of pea plants. Appl Environ Microbiol 69:4396–4402

Mamatha G, Bagyaraj DJ, Jaganath S (2002) Inoculation of field-established mulberry and papaya with arbuscular mycorrhizal fungi and a mycorrhiza helper bacterium. Mycorrhiza 12:313–316

Marschner P, Crowley DE (1996) Root colonization of mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum*) by *Pseudomonas fluorescens* 2–79RL. New Phytol 134:115–122

Mayo K, Davis RE, Hotta J (1986) Stimulation of germination of spores of *Glomus versiforme* by spore associated bacteria. Mycologia 78:426–431

Meyer JR, Linderman RG (1986) Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed *by Glomus fasciculatum*. Soil Biol Biochem 18:191–196

Michelsen A, Sprent J (1994) The influence of vesicular arbuscular mycorrhizal fungi on the nitrogen fixation of nursery grown *Ethiopian acacias* estimated by the ^{15}N natural abundance method. Plant Soil 160:249–257

Molla AH, Shamsuddin ZH, Halimi MS, Morziah M, Pateh AB (2001) Potential for the enhancement of root growth and nodulation of soyben co-inoculated with Azospirillum and Bradyrhizobium in laboratory systems Soil Biol Biochem 33:457–463

Muthukumar T, Udaiyan K, Rajeshkannan V (2001) Response of neem (*Azadirachta indica* A. Juss) to indigenous arbuscular mycorrhizal fungi, phosphate-solubilizing and asymbiotic nitrogen-fixing bacteria under tropical nursery conditions. Biol Fertil Soils 34:417–426

Niemann S, Keel C, Puhler A, Selbitschka W (1997) Biocontrol strain *Pseudomonas fluorescens* CHAO and its genetically modified derivative with enhanced biocontrol capability exert comparable effects on the structure of *a Sinorhizobium meliloti* population in gnotobiotic systems. Biol Fertil Soils 25:240–244

Noel TC, Sheng C, Yost CK, Pharis RP, Hynes MF (1996) *Rhizobium leguminosarum* as a plant growth promoting rhizobacterium: direct growth promotion of canola and lettuce. Can J Microbiol 42:279–283

Olesniewicz KS, Thomas RB (1999) Effects of mycorrhizal colonization on biomass production and nitrogen fixation of black locust (*Robinia pseudoacacia*) seedlings grown under elevated atmospheric carbon dioxide. New Phytol 142:133–140

Pacovsky RS (1986) Micronutrient uptake and distribution in mycorrhizal and phosphorus fertilized soybeans. Plant Soil 95:379–385

Parmar N, Dadarwal KR (1999) Stimulation of nitrogen fixation and incubation of flavonoid-like compounds by rhizobacterium. J Appl Microbiol 86:36–44

Podile AR (1995) Seed bacterization with *Bacillus subtilis* AF1 enhances seedling emergence, growth and nodulation of pigeonpea. Indian J Microbiol 35:199–204

Poi SC, Ghosh G, Kabi MC (1989) Response of chickpea (*Cicer arietinum* L.) to combined inoculation with *Rhizobium*, phosphobacteria and mycorrhizal organisms. Zentralbl Microbiol 114:249–253

Relic B, Perret X, Estrada-Garcia MT, Kopcinska J, Golinowski W, Krishnan HB, Pueppke SG, Broughton WJ (1994) Nod factors of *Rhizobium* are a key to the legume door. Mol Microbiol 13:171–178

Requena N, Jimenez I, Toro M, Barea JM (1997) Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in meditenanean semi-arid ecosystems. New Phytol 136:667–677

Samac DA, Willert AM, McBride MJ, Kinkel LL (2003) Effects of antibiotic producing *Streptomyces* on nodulation and leaf spot in alfalfa. Appl Soil Ecol 22:55–66

Saxena AK, Rathi SK, Tilak KVBR (1997) Differential effect of various endomycorrhizal fungi on nodulating ability of green gram by *Bradyrhizobium* sp. (*Vigna*) strain S 24. Biol Fertil Soils 24:175–178

Saxena AK, Shende R, Tilak KVBR (2002) Interaction of arbuscular mycorrhiza with nitrogen fixing bacteria. In: Sharma AK, Johri BN (eds) Arbuscular mycorrhizae: interactions in plants, rhizosphere and soils, Scientific Publishers, USA, pp 47–68

Schloter M, Wiehe W, Assmus B, Steindl H, Bekke H, Hoflick G, Hartman A (1997) Root colonization of different plants by plant-growth promoting *Rhizobium leguminosarum* bv. *trifolii* R39 studied with monospecific polyclonal antisera. Appl Environ Microbiol 63:2038–2046

Schreiner RP, Mihara KL, Mc Daniel H, Bethlenfalvay GJ (1997) Mycorrhizal fungi influence plant and soil functions and interactions. Plant Soil 188:199–209

Schroth MN, Ole Becker J (1990) Concepts of ecological and physiological activities of rhizobacteria related to biological control and plant growth promotion. In: Hornby D (ed) Biological control of soil-borne plant pathogens. CAB International, Wallingford, UK, pp 389–414

Secilia J, Bagyaraj DJ (1987) Bacteria and actinomycetes associated with pot cultures of vesicular-arbuscular mycorrhizas. Can J Microbiol 33:1069–1073

Sengupta A, Choudhuri S (1995) Effect of dual inoculation of *Rhizobium* and mycorrhiza on growth response of *Sesbania grandiflora* L. in coastal saline and sand dune soil. Ind J Forestry 18:35–37

Sindhu SS, Gupta SK, Dadarwal KR (1999) Antagonistic effect of *Pseudomonas* spp. on pathogenic fungi and enhancement of plant growth in green gram (*Vigna radiata*). Biol Fertil Soils 29:62–68

Sindhu SS, Suneja S, Goel AK, Parmar Nand, Dadarwal KR (2002) Plant growth promoting effects of Pseudomonas sp. on coinoculation with *Mesorhizobium* sp. *Cicer* strain under sterile and 'wilt sick' soil conditions. Appl Soil Ecol 19:57–64

Singh S, Gaur YD (1995) Soil microbes as inducers of nod-gene expression. Indian J Microbiol 35:317–325

Smith S, Gianinazzi-Pearson V (1988) Physiological interactions between symbionts in vesicular-arbuscular mycorrhizal plants. Ann Rev Plant Physiol Plant Mol Biol 39:221–244

Smith RS, Miller RH (1974) Interactions between *Rhizobium japonicum*, and soybean rhizosphere bacteria. Agronomy J 66:564–567

Somasegaran P, Hoben HJ, Lewinson L (1991) Symbiosis interactions *of Phaseolus acutifolius* and *P. acutifolius* x *P. vulgaris* hybrid progeny in symbiosis with *Bradyrhizobium* spp. and *Rhizobium leguminosarum* bv. *Phaseoli*. Can J Microbiol 37:497–503

Sreenivasa MN, Basavarga GT, Kulkarni JM (1995) Vesicular-arbuscular mycorrhiza assist in nodulation and N_2-fixation in soybean. J Maharashtra Agric Univ 20:292–293

Srinivasan M, Petersen DJ, Holl EB (1996) Influence of indole acetic acid producing *Bacillus* isolates on the nodulation of *Phaseolus vulgaris* by *Rhizobium etli* under gnotobiotic conditions. Can J Microbiol 42:1006–1014

Thiagarajan TR, Ahmad MH (1993) Influence of a VAM on the competitive ability of *Bradyrhizobium* spp. for nodulation of cowpea (*Vigna unguiculata* L. Walp) in non-sterilized soil. Biol Fertil Soils 12:112–116

Tilak KVBR, Dwivedi A, Li CY (1990) Enhancement of spore germination of *Glomus fasciculatum* by bacterial cell free extracts. Indian J Exp Biol 28:373–375

Tokala RK, Strap JL, Jung CM, Crawford DL, Salove MH, Deobald LA, Bailey JF, Morra MJ (2002) Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). Appl Environ Microbiol 68:2161–2171

Toro M, Azcon R, Barea JM (1997) Improvement of arbuscular mycorrhiza development by inoculation os soil with phosphate solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient recycling. Appl Environ Microbiol 63:4408–4412

Toro M, Azcon R, Barea JM (1998) The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotype, mycorrhizal fungi, phosphate-solubilizing rhizobacteria and rock phosphate on nitrogen and phosphorus aquisition by *Medicago sativa*. New Phytol 138:265–273

Turner JT, Backman PA (1991) Factors relating to peanut yield increases following *Bacillus subtilis* seed treatment. Plant Dis 75:347–353

Valdenegro M, Barea JM, Azcon R (2001) Influence of arbuscular-mycorrhizal fungi, *Rhizobium meliloti* strains and PGPR on the growth of *Medicago arborea* used as model legume for re-vegetation and biological reactivation in a semi-arid mediterreanean area. Plant Growth Regulation 34:233–240

Vessey JK, Buss TJ (2002) *Bacillus cereus* UW85 inoculation effects on growth, nodulation, and N accumulation in grain legumes: controlled-environment studies. Can J Plant Sci 82:282–290

Villacieros M, Power B, Sanchez-Contreras M, Lloret J, Cruezabal RI, Martin M, Fernande-Pinas F, Bonilla I, Wheln C, Dowling DN, Riilla R (2003) Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. Plant Soil 251:47–54

Vivas A, Azcon R, Biro B, Barea JM, Ruiz-Lozano JM (2003) Influence of bacterial strains isolated from lead-polluted soil and their interations with arbuscular mycorhizae on the growth of *Trifolium pratense* L. under lead toxicity. Can J Microbiol 49:577–588

Walley FL, Germida JJ (1997) Response of spring wheat (*Triticum aestivum*) to interactions between *Pseudomonas* species and *Glomus clarum* NT4. Biol Fertil Soils 24:365–371

Xavier LJC, Germida JJ (2003) Selective interactions between arbuscular mycorrhizal fungi and *Rhizobium leguminosarum* bv. *viceae* enhance pea yield and nutrition. Biol Fertil Soils 37:261–267

Zhao B, Trouvelot A, Gianianzzi S, Gianinazzi-Pearson V (1997) Influence of two legume species on hyphal production and activity of two arbuscular mycorrhizal fungi. Mycorrhiza 7:179–185

8 Bacterial Community Composition and Activity in Rhizosphere of Roots Colonized by Arbuscular Mycorrhizal Fungi

Petra Marschner, Sari Timonen

8.1 Introduction

Rhizosphere microbial communities can be regarded as a subset of the soil microbial community. As the root tip grows through the soil, microorganisms in its pathway will be the first colonisers. During rapid root growth, the zone of elongation immediately behind the root tips is only sparsely colonised by soil microorganisms. Thereafter, microbial population densities increase rapidly in the zone behind the root tips, where high concentrations of soluble, insoluble and volatile root exudates can be utilised for microbial growth and metabolism. In contrast, along the older root parts, the compounds present in the rhizosphere are dominated by cellulose and other recalcitrant cell wall materials from sloughed root cortex tissues. Here the population density is often lower than in the younger root regions closer to the rot tip. The species composition of microbial communities in rhizosphere differs from that in the bulk soil (Foster 1986; Marilley and Aragno 1999). This is a clear indication that plants have a strong influence on the microbial populations on their roots. Indeed, in many cases the rhizosphere communities of different plant species growing in the same soil are distinct (Ibekwe and Kennedy 1998) and plants may even have very similar microbial community composition in different soils (Grayston et al. 1998; Miethling et al. 2000).

Plant roots release 1–25% of the net photosynthesis as soluble and insoluble compounds into the rhizosphere (Merbach et al. 1999). Among rhizosphere microbial ecologists there is currently a consensus that differences in exudate amount and composition are likely to affect microbial community composition because microbial species differ in their ability to metabolise and compete for different carbon sources. Therefore, differences in exudate amount and composition will affect the competitiveness and hence the survival of microbial species. A wide range of factors have

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been shown to affect root exudation, including plant genotype (Rovira 1959; Rengel 1997; Grayston et al. 1998), plant age (Martin 1971; van Veen et al. 1991; Marschner et al. 2001), nutritional status (Hoffland et al. 1989; Liljeroth et al. 1990; Marschner and Crowley 1998; Fan et al. 2001) and colonization by mycorrhizal fungi (Po and Cumming 1997; Marschner et al. 1997). In addition to being easily available substrates for soil microorganisms, certain components of root exudates can also have a selective influence on rhizosphere microorganisms by repelling some species and increasing the competitive ability of others (Geurts and Franssen 1996).

Mycorrhizal plants transfer more assimilates to the roots than non-mycorrhizal plants (Wang et al. 1989; Eissenstat et al. 1993), which may be explained by the carbon demand of the fungus for growth and respiration (Fitter 1991; Kucey and Paul 1982). Arbuscular mycorrhizal (AM) colonization has been shown to decrease root exudation (Dixon et al. 1989; Graham et al. 1981; Marschner et al. 1997) although no effect on exudation has also been reported (Azaizeh et al. 1995). Mycorrhizal colonization may also affect root exudate composition (Marschner et al. 1997; Po and Cumming 1997) and the carbohydrate metabolism of the roots (Buwalda and Goh 1982; Shachar-Hill et al. 1995). These changes could be related to the carbon uptake by the fungus and/or the effect of mycorrhizal colonization on host plant physiology. And mycorrhizal fungi themselves may release exudates that selectively influence the microorganisms in the rhizosphere. Exudates from mycorrhizal fungi have not yet been investigated in detail; however there are reports of compounds such as glomalin, which may increase soil aggregation (Rillig et al. 2002). As microorganisms are mainly found in soil aggregates, glomalin can have positive effects on microbial population density (Andrade et al. 1998b).

Mycorrhizal colonization could also indirectly affect the microbial community in the rhizosphere by its effects on root morphology (Berta et al. 1990, 1993; Hetrick 1991), rhizosphere pH (Bago and Azcón-Aguillar 1997), nutrient content (Li et al. 1991) and enzyme activity (Tarafdar and Marschner 1994) as well as on soil structure (Neergaard-Bearden and Petersen 2000; Rillig et al. 2002; Tisdall 1991). As discussed in Chap. 9, the hyphae of AM fungi create an additional habitat for soil microorganisms which is distinct from that of the rhizosphere of non-mycorrhizal roots and will exert its own influence on the microbial communities.

Creating a mycorrhizosphere habitat may be beneficial for mycorrhizal fungi because some microorganisms specific for the rhizosphere of mycorrhizal roots can stimulate mycorrhizal formation and change mycorrhizal gene expression (Becker et al. 1999; Poole et al. 2001). If plant growth is increased by certain rhizosphere microorganisms this would also benefit the AM fungus because the larger plants could supply the fungus with more carbohydrates.

In this chapter, we will outline the effect of AM colonization on bacterial rhizosphere colonization, community structure and activity and the possible causes of these effects.

8.2 Rhizosphere Colonization by Bacteria

8.2.1 Soil Bacteria

AM colonization affects the colonization pattern of roots by bacteria, resulting in a greater spatial variability of bacterial distribution on AM roots (Christensen and Jacobsen 1993). It can increase the population density of bacteria in the rhizosphere (Abdel-Fatah and Mohamedin 2000; Andrade et al. 1998a; Bagyaraj and Menge 1978; Medina et al. 2003; van Aarle et al. 2003), have no effect on bacterial density (Andrade et al. 1997; Mansfeld-Giese et al. 2002; Meyer and Lindermann 1986; Olsson et al. 1998), or decrease it (Ames et al. 1984; Christensen and Jakobsen 1993). These apparently contradictory findings may be due to AM fungal species-specific interactions, because, as shown in a number of studies (Krishnaraj and Sreenivasa 1992; Marschner et al. 2001; Marschner and Baumann 2003; Secilia and Bagyaraj 1987), AM fungal species differ in their effect on the microorganisms in the rhizosphere. There are also indications that the interactions between AM and rhizosphere bacteria are plant species-specific (Marschner and Timonen 2004; Medina et al. 2003; Vancura et al. 1989) (Fig. 8.1). AM

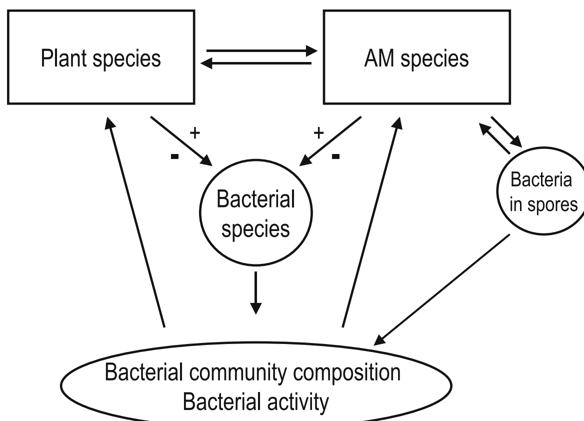

Fig. 8.1. Interactions between plant species, AM colonization and bacteria in the rhizosphere (see text for details)

colonization may also indirectly affect bacteria via changes in population density of bacterial predators such as protozoa (Wamberg et al. 2003).

Compared to non-mycorrhizal roots, roots colonized by AM fungi offer soil microorganisms an additional habitat: the extraradical fungal structures (see Chap. 9). Bianciotto et al. (1996) showed that bacteria form biofilms on spores and hyphae, indicating that these fungal structures can be important habitats for soil bacteria. Our own results (Marschner and Timonen 2004) suggest that the bacterial community colonizing the external mycelium of the outer mycorrhizosphere may be different from that colonizing the inner mycorrhizosphere of AM plants.

8.2.2
Pseudomonads

Of the many soil bacterial genera, the interactions between AM colonization and pseudomonads has received the most interest. The reasons for this are that pseudomonads are considered to be typical rhizosphere bacteria, easily cultured in laboratory media and many are known to be pathogens, biocontrol agents or plant growth-promoting bacteria.

AM colonization often decreases the rhizosphere colonization by *Pseudomonas* sp. (Marschner and Crowley 1996; Marschner et al. 1997; Meyer and Linderman 1986; Paulitz and Linderman 1989). In the study by Ravenskov et al. (1999), the population density of a fluorescent pseudomonad was not affected by AM colonization but its culturability was decreased; suggesting that the cells are more starved in the rhizosphere of AM roots (Ramos et al. 2000). This confirmed the studies by Marschner and Crowley (1996) and Marschner et al. (1997) who reported that AM colonization can reduce the physiological activity of a fluorescent pseudomonad in the rhizosphere. Ravnskov et al. (1999) found that their isolate did not attach to AM hyphae. AM fungal species differ in their suppressive effect towards *Pseudomonas* sp. (Marschner and Crowley 1996; Marschner et al. 1997; Paulitz and Linderman 1989). However, Paulitz and Linderman (1989) argued that this apparent AM fungal species effect may be related to differences in the extent of root colonization by the different AM fungal species. Hence, AM species with a greater percentage root length colonized would be expected to have a stronger suppressive effect.

8.2.3
N_2 Fixing Bacteria and P Solubilizers

Only very few studies have examined the effect of AM colonization on associative N_2 fixing bacteria. Klyuchnikov and Kozhevin (1990) found that

AM colonization increased the rhizosphere population density of *Azospirillum brasiliense*. Far more studies investigated the interactions between AM colonization and the agronomically very important symbiotic N_2 fixers.

In legumes colonized by AM fungi and *Rhizobium* both symbionts represent a significant carbon sink (Kucey and Paul 1982) and competition for host carbohydrates may explain negative interactions between AM fungi and *Rhizobium*. For example, Reinhard et al. (1992) found that the presence of *Rhizobium* decreased AM colonization. The competition between the two symbionts may be particularly expressed under low light conditions when less carbohydrates are translocated to the roots (Bethlenfalvay et al. 1982).

On the other hand, positive interactions between AM and *Rhizobium* have also been frequently reported. AM colonization can increase nodulation (Abbott and Robson 1977) and enhance plant yield and N uptake (Barea et al. 1987; Xavier and Germida 2003). AM colonization can also stimulate colonization of alder by *Frankia*, the N_2 fixing actinomycete (Fraga-Beddiar and Le Tacon 1990).

The apparent contrasting results, negative interactions on the one hand and positive interactions on the other, could be due to compatibility between the two symbionts as well as between the microbial partners and the host plant. Evidence for the former was given by Xavier and Germida (2003), who showed recently that some combinations of AM fungal species and *Rhizobium* species had a negative effect on yield and N uptake while others have a positive effect. The importance of the combination of AM species and N_2 species was also evident in a study by Subba Rao et al. (1985) where the extent of synergism between *A. brasiliense* and AM colonization in terms of plant growth strongly depended on the AM fungal species. The contrasting results may also be due to the benefit gained by AM colonization for P uptake. Under conditions of low P supply, AM colonization can increase P supply to plants and nodules and thus positively affect nodulation and N_2 fixation. If P is not limiting growth of plants and *Rhizobium*, AM fungi will represent a carbon drain with little or no benefit. Then, negative interactions between *Rhizobium* and AM colonization may be expected. It should, however, be noted that AM fungi not only increase uptake of P, but also of other poorly mobile nutrients such as Zn (George et al. 1994; Ryan 2003) and can improve soil structural stability (Andrade et al. 1998b; Neergaard-Bearden and Petersen 2000), thus improving plant growth and thereby also carbohydrate supply to *Rhizobium*. Additionally, AM colonization can suppress soil-borne plant pathogens (see Chap. 9), which would also result in improved plant growth. Positive effects of AM on *Rhizobium* could be related to the suppression of microorganisms that inhibit root colonization by *Rhizobium,* while negative interactions may be expected if such microorganisms are stimulated by the presence of AM.

Many soil microorganisms can increase the solubility of sparingly soluble P minerals. If AM fungi have a stimulating effect on such microorganisms, plant P uptake could potentially be increased. Indeed, AM colonization can increase the population density of P solubilizers in the rhizosphere and co-inoculation of plants with AM fungi and P solubilizers can increase plant P uptake compared to inoculation with AM fungi alone (Sreenivasa and Krishnaraj 1992) or P solubilizers alone (Azcón et al. 1976).

8.2.4 Biological Control Organisms

Biocontrol agents such as pseudomonads, which produce antibiotics or lyse fungal cell walls could potentially have a negative impact on mycorrhizal colonization. There are reports that biocontrol agents such as *Azospirillum*, *Pseudomonas* or *Trichoderma* have no negative effect on AM colonization (Barea et al. 1998; Vazquez et al. 2000). On the other hand, Wyss et al. (1992) showed that two biological control organisms, *Trichoderma harzianum* and *Streptomyces griseoviridis*, decreased colonization by *Glomus mosseae*. This was also the case for *Glomus intraradices* (Green et al. 1999). With respect to the effect of AM colonization on biocontrol organisms, Green et al. (1999) showed that *G. intraradices* decreased the population density and activity of *Trichoderma harzianum*. From the data presented so far in this chapter, it seems very likely that the interaction between biocontrol agents with AM fungi would by highly specific for a given biocontrol agent/AM fungus combination which may be further affected by the plant species.

8.3 Bacterial Community Composition

The studies with single isolates suggest that the density of some bacterial species is lower in AM roots than in non-mycorrhizal roots. However, these studies are highly artificial because only one bacterial species is used. This is in contrast to the soil environment, where the microbial community is highly complex and consists of species with different growth rates and substrate preferences. Nevertheless, experiments conducted with complex microbial communities show that AM colonization can change the bacterial community composition in the rhizosphere by stimulating the population density of certain bacterial species or functional groups, while depressing others (Amoralazcano et al. 1998; Andrade et al. 1997; Marschner et al. 2001; Marschner and Baumann 2003; Meyer and Linderman 1986; Posta et al. 1994; Secilia and Bagyaraj 1987; Wamberg et al. 2003) (Table 8.1). In many

Table 8.1. Interactions between plant species, AM colonization and bacteria in the rhizosphere (see text for details)

Microorganism	AM fungus	Effect of AM	Reference
Indigenous bacteria	*Indigenous fungi*	Increase	van Aarle et al. (2003)
Gram-positive *Bacillus* sp	*Glomus sp.*	Decrease	Secilia and Bagyaraj (1987)
	Glomus intraradices	Decrease	Posta et al. (1994)
Gram-negative	*Glomus sp.*	Increase	Secilia and Bagyaraj (1987)
	Glomus mosseae	Increase	Posta et al. (1994)
Fluorescent pseudomonads	*Glomus desterticola*	Decrease	Marschner et al. (1997); Vázquez et al. (2000)
	Glomus fasciculatus	Decrease	Meyer and Linderman (1986)
	Glomus mosseae	Decrease	Waschkies et al. (1994); Posta et al. (1994); Vázquez et al. (2000); Marschner et al. (1997)
Azotobacter	*Glomus fasciculatus*	Increase	Bagyaraj and Menge (1978)
Azospirillum brasiliense	*Glomus mosseae*	Increase	Klyuchnikov and Kozhevin (1990)
Actinomycetes	*Glomus fasciculatus*	Increase	Bagyaraj and Menge (1978)
	Glomus intraradices	Increase	Abdel-Fatah and Mohamedin (2000)
	Glomus mosseae	Increase	Posta et al. (1994)
	Glomus sp.	Increase	Secilia and Bagyaraj (1987)
Streptomyces sp	*Glomus deserticola*	No effect	Vázquez et al. (2000)
	Glomus fasciculatus	Decrease	Meyer and Linderman (1986)
	Glomus mosseae	No effect	Vázquez et al. (2000)
Ammonia oxidizers	*Glomus mosseae*	Increase	Amoralazcano et al. (1998)
	Glomus fasciculatus	Increase	Amoralazcano et al. (1998)
Ammonifiers	*Glomus fasciculatus*	Decrease	Amoralazcano et al. (1998)
	Glomus mosseae	Decrease	Amoralazcano et al. (1998)
Chitinase producers	*Glomus fasciculatus*	Decrease	Meyer and Linderman (1986)
Mn reducers	*Glomus mosseae*	Increase	Posta et al. (1994); Kothari et al. (1991)
N_2 fixers	*Glomus sp.*	Increase	Secilia and Bagyaraj (1987)
Nitrifiers	Glomus fasciculatus	Decrease	Amoralazcano et al. (1998)
	Glomus mosseae	Decrease	Amoralazcano et al. (1998)

studies it has been shown that the population density of Gram-negative bacteria (Secilia and Bagyaraj 1987; Posta et al. 1994) and actinomycetes (Bagyaraj and Menge 1978; Abdel-Fatah and Mohamedin 2000) is increased in the rhizosphere of AM roots. Kothari et al. (1991) and Posta et al. (1994) found that AM colonization increased the population density of Mn reducers in the rhizosphere, thus increasing Mn availability to the plants and plant Mn uptake. In agreement with the studies with single isolates mentioned above, fluorescent pseudomonads, are generally inhibited by AM colonization (Meyer and Linderman 1986; Posta et al. 1994; Waschkies et al. 1994). In the study by Waschkies et al. (1994) inoculation with AM fungi was associated with an alleviation of grape vine replant disease and the authors argued that this was due to the decreased population density of fluorescent pseudomonads which appear to be one of the causative agents of the disease.

AM colonization can also affect microorganisms involved in N mineralisation in soil. The population density of ammonia oxidizers was higher, while those of ammonifiers and nitrifiers was lower in pot cultures of *Glomus mosseae* and *G. fasciculatum* than in non-mycorrhizal pot cultures (Amoralazcano et al. 1998).

It should be noted that most studies investigating the effect of AM colonization on bacteria in the rhizosphere have relied on culture-dependent methods such as dilution plating. However, less than 5% of soil microorganisms are assessed with culture-dependent methods (Bakken 1985). The main reasons for this low recovery are that (i) many microbial species do not grow or grow very slowly on conventional culture media (Janssen et al. 2002) and (ii) a large fraction of cells are in a viable but non-culturable state (Oliver 1993) or in a state of starvation (Ramos et al. 2000) and therefore do not form visible colonies on standard laboratory media.

Bacterial community composition as affected by AM colonization has also been studied using culture-independent methods such as those based on differences in gene sequence or fatty acid profiles. In maize, Marschner et al. (2001) showed that the bacterial community composition in the rhizosphere (assessed by denaturing gradient gel electrophoresis) of plants inoculated with *Glomus mosseae* or *G. intraradices* differed from that of non-mycorrhizal plants. The two fungal species differed in their effect on bacterial community composition and this was not related to the P status of the plant. In this study, the effect of AM colonization was more pronounced after six weeks than after three weeks and during this time the percentage root length colonized by the fungi increased more than twofold. This suggests that the extent of AM effect maybe related to the percent root length colonized (Paulitz and Linderman 1989). However, we found recently that AM colonization had a strong effect on bacterial community composition in canola (*Brassica napus*) with less than 10% of root length colonized while

it had no effect in clover (*Trifolium subterraneum*) where more than 50% root length were colonized (Marschner and Timonen 2004). Hence, the bacterial community composition can even be affected when only a small fraction of the root system is colonized by AM fungi.

The results of a study with split-root maize plants (Marschner and Baumann 2003) indicate that the AM effect is, at least in part, plant-mediated because AM colonization changed the bacterial community composition in the rhizosphere on both the root half colonized by AM and the non-mycorrhizal half. In agreement with the earlier study (Marschner et al. 2001), the rhizosphere bacterial community composition was fungal species-specific.

On the other hand, there are reports that AM colonization has no effect on bacterial community composition (Mansfeld-Giese et al. 2002; Olsson et al. 1996; Søderberg et al. 2002). As mentioned above, these contrasting results indicate that the effect may be fungal species-specific (Marschner and Baumann 2003; Marschner et al. 2001; Secilia and Bagyaraj 1987) or plant species-specific (Vancura et al. 1989). This is supported by our own results (Marschner and Timonen 2004), which showed complex interactions of plant and AM fungal species on the bacterial community composition in the rhizosphere.

8.4 Bacterial Activity

As mentioned above, AM colonization can either increase N_2 fixation (Azcon et al. 1991; Barea et al. 1987) or decrease it (Reinhard et al. 1992). Bethlenfalvay et al. (1982) showed that although nodule dry weight may be decreased by AM colonization under low light conditions, specific activity of nodules (N_2 fixation per dry weight of nodule) is increased. Thus, the development of nodules was inhibited by the presence of AM fungi, but once the nodules had reached maturity they were capable of competing effectively with the AM fungus for host assimilates and may have benefited from the improved P nutrition of AM plants. This suggests that the interactions between AM fungi and *Rhizobium* are complex and may change during the development of the nodules.

Besides the well-studied effects on N_2 fixation there are only a limited number of studies that have examined the effect of AM colonization on other microbial activities in the rhizosphere. AM colonization increased chitinase activity in the rhizosphere (Abdel-Fatah and Mohamedin 2000), suggesting that the presence of AM hyphae stimulates the capacity of the rhizosphere microflora to decompose fungal cell walls. Christensen and Jacobsen (1993) found that AM colonization decreased the growth rate of

bacteria in the rhizosphere. In agreement, the studies by Marschner and Crowley (1996) and Marschner et al. (1997) indicate that AM colonization induces a state of starvation in a genetically modified bioluminescent pseudomonad and the degree of inhibition was fungal species-specific. Søderberg et al. (2002) showed that this decrease may be also be plant-species specific.

The increased P uptake by plants inoculated by both P solubilizers and AM fungi compared to inoculation with each microorganism separately (Azcon-Aguilar et al. 1986b; Sreenivasa and Krishnaraj 1992) suggests that presence of AM fungi may stimulate the activity of P solubilizers.

8.5 Effects of Bacteria on AM Fungi

The effects of bacteria on AM fungi will only be briefly outlined here. For a more detailed discussion the reader is referred to Duponnois (Chap. 15) on mycorrhizal helper bacteria.

Certain bacterial species may stimulate AM spore germination (Azcon-Aguilar et al. 1986a, Hildebrand et al. 2002), AM colonization (Fester et al. 1999; Vivas et al. 2003) or the proliferation of the extraradical mycelium (Gryndler et al. 2002). It appears, however, that this effect is bacterial and fungal species-specific (Gryndler et al. 2002; Medina et al. 2003). Bacteria isolated from spores can also inhibit spore germination (Xavier and Germida 2003).

8.6 Bacteria in AM Fungi

Evidence is now emerging that bacteria can also live within AM fungi. Bacteria-like objects (BLOs) in AM fungi were first reported by Mosse (1970) and MacDonald et al. (1983). More recently it was confirmed that these BLOs are indeed bacteria (Scannerini and Bonfante 1991). Xavier and Germida (2003) found Gram-negative and Gram-positive bacteria on the surface of AM spores, but only Gram-positive bacteria within the spores. Bacteria appear to colonize fungal spores intracellularly (Bianciotto et al. 2000), where they are associated with protein and lipid bodies (Minerdi et al. 2002). AM hyphae also contain bacteria intracellularly (Bianciotto et al. 1996).

Some bacterial species found in spores seem to be ubiquitous; they are found in the spores of the same fungal species isolated from different areas (Minerdi et al. 2002) and even in spores of different AM fungal species

(Bianciotto et al. 2000). Minerdi et al. (2002) argued that this suggests co-evolution of the intracellular bacteria and AM fungi. These bacteria may be transferred from one generation of AM fungi to the next via asexual AM spores. Interestingly, the spores of some AM species do not seem to contain bacteria (Bianciotto et al. 2000).

The information on the bacterial species found in AM fungi is just emerging. *Burkholderia* sp. appear to be one of the major groups of bacteria found inside AM spores (Minerdi et al. 2002). Xavier and Germida identified one spore isolate as *Bacillus patsuli*. *Burkholderia* sp. carry *nif* genes, could therefore potentially fix N_2 and thus contribute to the N nutrition of the fungus (Minerdi et al. 2001). However it remains to be shown that the bacteria in spores actually fix nitrogen. The chitinolytic bacteria isolated from spores (Filippi et al. 1998) could have a role in spore germination. Clearly more information about bacterial species from within spores and their role and significance for spore survival and germination is needed (Filippi et al. 1998; Bianciotto et al. 2000).

8.7 Conclusions

Root colonization by AM fungi can affect the bacterial community composition in the rhizosphere by stimulating some species while suppressing others. These effects may be due directly to the fungus or could be plant and/or soil-mediated. They appear to be the result of complex fungus-plant-environment interactions which we are just beginning to understand (Fig. 8.1).

References

Abbott LK, Robson AD (1977) Growth stimulation of subterranean clover with vesicular-arbuscular mycorrhizas. Austr J Agric Res 28:639–649

Abdel-Fatah GM, Mohamedin AH (2000) Interactions between a vesicular-arbuscular mycorrhizal fungus (*Glomus intraradices*) and *Streptomyces coelicolor* and their effects on sorghum plants grown in soil amended with chitin of brawn scales. Biol Fertil Soils 32:401–409

Ames RN, Reid CPP, Ingham ER (1984) Rhizosphere bacterial population responses to root colonization by a vesicular-arbuscular mycorrhizal fungus.New Phytol 96:555–563

Amoralezcano E, Vazquez MM, Azcon R (1998) Response of nitrogen-transforming microorganisms to arbuscular mycorrhizal fungi. Biol Fertil Soils 27:65–70

Andrade G, Mihara KL, Linderman RG, Bethlenfalvay GJ (1997) Bacteria from rhizosphere and hyphosphere soils of different arbuscular mycorrhizal fungi. Plant Soil 192:71–79

Andrade G, Linderman RG, Bethlenfalvay GJ (1998a) Bacterial associations with the mycorhizosphere and hyphosphere of the arbuscular mycorrhizal fungus *Glomus mosseae*. Plant Soil 202:79–87

Andrade G, Milhara KL, Linderman RG, Bethlenfalvay GJ (1998b) Soil aggregation status and rhizobacteria in the mycorrhizosphere. Plant Soil 202:89–96

Azaizeh HA, Marschner H, Römheld V, Wittenmayer L (1995) Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth, nutrient acquisition and root exudation of soil-grown maize plants. Mycorrhiza 5:321–327

Azcón R, Barea JM, Hayman DS (1976) Utilisation of rock phosphate in alkaline soils by plants inoculated with mycorrhizal fungi and phosphate solubilizing bacteria. Soil Biol Biochem 8:135–138

Azcón R, Rubio R, Barea JM (1991) Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N_2-fixation (^{15}N) and nutrition of *Medicago sativa* L. New Phytol 117:399–404

Azcón-Aguilar C, Diaz RRM, Barea JM (1986a) Effect of soil micro-organisms on spore germination and growth of the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. Trans Br Mycol Soc 86:337–340

Azcón-Aguilar C, Gianinazzi-Pearson V, Fardeau JC, Gianinazzi S (1986b) Effect of vesicular-arbuscular mycorrhizal fungi and phosphate-solubilizing bacteria on growth and nutrition of soybean in a neutral-calcareous soil amended with ^{32}P–^{45}Ca-tricalcium phosphate. Plant Soil 96:3–15

Bago B, Azcón-Aguillar C (1997) Changes in the rhizosphere pH induced by arbuscular mycorrhiza formation in onion (*Allium cepa* L.). Zt Pflanzenern Bodenkde 160:333–339

Bagyaraj DJ, Menge JA (1978) Interaction between a VA mycorrhiza and *Azotobacter* and their effects on rhizosphere microflora and plant growth. New Phytol 80:567–573

Bakken LR (1985) Separation and purification of bacteria from soil. Appl Environ Microbiol 49:1482–1487

Barea JM, Azcón-Aguillar C, Azcón R (1987) Vesicular-arbuscular mycorrhiza improve both symbiotic N_2 fixation and N uptake from soil as assessed with a ^{15}N technique under field conditions. New Phytol 106:717–725

Barea JM, Andrade G, Bianciotto V, Dowling D, Lohrke S, Bonfante P, O'Gara F, Azcón-Aguillar C (1998) Impact on arbuscular mycorrhiza formation of Pseudomonas strains used as inoculants for biocontrol of soil-borne fungal plant pathogens. Appl Environ Microbiol 64:2304–2307

Becker DM, Bagley ST, Podila GK (1999) Effects of mycorrhizal-associated *Streptomyces* on growth of *Laccaria bicolor, Cenococcum geophilum*, and *Armillaria* species and on gene expression in *Laccaria bicolor*. Mycologia 91:33–40

Berta G, Fusconi A, Trotta A, Scannerini S (1990) Morphogenetic modifications induced by the mycorrhizal fungus *Glomus* strain E3 in the root system of *Allium porrum* L. New Phytol 114:207–215

Berta G, Fusconi A, Trotta A (1993) VA mycorrhizal colonization and the morphology and functioning of root systems. Environ Experim Bot 33:159–173

Bethlenfalvay GJ, Pacovsky RS, Bayne HG, Stafford AE (1982) Interactions between nitrogen fixation, mycorrhizal colonization, and host plant growth in the *Phaseolus-Rhizobium-Glomus* symbiosis. Plant Physiol 70:446–450

Bianciotto V, Bandi C, Minerdi D, Sironi M, Tichy HV, Bonfante P (1996) An obligately endosymbionitic mycorrhizal fungus itself habors obligately intracellular bacteria. Appl Environ Microbiol 62:3005–3010

Bianciotto V, Lumini E, Lanfranco L, Minerdi D, Bonfante P, Perotto S (2000) Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to the family *Gigasporaceae*. Appl Environ Microbiol 66:4503–4509

Buwalda JG, Goh KM (1982) Host-fungus competition for carbon as a cause of growth depressions in vesicular-arbuscular mycorrhizal ryegrass. Soil Biol Biochem 14:103–106

Christensen H, Jakobsen I (1993) Reduction of bacterial growth by a vesicular-arbuscular mycorrhizal fungus in the rhizosphere of cucumber (*Cucumis sativus* L.). Biol Fertil Soils 15:253–258

Dixon RK, Garrett HE, Cox GS (1989) Boron fertilization, vesicular-arbuscular mycorrhizal colonization and growth of *Citrus jambhiri* Lush. J Plant Nutrit 12:687–700

Eissenstat DM, Graham JH, Syvertsen JP, Drouillard DL (1993) Carbon economy of sour orange in relation to mycorrhizal colonization and phosphorus status. Ann Bot 71:1–10

Fan TWM, Lane AN, Shenker M, Bartley JP, Crowley DE, Higashi RM (2001) Comprehensive chemical profiling of gramineous plant root exudates using high-resolution NMR and MS. Phytochem 57:209–221

Fester T, Maier W, Strack D (1999) Accumulation of secondary compounds in barley and wheat roots in response to inoculation with an arbuscular mycorrhizal fungus and co-inoculation with rhizosphere bacteria. Mycorrhiza 8:241–246

Filippi C, Bagnoli G, Citernesi AS, Giovannetti M (1998) Ultrastructural spatial distribution of bacteria associated with sporocarps of *Glomus mosseae*. Symbiosis 24:1–12

Fitter AH (1991) Costs and benefits of mycorrhizas: implications for functioning under natural conditions. Experimentia 47:350–355

Foster RC (1986) The ultrastructure of the rhizoplane and rhizosphere. Ann Rev Phytopathol 24:211–234

Fraga-Beddiar A, Le Tacon F (1990) Interactions between a VA mycorhizal fungus and *Frankia* associated with alder (*Alnus glutinosa* (L.) Gaetn.). Symbiosis 9:247–258

George E, Römheld V, Marschner H (1994). Contribution of mycorrhizal fungi to micronutrient uptake by plants. In: Manthey JA, Crowley DE, Luster DG (eds) Biochemistry of metal micronutrient in the rhizosphere. Lewis Publishers, Boca Raton, pp 93–109

Geurts R, Franssen H (1996) Signal transduction in *Rhizobium*-induced nodule formation. Plant Physiol 112:447–453

Graham JH, Leonard RT, Menge JA (1981) Membrane-mediated decrease of root-exudation responsible for phosphorus inhibition of vesicular-arbuscular mycorrhiza formation. Plant Physiol 68:548–552

Grayston SJ, Wang S, Campbell CD, Edwards AC (1998) Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol Biochem 30:369–378

Green H, Larsen J, Olsson PA, Jensen DF, Jakobsen I (1999) Suppression of the biocontrol agent *Trichoderma harizianum* by mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* in root-free soil. Appl Environ Microbiol 65:1428–1434

Gryndler M, Vosatka M, Hrselova H, Catska V, Chvatalova I, Jansa J (2002) Effect of dual inoculation with arbuscular mycorrhizal fungi and bacteria on growth and mineral nutrition of strawberry. J Plant Nutrit 25:1341–1358

Hetrick BAD (1991) Mycorrhizas and root architecture. Experimentia 47:355–362

Hildebrand U, Janetta K, Bothe H (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl Environ Microbiol 68:1919–1924

Hoffland E, Findenegg GR, Nelemans JA (1989) Solubilization of rock phosphate by rape. II. Local root exudation of organic acids as a response to P starvation. Plant Soil 113:161–165

Ibekwe AM, Kennedy AC (1998) Fatty acid methyl ester (FAME) profiles as a tool to investigate community structure of two agricultural soils. Plant Soil 206:151–161

Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Verrumicrobia*. Appl Environ Microbiol 68:2391–2396

Klyuchnikov AA, Kozhevin PA (1990) Dynamics of *Pseudomonas fluorescens* and *Azospirillum brasiliense* populations during the formation of the vesicular-arbuscular mycorrhiza. Microbiol 59:449–452

Kothari SK, Marschner H, Römheld V (1991) Effect of a vesicular-arbuscular mycorrhizal fungus and rhizosphere micro-organisms on manganese reduction in the rhizosphere and manganese concentrations in maize (*Zea mays* L.). New Phytol 117:649–655

Krishnaraj PU, Sreenivasa MN (1992) Increased root colonization by bacteria due to inoculation of vesicular-arbuscular mycorrhizal fungus in chilli (*Capsicum annuum*). Zbl Mikrobiol147:131–133

Kucey RMN, Paul EA (1982) Carbon flow, photosynthesis, and N_2 fixation in mycorrhizal and nodulated faba beans (*Vicia faba* L.). Soil Biol Biochem 14:407–412

Li XL, George E, Marschner H (1991) Extension of the phosphorus depletion zone in VA-mycorrhizal white clover in a calcareous soil. Plant Soil 136:41–48

Liljeroth E, Bååth E, Mathiasson I, Lundborg T (1990) Root exudation and rhizoplane bacterial abundance of barley (*Hordeum vulgare* L.) in relation to nitrogen fertilization and root growth. Plant Soil 127:81–89

MacDonald RM, Chandler MR, Mosse B (1983) The occurrence of bacterium-like organelles in vesicular-arbuscular mycorrhizal fungi. New Phytol 90:659–663

Mansfeld-Giese K, Larsen J, Bodker L (2002) Bacterial populations associated with mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices*. FEMS Microbiol Ecol 41:133–140

Marilley J, Aragno M (1999) Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. Appl Soil Ecol 13:127–136

Marschner P, Baumann K (2003) Changes in bacterial community structure induced by mycorrhizal colonization in split-root maize. Plant Soil 251:279–289

Marschner P, Crowley DE (1996) Root colonization of mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum*) by *Pseudomonas fluorescens* 2–79RL. New Phytol 134:115–122

Marschner P, Crowley DE (1998) Phytosiderophore decrease iron stress and pyoverdine production of Pseudomonas fluorescens Pf-5 (*pvd-inaZ*). Soil Biol Biochem 30:1275–1280

Marschner P, Timonen S (2004) Interactions between plant species, mycorrhizal colonization and light intensity on the bacterial community composition in the rhizosphere. Appl Soil Ecol 28:23–36

Marschner P, Crowley DE, Higashi RM (1997) Root exudation and physiological status of a root-colonizing fluorescent pseudomonad in mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum* L.). Plant Soil 189:11–20

Marschner P, Crowley DE, Lieberei R (2001) Arbuscular mycorrhizal infection changes the bacterial 16S rDNA community composition in the rhizosphere of maize. Mycorrhiza 11:297–302

Martin JK (1971) Influence of plant species and plant age on the rhizosphere microflora. Aust J Biol Sci 24:1143–1150

Medina A, Probanza A, Gutierrez Mañero FJ, Azcón R (2003) Interactions of arbuscular-mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin). App Soil Ecol 22:15–28

Merbach W, Mirus E, Knof G, Remus R, Ruppel S, Russow R, Gransee A, Schulze J (1999) Release of carbon and nitrogen compounds by plant roots and their possible ecological importance. J. Plant Nutr Soil Sci 162:373–383

Meyer JR, Linderman RG (1986) Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. Soil Biol Biochem 18:191–196

Miethling R, Wieland G, Backhaus H, Tebbe CC (2000) Variation of microbial rhizosphere communities in response to crop species, soil origin, and inoculation with *Sinorhizobium meliloti* L 33. Microb Ecol 40:43–56

Minerdi D, Fani R, Gallo R, Boarino A, Bonfante P (2001) Nitrogen fixation genes in a endosymbiotic Burkholderia strain. Appl Environ Microbiol 67:725–732

Minerdi D, Bianciotto V, Bonfante P (2002) Endosymbiontic bacteria in mycorrhizal fungi: from their morphology to genomic sequences. Plant Soil 244:211–219

Mosse B (1970) Honey coloured sessile *Endogone* spores. Changes in fine structure during spore development. Arch Microbiol 74:146–159

Neergaard-Bearden B, Petersen L (2000) Influence of arbuscular mycorrhizal fungi on soil structure and aggregate stability of a vertisol. Plant Soil 218:173–183

Oliver JD (1993) Formation of viable but nonculturable cells. In: Kjelleberg S (ed) Starvation in bacteria. Plenum Press, New York, pp 239–272

Olsson PA, Bååth E, Jakobsen I, Söderstrom B (1996) Soil bacteria respond to presence of roots but not to mycelium of arbuscular mycorrhizal fungi. Soil Biol Biochem 28:463–470

Olsson PA, Francis R, Read DJ, Söderström B (1998) Growth of arbuscular mycorrhizal mycelium in calcareous dune sand and its interaction with other soil microorganisms as estimated by measurement of specific fatty acids. Plant Soil 201:9–16

Paulitz TC, Linderman RG (1989) Interactions between fluorescent pseudomonads and VA mycorrhizal fungi. New Phytol 113:37–45

Po C, Cumming JR (1997) Mycorrhizal fungi alter the organic acid exudation profile of red clover rhizospheres. In: Flores HE, Lynch JP, Eissenstat D (eds) Radical biology: advances and perspectives of the function of plant roots. American Society of Plant Physiology, pp 517–519

Poole EJ, Bending GD, Whipps JM, Read DJ (2001) Bacteria associated with *Pinus sylvestris-Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation in vitro. New Phytol 151:743–751

Posta K, Marschner H, Römheld V (1994) Manganese reduction in the rhizosphere of mycorrhizal and non-mycorrhizal maize. Mycorrhiza 5:119–124

Ramos C, Molbak L, Molin S (2000) Bacterial activity in the rhizosphere analysed at the single-cell level by monitoring ribosome contents and synthesis rates. Appl Environ Microbiol 66:801–809

Ravnskov S, Nybroe O, Jakobsen I (1999) Influence of an arbuscular mycorrhizal fungus on *Pseudomonas fluorescens* DF57 in rhizosphere and hyphosphere soil. New Phytol 142:113–122

Reinhard S, Martin P, Marschner H (1992) Interactions in the tripartite symbiosis of pea (*Pisum sativum* L.), *Glomus* and *Rhizobium* under non-limiting phosphorus supply. J Plant Physiol 141:7–11

Rengel Z (1997) Root exudation and microflora populations in the rhizosphere of crop genotypes differing in tolerance to micronutrient deficiency. Plant Soil 196:255–260

Rillig MC, Wright SF, Eviner VT (2002) The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. Plant Soil 238:325–333

Rovira D (1959) Root excretions in relation to the rhizosphere effect. IV. Influence of plant species, age of plant, light, temperature, and calcium nutrition on exudation. Plant Soil 11:53–64

Ryan MH (2003) Arbuscular mycorrhizae in wheat and field pea crops on a low P soil: increased Zn uptake but no increase in P uptake or yield. Plant Soil 250:225–239

Scannerini S, Bonfante P (1991) Bacteria and bacteria like objects in endomycorrhizal fungi (*Glomaceae*). In: Margulis L, Fester R (eds) Symbiosis as source of evolutionary innovation: speciation and morphogenesis. MIT Press, Cambridge MA, pp 273–287

Secilia J, Bagyaraj DJ (1987) Bacteria and actinomycetes associated with pot cultures of vesicular-arbuscular mycorrhizas. Can J Microbiol 33:1069–1073

Shachar-Hill Y, Pfeffer PE, Douds D, Osman SF, Doner LW, Ratcliffe RG (1995) Partitioning of intermediary carbon metabolism in vesicular-arbuscular mycorrhizal leek. Plant Physiol 108:7–15

Søderberg KH, Olsson PA, Bååth E (2002) Structure and activity of the bacterial community in the rhizosphere of different plant species and the effect of arbuscular mycorrhizal colonization. FEMS Microbiol Ecol 40:223–231

Sreenivasa MN, Krishnaraj PU (1992) Synergistic interaction between VA mycorrhizal fungi and a phosphate solubilizing bacterium in chilli (*Capsicum annuum*). Zentralbl Microbiol 147:126–130

Subba Rao NS, Tilak KVBR, Singh CS (1985) Synergistic effect of vesicular-arbuscular mycorrhizas and *Azospirillum brasilense* on the growth of barley in pots. Soil Biol Biochem 17:119–121

Tarafdar JC, Marschner H (1994) Phosphatase activity in the rhizosphere and the hyphosphere of VA mycorrhizal wheat supplied with inorganic and organic phosphorus. Soil Biol Biochem 26:387–395

Tisdall JM (1991) Fungal hyphae and structural stability of soil. Austr J Soil Res 29:729–743

van Aarle IM, Söderström B, Olsson PA (2003) Growth and interactions of arbuscular mycorrhizal fungi in soils from limestone and acid rock habitats. Soil Biol Biochem 35:1557–1564

van Veen JA, Liljeroth E, Lekkerkerk LJA (1991) Carbon fluxes in plant-soil systems at elevated atmospheric CO_2 levels. Ecol Applic 1:175–181

Vancura V, Orozco MO, Graunova O, Prikryl Z (1989) Properties of bacteria in the hyphosphere of a vesicular-arbuscular mycorrhizal fungus. FEMS Microb Ecol 29:421–427

Vázquez MM, César S, Azcón R, Barea JM (2000) Interactions between arbuscular mycorrhzizal fungi and other microbial inoculates (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Appl Soil Ecol 15:261–272

Vivas A, Marulanda A, Gomez M, Barea JM, Azcon R (2003) Physiological characteristics (SHD and ALP activities) of arbuscular mycorrhizal colonization as affected by *Bacillus thurigiensis* inoculation under two phosphorus levels. Soil Biol Biochem 35:987–996

Wamberg C, Christensen S, Jacobsen I, Mueller AK, Sørensen SJ (2003) The mycorrhizal fungus (*Glomus intraradices*) affects microbial activity in the rhizosphere of pea plants (*Pisum sativum*). Soil Biol Biochem 35:1349–1357

Wang GM, Coleman DC, Freckman DW, Dyer MI, McNaughton SJ, Acra MA, Goeschl JD (1989) Carbon partitioning patterns of mycorrhizal versus non-mycorrhizal plants: real-time dynamic measurements using $^{11}CO_2$. New Phytol 112:489–493

Waschkies C, Schropp A, Marschner H (1994) Relations between grapevine replant disease and root colonization of grapevine (*Vitis* sp.) by fluorescent pseudomonads and endomycorrhizal fungi. Plant Soil 162:219–227

Wyss P, Boller T, Wiemken A (1992) Testing the effect of biological control agents on the formation of vesicular arbuscular mycorhiza. Plant Soil 147:159–162

Xavier LJC, Germida JJ (2003) Selective interactions between arbuscular mycorrhizal fungi and *Rhizobium* leguminosarum bv. viceae enhance pea yield and nutrition. Biol Fertil Soils 37:261–267

9 Mycorrhizosphere Concept

Sari Timonen, Petra Marschner

9.1 Introduction

The roots of most terrestrial plants are colonised by mycorrhiza-forming symbiotic fungi (Molina et al. 1992). Mycorrhizal plants include the majority of our crop plants and almost all woody plants. During mycorrhizal colonisation roots become internally colonised by mycorrhizal fungi, which establish an intimate contact with plant cells and a direct link to the carbohydrate reserves of the colonised plants. The fungi also grow on the root surface and extend into the surrounding soil, significantly expanding the functional space and capabilities of the rhizosphere (Allen 1992). The root-mycelial system operates as a continuum in the soil and it is difficult to study rhizosphere without taking the mycorrhizal fungi into account. Thus the concept of rhizosphere could easily be enlarged into the concept of mycorrhizosphere in the case of most land plants.

This chapter outlines the structure and prevalence of mycorrhizospheres in nature. The implications of mycorrhizosphere formation on inorganic, organic and biological properties of soil as well as plant are discussed. This chapter provides a general introduction to the different types of mycorrhizospheres and associated organisms, which are portrayed in detail within other chapters of this book.

9.2 Mycorrhizosphere Structure

The rhizosphere can be defined as roots and their immediate zone of influence (Hiltner 1904) (Fig. 9.1). The mycorrhizosphere comprises of roots, hyphae of the directly connected mycorrhizal fungi, associated microorganisms and the soil within their direct influence (Rambelli 1973),

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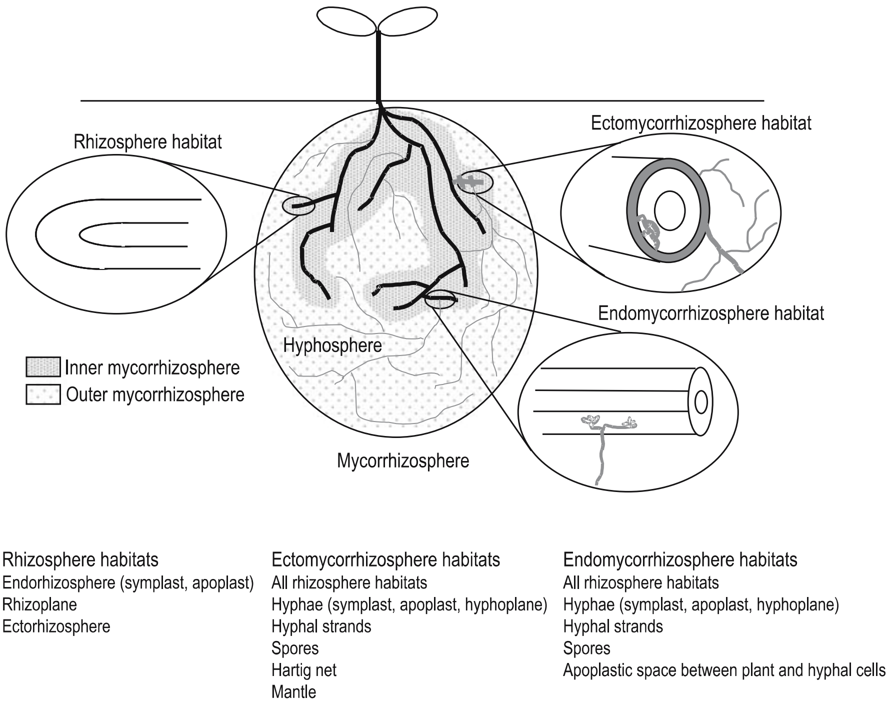

Fig. 9.1. Mycorrhizosphere habitats

thus including both rhizosphere and hyphosphere. Hiltner referred to the rhizosphere only with respect to the biological changes induced by roots, but subsequently the chemical and physical changes in this soil compartment have also been intensively studied. The rhizosphere differs from the bulk soil in regard to pH, redox potential and concentration of nutrients and water (Marschner 1995). These changes are induced by uptake of nutrients and water by the roots as well as exudation of compounds from the roots. The rhizosphere of most plants growing in natural habitats always includes not only roots but also the intimately intertwined mycelium of mycorrhizal fungi and it can be difficult to define the limits of rhizosphere and hyphosphere (Fig. 9.2). Thus, in most cases rhizosphere can be defined as inner mycorrhizosphere (Fig. 9.1). The hyphosphere consists of the external mycelium of mycorrhizal fungi, the associated microorganisms and the soil surrounding them. The external mycelium of mycorrhizal fungi forms the outer mycorrhizosphere and usually extends at least several centimetres into the surrounding soil (Johnson et al. 2002).

The structure of the mycorrhizosphere differs between types of mycorrhizal symbiosis. In the case of most endomycorrhizal symbioses the hyphae penetrate the root tissue and form a thin network of runner hyphae on

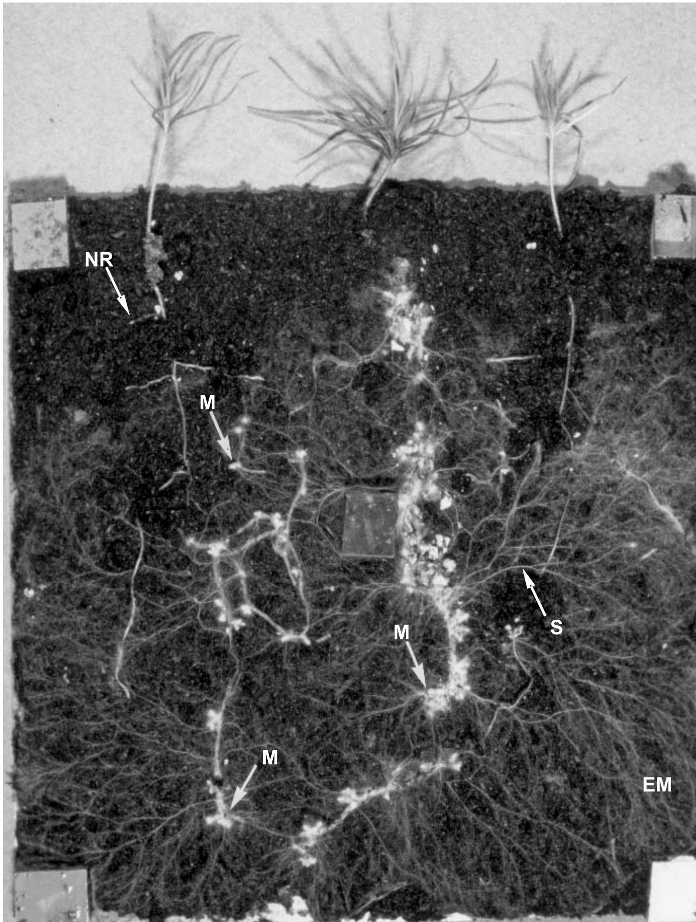

Fig. 9.2. Ectomycorrhizosphere with three pine seedlings connected by external mycelium of mycorrhizal fungus *Suillus variegatus*. M, mycorrhiza; NR, non-mycorrhizal root; S, strand; EM, external mycelium

the root surface. In ectomycorrhizal symbiosis the root tips are ensheathed by a thick mantle of hyphae (Fig. 9.2). Ectomycorrhizal fungi often also form strands and rhizomorphs, which can conduit plant derived carbon compounds up to meters away from the plant roots into the distal hyphal tips (Finlay and Söderström 1992).

We may choose to call the mycorrhizosphere just rhizosphere with its associating microorganisms, these including the mycorrhizal fungi. However, this can easily be misleading in the case of mycorrhizal roots, which are an intimate relationship of a plant and a multicellular organism reaching far beyond the immediate vicinity of roots.

9.3 Evolution and Occurrence of Mycorrhizospheres

When plants first colonised the land, those aquatic plants capable of forming mutualistic interactions with filamentous hyphae were probably best equipped to make the transition from water to land. Aquatic plants are adapted to living in an environment where roots are poorly developed and only needed for attachment to the substrate, since water and nutrients can be acquired through surfaces of leaves and stems. In the land environment, on the other hand, roots are essential for sufficient water and nutrient uptake. On land, those plants associated with filamentous fungi would have had a clear advantage to the plants without them. Via the fungal partner they could exploit the temporarily dry soil and slowly take over the dry land. It is the current hypothesis that all contemporary land plants stem from these mycorrhizal ancestors (Smith and Read 1997). Indeed, even today ca. 80% of land plants are colonised by arbuscular mycorrhizal fungi, which are phylogenetically closely related to the early mycorrhizal fungi (Simon et al. 1993). Moreover, the symbiotic tissues and cells typical of contemporary arbuscular mycorrhizal roots are very similar to those found in fossils from Rhynie chert formed four million years ago (Remy et al. 1994). Land plant species able to survive without mycorrhizal symbionts are believed to have emerged later and are usually found in relatively recent plant families such as Chenopodiaceae, Brassicaceae, Cyperaceae and Juncaceae (Brundrett 1991). Thus it appears a valid claim that mycorrhizospheres developed first and pure rhizospheres later.

Although mycorrhizospheres dominate in natural ecosystems, pure rhizospheres do exist in some environments. Non-mycorrhizal plant species occur and thrive in wetlands and disturbed habitats. The disturbance can be a natural catastrophe such as volcanic eruption, melting of glaciers or severe forest fire. In the succession after disturbance, the pioneer plants are often non-mycorrhizal while mycorrhizal plants follow later in the succession after the destroyed hyphal network is re-established (Allen 1991). The majority of soil disturbance is caused by human activities. Man-made disturbances include agricultural management practices such as tilling, fertiliser and pesticide application. Consequently, agricultural plants often are colonised to a lesser extent than plants in a natural environment (Gianinazzi et al. 2002; Sturz et al. 1997).

9.4 Mycorrhizospheres as Habitats

Mycorrhizal colonisation changes the metabolism of the roots and modifies the amount and composition of compounds released by the roots into the soil. The few results available on the effect of mycorrhizal colonisation on the root exudates show that the exudation patterns vary depending on plant species, mycorrhizal fungus, plant age and nutritional condition (Laheurte et al. 1990; Leyval and Berthelin 1993). Mycorrhizal fungi may reduce the amount of root exudates in the rhizosphere by taking up carbohydrates directly from root cells before they reach the surrounding soil. The few results gained from arbuscular mycorrhizal systems generally support this hypothesis (Laheurte et al. 1990; Mada and Bagyaraj 1993; Marschner et al. 1997). However, Azaizeh et al. (1995) found no effect of arbuscular mycorrhizal colonisation on exudation. Ectomycorrhizal colonisation by *Laccaria laccata* significantly reduced the amount of soluble carbon compounds in the inner mycorrhizospheres of Scots pine but had the opposite effect in beech (Leyval and Berthelin 1993). Mycorrhizal colonisation also modified sugar, amino acid and organic acid composition of root exudates (Laheurte et al. 1990; Leyval and Berthelin 1993). The carbohydrates taken up by mycorrhizal fungi are partially metabolised into fungi-specific compounds such as trehalose, mannitol and arabitol (Söderström et al. 1988). These and other fungal metabolites such as lactic acid and oxalic acid released by fungi further modify the mycorrhizosphere (Lapeyrie et al. 1987; Leyval and Berthelin 1993; Wallander 2000).

The types of physical of habitats offered by mycorrhizospheres are far more varied than those provided by rhizospheres (Fig. 9.1). The mycorrhizal fungi themselves include surfaces, apoplastic and even symplastic spaces in their hyphae and spores (Bianciotto et al. 1996; Linderman and Paulitz 1990; Scannerini and Bonfante-Fasolo 1991). Additionally, mycorrhizospheres provide unique habitats that cannot be found in either partner alone. This is particularly the case in ectomycorrhizas, which consist of many different types of habitats ranging from plant-fungal surfaces within the mycorrhizas to the purely fungal mantle surfaces.

Many of these mycorrhizosphere habitats may be unfavourable for soil organisms due to high concentration of organic acids and secondary metabolites produced by both the host plants and fungi. Yet, living in these environments brings benefits. Both plants and fungi provide easily available nutrients in form of exudates and lysates. The root and hyphal structures offer surfaces for attachment. Some of these are enclosed habitats in which the microorganisms are protected from predation and physical environmental stress such as desiccation.

Hyphal strands connect fungal patches growing on different soil microhabitats as well as roots of plants of the same or different species. Thus, the outer mycorrhizospheres may provide microorganisms with an avenue to spread within the soil and between plants. It has not yet been vigorously tested if different microbial species could use these mycelial connections for spreading in the underground network of mycorrhizal fungi and roots. Could the hyphae operate as connectors of different metapopulations of microorganisms in otherwise physically separated mycorrhizas or fungal patches?

9.5 Microorganisms Involved in Forming Mycorrhizospheres

Mycorrhizospheres can be regarded as a community of different organisms, which include plants, mycorrhizal fungi, other microorganisms and soil fauna. The plant roots are the dominating members of these communities, but many of the rhizosphere functions are in fact carried out or enhanced by the associated microorganisms (Fig. 9.3). The microbial community is responsible for the majority of nutrient mobilisation and uptake. The rhizosphere microorganisms also have a major effect on defence against various pathogens, maintaining soil structure as well as retaining water and nutrients. Most of the organisms involved in these vital functions are as yet

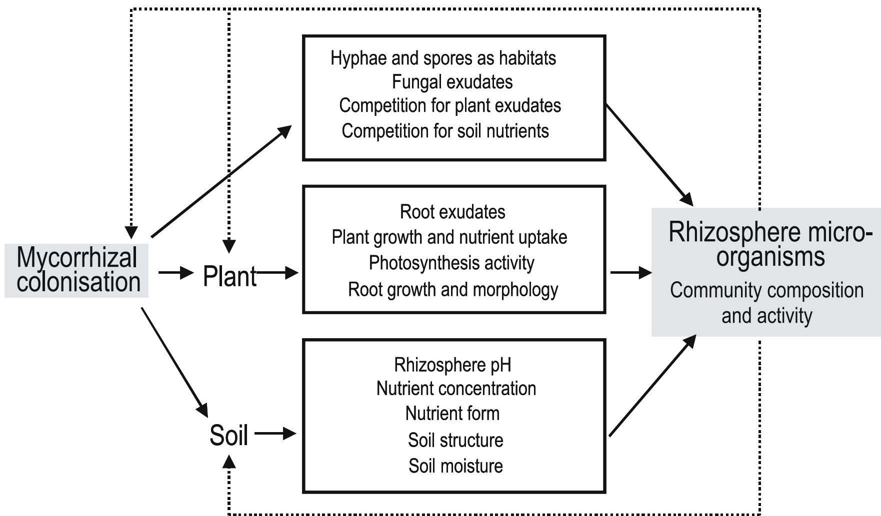

Fig. 9.3. Direct and indirect effects of mycorrhizal colonisation on community composition and activities of other microorganisms in the rhizosphere (see text for details)

unknown species. We are, nevertheless, slowly starting to build a picture about the groups of organisms involved.

9.5.1 Mycorrhizal Fungi

Mycorrhiza-forming fungi belong to three divisions of true fungi: Zygomycota, Ascomycota and Basidiomycota (Harley and Smith 1983). The mycorrhizal fungi in the division of Zygomycota belong in the order Glomales. In the light of recent molecular evidence it has been suggested that this order should gain a status of a new division Glomeromycota (Schüßler et al. 2001). The aseptate, obligate symbiotic fungi in this taxon form arbuscular mycorrhizal symbiosis with plants belonging into angiosperms, gymnosperms, pteridiophytes, mosses, lycopods and Psilotales (Smith and Read 1997). Fungi belonging to the division Ascomycota are involved in forming ericoid mycorrhizas with plants of the order Ericales and ectomycorrhizas with trees (Straker 1996; Tedersoo et al. 2003). Fungi forming ecto-, orchid, monotropoid, arbutoid and some ericoid mycorrhizal associations belong to Basidiomycota (Harley and Smith 1983). Mycorrhiza forming fungi have been most intensively studied in plant species forming arbuscular and ectomycorrhiza. Thus the lists of fungal species forming these symbioses are most advanced but still far from complete (see, e.g. http://www.tu-darmstadt.de/fb/bio/bot/schuessler/amphylo/amphylo_home.html; http://unite.zbi.ee/about.php3).

Mycorrhizal fungi facilitate nutrient and water uptake from soil. Fungal hyphae are thinner than plants roots, having roughly a ten times smaller diameter, which allows them to penetrate soil pores inaccessible to plant roots. Mycorrhizal hyphae also grow faster than plant roots into the soil beyond the nutrient depletion zone created by roots. The uptake, translocation and storage of the macronutrients, nitrogen and phosphorus, have thus far been most extensively studied (George and Marschner 1996; George et al. 1995). Other plant health promoting effects of mycorrhizal colonisation include protection against root pathogens and the production of substances affecting plant growth (Borowicz 2001; Ng et al. 1982; Rudawska and Gay 1989; Singh and Singh 1996).

9.5.2 Other Fungi

Besides mycorrhiza-forming fungi, other fungi also inhabit mycorrhizospheres. While the majority of these fungi are still unknown, the interactions between mycorrhizal and pathogenic fungi have been investigated

in some studies. One hypothesis of researchers has been, that mycorrhizal fungi can deter pathogens or at least alleviate their deleterious effects on plants. Indeed, in many cases this has been found to be true (Azcón-Aguilar and Barea 1996; Chakravarty and Unestam 1987; Duchesne and Peterson 1987; Hooker et al. 1994; Marx 1972). The antagonistic effects of mycorrhizal fungi against plant pathogenic fungi can be caused by several factors: (i) decreased sugar exudation caused by mycorrhizal colonisation and consequent evasion from detection by pathogens, (ii) physical exclusion of the pathogen from root surface by the mycorrhizal fungus, (iii) direct fungicidal products released by the mycorrhizal fungi, (iv) accelerated defence responses of plants colonised by mycorrhizal fungi, or (v) improved general condition of the plant. The interaction between mycorrhizal fungi and other fungi can be rather specific. The extract of arbuscular mycorrhizal fungus *Glomus intraradices* reduced germination of the pathogen *Fusarium oxysporum* but stimulated germination of a non-pathogenic *Trichoderma harzianum* (Filion et al. 1999).

Qian et al. (1998) demonstrated a high diversity of non-pathogenic microfungi in the rhizoplanes of mycorrhizal spruce and beech. The fungal population patterns indicated antagonistic interactions between pathogenic fungi and microfungi that may have reduced root disease. Distinct communities of microfungi have been shown to colonise the surface and in the interior of beech ectomycorrhizas (Varese and Luppi-Mosca 1996). From these few studies it is difficult to extrapolate the significance of microfungi on the mycorrhizosphere community. Many of them may have no particular effect. However, those species that produce antibiotics are most likely to have an effect on the bacterial and fungal community. The saprophytic soil fungi in the vicinity of the mycorrhizosphere may contribute to the nutrient pool of mycorrhizal plants since mycorrhizal fungi have been shown to extract nutrients from them (Lindahl 2000).

9.5.3 Bacteria

Bacteria in the mycorrhizospheres have been shown to play a role in plant nutrient uptake, growth promotion, protection against pathogens (Schelkle and Peterson 1996). They also influence microbial predators (Siddiqui and Mahmood 1995), nitrogen fixation (Li et al. 1992; Yamanaka et al. 2003) and mycorrhizal colonisation (Garbaye 1994). Additionally, they have been implied to have profound effect on mineral weathering (Leyval et al. 1990). Due to these putatively plant-beneficial functions bacteria are, together with mycorrhizal fungi, the best-studied group of mycorrhizosphere organisms.

Mycorrhizosphere-associated bacteria have been studied most intensively in arbuscular mycorrhizal systems. The composition and effects of bacterial communities associated with arbuscular mycorrhizal fungi are described in detail in Marschner and Timonen (Chap. 8). The other endomycorrhizal systems have received only marginal interest, but a few reports exist on bacteria in orchid and monotropoid mycorrhizospheres. Bacteria belonging to the genera *Arthrobacter*, *Bacillus*, *Mycobacterium* and *Pseudomonas* were detected on underground roots of the terrestrial orchid *Calanthe vestita*. Bacterial strains of the genera *Bacillus*, *Curtobacterium*, *Flavobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus* and *Xanthomonas* have been isolated from aerial roots of the epiphytic orchid *Dendrobium moschatum* (Tsavkelova et al. 2001). Distinct cyanobacterial populations were found on the underground roots of *Calanthe vestita* as well as on aerial roots of three epiphytic orchids forming a sheath of interlaced fungal hyphae and filamentous cyanobacteria (Tsavkelova et al. 2003a,b). Many cyanobacteria are able to fix atmospheric nitrogen and could thus assist in plant nitrogen acquisition. Strains of *Pseudomonas putida*, *Xanthomonas maltophilia*, *Bacillus cereus* and *Arthrobacter* sp. have been isolated from underground tissues of Western Australian orchids and they have been shown to have variable effects on mycorrhiza-assisted germination of the orchid *Pterostylis vittata* (Wilkinson et al. 1994). This may be due to the production of growth factors such as indole acetic acid by the bacterial strains. The bacterial community associated with orchid root tissue colonised by mycorrhizal fungi appears to vary with orchid genera and season (Wilkinson et al. 1989). Monotropoid mycorrhizas have been shown to host bacteria on surfaces of their mantles, but they have not been identified (Massicotte et al. 2005).

Ectomycorrhizosphere-associated bacteria were reported for the first time in the beginning of the 1960s by Katznelson et al. (1962). Since then, information about species diversity and occurrence of different types of ectomycorrhizosphere bacteria in nursery systems and forest soils has accumulated rather slowly (Garbaye 1994; Timonen 2000 and references therein). Ectomycorrhizal fungi can either suppress or promote bacterial activity depending on the prevailing conditions (Olsson and Wallander 1998; Ponge 1991). Microscopic evidence of ectomycorrhizosphere bacteria show that different fungal species have distinct effects on the associated bacteria; some ectomycorrhizas host copious populations of diverse bacteria on all surfaces, whereas others appear to be devoid of bacteria (Foster and Marks 1966; Nurmiaho-Lassila et al. 1997). In mycorrhizal Scots pine, population density of culturable bacteria increases towards the inner mycorrhizosphere, probably due to larger pool of available carbohydrates (Timonen et al. 1998). Pseudomonads and *Burkholderia* sp. were mainly found in the inner mycorrhizospheres of pine, whereas the distribution of

Bacillus sp. was more uniform (Bending et al. 2002; Timonen et al. 1998). The most thoroughly studied ectomycorrhiza-associated bacterial system is the Douglas fir-*Laccaria laccata-Pseudomonas fluorescens* combination of which detailed functional data exists and successful nursery applications have been carried out (Brule et al. 2001 and references therein). Ectomycorrhiza-associated bacteria are described in more detail in Reddy and Satyanarayana (Chap. 13) and the particular issue of mycorrhiza development helper effects of bacteria is covered in Duponnois (Chap. 15).

9.5.4 Archaea

Members of Archaea have only recently been detected in mycorrhizospheres (Bomberg et al. 2003). The diversity of Archaea in ectomycorrhizospheres appears to be affected by the species of mycorrhizal fungus as well as by location within the mycorrhizosphere. It is not yet clear to what extent Archaea interact with the other organisms in the mycorrhizosphere. Archaea have the unique capability to produce methane from organic acids and other organic substrates. They can also fix atmospheric nitrogen and produce various antibiotic substances. These are characteristics, which could alter the environment of the mycorrhizosphere community.

9.5.5 Protozoa

Most soil protozoa prey on bacteria, although the testate amoebae are omnivorous and some species consume hyphae (Darbyshire 1994; Ogden and Pitta 1990). It is also known that protozoa differ in their preference for bacterial species (Rønn et al. 2001, 2002b). Considering the complexity of interactions between plant species, mycorrhizal fungus, environmental factors and bacterial communities, it is not surprising that the scant information we currently have about protozoan populations in mycorrhizospheres is far from clear. It has been demonstrated that the effect of arbuscular mycorrhizal colonisation on protozoa depends on the physiological status of the plant (Rønn et al. 2002a; Wamberg et al. 2003). In young, rapidly growing plants, mycorrhizal colonisation had a negative effect on density of protozoa, whereas during flowering the effect was positive or neutral. The studies carried out in ectomycorrhizospheres indicate that the species of mycorrhizal fungus may have a profound effect on the protozoa populations. *Paxillus involutus* reduced the density of naked amoebae and flagellates in conifer rhizospheres, whereas *Lactarius rufus* and *Suillus bovinus* appear to have an opposite effect (Bonkowski et al. 2001; Jentschke et

al. 1995; Timonen et al. 2004). Additionally, different species of mycorrhizal fungi have been demonstrated to favour certain protozoa types in conifer mycorrhizospheres (Ingham and Massicotte 1994). In pine ectomycorrhizospheres the density of omnivorous testate amoebae was higher in the external mycelia than in mycorrhizas (Timonen et al. 2004).

Other multicellular soil animals such as nematodes, mites and earthworms live within mycorrhizospheres and may also have a profound effect on the mycorrhizosphere communities. They forage on roots and hyphae, and prey on the associated microorganisms. They also cause severe disturbance by destroying mycelial networks through burrowing and by leaving behind nutrients in form of faeces and corpses.

9.6 Ecological Significance of Mycorrhizospheres

The microorganisms present in the mycorrhizospheres may compete with each other for resources but they can also complement each other's functions (Fig. 9.3). Together, plant roots, mycorrhizal fungi and the other associating microorganisms with their unique set of enzymes and metabolites can acquire nutrients and overcome adverse conditions more effectively than alone.

A good example of the synergism between mycorrhizosphere organisms is nutrient acquisition. Plants have effective mechanisms such as organic acids and transporters for nutrient extraction and uptake. Mycorrhizal fungi add to these traits through the expanded surface area, pore penetration, exudates and enzyme activities. Many ericoid and ectomycorrhizal fungi have well developed saprophytic capabilities and potential to extract nitrogen and phosphorus from organic sources (Read and Perez-Moreno 2003). Mycorrhizal fungi of all the three divisions forming arbuscular, ericoid and ectomycorrhizal associations have been shown to take up organic nitrogen (Hawkins et al. 2000; Näsholm et al. 1998; Xiao and Berch 1999). However, in arbuscular mycorrhizal systems the effect of the mycorrhizal fungus in the efficiency of nitrogen capture from organic sources appears predominantly indirect (Hodge 2001, 2003). Mycorrhizal fungi have also been shown to hydrolyse organic phosphorus to inorganic phosphorus, which can then be transported to host roots (Koide and Kabir 2000; Read 1996; Wallander 2000).

In addition to plants and mycorrhizal fungi, the bacteria in the mycorrhizosphere also facilitate nutrient uptake. They are particularly important in extracting phosphorus from recalcitrant minerals. Bacterial weathering of mica and rock phosphate to release cations and phosphorus has been observed both in ecto and arbuscular mycorrhizospheres (Berthelin

and Leyval 1982; Kim et al. 1998; Leyval and Berthelin 1991; Leyval et al. 1990, 1993; Sreenivasa and Krishnaraj 1992). A synergistic interaction between mycorrhizal fungi and bacteria in extracting nutrients was observed in these studies. Mycorrhizosphere bacteria also include nitrogen-fixing species. Some of these live in root nodules produced by the plants, but some have been reported from within mycorrhizas (Li and Hung 1987). There are numerous reports of plant growth promotion and enhancement of mycorrhizal colonisation in tripartite systems with plant, mycorrhizal fungus and nitrogen fixing bacteria (Chanway and Holl 1991; Khan et al. 1995; Mosse et al. 1976; Sturz et al. 2000; Xie et al. 1995) and Marschner and Timonen (Chap. 8) for details.

The multi-species microbial community within the mycorrhizosphere could be important for the survival of the sessile and often long-lived plants in a changing environment. Particularly trees, which live in the same location for decades, may be able to adjust to the changes in the external conditions better with the help of altering mycorrhizal fungi and associating bacteria. The genetic structure and metabolic capabilities of a tree change very slowly, if at all, during the life of a tree. On the other hand, the genetic composition and physiological abilities of the associated microbial community can change relatively rapidly. This can alleviate the putatively harmful effects of changes within the environment. The microbial communities of mycorrhizospheres are also likely to help plants to overcome the challenges posed by spatial heterogeneity of soils. Distinct microbial communities within the hyphosphere could allow plants to exploit the different types of soil microsites more effectively.

The hyphal network of mycorrhizal fungi connects plants with each other (Fig. 9.2). The interconnecting hyphae have been shown to be able to transfer carbon from one plant to another (Simard et al. 1997). This is striking and may be important in keeping severely shaded plants alive. Yet, probably even more important is the possibility that smaller, weaker or shaded plants may utilise the nutrients and water in the fungal network supported by other plants. An extreme example of this are achlorophyllous parasitic plants, which obtain even their carbohydrates via the mycorrhizal mycelia connected to a host plant (Furman 1966; Hibbett 2002).

Mycorrhizospheres have an effect on soil structure both at microscopic and macroscopic levels. Arbuscular mycorrhizal fungal hyphae produce sticky substrates, such as glomalins, which effectively bind soil particles into aggregates (Thomas et al. 1993; Tisdall et al. 1997). The increased aggregation is beneficial by maintaining a porous but stabile soil structure and preventing erosion (Tisdall 1994). Glomalin appears to be very stable and may remain in the soil even after the death of the mycorrhizal fungus (Steinberg and Rillig 2003). Mycorrhizospheres and their composition have a significant effect on both the quality and the concentration of organic

acids in the soil solution and consequently on acid cycling, mineralisation and nutrient cycling in forest soils (Read and Perez-Moreno 2003; van Hees et al. 2003). These nutrient-releasing activities together with water movement in soil are among the key components determining the vertical movement of elements in the soil profile. Roots and mycorrhizal fungi also transport nutrients for considerable distances and thus create lateral movement of substrates through soil.

9.7 Conclusions

Mycorrhizospheres are fascinating systems. Their composition and functions are of great importance for the survival and sustainability of almost all terrestrial plant communities. Unfortunately, the inaccessibility of the underground roots and the hyphae attached to them makes the study of undisturbed mycorrhizospheres very difficult. Mycorrhizosphere communities are highly complex, with organisms from practically all the kingdoms of life and most of these organisms still remain to be identified. In these tightly woven communities, organisms have multiple-level interactions with each other and the environment. The untangling of species composition and physiological functions of mycorrhizospheres has only just started, but is now proceeding quicker than ever, particularly with the help of the new molecular tools.

References

Allen MF (1991) The ecology of mycorrhizae. Cambridge University Press, Cambridge

Allen MF (1992) Mycorrhizal functioning: an integrative plant-fungal process. Chapman and Hall, London

Azaizeh HA, Marschner H, Romheld V, Wittenmayer L (1995) Effects of a vesicular-arbuscular mycorrhizal fungus and other soil-microorganisms on growth, mineral nutrient acquisition and root exudation of soil-grown maize plants. Mycorrhiza 5:321–327

Azcón-Aguilar C, Barea JM (1996) Arbuscular mycorrhizas and biological control of soil-borne plant pathogens – an overview of the mechanisms involved. Mycorrhiza 6:457–464

Bending GD, Poole EJ, Whipps JM, Read DJ (2002) Characterisation of bacteria from Pinus sylvestris-Suillus luteus mycorrhizas and their effects on root-fungus interactions and plant growth. FEMS Microbiol Ecol 39:219–227

Berthelin J, Leyval C (1982) Ability of symbiotic and non-symbiotic rhizospheric microflora of maize (Zea mays) to weather micas and to promote plant growth and plant nutrition. Plant Soil 68:369–377

Bianciotto V, Bandi C, Minerdi D, Sironi M, Tichy HV, Bonfante P (1996) An obligately endosymbiotic mycorrhizal fungus itsel harbors obligately intracellular bacteria. Appl Environ Microbiol 62:3005–3010

Bomberg M, Jurgens G, Saano A, Sen R, Timonen S (2003) Nested PCR detection of Archaea in defined compartments of pine mycorrhizospheres developed in boreal forest humus microcosms. FEMS Microbiol Ecol 43:163–171

Bonkowski M, Jentschke G, Scheu S (2001) Contrasting effects of microbial partners in the rhizosphere: interactions between Norway spruce seedlings (*Picea abies* Karst.), mycorrhiza (*Paxillus involutus* (Batsch) Fr.) and naked amoebae (protozoa). Appl Soil Ecol 18:193–204

Borowicz VA (2001) Do arbuscular mycorrhizal fungi alter plant-pathogen relations? Ecology 82:3057–3068

Brule C, Frey-Klett P, Pierrat J, Courrier S, Gerard F, Lemoine M, Rousselet J, Sommer G, Garbaye J (2001) Survival in the soil of the ectomycorrhizal fungus Laccaria bicolor and the effects of a mycorrhiza helper *Pseudomonas fluorescens*. Soil Biol Biochem 33:1683–1694

Brundrett M (1991) Mycorrhizas in natural ecosystems. Adv Ecol Res 21:171–313

Chakravarty P, Unestam T (1987) Mycorrhizal fungi prevent disease in stressed pine seedlings. J Phytopath 118:335–340

Chanway CP, Holl FB (1991) Biomass increase and associative nitrogen fixation of mycorrhizal *Pinus contorta* seedlings inoculated with a plant growth promoting *Bacillus* strain. Can J Bot 69:507–511

Darbyshire JF (1994) Soil protozoa. CAB International, Wallingford, UK

Duchesne LC, Peterson RL (1987) The accumulation of plant-produced antimicrobial compounds in response to ectomycorrhizal fungi: a review. Phytoprotection 68:17–27

Filion M, StArnaud M, Fortin JA (1999) Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. New Phytol 141:525–533

Finlay RD, Söderström B (1992) Mycorrhiza and carbon flow to the soil. In: Allen M (ed) Mycorrhizal functioning: an integrative plant-fungal process. Chapman and Hall, London, UK, pp 134–160

Foster RC, Marks GC (1966) The fine structure of the mycorrhizas of *Pinus radiata* D. Don. Austral J Biol Sci 19:1027–1038

Furman TE (1966) Symbiotic relationships of Monotropa. Am J Bot 53:627

Garbaye J (1994) Tansley Review No. 76: Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

George E, Marschner H (1996) Nutrient and water uptake by roots of forest trees. Zeitsch Pflanzenern Bod 159:11–21

George E, Marschner H, Jakobsen I (1995) Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. Critic Rev Biotech 15:257–270

Gianinazzi S, Schüepp H, Barea M, Haselwandter K (2002) Mycorrhizal technology in agriculture – from genes to bioproducts. Birkhäuser, Basel

Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic Press, London

Hawkins H, Johansen A, George E (2000) Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant Soil 226:275–280

Hibbett DS (2002) Plant-fungal interactions: when good relationships go bad. Nature 419:345–346

Hiltner L (1904) Über neue Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie unter besonderer Berüksichtigung der Gründüngung und Brache. Arb Dtsch Lanwirt Ges 98:59–78

Hodge A (2001) Arbuscular mycorrhizal fungi influence decomposition of, but not plant nutrient capture from, glycine patches in soil. New Phytol 151:725–734

Hodge A (2003) Plant nitrogen capture from organic matter as affected by spatial dispersion, interspecific competition and mycorrhizal colonization. New Phytol 157:303–314

Hooker JE, Jaizme VM, Atkinson D (1994) Biocontrol of plant pathogens using arbuscular mycorrhizal fungi. In: Gianinazzi S, Schuepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhauser Verlag Basel, pp 191–200

Ingham ER, Massicotte HB (1994) Protozoan communities around conifer roots colonised by ectomycorrhizal fungi. Mycorrhiza 5:53–61

Jentschke G, Bonkowski M, Godbold DL, Scheu S (1995) Soil protozoa and forest tree growth – non nutritional effects and interaction with mycorrhizae. Biol Fertil Soils 20:263–269

Johnson D, Leake JR, Ostle N, Ineson P, Read DJ (2002) In situ (CO_2)-C-13 pulse-labelling of upland grassland demonstrates a rapid pathway of carbon flux from arbuscular mycorrhizal mycelia to the soil. New Phytol 153:327–334

Katznelson H, Rouatt JW, Peterson EA (1962) The rhizosphere effect of mycorrhizal and non-mycorrhizal roots of yellow birch seedlings. Can J Bot 40:377–382

Khan MK, Sakamoto K, Yoshida T (1995) Dual inoculation of peanut with *Glomus* sp. and *Bradyrhizobium* sp. enhanced the symbiotic nitrogen fixation as assessed by N-15-technique. Soil Sci Plant Nutr 41:769–779

Kim KY, Jordan D, McDonald GA (1998) Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. Biol Fertil Soils 26:79–87

Koide RT, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus Glomus intraradices can hydrolyse organic phosphate. New Phytol 148:511–517

Laheurte F, Leyval C, Berthelin J (1990) Root exudates of maize, pine and beech seedlings influenced by mycorrhizal and bacterial inoculation. Symbiosis 9:111–116

Lapeyrie F, Chilvers GA, Bhem CA (1987) Oxalic-acid synthesis by the mycorrhizal fungus Paxillus-involutus (Batsch ex Fr) Fr. New Phytol 106:139–146

Leyval C, Berthelin J (1991) Weathering of a mica by roots and rhizospheric microorganisms of pine. Soil Sci Soc Am J 55:1009–1016

Leyval C, Berthelin J (1993) Rhizodeposition and net release of soluble organic compounds by pine and beech seedlings inoculated by rhizobacteria and ectomycorrhizal fungi. Biol Fertil Soils 15:259–267

Leyval C, Laheurte F, Belgy G, Berthelin J (1990) Weathering of micas in the rhizospheres of maize, pine and beech seedlings influenced by mycorrhizal and bacterial inoculation. Symbiosis 9:105–109

Leyval C, Surtiningsih T, Berthelin J (1993) Mobilization of P and Cd from rock phosphates by rhizospheric microorganisms (phosphate-dissolving bacteria and ectomycorrhizal fungi). Phosphorus Sulfur Silicon Relat Elem 77:685–688

Li CY, Hung LL (1987) Nitrogen-fixing (acetylene-reducing) bacteria associated with ectomycorrhizae of Douglas-fir. Plant Soil 98:425–428

Li CY, Massicotte HB, Moore LVH (1992) Nitrogen-fixing *Bacillus* sp. associated with Douglas-fir tuberculate ectomycorrhizae. Plant Soil 140:35–40

Lindahl B (2000) Ectomycorrhizal fungi raid saprotrophic ones. Mycol Res 104:386–387

Linderman RG, Paulitz TC (1990) Mycorrhizal-rhizobacterial interactions. In: Hornby DJ (ed) Biological control of soil-borne plant pathogens. CAB International Wallingford, Oxon, pp 261–283

Mada RJ, Bagyaraj DJ (1993) Root exudation from Leucaena-Leucocephala in relation to mycorrhizal colonization. World J Microbiol Biotech 9:342–344

Marschner H (1995) Mineral nutrition of higher plants. Academic Press, London

Marschner P, Crowley DE, Higashi RM (1997) Root exudation and physiological status of a root-colonizing fluorescent pseudomonad in mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum* L). Plant Soil 189:11–20

Marx DH (1972) Ectomycorrhizae as biological deterrents to pathogenic root infection. Phytopathol 10:429–454
Massicotte HB, Melville LH, Peterson RL (2005) Structural features of mycorrhizal associations in two members of the Monotropoideae, Monotropa uniflora and Pterospora andromedea. Mycorrhiza 15:101–110
Molina R, Massicotte H, Trappe JM (1992) Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In: Allen M (ed) Mycorrhizal functioning: an integrative plant-fungal process. Chapman and Hall London, UK, pp 357–423
Mosse B, Powell CL, Hayman DS (1976) Plant growth responses to vesicular-arbuscular mycorrhiza. IX. Interactions between V.A. mycorrhiza, rock phosphate and symbiotic nitrogen fixation. New Phytol 76:331–342
Näsholm T, Ekblad A, Nordin A, Giesler R, Högberg M, Högberg P (1998) Boreal forest plants take up organic nitrogen. Nature 392:914–916
Ng PP, Cole ALJ, Jameson PE, McWha JA (1982) Cytokinin production by ectomycorrhizal fungi Rhizopogon luteolus, Boletus elegans and Suillus luteus commonly forming mycorrhizal associations with Pinus radiata. New Phytol 91:57–62
Nurmiaho-Lassila E-L, Timonen S, Haahtela K, Sen R (1997) Bacterial colonization patterns of intact Scots pine mycorrhizospheres in dry pine forest soil. Can J Microbiol 43:1017–1035
Ogden CG, Pitta P (1990) Biology and ultrastructure of the mycophagous, soil testate amoeba, *Phryganella acropodia* (Rhizopoda, Protozoa). Biol Fertil Soils 9:101–109
Olsson PA, Wallander H (1998) Interactions between ectomycorrhizal fungi and the bacterial community in soils with applications of different primary minerals. FEMS Microbiol Ecol 27:195–205
Ponge JF (1991) Succession of fungi and fauna during decomposition of needles in a small area of Scots pine litter. Plant Soil 138:99–113
Qian X, ElAshker A, Kottke I, Oberwinkler F (1998) Studies of pathogenic and antagonistic microfungal populations and their potential interactions in the mycorrhizoplane of Norway spruce (Picea abies (L.) Karst.) and beech (Fagus sylvatica L.) on acidified and limed plots. Plant Soil 199:111–116
Rambelli A (1973) The rhizosphere of mycorrhizae. In: Marks GL, Koslowski TT (eds) Ectomycorrhizae. Academic Press, New York, pp 299–343
Read DJ (1996) The structure and function of the ericoid mycorrhizal root. Ann Bot 77:365–374
Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? New Phytol 157:475–492
Remy W, Taylor TN, Hass H, Kerp H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizas. Proc Nat Acad Sci 91:11841–11843
Rudawska M, Gay G (1989) IAA-synthesizing activity of different ectomycorrhizal fungi in relation to nitrogen nutrition. Agric Ecosys Envir 28:425–430
Rønn R, Ekelund F, Grunert J (2001) Protozoan response to addition of the bacteria *Mycobacterium chlorophenolicus* and *Pseudomonas chloraphis* to soil microcosms. Biol Fertil Soils 33:126–131
Rønn R, Gavito M, Larsen J, Jakobsen I, Fredriksen H, Christensen S (2002a) Response of free-living protozoa and microorganisms to elevated atmospheric CO_2 and presence of mycorrhiza. Soil Biol Biochem 34:923–932
Rønn R, McCaig AE, Griffiths BS, Prosser JI (2002b) Impact of protozoan grazing on bacterial community structure in soil microcosms. Appl Environ Microbiol 68:6094–6105

Scannerini S, Bonfante-Fasolo P (1991) Bacteria and bacteria like objects in endomycorrhizal fungi (Glomaceae). In: Margulis L, Fester R (eds) Symbiosis as a source of evolutionary innovation: speciation and morphogenesis. MIT Press, Cambridge, Massachusetts, pp 273–287
Schelkle M, Peterson RL (1996) Suppression of common root pathogens by helper bacteria and ectomycorrhizal fungi *in vitro*. Mycorrhiza 6:481–485
Schüßler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1421
Siddiqui ZA, Mahmood I (1995) Biological control of Heterodera cajani and Fusarium udum by Bacillus subtilis, Bradyrhizobium japonicum and Glomus fasciculatum on pigeonpea. Fund Appl Nemat 18:559–566
Simard SW, Perry DA, Jones MD, Myroid DD, Durall DM, Molina R (1997) Net transfer of carbon between ectomycorrhizal tree species in the field. Nature 388:579–582
Simon L, Bousquet J, Levesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69
Singh S, Singh S (1996) Interaction of mycorrhizae with plant disease pathogens. Part I. Efficacy of mycorrhizal fungi as disease control agents. Mycorrhiza News 8:1–9
Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic Press, London
Söderström B, Finlay RD, Read DJ (1988) The structure and function of the vegetetive mycelium of ectomycorrhizal plants. IV. Qualitative analysis of carbohydrate contents of mycelium interconnecting host plants. New Phytol 109:163–166
Sreenivasa M, Krishnaraj P (1992) Synergistic interaction between VA mycorrhizal fungi and a phosphate solubilizing bacterium in chilli (*Capsicum annuum*). Zentralbl Microbiol 147:126–130
Steinberg PD, Rillig MC (2003) Differential decomposition of arbuscular mycorrhizal fungal hyphae and glomalin. Soil Biol Biochem 35:191–194
Straker CJ (1996) Ericoid mycorrhiza: ecological and host specificity. Mycorrhiza 6:215–225
Sturz AV, Carter MR, Johnston HW (1997) A review of plant disease, pathogen interactions and microbial antagonism under conservation tillage in temperate humid agriculture. Soil Till Res 41:169–189
Sturz AV, Christie BR, Nowak J (2000) Bacterial endophytes: potential role in developing sustainable systems of crop production. Critic Rev Plant Sci 19:1–30
Tedersoo L, Kõljalg U, Hallenberg N, Larsson K-H (2003) Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. New Phytol 159:153–165
Thomas RS, Franson RL, Bethlenfalvay GJ (1993) Separation of vesicular-arbuscular mycorrhizal fungus and root effects on soil aggregation. Soil Sci Soc Am J 57:77–81
Timonen S (2000) Pine mycorrhizospheres as variable habitats for bacterial communities. In: Benedetti A, Tittarelli F, DeBertoldi S, Pinzari F (eds) Biotechnology of soil: monitoring, conservation and remediation. EurOp, Luxembourg, pp 101–106
Timonen S, Jørgensen KS, Haahtela K, Sen R (1998) Bacterial community structure of Scots pine-*Suillus bovinus* and -*Paxillus involutus* mycorrhizospheres in dry pine forest soil and nursery peat. Can J Microbiol 44:499–513
Timonen S, Christensen S, Ekelund F (2004) Distribution of protozoa in Scots pine mycorrhizospheres. Soil Biol Biochem 36:1087–1093
Tisdall JM (1994) Possible role of soil micro-organisms in aggregation of soils. Plant Soil 159:115–121
Tisdall JM, Smith SE, Rengasamy P (1997) Aggregation of soil by fungal hyphae. Austr J Soil Res 35:55–60
Tsavkelova EA, Cherdyntseva TA, Lobakova ES, Kolomeitseva GL, Netrusov AI (2001) Microbiota of the orchid rhizoplane. Microbiol 70:492–497

Tsavkelova EA, Lobakova ES, Kolomeitseva GL, Cherdyntseva TA, Netrusov AI (2003a) Localization of associative cyanobacteria on the roots of epiphytic orchids. Microbiology 72:86–91

Tsavkelova EA, Lobakova ES, Kolomeitseva GL, Cherdyntseva TA, Netrusov AI (2003b) Associative cyanobacteria isolated from the roots of epiphytic orchids. Microbiology 72:92–97

van Hees PAW, Godbold DL, Jentschke G, Jones DL (2003) Impact of ectomycorrhizas on the concentration and biodegradation of simple organic acids in a forest soil. Eur J Soil Sci 54:697–706

Varese GC, Luppi-Mosca AM (1996) Surface and inner microfungal communities of *Fagus sylvatica* L. ectomycorrhizae. In: Szaro TM, Bruns TD (eds) First International Conference on Mycorrhizae. Springer, Berkeley, California, USA, p 121

Wallander H (2000) Uptake of P from apatite by Pinus sylvestris seedlings colonised by different ectomycorrhizal fungi. Plant Soil 218:249–256

Wamberg C, Christensen S, Jakobsen I, Müller AK, Sørensen SJ (2003) The mycorrhizal fungus (*Glomus intraradices*) affects microbial activity in the rhizosphere of pea plants (*Pisum sativum*). Soil Biol Biochem 35:1349–1357

Wilkinson KH, Dixon KL, Sivasithamparam K (1989) Interaction of soil bacteria, mycorrhizal fungi and orchid seed in relation to germination of Australian orchids. New Phytol 112:429–435

Wilkinson KG, Dixon KW, Sivasithamparam K, Ghisalberti EL (1994) Effect of IAA on symbiotic germination of an Australian orchid and its production by orchid-associated bacteria. Plant Soil 159:291–295

Xiao G, Berch S (1999) Organic nitrogen use by salal ericoid mycorrhizal fungi from northern Vancouver Island and impacts on growth in vitro of *Gaultheria shallon*. Mycologia 9:145–149

Xie ZP, Staehelin C, Vierheilig H, Wiemken A, Jabbouri S, Broughton WJ, Vogellange R, Boller T (1995) Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. Plant Physiol 108:1519–1525

Yamanaka T, Li C, Bormann B, Okabe H (2003) Tripartite associations in an alder: effects of Frankia and *Alpova diplophloeus* on the growth, nitrogen fixation and mineral acquisition of *Alnus tenuifolia*. Plant Soil 254:179–186

10 Molecular Techniques for Understanding the Microbial Community Structure in Mycorrhizosphere

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10.1 Introduction

The rhizosphere, a soil-root interface, is a dynamic microcosm where microorganisms, plant roots and soil constituents interact (Lynch 1990; Azcon-Aguilar and Barea 1992; Barea et al. 2002). Carbon fluxes are critical for rhizosphere functioning (Toal et al. 2000). The supply of photosynthates and decaying plant material to the root-associated microbiota, together with microbially induced changes in rooting patterns, and the supply of available nutrients to plants derived from microbial activities, are the key issues in rhizosphere formation and functioning. Rhizosphere functioning is known to influence markedly plant fitness and soil quality because microbial developments in such environment can help the host plant to adapt to stress conditions concerning water and mineral deficits, and the presence of soil- borne plant pathogens (Lynch 1990; Bowen and Rovira 1999).

Mycorrhizal fungi are relevant members of the rhizosphere mutualistic mycosymbiont populations that are known to carry out many critical ecosystem functions such as improvement of plant establishment, enhancement of plant nutrient uptake, plant protection against cultural and environmental stresses and improvement of soil structure (Smith and Read 1997). Mycorrhizal forms have been classified and grouped together based on their structural characteristics as ecto-, endo- (AM and VAM), ericaceous and orchidaceous mycorrhizas. Mycorrhiza establishment is known to modify several aspects of plant physiology including mineral nutrient composition, hormonal balance, and carbon allocation patterns (Azcon-Aguilar and Bago 1994). Consequently, the mycorrhizal symbiotic status changes the chemical composition of root exudates, while the fungal mycelium serves as a carbon source to rhizosphere microbial communities, and further, introduces physical modifications in the envi-

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ronments surrounding the roots (Barea et al. 2002). These changes that affect the microbial populations in the rhizosphere of mycorrhizal plants, both quantitatively and qualitatively, were considered in proposing the term 'mycorrhizosphere' to describe the microbial ambience around mycorrhizas (Linderman 1992, 2000; Barea 2000; Gryndler et al. 2000). The mycorrhizosphere is a truly dynamic region of immense importance to the functioning and the ecological success of the plant, and comprises mycorrhizas surrounding soil, soil microorganisms and mycorrhiza-associated microorganisms. Two main groups of microorganisms interact with mycorrhizal fungi in the rhizosphere environments, saprophytes and symbionts. These comprise detrimental, neutral and beneficial bacteria, and fungi. Detrimental microbes include the major plant pathogens as well as minor parasitic and non-parasitic deleterious rhizosphere organisms. Mycorrhizal fungi interact with microbes colonizing root tissues and the endophytic microorganisms, which are involved in plant growth promotion and plant protection (Kloepper 1994; Sturz and Novak 2000). Mycorrhizal fungi also interact with both symbiotic and free living N_2 fixing microbes (Barea 1997).

In order to comprehend the diversity of microbes (culturable and non-culturable) present in the mycorrhizosphere, a variety of techniques and tools have been employed. In this chapter, an attempt has been made to describe various techniques that are useful for understanding the diversity of microbes present in the mycorrhizosphere, and their merits and demerits.

10.2 Culture-Dependent Methods for Identification of Bacteria

The historic way to characterize bacteria is to describe quantitatively as many phenotypic properties as possible, such as morphology, structure, cultivation, nutrition, biochemical metabolism, pathogenicity, antigenic properties, and ecology (Table 10.1). Phenotypic similarities do not necessarily indicate phylogenetic relationships (relationship based on the ancestry of organisms) (Gillis and De Leg 1992). In contrast to animals and plants, the morphology of microorganisms is, in general, too simple to serve as a basis for a sound classification and to allow for reliable identification. Thus, until very recently, microbial identification required the isolation of pure cultures (or defined cocultures) followed by testing for multiple physiological and biochemical traits (Amann et al. 1995). For isolation of microbes, enrichment culture techniques are appropriate. Cultural methods will reveal only those physiological and nutritional types compatible with the cultural environment. The potential limitations of this approach are widely acknowledged and accepted (Torsvik et al. 1996).

Table 10.1. Some characters of microbes used in the identification and systematics[a]

Categories	Example
Cultural	Colony morphology
	Color of colonies
	Fruiting bodies
	Mycelia
Morphological	Cell morphology
	Cell size
	Motility
	Flagellation type
	Reserve materials
	Gram Stain
	Acid-fast stain
Physiological	Temperature range
	pH range
	Salinity tolerance
Biochemical	Carbon source utilization
	Oxidization of carbohydrates
	Fermentation of carbohydrates
	Enzyme profile
Inhibitory test	Selective media
	Antibiotics
	Dyes
Serological	Agglutination
	Immunodiffusion
Chemotaxonomic	Fatty acids
	Polar lipids
	Mycolic acids
	Lipopolysaccharide composition
	PAGE of lipopolysaccharide
	Cell wall D-amino acids
	Cell wall amino acid composition
	Whole cell sugars
	Cell wall sugars
	Cellular pigments
	Quinone system
	Polyamine content
	Whole cell protein PAGE
Genotypic	DNA base ratio (G+C content)
	Random amplified polymorphic DNA (RAPD)
	Restriction fragment length polymorphism (RFLP)
	Terminal restriction fragment length polymorphism (T-RFLP)
	Amplified ribosomal DNA restriction analysis (ARDRA)
	Pulsed field gel electrophoresis (PFGE) of DNA fragment
	DNA probes
	Denaturing gradient gel electrophoresis (DGGE)
	Temperature gradient gel electrophoresis (TGGE)

Table 10.1. (continued)

Phylogenetic	DNA: DNA hybridization
	DNA: rRNA hybridization
	16S rRNA sequence
	23S rRNA sequence
	18S rRNA sequence
	28S rRNA sequence
	Internal transcribed spacer (ITS) sequence
	Intergenic spacer (IGS) sequence
	Sequence of β-subunit of ATP-synthase
	GroEL (Chaperionin) sequence

[a] (Source: Busse et al. 1996)

Many biochemical, nutritional and physiological characterization tests have been established in bacterial systematics, e.g. tests for indole (Kovacs 1928) oxidation-fermentation (Hugh and Leifson 1953), cytochrome-oxidase (Kovacs 1956; Gaby and Hadley 1957; Cowan and Steel 1964), catalase (Gagnon and Hunting 1959), aminopeptidase (Cerny 1976; Manafi and Kneifel 1990), and KOH-test (Gregersen 1978; Halebian et al. 1981; Bourgault and Lamothe 1988). Usually, these tests are carried out in solid and/or liquid media. The tests are laborious, time consuming, and often difficult to standardize and interpret. It must, however, be emphasized that the traditional tests continue to be valuable as these phenotypic characters are important for delineation of taxa (Busse et al. 1996). Since the pioneering work of Buissiere and Nardon (1968) on single substrate multi-test methods, from which the API system evolved, miniaturized identification systems have been developed (D'Amato et al. 1991). These miniaturized systems are commercially available. Identification of bacteria is carried out using a numerical manual or a computer-assisted system based on numerical taxonomy (Sneath and Sokal 1973). This procedure involves the comparison of a large number of characteristics of the organism to be identified with those of the known organisms. The overall degree of similarity between test strains is then estimated by computation and a similarity index value is calculated, e.g. the 'simple matching coefficient' (S_{SM}) or the 'Jaccard coefficient' (S_J). Such test kits are available to identify a wide range of bacteria, which are relevant in clinical bacteriology and food hygiene, e.g. anaerobes, *Bacillus*, *Campylobacter*, *Corynebacteria*, members of Enterobacteriaceae, non-fermenting Gram-negatives, *Lactobacillus*, *Listeria*, *Staphylococcus*, and*Streptococcus*. Miniaturized identification systems are supplied by API ATB system, Biolog system, Vitek AutoMicrobic System, and Sensititre system (Stager and Davis 1992).

These methods are easy to apply and the results are obtained within a short time. However, for identifying new isolates from nature, the result

obtained by such tests can be misleading, as there is a high probability of selecting a new species, which has not been included in the database of such systems, but possessing a substrate utilization profile similar to a strain within the database (Busse et al. 1996).

10.3 Culture-Independent (Molecular) Methods

Microbes are the significant contributors to the biomass of our planet, and it is believed that we know only a minute percentage (0.001–0.3%) of the entire profile of species and activities (Amann et al. 1995; Curtis et al. 2002). Our inability to grow most bacteria and archaea in the laboratory (Table 10.2) (Torsvik and Ovreas 2002; Torsvik et al. 2002) frequently conceals the fact that prokaryotes are the most diverse of the microbes (Curtis et al. 2002), an award frequently given to Protista and fungi (Margulis and Schwartz 1998).

Staley and Konopka (1985) coined the term 'great plate count anomaly' to describe this phenomenon, which has been known to microbiologists for generations. By now there is little doubt that, in most cases, the majority of microscopically visualized cells are viable but do not form visible colonies on plates. Two different types of cells contribute to this silent but active majority: (i) known species for which the applied cultivation conditions are just not suitable or which have entered a non-culturable state, and (ii) unknown species that have never been cultured before due to lack of suitable methods. It has been well documented for pathogens like *Salmonella enteritidis*, *Vibrio cholerae* and *V. vulnificus* that bacteria may quickly enter a non-culturable state upon exposure to salt, water, fresh water or low temperature (Amann et al. 1995).

In order to sort out the problems associated with cultivation based approach, genotypic methods are applied, which basically depend upon the

Table 10.2. Culturability determined as a percentage of culturable bacteria in comparison with the total cell count[a]

Habitat	% Culturability (in CFU)
Activated sludge	1–15
Soil	0.3
Freshwater	0.25
Sediments	0.25
Unpolluted estuarine waters	0.1–3
Mesotrophic lake	0.1–1
Seawater	0.001–0.1

[a](Source: Amann et al. 1995)

information derived from the nucleic acid (DNA and RNA). It is of primary interest to understand at which level various methods carry information and to realize the amount of time and work required. The taxonomic information level of some of these techniques is illustrated in Fig. 10.1 (Vandamme et al. 1996). Obviously, genotypic methods presently dominate modern taxonomic studies and some of them are described below.

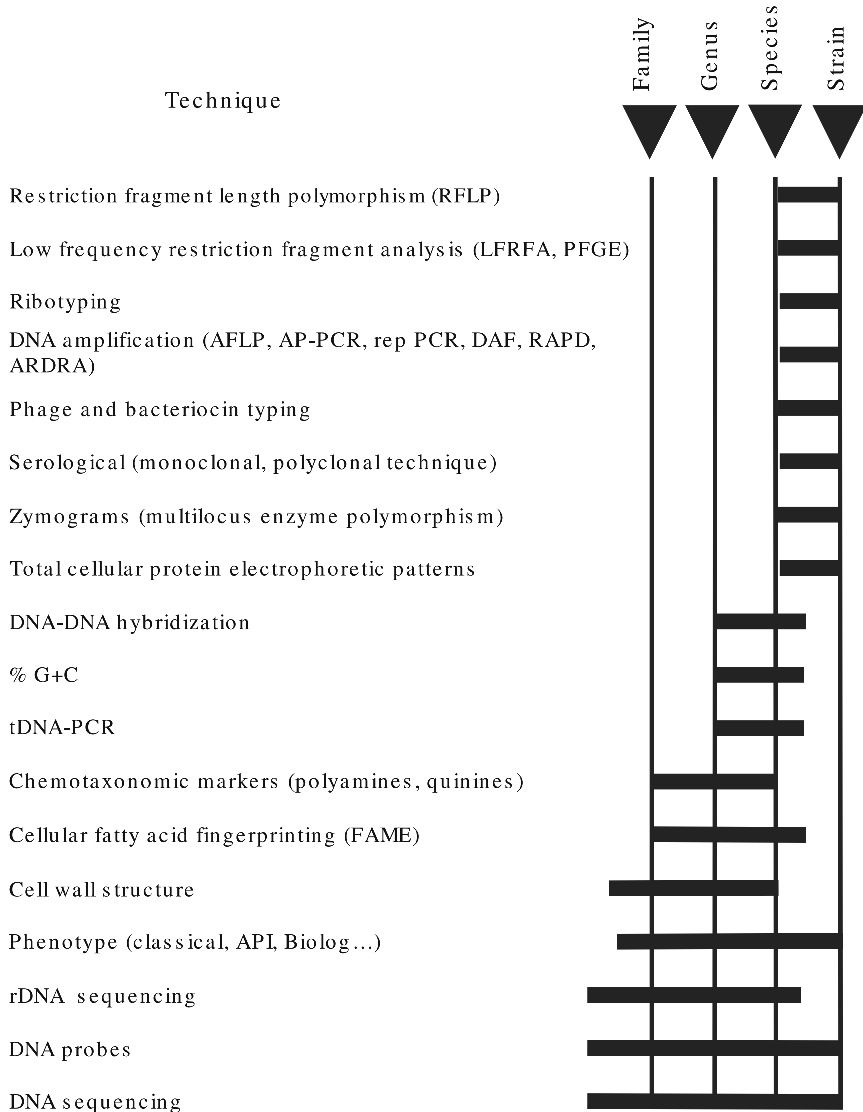

Fig. 10.1. Taxonomic resolution of some of the currently used techniques (source: Vandamme et al. 1996)

10.3.1 Determination of the DNA Base Ratio (Mole Percent G+C)

Determination of the mole percent guanosine plus cytosine is one of the classical genotypic methods and is considered as a part of the standard description of bacterial taxa (Table 10.1). Generally, the range observed is not more than 3% within a well-defined species and not more than 10% within a well-defined genus (Stackebrandt and Goebel 1994). It varies between 24 and 76% in the bacterial world (Vandamme et al. 1996). Different methods for the analysis of the G+C content have been employed, including the buoyant density method (Schildkraut et al. 1962; Mandel et al. 1968), thermal denaturation method (Marmur and Doty 1962; De Ley 1970) and HPLC method (Katayama-Fujimua et al. 1982; Tamaoka and Komagata 1984; Mesbah et al. 1989). To analyze the G+C content by HPLC, purified DNA (10 μg) is first digested with nuclease P_1, then with alkaline phosphate. The digest is then subjected to HPLC analysis using an eluent consisting of 0.2 mol/l $NH_4H_2PO_4$ and CH_3CN (20:1; v/v) (Kaneko et al. 1986). The G+C content is calculated from the corresponding peak areas of the nucleosides (Busse et al. 1996).

10.3.2 DNA:DNA Hybridization

The percent DNA:DNA hybridization and the decrease in thermal stability of the hybrid are used to delineate species (Wayne et al. 1987). The percent DNA binding (De Ley et al. 1970) of the DNA:DNA hybridization value or the relative ratio is an indirect parameter of the sequence similarity between two entire genomes. These techniques allowed the entire genome of one bacterial strain to be compared with that of other strains in terms of nucleotide base sequence. DNA hybridization is most useful at the species level. Its practical value lies in the fact that the bacterial strains usually tend to be either very closely related or not. This eliminates many of the problems that often occur between species that are defined by their phenotypic characteristics. In fact, DNA-DNA duplexes do not even form if the base pair mismatches between two DNA molecules exceed 10–20%. A species that is based on DNA:DNA hybridization can usually be readily defined in phenotypic terms, because the strains in the species tend to be very similar to each other not only in the genotype but also in the phenotype. The species is presently the only bacteriological taxonomic unit that can be defined in the phylogenetic terms (Wayne et al. 1987). DNA:DNA hybridization is much less useful at the genus level of classification. This is because the hybridization values between different species are usually too

low to provide much information about the relatedness among the species within a genus (Krieg and Holt 1984). Comparative studies involving both 16S rRNA sequencing and DNA-DNA hybridization indicated that a 50% DNA-DNA pairing typically corresponds to about 99% 16S rRNA similarity. Some researchers reported 16S rRNA sequence identity between *Bacillus globisporus* and *B. psychrophilus*, two species that are clearly justified by DNA-DNA similarity of less than 50%. Therefore, these retrieved new rRNA sequences with similarity values below 95% should at least be regarded as good evidence that microbiologists have discovered the presence of novel species.

It is highly debatable whether data, which were obtained with short oligonucleotides and experimentally induced mispairing can be extrapolated to entire genomes. At present, it, therefore, remains impossible to convert a present DNA-DNA hybridization value into a percent of whole genome sequence similarity (Vandamme et al. 1996).

10.3.3
DNA:rRNA Hybridization

Using this approach, the relatedness of different taxa, e.g. *Rhizobium* and *Agrobacterium* (De Smedt and De Leg 1997) or the heterogeneity within a taxa, e.g. within the genera *Pseudomonas*, *Xanthomonas* and *Alcaligenes* (De Vos and De Leg 1983) was demonstrated. Based on DNA:rRNA hybridization, in combination with other characteristic features, numerous new genera have been established, especially for the species of the genus *Pseudomonas*. This method could also be used to exclude certain strains from other genera e.g. *Rubrivivax gelatinosum* from the genus *Rhodocyclus* (Willems et al. 1991).

10.3.4
Ribosomal RNA (rRNA) and Spacer Region

Since protein synthesis is a very ancient process and present in all living cells, rRNA molecules are excellent targets to estimate evolutionary relationships. Zuckerkandl and Pauling (1965) proposed that macromolecules such as nucleic acids and proteins could serve as evolutionary chronometers. The sequence of the units should contain information about the evolutionary distances among the organisms, including those of bacteria (Akkermanns et al. 1998). Analysis of 16S rRNA gene is now widely used for the analysis of bacterial populations, and the analysis of 18S rRNA genes and internal transcribed spacer (ITS) regions is increasingly being used to analyze fungal populations. The macromolecules that are most suitable for

this would require the following prerequisites: (i) universal occurrence, (ii) functionally homologous in all organisms, and (iii) the sequence in the molecule should equally change as the wider the evolutionary distance the lower the mutation rate in the sequence.

Ribosomal RNA is proposed as one of the best candidates, and was first used by Woese et al. (1990) in his studies on bacterial evolution. Major properties of rRNA are: (i) rRNAs are the molecules present in all ribosomes, (ii) they are functionally constant, (iii) they have a wide distribution, (iv) they are well conserved over large phylogenetic distances, (v) they occur in large number in cells (1000–100,000 /cell), (vi) in bacteria three types of rRNA molecules are present with different chain length and sedimentation rate(s): 5S rRNA (about 120 nucleotides), 16S rRNA (~1600 nucleotides) and 23S rRNA (~3000 nucleotides). The 5S rRNA molecule is too small and only suitable to distinguish major phylogenetic groups. 23S rRNA is excellent for phylogenetic studies, but so far few studies are available. 16S rRNA has been given the most attention. Some fungal diversity studies are based on small subunit rRNA (18S rRNA), but this region possesses less useful information than 16S rRNA genes of prokaryotes. To obtain greater specificity, the analysis of amplification of ITS region is required (Viaud et al. 2000) (Fig. 10.2). Ribosomal DNA (rDNA) in eukaryotes is arranged in tandemly repeated units containing the coding regions for 18S, 5.8S, and 28S ribosomal RNA separated by spacers. The large intergenic spacer, or IGS, which separates the 28S and 18S coding regions, contains signals for transcription, initiation and termination. It often contains repetitive regions, which can vary in length due to variation in the number of repeated sequences. The internal transcribed spacers (ITS), which separate the 5.8S gene from the 18S and 28S genes on either side of it, contain motifs responsible for the correct splicing of the mature 18S, 28S and 5.8S rRNA molecules from the primary rRNA transcript (Fig. 10.2). The internal transcribed spacers (ITS) are noncoding regions of DNA sequence that separate genes coding for 28S, 5.8S, and 18S ribosomal RNAs. These ribosomal RNA (rRNA) genes are highly conserved across the taxa, while the spacers between them may be species-specific. The conservation of the rRNA genes allows easy access to the ITS regions with "universal" primers for polymerase chain reaction (PCR) amplification. The variation in the spacers has proven useful for distinguishing a wide diversity of difficult-to-identify taxa. The ITS region is expected to show intraspecific variation because (a) it evolves rapidly and (b) populations of a species can be reproductively isolated on large scales. The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other regions of rDNA, the variation among individual rDNA repeats can

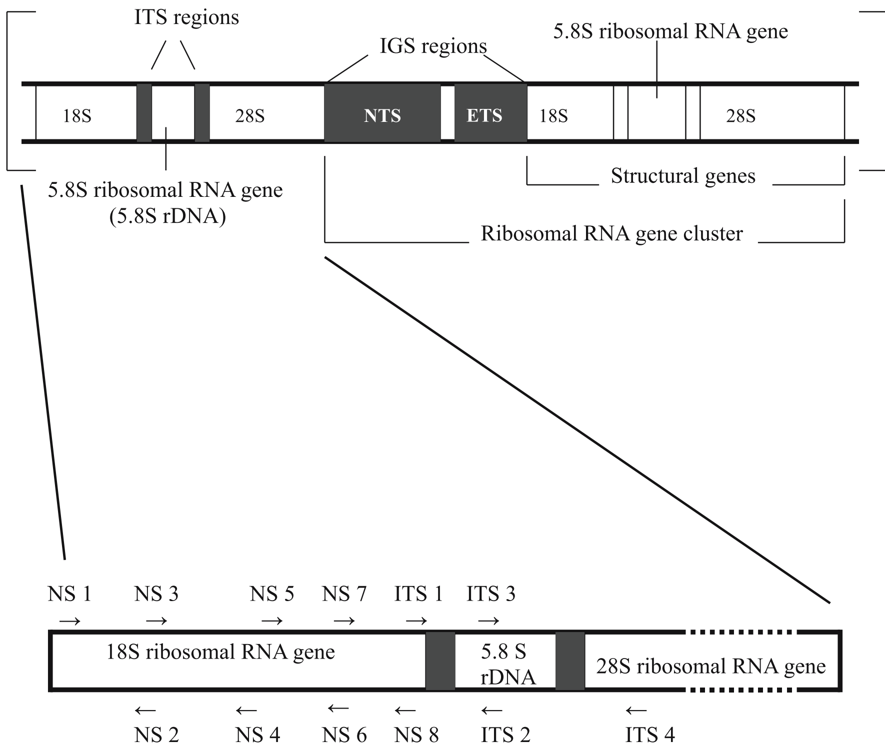

Fig. 10.2. Structure of the ribosomal RNA gene cluster showing the positions of fungal PCR primers. The cluster is split into coding (18S, 5S and 28S genes) and non-coding (Inter-Genic Spacer or IGS and Internally Transcribed Spacer or ITS sequences) regions. Some of the non-coding areas are transcribed (Externally Transcribed Spacer or ETS and ITS) while others are not (Non-Transcribed Spacer or NTS sequence). The positions of the primers and their direction of replication are indicated by *arrows*. The IGS and ITS regions usually exhibit the most sequence variation while the coding areas are the most conserved (source: Mitchell et al. 1995)

sometimes be observed within both the ITS and IGS regions (Fig. 10.2). In addition to the standard ITS1+ITS4 primers used by many researchers (Pandey et al. 2003; Singla et al. 2004), several taxon-specific primers have been described that allow selective amplification of fungal sequences.

10.3.5 Amplification by Polymerase Chain Reaction (PCR)

Out of many molecular methods that are currently available, the polymerase chain reaction (PCR) based techniques have become very powerful (Innis et al. 1990; McPherson et al. 1992). This approach was first applied

by Giovannoni et al. (1990a,b) in the analysis of Sargasso Sea picoplankton. Results indicated again the presence of defined clusters of proteobacterial and cyanobacterial origin. PCR amplification of 16S rRNA from organisms, which are not yet culturable, has provided an invaluable insight into the understanding of community structure, especially of communities inhabiting extreme environments where growth conditions may be difficult to mimic in the laboratory. The PCR is a technique to amplify (in vitro) specific DNA sequences with the help of a DNA polymerase enzyme and specific primers. Over the past 25 years, a large number of primer sequences for amplification and sequencing of SSU rRNA genes has been published (Das Sarma and Fleischmann 1995; Elwood et al. 1985, Kolganova et al. 2002; Watanabe et al. 2001). Some of these primers have been designed as taxa specific, whilst others have been designed to amplify all prokaryotic SSU-rRNA genes and are referred to as universal (Barns et al. 1994; Hugenholtz et al. 1998; Nielsen et al. 1999).

10.3.6 Primers

Primers are required that should be both universal and specific. Ideally, they must be specific to the domains in question, whilst complimentary to sequences in all taxa within that domain. A list of 16S rRNA primers has been published (Das Sarma and Fleischmann 1995; Reysenbach and Pace 1995) that are alluded to as universal or domain specific, but little empirical evidence supporting these specificities are available.

16S rDNA Primers

According to variability map, over 10% of bases in the 16S rRNA gene are totally conserved (within a sample of 500 bacterial sequences). The majority of these conserved bases are, however, not adjacent to each other and thus form no continuous conserved regions for universal priming. The longest string of totally conserved bases is between positions 788 and 798, but in most areas of the gene, absolutely conserved bases are found in strings of less than 4. Thus, no primer of sufficient length can be designed that is a 100% match to all bacterial, let alone, all prokaryotic 16S rRNA gene sequences. Furthermore, sequences of recently discovered taxa are not adequately represented in the variability map (Watanabe et al. 2001).

Universal primer design is a compromise between complementarities and other primer attributes, such as primer-primer complementarities, annealing template and G/C ratio (McPherson et al. 1995) (Table 10.3). Ideal annealing temperatures and lack of self-complementation are sacrificed in 16S rRNA gene primer design in order to obtain optimal specificity (Baker et

al. 2003). None of the primers in current use are 'truly universal', and the introduction of inosine residue in the primers has broader specificity, but excessive use can lead to amplification of non-target groups (Baker et al. 2003).

Bias in the phylogenetic analysis is introduced through differential amplification caused by differences in the efficiency of primer binding, interference by sequences flanking primer regions (Hansen et al. 1998) and differences in the kinetics of the PCR reaction (Brunk and Eis 1998; Reysenbach and Pace 1995). As a consequence, many, if not all, 16S rDNA libraries will not be totally representative of microbial communities, especially on a quantitative level (Farelly et al. 1995). However, pooling of several PCR reactions utilizing slightly different primers may significantly reduce bias and provide a more accurate understanding of microbial community.

18S rDNA and ITS Primers

A major challenge in applying rRNA technology to fungal communities is the design of suitable PCR primers with specificity for fungal DNA, while reducing co-amplification of similar target DNA from non-fungal sources. Numerous PCR primers have been described (Table 10.3) that amplify fungal rDNA from a wide range of taxonomic groups (White et al. 1990),

Table 10.3. Primer sequences specific to ITS region of fungi and 16S rDNA of bacteria[a]

Primer name	Sequence ($5' \rightarrow 3'$)
ITS Specific	
ITS1	TCCGTAGGTGAACCTGCGG
ITS2	GCTGCGTTCTTCATCGATGC
ITS3	GCATCGATGAAGAACGCAGC
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG
ITS1-F	CTTGGTCATTTAGAGGAAGTAA
ITS4-B	CAGGAGACTTGTACACGGTCCAG
Bacteria specific 16S rDNA sequence	
E8F	AGAGTTTGATCCTGGCTCAG
E9F	GAGTTTGATCCTGGCTCAG
E334F	CCAGACTCCTACGGGAGGCAGC
E341F	CCTACGGGIGGCIGCA
E786F	GATTAGATACCCTGGTAG
E533R	TIACCGIIICTICTGGCAC
E926R	CCGICIATTIITTTIAGTTT
E939R	CTTGTGCGGGCCCCCGTCAATTC
E1115R	AGGGTTGCGCTCGTTG
E1541R	AAGGAGGTGATCCANCCRCA

[a] White et al. 1990; Baker et al. 2003

although very few were designed for use with DNA extracted from mixed communities, and their lack of selectivity may lead to complex environmental samples. There have also been conflicting reports about the specificity of newly designed fungal 18S rRNA primers (Smit et al. 1999; Borneman and Hartin 2000). For example, although the PCR primer pairs EF-4/EF-3 and EF4/Fung5 were shown in one study to amplify only fungal 18S rDNA sequence from wheat rhizospheric soil (Smit et al. 1999), other investigators have shown that the same primers can also amplify some fungal template (Borneman and Hartin 2000). In addition, 18S rRNA gene sequences are generally only able to resolve taxonomic groups to the level of genus, and the taxonomic resolution of fungal 18S rRNA and ITS sequence is limited by the current availability of information held within databases. However, this problem is decreasing, and molecular identification, wherever possible, is more rapid.

Although, primer specificity is desirable, in certain situations with low non-fungal diversity, less specific primers may be useful. For example, Vainio and Hantula (2000) analyzed fungi colonizing Norway spruce stumps. The lack of non-fungal eukaryotic contaminants enabled comparison of wood colonizers isolated in pure culture and those detected by molecular technique. 18S rRNA genes provide good coverage of fungal taxa, but do not enable fine scale analysis.

This can be analyzed by ITS sequences, and primers have been developed for amplification of ITS regions from the extracted DNA (Viaud et al. 2000).

10.3.7 Random Amplified Polymorphic DNA (RAPD)

In this technique random primers anneal randomly to DNA, and thus, the PCR products are mixtures of fragments with differences in length and/or sequences. The differentiation of closely related strains by comparing random amplified polymorphic DNA patterns has been introduced. This approach has been applied to different groups of prokaryotes including halophilic archaeal isolates (Martinez-Murcia et al. 1995), but mainly to bacteria of clinical relevance for the differentiation of closely related species that are difficult to distinguish, such as *Listeria monocytogenes* and *Listeria innocua* or different strains of '*Haemophilus somnus*' and *Staphylococcus aureus*.

In comparison between randomly amplified polymorphic DNAs (RAPD) with restriction fragment length polymorphism (RFLP), it is found that: (i) for RAPD, less quantity of DNA is required (~10–50 ng), while for RFLP large quantity of DNA is required, i.e. 2–10 μg, (ii) the same primers with arbitrary sequences can be used for different species in RAPD, but for

RFLP different species specific probes are required, (iii) RAPD is five times quicker than RFLP, and (iv) RAPD is comparatively less reliable.

10.3.8 Amplification Fragment Length Polymorphism (AFLP)

The amplification reaction is stringent, versatile and robust, and appears to be quantitative. While AFLP is capable of producing very complex fingerprints (100 bands where RAPD produces 20), it is a technique that requires DNA of reasonable quality and reasonable quantity and is more experimentally demanding (Karp et al. 1996). AFLP can be used for mapping, fingerprinting and genetic distance calculation between genotypes. The advantage of AFLP is its high multiplexity, and therefore, the possibility of generating high marker densities. One limitation of the AFLP technique is that fingerprints may share few common fragments when genome sequence homology is less than 90%. Therefore, AFLP cannot be used in comparative genomic analysis with hybridization based probes or when comparing genomes that are evolving rapidly such as those of microbes. Conversely, very homologous genomes may not be suitable for AFLP analysis (Karp et al. 1996).

The value of amplified rDNA restriction analysis for identification of the phylogenetically and phenotypically delineated genera and species within the Comamonadaceae has been investigated. Restriction analysis of the 16S rDNA, the 16S to 23S rDNA spacer region, and part of the 23S rDNA with *Hin*fI and *Cfo*I, resulted in consistent species-specific patterns, suggesting that identification at the species level is possible. Within *Comamonas terrigena*, the three genotypic groups could be differentiated by the combined use of *Hin*fI and *Nci*I patterns (Vandamme et al. 1996).

10.3.9 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another. Sample DNA is cut (digested) with one or more restriction enzymes and resulting fragments are separated according to molecular size using gel electrophoresis (Avise 1994). Molecular size standards are used to estimate fragment size. Restriction

fragment length polymorphism is most suited to studies at the intraspecific level or among closely related taxa (Pandey et al. 2003). Presence and absence of fragments resulting from changes in the recognition sites are used for identifying species or populations. RFLP markers have several advantages in comparison with the RAPD such as: (i) they are codominant and unaffected by the environment, (ii) any source DNA can be used for the analysis, and (iii) many markers can be mapped in a population that is not stressed by the effects of phenotypic mutations.

10.3.10 Gradient Gel Electrophoresis

Using this approach, members of a community making up only 1% of the total, can be detected. Muyzer et al. (1993) first applied denaturing gel electrophoresis (DGGE) techniques for the analysis of whole bacterial communities. Separation of DNA fragments in DGGE and TGGE is based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) (DGGE) or a linear temperature gradient (TGGE). The separation of DNA fragments, commonly up to 400 bp, is achieved as a function of their different G+C content and distribution (Muyzer 1999). For the amplification of the rDNA fragments of DNA extracted from a natural sample, one primer designed complementary to the respective conserved region of rRNA, but additionally consisting of a 40 nucleotide GC rich sequence (GC clamp) at its 5′ end is used in combination with a conventional primer (Busse et al. 1996). The separation of the fragments could very well be improved and even one or two mismatches in PCR products of 400 bp resulted in the distinct bands in gels. The sensitivity of DGGE analysis can be refined with the targeting of precise (and even non-dominant) taxonomic groups, by using specific PCR primers (Heuer et al. 1997; Nubel et al. 1997) or by identifying community members by hybridization of blotted DGGE gels with group-specific oligonucleotide probes (Heuer et al. 1999). In DGGE analysis, the generated banding pattern is considered as an image of the whole bacterial community. An individual discrete band refers to a unique sequence type or phylotype (Muyzer et al. 1995), which is treated in turn as a discrete bacterial population.

The DGGE approach is advantageous over the cultivation strategy or analysis of community structures, as more members of the community can be detected simultaneously. However, it can be assumed that all the strains of a given sample are amplified. Based on varying rigidity of the different bacterial cell walls, not all cells may become disrupted during the DNA extraction.

10.3.11
Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Avaniss-Aghajani et al. (1994) mated the automated sequencing technology and PCR amplification with the highly conserved phylogenetic marker 16S rRNA. These researchers described the technique as a 'highly sensitive and rapid protocol for identifying a broad spectrum of bacterial species'. It should be noted here that any genetic marker with conserved sequence domains appropriate for primer design could be used with this technique. The relatively large database of the 16S rRNA sequences makes it an ideal candidate (Marsh 1999).

As its name implies, terminal restriction fragments length polymorphism (T-RFLP) analysis measures the size polymorphism of terminal restriction fragments from a PCR amplified marker. It is a marriage at least of three technologies including comparative genomics/RFLP, PCR, and nucleic acid electrophoresis. Comparative genomics provide the necessary insight in to the design of primers for the amplification product (amplicon) of choice, and PCR amplifies the signal from high background of unrelated markers. Subsequent digestion with judiciously selected restriction endonucleases produces terminal fragment appropriate for sizing on high resolution (±1 base) sequencing gels. The latter step is conveniently performed on automated systems such as the ABI gel or capillary electrophoresis system that provide digital output.

T-RFLP has been used for the strain identification, comparative community analysis and to derive estimates of the diversity of a phylogenetic group within a community. Because of high throughput capacity and the supporting sequence databases, the technique will prove most valuable in comparative community analysis. Increasing levels of community dissection can be attained with the systematic use of phylogenetic specific primers in the T-RFLP protocol. The T-RFLP data, when juxtapositioned with complementary data on the diversity and distribution of fundamental physiological markers as well as physico-chemical data describing the particular ecosystem, will provide a level of insight into the demographic structure and function of microbial communities that are yet to be realized (Marsh 1999).

10.3.12
Screening of Libraries of Amplified 16S rDNA Sequence

Among the a number of fingerprinting techniques, denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993) and terminal restriction fragment length polymorphisms (t-RFLP) (Liu et al. 1997; Braker et al. 2001) are

used, which require amplification of specific DNA sequences, generally by PCR. The general procedure recommends the construction of a 16S rDNA library by cloning the amplified DNA fragments into a host vector and transformation in *E. coli* (Sambrook et al. 1989). Processing a DNA library requires the screening of the selected clones. 16S rDNA library screening is a costly, time-consuming process (Gonzalez et al. 2003). Since ribosomal RNA genes are highly conserved between microbial species, recently developed DNA subtraction techniques (Bjourson et al. 1992; Buehaille et al. 2000) do not work properly. Screening with the labeled oligomeric probes cannot be used since the sequence is to be targeted is unknown. The standard screening procedure consists of examining a number of clones using molecular fingerprinting techniques (e.g., DGGE analysis) until the clone carrying the DNA fragment of interest is found. When the clone of interest represents a minority in a 16S rDNA library, this procedure often misses the desired clone due to the difficulty, both in timing and costs, of processing a large enough set of clones (Hughes et al. 2001). Gonzalez et al. (2003) proposed a new strategy for the screening of the 16S rDNA library (Fig. 10.3).

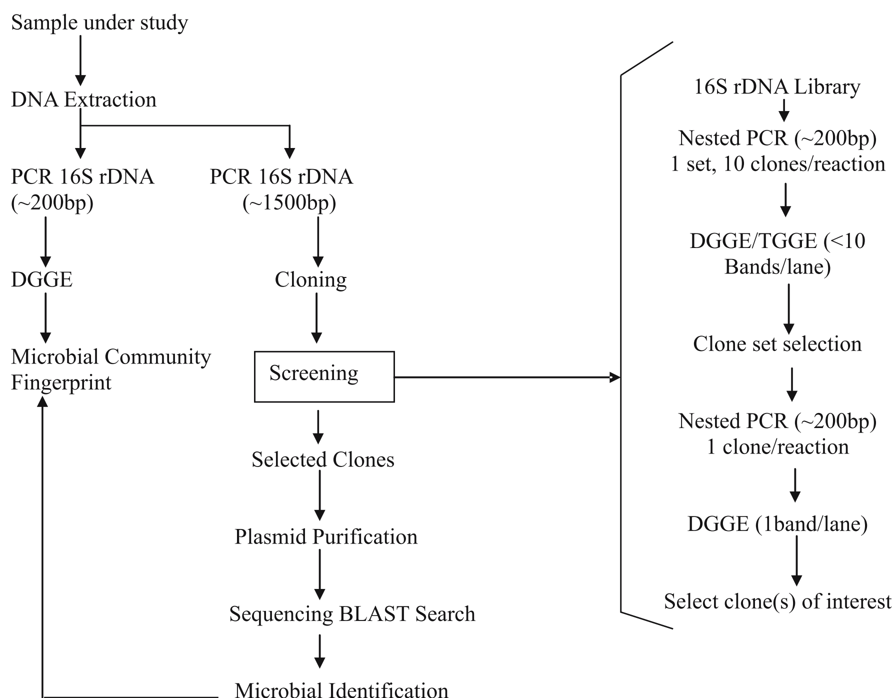

Fig. 10.3. Procedure for microbial diversity analysis of environmental samples using DGGE/TGGE and the proposed strategy for an efficient screening of 16S rDNA libraries (source: Gonzalez et al. 2003)

10.3.13 In Situ Hybridization

Quantification of the relative abundance of certain populations from the relative abundance of certain rDNA clones will always be biased by the fact that the number of rRNA gene operons present in a bacterial chromosome can range from one copy in mycoplasmas (Amikam et al. 1984) to ten copies in Bacilli (Jarvis et al. 1988). In situ identification and enumeration of microorganisms harboring a certain rRNA sequence requires a technique in which the rRNA is specifically detected within morphologically intact cells, a technique here referred to as whole-cell hybridization. One can not only determine the cell morphology of an uncultured microorganism and its abundance, but also analyze spatial distributions in situ (Amann et al. 1995). Quantification of the signal conferred by rRNA-targeted oligonucleotides should even allow the estimation of in situ growth rates of individual cells. The use of in situ hybridization for counting and identifying organisms had already been proposed by Olsen et al. (1986). The microscopic identification of single microbial cells with rRNA-targeted probes was first performed with radioactively labeled oligonucleotides (Giovannoni et al. 1988). As demonstrated several years ago by the immunofluorescence approach (Bohlool and Schmidt 1980), fluorescent probes yield superb spatial resolution and can be instantaneously detected by epifluorescence microscopy. Fluorescently labeled rRNA-targeted oligonucleotide probes were demonstrated to allow detection of individual cells (De Long et al. 1989). This made whole-cell hybridization, with rRNA-targeted probes, a suitable tool for determinative, phylogenetic, and environmental studies in microbiology (Amann et al. 1990). Like immunofluorescence, whole-cell hybridization with fluorescently labeled rRNA-targeted oligonucleotides can be combined with flow cytometry for a high-resolution automated analysis of mixed microbial populations (Wallner et al. 1993; Amann et al. 1990, 1995).

Autoradiography combined with 16S rRNA probing provides greater discriminatory power for targeted identification and detection of active cells belonging to phylogenetic groups with no known cultivated representatives. This approach termed STAR-FISH, has been used to detect the activity of marine plankton (Ouverney and Fuhrman 1999) and cells in activated sludge (Lee et al. 1999; Prosser 2002).

10.4 Limitation of rDNA Technology

Certain biases such as variation in lysis of cells in different microbial groups, between spores and mycelia and between regions of mycelia of different

ages, influence of DNA extraction are associated with this molecular technique. In any case, the 16S rRNA gene represents only 0.05% on average of the genome of a prokaryotic cell and has very little value to predict the activities (physiology), style of life (niche) or biotechnological properties of such cells (first to mention a few relevant characteristics) (Rodriguez-Valera 2002). Multiple 16S rRNA genes of the same isolate have identical or show only minor differences.

PCR amplification and the use of primers can lead to differences in the proportions of different rRNA genes and sequencing errors, formation of chimeric sequences and introduction of errors during cloning can influence results. DGGE/TGGE have also some specific limitations (Muyzer and Smalla 1998), for instance, the detection of heteroduplex molecules (Ferris and Ward 1997) and molecules produced by different rRNA operons of the same organism (Nubel et al. 1997). Furthermore, the separation of relatively small DNA fragments, and the co-migration of DNA fragment with different sequences (Vallaeys et al. 1997).

A potential risk of gene amplification by PCR from mixed-culture DNA is the formation of chimeric sequences assembled from sequences of different species. This is more than a theoretical threat as demonstrated by Liesack et al. (1991). A chimera can then be identified either by checking the complementarity of helical regions or by performing comparative sequence analyses of different sections of the rDNA amplification products (Liesack et al. 1991). Phylogenetic trees reconstructed for different sections, e.g., the 59 and 39 halves of a chimeric clone would indicate different affiliations. The rRNA database project at the University of Illinois supplies a chimera check program (Larsen et al. 1993).

In general, one particular rDNA sequence is associated with one species. Different isolates of some species such as *Mycobacterium gordonae* may show different rDNA sequences (Kirschner and Bottger 1992), but to our knowledge, no difference has ever been shown between different copies of the 16S rDNA genes of a single *Mycobacterium* isolate. Ninet et al. (1996) found a slowly growing *Mycobacterium* (*Mycobacterium* strain X) belonging to the *Mycobacterium terrae* complex based on biochemical criteria. The 16S rDNA sequence yielded ambiguous results because of the presence of two different 16S rDNAs.

10.5 Conclusions

The advent of rDNA (16S, 18S, 23S, 28S, ITS) and molecular fingerprinting techniques in the field of microbial ecology, which provide phylogenetic characteristics of the microorganisms, has revolutionized microbial

systematics. It is hoped that the technological advancements will continue and more data will be accumulated. There will be more automation and software development for the analysis of the data. However, potential bias is always associated irrespective of the techniques applied. The major challenge is to reduce the bias associated with the techniques so that there should not be under- or over-estimation of the biodiversity of different environments. The second most challenging task will be to process the mass information into useful speciation and classification concepts. The concept of polyphasic taxonomy is better to cultivate the useful information regarding identification and phylogeny of the microbes. Polyphasic taxonomy is basically a combination of methods based on phenotypes, genotypes and phylogenetics. The main problem in applying the polyphasic approach is the interpretation of the chemotaxonomic data because of the absence of a fatty acid profiles database. This can be sorted out with the knowledge of the published reports. In ectomycorrhizas, molecular methods alone may fail to separate well recognized phenotypic species because of the low level of divergence among species due to lack of sufficient divergence in the ITS region. Still, ITS contains useful information and has considerable utility in the identification of the distant genera. Further research requires focus on the mitochondrial small subunit rRNA gene, which also has the potential for identification at the species level and phylogeny.

References

Akkermanns ADL, Elsas JDV, De Bruijn FJ (eds) (1998) Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands

Amann RI, Krumholz L, Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172:762–770

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Amikam D, Glaser G, Razin S (1984) Mycoplasmas (*Mollicutes*) have a low number of rRNA genes. J Bacteriol 158:376–378

Avaniss-Aghajani E, Jones K, Chapman D, Brunk C (1994) A molecular technique for identification of bacteria using small subunit ribosomal RNA sequences. Biotechniques 17:144–149

Avise JC (1994) Molecular markers. Natural history and evolution. Chapman and Hall, New York

Azcon-Aguilar C, Bago B (1994) Physiological characteristics of the host plant promoting an undisturbed functioning of the mycorrhizal symbiosis. In: Gianiazzi S, Schuepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. ALS Birkhauser, Basel, Switzerland, pp 47–60

Azcon-Aguilar C, Barea JM (1992) Interactions between mycorrhizal fungi and other rhizosphere microorganisms. In: Allen MJ (ed) Mycorrhizal functioning: an integrative plant-fungal process. Routledge, Chapmann and Hill, New York, pp 163–198

Baker GC, Smith JJ, Cowan DA (2003) Review and re-analysis of domain specific 16S primers. J Microbiol Meth 55:541–555

Barea JM (1997) Mycorrhiza/bacteria interactions on plant growth promotion. In: Ogoshi A, Kobayashi L, Homma Y, Kodama F, Kondon N, Akino S (eds) Plant growth-promoting rhizobacteria, present status and future prospects. OECD, Paris, pp 150–158

Barea JM (2000) Rhizosphere and mycorrhiza of field crops. In: Toutant JP, Balaz E, Galante E, Lynch JM, Schepers JS, Werner D, Werry PA (eds) Biological resource management: connecting science and policy (OECD) INRA Editions and Springer, pp 110–125

Barea JM, Azcon R, Azcon-Aguilar C (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie van Leeuwenhoek 81:343–351

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in Yellowstone National Park hot spring environment. Proc Natl Acad Sci USA 91:1609–1613

Bjourson AJ, Stone CE, Cooper JE (1992) Combined subtraction hybridization and polymerase chain reaction amplification procedure for isolation of strain-specific *Rhizobium* DNA sequences. Appl Environ Microbiol 58:296–301

Bohlool BB, Schmidt EL (1980) The immuno fluorescence approach in microbial ecology. Adv Microb Ecol 4:203–241

Borneman J, Hartin RJ (2000) PCR primers that amplify fungal rRNA genes from environmental samples. Appl Environ Microbiol 66:4356–4360

Bourgault AM, Lamothe F (1988) Evaluation of the KOH test and the antibiotic disk test in routine clinical anaerobic bacteriology. J Clin Microbiol 26:2144–2146

Bowen GD, Rovira AD (1999) The rhizosphere and its management to improve plant growth. Adv Agron 66:1–102

Braker G, Ayala-del-Rio HL, Devol AH, Fesefeldt A, Tiedje JM (2001) Community structure of denitrifiers, bacteria, and archaea along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (nirS) and 16S rRNA genes. Appl Environ Microbiol 19:421–430

Brunk CF, Eis N (1998) Quantitative measure of small subunit rRNA gene sequences of the Kingdom Korarchaeota. Appl Environ Microbiol 64:5064–5066

Buehaille R, Couble ML, Magloire H, Bleicher F (2000) A subtractive PCR based cDNA library from human odontoblast cells: identification of novel genes expressed in tooth forming cells. Matrix Biol 19:421–430

Buissiere J, Nardon P (1968) Micromethode d'identification des batteries. I. Iniret de la quantification des characttres biochimiques. Ann Inst Pasteur, Paris 115:218

Busse H, Denner EBM, Lubitz WJ (1996) Classification and identification of bacteria: current approaches to an old problem. Overview of methods used in bacterial systematics. J Biotechnol 47:3–38

Cerny G (1976) Method for distinction of the Gram-negative from Gram-positive bacteria. Eur J Appl Microbiol 3:223–225

Cowan ST, Steel KJ (1964) Comparison of differentiating criteria for Staphylococci and Micrococci. J Bacteriol 88:804

Curtis TP, Sloan WT, Scannell JW (2002) Estimating prokaryotic diversity and its limits. Proc Natl Acad Sci USA 99:10494–10499

D'Amato RF, Bottone EJ, Amsterdam D (1991) Substrate profile systems for the identification of bacteria and yeasts by rapid and automated appoaches. In: Balows A, Hausler WJ Jr, Hermann KL, Isenberg HD Shadomy HJ (eds) Manual of clinical microbiology, 5th edn. America Society for Microbiology, Washington DC, pp 128–136

Das Sarma S, Fleischmann EF (1995) Archea: a laboratory manual – halophiles. Cold Springer Harbour Laboratory Press, New York, pp 269–272

DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363
De Ley J (1970) Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. J Bacteriol 101:738–754
De Ley J, Cattoir H, Reynaerts A (1970) The quantitative measurements of DNA hybridization from renaturation rates. Eur J Biochem 12:133–142
De Smedt J, De Ley J (1977) Intra- and intergeneric similarities of *Agrobacterium* ribosomal acid cistrons. Int J Syst Bacteriol 27:222–240
De Vos P, De Ley J (1983) Intra- and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. Int J Syst Bacteriol 33:487–509
Elwood HJ, Olsen GJ, Sogin ML (1985) The small subunit rRNA gene sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata*. Mol Biol Evol 2:399
Farelly V, Rainey FA, Stackebrandt E (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes mixture of bacterial species. Appl Environ Microbiol 61:2798–2801
Ferris MJ, Ward DM (1997) Seasonal distribution of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl Environ Microbiol 63:1375–1381
Gaby WL, Hadley L (1957) Practical laboratory test for the identification of *Pseudomonas aeruginosa.* J Bacteriol 74:356
Gagnon M, Hunting WM (1959) New method for the catalase determination. Anal Chem 31:144
Gillis PBM, De Leg J (1992) The genus *Aquaspirillum*. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH (eds.) The prokaryotes: a handbook on the biology of bacterial ecophysiology, isolation, identification, and applications. Springer, Berlin Heidelberg New York, pp 2569–2582
Giovannoni SJ, DeLong EF, Olsen GJ, Pace NR (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J Bacteriol 170:720–726
Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990a) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:60–63
Giovannoni SJ, DeLong EF, Schmidt TM, Pace NR (1990b) Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. Appl Environ Microbiol 56:2572–2575
Gonzalez JM, Ortiz-Martinez A, Gonzalez-delvalle MA, Laiz L, Saiz-Jimenez (2003) An efficient strategy for screening large cloned libraries of amplified 16S rDNA sequence from complex environmental communities J Microbiol Meth 55:459–463
Gregersen T (1978) Rapid method for distinction of Gramnegative from Grampositive bacteria. Eur J Appl Microbiol Biotechnol 5:123–127
Gryndler M, Hrselova H, Striteska D (2000) Effect of soil bacteria on growth of hyphae of the arbuscular mycorrhizal (AM) fungus Glomus claroideum. Folia Microbiol 45:545–551
Halebian S, Harris B, Finegold M, Rolfe RD (1981) Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. J Clin Microbiol 13:444–448
Hansen MC, Tolker-Neilson T, Givskov M, Molin S (1998) Biased 16S rDNA PCR amplification caused by interference from DNA flanking template region. FEMS Microbiol Ecol 26:141–149
Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH (1997) Analysis of actinmycetes communities by specific amplification of genes encoding 16S rDNA and gel electrophoretic separation in denaturing gradients. Appl Env Microbiol 63:3233–3241
Heuer H, Hartung K, Wieland G, Kramer I, Smalla K (1999) Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. Appl Environ Microbiol 65:1045–1049

Hugenholtz P, Pitulle C, Hershberger KL, Pace NR (1998) Novel division level bacterial diversity in a Yellowstone hot spring. J Bacteriol 180:366–376

Hugh R, Leifson E (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J Bacteriol 66:24–26

Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJM (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol 67:4399–4406

Innis MA, Gelfand DH, Sninsky JJ, White TJ (1990) PCR protocols: a guide to methods and applications. Academic Press, New York

Jarvis ED, Widom RL, LaFauci G, Setoguchi Y, Richter IR, Rudner R (1988) Chromosomal organization of rRNA operons in *Bacillus subtilis*. Genetics 120:625–635

Kaneko T, Katoh K, Fujimoto M, Kumagai M, Tamaoka J, Kataydma-Fujimura Y (1986) Determnation of the nucleotide composition of a deoxyribonucleic acid by high-performance liquid chromatography of its enzymatic hydrolysate: a review. J Microbial Meth 4:229–240

Karp A, Seberg O, Buiatti M (1996) Molecular techniques in the assessment of botanical diversity. Annals Bot 78:143–149

Katayama-Fujimura Y, Tsuzaki N, Kuraishi H (1982) Ubiquinone, fatty acid and DNA base composition determination as a guide to the taxonomy of the genus *Thiobucillus.* J Gen Microbial 128:1599–1611

Kirschner P, Bottger EC (1992) Microheterogenisity within rRNA of *Mycobacterium gordonae.* J Clin Microbiol 30:1049

Kloepper JW (1994) Plant growth promoting rhizobacteria (other systems) In: Okon Y (ed) *Azospirillum*/plant associations. CRC Press, Boca Raton, pp 111–118

Kolganova TV, Kuznetsov BB, Tourova TP (2002) Designing and testing oligonucleotide primers for amplification and sequencing of Archael 16S rRNA genes. Mikrobiologia 71:283–286

Kovacs N (1928) Eine vereinfachte Methode zum Nachweis der Indolbildung durch Bakterien. Z Immunitaetsforsch Exp Ther 55: 311–315

Kovacs N (1956) Identification of *Pseudomonas pyocyanae* by the oxidase reaction. Nature 178:703

Krieg NR, Holt JG (1984) Bergey's manual of systematic bacteriology, vol. I. Williams and Wilkins, Baltimore

Larsen N, Olsen GJ, Maidak BL, McCaughey MJ, Overbeek R, Macke TJ, Marsh TL, Woese CR (1993) The ribosomal database project. Nucleic Acids Res 21:3021–3023

Lee N, Neilsen PH, Andreasen KH, Juretschko S, Schleifer JL, Wagner M (1999) Combination of fluorescent in situ hybridization and microautoradiography – a new tool for structure-function analysis in microbial ecology. Appl Environ Microbiol 65:1289–1297

Liesack W, Weyland H, Stackebrandt E (1991) Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed culture of strict barophilic bacteria. Microb Ecol 21:191–198

Linderman RG (1992) Vescicular-arbuscular mycorrhizae and soil microbial interactions. In: Bethlenfalvay GJ, Linderman RG (eds) Mycorrhizae in sustainable agriculture. ASA Spec Publ, Madison, Wisconsin, pp 45–70

Linderman RG (2000) Effects of mycorrhizas on plant tolerance to diseases. In: Kapulnick Y, Douds DD (eds) Arbuscular mycorrhizas: physiology and function. Kluwer Academic Press, pp 345–366

Liu W, Marsh TL, Cheng H, Fomey LJ (1997) Charecterization of microbial diversity by determining terminal restriction length polymorphism of genes encoding 16S rDNA. Appl Environ Microbiol 63:4516–4522

Lynch JM (1990) The rhizosphere. John Wiley, New York

Manafi M, Kneifel W (1990) Rapid methods for differentiating Gram-positive from Gram-negative aerobic and facultative anaerobic bacteria. J Appl Bacteriol 69:822–827
Mandel M, Schildkraut CL, Marmur J (1968) Use of CsCl density gradient analysis for determining the guanine plus cytosine content of DNA. Meth Enzymol 12:184–195
Margulis L, Schwartz KV (1998) Five kingdoms: an illustrated guide to the phyla of life on earth. Freeman, New York
Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118
Marsh TL (1999) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplified products. Cur Opin Microbiol 2:323–327
Martinez-Murcia AJ, Acinas SG, Rodriguez-Valera F (1995) Evalution of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol Ecol 17:247–256
McPherson MJ, Hames BD, Taylor GR (1995) PCR II. Oxford University Press, UK
McPherson PQ, Taylor GR (1992) PCR. A practical approach. The practical approach series. IRL Press at Oxford University Press, Oxford, UK
Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. Int J Syst Bacteriol 39:159–167
Mitchell JI, Roberts PJ, Moss ST (1995) Sequence or structure? A short review on the applications of nucleic acid sequence information to fungal taxonomy. Mycologist 9:67–75
Muyzer G (1999) Genetic fingerprinting of microbial communities-present status and future perspectives. In: Microbial biosystems: new frontiers. Proceedings of the 8th International Symposium on Microbial Ecology, Halifax, Canada
Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73:127–141
Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16 s rRNA. Appl Environ Microbiol 59:695–700
Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995) Phylogenetic relationship of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresois of 16S rDNA fragment. Arch Microbiol 164:165–172
Nielsen AT, Liu WT, Filipe C, Grady L, Molin S, Stahl DA (1999) Identification of a novel group of bacteria in a sludge from a deteriorated biological phosphorus removal reactor. Appl Environ Microbiol 65:1251–1258
Ninet B, Monod M, Emler S, Pawlowski J, Metral C, Rohner P, Auckenthaler R, Hirschel B (1996) Two different 16S rRNA genes in a Mycobacteria strain. J Clin Microbiol 34:2531–2536
Nubel U, Garcia-Pichel F, Muyzer G (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl Environ Microbiol 63:3327–3332
Olsen GJ, LaneDJ, GiovannoniSJ, Pace NR, Stahl DA (1986) Microbial ecology and evolution: a ribosomal RNA approach. Ann Rev Microbiol 40:337–365
Ouverney CC, Fuhrman JA (1999) Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types *in situ*. Appl Environ Microbiol 65:1746–1752
Pandey AK, Reddy MS, Suryanarayan TS (2003) ITS-RFLP and ITS sequence analysis of a foliar endophytic *Phyllosticta* from different tropical trees. Myco Res 107:439–444

Prosser JI (2002) Molecular and functional diversity in soil microorganisms. Plant Soil 244:9–17
Reysenbach AL, Pace NR (1995) In: Robb FT, Place AR (eds) Archea a laboratory manual thermophiles. Cold Spring Harbour Laboratory Press, New York, pp 101–107
Rodriguez-Valera F (2002) Approaches to prokaryotic biodiversity: a population genetics perspective. Environ Microbiol 4:628–633
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbour Laboratory Publ., NY
Schildkraut CL, Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J Mol Biol 4:430–443
Singla S, Reddy MS, Marmeisse R, Gay G (2004) Genetic variability and taxonomic position of ectomycorrhizal fungus *Pisolithus* from India. Microbiol Res 159:203–210
Smit E, Leeflang P, Glandorf B, van Elsas JD, Wernars K (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Appl Env Microbiol 65:2614–2621
Smith DE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, London
Sneath PHA, Sokal RR (1973) Numerical taxonomy: the principles and practice of numerical classification. W.H. Freeman, San Francisco
Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA-DNA reassociation and 16 s rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849
Stager CE, Davis JR (1992) Automated systems for identification of microorganisms. Clin Microbiol Rev 5:302–307
Staley JT, Konopka A (1985) Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Ann Rev Microbiol 39:321–346
Sturz AV, Nowak J (2000) Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. Appl Soil Ecol 15:183–190
Tamaoka J, Komagata K (1984) Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol Lett 25:125–128
Toal ME, Yeomans C, Killham K, Meharg AA (2000) A review of rhizosphere carbon flow modelling Plant Soil 222:263–281
Torsvik V, Ovreas L (2002) Microbial diversity and function in soil: from genes to ecosystems. Curr Opin Microbiol 5:240–245
Torsvik V, Sorheim R, Gokoyr J (1996) Total bacterial diversity in soil and sediment communities – a review. J Indust Microbiol 17:170–178
Torsvik V, Ovreas L, Thingstad TF (2002). Prokaryotic diversity – magnitude, dynamics, and controlling factors. Science 296:1064–1066
Vainio EJ, Hantula J (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol Res 104:927–936
Vallaeys T, Topp E, Muyzer G, Macheret V, Laguerre G, Rigaud A, Soulas G (1997) Evaluation of denaturing gradient gel electrophotresis in the detection of 16S rRNA sequence variation in rhizobia and methanotrophs. FEMS Microbiol Ecol 24:279–285
Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 60:407–438
Viaud M, Pasquier A, Brygoo Y (2000) Diversity of soil fungi studied by PCR-RFLP of ITS. Mycol Res 104:1027–1032
Wallner G, Amann R, Beisker W (1993) Optimizing fluorescent in situ hybridization of suspended cells with rRNA-targeted oligonucleotide probes for the flow cytometric identification of microorganisms. Cytometry 14:136–143

Watanabe K, Kodama Y, Harayama S (2001) Design and evaluation of PCR primers to amplify 16S rDNA fragments used for community fingerprinting. J Microbiol Methods 44:253–262

Wayne LG, Brenner DJ, Cowell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Tripper HG (1987) Report of the ad hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int J Syst Bacteriol 37:463–464

White TJ, Bruns TD, Lee S, Taylor J (1990) Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academy Press, New York, pp 315–322

Willems A, Gillis M, De Ley J (1991) Transfer of *Rhodocyclus gelatinosus* to *Rubriviwu* gen. nav., comb. nov., and phylogenetic relationships with *Leptothrix, Sphaerotilus natans. Pseudomonas saccharophila,* and *Alcaligenes latus.* Int J Syst Bacteriol 41:65–73

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucaryota. Proc Nat Acad Sci USA 87:4576–4579

Zuckerkandl E, Pauling L (1965) Molecules as documents of evolutionary history. J Theoret Biol 8:357–366

11 Arbuscular Mycorrhizal Fungi and the Form and Functioning of the Root System

David Atkinson

11.1 Introduction

That micro-organisms such as arbuscular mycorrhizal fungi (AMF) can influence the functioning of the plant root system has been known since mycorrhizas were first identified in the nineteenth century (Frank 1887; Hiltner 1904). Their value in aiding plants in the absorption of phosphorus has often resulted in this being identified as their most important characteristic. Koide (1991) commented "I have assumed that the most important effects on the host plant due to mycorrhizal infection is an increase in phosphorus acquisition" and "much of the variation in response can be attributed to variation in the magnitude of the phosphorous deficit". He did however also acknowledge "there are ... effects of infection which are apparently not directly related to improved phosphorus nutrition". Information on the effects of AMF on root system form are of more recent lineage. For many years it was considered that AMF did not affect the development of the root system (Harley and Smith 1983). This assumption is understandable as the modifications of which we are now aware are of a subtle nature (Berta et al. 2002). The types of modification are however of a form usually predicted as important to enhancing a plant's ability to access sparsely soluble nutrients such as phosphate (Silberbush and Barber 1983). However it is now clearly established that AMF can and do modify root form and that such effects may, under some conditions, be independent of phosphate supply (Atkinson et al. 1994).

For much of the period since their discovery AMF have been viewed as a component in the plants acquisition of resources from the soil. Against this background they have too often been seen as necessary only under conditions where the plants need extra capacity to source such nutrients. On this basis they are commonly seen as "an extra" for the plant, useful under nutrient limiting conditions but unnecessary at other times. However the relation between AMF and plants is an ancient one; AMF were a significant factor in the ability of plants to grow on land. As such the mycorrhizal state should be regarded as normal (Dunsiger et al. 2003). This historical

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perspective recognises a key role for AMF in helping to link the performance of a plant to its environment and in regulating plant growth in line with environmental conditions. In this role a major function of AMF becomes the provision of information on its environment to the plant, with this role being as important as that of resource acquisition.

This chapter details the ways in which AMF may influence the form of the plant root system both through effects on root growth, branching and death and how they influence the functioning of the root system both as a means of the acquisition of resources such as nutrients but also as a source of information which causes the plant to modify its growth so as to adapt better to prevailing environmental conditions.

11.2 AMF Infection and Whole Plant Functioning

AMF exert their primary effects on the functioning of the root system but they are of interest because these root system effects modify the ability of the plant or crop as a whole to cope with an environment which is commonly sub-optimal. While effects of AMF on plant growth mediated through improved nutrient supply have been known for many years (Harley and Smith 1983) the significance of infection and its value in a range of practical situations remains a matter for detailed experimentation. Large numbers of papers are published every year on this topic. Although it remains important to identify the effects of AMF on the physiology of individual roots and whole root systems it matters also to explore the limits of such effects at a whole-plant level. These may become manifest either through a whole plant performance which might otherwise only have been achieved by the supply of large amounts of external resources, e.g. fertilisers, pesticides, irrigation, etc. or through a performance which greatly exceeds that able to be achieved by non-mycorrhizal individuals of the same species. Basic growth and development studies thus remain important to help scale the impact of AMF on function. The results of some recent studies are detailed in Table 11.1 as a means of indicating some remaining key practical information gaps. Examples have been selected to show issues in cereals, legumes and perennial species. Together these show refinement in understanding of conditions in which AMF work. Several studies (Galal et al. 2003; Setua 1999) show that AMF can be effective on both growth and production even when P fertilisers are being applied or when the soil P status is significant. The impact of AMF are influenced by a range of environmental characteristics, e.g. light intensity (Zhu and Smith 2001) and CO_2 concentration (Jifon et al. 2002) and nutrition (Melloni et al. 2001; Song et al. 1999). When carbon supply becomes limiting the impact of AMF

Table 11.1. The effect of AMF on nutrition and whole plant performance

Plant species	Experimental conditions	Effects on nutrition and growth	Reference
Wheat	Effects of P levels to 7 mg/kg and rhizobium co-inoculation	Rhizobium increased AMF effect on growth. AMF increased P uptake at highest levels	Galal et al. (2003)
Wheat	Effect of P levels to 100 mg/kg and G intraradices on high/low P seeds	At low light AMF no effect P uptake. High light AMF increased P AMF eliminated high P seed advantage	Zhu and Smith (2001)
Barley	Effect of P levels to 48 mg/kg on soils with salinity to 17 d S m^{-1} and G intraradices	Both AMF and P increased growth in saline soils. AMF P, Fe, Zn increased	Mohammad et al. (2003)
Maize	Effect of P levels to 40 mg/kg on acid and alkaline soils and G intradices	Infection varied from 48 to 68% and increased weight but P only on acid soil. Cu, Mn, Zn both soils	Ozcan and Taban (2000)
Bean	Effect of organic and mineral fertiliser, Glomus spp and Rhizobium	Dual inoculation inc AMF infection, yield and weight in control and organic treatment, N and P in all treatments and Mg in organic	Aryal et al. (2003)
Bean	Effect G mosseae and G spurcum on soils (a) PH5, 4 mg P kg^{-1} (b) PH7, 8 mg P kg^{-1}	G mosseae increase growth and P in higher PH soil	Izaguirre-Mayoral et al. (2000)
Chick Pea	Acid soil with soluble P to 50 mg/kg or rock P to 200 mg/kg and G clarum	AMF inoculation doubled infection and increased P, K, Mg and micro-nutrients regardless of P treatment	Alloush et al. (2000)
Casuarina	Effect of range of AM species on soil with 3 kg P ha^{-1}	AM colonisation varied from 60 to 71%. Growth increased most by G fasciculatum (2×) as was P uptake	Sempavalan et al. (2001)
Eucalyptus	Effects of variation in AM spore numbers and P to 30 ppm	Efficiency of inoculation best at zero P, growth best at medium spore numbers and P. P uptake best high spore numbers	Sastry et al. (2000)
Citrus	Effects CO_2 levels on high P soil with G intraradices	At ambient CO_2 AMF reduced growth (−18%). At elevated CO_2 increased growth (+15%)	Jifon et al. (2002)
Mulberry	Effects of P treatment to 180 kg/ha on alluvial soil with Glomus sp^s	G mosseae increased growth and P uptake in low P treatment became equal to high P	Setua et al. (1999)

on growth is negative, a point emphasised by Muthukumar and Udaiyan (2000) who found a negative correlation between root carbohydrate and AM infection. However it is also clear that AMF do influence the ability of plants to grow in difficult conditions such as saline soils (Mohammad et al. 2003) and acid soils (Ozcan and Taban 2000) and through mechanisms beyond "just P supply". The effectiveness of AM is clearly influenced by the fungal partner (Sempavalam et al. 2001), the efficiency of the infection process (Izaguirre-Mayoral et al. 2000) and the impact of other organisms. These whole plant studies confirm that many long standing key issues in relation to the functioning of AMF remain unsolved at a practical level (Koide 1985). The definition of the circumstances under which AMF will have a critical role in growth or survival, their interaction with other micro-organisms, e.g. *Rhizobium* (Galal et al. 2003; Aryal et al. 2003) and the means by which AMF modify a plants interaction with its environment will ultimately influence the need to either add AMF, through inoculation, or to manage the soil so as to promote AMF infection. These issues provide the scale and scope for the subsequent sections of this review.

11.3 AMF and Root Form and Function

The effect of AMF on nutrient supply was reviewed by Koide (1991) who focused on the role of AMF in enhancing phosphorus absorption, especially under conditions in which P supply was limiting, i.e. when potential demand from the plant is likely to be greater than potential soil supply. AMF have the ability to modify both the supply and demand elements of this balance. Most of the research focus to date has related to effects on supply. The ability of AMF to influence both elements of the balance leads to the variation in response illustrated in Table 11.1. AMF can and do increase P accumulation. This may be used for current or future growth. The past and current focus upon P is a consequence of its being a key element needed for plant growth, an element frequently limiting in relation to availability in soils and because of its limited mobility and restricted availability: an aspect where the enhancement of root surface area, which occurs with AMF infection, but which is also aided by microbial products, is likely to have the greatest effect.

The relationship between a plant's ability to absorb an immobile nutrient such as P has been reviewed by Nye and Tinker (1997) and Newman and Andrews (1973) among others. Where soil P availability is low, root length can be a good predicator of P availability to the plant (Silberbush and Barber 1983). In some models root hair length has also been incorporated as a variable (Jungk 1987). P inflow has been shown to be low in species with

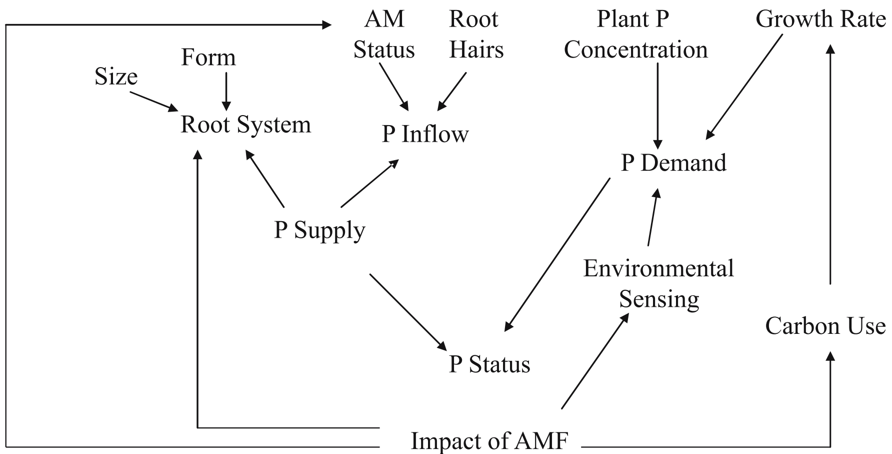

Fig. 11.1. Factors influencing the impact of AM infection. The P status of the plant is a result of demand and supply. AMF can influence both of these. Modified from Koide (1991)

short root hairs, e.g. onions, intermediate in species with medium (approximately 0.25 mm) root hairs, e.g. tomato and high in species with long (greater than 0.4 mm) root hairs, e.g. rape, ryegrass. Too many appraisals are based on assessments without AMF presence or without consideration of AMF. Common species with short root hairs, e.g. onion are those which are highly mycotrophic while those with long root hairs, especially when associated with high root length density, tend not to be greatly infected with AMF, a factor ignored in much of the earlier literature. As an example of a resource dominated model the relationship between plant P requirement and the potential role of AMF is summarised in Fig. 11.1. AMF impact both the supply and demand sides of the balance. P demand is a function of the addition of new dry matter and the minimum concentration of P within it. AMF need carbon for their growth and maintenance so that under conditions of restricted C supply, e.g. Jifon et al. (2002), AMF reduce plant growth. They may also, through their role in sensing the environment, reduce growth so that growth is better related to environmental capacity (Dunsiger et al. 2003). The role of AMF in influencing supply has been more extensively studied (Aikil and Ruotsalainen 2002). AMF can influence the size of the root system, the form or architecture of the root system, the survival of individual roots, the ability of a length of root to absorb P from the soil and the availability of P from the soil for absorption. Many of these interact; e.g. to interpret the significance of AMF effects on root methodology it is helpful to review the variables which can be used to describe a plant root system as these infer related groups of properties (Atkinson 1992). Root systems can be described in terms of:

1. Quantitative parameters, e.g. length
2. Structural elements, the way individual roots are connected, e.g. morphology or architecture
3. Temporal aspects, when growth and death occur

These aspects are now reviewed against their contribution to resource acquisition.

11.3.1 Root System Size

As the development and maintenance of AMF use resources which might otherwise have been devoted to root system development, it is inevitable that they interact. Azcon and Ocampo (1981) found that the response to AMF infection was inversely related to root system size in wheat. Similarly in tomato Bryla and Kordis (1990) found that root density was negatively related to AMF infection. Fitter (1977) reported decreases in root length as a consequence of AMF infection. It has been suggested that some of these effects may be a consequence of an increased P status as this is also known to reduce root weight (Schjorring and Jensen 1984). In contrast Ortas et al. (2002) found that *G. clarum* infection increased root length in citrus while Bagayoko et al. (2000) found that P and AMF together increased root growth. A number of studies, e.g. Hooker et al. (1992) and Atkinson et al. (2003) have found AMF to have no significant effect on root weight or length. Effects of AMF on both root mass and length and their functional significance thus vary with, at times, other factors having a greater effect than AMF. On the basis that the natural status for most plant species is to be infected with AMF, just as most plants have chloroplasts, it is unsurprising that there will be situations where AMF are associated with both increases and decreases in root system size and others where treatments have no influence. A focus on AMF as a "treatment" is the product of a reductionist approach (Read 2002) rather than one which aims to place AMF in a wider ecological contest.

11.3.2 Root and Root System Morphology

Awareness that AMF can influence the form and architecture of the root system is a relatively recent discovery. That ectomycorrhizas influence root system form has long been known, with the shorter root tips visually obvious (Harley and Smith 1983). While the modifications to the root system

caused by AMF can be extensive they cannot be quantified without careful analysis. With AMF frequently the root system is more highly branched, i.e. a greater number of smaller diameter high order roots. Demonstrating conclusive effects requires the comparison of infected and non-infected plants and the use of image analysis techniques (Hooker et al. 1992, 1998; Hooker and Atkinson 1992). The subject has been reviewed by Atkinson et al. (1994) and Berta et al. (2002).

Previous reviews have emphasised the importance of effects on root system morphology for both the ability of the root system to function in absorption and the energy needed to construct a root system. Berta et al. (1993) found, in *Allium porrum*, that infection with *Glomus* E3 led to a root system with more shorter, more branched adventitious roots of greater diameter but with a relatively unchanged overall pattern. A more branched root system can lead to a range of arrangements in the soil. A herring-bone form, with effectively many branches arising from a central axis, can be effective in soil exploration but has a significant cost in terms of carbon resources (Berta et al. 1993). Mycorrhizal plants, e.g. *Vitis vinifera* (Schellenbaum et al. 1991) and *Platanus acerifolia* (Tisserant et al. 1991) develop a resource efficient random rooting pattern. It seems clear that, while the scope to vary branching within an individual species is not unlimited, the branching pattern actually produced can be influenced by a range of factors of which AMF may well be one of the more significant. Berta et al. (1993) suggested that the scope for modification was greater in dicot species than in grasses. A much branched root system can be both a response to a limited soil nutrient supply and a strategy for maximising infection. The relative importance of this will vary between situations. Schellenbaum et al. (1991) suggested the differences in branching between control and AMF infected roots increased with increasing root order. The number of laterals developed per unit root length were increased by 125%, 185% and 230% for increasing root orders. Hooker et al. (1992) reported a similar effect with the branching of secondary roots increased by 81% and that of tertiary roots by 616% in poplar. Here the level of AMF infection seemed to influence branching. Twenty-three percent infection by *Scutellespora calospora* resulted in a 40% increase in lateral production while a 52% infection by *G. caledonium* gave a 60% increase in branching. The effect of AMF infection with *Glomus* E3 on poplar is shown in Table 11.2 using data from Hooker et al. (1992). This study used a soil whose fertility was such that neither AMF or nutrient additions influenced total growth. While the addition of phosphorous had a small effect on branching, an increase of 32% compared to the control for the branching of secondary roots, this effect was smaller than that induced by AMF, 81%, and less pronounced in respect of the branching of tertiary or quaternary roots. Forbes et al. (1996) assessed the effect of AMF infection on *Plantago lanceolata*. In this species AMF re-

Table 11.2. The effect of AMF (Glomus E3) or a high phosphate treatment on the number of lateral roots produced from the various root orders and the contribution to the length of the root system of those laterals. Data from Hooker et al. (1992)

Treatment	Root order							
	1°		2°		3°		4°	
	No	(%)	No	(%)	No	(%)	No	(%)
Control	342	(59)	356	(38)	18	(3)	0	(0)
Glomus E3	336	(21)	646	(58)	129	(18)	21	(2)
High Nutrient	315	(35)	469	(62)	32	(3)	0	(0)
LSD	197	(15)	178	(14)	70	(9)	30	(2)

duced branching, at 21 °C by around 15%. Temperature also changed both branching and infection. Infection was 52% at 15 °C but only 36% at 27 °C. Higher root orders were more heavily colonised. At 27 °C colonisation increased from < 30% in secondary roots to 50% in quaternary roots. The combination of these effects meant that while at 15 °C > 50% of total root length was colonised, at 21 and 27 °C < 40% was colonised (Atkinson et al. 2003).

The effect of AMF on activity in root apices has been discussed by Berta et al. (2000, 2002). When colonised by AMF advantitious roots showed determinant growth. All root apices show a reduction in root diameter, root cap size, the distance between initials and the differentiated zone and in mitotic activity. In AMF colonised plants the percentage of active apices decreased faster than in non-infected plants. In tomato (Berta et al. 2000) the nuclei of AMF infected cortex cells were large with more de-condensed chromatin. Ploidy varied between the nuclei of AM and control roots with nuclear polyploidisation and AMF colonisation strongly correlated. Polyploidy is usually associated with high metabolic activity.

Given the variation which occurs in the magnitude and type of AMF effects on morphology a range of mechanisms are possible. Currently there is no clear consensus as to the mechanism for the effects. Traditionally it had been suggested that the primary mechanism was to improve phosphorus nutrition (Amijee et al. 1989). However the data of Hooker et al. (1992) where the effects of AMF infection were compared directly with those of a series of high P treatments (Table 11.2) suggest that factors beyond improved P nutrition may be important and that the effects of AMF cannot be explained solely in terms of P effects.

AMF induced modifications to root system development and architecture may modify plant function. That the AMF infected root system is commonly made up of more smaller diameter high order roots is likely to impact on root longevity and turnover. This is reviewed in the next section. This

form of root system is appropriate to the exploitation of scarce resources especially where the availability of resources is transient. High order roots which are more heavily colonised allow the fungi to be placed close to nutrient sources whilst the shorter life of high order roots reduces the carbon maintenance expenditure on roots in areas where nutrients have been depleted (Eissenstat et al. 2000): roots of small diameter have shorter lives.

A major area where the effects of AMF infection on root system form may be important is in relation to the impact of plant pathogens. AMF have been shown to reduce the severity of infection by pathogens with some consequences for changes in the root system (Hooker et al. 1994). Salami (1999) found that inoculation with *G. etunicatium* reduced the impact of *Phytophthora infestans*. The effect was greatest when AMF infection occurred in advance of, or simultaneously with, the impact of the pathogen. Similarly Elwan et al. (2002) found that AMF could reduce the impact of *Rhizoctonia* on cotton and Yao et al. (2002) found that *G. etunicatium* reduced the impact of Rhizoctonia on potato by 77% so increasing yield by 140%. Karagiannidis et al. (2002) found that *G. mosseae* eliminated the pathogenic effects of verticillium wilt in both egg plant and tomato.

Most of the AMF pathogen interactions studied have been in relation to *Phytophthora*. As *Phytophthora* usually infects roots behind the tip the increased branching produced by AMF infection might be assumed to increase susceptibility. Norman et al. (1996) tested this. In non-mycorrhizal plants increased branching does increase infection. In AMF infected plants increased branching reduced infection by *Phytophthora* as a result of a modified pattern of exudate production. The effect of AMF is influenced by both time and the extent of colonisation. Henry and Kosola (1999) found the extent of colonisation in one-week-old roots increased with the age of a plant in the grass *Andropogan gerardii*. Colonisation was not generally affected by soil P status although in low P status soils it increased with increasing root age. Atkinson et al. (2003) found that environmental factors such as temperature influence the percent colonisation and the length of individual tertiary roots.

A key need in studies of the effects of AMF on root morphology has been to distinguish effects due to plant size from those due to AMF. Bressan and Vasconcellos (2002) assessed the effect of inoculation with *G. etunicatum* and *G. clarum* and P levels varying from 0 to 200 mg/kg on maize. They found AMF increased root weight, the number of first and second order laterals and P concentration but decreased root-shoot ratio and the number of root hairs. Root weight was significantly correlated with root colonisation. High P levels reduced AMF colonisation. Fusconi et al. (2001) assessed the effect of AMF on the root meristem of *Allium porrum*. They found that the effects of AMF could appear similar to those of improved P nutrition. However at a detailed level AMF blocked meristem activity leading to more

Table 11.3. The effect of AMF infection on the components of root system development (derived from Atkinson et al. 1994)

Process	Effect of AMF	Consequence for root system
Mitotic cycle in root apice cells	Lengthened especially interphase: metaphase increases with increasing colonisation	Modified architecture
Meristematic activity	Blocked by colonisation	Modified root system form
All death in apical meristem	No of abscised apices increased	Short roots
Variation in size of root apices	Diameter and base of meristem and meristem length increased	Larger root apices and thicker roots
Prim ordeal formation	Increased	More numerous and more branched roots. Shorter lived roots

inactive apices and meristems in metaphase. A high supply of P lengthen the mitotic cycle but without blocking the apices, thus resulting in slowed but continued root growth. The effects of AMF on root morphology are summarised in Table 11.3.

11.3.3 Root Longevity

Several recent studies have assessed the effect of AMF on root longevity. Until recently it was assumed that most of the root system survived at least as long as the leaves. Black et al. (1998), Atkinson and Watson (2000) and Watson et al. (2000) showed that, for tree crops and a range of agricultural species, the longevity of many roots could be measured in days rather than weeks. In pea and oats less than 50% of roots survive over seven days. In *Lolium perenne* only 15% of new roots survive over 21 days in warm soils. Soil temperature markedly influences survival (Forbes et al. 1997). Effects of AMF thus needs to be set in the context of a range of other factors which can change root longevity in a major way and the impact of environmental variables such as temperature.

Hooker et al. (1992, 1995) assessed the effect of AMF infection on poplar. They found that infected roots lived a shorter period of time than did control roots. They attributed this to the more pronounced root branching in mycorrhizal poplar. In contrast, with clover, Atkinson et al. (2003) found longevity to be increased in mycorrhizal roots (Fig. 11.2). In *Lolium perenne* AMF had no effect on survival which was lower than that in clover, 40% at 20 days compared to 80% (Fig. 11.3).

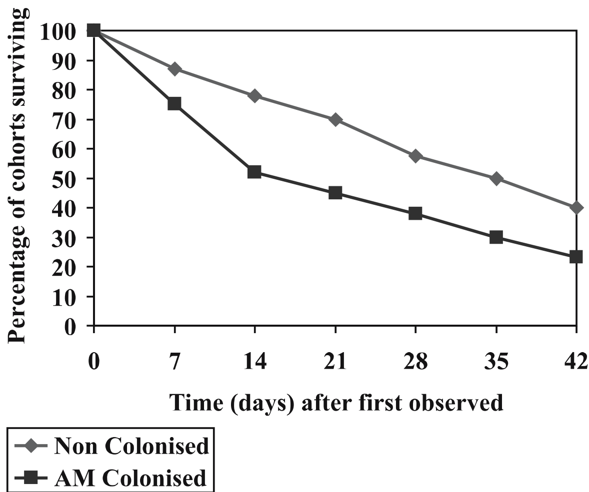

Fig. 11.2. The impact of AMF colonisation on root survival in *Trifolium repens* AM – colonised. Reproduced from Atkinson et al. (2003)

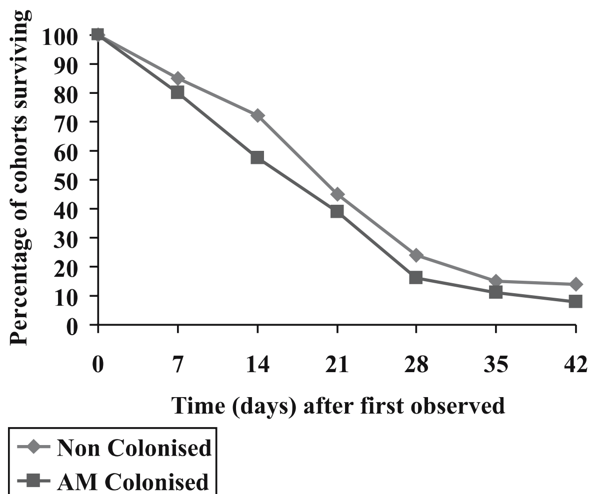

Fig. 11.3. The impact of AMF colonisation on root survival in *Lolium perenne*. Reproduced from Atkinson et al. (2003)

Infection with AMF can thus increase, decrease or have no influence on root longevity. While plant species is important the extent of root colonisation will also be a significant factor (Table 11.2). Different species are naturally colonised to different extents. Grass species tend to be infected to only a limited extent. Here AMF had little effect on longevity. Clover is naturally well infected and AMF increased longevity. Root morphology, as

discussed above, is influenced by infection to a varying degree in general with increased infection leading to greater branching. Fitter (1985) found that AMF infection reduced branching in clover. In contrast, in poplar where root longevity was reduced (Hooker et al. 1995), AMF resulted in a major increase in root branching (Hooker et al. 1992). Whether AMF influence or decrease root longevity may well be linked to its effect on the degree of branching. Where branching is increased, as in poplar, then mean root longevity falls; where branching is reduced as in clover then mean root longevity increases. This is clearly a sector where more research would be helpful.

11.4
Infection and Root Function

It is clear that the degree of root infection modifies root morphology, survival and functioning, for example, Isobe and Tsuboki (1998). The impact of level of infection and the distribution of infection in different parts of the root system are not however well understood. This and the functioning of the individual hyphae are considered here.

11.4.1
Effects on Infection

Citrus seedlings plants grown with high P (2 mmol/l) showed reduced colonisation, an effect countered if the treatment was applied in an elevated (2×) CO_2 regime. High P, high CO_2 and *G. intraradices* all increased growth but decreased root to shoot ratio. *G. intraradices* reduced starch concentrations in structural roots especially under low P supply (Syvertsen and Graham 1999). Light intensity also influences the contribution of AMF. Olsen et al. (1999) found that when phosphate was more limiting than carbon, then AMF improved crop growth, but not when the reverse occurred. While an increased level of infection is usually associated with an increased growth effect the responsiveness of plants to AMF infection is not always correlated with the levels of infection found (Kaeppler et al. 2000). Dunsiger (1999) studied the relationship between the length of extra-matricular hyphae in the soil and mycorrhizal colonisation, root and shoot weight. Hyphal length and both percentage infection and root weight were poorly correlated. The relationship with shoot weight was better. The impact of percentage infection may be modified by the functioning of individual hyphae both within the plant and the extra-matricular hyphae.

11.4.2 Hyphae Functioning

Fungal species differ in their ability to access soil P and to transfer it to the plant. Solaiman and Abbott (2003) assessed the effectiveness of AMF in the under-story species of a Jarrah forest. The proportion of root length colonised was positively related to plant biomass in *Phyllanthus colycinus* but not in *Trifolium subterraneum*. In *Phyllanthus colycinus* hyphae accessed more P 2 cm from the root than from greater distances. Drew et al. (2003) assessed the impact of soil structure on the functioning of the extra-matricular hyphae. They found that *G. intraradices* used distant P more than did *G. mosseae* and that P acquisition was not related to the amount of external hyphae. For both fungi more hyphae were produced in a soil medium compared to quartz sand with pore size having a greater impact on *G. intraradices*. The absence of a quantitative link between the level of infection and nutrient uptake was also found by Rubio et al. (2002) in a study using wheat and *G. etunicatum*. Nadian et al. (1998) assessed the impact of soil compaction on AMF infection and functioning. They found that different AMF species were effected by different levels of soil compaction. Compaction to a bulk density of 1.6 mg/m^3 had no effect on colonisation percentage but reduced the total length of root colonised.

A factor which has received relatively little attention is the longevity of the extra-matricular hyphae in soil. Using a Rhizotron method Atkinson and Watson (2000) assessed hyphal longevity in a mixed plant community. They found that 60% of the external hyphae visible turned-over in 7 days and that by 21 days less than 10% of hyphae remained. Hyphae appear to be more ephemeral than roots.

11.5 AMF and Resource Acquisition

In the proceeding sections I have discussed the practical impact of AMF on the growth of a range of crop types and the effect on AMF on root system development and the survival of individual roots. Unequivocally an important factor for AMF is that they facilitate the plant gaining resources which would not otherwise be available. This has most substantially been assessed for phosphorus. As this has been substantially reviewed elsewhere, e.g. Harley and Smith (1983), Smith and Smith (1990), Koide (1991) and van der Heijden and Sanders (2002), it is unnecessary to review it again in any detail. A review of AMF effects on functioning, however, would be incomplete without some mention of recent studies on the impact of AMF on the uptake of P. This is important to the supply-side aspects identified in Fig. 11.1.

11.5.1
Effects on Nutrient Uptake from the Soil

Tibbett (2000) suggested that the relatively slow rate of root proliferation within a nutrient rich patch of soil (Hodge et al. 1998) implied that the primary mechanism for acquiring nutrients from such patches was for the plant roots to support a network of mycorrhizal hyphae. The structure of the vegetative mycelia allows rapid colonisation of the area (Boddy 1993). Simard et al. (2002) reviewed literature on the length of mycorrhizal hyphae which could be found in soil and the relative surface they provided for uptake, compared to that provided by a root. They identified a range of hyphal lengths from1 300 m m^{-1} (Jones et al. 1990) to 8,000 mm^{-1} (Read and Boyd 1986) and concluded that mycorrhizae could increase the effective root surface area 60-fold. Although these estimates were for ectomycorrhizal fungi, effects of AMF are also substantial (Tisdall and Oades 1979).

Hawkes and Casper (2002) found that shrubs with AMF absorbed more rubidium than non-infected plants but without a change in the volume of soil exploited. This suggested more intensive soil exploitation. Hodge et al. (2000) found that AMF enhanced root development especially within organic patches but did not significantly increase N uptake. P concentration in the plant was increased.

P uptake is affected by a range of factors, e.g. root age, soil temperature and so effects of AMF must be seen in a wider context. Staddon et al. (1999) studied the interaction between CO_2 availability from the atmosphere and AMF infection on P inflow. They found that a range of herbaceous species all responded similarly to elevated (610 μMol Mol^{-1}) CO_2. This did not change the percentage root length colonised by AMF but increased the total colonised length, because root length was increased. P inflow was stimulated in total but not on a root length basis. A recent study of the effect of root hairs on the functioning of barley also emphasises the importance of “root” surface area in general, for phosphorus uptake: root hairs and AMF can be seen as alternative evolutionary solutions to the same problem. Both increase plant contact with soil and reduce the length of the external transfer path for nutrients moving to the root. Gahoonia et al. (2001) found that a mutant of the barley variety Pallas, which did not have root hairs, absorbed only half the P of the original variety with root hairs. The original variety absorbed most of the P contained in the 0.8 mm radius root hair zone, produced more acid phosphatase and mobilised more organic P. ‘Root’ surface area is important to P uptake in low P availability soils.

While it is generally accepted that AMF allow the more efficient uptake of labile and sparsely soluble sources of P from the soil it remains unclear as to just how they do this. Whether they make available to the plant P from insoluble mineral sources and from the organic sources, which are

increasingly being recognised as a major component of the soil's total P reserves, or whether the effect is simply due to more efficient uptake, is the key question. The latter has long been the prevailing consensus and recent studies have produced more evidence to support this view. Yao et al. (2001) assessed the ability of *Trifolium pratense* with *G. versiforme* to access P from calcium phosphates varying from $CaHPO_4$ to $Ca_{10}(PO_4)_6F_2$. AMF promoted the uptake of P but effectiveness was greater for Ca_2P and least for Ca_{10} forms. The external hyphae were efficient at mobilising P from Ca_2P, Ca_8P and AlP. In this study AMF mobilised P, also available to uninfected roots, but were more effective. In addition to organic compounds acting as P sources, Aryal et al. (2003) and Ravnskov et al. (1999) found that organic additions to the soil could influence the functioning of extra-matricular hyphae. Hyphal growth of *G. intraradices* was promoted by yeast and bovine serum albumin but depressed by starch and cellulose. P uptake was decreased by cellulose.

11.5.2 AMF and Water Relations

While soil water can be considered as a resource in the same way as is P it differs from P in that only a small percentage of the water absorbed by a plant is retained within the plant. Most absorbed water is transpired. In assessing the effects of AMF on water relations a wider range of aspects are therefore of importance.

Over the years during which the effects of AMF have been studied, suggestions have been made that mycorrhizas influence the plants ability to cope with water stress. Following the logic of the role of AMF in improving P supply it is commonly suggested that AMF function by increasing the ability of the plant to absorb water. Whether this occurs remains an open question. It is now clear however that AMF can influence an infected plant's response to water stress in a number of other ways (Fig. 11.4), e.g. through effects on soil aggregation and water holding capacity (Sutton and Sheppard 1976).

Souza et al. (1999) assessed the growth of the tropical grasses *Brachiaria brizantha* and *Stylosanthe guianensis* on a low fertility soil under water stressed conditions. They found that increasing P supply to 300 mg/kg increased drought resistance which was further enhanced by the presence of AMF (*G. etunicatum*). The same fungus had little impact on the performance of *Eucalyptus grandis* seedlings which under some conditions benefited from inoculation with the ecotomycorrhizal fungus *Pisolithus tinctorius* (Fernandes et al. 1999). In soyabean Barakah and Heggo (1998) found that the dual inoculation of plants with *Bradyrhizobium*, *Glomus* and *Gigaspora* increased growth by 400% under conditions of water stress which

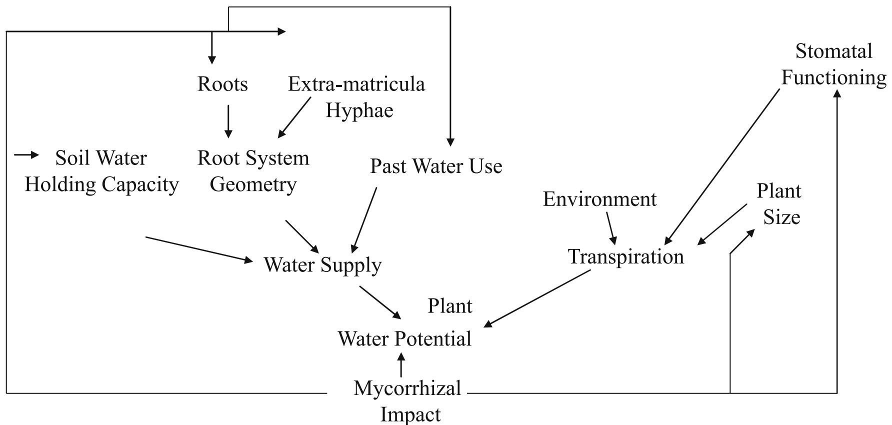

Fig. 11.4. Factors which determine the impact of AMF on plant water potential. The balance of water absorption and transpiration determine plant water potential and water deficits. AMF can influence both of these in a range of ways

increased infection compared to that found under non-stressed conditions. AMF increased NPK concentrations under stressed and non-stressed conditions alike. Similar results were found for barley and wheat by Al-Karaki and Clark (1999) and Al-Karaki et al. (1998). They found that AMF increased growth regardless of soil water potential. Concentrations of P, Mn and Cu were increased by AMF on all soils.

Recent studies on the performance of AMF infected plants under conditions of water stress thus indicate that AMF favourably influence plant response to a limited water supply. The resource acquisition model would assume that AMF would affect a plant's ability to cope with water stress by increasing the plant's ability to obtain water. Whether or not AMF do this, it is now clear that AMF can influence water relations in other ways (Fig. 11.4). AMF influence both the supply and demand sides of the balance. Dunsiger et al. (2003) suggested that AMF influence how a plant responds to a decreasing soil water potential, i.e. increasing water stress, in three ways:

1. In a study of the impact of AMF on the relationship between transpiration and soil water potential they found that AMF infected plants reduced their transpiration more than did non-infected plants as soil water potential decreased. This would reduce plant water stress and permit available soil water to be available for use over a longer period. This relationship is shown in Fig. 11.5. This effect can be mediated in a range of ways including the decreased production of aquaporins (water channels) in the cells of the root cortex or by an increased production of ABA (Steudle 2000).

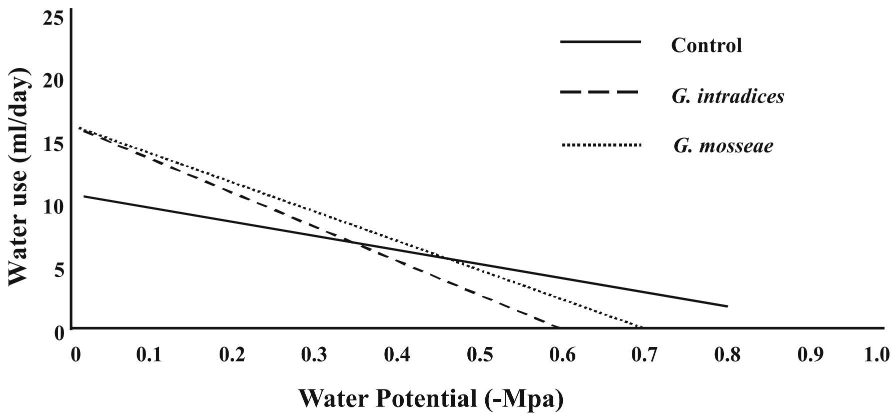

Fig. 11.5. Plant water use compared with soil water potential when inoculated with different mycorrhizal species – one month after inoculation. Trend lines are fitted by linear regression. Reproduced from Dunsiger et al. 2003

2. Through an increase in the direct transport of water from the soil by the extra-matricular hyphae as suggested earlier by Safir et al. (1972). Dunsiger et al. (2003) related water removed from a hyphal area in a compartmentalised root box to the length of hyphae found in the soil in that section. The relationship was not good (Fig. 11.6). This does not wholly discount this as a mechanism among a wider range of possibilities. An increased absorption of water, in this way, would not however easily relate to the more conservative pattern of water use described in 1. above.

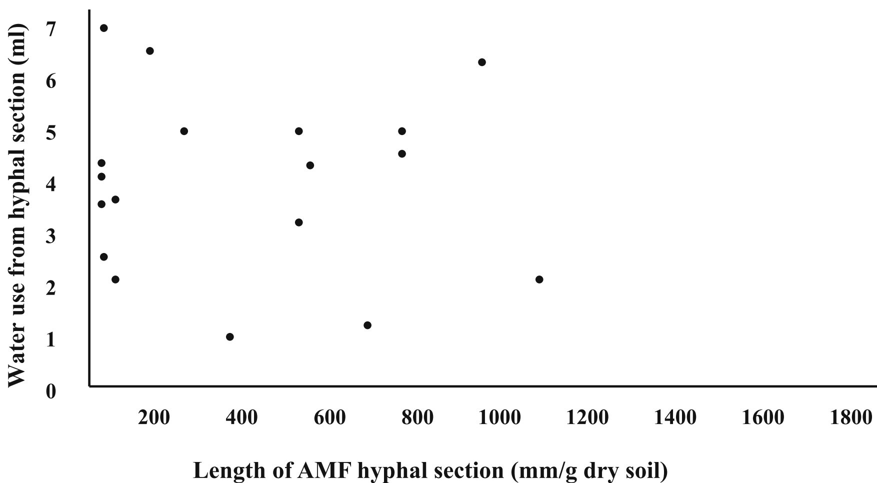

Fig. 11.6. Relationship between AMF hyphal length in the hyphal section of rhizoboxes and water loss from hyphal sections over five days. Reproduced from Dunsiger et al. 2003

3. The most radical suggestion of Dunsiger et al. (2003) was that hyphae signalled a decreasing water availability to the plant which then down-regulated its water use by premature stomatal closure, a feature recorded in their study. Growing plants in a root box, providing a root plus hyphal and hyphal only compartments, showed that the relationship between leaf stomatal conductance and soil water potential in both compartments was less good following the severing of the hyphal connections with the hyphal compartment. While this did not prove the signalling mechanism it did provide support for the hypothesis and suggested signalling as an aspect of the total role of AMF in relation to plant water use. Mechanisms by which roots and soil organisms communicate have recently been discussed by Bais et al. (2004). They suggested that a range of root exudates could, as chemical signals, have a significant role in the infection by symbionts such as AMF, resistance to pathogens and the soil water release characteristics and hydraulic conductivity of rhizosphere soil.

The relationship between different plant species, their stomata and soil water potentials have always been complex in practice (Hsiao et al. 1976). That AMF do not have a simple controlling role is to be expected. The results obtained through recent research do however suggest that AMF infected plants are more acutely coupled to the soil environment and so are better equipped to deal with stressful situations, i.e. their use of a potentially scarce resource is better coupled to availability. This must require an improved ability to sense the environment and so implies a role for AMF beyond more resource acquisition.

11.6 Conclusions

With the development of the breadth of current experimental work on AMF we became aware of the range of aspects of plant and crop development in which they are important. More than perhaps any other rhizosphere organism, AMF are a part of the plant. AMF cannot be regarded as an additive to crops. Many of the agricultural treatments applied in recent years (Dunsiger et al. 2003) have however resulted in a loss of AMF from agricultural soils and from current cropping systems necessitating inoculation so as to restore AMF status to what it would naturally have been. The complexity of fungal-plant structures is illustrated by the recent study of van der Heijden and Kuyper (2001) who found fungus-plant compatibility had a major influence on AMF effectiveness. Plant origin influenced symbiosis effectiveness, mediated through genetic difference in root parameters. Fungal origin had a lesser effect. Miller and Kling (2000) identified the range of

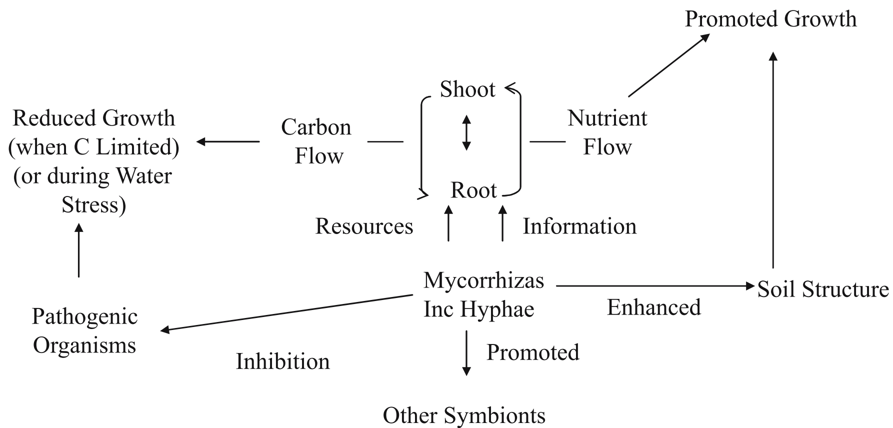

Fig. 11.7. Effects of AM fungi on root form and function

factors which AMF could influence and the range of scales at which they were operational. Currently we can determine the colonisation of a root segment by AMF. With more difficulty the same can be done for an individual root system. We know little of how these parameters vary with time. The information presented here indicates that the amount of fungus, and where it is in the root system, will change with time and may even change over a period of days. Being able to characterise this is one of the major challenges for the future, roots and their AMF symbionts are not physiologically static. In addition, many factors which are influenced by AMF are also influenced by other parameters, e.g. temperature and so the impact of AMF must be viewed against this wider background of environmental conditions. This is summarised in Fig. 11.7 which provides a framework for assessing some of our current gaps in information. While much remains unknown about resource provision, even more remains unknown about information supply. Much of what is needed has a molecular basis. Current developments in this sector are likely therefore to aid the development of a more definite view of the role of AMF in root functioning.

References

Aikio S, Ruotsalainen AL (2002) The modelled growth of mycorrhizal and non-mycorrhizal plants under constant versus variable soil nutrient concentration. Mycorrhiza 12:257–261

Al-Karaki GN, Clark RB (1999) Varied rates of mycorrhizal inoculum on growth and nutrient acquisition by barley grown with drought stress. Plant Nutr 22:1775–1784

Al-Karaki GN, Al-Raddad A, Clark RB (1998) Water stress and mycorrhizal isolate effects on growth and nutrient acquisition of wheat. Plant Nutr 21:891–902

Alloush GA, Zeto SK, Clark RB (2000) Phosphorus source, organic matter, and arbuscular mycorrhiza effects on growth and mineral acquisition of chickpea grown in acidic soil. Plant Nutr 23:1351–1369

Amijee F, Tinker PB, Stribley D (1989) The development of endomycorrhizal root systems. VII. A detailed study of effects of soil phosphorous on colonisation. New Phytol 111:435–446

Aryal UK, Xu HL, Fujita M (2003) Rhizobia and AM fungal inoculation improve growth and nutrient uptake of bean plants under organic fertilization. Sustain Agric 21:29–41

Atkinson D (1992) Tree root development: the role of models in understanding the consequences of arbuscular endomycorrhizal infection. Agronomie 12:817–820

Atkinson D, Watson CA (2000) The beneficial rhizosphere: a dynamic entity. Appl Soil Ecol 15:99–104

Atkinson D, Berta G, Hooker JE (1994) Impact of mycorrhizal colonisation on root architecture, root longevity and the formation of growth regulators. In: Gianinazzi S, Scheu H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhauser, Switzerland, pp 89–99

Atkinson D, Black KE, Forbes PJ, Hooker JE, Baddeley JA, Watson CA (2003) The influence of arbuscular mycorrhizal colonisation and environment on root development in soil. Eur Soil Sci 54:751–757

Azcon R, Ocampo JA (1981) Factors affecting the vesicular – arbuscular infection and mycorrhizal dependency of thirteen wheat cultivars. New Phytol 87:677–685

Bagayoko M, George E, Romheld V, Buerkest A (2000) Effect of mycorrhizae and phosphorus on growth and nutrient uptake of millet, cowpea and sorghum on a West African soil. J Agric Sci 135:399–407

Bais HP, Park SW, Weir TL, Callaway RM, Vivenco JM (2004) How plants communicate using the underground information super highway. Trends Plant Sci 9:26–32

Barakah FN, Heggo AM (1998) Moisture stress, bradyrhizobia, vesicular-arbuscular mycorrhiza and P-fertilizers effects on soybean growth, nutrient content and phosphatase activity under calcareous soil. Ann Agric Sci (Cairo) 43:461–475

Berta G, Fusconi A, Trotta A (1993) VA-mycorrhizal infection and the morphology and function of root system. Environ Exp Bot 33:159–173

Berta G, Fusconi A, Sampo S, Lingua G, Perticone S, Repetto O (2000) Polyploidy in tomato roots as affected by arbuscular mycorrhizal colonisation. Plant Soil 226:37–44

Berta G, Fusconi A, Hooker JE (2002) Arbuscular mycorrhizal modification to plant root systems: scale, mechanism and consequences. In: Gianinazzi S, Schue H, Barea JM, Hesslwandter K (eds) Mycorrhizal technology in agriculture. Birkhauser, Switzerland, pp 71–85

Black KE, Harbron CG, Franklin M, Atkinson D, Hooker JE (1998) Differences in root longevity of some tree species. Tree Physiol 18:259–264

Boddy L (1993) Saprophytic cord forming fungi: welfare strategies and other ecological aspects. Mycol Res 97:641–655

Bressan W, Vasconcellos CA (2002) Morphological alterations on root system of maize induced by mycorrhizal fungi and phosphorus. Pesqui Agropecu Bras 37:509–517

Bryla DR, Koide RT (1990) Role of mycorrhizal infection in the growth and reproduction of wild vs cultivated plants. II. Eight wild accessions and two cultivars of *Hycopersicon esculentum*. Oecologia 84:82–92

Drew EA, Murray RS, Smith SE, Jakobsen I (2003) Beyond the rhizosphere: growth and function of arbuscular mycorrhizal external hyphae in sands of varying pore sizes. Plant Soil 251:105–114

Dunsiger Z (1999) The influence of arbuscular mycorrhizas on the water relation of trees. PhD Thesis, University of Edinburgh

Dunsiger Z, Atkinson D, Watson CA (2003) Arbuscular mycorrhizal fungi: their role in the ability of crops to cope with stress. In: Proc BCPC Intl Congr Crop Sci and Technol, pp 433–439

Eissenstat DM, Wells CE, Yanai RD, Whitbeck JL (2000) Building roots in a changing environment: implications for root longevity. New Phytol 147:33–42

Elwan IM, Omran SEH, Mohamed HZ (2002) Effect of phosphorus and micronutrients fertilization accompanied with VA-mycorrhizal fungi in controlling Rhizoctonia damping-off on the growth and nutritional status of cotton plants. Ann Agric Sci (Cairo) 47:1139–1157

Fernandes MF, Ruiz HA, Neves JCL, Muchovej RMC (1999) Growth and phosphorus uptake by Eucalyptus grandis seedlings associated to mycorrhizal fungi in different phosphorus rates and soil water potentials. Rev Bras Cienc do Solo 23:617–625

Fitter AH (1977) Influence of mycorrhizal infection on competition for phosphorous and potassium by two grasses. New Phytol 79:199–225

Fitter A (1985) Functional significance of root morphology and root system architecture. In: Fitter AH, Atkinson D, Reid DJ, Usher MB (eds) Ecological interaction in soil. Blackwell, Oxford, pp 87–106

Forbes PJ, Ellison CH, Hooker JE (1996) The impact of arbuscular mycorrhizal fungi and temperature on root system development. Agronomie 16:617–620

Forbes PJ, Black KE, Hooker JE (1997) Temperature-induced alteration to root longevity in *Lolium perenne*. Plant Soil 190:87–90

Frank AB (1887) Ueber neue mykorrhiza – former Ber Dtsch Bot Ges 5:395

Fusconi A, Tagliasacchi AM, Berta G, Trotta A, Brazzaventre S, Ruberti F, Scannerini S (2001) Root apical meristems of *Allium porrum* L. as affected by arbuscular mycorrhizae and phosphorus. Protoplasma 214:219–226

Gahoonia TS, Neilsen NE, Priyavadan AJ, Jahoor A (2001) A root hairless barley mutant for elucidating genetics of root hairs and phosphorous uptake. Plant Soil 235:211–219

Galal YGM, El-Ghandour IA, Osman ME, Raouf AMNA (2003) The effect of inoculation by mycorrhizae and *Rhizobium* on the growth and yield of wheat in relation to nitrogen and phosphorus fertilization as assessed by 15 N techniques. Symbiosis (Rehovot) 34: 171–183

Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic Press, London

Hawkes CV, Casper BB (2002) Lateral root function and root overlap among mycorrhizal and non-mycorrhizal herbs in a Florida shrubland, measured using rubidium as a nutrient analog. Am J Bot 89:1289–1294

Henry A, Kosola KR (1999) Root age and phosphorus effects on colonization of Andropogon gerardii by mycorrhizal fungi. Soil Biol Biochem 31:1657–1660

Hiltner L (1904) Über neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache. Arb. dtsch. Landwirt. Ges. 98:59–78

Hodge A, Stewart J, Robinson D, Griffiths BS, Fitter AH (1998) Root proliferation, soil fauna and plant nitrogen capture from nutrient rich pockets in soil. New Phytol 139:479–494

Hodge A, Robinson D, Fitter AH (2000) An arbuscular mycorrhizal inoculum enhances root proliferation in, but not nitrogen capture from, nutrient-rich patches in soil. New Phytol 145:575–584

Hooker JE, Atkinson D (1992) Application of computer aided image analysis to studies of Am fungi effects on plant root systems architecture and dynamics. Agronomie 12:821–824

Hooker JE, Munro M, Atkinson A (1992) Vesicular – arbuscular mycorrhizal fungi induced alteration in poplar root system morphology. Plant Soil 145:207–214

Hooker JE, Jaizme-Voga M, Atkinson D (1994) Biocontrol of plant pathogens using arbuscular mycorrhizal fungi. In: Gianinazzi S, Schue H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhuaser, Basel, pp 191–200

Hooker JE, Black KE, Perry RL, Atkinson D (1995) Arbuscular mycorrhizal fungi in dual alteration to root longevity of poplar. Plant Soil 172:327–329

Hooker JE, Berta G, Lingua G, Fusconni A, Sgorbate S (1998) Quantification of AMF-induced modifications to root system architecture and longevity. In: Varma A (ed) Mycorrhizal manual. Springer, Berlin Heidelberg New York, pp 515–531

Hsiao TC, Acevdeo E, Fereres E, Henderson DW (1976) Water stress, growth and osmotic adjustment. Philos Trans R Soc London 273:479–500

Isobe K, Tsuboki Y (1998) The relationship between growth promotion by arbuscular mycorrhizal fungi and root morphology and phosphorus absorption in gramineous and leguminous crops. Jpn J Crop Sci 67:347–352

Izaguirre-Mayoral ML, Carballo O, Carreno L, Mejia MG de (2000) Effects of arbuscular mycorrhizal inoculation on growth, yield, nitrogen, and phosphorus nutrition of nodulating bean varieties in two soil substrates of contrasting fertility. J Plant Nutr 23:1117–1133

Jifon JL, Graham JH, Drouillard DL, Syvertsen JP (2002) Growth depression of mycorrhizal Citrus seedlings grown at high phosphorus supply is mitigated by elevated CO. New Phytol 153:133–142

Jones MD, Durall DM, Tinker PB (1990) Phosphorous relationships and production of extramatricular hyphae by two types of willow ectomycorrhizas and different soil phosphorus levels. New Phytol 115:259–267

Jungk A (1987) Soil-root interactions in the rhizosphere affecting plant availability of phosphorus. J Plant Nutr 10:1197–1204

Kaeppler SM, Parker JC, Mueller SM, Senior L, Stuber C, Tracy WF (2000) Variation among maize inbred lines and deliction of quantitative trait loci for growth of low phosphorus and responsiveness to arbuscular mycorrhizal fungi. Crop Sci 40:358–364

Karagiannidis N, Bletsos F, Stavropoulos N (2002) Effect of Verticillium wilt (Verticillium dahliae Kleb.) and mycorrhiza (*Glomus mosseae*) on root colonization, growth and nutrient uptake in tomato and eggplant seedlings. Sci Hortic 94:145–156

Koide RT (1985) The nature of growth depressions in sunflower by vesicular-arbuscular mycorrhizal infection. New Phytol 99:449–462

Koide RT (1991) Nutrient supply, nutrient demand an plant response to mycorrhizal infection. New Phytol 117:365–386

Melloni R, Nogueira MA, Freire VF, Cardoso EJBN (2001) Phosphorus levels and arbuscular mycorrhizal fungi on growth and mineral nutrition of Citrus limonia (L.) Osbeck. Rev Bras Cienc do Solo 24:767–775

Miller RM, Kling M (2000) The importance of integration and scale in the arbuscular mycorrhizal symbiosis. Plant Soil 226:295–309

Mohammad MJ, Malkawi HI, Shibli R (2003) Effects of arbuscular mycorrhizal fungi and phosphorus fertilization on growth and nutrient uptake of barley grown on soils with different levels of salts. J Plant Nutr 26:125–137

Muthukumar T, Udaiyan K (2000) Influence of organic manures on arbuscular mycorrhizal fungi associated with Vigna unguiculata (L.) Walp. in relation to tissue nutrients and soluble carbohydrate in roots under field conditions. Biol Fertil Soils 31:114–120

Nadian H, Smith SE, Alston AM, Murray RS, Siebert BD (1998) Effects of soil compaction on phosphorus uptake and growth of Trifolium subterraneum colonized by four species of vesicular-arbuscular mycorrhizal fungi. New Phytol 140:155–165

Newman EI, Andrews RE (1973) Uptake of phosphorous and potassium in relation to root growth and root density. Plant Soil 38:49–69

Norman JR, Atkinson D, Hooker JE (1996) Arbuscular mycorrhizal fungal induced alteration to root architecture in strawberry and induced resistance to the root pathogen *Phytophthora fragariae*. Plant Soil 185:191–198

Nye PH, Tinker PB (1977) Solute movement in the soil-root system. Blackwell, Oxford

Olsen JK, Schaefer JT, Edwards DG, Hunter MN, Galea VJ, Muller LM (1999) Effects of mycorrhizae established from an existing intact hyphol network on the growth resistance of capsicum (*Capsicum annuum* L) and tomato (*Lycopersicon esculentum* Mill) to five ratios of applied phosphorus. Austral J Agric Res 50:223–237

Ortas I, Ortaka D, Kaya Z (2002) Variation mycorrhizal fungi propagated on different hosts have different effects on citrus growth and nutrient uptake. Com Soil Sci Plant Anal 33:259–272

Ozcan H, Taban S (2000) Effects of VA-mycorrhiza on growth and phosphorus, zinc, iron, copper and manganese concentrations of maize grown in acid and alkaline soils. Turk J Agric For 24:629–635

Ravnskov S, Larsen J, Olsson PA, Jakobsen I (1999) Effects of various organic compounds on growth and phosphorus uptake of an arbuscular mycorrhizal fungus. New Phytol 141:517–524

Read DJ (2002) Towards ecological deliverance – progress and pitfalls in the path towards an understanding of mycorrhizal functions in nature. In: Van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Berlin Heidelberg New York, pp 3–29

Read DJ, Boyd R (1986) Water relations of mycorrhizal fungi and their host plants. In: Ayres PG, Boddy L (eds) Water fungi and plants. CUP, pp 287–303

Rubio R, Borie F, Schalchli C, Castillo C, Azcon RAA (2002) Plant growth responses in natural acidic soil as affected by arbuscular mycorrhizal inoculation and phosphorus sources. J Plant Nutr 25:1389–1405

Safir GR, Bayer JS, Gerdemann JW (1972) Nutrient status and mycorrhizal enhancement of water transport in soyabean. Plant Physiol 49:700–703

Salami AO (1999) Influence of mycorrhizal biotechnology on disease severity and growth of pepper (*Capsicum annum* Linn). Int J Trop Plant Dis 17:51–60

Sastry MSR, Sharma AK, Johri BN (2000) Effect of an AM fungal consortium and *Pseudomonas* on the growth and nutrient uptake of *Eucalyptus* hybrid. Mycorrhiza 10:55–61

Schellenbaum L, Berta G, Ranirina F, Tisserant B, Gianinazzi S, Fitter AH (1991) Influence of endomycorrhizal infection on root morphology in a micropropagated woody plant species (*Vitis vinifera* L). Ann Bot 68:135–141

Schjorring JK, Jensen P (1984) Phosphorus nutrition of barley, buckwheat and rape seedlings. II. Influx and efflux of phosphorous by intact roots of different P status. Physiol Plant 61:584–590

Sempavalan J, Kumutha K, Santhanakrishnan P (2001) Relative effect of vesicular arbuscular mycorrhizal fungi on growth and nutrient uptake of *Casuarina equisetifolia*. Madras Agric J 88:700–703

Setua GC, Kar R, Ghosh JK, Das NK, Saratchandra B (1999) Response of direct inoculation of VAM on growth, leaf yield and phosphorus uptake in mulberry (*Morus alba*). Indian J Agric Sci 69:444–448

Silberbush M, Barber SA (1983) Sensitivity of simulated phosphorus uptake to parameters used by a mechanistic mathematical model. Plant Soil 74:93–100

Simard SW, Jones MD, Durall DM (2002) Carbon and nutrient fluxes within and between mycorrhizal plants. In: Van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Berlin Heidelberg New York, pp 34–74

Smith SE, Smith FA (1990) Structure and function of the interfaces in biotrophic symbiosis as they relate to nutrient transport. New Phytol 114:1–38

Solaiman MZ, Abbott LK (2003) Phosphorus uptake by a community of arbuscular mycorrhizal fungi in jarrah forest. Plant Soil 248:313–320

Song Yong Chun, Zhang Jun Ling, Li Xiao Lin, Feng Gu, Yang Zhi Fu AA (1999) Effect of VA mycorrhizal hyphae links on growth and P nutrient of red clover. J Chin Agric Univ 4:26–32

Souza RF de, Pinto JC, Siqueira JO, Rezende VF (1999) Effects of mycorrhizae and phosphorus fertilizer on growth of *Brachiaria brizantha* and *Stylosanthes guianensis* on soil of low fertility. 3. Dry matter yield under water stress. Pasturas Tropic 21:31–35

Staddon PL, Graves JD, Fitter AH (1999) Effect of enhanced atmospheric CO_2 on mycorrhizal colonisation and phosphorus inflow in 10 herbaceous species of contrasting growth strategies. Func Ecol 13:190–199

Steudle E (2000) Water uptake by plant roots an integration of views. Plant Soil 226:45–56

Sutton JC, Sheppard BR (1976) Aggregation of sand-dune soil by endomycorrhizal fungi. Can J Bot 54:326–333

Syvertsen JP, Graham JH (1999) Phosphorus supply and arbuscular mycorrhizas increase growth and net gas exchange responses of two Citrus spp. grown at elevated [CO_2]. Plant Soil 208:209–219

Tibbett M (2000) Roots, foraging and the exploitation of soil nutrient patches: the role of mycorrhizal symbiosis. Func Ecol 14:397–399

Tisdall JM, Oades JM (1979) Stabilisation of soil aggregates by the root system of rye grass. Aust Soil Res 17:429–441

Tisserant B, Schellenbaum L, Gianinazzi-Pearson V, Gianinazzi S, Berta G (1991) Influence of infection by an endomycorrhizal fungi on root development and architecture in *Plantanus acerifolia*. Allinia 30:171–181

van der Heijden EW, Kuyper TW (2001) Does origin of mycorrhizal fungus or mycorrhizal plant influence effectiveness of the mycorrhizal symbiosis. Plant Soil 230:161–174

van der Heijden MGA, Sanders I (2002) Mycorrhizal ecology. Springer, Berlin Heidelberg New York

Watson CA, Ross JM, Bagnaresi U, Minotta GF, Roffi F, Atkinson D, Black KE, Hooker JE (2000) Environment-induced modifications to root longevity in *Lolium perenne* and *Trifolium repens*. Ann Bot 85:397–401

Yao Q, Li X, Feng G, Christie P (2001) Mobilisation of sparingly soluble inorganic phosphates by the external mycillium of an arbuscular mycorrhizal fungus. Plant Soil 230:279–285

Yao MK, Tweddell RJ, Desilets H (2002) Effect of two vesicular-arbuscular mycorrhizal fungi on the growth of micropropagated potato plantlets and on the extent of disease caused by *Rhizoctonia solani*. Mycorrhiza 12:235–242

Zhu YG, Smith SE (2001) Seed phosphorus (P) content affects growth, and P uptake of wheat plants and their association with arbuscular mycorrhizal (AM) fungi. Plant Soil 231:105–112

12 Fungal Recognition Responses to Host Derived Signals by Arbuscular Mycorrhizal Fungi

Cristiana Sbrana

"after many millions of generations in which communication was refined and made selective enough to readily distinguish friend from foe, it is not surprising that the subtle signals exchanged between partners are not easily detected by root biologists."

(Koske and Gemma 1992)

12.1 Introduction

The arbuscular mycorrhizal (AM) symbiosis is a widespread plant-fungal association, involving 80% of land plants and soil fungi belonging to Glomeromycota, characterized by reciprocal benefit for partners, since part of plant photosynthate is translocated from root cells to the fungus, which, in turn, sustain plant mineral nutrition (Smith and Read 1997). AM fungi are obligate biotrophs, since their spores, released in the soil, are capable of germination and limited growth in the absence of host plants, but are unable to complete their life cycle without establishing a functional symbiosis.

Despite the lack of host-regulated spore germination, spores of these obligate biotrophs have been discovered in 460 million years old fossil roots: this finding supports the view of AM fungi as "living fossils", and represents the evidence of organisms owning successful survival strategies operating during their life cycle (Giovannetti 2001). Their wide host range maximizes the probability of germinating spores to encounter roots suitable for symbiosis establishment; the unambiguous regulation of infection structures differentiation hinders any energy dissipation in useless infection attempts with non-host roots; the ability of multiple germination when mycelium is detached from the mother spore and/or of protoplasm withdrawal from peripheral hyphae enhance the probability of survival even for spores germinating in the absence of the host (Giovannetti et al. 1993a,

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1994; Logi et al. 1998). In addition to these strategies, the development of wide and interconnected hyphal networks by both pre-symbiotic and symbiotic mycelium may represent a fundamental mechanism for increasing chances of gaining access to the carbon sink represented by host roots (Giovannetti et al. 1999, 2004). Although AM fungi extensively colonize the root cortex, host defence responses, if elicited, are weak and transient (Morandi et al. 1984; David et al. 1998).

Since the association between Glomeromycota and roots of vascular plants, evolved over hundreds of million years, requires full functional compatibility and adaptation to symbiotic life of both host and fungal cells, these changes clearly involve a process of coordinated recognition events between symbionts.

One of the major questions in the study of AM symbiosis is which are the cues responsible of the initiation and progression of partner's recognition process. Although many aspects of the symbiosis are well studied from physiological and ecological perspectives, the mechanisms by which the fungus and the plant establish their relationship are largely unknown.

In this chapter pre- and post-contact events, fundamental steps in the life cycle of these obligate biotrophs, will be reviewed.

12.2 AM Fungal Life Cycle

AM symbiosis is established through a sequence of events representing key developmental changes in the life cycle of the fungus. After spore germination, a non-symbiotic phase occurs, characterized by limited growth of mycelium, switching to pre-symbiotic stages when host root signals are perceived. Differentiation of hyphal growth pattern precede the formation of infection structures on the surface of host root cells, then hyphae penetrate root tissues, triggering the formation of arbuscules, intracellular haustoria which are considered the main site of nutrient exchange, in inner cortical cells. The formation of additional infection units extending fungal colonization of host root cortex enables the fungus to drain enough carbon to develop an extensive extraradical hyphal network which explore the surrounding environment, absorbing mineral nutrients from the soil, and to complete its life cycle with formation of new spores by the external mycelium.

Germination of AM fungal spores is not regulated by host-derived signals, since they are capable of germination and growth, under adequate edaphic conditions, in the absence of host plants. Molecular signals involved in spore germination and cell cycle activation still remain unknown, though it has been observed that these processes may be affected by differ-

ent environmental factors like pH, temperature, moisture, nutrients, host plants, presence of microorganisms (Mosse 1959; Hepper 1979).

Non-symbiotic growth of the germ tube produce a coenocytic mycelial network, containing many nuclei, which is not capable of extensive hyphal development and, in the absence of the host, ceases growth within 8–20 days of germination (Hepper 1984; Bécard and Piché 1989; Logi et al. 1998). During spore germination and growth, protoplasm movements towards the emerging germ tube and nuclear/organelles movements along hyphae suggested redistribution of spore content into the growing mycelium (Sward 1981; Logi 1998; Bago et al. 1998). Other findings have confirmed a process of resources reallocation during the early days of spore germination: the number of nuclei per spore was shown to decrease from 2000 to 800, suggesting nuclear migration towards the growing hyphae, and organized cytoskeletal components, both microtubules and microfilaments, were localized in actively growing mycelium (Bécard and Pfeffer 1993; Åström et al. 1994; Logi et al. 1998).

Though host-derived signals do not regulate fungal spore germination, the presence of host roots or root exudates may positively affect spore germination and germ tube growth, but different results have been observed depending on experimental conditions (Tommerup 1984a; Gemma and Koske 1988; Bécard and Pichè 1989; Giovannetti et al. 1993a; Suriyapperuma and Koske 1995; Tawaraya et al. 1996). Studies on the influence of genetically modified plants on growth and infectivity of the arbuscular mycorrhizal fungus *G. mosseae,* performed in an in vivo experimental system, showed that the stimulation of pre-symbiotic hyphal growth by host root exudates may be affected by genetic modification (corn transformation event *Bt* 176) or not (corn transformation event *Bt* 11 and defensin expressing aubergine) (Turrini et al. 2004).

Controversial results have also been reported on the activation of the cell cycle in AM fungi growing in the absence of the host, since dividing nuclei, nuclei with highly condensed chromatin and BrdU-incorporating nuclei were observed in germinating spores of *A. laevis* and *G. margarita* (Mosse 1973; Sward 1981; Bianciotto and Bonfante 1993) though the absence of nuclear division was suggested by other authors (Burggraaf and Beringer 1989). No information is available on nuclear behaviour in the presence of host root exudates.

Non-symbiotic mycelial network extension is usually lower than 200 mm, since a growth arrest has been shown to occur at different times after spore germination, when retraction septa are produced, separating viable from empty mycelium (Fig. 12.1a) (Logi et al. 1998; Bago et al. 1998). Such behaviour seems not to be due to any block in metabolic pathways or to exhaustion of spore resources. All authors agree with the evidence of a progressive reduction of mycelial viability, which decreases with

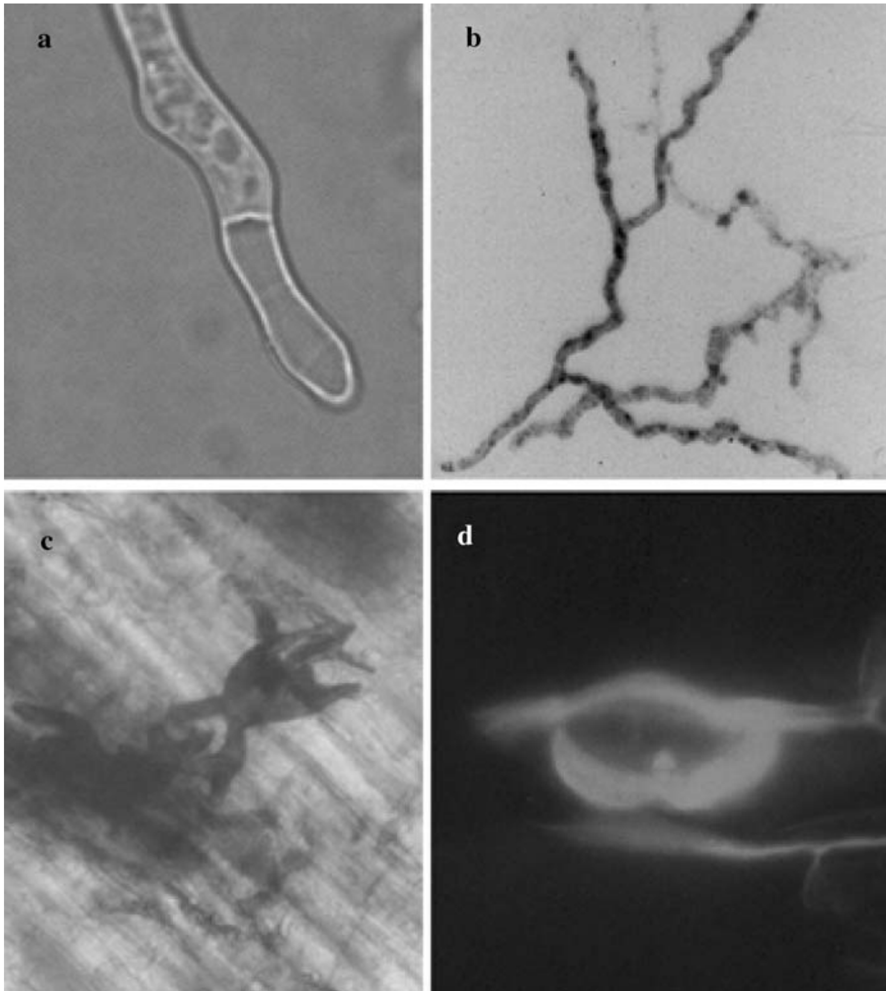

Fig. 12.1. Bright field and epifluorescence micrographs showing: **a** protoplasm retraction and septum formation in a *Glomus caledonium* hyphal tip; **b** branching enhancement in *Glomus mosseae* mycelium grown on a membrane overlying host roots; **c** *G. mosseae* appressorium developed on *Malus pumila* roots; **d** autofluorescent root cell wall thickening below a *G. mosseae* appressorium in the Myc^{-} pea mutant P2

increasing distance from germ tube base and with the age of mycelium, since protoplasm retraction, initiating at hyphal tips and isolating distal (empty) from proximal (viable) mycelium to the mother spore, allocates resources towards the principal germ tube. Intense signals for cytoskeletal proteins were found during protoplasm retraction, and this suggests that migration of nuclei and cellular organelles from hyphal tips towards the

mother spore is a process of resource reallocation, functional to maintain the limited energy resources of germlings, rather than a senescence phenomenon (Logi et al. 1998). The detection of infectivity in *G. caledonium* and *A. laevis* germinated spores incubated four months in soil and of metabolic activity and infectivity in hyphae proximal to the mother spore of *G. caledonium* maintained for six months in vitro support this hypothesis and place the occurrence of growth stasis among previously mentioned survival behaviours of AM fungi, as a strategy for long-term infectivity retention (Tommerup 1984b; Logi et al. 1998).

If this strategy is really operating in nature, an alternative/optional life cycle, activating when spores germinate in the absence of the host may be drawn beside the known life cycle: when host stimuli are lacking, germlings undergo protoplasm withdrawal and mycelial septation leading to a metabolic stasis which may be broken when host roots are available (Fig. 12.2). Recent results obtained after suppressive-subtractive library construction from *G. mosseae* mycelium suggest that the gene *GmGIN* 1, which is mostly expressed in non-symbiotic mycelium and codifies for

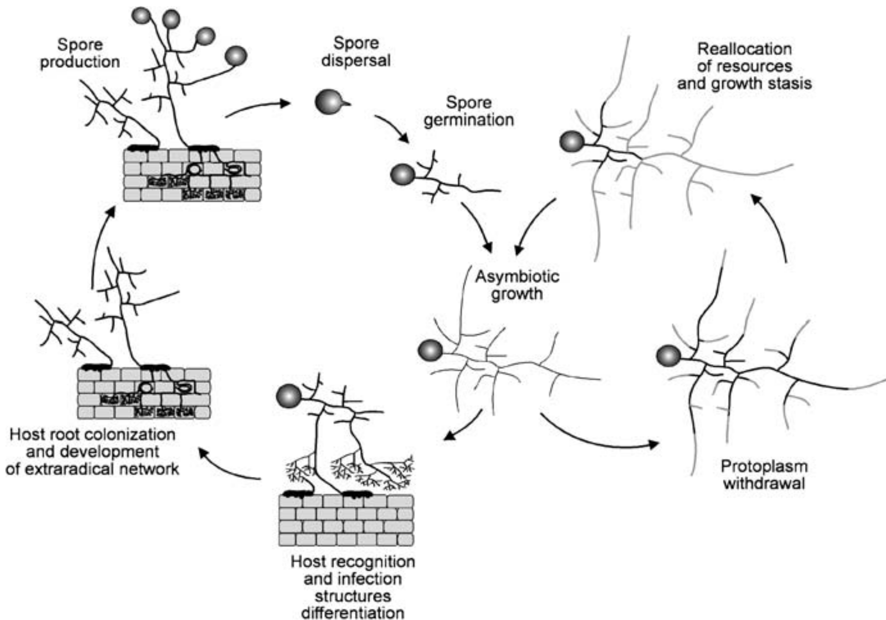

Fig. 12.2. Line drawing showing an alternative cycle in the life of arbuscular mycorrhizal fungi: when germinated spores do not perceive any host signal a resource reallocation pathway may activate, retracting protoplasm from hyphal tips to maintain long-term viability and infectivity with a limited energy dissipation. Modified from Logi (1998), with permission

a protein having putative self-splicing activity and an N-terminal part with GTP-binding activity, could be involved in the signalling cascade controlling growth arrest in the absence of the host plant, though no evidence is available on the function of this gene (Requena et al. 2002).

It also remains to be unravelled which is the signal acting as trigger for fungal growth arrest: limited time from germination or hyphal length, and/or exceeded ration of energy consumption may represent the prompt of an energy-saving mechanism operating when germinated spores of the obligately biotrophic AM fungi fail to contact a suitable host.

12.3 Chemotropic Responses

The hypothesis that contact between AM fungal hyphae and host roots results by chance encounters seems unlikely when considering the obligate nature of the symbiosis. Since the exchange of pre-contact signals represents the earliest step in a plant-microbe interaction, in a complex environment such as soil, the detection of specific plant-derived molecules by microbes may be critical to recognition and subsequent colonization of hosts. Non-chemotactic mutants of *Rhizobium* are able to nodulate roots, but their efficiency and competitiveness are reduced, and similar results were obtained with *Azospirillum* (Caetano-Anolles et al. 1988; Vande Broek et al. 1998). Consistently, the loss of normal chemotactic capabilities appears to be a crucial limiting factor in pathogenic interactions with host by *Agrobacterium tumefaciens* (Hawes and Smith 1989). These results suggest that chemotaxis/chemotropism phenomena may be important when symbioses have to be established in natural environment, where competition and infectivity are the results of a combination of factors, including the ability of contacting host roots, whereas these phenomena may appear to be superfluous in experiments carried out in laboratory conditions.

Some authors reported the occurrence of directional growth of AM fungal hyphae towards host roots in different experimental systems, without unequivocal detection of chemotropic growth (Mosse and Hepper 1975; Mugnier and Mosse 1987). Host-specific chemotropic growth has been demonstrated to occur in aerial hyphae of the AM fungus *Gigaspora gigantea*, which were attracted by volatile host roots factors, and were able to contact maize roots over a distance of up to 11 mm (Koske 1982). Preliminary results obtained with the arbuscular fungus *Glomus mosseae* suggest that fungal mycelium is able to perceive host signals and reorient hyphal growth toward host roots up to a distance of about 1 mm (Sbrana and Giovannetti 2005).

High phosphorus concentrations decreased the attraction of *G. gigantea* hyphae towards roots (Suriyapperuma and Koske 1995) and this finding seems consistent with the relationship between plant phosphorus content and root exudation quantity and quality, suggesting that high exudation and/or the presence in exudates of a large variety of compounds may be responsible for strong chemotropic responses, as observed in different fungal species.

Increased exudation due to reduced phosphorus availability is also responsible for enhanced stimulation of pre-infection hyphal branching, suggesting that similar host-derived compounds could act as signals for both chemotropism and branching. Connection between chemotropism and branching has been previously reported to occur in other fungal organisms, suggesting that hyphal tip reorientation and new branches development have some receptors and/or the generating mechanism in common (Schreurs et al. 1989).

12.4 Pre-Contact Host Recognition

Symbioses establishment is the result of a cascade of events during which the results of different interaction steps between symbiont and host lead to a specific union. A fundamental role is played here by pre-contact partner recognition events, an early step preceding infection during which the fungus discriminates between host and non-host roots and switches its developmental programme towards hyphal differentiation.

In the pre-symbiotic stage, the earliest defined recognition phenomenon in AM fungal hyphae is the hyphal growth enhancement and branching response to the perception of host-derived signals: this event has been observed whenever fungal mycelium approaches host roots in many different experimental systems (Fig. 12.1b).

Volatile compounds released by host roots, included CO_2, increase growth of AM spore germ tubes, and this observation has allowed the use of methods for increasing the CO_2 percentage in atmosphere to stimulate germination and mycelial development during in vitro culturing of different AM fungal species (Bécard and Piché 1989; Bécard et al. 1992).

Many reports on the effects of host root exudates on mycelial growth suggested that these compounds induced hyphal growth increase (Gemma and Koske 1988; Bécard and Piché 1989). Enhanced branching (differential morphogenesis) was also observed to be induced in AM fungal hyphae growing on membrane filters overlying host root system, and it was shown that this phenomenon occurred only in membrane areas exactly corresponding to host roots growing underneath: such localised differential growth pattern

could be due either to limited-diffusing/unstable compounds, or to a high concentration of signals needed for the initiation of branching response (Giovannetti et al. 1993b, 1994). The occurrence of a recognizable and unambiguous morphogenetical change in mycelium, which appeared to be strictly dependent on the perception of host-derived signals, suggested that it was likely to represent the change from an "asymbiotic" stage to a truly "pre-symbiotic" state of mycelium: the profuse hyphal branching occurring in AM hyphae in the presence of host-derived compounds may be functional to locate suitable infection sites on the root surface, but it could also represent a sign of fungal commitment to infective status (Giovannetti and Sbrana 1998).

Successive investigations showed that hyphal branching enhancement elicited in AM fungal mycelium by host-derived signals passing through a membrane was accomplished within 24 h since the beginning of the plant/fungus interaction and that of non-host plants or plant hosts of mycorrhizas other than arbuscular did not exert any effect on fungal morphogenesis, confirming that host-derived signals are the cues enabling AM symbionts to discriminate unambiguously hosts from non-hosts (Giovannetti et al. 1994). Subsequently, in vitro experiments confirmed the occurrence of the phenomenon in the presence of host plants (Giovannetti et al. 1996) and in the presence of crude or semi-purified fractions of root exudates collected from *Agrobacterium rhizogenes*-transformed carrot roots (Buee et al. 2000). Different species of *Gigaspora* were found to respond to host exudates with hyphal branching induction between 5 and 8 h after application, leading to visible enhanced branching pattern after 16–24 h and to larger mycelial growth four days after application (Nagahashi and Douds 1999; Buee et al. 2000). Many different plant exudates, both from seedlings and from transformed roots, were found to be active in inducing hyphal branching in *Gigaspora rosea* in this system, whereas no fungal response was obtained with exudates from non-host plants (Buee et al. 2000). Moreover, the appearance of hyphal branching was found to change with the exudate dosage applied: few and long hyphal branches were observed with lowest exudate concentration whereas a finger-like appearance of branches, highly reminiscent of arbuscules, was induced by maximum exudate concentration (Nagahashi and Douds 1999). Accordingly, fan-like or arbuscule-like structures have been primarily observed to develop in close proximity of host roots, either on membranes or on agar, by many authors, suggesting fungal sensing of locally high concentration of signals (Mosse and Hepper 1975; Giovannetti et al. 1994).

Many works have shown that hyphal growth and/or hyphal branching induction are depressed when plants grew at high P content (Nagahashi et al. 1996; Tawaraya et al. 1998), and in vitro studies on branching factors, as seen above for *Gigaspora* chemoattraction, confirm this finding by evidencing

that exudates from transformed host root grown under phosphorus stress display enhanced branching signal (Buee et al. 2000).

Host plant mutants of the leguminous species *Pisum sativum, Medicago sativa, Medicago truncatula, Lotus japonicus, Vicia faba, Phaseolus vulgaris* and *Glycine max,* defective in different genes and unable to establish AM symbiosis, have been studied with the aim of dissecting the process of recognition between fungus and plant (Bradbury et al. 1993a,b; Sagan et al. 1995; Shirtliffe and Vessey 1996; Wegel et al. 1998). Many plant genes involved at different stages of symbiotic interaction have been identified by using these mutants, and they would have also been very useful for experiments aimed at the identification in root exudates of molecules acting as host roots recognition signals for AM fungi. Unfortunately, all these mutants were impaired at stages following appressorium differentiation: both differential hyphal morphogenesis and appressoria were formed on the roots of Myc^{-} mutants of *Pisum sativum* (Gollotte et al. 1993) and Buee et al. (2000) showed that root exudates from a Myc^{-} pea mutant are able to elicit fungal branching.

No mutants lacking appressorium formation have been described among leguminous plants, probably due to the previous screening for nodulation defects, by which nodulation-independent mutations could have been discarded. A recent approach, selecting mutants directly for their susceptibility to mycorrhizal symbiosis, has provided evidence that some recognition steps unique for mycorrhizas establishment may be detected in non-leguminous plants, as reported for *Lycopersicon esculentum*.

Results obtained with this mutant of *L. esculentum* (*pmi*), which shows susceptibility to infection when challenged with extraradical hyphae, whereas it hinders infection and further colonization of AM fungi when inoculated with spores as the main inoculum source, evidence the need of pre-contact signals to perform subsequent developmental stages by germinated spores (David-Schwartz et al. 2001, 2003). This suggests that while properties ruling post-infection stages of AM colonization are normal, this mutant could lack signals needed to make successful spore mycelial differentiation towards the infective stage. In fact, tests aimed at examining pre-symbiotic AM fungal behaviour showed lower spore germination and growth and appressorium development in the presence of *pmi* roots with respect to wild type (David-Schwartz et al. 2001). Moreover, it has been recently reported that semipurified fractions of root exudates of the *pmi* mutant (M161) inhibit hyphal growth of *G. gigantea* and *G. intraradices* in vitro, though results about hyphal branching are not clear (Gadkar et al. 2003). It has been claimed that spores are not the main inoculum source in nature, since their infective ability has been observed to be lower than 'whole inoculum', and these findings strongly suggest that spore mycelial colonization of host roots may be affected by the requirement of

a developmental switch, at the early stages of fungus-plants interaction, for subsequent formation of appressoria.

Recent studies, reporting pre-branching activation of genetic and physiological machinery involved in fungal respiration in *G. rosea* and *G. intraradices* treated with host root exudates, also support the hypothesis that enhanced branching represent a developmental switch, induced by host-derived signals, fundamental for symbiosis establishment (Tamasloukht et al. 2003). Results of this work showed that activation of fungal genes, encoding for putative pyruvate carboxylase and mitochondrial ADP/ATP translocase, and oxygen consumption, were induced after 0.5–1 h and 1.5–3 h treatment with semipurified exudates, respectively, when no morphological change in growth pattern of germinated spores was detectable yet, suggesting that a switch of fungal respiration from basal to high level, functional to support an increase in mycelial growth and branching, could be the target of host-derived signals.

Another study confirms that host signals induce not only a physiological fungal switch, but also the release of fungal signals for host plants. In fact, it has been reported that a diffusible factor released by AM fungal germinated spores triggers the expression of one of the early nodulin genes involved in both nodulation and arbuscular mycorrhization early steps, MtENOD11. In this study, germinated spores of the AM fungal species *Gigaspora rosea, G. gigantea, G. margarita, Glomus intraradices,* growing onto a dialysis membrane overlying roots, were able to induce MtENOD11 expression in roots of both wild type and Myc^{-} mutants of *Medicago truncatula* (Kosuta et al. 2003). MtENOD11 induction correlated both spatially and temporally with the appearance of the hyphal branching indicating host recognition, and it was always detected after branching enhancement appearance and only in roots located in membrane areas corresponding to mycelial differentiation zones. These findings suggest that host-derived signals may activate genes of the fungus which are responsible of subsequent root biochemical changes, inducing the release of putative "Myc" factors in the same way host flavonoids activate Nod factors production by rhizobia. Since Nod factors molecule possesses a chitin skeleton, this kind of compounds could be easily synthesized by fungal cells; moreover, since chito-oligosaccharides are able to induce phenomena involved in rhizobia recognition by host roots, simple chitin oligomers could also represent signals for the host released by the fungus (Catoira et al. 2000; Stracke et al. 2002; Kosuta et al. 2003). Recent results, obtained by using fluorescent sulfated Nod factors, show that these signalling compounds are absorbed by root hairs and strongly bound by root hairs cell walls: the immobilization is probably due to a Nod factor binding receptor, which is able to recognise the chitin chain of the compound, whereas the subsequent specific recognition of Nod factors features involves a signalling receptor which triggers the

signal transduction cascade leading to root hair deformation (Goedhart et al. 2003).

It is interesting to note that both branching enhancement in the fungal symbiont and MtENOD11 expression in host roots occur when host and fungal cells are only a short distance apart, and this strengthens the hypothesis that signalling compounds are slowly diffusible or unstable molecules, or that a high concentration of signal is needed for the induction of responses.

No expression of this Nod gene was observed when *M. truncatula* roots were challenged with the phytopathogenic fungi *Fusarium solani*, *Phoma medicaginis, Phytophthora medicaginis* and *Rhizoctonia* sp., indicating that a specific AM fungal factor is responsible of MtENOD11 induction (Journet et al. 2001; Kosuta et al. 2003).

Pea and *M. truncatula* mutants defective in Sym19, Sym8 and dmi1, dm2 genes, respectively, all unable to nodulate and to form AM symbioses, do not exhibit the typical multiple oscillations of cytoplasmic Ca^{2+} concentration (Ca^{2+} spiking) which is an early cellular response of plant root to Nod factors, prerequisite to root hair deformation (Wais et al. 2000; Walker et al. 2000). These mutants do not respond with Ca^{2+} spiking when challenged with chitin oligomers, which usually induce in wild type and mutants of different types a Ca^{2+} spiking phenomenon similar to the one observed in the presence of Nod factors. The induction of ENOD genes also follows the Ca^{2+} spiking response, which can be considered another fundamental step shared by rhizobia and AM fungi early recognition processes. Since both pea and *M. truncatula* mutants, defective at Sym30, Sym19, Sym8 and at dmi3 loci, respectively, block both nodulation and mycorrhization, with (Sym30 and dmi3) or without (Sym 8 and 19) Ca^{2+} spiking, it may be argued that these genes are common to both symbioses and that their products act before or after this event. Mutants defective exclusively in nodulation showing regular calcium spiking are impaired in genes acting after this event and also after the divergence between the recognition pathways of Rhizobia and AM fungi.

A Myc^{+} mutant of *M. truncatula* (C31), lacking cytoplasmic Ca^{2+} influx which is induced immediately after Nod factors application and before Ca^{2+} spiking, provides evidence of an additional locus (NFP: Nod factor perception) involved in controlling both Ca^{2+} influx and Ca^{2+} spiking though not involved in AM fungal recognition (Ben-Amor et al. 2003).

Though much work has been performed on release of signals with root exudates by AM fungal hosts, limited information is available on the chemical nature of the compounds involved. Release of flavonoids by plant hosts of rhizobia induce the earliest bacterial response, Nod genes activation and lipo-chitin oligosaccharides (LCOs) synthesis, which determine symbiosis specificity and triggers all subsequent steps of the interaction between plant

and symbiont. Hyphal growth and branching enhancement have been observed in *Gigaspora* and *Glomus* spores grown in the presence of flavonoids, according to a signalling role of these compounds, though it has also been shown that chalcone synthase defective plants, where flavonoids synthesis was hindered, not only show regular infection pattern by AM fungi, but also release a branching factor in their exudates (Bécard et al. 1995; Buee et al. 2000). Moreover, quercetin, myricetin and kaempferol, strongly stimulating AM fungal growth, were also shown to occur in non-host root exudates.

Although their chemical nature remains to be unravelled, results obtained so far suggest that the signal molecules eliciting fungal recognition responses should have a maximum molecular weight of 500 Da and a lipophilic character (Giovannetti et al. 1996; Buee et al. 2000).*

12.5 Does Non-Host Signalling Occur?

Many studies aimed at explaining the inability of plants non-host to AM fungi to establish this kind of symbiosis have suggested different hypotheses, sometimes controversial, for this phenomenon. In many papers it is reported that plant species belonging to the families Chenopodiaceae, Brassicaceae, Caryophyllaceae and to the genus *Lupinus* do not induce fungal recognition responses, namely growth and branching enhancement, and consequently do not allow the differentiation of appressoria on their roots (Avio et al. 1990; Giovannetti et al. 1993a, 1994). These findings suggest that non-host plants lack factors triggering root recognition by AM fungi, which are essential for further mycorrhizas development. Some evidence on the release of inhibitory compounds by non-hosts has been reported, and a heat-labile factor able to reduce *G. gigantea* and *G. intraradices* growth in vitro has been recently detected in root exudates of a non-mycorrhizal tomato mutant (Vierheilig et al. 2000; Schreiner and Koide 1993; Oba et al. 2002; Gadkar et al. 2003). Thus, contradictory fungal behaviours have been observed in other works, either involving analysis of experimental trials or of natural samples (Ocampo et al. 1980; Glenn et al. 1985; Parra-Garcia et al. 1992). Some authors also observed AM fungal colonization of wild species, such *Capsella bursa-pastoris* and *Salsola kali*, though in the latter resistance/rejection responses by the plant were detected (Allen et al. 1989; Demars and Boerner 1994, 1995).

Some aut hors have reported development appressoria or entry points on non-host roots, often abortive in healthy non-host root cells, whereas

* Recently, a branching signal has been isolated (Akiyama et al. 2005).

senescent or dead cells could be unspecifically colonized by AM fungal mycelium, as previously reported for senescing plant epidermal tissues (Stasz and Sakai 1984; Glenn et al. 1985; Avio et al. 1990; Cazares and Smith 1992; Cazares and Trappe 1993; Giovannetti et al. 1994). We can deduce that development of hyphal swellings or appressoria-like structures on non-host roots should not be claimed as a sign of plant mycorrhizal status, since these structures are unable to give rise to intraradical colonization and may be associated with dead roots or root cell wall fragments.

12.6 Signalling at Host Contact

Although it is not clear whether chemical signalling preceding appressorium formation, to which the fungus respond with enhanced growth and branching, is a prerequisite for the subsequent development of mycorrhizal infection or it is only functional to enhance fungal chance of contacting suitable roots, a consistent occurrence of hyphal branching and appressoria development has been frequently observed. Within 24–36 h in in vivo systems and 4–5 days in in vitro cultures, appressoria and first intraradical hyphae have been observed in the majority of plant roots: successful interactions were always correlated with the formation of dense, highly ramified fungal fans near roots, suggesting that fungal differentiation is a prerequisite for appressorium formation, root penetration and colonization.

Though the most significant evidence indicating the successful recognition of a potential host plant is represented by the differentiation of fungal infection structures, the appressoria (Fig. 12.1c): these are swollen, inflated structures which can be detected only on the surface of host roots as early as 36 h after the beginning of plant/fungus interaction (Giovannetti and Citernesi 1993; Giovannetti et al. 1994). In fact, no "true" appressoria can be differentiated by AM fungi on the surface of non-host plant roots or on surfaces representing thigmotropic stimuli (nylon, silk, cellulose, polyamide or glass threads), even in the presence of host root exudates (Giovannetti et al. 1993b).

Nagahashi and Douds (1997) showed appressoria development on the surface of isolated and purified cell walls: their shape resembled structures, defined by other authors as "swellings" or "appressorium-like", formed on senescing or dead root tissues, on non-host roots or on heterologous hyphae (Glenn et al. 1985; Giovannetti et al. 1994, 1999). These structures have in common the lack of pre-infection branching enhancement, since no hyphal fans have been observed in these systems, where host-derived signals cannot be perceived by the fungus. Nagahashi (2000) has therefore attempted to distinguish between two kinds of appressoria: "functional"

appressoria, defined by Garriock et al. (1989) as those which adhere to the host root surface and initiate penetration hyphae, and “non-functional” appressoria, which are formed on non-host roots (Giovannetti et al. 1993a) or on isolated host epidermal cell walls (Nagahashi and Douds 1997) and never give rise to successful penetration hyphae. Development of infection pegs in senescing or non-host tissues, reported to occur by unspecific colonization, lead to early septation and abortion of mycelial entry. Appressoria formed on roots of Myc^{-1} mutants have also been defined as “non-functional” in this view, though in this system pre-infection branching of AM fungal hyphae, leading to appressoria overproduction, has been observed, and the lack of successful penetration of hyphae is due to plant cells defence responses (Fig. 12.1d) (Gollotte et al. 1993; Calantzis et al. 2001).

Which are the signals from host roots triggering appressorium formation? Some parasitic fungi utilize the physical surface of the host to localize the infection site: ridges and troughs of particular sizes on the leaf surfaces are topographic cues to which hyphae respond by swelling their apices and differentiating the infection structures (Hoch et al. 1987; Jelitto et al. 1994). Alternation of ridges and troughs marks the break between cells, which might offer less resistance to infection tubes and might avoid direct initial contact with cell protoplasm, reducing the risk of triggering plant defence mechanisms before infection establishment. The ability of AM fungal hyphae to recognise surface topology has been stated, since growth and apical swelling, irrespective of functional appressoria formation, always occur in grooves between cells. Plant lectins, which are known to be involved in recognition between *Rhizobium* and legumes, could represent additional recognition clues, since it has been reported that genes encoding proteins with similarities to lectins are exclusively activated in *M. truncatula* plants during interaction with AM fungi (Wulf et al. 2003).

Genetic studies on different legume species have provided a number of mutants useful for AM studies, since some of the nodulation-defective (Nod^-) mutants were also unable to be colonized by AM fungi (Myc^-). These mutants might have been useful for determining the genetic basis of appressorium differentiation, but *Pisum sativum, Medicago sativa*, *Medicago truncatula* and *Lotus japonicum* Nod^-/Myc^- mutants suppress mycorrhizal formation during the colonization phase, after appressorium development (Gollotte et al. 1993; Sagan et al. 1995; Shirtliffe and Vessey 1996; Wegel et al. 1998; Calantzis et al. 2001). The ability of AM fungi to form appressoria on roots of Myc^- mutants indicates that these plants are not altered in genes producing signals for AM fungi. In Myc^{-1} mutants of *P. sativum* and *M. sativa*, induction of infection structures is followed by plant defence reactions, since fungal root penetration of the epidermal cell layer is hindered by deposition of cell walls reinforcements: cytological

studies have shown that deposition of ß-glucans and increased phenolics occur only in cells beneath appressoria, and this phenomenon often leads to record high numbers of deformed or highly branched appressoria on roots, since AM fungi, perceiving stimuli triggering infection, enhance branching and infection structures differentiation (Gollotte 1993; Bradbury et al. 1993a,b).

The Myc^{-1} mutation has been shown to occur at a single locus and to be recessive and genetically stable in pea; moreover, this genetic trait did not segregate from the Nod^{-} character (unable to form nodules) in F2 populations, suggesting that one gene controls both characters (Sagan et al. 1993). The three Nod^{-}/Myc^{-} mutants characterized in the species *M. truncatula* are blocked in a signalling pathway related to *Rhizobium* Nod factor perception/transduction, suggesting that early signaling steps are in common between the two symbioses (Catoira et al. 2000). Plant genes specifically expressed during different stages of nodulation, such as MsENOD, PsENOD, VfENOD and VfLb, are also transcribed during AM fungal interaction with hosts (van Rhijn et al. 1997; Journet et al. 2001; Frühling et al. 1997). During root nodulation, MtENOD11 expression correlates with preinfection responses and infection thread formation in root hairs (Journet et al. 2001). In interactions with arbuscular mycorrhizal fungi, this early nodulin gene is only expressed in successful associations that lead to the formation of penetrating hyphae at the interface between appressoria and epidermal cells, and these findings suggest that the transcriptional activation of MtENOD11 is linked to the penetration of fungal hyphae across the root epidermis and through the outer cortex, similar to what is observed for pea ENOD12A gene. Contrary to findings on pea ENOD12A gene, root cortical cells of *M. truncatula* containing arbuscules also showed MtENOD11 expression, suggesting that this gene is expressed throughout the infection process in all the root cells contacted by the fungus. In contrast, no gene expression was detected in epidermal cells in contact with appressoria on Ri T-DNA-transformed roots of the Pen^{-} mutant TR26, confirming that hyphal penetration is required for MtENOD11 induction in root epidermis. The comparison of MtENOD11 expression during rhizobial and endomycorrhizal root infection suggests that transcription is triggered in both cases in epidermal and cortical cells physically associated with fungal penetration: the infection thread in the case of *S. meliloti*, internal hyphae in the case of the endomycorrhizal fungus. MtENOD11 is predicted to encode a repetitive proline rich protein, which could play a role in cell wall plasticity modification, allowing fungal penetration of root cells (Journet et al. 2001).

Hyphae produced by appressoria release localized hydrolytic enzymes able to degrade the plant cell wall at the point of contact, and penetrate through the epidermal cell layer into the cortical tissue where arbuscules are differentiated. In *Medicago* mutants, deposition of phenolic compounds

and cell wall thickening have been observed in the Myc^{-1} mutants, with the Myc^{-2} mutants producing electron dense deposits in conjunction with fungal colonization failure sites (Smith and Read 1997).

Recent results obtained with the SYMRK mutant of *Lotus japonicus,* in which mycorrhizal development is arrested at the epidermal cell layer, showed that leghaemoglobin synthesis, activated by Nod factors in wild type plants, is not activated in this mutant even though Nod factor is produced (Stracke et al. 2002). Moreover, independent mutation events in the receptor-like kinase (RLK) gene from different mutant alleles have been found to suppress AM fungal and rhizobial root colonization, and this finding support the requirement of RLK gene for both symbioses, suggesting that the mutated gene is involved in this shared pathway of signal transduction (Endre et al. 2002; Stracke et al. 2002).

In *Lotus japonicus*, mutants Ljsym71 and Ljsym72 show poor development of the external mycelium, formation of extremely branched deformed appressoria and blocking of hyphal penetration at the root epidermis by *Glomus* sp. Small amounts of internal colonization including degenerated arbuscule formation occur infrequently in these types of mutants, which have been defined with the term Coi- (cortex invasion phenotypes) (Wegel et al. 1998). These mutants appear to belong to a different class with respect to pea Myc^{-2}, in which early stages of infection and intercellular fungal growth in roots are allowed but arbuscules are aborted (Gianinazzi-Pearson 1996).

It can be concluded that signals involved in appressorium differentiation do not trigger a cascade of unregulated events leading to root colonization, but each new step in fungal-plant cell interaction requires an additional recognition event, by which the host plant control fungal spreading to ensure a balance of symbiosis costs and benefits.

12.7 Conclusions

Since mycorrhizal fungi, unlike other symbionts, are not vertically transmitted from host parent to offspring, successful mycorrhizal synthesis is established by complex communication events between fungi and plants. It is still not well understood which are the signals responsible of growth stimulation and branching of AM fungi (Akiyama et al. 2005), though it has been stated that this recognition step represents the sign of a metabolic switch changing fungal state from asymbiotic to presymbiotic or infective. Interactions between incompatible partners, particularly those with host plant mutants, have provided useful model systems for the dissection of the recognition pathway and the detection of genes responsible of control

points. Results have shown that early recognition steps do not activate an unregulated cascade of events leading to symbiosis establishment, but that each new morphogenetic change in both partners requires successful biochemical communication stages. Progress in this field will allow one to draw a flow diagram of recognition steps and plant/fungal genes involved in the establishment of mycorrhizal symbioses, also revealing which is the weight each partner has in determining the success of interaction.

References

Akiyama K, Matsuzaki K, Hayashi H (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435:824–827

Allen MF, Allen EB, Friese CF (1989) Responses of the non-mycotrophic plant *Salsola kali* to invasion by vesicular-arbuscular mycorrhizal fungi. New Phytol 111:45–49

Åström H, Giovannetti M, Raudaskoski M (1994) Cytoskeletal components in the arbuscular mycorrhizal fungus *Glomus mosseae*. Mol Plant Microbe Interact 7:309–312

Avio L, Sbrana C, Giovannetti M (1990) The response of different species of *Lupinus* to VAM endophytes. Symbiosis 9:321–323

Bago B, Zipfel W, Williams RM, Chamberland H, Lafontaine JG, Webb WW, Piche Y (1998) In vivo studies on the nuclear behavior of the arbuscular mycorrhizal fungus *Gigaspora rosea* grown under axenic conditions. Protoplasma 203:1–15

Bécard G, Pfeffer PE (1993) Status of nuclear division in arbuscular mycorrhizal fungi during *in vitro* development. Protoplasma 174:62–68

Bécard G, Piché Y (1989) Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2320–2325

Bécard G, Douds DD, Pfeffer PE (1992) Extensive in vitro hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. Appl Environ Microbiol 58:821–825

Bécard G, Taylor LP, Douds DD, Pfeffer PE, Doner LW (1995) Flavonoids are not necessary plant signal compounds in arbuscular mycorrhizal symbioses. Mol Plant Microbe Interact 8:252–258

Ben-Amor B, Shaw SL, Oldroyd GED, Maillet F, Penmetsa RV, Cook D, Long SR, Denarie J, Gough C (2003) The NFP locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. Plant J 34:495–506

Bianciotto V, Bonfante P (1993) Evidence of DNA replication in an arbuscular mycorrhizal fungus in the absence of the host plant. Protoplasma 176:100–105

Bradbury SM, Peterson RL, Bowley SR (1993a) Colonization of three alfalfa (*Medicago sativa* L.) nodulation phenotypes by indigenous vesicular-arbuscular mycorrhizal fungi. Symbiosis 15:207–215

Bradbury SM, Peterson RL, Bowley SR (1993b) Further evidence for a correlation between nodulation genotypes in alfalfa (*Medicago sativa* L.) and mycorrhiza formation. New Phytol 124:665–673

Buee M, Rossignol M, Jauneau A, Ranjeva R, Bécard G (2000) The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol Plant Microbe Interact 13:693–698

Burggraaf JP, Beringer JE (1989) Absence of nuclear DNA synthesis in vesicular-arbuscular mycorrhizal fungi during in vitro development. New Phytol 111:25–33

Caetano-Anolles G, Wall LH, DeMicheli AT, Macchi EM, Bauer WD, Favelukes G (1988) Role of motility and chemotaxis in efficiency of nodulation by *Rhizobium meliloti*. Plant Physiol 86:1228–1235

Calantzis C, Morandi D, Arnould C, Gianinazzi PV (2001) Cellular interactions between G-mosseae and a Myc$^-$ dmi2 mutant in *Medicago truncatula*. Symbiosis 30:97–108

Catoira R, Galera C, de-Billy F, Penmetsa RV, Journet EP, Maillet F, Rosenberg C, Cook D, Gough C, Denarie J (2000) Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. Plant Cell 12:1647–1665

Cázares E, Smith JE (1992) Occurrence of vesicular-arbuscular mycorrhiza on Douglas fir and Western hemlock seedlings. In: Read DJ, Lewis DH, Fitter AH (eds) Mycorrhizas in ecosystems. CAB International, Wallingford, UK, pp 370–374

Cázares E, Trappe JM (1993) Vesicular endophytes in roots of Pinaceae. Mycorrhiza 2:153–156

David R, Itzhaki H, Ginzberg I, Gafni Y, Galili G, Kapulnik Y (1998) Suppression of tobacco basic chitinase gene expression in response to colonization by the arbuscular mycorrhizal fungus *Glomus intraradices*. Mol Plant Microbe Interact 11:489–497

David-Schwartz R, Badani H, Smadar W, Levy AA, Galili G, Kapulnik Y (2001) Identification of a novel genetically controlled step in mycorrhizal colonization: plant resistance to infection by fungal spores but not extra-radical hyphae. Plant J 27:561–569

David-Schwartz R, Gadkar V, Wininger S, Bendov R, Galili G, Levy AA, Kapulnik Y (2003) Isolation of a premycorrhizal infection (pmi2) mutant of tomato, resistant to arbuscular mycorrhizal fungal colonization. Mol Plant Microbe Interact 16:382–388

Demars BG, Boerner REJ (1994) Vesicular-arbuscular mycorrhizal fungi colonization in *Capsella bursa-pastoris* (Brassicaceae). Am Midland Natur 132:377–380

Demars BG, Boerner REJ (1995) Arbuscular development in 3 crucifers. Mycorrhiza 5:405–408

Endre G, Kereszt A, Kevei Z, Mihacea S, Kaló P, Kiss GB (2002) A receptor kinase gene regulating symbiotic nodule development. Nature 417:962–966

Frühling M, Roussel H, Gianinazzi-Pearson V, Puhler A, Perlick AM (1997) The Vicia faba leghemoglobin gene VfLb29 is induced in root nodules and in roots colonized by the arbuscular mycorrhizal fungus *Glomus fasciculatum*. Mol Plant Microbe Interact 10:124–131

Gadkar V, David SR, Nagahashi G, Douds DD, Wininger S, Kapulnik Y (2003) Root exudate of pmi tomato mutant M161 reduces AM fungal proliferation in vitro. FEMS Microbiol Lett 223:193–198

Garriock ML, Peterson RL, Ackerley CA (1989) Early stages in colonization of *Allium porrum* (leek) roots by the vesicular-arbuscular mycorrhizal fungus, *Glomus versiforme*. New Phytol 112:85–92

Gemma JN, Koske RE (1988) Pre-infection interactions between roots and the mycorrhizal fungus *Gigaspora gigantea*: chemotropism of germ-tubes and root growth response. Trans Br Mycol Soc 91:123–132

Gianinazzi-Pearson V (1996) Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. Plant Cell 8:1871–1883

Giovannetti M (2001) Survival strategies in arbuscular mycorrhizal symbionts. In: Seckbach J (ed) Symbiosis mechanisms and model systems. Kluwer Academic Publisher, Dordrecht, The Netherlands, pp 185–196

Giovannetti M, Citernesi AS (1993) Time-course of appressorium formation on host plants by arbuscular mycorrhizal fungi. Mycol Res 98:1140–1142

Giovannetti M, Sbrana C (1998) Meeting a host: the behaviour of AM fungi. Mycorrhiza 8:123–130

Giovannetti M, Avio L, Sbrana C, Citernesi AS (1993a) Factors affecting appressorium development in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe. New Phytol 123:114–122

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993b) Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. New Phytol 125:587–594

Giovannetti M, Sbrana C, Logi C (1994) Early processes involved in host recognition by arbuscular mycorrhizal fungi. New Phytol 127:703–709

Giovannetti M, Sbrana C, Citernesi AS, Avio L (1996) Analysis of factors involved in fungal recognition responses to host derived signals by arbuscular mycorrhizal fungi. New Phytol 133:65–71

Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:5571–5575

Giovannetti M, Sbrana C, Avio L, Strani P (2004) Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal networks. New Phytol 164:175–181

Glenn MG, Chew FS, Williams PH (1985) Hyphal penetration of *Brassica* (Cruciferae) roots by a vesicular-arbuscular mycorrhizal fungus. New Phytol 99:463–472

Goedhart J, Bono JJ, Bisseling T, Gadella TWJ Jr (2003) Identical accumulation and immobilization of sulphated and nonsulfated Nod factors in host and nonhost root hair cell walls. Mol Plant Microbe Interact 16:884–892

Gollotte A, Gianinazzi-Pearson V, Giovannetti M, Sbrana C, Avio L, Gianinazzi S (1993) Cellular localization and cytochemical probing of resistance reactions to arbuscular mycorrhizal fungi in a 'locus a' Myc^- mutant of *Pisum sativum* L. Planta 191:112–122

Hawes MC, Smith LY (1989) Requirement for chemotaxis in patogenicity of *Agrobacterium tumefaciens* on roots of soil-grown pea plants. J Bacteriol 171:5668–5671

Hepper CM (1979) Germination and growth of *Glomus caledonium* spores: the effects of inhibitors and nutrients. Soil Biol Biochem 11:269–277

Hepper CM (1984) Regulation of spore germination of the vesicular-arbuscular mycorrhizal fungus *Acaulospora laevis* by soil pH. Trans Br Mycol Soc 83:154–156

Hoch HC, Staples RC, Whitebread B, Comeau J, Wolf ED (1987) Signaling for growth orientation and cell differentiation by surface topography in Uromyces. Science 235:1659–1662

Jelitto TC, Page HA, Read ND (1994) Role of external signals in regulating the pre-penetration phase of infection by the rice blast fungus, *Magnaporthe grisea*. Planta 194:471–477

Journet EP, El-Gachtouli N, Vernoud V, de-Billy F, Pichon M, Dedieu A, Arnould C, Morandi D, Barker DG, Gianinazzi PV (2001) *Medicago truncatula* ENOD11: A novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. Mol Plant Microbe Interact 14:737–748

Koske RE (1982) Evidence for a volatile attractant from plant roots affecting germ tubes of a VA mycorrhizal fungus. Trans Br Mycol Soc 79:305–310

Koske RE, Gemma JN (1991) Fungal reactions to plants prior to mycorrhizal formation. In: Allen MF (ed) Mycorrhizal functioning. Chapman and Hall, London, pp 3–36

Kosuta S, Chabaud M, Lougnon G, Gough C, Denarie J, Barker DG, Becard G (2003) A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific MtENOD11 expression in roots of *Medicago truncatula*. Plant Physiol 131:952–962

Logi C (1998) Eventi precoci del riconoscimento ospite-simbionte nelle micorrize arbuscolari. PhD thesis, University of Pisa

Logi C, Sbrana C, Giovannetti M (1998) Cellular events involved in survival of individual arbuscular mycorrhizal symbionts growing in the absence of the host. Appl Environ Microbiol 64:3473–3479

Morandi D, Bailey JA, Gianinazzi-Pearson V (1984) Isoflavonoid accumulation in soybean roots infected with vesicular-arbuscular mycorrhizal fungi. Physiol Plant Pathol 24:357–364

Mosse B (1959) The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. Trans Br Mycol Soc 42:273–286

Mosse B (1973) Advances in the study of vesicular-arbuscular mycorrhiza. Annu Rev Phytopathol 11:171–196

Mosse B, Hepper CM (1975) Vesicular-arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA root grown axenically. Phytopathology 77:1045–1050

Nagahashi G (2000) *In vitro* and *in situ* techniques to examine the role of roots and root exudates during AM fungus-host interaction. In: Kapulnick Y, Douds DD Jr (eds) Arbuscular Mycorrhizas: physiology and function. Kluwer Academic Press, pp 287–306

Nagahashi G, Douds DD (1997) Appressorium formation by AM fungi on isolated cell walls of carrot roots. New Phytol 136:299–304

Nagahashi G, Douds DD (1999) Rapid and sensitive bioassay to study signals between root exudates and arbuscular mycorrhizal fungi. Biotechnol Techn 13:893–897

Nagahashi G, Douds DD Jr, Abney GD (1996) Phosphorus amendment inhibits hyphal branching of the VAM fungus *Gigaspora margarita* directly and indirectly through its effect on root exudation. Mycorrhiza 6:403–408

Oba H, Tawaraya K, Wagatsuma T (2002) Inhibition of pre-symbiotic hyphal growth of arbuscular mycorrhizal fungus *Gigaspora margarita* by root exudates of *Lupinus* spp. Soil Sci Plant Nutr 48:117–120

Ocampo JA, Martin J, Hayman DS (1980) Influence of plant interactions on vesicular-arbuscular mycorrhizal infection. I. Host and non-host plants grown together. New Phytol 84:23–25

Parra-Garcia MD, Lo Giudice V, Ocampo JA (1992) Absence of VA colonization in *Oxalis pes-caprae* inoculated with *Glomus mosseae*. Plant Soil 145:298–300

Requena N, Mann P, Hampp R, Franken P (2002) Early developmentally regulated genes in the arbuscular mycorrhizal fungus *Glomus mosseae*: identification of GmGIN1, a novel gene with homology to the C-terminus of metazoan hedgehog proteins. Plant Soil 244:129–139

Sagan M, Huguet T, Barker D, Duc G (1993) Characterization of two classes of non-fixing mutants of pea plants (*Pisum sativum* L). Plant Sci 95:55–66

Sagan M, Morandi D, Tarenghi E, Duc G (1995) Selection of nodulation and mycorrhizal mutants in the model plant *Medicago trunculata* (Gaertn.) after γ-ray mutagenesis. Plant Sci 111:63–71

Sbrana C, Giovannetti M (2005) Chemotropism in the arbuscular mycorrhizal fungus *Glomus mosseae*. Mycorrhiza 15:539–545

Schreiner RP, Koide RT (1993) Mustards, mustard oils and mycorrhizas. New Phytol 123: 107–113

Schreurs WJA, Harold RL, Harold FM (1989) Chemotropism and branching as alternative responses of *Achlya bisexualis* to amino acids. J Gen Microbiol 135:2519–2528

Shirtliffe SJ, Vessey JK (1996) A nodulation (Nod^+/Fix^-) mutant of *Phaseolus vulgaris* L has nodule-like structures lacking peripheral vascular bundles (Pvb^-) and is resistant to mycorrhizal infection (Myc^-). Plant Sci 118:209–220

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, London

Stasz TE, Sakai WS (1984) Vesicular-arbuscular mycorrhizal fungi in scale-leaves of Zingiberaceae. Mycologia 76:754–757

Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, Parniske M (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 417:959–962
Suriyapperuma SP, Koske RE (1995) Attraction of germ tubes and germination of spores of the arbuscular mycorrhizal fungus *gigaspora gigantea* in the presence of roots of maize exposed to different concentrations of phosphorus. Mycologia 87:772–778
Sward RJ (1981) The structure of the spores of *Gigaspora margarita*. II. Changes accompanying germination. New Phytol 88:661–666
Tamasloukht M, Sejalon DN, Kluever A, Jauneau A, Roux C, Becard G, Franken P (2003) Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. Plant Physiol 131:1468–1478
Tawaraya K, Watanabe S, Yoshida E, Wagatsuma T (1996) Effect of onion (*Allium cepa*) root exudates on the hyphal growth of *Gigaspora margarita*. Mycorrhiza 6:57–59
Tawaraya K, Hashimoto K, Wagatsuma T (1998) Effect of root exudate fractions from P-deficient and P-sufficient onion plants on root colonisation by the arbuscular mycorrhizal fungus *Gigaspora margarita*. Mycorrhiza 8:67–70
Tommerup IC (1984a) Development of infection by a vesicular-arbuscular mycorrhizal fungus in *Brassica napus* and *Trifolium subterraneum*. New Phytol 98:497–502
Tommerup IC (1984b) Persistence of infectivity by germinated spores of vesicular-arbuscular mycorrhizal fungi in soil. Trans Br Mycol Soc 82:275–282
Turrini A, Sbrana C, Nuti MP, Pietrangeli B, Giovannetti M (2004) Development of a model system to assess the impact of genetically modified corn and aubergine plants on arbuscular mycorrhizal fungi. Plant Soil 266:69–75
van Rhijn P, Fang Y, Galili S, Shaul O, Atzmon N, Wininger S, Eshed Y, Lum M, Li Y, To V, Fujishige N, Kapulnik Y, Hirsch AM (1997) Expression of early nodulin genes in alfalfa mycorrhizae indicates that signal transduction pathways used in forming arbuscular mycorrhizae and Rhizobium-induced nodules may be conserved. Proc Nat Acad Sci USA 94:5467–5472
Vande Broek A, Lambrecht M, Vanderleyden J (1998) Bacterial chemotactic motility is important for the initiation of wheat root colonization by *Azospirillum brasilense*. Microbiology 144:2599–2606
Vierheilig H, Bennett R, Kiddle G, Kaldorf M, Ludwig MJ (2000) Differences in glucosinolate patterns and arbuscular mycorrhizal status of glucosinolate-containing plant species. New Phytol 146:343–352
Wais RJ, Galera C, Oldroyd G, Catoira R, Penmetsa RV, Cook D, Gough C, Denarié J, Long SR (2000) Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. Proc Nat Acad Sci USA 97:13407–13412
Walker SA, Viprey V, Downie JA (2000) Dissection of nodulation signaling using pea mutants defective for calcium spiking induced by Nod factors and chitin oligomers. Proc Nat Acad Sci USA 97:13413–13418
Wegel E, Schauser L, Sandal N, Stougaard J, Parniske M (1998) Mycorrhiza mutants of Lotus japonicus define genetically independent steps during symbiotic infection. Mol Plant Microbe Interact 11:933–936
Wulf A, Manthey K, Doll J, Perlick AM, Linke B, Bekel T, Meyer F, Franken P, Kuster H, Krajinski F (2003) Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula*. Mol Plant Microbe Interact 16:306–314

13 Interactions Between Ectomycorrhizal Fungi and Rhizospheric Microbes

Mondem Sudhakara Reddy, Tulasi Satyanarayana

13.1 Introduction

Microorganisms are essential for the functioning and sustainability of all natural ecosystems. The soil microbiota is a key component of sustainable system and used as natural resource tool. Microbial activities are crucial to the establishment, development, nutrition and health of plants (Azcon-Aguilar and Barea 1992; Linderman 1992). Microbial populations in soil actively develop around plant roots, within the rhizosphere, where they are stimulated by root exudates, plant residues and other organic substrates supplied by the plant. Carbon fluxes are critical for rhizosphere functioning (Toal et al. 2000). The rhizosphere is a physical, chemical and biological environment, which is clearly distinct from the bulk soil, where altered microbial diversity and activity are characteristic (Kennedy and Smith 1995). The supply of photosynthates and decaying plant materials to the root associated microbiota, together with microbial-induced changes in rooting patterns, and the supply of available nutrients to plants derived from microbial activities are key issues of rhizosphere formation and functioning (Barea et al. 2002). The beneficial activities of rhizosphere microbiota include the increased availability of plant nutrients, improvement of nutrient uptake and protection against root pathogens (Azcon-Aguilar and Barea 1992). Certain microorganisms can be used as inoculants to improve the growth and health. These can be grouped into two types: i) saprophytes such as plant growth promoting rhizobacteria (PGPR) that are involved in biological control, and in seedling establishment (Kloepper 1994), free living nitrogen fixing bacteria and phosphate solubilizing bacteria (Barea et al. 1992), and ii) mutualistic symbionts such as mycorrhizal fungi and bacteria involved in nitrogen fixation.

Research on the mycorrhizal associations over the past several decades has promoted understanding and appreciation of the important role of this

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symbiosis in the functioning and performance of plants in a wide array of terrestrial ecosystems. We now understand that the role of mycorrhizal fungi extends beyond the symbiotic acquisition of phosphorus for the host plant and reciprocal carbon provision from the host to fungus. Additional effects of mycorrhizal fungi on the functioning of their host plants including increased disease resistance, improved water relations, acquisition of other soil nutrients and alterations in other soil physico-chemical properties have been documented. The effects of mycorrhizal fungi on the function and growth of individual plants have numerous consequences that extend to the plant population and community levels. The costs and benefits of colonization by mycorrhizal fungi for plant resource availability can strongly influence the patterns of plant reproduction and demography, response to competitors, herbivores and other biotic interactions, and patterns of species composition, diversity and succession (Hartnett and Wilson 2002). In addition to their direct effects on their host plants, mycorrhizal fungi may influence plant communities indirectly through their effects on interactions between plants and their herbivores, pathogens, pollinators and other microbial mutualists (Finlay and Soderstom 1989; Fitter and Garbaye 1994; Hodge 2000).

Mycorrhizal fungi are relevant members of the rhizosphere mutualistic populations known to carry out many critical ecosystem functions. Mycorrhizal associations vary widely in form and function. Mycorrhizal forms have been classified and grouped together by the structural characteristics at maturity as ecto-, endo (AM), ericaceous and orchidaceous mycorrhizas. As this association forms, chemical and physical changes occur in the rhizosphere due to altered host physiology and the chemical and physical presence of mycorrhizal fungi in the rhizosphere and beyond. These changes were considered by Linderman (1988) in proposing the term "mycorrhizosphere" to describe the microbial ambience around mycorrhizas. The mycorrhizosphere is truly dynamic region of immense importance to the functioning and the ecological success of the plant. This is, in reality, the "envelope" of activities that provides the interface between the composite root-fungus structure and soil. The modifications of plant roots by symbiotic fungi into distinct structural characteristics of mycorrhization result in a unique and intriguing component of the rhizosphere. Mycorrhizosphere comprises mycorrhizas, surrounding soil, soil microorganisms and mycorrhiza-associated microorganisms (see Timonen and Marschner, this volume). The mycorrhizosphere is characterized by a high microbial activity and nutrient turnover since root exudates stimulate the organisms in the surrounding environment by providing 'ready to use' carbon and nitrogen sources. Two main groups of microorganisms interact with mycorrhizal fungi in the rhizosphere environments: saprophytes and symbionts. These comprise detrimental, neutral and beneficial bacteria and fungi.

Detrimental microbes include the major plant pathogens as well as minor parasitic and non-parasitic and other deleterious rhizosphere organisms (Nehl et al. 1996). Mycorrhizal fungi also interact with microorganisms colonizing root tissues and the endophytic microorganisms, which are involved in plant growth promotion and plant protection (Kloepper 1994; Sturz and Nowak 2000). Apart from these, mycorrhizal fungi also interact with both symbiotic and free living nitrogen fixing bacteria (Barea 1997).

Ectomycorrhizas are found on woody plants ranging from shrubs to forest trees. Many of the host plants belong to the families Pinaceae, Fagaceae, Betulaceae and Myrtaceae. Over 4000 fungal species, belonging primarily to the Basidiomycotina and fewer to the Ascomycotina, are well known to form ectomycorrhizas. Inoculating trees with selected ectomycorrhizal fungal strains is an efficient way to improve the growth of forest seedlings in nurseries and plantations. The success of fungal inoculation, i.e., fast and massive mycorrhizal establishment of the introduced fungal strain, depends on abiotic factors such as soil pH, fertility, moisture and temperature (Slankis 1974). However, it also depends on biotic factors such as soil microbial communities. Plants typically allocate 10–20% of their photosynthate to their ectomycorrhizal fungus partner, the mycelium of which represents a major route through which carbon flows between the plant and the soil microbial community (Smith and Read 1997). Carbon is subsequently released from the hyphae of ectomycorrhizal fungi as exudates (Sun et al. 1999) following senescence of hyphae (Bending and Read 1995). The region of soil surrounding ectomycorrhizal roots and extramatrical mycelium is, therefore, analogous to the plant rhizosphere (Linderman 1988). In this chapter, an attempt has been made to describe various interactions that are taking place among ectomycorrhizal fungi and other microbes in the mycorrhizosphere.

13.2 Interaction with MHBs

Microbial populations in the rhizosphere are known either to benefit or to interfere with the mycorrhizal establishment (Germida and Walley 1996), and many microorganisms can benefit from mycorrhiza formation and/or functioning (Barea 1997). Garbaye and Bowen (1987) described how mycorrhizal infection of *Pinus* seedlings by three ectomycorrhizal fungi could be enhanced or impeded depending on the soil microflora. Since then the promoting effect of bacteria called "Mycorrhiza Helper Bacteria (MHBs)" (Garbaye 1994) on mycorrhizal establishment has been clearly demonstrated in a range of ectomycorrhizal associations (Garbaye and Bowen 1989; Rozycki et al. 1994; Gagnon 1996; Dunstan et al. 1998). The occurrence of bacteria on

the surface of mycorrhizal roots and inter- and intracellular locations within the ectomycorrhizal mantle and Hartig net have been found (Nurmiaho-Lassila et al. 1997; Mogge et al. 2000). The distinct environment of the ectomycorrhizosphere ensures that its bacterial community is distinct from that of rhizosphere and its bulk soil (Timonen et al. 1998). Some of the bacterial isolates such as *Bacillus* spp, *Burkholderia* spp., and *Pseudomonas* spp. isolated from ectomycorrhizosphere have been shown to promote ectomycorrhizal development (Garbaye 1994; Poole et al. 2001). Plant growth promoting bacteria, including *Pseudomonas* sp. and *Bacillus* sp., have been isolated from rhizosphere soil around *Picea glauca* seedlings (Shishido et al. 1996a), although it is not clear whether these isolates were associated with the extramatrical mycelium of mycorrhizal fungi. Nitrogen fixing isolates of *Pseudomonas* and *Bacillus* have been isolated from roots of pine and oak (Rozyck et al. 1999). The characteristics of ectomycorrhiza inhabiting bacteria have largely been determined using physiological or phenotypic tests (Varese et al. 1996) and, as a consequence, there is very little definitive information regarding the diversity and relatedness of ectomycorrhizosphere bacteria. A range of bacteria associated with ectomycorrhizas and their role in improving the host plants are summarized in Table 13.1. Furthermore, the extent to which bacteria with different functional effects on plant and fungus growth can coexist within ectomycorrhizal roots is unclear. The proposed mechanisms of action by MHBs are improvement of receptivity of the root (mycorrhizal infection), modification of the rhizospheric soil, mediation of fungal propagules and enhancement of fungal growth in the rhizosphere (Garbaye 1994), where the most direct evidence is for enhancement of fungal growth (see Duponnois, this volume).

Mycorrhizal establishment changes, both quantitatively and qualitatively, the microbial populations in the rhizosphere (Azcon-Aguilar and Barea 1992). The number of bacterial groups associated with ectomycorrhizas varied from different mycorrhizal systems. Bending et al. (2002) reported *Bacillus* spp. to be the dominant members of the culturable community; 34 of the 55 isolates were with ectomycorrhizas of *Pinus sylvestris* and *Suillus luteus*. Timonen et al. (1998) found Gram-negative bacteria to be more abundant on mycorrhizas of *P. sylvestris – S. bovinus* and *Paxillus involutus* than were Gram-positive types. Similarly, using fluorescent oligonucleotide probes to investigate the in situ localization of bacteria associated with *Fagus sylvatica – Lactarius* sp. ectomycorrhizas, Mogge et al. (2000) recorded α- and β-subclass proteobacteria to be the dominant members of the bacterial community. Gram-positive bacteria with low DNA G+C content, which include *Bacillus* sp., were found in relatively low abundance. Among the *Bacillus* isolates, *B. cereus*, *B. psychrophilus* and *B. sphaericus* subgroups were reported (Bending et al. 2002). Among a diverse assemblage of *Bacillus* isolates from *P. sylvestris – S. bovinus* and

Table 13.1. A range of bacteria associated with ectomycorrhizas in different host plants

Bacteria	Ectomycorrhizal fungi	Host plant	Reference
Bacillus subtilis MB3			
Pseudomonas fluorescens Bbc6	*Laccaria bicolor*	*Pseudotsuga menziesii*	Duponnois and Garbaye (1991)
Bacillus spp.	*Suillus luteus*	*Pinus sylvestris*	Bending et al. (2002)
Burkholderia spp.			
Serratia spp.			
Pesudomonas spp.			
Proteobacteria	*Lactarius sp.*	*Fagus sylvatica*	Mogge et al. (2000)
Bacillus spp.	*L. rufus*	*P. sylvestris*	Poole et al. (2001)
Burkholderia spp.			
P. monteilli HR13	*Pisolithus alba*	*Acacia* spp.	Duponnois and Plenchette (2003); Founoune et al. (2002)
	Scleroderma sp.		
B. polymyxa (L6, pw-2, pw2R, S20)	*Wilcoxia* sp.	Pine & Spruce	Shishido et al. (1996b)
P. fluorescens Bbc6	*L. fraterna*	*Eucalyptus* spp.	Dunstan et al. (1998)
B. subtilis MB3			
Bacillus sp. Elf28			
Pseudomonad Elf29			
Pseudomonas spp.	*Suillus grevillei*	*Larix decidua*	Varese et al. (1996)
Strptomyces spp.			
Bacillus spp.			
Pseudomonas spp.	*S. bovinus*, *Paxillus involutus*	*P. sylvestris*	Timonen et al. (1998)

P. involutus mycorrhizas, and extramatrical hyphae, Timonen et al. (1998) did not find members of *B. cereus* subgroup. The dominant Gram-positive bacteria on roots of *P. sylvestris* – *Lactrarius rufus* were *Paenibacillus* sp. (Poole et al. 2001). The mycorhizospheres of the seedlings of Himalayan oak (*Quercus leucotrichophora*) and deodar (*Cedrus deodara*) were shown to contain *Bacillus subtilis*, *Erwinia* sp., *Pseudomonas fluorescens*, *Vibrio* spp. and *Xanthomonas* sp.(Yadav et al. 2001); the fluoresent pseudomonads were quite low in both the mycorhizospheres. These observations, therefore, suggest that Gram-positive spore formers are found widely on ectomycorrhizal roots and the specific type depends upon soil environment and the nature of the fungal symbionts.

Soil microorganisms are known to produce compounds that increase root cell permeability, and therefore, are able to increase the rates of root exudation. This, in turn, would stimulate mycorrhizal fungal mycelia in the rhizosphere or facilitate root penetration by the fungus. Biologically active substances such as amino acids, plant hormones, vitamins and other organic compounds are produced by soil microorganisms, which stimulate the growth rates of mycorrhizal fungi. Mycorrhiza helper bacteria benefit the plants under controlled mycorrhization in forest nurseries by reducing the need for soil fumigation due to specificity between the fungus and MHBs (Duponnois et al. 1993), increased mycorrhizal colonization of the plant stock and reducing the quantity of fungal inoculum. The MHB effect on ectomycorrhizal and endomycorrhizal symbioses has been investigated with a limited number of plant species (Duponnois and Plenchette 2003). Some isolates of bacteria, isolated from pine nursery soil or washed *P. radiata* mycorrhizal roots, were also found to have significant positive or negative effects on colonization of *P. radiata* by ectomycorrhizal fungi (Bowen and Theodorou 1979). Garbaye and Bowen (1989) found that most bacteria isolated from within the mantle of *P. radiata-Rhizopogon luteolus* ectomycorrhizas had a beneficial effect on mycorrhizal formation in the same plant-fungus system, and that the bacteria that enhanced mycorrhiza formation also stimulated fungal growth in soil and/or along *P. radiata* roots. Garbaye et al. (1990) selected a mycorrhiza helper bacterial strain *Pseudomonas fluorescens* (BBC_6) for improving the efficiency of *Laccaria bicolor* (S238 N) inoculation in French nurseries. The improvement of Douglas-fir under gnotobiotic conditions as well as in glass house and nursery experiments were also reported using the same strain of *L. bicolor* (Duponnois and Garbaye 1991, 1992). Three bacterial isolates, *Serratia* sp (EJP84), *Pseudomonas* sp. (EJP115) and *Bacillus* sp. (EJP130) stimulated root growth (Bending et al. 2002) of *P. sylvestris*. Bacterial inoculant *Pseudomonas monteilli* strain (HR13) significantly increased ectomycorrhizal colonization of root systems with the fungal symbionts (*Pisolithus alba* and *Scleroderma* sp.) and *Acacia* sp. and consequently the number and biomass of rhizobial nodules (Duponnois and Plenchette 2003; Founoune et al. 2002). Mycorrhizal formation by *Laccaria fraterna* (EJ10), as measured by percentage infected root tips, increased significantly up to 296% when coinoculated with MHB isolates BBc6, *Bacillus subtilis* (MB3) in *Eucalyptus diversicolor* seedlings (Dunstan et al. 1998). Plant growth promoting bacteria have been isolated from rhizosphere of pine and spruce and identified as *Bacillus polymyxa* and *P. putida* (O'Neil et al. 1992; Shishido et al. 1996b). Shishido et al. (1996b) reported that the ectomycorrhizal fungus community had at least six components, and effects of the growth promoting bacteria on the relative proportions of the different ectomycorrhizal types. The growth promotion resulted from shifts in the relative proportions of

fungi forming ectomycorrhizas, leading to changes in the functional benefits to the plant from its associated fungal community. Bending et al. (2002) recorded growth promotion by bacteria in the *P. sylvestris – S. luteus* symbiosis and this did not result from enhanced mycorrhiza formation, but the bacteria affected the functioning of the mycorrhizal symbiosis. Garbaye and Duponnois (1992) measured hyphal growth of fungi exposed to volatile compounds produced by bacteria; BBC_6 and MB3 increased hyphal growth of *L. bicolor* (S238), The MHBs stimulated hyphal growth in the rhizosphere, and therefore, increase the probability of a root-mycelium encounter (Frey-Klett et al. 1997; Garbaye 1994). Chanway et al. (1991) suggested that the growth promotion of pine by *B. polymyxa* resulted from the production of hormones, indole acetic acid, which promoted the formation of lateral roots. The induction of basidiospore germination by gluconic acid was recorded in the ectomycorrhizal fungus *Tricholoma robustum* (Iwase 1992).

The MHB effect is not plant specific (Garbaye 1994; Duponnois and Plenchette 2003). Garbaye et al. (1992) have shown an MHB (*P. fluorescens* BBc6) to promote mycorrhizal formation with *L. laccata* in four conifer species, Norway spruce (*Picea abies*), Austrian pine (*P. nigra*), Scots pine (*P. sylvestris*) and Douglas-fir (*Pseudotsuga menziesii*) and an angiosperm, pedunculate oak (*Quercus robur*). Duponnois and Plenchette (2003) have recently shown the MHB, *P. monteilli* (HR13) to improve the growth of *Acacia mangium, A. auriculiniformis* and *A. holosericea*. In contrast, it was demonstrated that the MHB effect was fungus specific. The intragenic fungal specificity of MHB was demonstrated in the Douglas fir-*L. laccata* S238 symbiotic combination (Garbaye and Duponnois 1992) and others (Duponnois et al. 1993; Dunstan et al. 1998). However, Duponnois and Plenchette (2003) demonstrated that the MHB, *P. monteilli* (HR13), isolated from *Pisolithus alba* stimulated mycorrhizal development of *A. holosericea* with two species of *Scleroderma* and also with arbuscular mycorrhizal fungus G*lomus intraradices*, contradicting the statement that MHBs are fungus specific. Further studies are, therefore, needed for clarifying whether MHBs are fungal specific.

13.3 Inhibitory Effects

Detrimental effects of soil microorganisms on mycelial growth and mycorrhiza formation have also been reported. Some of the ectomycorrhizosphere bacteria particularly *Streptomyces* sp. inhibited the growth of ectomycorrhizal fungi (Varese et al. 1996). The bacterial isolates *Burkholderia* and *Serratia* inhibited ectomycorrhiza formation (Bending et al. 2002). The

negative interactions of bacteria with symbionts could be due to bacterial chitinase, which plays a key role in the inhibition of soil fungi by bacteria (Chet et al. 1990; De Boer et al. 2001). The *Serratia* isolates were vigorous chitinase producers, and therefore, completely inhibited root colonization by *S. luteus* after four weeks of growth (Bending et al. 2002). Some strains of *Pseudomonas* and *Burkholderia* reduced the total mycorrhizal rate, but they were not able to produce chitinase. On the contrary, most of the *B. cereus* subgroup isolates that promoted growth of *S. luteus* along the root were able to produce chitinase. Hence, the ability to produce chitinase may not, therefore, be related to the capacity of ectomycorrhizosphere bacteria to inhibit the growth of ectomycorrhizal fungi. De Boer et al. (1998) reported that the chitinolytic potential of soil bacteria did not necessarily reflect their potential to inhibit the growth of a variety of soil born plant pathogens. Although the hyphae of ectomycorrhizal fungi have been shown to produce exudates (Sun et al. 1999), which could be C source for mycorrhizosphere bacteria, chitin available following senescence of ectomycorrhizal hyphae represents a particularly abundant C source (Bending and Read 1995). The ability to produce chitinase by *Serratia* sp., and *Bacillus* sp., suggests that they have the potential to use of C from living and/or senescent fungal tissues.

The mechanism of interactions of fungi with other soil microbes include the inhibition of the pathogen by antimicrobial compounds (antibiosis), competition for iron through production of siderophores, competition for colonization sites and nutrients, induction of plant resistance mechanisms, degradation of pathogenicity factors of the pathogen such as toxins and parasitism, which may involve production of extra cellular cell wall degrading enzymes (Whipps 2001). None of these mechanisms are necessarily mutually exclusive, and frequently several modes of action are exhibited by a single ectomycorrhizal fungus.

Although production of antibiotics by fungi involved in biocontrol is a well documented phenomenon (Howell 1998), there is very little recent work that clearly demonstrates the production of antibiotics by fungi in the rhizosphere. Antibiotic production by fungi exhibiting biocontrol activity has most commonly been reported for isolates of *Trichoderma* and *Gliocladium* (Howell 1998). There are also reports available about the production of antibiotics by ectomycorrhizal fungi. Tsantrizos et al. (1991) reported secretion of antifungal antibiotics by the ectomycorrhizal fungus *Pisolithus tinctorius*. The antibiotic compounds pisolithin A (*p*-hydroxybenzoylformic acid) and pisolithin B [(*R*)-(−)-*p*-hydroxymandelic acid)] were isolated from the culture filtrates of *P. tinctorius*. These and a few structurally related compounds were shown to inhibit spore germination and cause hyphal lysis of a significant number of phytopathogenic and dermatogenic fungi. Water extractable bioactive compounds from agaricoid

basidiomycetes inhibited growth of Gram-positive bacteria and/or yeasts. These compounds also exhibited activity against soil micromycetes and eliminated *Penicillium thomii, P. nigricans* and *Micromucor ramannianus* (Sidorova and Velikanov 2000). These compounds may act as regulators of the structure of microbial communities in forest soils and litter. These compounds however, have less effect on other organisms such as *P. purpurogenum, Pseudomonas flurosence* and some other bacteria. Rasanayagam and Jeffries (1992) studied the interaction of a variety of ectomycorrhizal fungi with *Pythium ultimum*, and they were of the opinion that the production of acid is responsible for antibiosis by some ectomycorrhizal fungi.

Although competition for nutrients, space or infection sites between microorganisms in rhizosphere exist, the greatest interest has recently been focused on the competition for iron. Under iron-limiting conditions, many of PGPR and mycorrhizal fungi produce a range of iron chelating compounds or siderophores, which have a very high affinity for ferric iron. These iron chelators are thought to sequester the limited supply of iron available in the rhizosphere making it unavailable to pathogenic fungi, thereby restricting their growth. Recent studies have clearly shown that the iron nutrition of the plant influences the rhizosphere microbial community structure (Yang and Crowley 2000). Though, there are reports available about the production of siderophores by ectomycorrhizal fungi (Watteau and Berthelin 1991; Haselwandter 1995; Haselwandter and Winkelmann 2001; Gupta and Satyanarayana 2002), their role in suppression of pathogens was not clearly demonstrated. Mycorrhizal fungi are strong candidates for providing biocontrol through competition for space by virtue of their ecologically obligate association with roots. Ectomycorrhizal fungi, because of their physical sheathing morphology, may well occupy normal pathogenic infection sites. Little work has been carried out to demonstrate this mechanism since it was first suggested by Marx (1972). Most of the biocontrol work was focused on antibiotic production and induced resistance (Perrin 1990; Duschesne 1994). It is not clear whether the biochemical responses similar to induced resistance following mycorrhizal infection affect disease control. Spatial or temporal separation experiments have, however, suggested that the increased levels of chitinases, β– $1-3$ glucanases, β–1. 4 glucosidase, PR-1 protein and peroxidase as well as cell wall appositions and phenolics could be associated with induced resistance (Benhamou et al. 1997; Xue et al. 1998).

The term mycoparasitism applies strictly to those relationships in which one living fungus acts as a nutrient source for another, but fungicolous relationships may also be included in which nutrient exchange has not been shown (Jeffries 1995). Mycoparasitic relationships can be necrotrophic or biotrophic. In natural ecosystems, it has been proposed that mycoparasitic relationships play an important role in the development of fungal

communities. Most of the microscopic observations concerning mycoparasitism have come from in vitro studies or sterile systems (Benhamou et al. 1999; Davanlou et al. 1999), and examples clearly demonstrating mycoparasitism in the rhizosphere are rare (Lo et al. 1998). The process involved in mycoparasitism may consist of sensing the host, followed by direct growth, contact, recognition, attachment, penetration and exit. Although not all these features occur in every fungal-fungal interaction, the key factor is nutrient transfer from host to mycoparasite. The evidence of mycoparasitism of the ectomycorrhizal fungus *L. laccata* against *Mucor hiemalis* in the rhizosphere of *P. sylvestris* was recently reported in vitro (Werner and Zadworny (2003). The growth of *M. hiemalis* was suppressed but no penetration of hyphae by *L. laccata* was reported. The sporangiospores germinated heavily and formed long hyphae in non-mycorrhizal roots, whereas their germination was totally inhibited on mycorrhizal roots.

Interactions between herbivores and mycorrhizal fungi are expected because both depend upon and influence the important plant resources. Above ground consumers may reduce photosynthate translocated to the root system and available to mycorrhizal fungi, resulting in reduction in mycorrhizal colonization and reduced development of the symbiosis (Gehring et al. 1997; Hetrick et al. 1990). Mycorrhizas, in turn, have many potential effects on plant-herbivore interactions. Under certain conditions, up to 40–50% of a plant's net production may be allocated to its fungal symbiont (Fogel and Hunt 1979; Harris and Paul 1987). Because mycorrhizal fungi consume photosynthate and at the same time enhance mineral nutrient acquisition and growth capacity, the cost-benefit relationships among mycorrhizal fungi, herbivores and host plants are likely to be complex. Mycorrhizas may affect herbivores through alteration of plant growth or foliar chemistry (Goverde et al. 2000; Koide 2000), and they may have large effects on plant responses to herbivores by influencing anti-herbivore defenses and/or herbivory tolerance.

13.4 Interactions with PGPRs

Many saprotrophic fungi, particularly certain isolates of *Trichoderma* species, caused plant growth promotion in the absence of any major pathogens (Whipps 1997). *Trichoderma harzianum* was shown to solubilize phosphate and micronutrients that could be made available to promote plant growth (Altomare et al. 1999). Mycorrhizal fungi interact with many of these PGPRs in the mycorrhizosphere. *T. harzianum* is an effective biocontrol agent against several fungal soil borne plant pathogens and possible adverse effects of this fungus on mycorrhizal fungi might be a drawback in its use

in plant protection. The reduction of hyphal length of *Glomus intraradices* in presence of *T. harzianum* was observed by Green et al. (1999), but had no effect on hyphal biomass. The external mycelium of *G. intraradices* was unaffected by antagonistic fungus *T. harzianum*, which on the other hand was suppressed by *G. intraradices*. Paulitz and Linderman (1991) also studied interaction of the fungal biocontrol agent *Gliocladium virens* with *Glomus etunicatum* and *G. mossae*. Their results also suggested that the fungal biocontrol agent *G. virens* did not have a detrimental effect on mycorrhizal fungi. Many reports are available on the interaction of different biocontrol agents and arbuscular mycorrhizal fungi, where the growth of mycorrhizal fungi was not affected by the biocontrol agents (Vazquez et al. 2000). Not many reports are available on the interactions of ectomycorrhizal fungi with the biocontrol agents.

The well known activities of nitrogen-fixing bacteria and phosphate solubilizing microorganisms improve the bioavailability of the major plant nutrients N and P. Synergetic interactions of such microorganisms with mycorrhizal fungi have been demonstrated (Barea et al. 2002). Management of such interactions is a promising approach for either low-input agricultural technologies (Bethlenfalvay and Linderman 1992), or for the re-establishment of the natural vegetation in a degraded area (Miller and Jastrow 1994; Barea and Jeffries 1995).

The inoculation of mycorrhizal fungi has been shown to improve nodulation and N_2 fixation. Although the main mycorrhizal effect in enhancing *Rhizobium* activity is mediated by a generalized stimulation of host nutrition, more localized effects may occur at the root or nodule level (Barea et al. 1992). Interactions can also take place at either the pre-colonization stages, when both microorganisms interact as rhizospheric inhabitants, or during the development of the tripartite symbiosis (Azcon-Aguilar and Barea 1992). Several legumes are able to form two types of mutualistic symbiotic associations with soil microorganisms: N_2 fixing rhizobial nodules and mycorrhizas. Legumes are typically coarse-rooted and, therefore, inefficient in extracting phosphorus from the soil. The two symbioses typically act synergistically, resulting in greater nitrogen and phosphorus content in combination than when each is inoculated onto the legume alone. The mycorrhizal fungi associated with legumes are an essential link for adequate phosphorus nutrition, leading to enhanced nitrogenase activity that in turn promotes root and mycorrhizal growth (Cornet and Diem 1982; Duponnois et al. 2001). Ba et al. (1994) investigated the complex interactions that occur in systems with more than one type of symbiosis using *Bradyrhizobium* sp. and the ectomycorrhizal fungus *P. tinctorius* on the roots of *Acacia holosericea*. After a single inoculation with *Bradyrhizobium* sp., bacteria typically entered the roots by forming infection threads in the root hair cells via the curling point of the root hair and/or after intercellular

penetration. Sheath formation and intercellular penetration were observed on *Acacia* roots after a single inoculation with *P. tinctorius*, but there was no radial elongation of epidermal cells. Simultaneous inoculation with both microorganisms resulted in nodules and ectomycorrhiza on the root system. These results suggested that simultaneous inoculation with both microorganisms inhibits infection thread development, thus conferring an advantage on fungal hyphae in the competition for infection sites. This further suggested that fungal hyphae can modify directly and/or indirectly the recognition factors leading to nodule meristem initiation and infection thread development. Nodule formation and functioning are dependent on mycorrhizal formation (Reddell and Warren 1986). The beneficial effect is generally attributed to the improvement of P uptake, which enhances nodulation and N_2 fixation (Cornet and Diem 1982). *Alnus* species forms multiple symbioses with ectomycorrhizal fungi and nitrogen fixing bacteria from the genus *Frankia*. *Frankia*, macronutrients and the mycorrhizal fungus together increased nitrogen fixation by 136% over the control in actinorrhizal plant *Alnus rubra* (Rojas et al. 2002). PGPR effects have also been reported between *Bacillus* spp. and *Alnus glutinosa* (Probanza et al. 1996). Tian et al. (2003) studied the effect of inoculation of ecto- and arbuscular mycorrhizal fungi and *Rhizobium* on the growth and nitrogen fixation by black locust *Robina pseudoacacia*. Their results indicated that inoculation with all three microbes together produced the most beneficial effects on nitrogen fixation, mycorrhizal development and plant growth. Multimicrobial interactions included not only mycorrhizal fungi and *Rhizobium* spp., but also PGPR. Li et al. (1992) tested the nitrogen fixing *Bacillus* sp. associated with Douglas-fir tuberculate ectomycorrhizas indicating a close nutritional relationship between the bacteria and tuberculate mycorrhizas. The nitrogen fixing *Azospirillum* spp. are know to benefit plant development and influence the morphology, geometry and physiology of the root system (Barea et al. 2002). *Azospirillum* enhanced mycorrhizal formation and response, while mycorrhizal fungi also improved *Azospirillum* establishment in the rhizosphere (Volpin and Kapulnik 1994). Rozycki et al. (1993) reported the association of three isolates of *Azospirillum* spp., with ectomycorrhizal fungi, *Rhizopogon vinicolor*, *L. laccata*, and *Hebeloma crustuliniforme*.

The interactions related to phosphate solubilizers have also received much attention. The species of the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers. The principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatases play a major role in the mineralization of organic phosphorus in the soil (Rodriguez and Fraga 1999). It has been postulated that some phosphate solubilizing bacteria behave as mycorrhizal helper bacteria (Garbaye 1994). Several studies have shown that phosphate solubilizing microbes interact with mycorrhizal fungi by

releasing phosphate ions in the soil, which causes a synergetic interaction that allows better exploitation of poorly available soluble P sources (Ray et al. 1981; Piccini and Azcon 1987). The fungal isolates of *Aspergillus niger* and *A. tubingensis* isolated from the rhizosphere soils of *Eucalyptus* plants improved the solubilization of insoluble phosphates specially rock phosphates (Reddy et al. 2002). It is likely that the phosphate solubilized by bacteria could be more efficiently taken up by the plant through a mycorrhizal mediated bridge between roots and surrounding soil that allows nutrient translocation from soil to plants (Jeffries and Barea 1994). In fact, Toro et al. (1997), using radioactive ^{32}P labeling, demonstrated that phosphate solubilizing bacteria associated with VAM improved mineral (N and P) accumulation in plant tissues. These authors suggested that the inoculated rhizobacteria could have released phosphate ions from insoluble rock phosphate and/or other P sources, which were then taken up by the external VAM mycelium.

13.5 Interations to Improve Soil Quality

There is an increasing interest in applying mycorrhizal fungi along with PGPRs to help revegetation of degraded and desertified ecosystems. A number of experiments on the long term benefits of inoculation with mycorrhizal fungi and *Rhizobium* were aimed at not only the establishment of target legume species, but also the benefit induced by the symbiotically tailored seedlings in key physical and chemical soil properties (Requena et al. 2001). As a result of the degradation/desertification processes, disturbance of natural plant communities is often accompanied or preceded by the deterioration of physical-chemical and biological soil properties, such as structure, nutrient availability, organic matter content, microbial activity (Barea et al. 2002). Therefore, it is becoming critical to recover these soil quality attributes by managing mycorrhizosphere interactions (Barea and Jeffries 1995). Another aspect of mycorrhizosphere interactions with regard to improving soil quality concern applications to the phytoremediation of heavy metal polluted soils. Several studies have demonstrated that the combined inoculations of mycorrhizal fungi and bacterial cultures, isolated from a contaminated environment, were able to increase absorption of metals from soil, and subsequent translocation of the metals to plant shoot (Leyval et al. 1997). Among soil microorganisms, mycorrhizal fungi are the only ones providing a direct link between soil and roots, and therefore are of great importance in heavy metal availability and toxicity to plants.

13.6 Conclusions

The interactions between ectomycorrhizal fungi and rhizospheric microorganisms operate through a variety of modes of action. There has been much interest in the recent years on the ectomycorrhizosphere, since the interactions among microorganisms play a pivotal role in improving plant growth and health and soil quality, which are key issues for the sustainability of natural as well as agroecosystems. Such interactions between ectomycorrhizal fungi and MHBs and PGPRs have recently been recorded, apart from the inhibitory effects. Not much work has been done on the role of microorganisms and ectomycorrhizal fungi in the ectomycorrhizosphere. The research efforts are needed on the application of modern molecular techniques and their integration with the conventional experimental procedures to understand and utilize soil-plant-microbe interactions.

References

Altomare C, Norvell WA, Björkman T, Harman GE (1999) Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295–22. Appl Environ Microbiol 65:2926–2933

Azcon-Aguilar C, Barea JM (1992) Interactions between mycorrhizal fungi and other rhizosphere microorganisms. In: Allen MJ (ed) Mycorrhizal functioning: an integrative plant-fungal process. Routledge, Chapmann and Hill, New York, pp 163–198

Ba AM, Balaji B, Piche Y (1994) Effect of time of inoculation on in-vitro ectomycorrhizal colonization and nodule initiation in *Acacia holosericea* seedlings. Mycorrhiza 4:109–119

Barea JM (1997) Mycorrhiza/bacteria interactions on plant growth promotion. In: Ogoshi A, Kobayashi L, Homma Y, Kodama F, Kondon N, Akino S (eds) Plant growth-promoting Rhizobacteria, present status and future prospects. OECD, Paris, pp 150–158

Barea JM, Jeffries P (1995) Arbuscular mycorrhizas in sustainable soil plant systems. In: Hock B, Varma A (eds) Mycorrhiza structure, function, molecular biology and biotechnology. Springer, Berlin Heidelberg New York, pp 521–559

Barea JM, Azcon R, Azcon-Aguilar C (1992) Vesicular-arbuscular mycorrhizal fungi in nitrogen fixing systems. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology. Academic Press, London, pp 391–416

Barea JM, Azcon R, Azcon-Aguilar C (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie van Leeuwenhoek 81:343–351

Bending GD, Read DJ (1995) The structure and function of the vegetative mycelium of ectomycorrhizal plants. V. Foraging behaviour and translocation of nutrients from exploited litter. New Phytol 130:401–409

Bending GD, Poole EJ, Whipps JM, Read DJ (2002) Characterisation of bacteria from *Pinus sylvestris-Suillus luteus* mycorrhizas and their effects on root-fungus interactions and plant growth. FEMS Microbiol Ecol 39:219–227

Benhamou N, Rey P, Cherif M, Hockenhull J, Tirilly Y (1997) Treatment with the mycoparasite *Pythium oligandrum* triggers induction of defense-related reactions in tomato

roots when challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici.* Phytopathol 87:108–122

Benhamou N, Rey P, Picard K, Tirilly Y (1999) Ultrastructural and cytochemical aspects of the interaction between the mycoparasite *Pythium oligandrum* and soil-borne plant pathogens. Phytopathol 89:506–517

Bethalenfalvay GJ, Linderman RG (1992) Mycorrhiza in sustainable agriculture. ASA Special Publication No 54, Madison, Wisconsin

Bowen GD, Theodorou C (1979) Interaction between bacteria and ectomycorrhizal fungi. Soil Biol Biochem 11:119–126

Chanway CP, Radley RA, Holl FB (1991) Inoculation of conifer seed with plant growth promoting Bacillus strains causes increased seedling emergence and biomass. Soil Biol Biochem 23:575–580

Chet I, Ordentlich A, Shapira R, Oppenheim A (1990) Mechanisms of biocontrol of soil-borne plant-pathogens by rhizobacteria. Plant Soil 129:85–92

Cornet F, Diem HG (1982) Etude comparative de l'efficacite des souches de *Rhizobium* d'Acacia isolees de sols du Senegal et effet de la double symbiose *Rhizobium-Glomus mosseae* sur la croissance de *Acacia holosericea* et *A. raddiana.* Bois Trop 198:3–15

Davanlou M, Madsen AM, Madsen CH, Hockenhull J (1999) Parasitism of microconidia, chlamydospores and hyphae of *Fusarium culmorum* by mycoparasitic *Pythium* species. Plant Pathol 48:352–359

De Boer W, Gunnewiek PJAK, Lafeber P, Janse JD, Spit BE, Woldendorp JW (1998) Anti-fungal properties of chitinolytic dune soil bacteria. Soil Biol Biochem 30:193–203

De Boer W, Gunnewiek PJAK, Kowalchuk GA, Van Veen JA (2001) Growth of chitinolytic dune soil beta-subclass Proteaobacteria in response to invading fungal hyphae. Appl Environ Microbiol 67:3358–3362

Dunstan WA, Malajczuk N, Dell B (1998) Effects of bacteria on mycorrhizal development and growth of container grown *Eucalyptus diversicolor* F. Muell seedlings. Plant Soil 201:243–251

Duponnois R, Garbaye J (1991) Mycorrhization helper bacteria associated with the Douglus-fir-*Laccaria laccata* synbiosis: effects in asceptic and in glasshouse conditions. Ann des Sci Forestieres 48:239–251

Duponnois R, Garbaye J (1992) Application des BAM (bacteries auxiliaries de la mycorhiza-tion) a l'inoculation du Douglas par *Laccaria laccata* S238 en pepiniere forestiere. Rev For Fr 44:491–500

Duponnois R, Plenchette C (2003) A mycorrhiza helper bacterium enhances ectomycorrhizal and endomycorrhizal symbiosis of Australian *Acaia* species. Myorrhiza 13:85–91

Duponnois R, Garbaye J, Bouchard D, Churin JL (1993) The fungus-specificity of mycor-rhization helper bacteria (MHBs) used as an alternative to soil fumigation for ectomy-corrhizal inoculation of bare-root Douglas-fir planting stocks with *Laccaria laccata.* Plant Soil 157:257–262

Duponnois R, Plenchette C, Ba AM (2001) Growth stimulation of seventeen fallow legumi-nous plants inoculated with *G. aggregatum* in Senegal. Eur J Soil Biol 37:181–186

Duschesne LC (1994) Role of ectomycorrhizal fungi in biocontrol. In: Pfleger FL, Linderman RG (eds) Mycorrhizae and plant health. American Phytopathological Society, St Paul, MN, pp 27–45

Finlay RD, Soderstrom B (1989) Mycorrhizal mycelia and their role in soil and plant commu-nities. In: Clarholm M, Bergstrom L (eds) Ecology of Arable Land. Kluwer, Amsterdam, pp 139–148

Fitter AH, Garbaye J (1994) Interactions between mycorrhizal fungi and other soil organ-isms. Plant Soil 159:123–132

Fogel R, Hunt G (1979) Fungal and arboreal biomass in a western Oregon Douglas-fir ecosystem: distribution patterns and turnover. Can J Forest Res 9:245–256

Founoune H, Duponnois R, Ba AM, Sall S, Branger I, Lorquin J, Neyra M, Chotte JL (2002) Mycorrhiza helper bacteria stimulate ectomycorrhizal symbiosis of *Acacia holocerecea* with *Pisolithus alba*. New Phytol 153:81–90

Frey-Klett P, Pierrat JC, Garbaye J (1997) Location and survival of mycorrhiza helper *Pseudomonas fluorescence* during establishment of ectomycorrhizal symbiosis between *Laccaria bicolor* and Douglus fir. Appl Environ Microbiol 63:139–144

Gagnon J (1996) Stimulation of mycorrhization and growth for containerized Jack pine seedlings inoculated with *Laccaria bicolor* and *Pseudomonas fluorescens*. In: Azcon C, Barea JM (eds) Mycorrhizas in integrated systems from genes to plant development. European Commission EUR 16728 EN, pp 634–637

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

Garbaye J, Bowen GD (1987) Effect of different microflora on the success of ectomycorrhizal inoculation of *Pinus radiata*. Can J For Res 17:941–943

Garbaye J, Bowen GD (1989) Stimulation of ectomycorrhizal infection of *Pinus radiata* by some microorganisms associated with the mantle of ectomycorrhizas. New Phytol 112:383–388

Garbaye J, Duponnois R (1992) Specificity and function of mycorrhization helper bacteria (MHB) associated with the *Pseudotsuga menziesii*-*Laccaria laccata* symbiosis. Symbiosis 14:335–344

Garbaye J, Duponnois R, Wahl JL (1990) The bacteria associated with *Laccaria laccata* ectomycorrhizas or sporocarps: effect on symbiosis establishment on Douglas-fir. Symbiosis 9:267–273

Garbaye J, Churin JL, Duponnois R (1992) Effect of substrate disinfection, fungicide treatments and mycorrhization helper bacteria (MHB) on ectomycorrhizal formation of pedunculate oak inoculated with *Laccaria laccata* in two bare root nurseries. Biol Fertil Soils 13:55–57

Gehring CA, Cobb NS, Whitham TG (1997) Three-way interactions among ectomycorrhizal mutualists, scale insects, and resistant and susceptible pinyon pines. Am Nat 149:824–841

Germida JJ, Walley FL (1996) Plant growth-promoting rhizobacteria after rooting patterns and arbuscular mycorrhizal fungi colonization of field-grown spring wheat. Biol Fertil Soils 23:113–120

Goverde M, van der Heijden MGA, Wiemken A, Sandres IR, Erhardt A (2000) Arbuscular mycorrhizal fungi influence life history traits of a lepidopteran herbivore. Oecologia 125:362–369

Green H, Larsen J, Olsson PA, Jensen DF, Jakobsen I (1999) Interaction between *Trichoderma harzianum* and the external mycelium of *Glomus intraradices*. ICOM2 (abstract)

Gupta V, Satyanarayana T (2002) Production of extracellular siderophores by ectomycorrhizal fungi. Indian J Microbiol 42:107–110

Harris KK, Paul EA (1987) Carbon requirements of vesicular arbuscular mycorrhizae. In: Safir GR (ed) Ecophysiology of VA mycorrhizal plants. CRC Press, Boca Raton, FL, pp 93–105

Hartnett DC, Wilson GWT (2002) The role of mycorrhizas in plant community structure and dynamics: lessons from grasslands. Plant Soil 244:319–331

Haselwandter K (1995) Mycorrhizal fungi-siderophore production. Crit Rev Biotechnol 15:287–291

Haselwandter K, Winkelmann G (2001) Ferricrocin – an ectomycorrhizal siderophore of *Cenococcum geophilum*. Biometals 15:73–77

Hetrick BAD, Wilson GWT, Owensby CE (1990) Mycorrhizal influences on big bluestem rhizome regrowth and clipping tolerance. J Range Manag 43:286–290
Hodge A (2000) Microbial ecology of the arbuscular mycorrhiza. Micro Ecol 32:91–96
Howell CR (1998) The role of antibiosis in biocontrol. In: Harman GE, Kubicek CP (eds) Tricoderma and Gliocladium. Vol 2. Enzymes, biological control and commercial applications. Taylor and Francis, London, pp 173–184
Iwase K (1992) Induction of basidiospore of germination by gluconic acid in the ectomyccorhizal fungus *Trichoderma robustum*. Can J Bot 70:1234–1238
Jeffries P (1995) Biology and ecology of microparasitism. Can J Bot 73:S1284–S1290
Jeffries P, Barea JM (1994) Biogeochemical cycling and arbuscular mycorrhizas in the sustainability of plant soil system. In: Gianinazzi S, Schuepp H (eds) Impact of Arbuscular mycorrhizas on sustainable agriculture and natural ecosystem. Birkhauser, Basel, Switzerland, pp 101–115
Kennedy AC, Smith KL (1995) Soil microbial diversity and the sustainability of agricultural soils. Plant Soil 170:75–86
Kloepper JW (1994) Plant growth promoting rhizobacteria (other systems) In: Okon Y (ed) *Azospirillum*/plant associations. CRC Press, Boca Raton, pp 111–118
Koide RT (2000) Mycorrhizal symbiosis and plant reproduction. In: Kapulnik Y, Douds DD (eds) Arbuscular mycorrhizas: physiology and functions. Kluwer Academic Publishers, Dordrecht, pp 19–46
Leyval C, Turnau K, Haselwandter K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza 7:139–153
Li CY, Massicote HB, Moore LVH (1992) Nitrogen-fixing Bacillus sp. associated with Douglas-fir tuberculate ectomycorrhizae. Plant Soil 140:35–40
Linderman RG (1988) Mycorrhizal interactions with the rhizosphere microflora: the mycorhizosphere effect. Phytopathology 78:366–371
Linderman RG (1992) Vescicular-arbuscular mycorrhizae and soil microbial interactions. In: Bethlenfalvay GJ, Linderman RG (eds) Mycorrhizae in sustainable agriculture. ASA Spec Publ, Madison, Wisconsin, pp 45–70
Lo C-T, Nelson EB, Hayes CK, Harman GE (1998) Ecological studies of transformed *Trichoderma harzianum* strain 1295–22 in the rhizosphere and on the phylloplane of creeping bentgrass. Phytopathology 88:129–136
Marx DH (1972) Ectomycorrhiza as biological deterrents to pathogenic root infections. Ann Rev Phytopathol 10:429–454
Miller RM, Jastrow JD (1994) Vesicular-arbuscular mycorrhizae and biogeochemical cycling. In: Pfleger FL, Linderman RG (eds) Mycorrhizae and plant health. APS Press, St Paul, MN, pp 189–212
Mogge B, Loferer C, Agerer R, Hutzler P, Hartmann A (2000) Bacterial community structure and colonization patterns of *Fagus sylvatica* L. ectomycorrhizospheres as determined by florescence in situ hybridization and confocal laser scanning microscopy. Mycorrhiza 9:271–278
Nehl DB, Allen SJ, Brown JF (1996) Deleterious rhizosphere bacteria: an integrating perspective. Appl Soil Ecol 5:1–20
Nurmiaho-Lassila EL, Timonen S, Haahtela K, Sen R (1997) Bacteria colonisation patterns of intact *Pinus sylvestris* micorrhizospheres in dry pine forest soil: an electron microscopy study. Can J Microbiol 43:1017–1035
O'Neil GA, Chanway CP, Axelrood PE, Radley RA, Holl FB (1992) An assessment of spruce growth response speficity after inoculation with coexistent rhizosphere bacteria. Can J Bot 70:2347–2353

Paulitz TC, Linderman RG (1991) Lack of antagonism between the biocontrol agent *Gliocladium-virens* and vesicular arbuscular mycorrhizal fungi. New Phytol 117:303–308
Perrin R (1990) Interactions between mycorrhizae and diseases caused by soil-borne fungi. Soil Use Manag 6:189–195
Piccini D, Azcon R (1987) Effect of phosphate-solubilizing bacteria and vesicular arbuscular mycorrhizal (VAM) on the utilization of bayoran rock phosphate by alfalfa plants using a Sand-vermiculite medium. Plant Soil 101:45–50
Poole EJ, Bending GD, Whipps JM, Read DJ (2001) Bacteria associated with *Pinus sylvestris-Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation in vitro. New Phytol 151:741–753
Probanza A, Lucas JA, Acero N, Gutierrez Manero FJ (1996) The influence of native rhizobacteria on european alder (*Alnus Glutinosa* (l.) Gaertn.) growth. Plant Soil 182:59–66
Rasanayagam S, Jeffries P (1992) Production of acid is responsible for antibiosis by some ectomycorrhizal fungi. Mycol Res 96:971–976
Ray J, Bagyaraj DJ, Manjunath A (1981) Influence of soil inoculation with vesicular arbuscular mycorrhizal (VAM) and a phosphate dissolving bacteria on plant growth and 32P uptake. Soil Biol Biochem 13:105–108
Reddell P, Warren R (1986) Inoculation of Acacia with mycorrhizal fungi: potential benefits. In: Turnbull JW (ed) Australian *Acaia* in developing countries. ACIAR, Canberra, pp 50–53
Reddy MS, Kumar S, Babita K, Reddy MS (2002) Biosolubilization of poorly soluble rock phosphates by *Aspegillus tubingensis* and *A. niger*. Bioresource Technol 84:187–189
Requena N, Perez-Solis E, Azcon-Aguilar C, Jeffries P, Barea JM (2001) Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Appl Environ Microbiol 67:495–498
Rodriguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17:319–339
Rojas NS, Perry DA, Li CY, Ganio LM (2002) Interactions among soil biology, nutrition and performance of actinorhizal plant species in the HJ. Andrew's experimental forest of Oregon. Appl Soil Ecol 19:13–26
Rozycki H, Strzelczyk E, Raczkowska E, Li CY (1993) Effects of different carbon and nitrogen-sources and vitamins on growth of *Azospirillum* spp. isolated from coniferous ectomycorrhizae and sporocarps of ectomycorrhizal fungi. Acta Microbiol Pol 41:193–201
Rozycki H, Kampert M, Strzelezyk E, Li CY, Perry DA (1994) Effect on different soil bacteria on mycorrhizae formation in Scots pine (*pinus sylvestris* L.) in vitro studies. Folia For Pol 36:92–102
Rozycki H, Dahm H, Strzelczyk E, Li CY (1999) Diazotrophic bacteria in root-free soil and in the root zone of pine (*Pinus sylvestris* L.) and oak (*Quercus robur* L.). Appl Soil Ecol 12:239–250
Shishido M, Massicotte HB, Chanway CP (1996a) Effect of plant growth promoting *Bacillus* strains on pine and spruce seedling growth and mycorrhizal infection. Ann Bot 77:433–441
Shishido M, Peterson DL, Massicote HB, Chanway CP (1996b) Pine and spruce seedling growth and mycorrhizal infection after inoculation with plant growth promoting *Pseudomonas* strains. FEMS Microbiol Ecol 21:109–119
Sidorova II, Velikanov LL (2000) Bioactive substances of agaricoid basidiomycetes and their possible role in regulation of myco- and microbiota structure in soils of forest ecosystems. I. Antibiotic activity of water extracts from basidioms of of several dominant agaricoid basidiomycetes. Mikol Fitopatol 34:11–17
Slankis V (1974) Soil factors influencing formation of mycorrhizae. Ann Rev Phytopath 12:437–457

Smith DE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, London
Sturz AV, Nowak J (2000) Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. Appl Soil Ecol 15:183–190
Sun YP, Unestam T, Lucas SD, Johanson KJ, Kenne L, Finlay RD (1999) Exudation-reabsorption in mycorrhizal fungi, the dynamic interface for interaction with soil and other microorganisms. Mycorrhiza 9:137–144
Tian CJ, He XY, Zhong Y, Chen JK (2003) Effect of inoculation with ecto- and arbuscular mycorrhizae and *Rhizobium* on the growth and nitrogen fixation by black locust, *Robinia pseudoacacia*. New For 25:125–131
Timonen S, Jorgensen KS, Haahtela K, Sen R (1998) Bacterial community structure at defined locations of *Pinus sylvestris-Suillus bovinus* and *Pinus sylvestris-Paxillus involutus* mycorrhizospheres in dry pine forest humus and nursery peat. Can J Microbiol 44:499–513
Toal ME, Yeomans C, Killham K, Meharg AA (2000) A review of rhizosphere carbon flow modelling. Plant Soil 222:263–281
Toro M, Azcon R, Barea JM (1997) Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (32P) and nutrient cycling. Appl Environ Microbiol 63:4408–4412
Tsantrizos YS, Kope HH, Fortin JA, Ogilvie KK (1991) Antifungal antibiosis from *Pisolithus tinctorius*. Phytochemistry 30:1113–1118
Varese GC, Portinaro S, Trotta A, Scannerini S, Luppi-Mosca AM, Martinotti MG (1996) Bacteria associated with *Suillus grevillei* sporocraps and ectomycorrhizae in vitro growth of the mycobiont. Symbiosis 21:129–147
Vazquez MM, Cesar S, Azcon R, Barea JM (2000) Interaction between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Appl Soil Ecol 15:261–272
Volpin H, Kapulnik Y (1994) Interaction of *Azospirillum* with beneficial soil microorganisms. In: Okon Y (ed) *Azospirillum*/plant associations. CRC Press, Boca Raton, pp 111–118
Watteau F, Berthelin J (1991) Iron solubilization by mycorrhizal fungi producing siderophores. Symbiosis 9:59–67
Werner A, Zadworny M (2003) In vitro evidence of mycoparasitism of the ectomycorrhizal fungus *Laccaria laccata* against *Mucor hiemalis* in the rhizosphere of *Pinus sylvestris*. Mycorrhiza 13:41–47
Whipps JM (1997) Developments in the biological control of soil-borne plant pathogens. Adv Bot Res 26:1–134
Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Expt Bot (Root special issue) 52:487–511
Xue L, Charest PM, Jabaji-Hare SH (1998) Systematic induction of peroxidases, 1,3-b-glucanases, chitinases and resistance in bean plants by binucleate *Rhizoctonia* species. Phytopathol 88:359–365
Yadav A, Bhatt M, Dubey AR (2001) Characterization of mycorhizosphere bacteria isolated from deodar and oak seedlings from Kumaun Himalaya. J Indian Bot Soc 80:209–211
Yang CH, Crowley DE (2000) Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. Appl Environ Microbiol 66:345–351

14 Role of Beneficial Microsymbionts on the Plant Performance and Plant Fitness

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14.1 Introduction

The number of microbes in the rhizosphere of higher plants is much higher in comparison to bulk soil (Hiltner 1904). Due to this positive rhizosphere-effect, there are active and passive physical and chemical changes in the root system of the higher plants, which may have a great impact both on their nutrient status and their growth (Biró 2003). Among the microbes present in the rhizosphere, the microsymbiont bacteria and fungi are the most important for the plant growth and development. Their role and capacity for the biological nitrogen fixation and phosphorous mobilisation is quite established (Barea et al. 2002). The translocation of macro- and micronutrients in these zones is influenced by the enhanced microbial activities. The effects are mediated by direct transfer of nutrients from plant by the increased root system, and also by improving the competitiveness of higher plants in the nutrient uptake.

Among the beneficial microbes, the associative and symbiotic N_2-fixing bacteria and the arbuscular mycorrhizal fungi (AMF) are the most common in the rhizosphere of higher plants. Artificial seed and soil inoculation techniques are used as a simple application of a mixed nodule extracts, or as a soil-root mixture (Hiltner 1904). However, the introduced microbes usually enter in competition with the native microflora in the soil (Graham 1992). The negative effects of the abiotic environmental stress factors (temperature, drought, acidity etc.) are also common (Graham 1992; Bayoumi et al. 1995). The final influence of any microbial inoculation in the rhizosphere, therefore, is the result of the complex interactions between the plants, the rhizosphere inhabitants and the different microbial and environmental components involved (Postma et al. 1989). The antagonistic and synergistic behaviours, of the beneficial microsymbionts is a crucial step considering the plant growth and their sustainability (Höflich 1993).

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Beside the element-translocations, the effect of the microsymbionts may reduce the severity of various environmental stress-factors, like heavy metals or toxic elements, the drought, the salinity or alkalinity and the soil-compaction etc. Symbiotic microorganisms form the so-called "tripartite" associations, where the interaction with the macro-symbiont leguminous host-plant could be beneficial for several reasons.

14.2 Managing the Beneficial Effect of Microsymbionts

Due to the positive effect of several microorganisms on plant growth and development, the manipulation of the rhizosphere by the introduction of several microbial species and isolates is currently increasing. This is done by using seed or soil inoculation. Such introductions improve the survival rate and the later growth of the micropropagated plants, (Monticelli et al. 2000; Sharmila et al. 2000).

The seed are inoculated depending on the actual agricultural practice. For soil inoculation, carrier materials are used, which influences the survival rate of the introduced microbes.

14.2.1 Isolation and Selection of Beneficial Microsymbionts

The different inoculants (Table 14.1) have been isolated, identified and selected as test organisms depending on their efficiency (i.e. N_2-fixing or P-mobilising ability, sensitivity against the environmental stress-factors, or better colonisation ability than the indigenous microflora etc.).

Selection of these strains or isolates as potential inocula is done in vivo conditions. These are inoculated into a sterilised substrate with the appropriate hosts. After a few weeks of growth, the biomass production and the symbiotic efficiency are assessed. Strains with a high performance ability, which produce more than 50% higher yield, are employed as inoculants for further investigations (de Leij et al. 1992). Sometimes sterilised soils are used as possible substrates.

Inoculants can also be procured from a commercial culture collection (Biró et al. 2000a). Beneficial microbes can be both the associative and the symbiotic N_2-fixers (*Azospirillum brasilense* and *Rhizobium meliloti*) used in combination with a local isolate of arbuscular mycorrhizal fungus (*Glomus fasciculatum*). The beneficial effect of *Rhizobium* strains is evaluated in a preliminary seedling experiment with the macrosymbiont hosts (i.e. *Medicago sativa*, *Pisum sativum* etc.). Seeds are sterilised with

Table 14.1. Possible combinations and some of the references of the dual- (tripartite) or multilevel coinoculations of the bacterial and fungal beneficial microbes in the rhizosphere of the monocotyledonous (grasses) and dicotyledonous (legumes) plants

	Bacteria			Fungi			References
	AN_2[a]	SN_2[a]	PGPR[a]	AMF[a]	EMF[a]	TF[a]	
Dual inoculations (tripartite systems)		x		x			Biró et al. (1993a,b); Sanchez-Diaz et al. (1990)
	x			x			Solaiman and Hirata (1997); Paula et al. (1992); Puppi et al. (1994)
			x		x		Garbaye (1994); Duponnois and Garbaye (1991)
Multilevel coinoculations[b]	x	x		x			Biró et al. (2000b); Köves-Péchy et al. (1999)
	x			x		x	Other potential possibilities
		x	x	x			Khan et al. (1997); Zaidi et al. (2003); Young et al. (1990)
			x	x		x	Other potential possibilities
			x		x	x	Other potential possibilities

[a] AN_2: Associative nitrogen fixing bacteria, SN_2: Symbiotic nitrogen fixing bacteria, PGPR: plant growth promoting rhizobacteria (*Pseudomonas* sp.), AMF: arbuscular mycorrhizal fungi, EMF: ectomycorrhizal fungi, TF: *Trichoderma* fungi

[b] Three microbial partners inoculated, potential possibilities

3% Chloramine T for 3 min, washed five times with sterilized tap water, and then germinated on Thorton agar. Depending on age and plant-size of the macrosymbionts, 1–5 ml of the *Rhizobium* inoculum is used (Vincent 1970). The microbial titre in these experiments is generally 10^8 CFU ml^{-1} doses, which is the "steady state" liquid suspension of a certain strain after 24 h of growth in liquid culture at 28 °C. In case of N_2-fixing bacterial inoculations, the nodule-number and/or the acetylene reduction activity (ARA) is measured by the method of Hardy et al. (1973). At least eight weeks of growth is necessary to assess the nodulation in the seedlings. After these procedures, the most effective *Rhizobium* strains are selected for further inoculations or for in situ applications. For inoculation of arbuscular mycorrhizal fungi, generally 3% of root-soil mixture previously inhabited by a *Glomus* or other AMF strains can be inoculated just below the seed-layer of the test plant. Several authors, however, used a much higher inoculum dose, such as 5 or 10% (Borchers and Perry 1987) or the increase of the spore density in the inoculum from 10 to 60 g^{-1} on a dry soil basis (Mukerji and Jagpal 1987).

14.2.2
Microcosm Experiments for the Inoculation Studies

It is always necessary to take pre-sterilised soil before sowing of macrosymbiont (test plants) hosts for inoculation of selected inoculants. The substrate should not contain any usual rhizosphere components (i.e. bacteria or fungi) including plant pathogens or symbionts (Postma et al. 1989; Bayoumi et al. 1999). After inoculation of the inoculum in the sterilised substrates a successful reconstruction of the original rhizosphere microflora can be observed in the full grown plant. Soil sterilisation can be done by Gamma irradiation as by this process the existing microbes in soil are killed but there is no effect on the element content and available metal fraction (Table 14.2).

Interactions are constantly going on between the root of higher plants and microbes including micro-symbionts and pathogens in the rhizosphere under normal soil conditions. The establishment of microflora and colonisation of roots by symbionts can be easily studied in microcosm experiments. The different types of microbes around the root can be effectively separated by sieving technique. The performance of introduced strains (inoculants) can be assessed by this method into fungal and bacterial

Table 14.2. Physical- and chemical characteristics of a calcareous chernozem soil originating from a natural, grass-type ecosystem (Erd, Hungary) before (C) and after (G) the gamma irradiation (15,000 J Co kg^{-1} soil)

Soil characteristics	Original (C)	Gamma-sterilised (G)
Soil texture (plasticity)[a]	39.0	39.0
Organic matter (g kg^{-1})	28.7	29.0
CEC (cmol kg^{-1})	16.0	16.4
$pH_{(H_2O)}$	7.5	7.5
Macronutrients (mg kg^{-1})		
P_2O_5	70.0	70.0
K_2O	208.0	182.0
NH_4	40.0	40.0
$NO_3 + NO_2$	20.0	20.0
Micronutrients (mg kg^{-1})		
Zn	6.3	6.5
Fe	20.5	20.9
Mg	98.2	99.0
Cu	4.9	4.8
S	44.6	45.0
Ca %	6.1	6.2

[a] See Buzas (1998)

components of rhizosphere microflora. The most widely used methodology is the development of a 'three substrate system', where three different potting mixtures are used for the plant performance analysis (Biró et al. 1999). The three different substrates used has the following features:

1. C – unsterilised soil, containing normal rhizosphere microflora in original, non-disturbed soil and serves as the control (C) for comparison purposes in the inoculation experiments.
2. G – gamma irradiated (15–20 k Gy Co kg^{-1}) sterilised soil where no microbes are present in the potting mixture. The native indigenous microflora has been eliminated. The soil physical and chemical however, remain unaltered (Table 14.2).
3. GB – gamma irradiated sterilised soil reinoculated with fungi free rhizosphere constituents that is only containing bacteria. To this is added soil suspension of microbes without indigenous AM fungi. For removing the propagules (spores and fragmented hyphae) of the indigenous AM fungi 80 ml pot^{-1} of a soil water suspension 2:1 v/v is passed through a Jena G5 (MN-5 μm pore-size) paper. Suspension prepared this way is used to reintroduce the natural native microbial community without indigenous AM fungi.

By using these three substrates effect of inoculated microsymbiont can be compared with the native microbes under different soil conditions (Biró et al. 1999). The inoculation of such strains might result in a stimulative effect on the growth, especially in sterilised substrates-G, where competitive native microflora has been eliminated. It is also possible to assess the effect of the bacterial component (using GB substrate) only, i.e. the "helper" microbes of the mycorrhizal fungi.

By using these substrates it is also possible to assess the effectivity of several microbial strains (inoculants) introduced separately or in combination in the potting substrates. There are several reports of assessing the effect of dual, tripartite or multiple inoculations of *Rhizobium*+ single or multiple species in Leguminous plants and AM fungi (Table 14.1).

14.2.3 Micropropagated Plants and Microsymbiont Inoculations

The effect of beneficial microsymbionts is being frequently assessed in micropropagated systems. Micro-propagation is a widely used method for mass production of several plant species, but it is especially important for the clonal multiplication of some fruit rootstocks and varieties. One of the most difficult problems during the procedure is the weaning stage,

when plants are transplanted from sterile to semi-sterile glasshouse conditions. Stress of the transplantation can be the reason of serious losses (Sharmila et al. 2000). Inoculation, therefore, with the selected beneficial microsymbionts may improve the survival rate and growth vigour of the plantlets (Nowak 1998). Fortuna et al. (1992) reported a two-week acclimation period for the micro-propagated and AMF inoculated plum rootstock in comparison to non AMF inoculated ones. The survival rate and growth of some micro-propagated trees has been reported (Balla et al. 1998). For this process healthy shoot-tips of the GF 677 peach rootstocks are collected. Actively growing 15–20 mm long tips are disinfected before in vitro establishment on vitamin- and auxin-supplied MS medium. In the later phases for the shoot elongation, the MS medium is diluted to one quarter by omitting the Cl^- ions and by the addition of 6-benzylaminopurine and adenin hemisulfate (0.75 mg l^{-1}) at cool and dark conditions. The rooted plantlets are further grown at high relative humidity under glasshouse conditions. Types and soil physical-chemical characteristics of the substrates (i.e. the humus content, compaction, available nutrients) are crucial for the appropriate acclimation processes.

14.3 Assessment of Plant Performance and Plant Fitness

Inoculation of beneficial microbes to the higher plants for better growth and establishment is now a common practice in agriculture for environmental protection (Burke et al. 2002). In tripartite systems, where the leguminous higher plants are inoculated with microsymbiont fungi and bacteria it is necessary to evaluate interaction between the macro- and the microsymbionts. For such studies it is always good to use sterilised substrates for comparable studies.

14.3.1 Plant Performance Parameters

In the inoculation trials, there is a need for frequent monitoring of the colonisation features of the introduced strains. Different methods are used to assess the abundance of microsymbionts during the plant growth and development.

For symbiotic N_2-fixers, the total nitrogen content and the nodule number are generally assessed to estimate their efficiency. N_2-free semisolid Nfb medium is used for the most probable number (MPN) counts of the associative *Azospirillum* bacteria (Okon et al. 1977). Specific selective media are used for the assessment of the culturable microbial populations.

Assessment of Rhizosphere Colonisation

Arbuscular Mycorrhizal Fungi

For estimating the root-colonisation by AM fungi a particular sub-sample of 1 g fresh lateral roots are randomly taken and cut for approximately 1 cm segments. They are cleared and stained with acid glycerol trypan blue (Phillips and Hayman 1970), and mounted on a microscopic slide for estimating the AMF colonisations (30 segments in three replicates). The frequency and intensity of the mycorrhizal infection (F%, M%) and the arbuscule content of the colonised roots (a%) are recorded and calculated (Trouvelot et al. 1985), using a five class system for the estimations.

The effects of mycorrhizal and bacterial treatments on the element uptake, the root-colonisation of AM fungi and other data regarding the dry matter production are tested by the analysis of variance (two-way ANOVA). Comparison of means can be possible by the calculating least significant differences (LSD = $P < 0.05$).

N_2-Fixing Bacteria

Both the total nitrogen content and the nodule number are assessed to estimate the efficiency of the rhizobium N_2-fixers. N_2-free semisolid Nfb medium was used for the MPN counts of the *Azospirillum* (Okon et al. 1977). Washed and cleared, 1 g root-sample is taken from each treatment and after smashing with sterilised quartz-sand a dilution series is prepared to inoculate the MPN tubes (10 ml of suitable semisolid media in three replicates). Growth records (development of the white, subsurface pellicle) are recorded after two days of incubation at 33 °C. Parallel root samples of 1 g (wet weight) are measured and placed in an oven at 70 °C to obtain the root moisture percentage.

Assessment of Plant Biomass Parameters

The effects of different biotic and abiotic factors on the host-plants are frequently studied for assessment of biomass-production beside the fresh-weight, the dry weight of the roots and shoots of the plants are measured, in pot-experiments. The pots are generally thinned for the same number of plants, after the emergence of seedlings. The dry matter measurements are required, to study effect of heavy metals. Depending on the types and doses of the metals, the water content of the plants may change considerably. After the growth periods, the fresh biomass of the plants is dried to a constant weight at 70 °C in an oven before the grinding and the element analysis.

Besides the root and shoot biomass, the nodulation potential of the plants is noted in relation to the nodule number or the nodule dry weight in the root systems. The first and the secondary infection capacity can be established, when the nodules on the main- and the lateral roots are sepa-

rately calculated. Due to the occurrence of large, "multi-fingered" nodules with small ones, the dry weight of the nodule biomass is more accurate parameter for the assessment of inoculation success. Long-term effect of the heavy metals, are known to delay the emergence of the first nodules and also the development of the AMF symbiosis by two to six days, depending on the metals and their concentrations.

In leguminous fodder plants, the dry weight of the shoots is calculated after the three cuttings (at the 6th, 9th and 12th weeks) of the fresh biomass production in the pot-experiments. Root biomass is measured after three months growth in the light chamber under controlled conditions at 22 °C during the day and 18 °C during the night, with an 18-h photo-period (photosynthetic photon flux density of 600 μmol m^{-2} s^{-1}, using metal halide lamps) and a relative humidity of 70–80%.

Analysis of the Plant Nutritional Status

Chemical Analysis

After recording the soil physical characteristics (Buzás 1998), the total element contents of soil-mixtures or substrates or the plant biomass samples are measured. Substrates are digested with conc. HNO_3 at 80 °C in a microwave oven. The plant element contents (Ca, Mg, K, P and S) are assessed after a wet digestion of the air-dried and ground plant samples with HNO_3^+ H_2O_2. For the measurement an inductively-coupled plasma atomic emission spectrometer (for instance ICP-AES, type FY-238) is used. Total N content in the shoot biomass is estimated by a modified Kjeldahl method after wet digestion with conc. $H_2SO_4 + H_2O_2$, measured by a "dead-stop" method.

14.3.2
Plant Fitness Parameters

Chlorophyll Fluorescence Techniques

Beneficial microsymbionts have multiple effects on the physiology of the plant at different stages. The bioenergetics of the photosynthetic system is noted by the analysis of the fast fluorescence kinetics O-J-I-P (Strasser et al. 1995). The analysis is based on a simple model and the 'Theory of energy fluxes in biomembranes', which provides a quantification of the behaviour and performance of the photosynthetic apparatus. In this system, the Chl *a* fluorescence transient can be recorded both in vivo and in situ by the analysis of the JIP test, which quantifies the photosystem II (PSII) behaviour.

The analysis of the fluorescence transient O-J-I-P by the JIP-test is also used to see the effects of mycorrhization on the behaviour and performance of the photosynthetic apparatus (Romano et al. 1996; Köves-Péchy et al.

1999; Calantzis et al. 2000). These studies demonstrate the beneficial role of the mycorrhizal symbiosis on the photosystem II activity by increased values of various activities and/or performance parameters.

The OJIP Test

The rise in fluorescence during the first second of illumination by oxygenic photosynthetic material, shows a sequence of phases from the initial (F_0) to the maximal (F_M) fluorescence. These phases have been labelled as O,J,I,P (Strasser and Govindjee 1992; Strasser et al. 1995). The shape of the OJIP-transient changes in different environmental conditions, such as light intensity (Tsimilli-Michael et al. 1995, 1996; Srivastava and Strasser 1996, 1997; Krüger et al. 1997), temperature and drought (Van Rensberg et al. 1996), or chemical influences. O-J-I-P transients are analysed using JIP-test (Strasser and Strasser 1995), which leads to quantification of the PSII behaviour (Strasser et al. 1996, 1999; Krüger et al. 1997; Tsimilli-Michael et al. 1998).

Chl *a* fluorescence transients of the intact leaves of a higher plant are measured by a PEA fluorimeter (Plant Efficiency Analyser, built by Hansatech Instruments Ltd. King's Lynn Norfolk, PE 30 4NE, GB) and recorded up to 1 s, with a data acquisition every 10 µs for the first 2 ms and every 1 ms thereafter, and 12-bit resolution (Strasser et al. 1999). The fluorescence transients are induced by a red light (peak at 650 nm) of 600 W m^{-2} by an array of six light-emitting diodes. Three leaves of each plant (15 per pot) are measured and the average fluorescence transients are used for the JIP-test analysis.

A typical Chl *a* fluorescence transient O-J-I-P is shown in Fig. 14.1, plotted on a logarithmic time scale so that the intermediate steps are clearly seen. Each transient is analysed by JIP-test (Strasser and Strasser 1995; Krüger et al. 1997; Strasser et al. 1999), using the original data (Fig. 14.1): the maximal measured fluorescence intensity, F_P, is equal here to F_M since the excitation intensity is high enough to ensure the closure of all reaction centres (RCs) of PSII; the fluorescence intensity at 50 µs is taken as the intensity F_0 when all RCs are open; the fluorescence intensity at 300 µs, required for the calculation of the initial slope $M_0 = (dV/dt)_0 \cong (\Delta V/\Delta t)_0$ (Fig. 14.1, Table 14.3); the fluorescence intensities at 2 ms (J step) denoted as F_J, and at 30 ms (I-step) denoted as F_I; the complementary area above the fluorescence induction curve (i.e. the area between the fluorescence transient and the line $F = F_M$); the time t_{Fmax} to reach F_M.

For the quantification of PSII behaviour the use of some formulae given in Table 14.3 are used. The biophysical parameters, started at time zero (onset of fluorescence induction), are recorded as:

1. The specific energy fluxes (per reaction centre) for absorption (ABS/RC), trapping (TR_0/RC), dissipation (DI_0/RC) and electron transport (ET_0/RC).

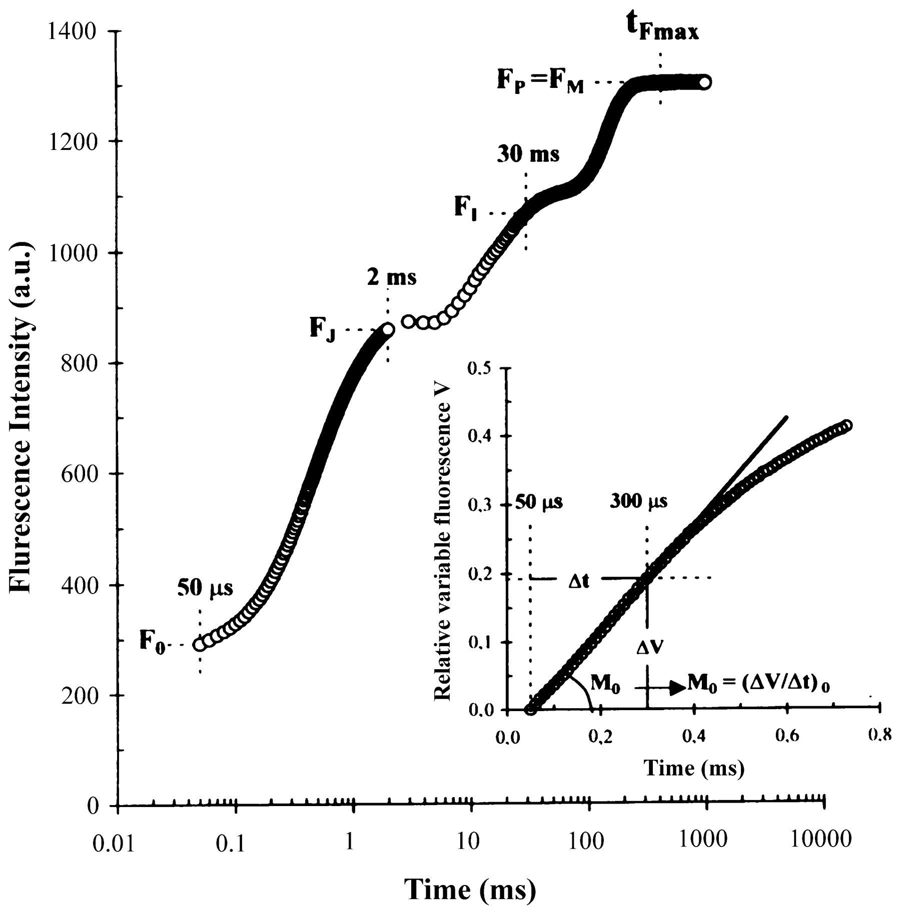


Fig. 14.1. A typical Chl *a* fluorescence transient O-J-I-P, plotted on a logarithmic scale from 50 ms to 1 s, with marked fluorescence intensities at selected times, utilised in the JIP-test: the fluorescence intensity F_0 (at 50 ms); the maximal fluorescence intensity, $F_P = F_M$ (at t_{Fmax}). The *insert* shows the relative variable fluorescence on a linear time scale, from 50 ms to 1 ms, demonstrating how the initial slope is calculated: $M_0 = (dV/dt)_0 = M_0 = (dV/dt)_0 \cong (\Delta V/\Delta t)0 = (V_{300ms})/(250\ \mu s)$

2. The flux ratios or yields, i.e. the maximum quantum yield of primary photochemistry ($\varphi_{Po} = TR_0/ABS$), the efficiency ($\psi_0 = ET_0/TR_0$) with which a trapped excitation can move an electron into the electron transport chain further than Q_A^-, and the quantum yield of electron transport ($\varphi_{Eo} = ET_0/ABS = \varphi_{Po} \cdot \psi_0$); the phenomenological energy fluxes (per excited cross section, CS_M) for absorption (ABS/CS_M), trapping (TR_0/CS_M), dissipation (DI_0/CS_M) and electron transport (ET_0/CS_M). The amount of active PSII reaction centres per excited cross section (RC/CS_M) can be

Table 14.3. A summary of the formulae for the calculation of the plant physiological parameters, such as the specific fluxes, the phenomenological fluxes and yields from the fluorescence data, according to the JIP-test

Specific fluxes	Phenomenological fluxes
$ABS/RC = (M_0/V_J)[1-(F_0/F_M)]$	$ABS/CS_M \approx F_M$
$TR_0/RC = (M_0/V_J)$	$TR_0/CS_M \approx F_M \times [1-(F_0/F_M)]$
$DI_0/RC = (ABS/RC)-(TR_0/RC)$	$DI_0/CS_M \approx (ABS/CS_M)-(TR_0/CS_M)$
$ET_0/RC = (M_0/V_J) \times (1-V_J)$	$ET_0/CS_M \approx F_M \times [1-(F_0/F_M)] \times (1-V_J)$
	Density of reaction centres
	$RC/CS_M \approx F_M \times [1-(F_0/F_M)]/(M_0/V_J)$
Yields	Yields as ratios of fluxes
$\phi_{Po} = [1-(F_0/F_M)]$	$\phi_{Po} = (TR_0/RC)/(ABS/RC)$
$\phi_{Eo} = [1-(F_0/F_M)] \times (1-V_J)$	$\phi_{Eo} = (ET_0/RC)/(ABS/RC)$
$\psi = (1-V_J)$	$\psi_0 = (ET_0/RC)/(TR_0/RC)$

where, $V_J = (F_J - F_0)/(F_M - F_0)$ and $M_0 = 4.\ (F_{300\,\mu s} - F_0)/(F_M - F_0)$

calculated. (Note: subscript "M" in CS_M indicates that the maximal fluorescence intensity was used as a measure of absorption per cross section: $ABS/CS_M \approx F_M$).

14.3.3 Interpretation of Data

Mean data on the effects of mycorrhizal and bacterial inoculation on the element uptake, the root-colonisation of AM fungi and data regarding the dry matter production are tested by the analysis of variance (two-way ANOVA). Correlation regression analysis of the nodulation data and the ARA measurements are done by using the Statgraphics 5.0 program.

Means of data for the colony forming units of the important beneficial microbes, such as the N_2-fixers, the phosphorus mobilising mycorrhiza or perhaps *Trichoderma* sp. can be distinguished by the analysis of variance (ANOVA). The input data of the MPN counts of *Azospirillum* is calculated using McGrady tables (Postgate 1969) and transformed as the logarithmic values. The colonisation values of AM fungi, i.e. the frequency (F%), the intensity (M%) or increase in the arbuscule numbers (a%) in the colonised roots are used as separate values. Comparison of mean data is possible by using the calculated least significant differences ($LSD = P < 0.05$). Relationships between the different parameters, i.e. the mycorrhizal colonisation values with other parameters, are used for correlation-regression analysis. In case of *Trichoderma*, the density and diversity of population or the abundance of a particular species is considered (Naár et al. 2000).

The difference between the two types of coefficients can also be calculated (correlation minus partial correlation coefficient) effects of heavy metal pollution, drought, salinity etc. if the values are above 0.5. The complex influence of a certain beneficial microbial group on the colonisation by a microsymbiont can be assessed with a multiple regression analysis. The selection of the contributing parameters is possible to test by a multiple partial correlation analysis (MPCA) by Statgraphics 5.0 statistical program (Naár et al 2000).

14.4 Plant Performance and Fitness by Microsymbiont Co-Inoculations

Many of the microbes in the soils interact specifically with the plant root systems and this soil-plant interface develops the specific microcosm, the rhizosphere. The microsymbionts (i.e. the mycorrhizal fungi and N_2-fixing bacteria) are generally most important as they help the plant in the nutrient supply to their co-symbionts.

The contribution of the Arbuscular mycorrhizal fungi (AMF) and the associative or obligate N_2-fixing microsymbionts (*Azospirillum*- and *Rhizobium*) to the soil fertility, productivity and to the crop yield is well-documented (Biró et al. 1993a; Bethlenfalvay and Schüepp 1994; Jeffries and Dodd 1991). They are also frequently used therefore to evaluate the functioning of the various soil-plant ecosystems, especially under nutrient unbalanced conditions (Barea et al. 1983). The arbuscular mycorrhizal fungi improve the uptake of water (Sanchez-Diaz et al. 1990; Tobar et al. 1994), phosphorus and other macro- and microelements in non-optimal situations (Pacovsky et al. 1985). Rhizosphere-mycorrhizosphere systems, therefore, can be tailored to help plants to establish and survive in nutrient-deficient, degraded habitats or during the stress periods (Sanchez-Diaz et al. 1990).

Seed- or soil inoculations for the above-mentioned reasons are common agricultural practices. The success of these technologies is highly dependent on the effectively and infectivity of the indigenous microbes and on the interactions between the main participants, such as the mycorrhizal fungi in the rhizosphere (Lindermann 1983; Puppi et al. 1994). Compatible combinations of the inoculated microbes, such as the symbiotic N_2-fixing rhizobium bacteria and the AM fungi, may result, therefore, in an enhanced effect on the plant development (Biró et al. 1993b; Paula et al. 1992) in various legumes. Positive influence of the associative *Azospirillum* diazotrophs on the AM fungal activity is also reported (Garbaye 1994), especially on the monocotyledonous host plants. In contrast differences were not found

in total dry weight in another *Glomus+Azospirillum* tripartite system (Pacovsky 1988).

Spores and the hyphosphere of the AM fungi may harbour the so-called "helper bacteria", such as *Burkholderia* or the associative diazotrophs (Minerdi et al. 1999), which can result in the so-called beneficial effects in various soil-plant ecosystems.

14.4.1
Establishment of Inoculated Microsymbionts in the Rhizosphere

In the rhizosphere many types of microbe-microbe interactions take place and these are highly dependent on the root exudation patterns of a certain soil-plant systems. The exudation of a specific host changes during the vegetation periods and is influenced by several biotic and abiotic environmental factors resulting in increase or decrease of the microsymbionts in the rhizosphere (Bansal and Mukerji 1994). In case of the *Azospirillum* associative N_2-fixers, the wheat germ agglutinin (WGA) acts as an attractive chemotactic factor in the rhizosphere (Antonyuk and Ignatov 2001). Not only the growth, but also the IAA production, the dinitrogen-fixation, the ammonium excretion and the protein synthesis (both old and new in the cell walls) also increase (Sadovnikova et al. 2003).

The diazotroph bacteria *Azospirillum* sp. are mainly dominant in the rhizosphere of monocots (and not the leguminous plants) and so are employed as inocula for grass-type hosts (Pacovsky 1988; Belimov et al. 1999) and not for legumes.

The introduced AM fungi and the N_2-fixing bacteria *Rhizobium* are used for the inoculation of leguminous hosts only in tripartite systems (Biró et al. 2000b). The association of microbes in the rhizosphere of monocots and legumes depends on the variability of the root-exudates etc.

When phosphorus-mobilising microbes like *Agrobacterium* or *Flavobacterium* sp. are inoculated independently in wheat or maize roots they are not established but if later AM fungi are also inoculated they are established. This supports the theory that physiologically the bacteria are better functional in the mycorrhizosphere (Biró 1992a,b).

Rhizosphere Components and Environmental Stress

The colonisation of the rhizosphere is highly influenced by the different biotic and abiotic factors in the rhizosphere. Under stress conditions the severity of the stressors is the most crucial. There are differences between the impact of the short- and long-term effects on the microbial abundance and their functioning in the rhizosphere (Table 14.4).

Table 14.4. Abundance of some countable microbial groups in the rhizosphere of clover (*Trifolium pratense* L.) at mycorrhizated (M) and non-mycorrhizated (NM) situations in case of the different doses of Zn, Ni and Cd salts (as sulphates). Heavy metal contaminated soil-samples were originating from the long-term field experiment of the RISSAC, where 13 heavy metals were spiked into the calcareous chernozem soil seven years prior the pot-experiment

Microbes		Control	Zn (kg ha^{-1})			Ni (kg ha^{-1})			Cd (kg ha^{-1})		
			90	270	810	90	270	810	90	270	810
Heterotrophs ($\times 10^6$)	M	8.2	4.3	2.9	9.0	5.0	8.0	4.1	4.3	1.3	1.1
	NM	9.5	7.5	4.8	5.0	3.9	6.1	4.3	3.6	1.7	1.0
P-mobilizers ($\times 10^6$)	M	10.3	9.1	6.3	10.0	4.6	3.0	5.4	5.1	1.3	2.0
	NM	2.7	2.1	5.9	1.9	2.8	1.2	2.3	6.5	3.0	1.9
Pseudomonads ($\times 10^4$)	M	2.3	3.1	5.0	1.2	1.6	1.1	0.2	3.2	2.2	0
	NM	4.8	2.9	7.3	5.8	1.5	1.9	1.3	4.1	3.0	0

More data from the experiment at Kádár (1995); Vörös et al. (1998)

For *Rhizobium* the long-term effect of the permanent sewage sludge application may result in the loss of the N_2-fixing function and also their abundance in the soil-legumes systems (Biró et al. 1999). After 15 years land-deposition of municipal or industrial sewage sludge (at 100 or 300 kg ha^{-1} rates) the natural, effective nodulation of the *Rhizobium leguminosarum* bv. *phaseoli* bacteria was reduced with the diversity of the *Rhizobium* population (Table 14.5). In such lands artificial inoculation of *Rhizobium* becomes necessary.

Table 14.5. Abundance and functioning of the *Rhizobium leguminosarum* bv. *phaseoli* and their diversity index (as calculated from the plasmid profile groups) in old arable and woodland soil after a 15 years of sewage sludge application (in 100-, or 300 kg ha^{-1} $year^{-1}$ doses)

Type and quantity of sewage sludge (kg ha^{-1} $year^{-1}$)		*Rhizobium* abundance ($\log_{10} g^{-1}$)	N-fixing ability	First nodules (days)	Diversity index	
					Old arable soil	Woodland soil
Control		5.204a	+++	12.6a	1.27ab	3.10c
100	Municipal	4.602b	+++	13.6b	1.37ab	1.60b
	Industrial	4.157b	++	13.2ab	2.73c	1.53b
300	Municipal	2.478c	+	16.3c	1.82b	1.03b
	Industrial	0.196d	–	18.0d	0.72a	0.26a

In Biró et al. (1999) the same letters in a column are not significantly different at P = 0.05% level

Rhizosphere Colonisation and Micro-Propagation

The inoculation with AM fungi can retard the initial growth of the higher plants if its physiological status is not sufficient enough to supply the fungi with the required nutrients.

The total nutrient status of the substrates and its actual availability, is influenced by several soil physical-, chemical- and climatic factors, and is important to develop a beneficial association between soil-plant root (Balla et al. 2003). Therefore microsymbiont co-inoculation is an important requirement for the micro-propagated plants, to adapt to the stress environmental conditions during the acclimation process. It is a essential requirement for the development of an optimal physiological- or energetic condition for the plantlets preceding the introduced symbiosis by the AM fungal strains (Harris et al. 1989).

Efforts were made earlier in the past to improve the soil properties and soil fertility by various soil-amendments, with organic manures and sewage sludge and the inorganic fertilisers (Noyd et al. 1996; Vörös et al. 1995). These additions enhance the pH, the nitrate- and ammonium availability or the cation exchange capacity providing much better conditions for the hosts and their micro-symbionts. Colonisation of AM fungi is much higher at the initial stages of growth of micro-propagated plantlets in nutrient poor conditions (Vestberg and Estaun 1994; Vestberg et al. 2002). Low phosphorus availability enhances the symbiosis function with the AM fungi (Harvey and Smith 1983).

AM symbiosis is dependent on nutrient availability (Biró et al. 1993a). In recultivated dump spoils a positive correlation exists between the amount of humus accumulation in the soils and colonisation of AM fungi (F%). Humus accumulation helps in nutrient availability, stabilisation and the quality of soil aggregates (Bouwman 1989). This AMF colonisation however, dose not depend on the types and abundance of mycorrhizal spores in the dump spoils (Biró et al. 1993a; Diaz and Honrubia 1993; Vörös et al. 1995). The number of spores in the rhizosphere is highly variable in the multifactorial soil-plant systems (Landwehr et al. 2002). Besides the type of higher plants and the vegetation period, the soil compaction (Biró et al. 1993b), the water content (Zak and Parkinson 1982) and the soil acidity or cation exchange capacity (Ho 1987) influence the spore number in the rhizosphere.

In the earlier stages of symbiosis the root structure is constantly changing and requires high energy-inputs for the macro-symbionts. At this stage the AM fungi may even become a parasitic partner without the development of the arbuscule. This also happens at very poor light intensity and early vegetation period of the barley (Zolnikova, personal communication; Scullion 1992).

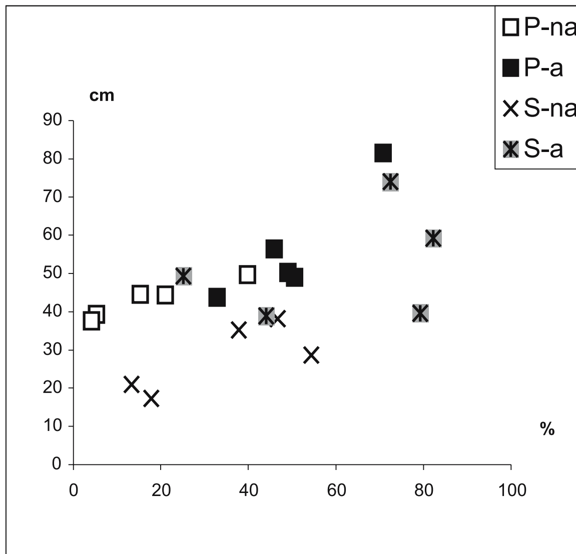


Fig. 14.2. Results of the correlation regression analysis between the plant height (cm) of one-year-old micropropagated peach and the arbuscule richness (a%) of the roots, when inoculated with several arbuscular mycorrhizal fungal (AMF) strains. Two types of inoculation methods and two potting substrates were used. P – nutrient-rich "Pindstrup" substrate; S – nutrient-poor loamy chernosem soil, érd, Hungary; na – mycorrhizal inoculation of the non-acclimatized plantlets, a – mycorrhizal inoculation of the acclimatized peach rootstocks

Best time of AMF-infection, is highly influenced by the growth activity of the host, which depends on the genotype or its genetic potential (Azcon-Aguilar and Ocampo 1981). In artificial systems, the growth substrates with real soil content is more effective to support beneficial symbiosis, due to the initial requirements of low nutrients (Vestberg and Estaun 1994; Estaun et al. 1994; Balla et al. 2003).

A better growth and mycorrhizal colonisation status of the peach (GF 77) root-stocks was observed when the micro-propagated plantlets were inoculated with AMF after and not before the weaning stage. Figure 14.2 shows better mycorrhization and growth in one-year-old peach plantlets (length in cm) and arbuscules formed (%).

14.4.2 Performance of Microsymbiont Inoculated Plants

Dry Weight of the Plant

Inoculated indigenous beneficial microsymbionts exert a positive influence on the performance and fitness of the higher plants. This can be shown by increase in plants fresh weight which depends on the water status of

Table 14.6. Shoot dry weight (Sh), root dry weight (Rt) and the total dry matter production (Sh+Rt) of alfalfa (*Medicago sativa L.*). Plants were grown for three months in the original-(C), in the gamma-sterilized-(G) or in the AM-free-resuspended (GB) chernozem soil and affected by single-, dual- or multilevel microbial treatments. Dry matter production was collected at the 6th, 9th and 12th weeks during the vegetation period, which is summarised in this table (g DW pot^{-1}). Each value is the mean of three pots

	Substrates and dry matter yield (g pot^{-1})								
	C			G			GB		
Strains[a]	Sh	Rt	Sh+Rt	Sh	Rt	Sh+Rt	Sh	Rt	Sh+Rt
Control	1.56a	1.72a	3.28a	0.97a	1.04a	2.35a	1.66a	1.80a	3.46a
M	1.72b	2.51b	4.23b	2.33b	2.84b	5.17b	2.49c	2.33b	4.82b
MR	1.60a	1.59a	3.19a	2.07b	3.93b	5.25c	2.27b	2.94b	5.21c
MS	1.71b	2.10a	3.81a	2.01b	2.71b	4.72b	2.10b	2.76b	4.86b
MRS	1.59a	2.38a	3.97a	2.50b	3.38b	5.87c	2.04b	3.29c	5.33c

[a]M = mycorrhiza (*Glomus fasciculatum* M107), R = rhizobium (*Rhizobium meliloti* S 5/7+Lu-41+K 4/1), S = spirillum (*Azospirillum brasilense* Km5) or their combinations, respectively

the soil/substrate where test plants grow. Dry weight of the root/shoot of the plant is also an important criterion for indicating plant growth. This is significant in relation to effects of heavy metals which controls the water balance of plants. Dry matter of the plants is enhanced due to interaction with microsymbiont fungi or bacteria. Visual observation of the inoculated plant also indicates development by showing changes in colour, height and robustness of the plant. In situ performance can also be adjudged by remote sensing in arable agricultural fields.

In the microcosm, experiments using single-, dual (tripartite) or multiple inoculations were carried out with alfalfa at various soil conditions (Biró et al. 2000a). Inoculations were made singly with *Azospirillum* or *Rhizobium* (S and R, respectively), dual (MR, MS) or multiple (MRS) combinations with the arbuscular mycorrhizal fungi (M) which increase the dry weight of the host. Better results are found using sterilised or AM free substrates (Table 14.6).

Resuspension of the gamma-sterilised soils by the AM-free soil extract has resulted in the reconstruction of the microflora in the rhizosphere of the untreated plants and substrates. The same dry matter yield could be found, therefore at those treatments in the control and the reincubated substrates.

Nutritional Status of the Inoculated Plants

Plants inoculated with microsymbionts show distinct improvement in its micro- and macroelement content (Balla et al. 2003); when AM fungus

Table 14.7. Macronutrients (N, P, K) in the shoot biomass of alfalfa in a pot experiment. Plants were grown in a non-sterilised calcareous chernosem soil (C = all microbes present), in a gamma sterilised soil (G = no microbes) and in the sterilised substrate, which was re-suspended with a mycorrhiza-free soil extract (GB = no mycorrhiza). Effect of the tripartite (MR, MS, RS) and multilevel (MRS) co-inoculations of bacterial and fungal micro-symbionts. M = *Glomus fasciculatum* M 107, R = *Rhizobium meliloti* S 5/7+Lu 41+K 4/1, S = *Azospirillum brasilense* Km 5 (strain collection of the RISSAC, Budapest)

Strain Comb.	Substrates and macro-elements								
		C			G			GB	
	N	P	K	N	P	K	N	P	K
Contr.	39.8a	2.6b	27.7a	18.1a	2.2a	17.8a	37.1a	2.5a	29.7a
M	38.2a	2.0a	24.9a	73.2c	4.4b	35.0b	65.5c	4.1b	36.8b
MR	39.1a	2.5ab	28.7a	56.2b	4.0b	32.8b	55.2b	3.8b	33.4ab
MS	49.6b	2.4ab	27.5a	51.3b	4.0b	30.5b	61.6c	4.2b	34.4ab
MRS	50.7b	2.2ab	26.2a	71.4c	5.0c	37.0b	64.2c	4.2b	34.4ab

Values denoted by the same letter are not significantly different at $P_{5\%}$ level

Glomus fasciulatum strain G 107 was inoculated in alfalfa plants growing on sterilised substrate there was distinct increase in N, P, K content in shoots of alfalfa (Table 14.7).

In unsterilised soil condition, N_2 fixation was better after *Rhizobium* inoculations in combinations of diazotrophs and AM fungi. Nitrogen content of soil was higher when *Azospirillum* was coinoculated with mycorrhizal fungi (Table 14.7).

Uptake of P and K increased after *Rhizobium* was inoculated/singly or in dual inoculation with *Azospirillum*. No such positive effect was observed with mycorrhizal partner (Table 14.7). In green-pea the macroelement uptake was enhanced when AM fungus and *Rhizobium* were inoculated together. However, P uptake was only slightly effected (Table 14.8).

There are, however, reports that there is not always an increase in macroelements uptake after inoculation with microsymbionts; sometimes there is decrease in metal uptake of the plants. The reduction in uptake of elements after symbiosis may be due to the root-adsorption or the dilution effect of the higher biomass production. However, sometimes enhanced heavy metal uptake is reported in some hyper-accumulator plants which are metal tolerant varieties. The increased heavy metal uptake is possible when the microsymbiont is a sensitive variety or has got less favourable physiological properties (less extraradical mycelia, excess arbuscules formed etc.). At the long-term toxic metal stress, the symbiosis may develop in non-mycotrofic plants like *Viola* sp. and *Thlapsi coerulea* (Landwehr et al. 2002). Concomitant with the metal uptake, other elements, such as P, N, Fe and Mn, also increase, which are favourable for the growth of plants (Posta

Table 14.8. Shoot dry-weight of green-pea and macroelement (N, P, K) content according to the microbial status of the calcareous loamy chernozem soil (c = control, without microbial inoculation, M = inoculated with a *Glomus* sp. strain, M+R = inoculated with the *Glomus* and the *Rhizobium leguminosarum* nitrogen-fixing strain)

Soil status		SW (g pot^{-1})	N (%)	P (%)	K (%)
Control	c	0.15	3.41	0.357	2.37
	M	0.24	3.70[a]	0.398[a]	2.62[a]
	M+R	0.22	4.08[a]	0.375	2.62[a]
Sterilised	c	0.21	3.52	0.336	2.01
	M	0.25	3.65	0.342	2.09
	M+R	0.34[a]	3.79[a]	0.339	2.47[a]

[a]Significant increase (at $P = 0.05\%$ level), further details at Kádár et al. (2001)

et al. 1994; Vivas et al. 2003a,b,c). Symbiosis with arbuscular mycorrhizal fungi therefore is able to protect the macrosymbiont from the phytotoxic effects.

The development of symbiosis between the metal-adapted microsymbionts and the higher plants is therefore beneficial, whether they increase or decrease the element-translocations in case of excess amounts of heavy metals or toxic elements. Such interactions may be crucial for the plant performance and plant fitness under environmental stress conditions, such as presence of heavy metals, drought and salinity, etc.

Microbial isolates like *Trichoderma* sp. with different metal-tolerant abilities (to Cd, Zn and Ni) may affect the metal uptake. Beside the abiotic stress-factors, there is a great impact of the biotic factors as well in the element-translocation, the detection and the augmentation of the environment.

The amount of colonisation by rhizobia is indicated by nodule formation and abundance of arbuscules in the inoculated roots (Vörös et al. 1998; Morvai et al. 1999; Kádár et al. 2001; Mikanova et al. 2001; Rajkai et al. 2002).

The macro-element uptake in green pea plants is related to the microbes in the rhizosphere of normal/control and gamma sterilised soils (Table 14.8).

Influence of the Macro- and Microsymbiont Compatibility

Now the increasing importance of the use of beneficial microbes as microbial inocula is widely realised not only for sustainable agriculture, but also for environmental protection. The use of microsymbionts is especially active, due to the fact that they can have a great impact on the soil-plant-animal-human food chain. The development of artificial symbiosis is in-

fluenced by several environmental and biotic factors. The constituents of the rhizosphere and their interactions is a important act for the element translocations (Kádár 1995).

The rhizosphere microflora is constantly changing with in the ecosystem. The microbial population of the rhizosphere (especially the endo-symbiont N_2-fixing microbes and the AM fungi) improves the nutrient-supply and the adaptation of the higher plants. The introduced microbes further enhances the growth of the plants (Kádár 1995).

Inoculation production of AM fungi requires the use of appropriate host-plants, which are sensitive enough to develop the symbiosis with the compatible partner, demonstrating evolutionary and phylogenetic relationships (Trappe 1987). Larger amounts of infective propagules (spores, intra- or extraradical hyphae, root segments, etc.) are required for better colonisation of AM fungi. In field experiments, however, the use of a mixed inoculum is suggested for better colonisation than a single AMF spore inoculum as this has a higher potential and is more effective than the indigenous microflora.

The success of inoculation can be evaluated by using different parameters like the rate of fungal colonisation and also the plant yield, i.e. dry weight, height of shoots, length of roots, etc. Beside these, several other parameters are also taken, such as the nutrient content, photosynthetic activity, carbon-balance studies, etc. A higher colonisation is obtained by using AM fungal strains originating from the same soil or sometimes from the rhizosphere of the same hosts (Balla et al. 1998; Biró et al. 1998).

14.4.3 Plant Physiological Parameters

The chlorophyll-fluorescence technique, particularly the OJIP test, is used to assess plant physiological status under various environmental conditions (Strasser and Govindjee 1992, Strasser and Strasser 1995). This method can be carried out quickly, that is in less than few seconds, and can be easily applied in field conditions for rapid screening of many samples (Table 14.3).

Three independent parameters, i.e. RC/ABS, φ_{Po} and ψ_o, are used to calculate the responses of PSII (Tsimilli-Michael et al. 1998, 2000). This represents an index combining functional and structural criteria, and is therefore taken as structure-function-index (SFI). Beside this the parameters favouring photosynthesis are indicated as SFI_{Po} and parameters related to dissipation (non-photosynthesis) are denoted as SFI_{No}:

$$\left(Chl_{RC}/Chl_{tot}\right) \cdot \varphi_{Po} \cdot \psi_o = SFI_{Po(ABS)} \qquad (14.1)$$

$$\left[1 - \left(Chl_{RC}/Chl_{tot}\right)\right] \cdot \left(1 - \varphi_{Po}\right) \ \left(1 - \psi_o\right) = SFI_{No(ABS)} \qquad (14.2)$$

where Chl_{tot} refers to the total chlorophyll *a* concentration ($Chl_{tot} = Chl_{antenna} + Chl_{RC}$). The so-called performance index, denoted as PI_{Po}, is defined as the ratio of the two structure-function-indexes.

The ratio $(Chl_{RC})/(Chl_{antenna})$ can be replaced by the ratio RC/ABS which can be calculated via the JIP-test equations. The performance index is then denoted as $PI_{ABS,Po}$ (or PI_{ABS} for simplicity, where the subscript "ABS" specifies the RCs' density, which is expressed per absorption):

$$PI_{ABS} = \frac{SFI_{Po}}{SFI_{No}} = \frac{RC}{ABS} \cdot \frac{\varphi_{Po}}{1-\varphi_{Po}} \cdot \frac{\psi_o}{1-\psi_o} \tag{14.3}$$

Substituting the biophysical by the experimental parameters (see Table 14.1) PI_{ABS} can be calculated as follows:

$$PI_{ABS} = \frac{1-(F_0/F_M)}{M_0/V_J} \cdot \frac{F_M - F_0}{F_0} \cdot \frac{1-V_J}{V_J} \tag{14.4}$$

The PI_{CS} can also be used, which can be explained on a cross section basis (CS_M):

$$PI_{CS} = \frac{RC}{CS_M} \cdot \frac{\varphi_{Po}}{1-\varphi_{Po}} \cdot \frac{\psi_o}{1-\psi_o} = \frac{ABS}{CS_M} \cdot PI_{ABS} \tag{14.5}$$

and basing on experimental parameters:

$$PI_{CS} \approx F_M \cdot PI_{ABS} = F_M \cdot \frac{1-(F_0/F_M)}{M_0/V_J} \cdot \frac{F_M - F_0}{F_0} \cdot \frac{1-V_J}{V_J} \tag{14.6}$$

The performance indexes $[p_i/(1-p_i)]$ are products where the several p_i stand for probabilities or fractions. Such expressions are well-known in chemistry, where p_i represents the fraction of the reduced and $(1-p_i)$ the fraction of the oxidised form of a compound, in which case $\log[p_i/(1-p_i)]$ expresses the potential or driving force for the corresponding oxido-reduction reaction (Nernst's equation). The log(PI) can be defined as the total driving force (DF) for photosynthesis of the observed system, created by summing up the partial driving forces for each of the several energy bifurcations (all at the onset of the fluorescence rise O-J-I-P). The $\log(PI_{ABS})$ and $\log(PI_{CS})$ give, respectively, the DF_{ABS} and DF_{CS}:

$$DF_{ABS} = \log(PI_{ABS}) = \log\left(\frac{RC}{ABS}\right) + \log\left(\frac{\varphi_{Po}}{1-\varphi_{Po}}\right) + \log\left(\frac{\psi_o}{1-\psi_o}\right) \tag{14.7}$$

$$DF_{CS} = \log\left(PI_{CS}\right) = \log\left(\frac{ABS}{CS_M}\right) + \log\left(\frac{RC}{ABS}\right) + \log\left(\frac{\varphi_{Po}}{1-\varphi_{Po}}\right) + \log\left(\frac{\psi_o}{1-\psi_o}\right) = \log\left(\frac{ABS}{CS_M}\right) + DF_{ABS} \quad (14.8)$$

Synergistic Interactions in the Rhizosphere

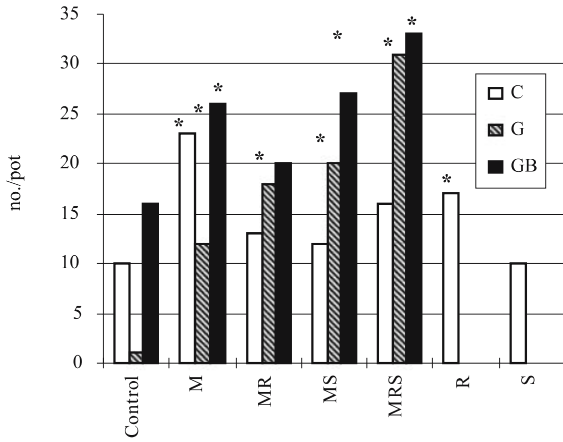
In microbial coinoculations, interaction occurs between the introduced microbes, the macrosymbionts and also among the microbial partners in the soil. When the beneficial microbes are preselected in vitro for certain stress tolerance abilities, the multiple coinoculations of such partners can result in enhanced beneficial effects, in comparison to single, separated inoculation. Such enhancement is due to additive effect or synergism, that means the use of several partners have a great advantage in the agriculture and for the environmental protection.

Figure 14.3 shows the synergistic effects that can be realised by arbuscule richness (*a*%) in mycorrhiza (AMF) inoculated roots, when all of the introduced microsymbionts were present in the root systems in the gamma-sterilised substrates. In the non-sterilised soil, however, significantly the lowest colonisation of AM fungi was observed after mycorrhiza and *Azospirillum* coinoculations. This highlights the significance of indigenous microbial populations in the rhizosphere, which may reduce the activity of the introduced strains, if they are not efficient enough.

The N_2-fixers and the AM fungi applied as dual-, tripartite- or multiple inocula may result in a synergistic effect on the growth and the survival of the host-plants under different environmental stress situations (Barea et al. 2002; Várallyay 2001). In agricultural field conditions or in technogenous areas, like surface coal-mining sites, artificial development of symbiosis enhances the plant-growth and development (Biró et al. 1993b, 2000a).

Beneficial rhizobacteria and fungi improves the uptake of macro- and microelements when nutrient supply is poor (Biró 2003). In such areas the success of the symbiosis formation is enhanced by the addition of some “starter” low quantity fertilizer doses (mainly nitrogen).

▶ **Fig. 14.3.** Nodule number, arbusculum richness and abundance of the associative diazotrophs on the alfalfa roots after three months of growth in a calcareous loamy chernozem soil (C), in gamma-sterilised soil (G) and in the sterilised substrate, which was resuspended with mycorrhiza free soil extract (GB). The effect of the single- (M, R, S), dual- (MR, MS – tripartite) and multiple (MRS) coinoculations were noted after three months of growth in a pot experiment under controlled conditions. M: vesicular arbuscular mycorrhizal fungi (*G. fasciculatum* M 107), R: *Rhizobium meliloti* strain-mixture, S: *Azospirillum brasilense* Km 5; and their combinations, respectively. Note: the synergistic effects of the MRS coinoculations are demonstrated at each parameter, investigated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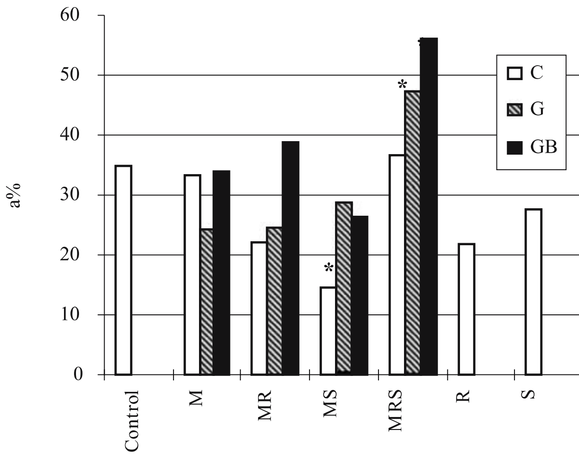
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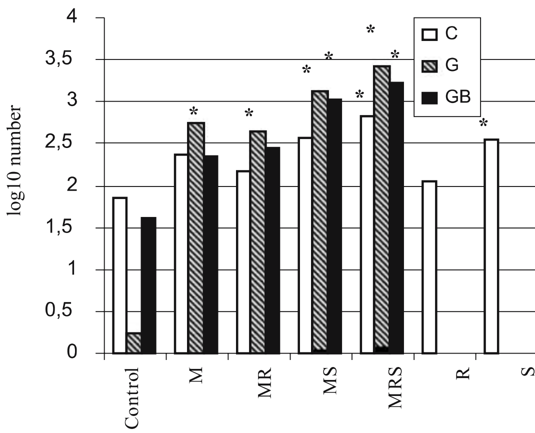
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Analysis of the rhizosphere microflora is a important criteria after inoculation and the plant growth.

When AM fungus *Glomus fasciculatum* strain M107 was inoculated singly or in combination with *Rhizobium meliloti* strains, resulted a multiple beneficial effect on the growth and yield of clover (Biró et al. 2000a). Growth further improved with triple inoculation with AM fungi and N_2 fixers *Azospirillum* and *Rhizobium* (Fig. 14.3). The data is generally noted by most probable numbers (MPN) of microbes in the rhizosphere of inoculated plants.

Azospirillum number increases in the rhizosphere with single- or dual mycorrhizal coinoculations with the addition of "helper" bacteria especially in sterile or AM-free soils (Fig. 14.3).

A further synergistic effect on the plant physiological parameters is shown in Fig. 14.4, where coinoculation of the beneficial microbes resulted in a physiological stress-buffer effect on the growth of alfalfa.

The specific electron transport (ET_0) per reaction centre (RC), i.e. the ET_0/RC value, remains nearly constant for all samples (Fig. 14.4). However, electron transport (per active cross section CS) ET_0/CS increases in the presence of mycorrhiza and helper bacteria. This increase parallels the increase in the density of reaction centres per cross section (RC/CS). The turnover number (N_T) indicates how many times Q_A gets reduced, oxidized and again reduced until all RCs are closed and F_M is reached. Therefore, N_T becomes a measure of the activity of the photosynthetic metabolism. N_T increases in the presence of mycorrhizal strain both in the control or sterile, as well as with the bacteria supplemented soil conditions (Köves-Péchy et al. 1999).

In multiple inoculations of *Azospirillum, Rhizobium* and AM fungi, *Azospirillum* sp. produces plant growth regulating (PGR) hormones, *Rhizobium* sp. fixes N_2, and AM fungi has the phosphorus solubilising ability. In combination they have synergistic or additive effect and supply nutrient and growth factors to host plant resulting in better plant growth and development.

Antagonistic Interactions in the Rhizosphere of Inoculated Plants

Introduced strains in the rhizosphere of higher plants must be competitive enough to withstand the antagonistic interactions with the particular components of autochtonous microflora. The effectivity and the best competitive ability is a prerequisite, when the beneficial microbes are introduced to the environmentally stressed ecosystems. Here the use of stress-tolerant microbes are more efficient (Vivas et al. 2003a,b,c).

The dual or multiple inoculations of the beneficial microbes increases their efficiency in the soil plant ecosystems. The JIP-test of the chlorophyll

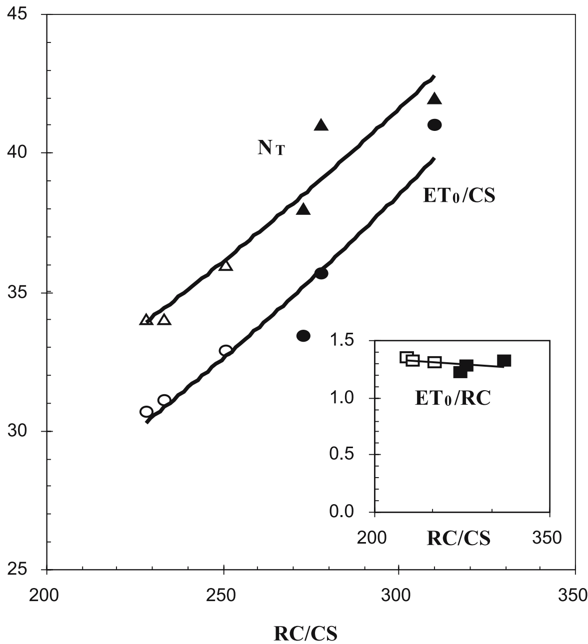

Fig. 14.4. Effect of the arbuscular mycorrhizal fungi (AMF) and the obligate or associative diazotroph bacteria (*Rhizobium* and *Azospirillum*) inoculation on some physiological parameters on the alfalfa plants after three months of growth in a pot experiment. Beside the electron transport per reaction centre (ET_0/RC), the electron transport per active cross sections (ET_0/CS) are also shown in case of the mycorrhiza and/or the bacteria co-inoculations. *Open symbols* indicates the control, the gamma sterilised or the bacterial reincubated conditions (*from left to right*), and *closed symbols* for the mycorrhiza inoculated treatments. The turnover number (N_T) indicates how many times Q_A gets reduced, oxidized and again reduced until all reaction centres (RC) are closed and the maximum yield (F_M) is reached. N_T in such a way becomes a measure of the activity of the photo-synthetic metabolism. Note: N_T increases in the presence of arbuscular mycorrhiza fungi both in control or sterile, as well as with bacteria reincubated soil

fluorescence technique is the method used to study the impact of such introduced beneficial microsymbionts in the rhizosphere (Strasser et al. 1996; Krüger et al. 1997).

The success and the failure of *Azospirillum* and AM fungal coinoculations depends greatly on the physiological status of the hosts, the time of the infections or the nutrient demands of the microsymbiont partners. This provides such plants with stress-tolerant ability.

Influence of microsymbiont inoculation is shown on the energy fluxes by means of the energy pipeline models of the photosynthetic apparatus (Strasser 1997; Strasser et al. 1996; Krüger et al. 1997).

In case of bacterial and fungal microsymbionts special functional relationships may exist in the rhizosphere of the higher plants (Paula et al. 1992).

Single inoculation of AM fungi in gamma-sterilised soil, results in an increased stress-tolerant ability of the test plants. It is further enhanced when the mycorrhizal strain is coinoculated with *Rhizobium* and even more enhanced by the coinoculation with both, *Rhizobium and Azospirillum* diazotrophs (Fig. 14.5).

In contrast, single inoculation of AM fungi results in an increase of the electron transport per leaf area (ABS/CS), and the leaf chlorophyll content. The mycorrhiza-induced increase of ABS/CS is completely com-

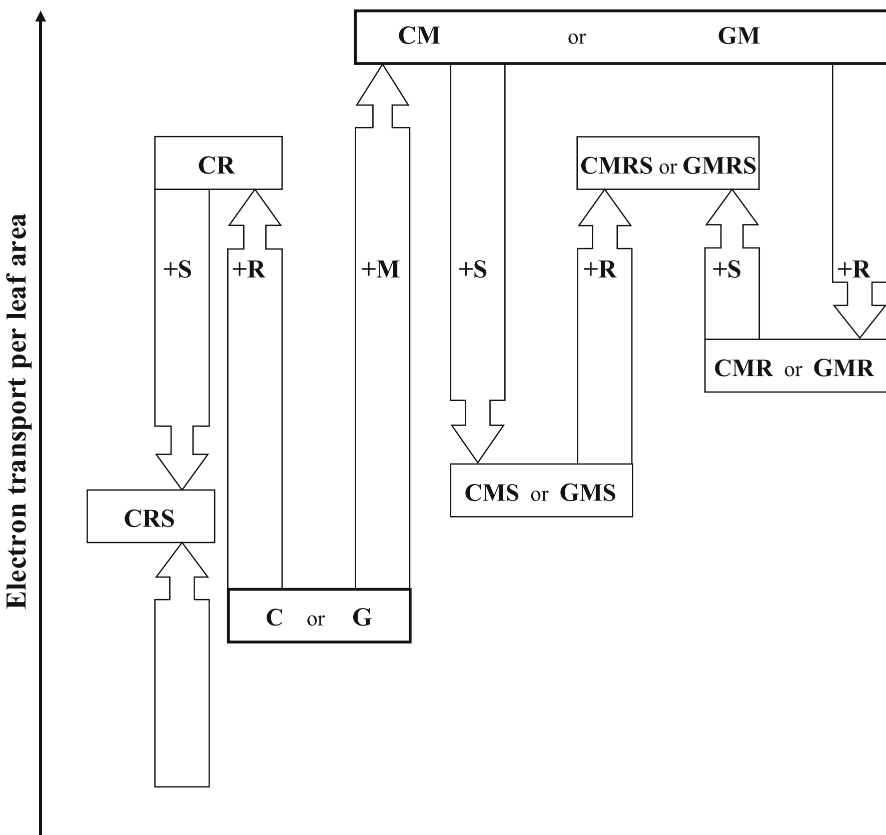

Fig. 14.5. Changes in the electron transport per leaf area of alfalfa, induced by inoculation with arbuscular mycorrhizal fungi (M) without and with *Azospirillum* (S) and/or *Rhizobium* (R) as diazotroph bacteria in control soil carrying all the native microflora (C) or gamma-sterilised soil (G), as well as by inoculation only with S and/or R in control soil. The electron transport values are normalised on the value exhibited with the G soil with no inoculation

pensated in the presence of the diazotrophs, both for the dual (mycorrhiza+*Azospirillum* or mycorrhiza+*Rhizobium*) and the tripartite (mycorrhiza+both diazotrophs) co-inoculations.

The growth of the plants was better when singly inoculated with AMF, while either *Rhizobium* or *Azospirillum* as coinoculants exhibited an antagonistic effect, though only partially compensating the effect of AMF. However, the two diazotrophs appear antagonistic to one another with respect to their antagonism to the mycorrhiza, but the multiple coinoculation resulted in a higher energetic status.

From Fig. 14.5, it is clear that the antagonistic effects due to changes in the electron transport activity per leaf area are the net results of the synergistic and the antagonistic effects of the symbiotic and associative diazotrophs, which regulate differently the PSII behaviour at different levels. These results demonstrated that the different combinations of AMF and diazotroph bacteria inoculations can be well distinguished by means of the JIP test parameters. They thus suggest that the JIP test, which is very helpful in stress studies, can be used to screen in the field and in a quick and non-invasive way through the PSII behaviour of the target plants, the effect and interactions of the microbial activities in their rhizosphere.

14.5 Conclusions

The paper is intended to show the beneficial interactions in the rhizosphere of higher plants, which help to cope with the abiotic environmental stress-factors in the various soil-plant systems. The beneficial microbes, such as the arbuscular mycorrhizal fungi (AMF), associative (*Azospirillum*) and symbiotic (*Rhizobium*) bacteria may result in a stress-buffer effect and a synergistic interaction in the plant growth and development. Beside the biomass measurements, those effects were assessed by an in situ chlorophyll-fluorescence OJIP test. The use of such microorganisms, as introduced microbial inocula is being highlighted in the study.

References

Antonyuk LP, Ignatov VV (2001) The role of wheat germ agglutinin in plant-bacteria interactions: a hypothesis and the evidence in its support. Russ J Plant Physiol 48:364–369

Azcón-Aguilar C, Ocampo JA (1981) Factors affecting the vesicular-arbuscular infection and mycorrhizal dependency of thirteen wheat cultivars. New Phytol 87:677–685

Balla I, Vértesy J, Biró B, Vörös I (1998) Survival and growth of micro-propagated peach, inoculated with endo-mycorrhizal strains. Acta Hortic 477:115–118

Balla I, Vértesy J, Végvári G, Szûcs E, Kállay T, Vörös I, Biró B (2003) Nutrition of the micropropagated fruit trees *in vitro* and *ex vitro*. Int J Hortic Sci 9:43–46

Bansal M, Mukerji KG (1994) Positive correlation between AMF-induced changes in root exudation and mycorrhizosphere mycoflora. Mycorrhiza 5:39–44

Barea JM, Bonis AF, Olivares J (1983) Interactions between *Azospirillum* and VA mycorrhiza and their effects on growth and nutrition of maize and ryegrass. Soil Biol Biochem 15:706–709

Barea JM, Azcón R, Azcón-Aguilar C (2002) Mycorrhizosphere interactions to improve plant fittness and soil quality. Antonie van Loeuwenhoek 81:343–351

Bayoumi HEAF, Biró B, Balázsy S, Kecskés M (1995) *Rhizobium* and *Bradyrhizobium* strains affected by inhibitory environmental factors. Acta Microbiol Immunol Hung 42:61–69

Bayoumi HEAF, Abdorhim H, Khalif A, Godwar H, Ködöböcz L, Jevcsák I, Biró B, Kecskés M (1999) Soil physical factors affecting the survival of *Rhizobium leguminosarum* bv *viceae* populations in vivo. Sci Bull North Univ Baia Mare C 13:8–28

Belimov AA, Serebrennikova NV, Stepanok VV (1999) Interaction of associative bacteria and endomycorrhizal fungus with barley upon dual inoculation. Microbiology 68:104–108

Bethlenfalvay GJ, Schüepp H (1994) Arbuscular mycorrhizas and agrosystem stability. In: Gianinazzi S, Schüepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhauser, Berlin, pp 117–131

Biró B (1992a) N_2-fixing, plant-growth promoting *Azospirillum* bacteria – a review (in Hungarian). Agrokem Talajtan 39:363–367

Biró B (1992b) Possibility of *Azospirillum* plant inoculations – a review (in Hungarian). Agrokem Talajtan 41:390–399

Biró B (2003) Soil- and rhizobiological tools in the service of sustainable agriculture and environmental protection. Acta Agronom Hung 50:77–85

Biró B, Köves-Péchy K, Szili-Kovács T, Szegi J (1993a) Effect of fertilizer on spontaneous *Rhizobium* infection in Hungarian soils. In: Szabolcs I (ed) Soil resilience and sustainable land use. Agrokem Talajtan 42:207–212

Biró B, Vörös I, Köves-Péchy K, Szegi J (1993b) Symbiont effect of *Rhizobium* bacteria and AM fungi on *Pisum sativum* in recultivated mine spoils. Geomicrobiol J 11:275–284

Biró B, Magyar K, Várady G, Kecskés M (1998) Specific replant diseases reduced by PGPR rhizobacteria on apple seedlings. Acta Hortic 477:75–81

Biró B, Köves-Péchy K, Vörös I, Kádár I (1999) Intensification of nodulation and nitrogen-fixing activity preceding the "loss of function" by the long-term application of some toxic metal rates. In: Wenzel WW et al. (ed) Proc 5th International Conference on the Biogeochemistry of Trace Elements, pp 178–179

Biró B, Vörös I, Takács T, Köves-Péchy K (2000a) Three-substrate method to monitor microbial competetiveness of inoculated microsymbionts. In: International COST Action 8.30 Workshop on "Methods to monitor microbial inoculants to improve their success" (Abstracts), p 8

Biró B, Köves-Péchy K, Vörös I, Takács T, Eggenberg P, Strasser RJ (2000b) Interrelation between *Azospirillum* and *Rhizobium* nitrogen-fixers and arbuscular mycorrhizal fungi in the rhizosphere of alfalfa at sterile, AMF-free or normal soil conditions. J Appl Soil Ecol 15:159–168

Borchers JG, Perry DA (1987) Mycorrhizal inoculum potential in site fractions of Southwest Oregon forest soils. In: Sylvia DM, Hung LL, Graham JH (eds) Mycorrhizae in the next decade. IFAS, Univ Florida, pp 188–189

Bouwman AF (1989) Modelling soil organic matter decomposition and rainfall erosion in two tropical soils after forest clearing for permanent agriculture. Land Degrad Rehab 1:125–140

Burke D, Hamerlynck E, Hahn D (2002) Effect of AM fungi on soil microbial populations and associated plant performance of the saltmarsh grass *Spartina patens.* Plant Soil 239:141–154

Buzás I (ed) (1998) Handbook of soil and agricultural practical studies. Vol. 1: Soil physical and chemical characteristics (in Hungarian). Mezõgazd Kiadó, Budapest

Calantzis C, Trouvelot S, van Tuinen D, Gianinazzi-Pearson V, Gianinazzi S, Strasser RJ (2000) A non destructive method for evaluating the mycorrhizal status of Micropropagated vine rootstock. In: Mycorrhiza and root research in Switzerland, Bulletin SGPW/SSA 14:8

de Leij FAAM, Kerry BR, Denneby JA (1992) The effect of fungal application rate and nematoda density on the effectiveness of *Verticillium chlamydosporium* as a biological control agent of *Meloidogyne incognita.* Nematology 38:112–122

Diaz G, Honrubia I (1993) Infectivity of mine soils from Southeast Spain. II. Mycorrhizal population levels. Mycorrhiza 4:85–88

Duponnois R, Garbaye J (1991) Mycorrhization helper bacteria associated with the Douglas fir-*Laccaria laccata* symbiosis. Ann Sci For 48:239–251

Estaun V, Calvet C, Camprubi A (1994) Arbuscular mycorrhizae and growth enhancement of micropropagated Prunus rootstock in different soilless potting mixes. Agric Sci Fin 3:17–26

Fortuna P, Citernesi S, Morini S, Giovanetti M, Loreti F (1992) Infectivity and effectiveness of different species of AM fungi in micropropagated plants of Mr S 2/5 plum rootstock. Agronomie 12:825–829

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

Graham PH (1992) Stress tolerance in *Rhizobium* and *Bradyrhizobium* and nodulation under adverse soil conditions. Can J Microbiol 38:475–484

Hardy RWF, Bums RC, Hosten RD (1973) Application of the acetylene – ethylene assay for measurement of nitrogen fixation. Soil Biol Biochem 5:47–81

Harris JA, Birch P, Short KC (1989) Changes in the microbial community and physico-chemical characteristics of topsoils stockpiled during opencast mining. Soil Use Manag 5:161–168

Harvey JL, Smith SE (1983) Mycorrhizal symbiosis. Academic Press, London

Hiltner L (1904) Zur frage der stickstoffernahrung in Pflanzen. Landw Vers Stat 51:92–97

Ho I (1987) Vesicular-arbuscular mycorrhizae of halophytic grasses in the alvord desert of Oregon. Northwest Sci 61:148–151

Höflich G (1993) Effect of variety and environmental factors on the photo-effectivity of bacterial inoculations in peas. Zentralbl Mikrobiol 148:315–324

Jeffries P, Dodd CJ (1991) The use of mycorrhizal inoculants in forestry and agriculture. In: Arora DK, Rai B, Mukerji KG, Knudsen GR (eds) Handbook of applied mycology. Vol. 1. Soil and plants. Marcel Dekker, New York, pp 77–129

Kádár I (1995): Effect of microelement loadings on the soil-plant-animal-human food chaine (in Hungarian) Akaprint, Budapest

Kádár I, Köves-Péchy K, Vörös I, Biró B (2001) Effect of microelement loadings on the pea at carbonaceous chernozem soil. II. Microsymbiont colonisation and heavy metal uptake. Agrokém Talajt 50:83–101

Khan MS, Zaidi A, Amil M (1997) Associative effect of *Bradyrhizobium* sp. (vigna) and phosphate solubilizing bacteria on mungbean (*Vigna radiata* L Wilczek). Biojournal 9:101–106

Köves-Péchy K, Biró B, Vörös I, Takács T, Osztoics E, Strasser RJ (1999) Enhanced activity of microsymbiont-host systems probed by the OJIP test. In: Garab G (ed) Photosynthesis. Kluwer Academic, Dordrecht, vol 4, pp 2765–2770

Krüger GHJ, Tsimilli-Michael M, Strasser RJ (1997) Light stress provokes plastic and elastic modifications in structure and function of photosystem II in camellia leaves. Physiol Plant 101:265–277

Landwehr M, Hildebrandt U, Wilde P, Nawrath K, Tóth T, Biró B, Bothe H (2002) The arbuscular mycorrhizal fungus *Glomus geospoum* in European saline, sodic and gypsum soils. Mycorrhiza 12:199–211

Lindermann RG (1983) Mycorrhizal interactions with the rhizosphere microflora: the mycorhizosphere effect. Phytopathology 78:366–371

Mikanova O, Kubát J, Mikhailovskaya N, Vörös I, Biró B (2001) Influence of heavy metal pollution on some soil-biological parameters in the alluvium of the Litavka river. Rostlynna Vyroba 47:117–122

Minerdi D, Fani R, Bonfante P (1999) Nitrogen fixation genes in a *Burkholderia*, living in symbiosis with an arbuscular mycorrhizal fungus. In: Sixth Symposium on Bacterial Genetics and Ecology, Florence, Italy, (Abstract), p 80

Monticelli S, Puppi G, Damiano C (2000) Effects of in vivo mycorrhization on Micropropagated fruit tree rootstocks. Appl Soil Ecol 15:105–111

Morvai B, Kádár I, Bujtás K, Biró B (1999) Heavy metal and sewage sludge research in progress. In: Proc. 13th National Conference on Environmental Protection, Siófok, Hungary, pp 192–196

Mukerji KG, Jagpal R (1987) VAM in reforestation in Indian waste lands. In: Sylvia DM, Hung LL, Graham JH (eds) Mycorrhizae in the next decade. Practical applications and research priorities. IFAS, Univ. Florida, pp 161–162

Naár Z, Román F, Füzy A (2000): Correlations between indigenous mycoparasitic and symbiotic beneficial fungi at heavy metal stress. Agrokém Talajtan 51:115–123

Nowak J (1998) Benefits of *in vitro* "biotization" of plant tissue cultures with microbial inoculants. In Vitro Cell Dev Biol Plant 34:122–130

Noyd RK, Pfleger FL, Norland MC (1996) Field responses to added organic matter, arbuscular mycorrhizal fungi, and fertilizer in reclamation of taconite iron ore tailing. Plant Soil 179:89–97

Okon Y, Albrecht SL, Burris RH (1977) Methods for growing *Spirillum lipoferum* and for couting it in pure culture and in association with plants. Appl Environ Microbiol 33:85–88

Pacovsky RS (1988) Influence of inoculation with *Azospirillum brasilense* and *Glomus fasciculatum* on sorghum nutrition. Plant Soil 110:283–287

Pacovsky RS, Fuller G, Paul EA (1985) Influence of soil on the interactions between endomycorrhizae and *Azospirillum* on sorghum. Soil Biol Biochem 17:521–531

Paula MA, Urquiaga S, Siqueira JO, Döbereiner J (1992) Synergistic effects of vesicular-mycorrhizal fungi and diazotrophic bacteria on nutrition and growth of sweet potato (*Ipomea batatas*). Biol Fert Soils 14:61–66

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and VAM fungi for rapid assessment of infection. Trans Brit Mycol Soc 55:158–161

Posta K, Marschner H, Römheld V (1994) Manganese reduction in the rhizosphere of mycorrhizal and non-mycorrhizal maize. Mycorrhiza 5:119–124

Postgate JR (1969) Viable counts and viability. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 1. Academic Press, New York, pp 611–618

Postma J, Hok-A-Hin H, Oude Voshaar JH (1989) Influence of the inoculum density on the growth and survival of *Rhizobium leguminosarum* biovar *trifolii* introduced into sterile and non-sterile loamy sand and silt loam. FEMS Microbiol Ecol 73:49–58

Puppi G, Azcón R, Höflich G (1994) Management of positive interactions of arbuscular mycorrhizal fungi with essential groups of soil microorganisms. In: Gianinazzi S, Schüepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhäuser-Verlag, Basel, pp 201–215

Rajkai K, Végh KR, Nacsa T (2002) Electrical capacitance as the indicator of root size and activity. Agrokém Talajt 51:89–98

Romano A, Strasser RJ, Eggenberg P, Martins-Loucao MA (1996) Mycorrhization of cork oak micropropagated plantlets: its vitality during acclimation measured with fast fluorescence techniques. In: Azcon-Aquilar C, Barea JM (eds) European Commission – EUR 16728, Luxemburg, pp 373–376

Sadovnikova YN, Bespalova LA, Antonyuk LP (2003) Wheat germ agglutinin is a growth factor for the bacterium *Azospirillum brasilense*. Dokl Biochem Biophys 389:103–105

Sanchez-Diaz M, Pardo M, Antolin M, Pena J, Aguirreola J (1990) Effect of water stress on photosynthetic activity in the *Medicago-Rhizobium-Glomus* symbiosis. Plant Sci 71:215–221

Scullion J (1992) Re-establishing life in restored topsoils. Land Degrad Rehab 3:161–168

Sharmila P, Jos TP, Pardha Saradhi P (2000) Vesicular arbuscular mycorrhizal fungi improves estalishment of micropropagated plants. In: Mukerji KG, Chamola BP, Singh J (eds) Mycorrhizal biology. Kluwer Academic/Plenum Publishers, New York, Dordrecht, pp 2356–250

Solaiman MZ, Hirata H (1997) Effect of AM fungi inoculation of rice seedlings at the nursery stage upon performance in the paddy field and greenhouse. Plant Soil 191:1–12

Srivastava A, Strasser RJ (1996) Stress and stress management of land plants during a regular day. J Plant Physiol 148:445–455

Srivastava A, Strasser RJ (1997) Constructive and destructive actions of light on the photosynthetic apparatus, J Sci Ind Res 56:133–148

Strasser BJ (1997) Donor side capacity of photosystem II probed by chlorophyll *a* fluorescence transients. Photosynth Res 52:147–155

Strasser BJ, Strasser RJ (1995) Measuring fast fluorescence transients to address environmental questions: The JIP test. In: Mathis P (ed) Photosynthesis: from light to biosphere. Kluwer Academic Publisher, The Netherlands, pp 977–980

Strasser RJ, Govindjee (1992) The F and the O-J-I-P fluorescence rise in higher plants and algae. In: Argyroudi-Akoyunoglou JH (ed) Regulation of chloroplast biogenesis. Plenum Press, New York, pp 423–426

Strasser RJ, Srivastava A, Govindjee (1995) Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. Photochem Photobiol 61:32–42

Strasser RJ, Eggenberg P, Strasser BJ (1996) How to work without stress but with fluorescence. Bull Soc Roy Sci Liege 65:330–349

Strasser RJ, Srivastava A, Tsimilli-Michael M (1999) The fluorescence transient as a tool to characterise and screen photosynthetic samples. In: Yunus M, Pathre U, Mohanty P (eds) Probing photosynthesis: mechanism, regulation and adaptation. Taylor and Francis, London, UK

Trouvelot A, Kough JL, Gianinazzi-Pearson V (1985) Mesure du Taux de mycorrhization VA d'un systeme radiculaire. In: Symposium Europeen sur les Mycorrhizes. INRA Paris, pp 217–221

Tobar R, Azcon R, Barea JM (1994) Improved nitrogen uptake and transport from ^{15}N-labelled nitrate by external hyphae of arbuscular mycorrhizal under water-stressed conditions. New Phytol 126:119–122

Trappe JM (1987) Phylogenetic and ecologicaal aspects of mycotrophy in the angiosperms from an evolutionary standpoint. In: Safir GR (ed) Ecophysiology of VA mycorrhizal plants. CRC Press, Boca Raton, Florida, pp 5–25

Tsimilli-Michael M, Krüger GHJ, Strasser RJ (1995) Suboptimality as driving force for adaptation: a study about the correlation of excitation light intensity and the dynamic fluorescence emission in plants. In: Mathis P (ed) Photosynthesis: from light to biosphere. Kluwer Academic Publisher, The Netherlands, pp 981–984

Tsimilli-Michael M, Krüger GHJ, Strasser RJ (1996) About the perpetual state changes in plants approaching harmony with their environment. Arch Sci Genève 49:173–203

Tsimilli-Michael M, Pêcheux M, Strasser RJ (1998) Vitality and stress adaptation of the symbionts of coral reef and temperate foraminifers probed by the fluorescence kinetics O-J-I-P. Arch Sci Genève 51:1–36

Tsimilli-Michael M, Eggenberg P, Biró B, Köves-Péchy K, Vörös I, Strasser RJ (2000) Synergistic and antagonistic effects of arbuscular mycorrhizal fungi, *Azospirillum* and *Rhizobium* nitrogen-fixers on the photosynthetic activity of alfalfa, probed by the chlorophyll *a* polyphasic fluorescence transient O-J-I-P. Appl Soil Ecol 15:168–179

Van Rensberg L, Krüger GHJ, Eggenberg P, Strasser RJ (1996) Can screening criteria for drought resistance in *Nicotiana tabacum* L. be derived from the polyphasic rise of the chlorophyll *a* fluorescence transient (OJIP)? S Afr J Bot 62:337–341

Várallyay G (2001) New concepts in the Hungarian soil-rehabilitation techniques (in Hungarian). Agrokém Talajt 50:119–135

Vestberg M, Estaun V (1994) Micropropagated plants, an opportunity to positively manage mycorrhizal activities. In: Gianinazzi S, Schüepp H (eds) Impact of AMF on sustainable agriculture and natural ecosystems. Birkhauser Verlag Basel/Switzerland, pp 217–225

Vestberg M, Cassels AC, Schubert A, Cordier C, Gianninazzi S (2002) Arbuscular mycorrhizal fungi and micropropagation of high value crops. In: Gianinazzi S, Shüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser Verlag/Switzerland, pp 223–233

Vincent JM (1970) A manual for the practical study of root-nodule bacteria. IBP Handbook No. 15. Blackwell Scientific Publications, Oxford Edinburgh

Vivas A, Vörös I, Biró B, Barea JM, Ruiz-Lozano JM, Azcón R (2003a) Beneficial effects of indigenous Cd-tolerant and Cd-sensitive *Glomus mossae* associated with a Cd-adapted strain of *Brevibacillus* sp. In improving plant tolerance to Cd contamination. Appl Soil Ecol 24:177–186

Vivas A, Vörös I, Biró B, Campos E, Barea JM, Azcon R (2003b) Symbiotic efficiency of autochtonous arbuscular mycorrhizal fungus (*G. mossae*) and *Brevibacillus* sp. Isolated from cadmium polluted soil under increasing cadmium levels. Environ Pollut 126: 179–189

Vivas A, Azcon R, Biró B, Barea JM, Ruiz-Lozano JM (2003c) Influence of bacterial strains isolated from lead-polluted soil and their interactions with arbuscular mycorrhizae on the growth of *Trifolium pratense* L. under lead toxicity. Can J Microbiol 49:577–588

Vörös I, Biró B, Köves-Péchy K, Molnár J, Szegi J (1995) Effect of various organic and inorganic additives on humus accumulation and mycorrhizal recolonization of dump spoils in Hungary. In: Turnaw K (ed) AM fungi in sustainable soil-plant systems. Krakow, Poland, pp 34–44

Vörös I, Biró B, Takács T, Köves-Péchy K, Bujtás K (1998) Effect of arbuscular mycorrhizal fungi on heavy metal toxicity to *Trifolium pratense* in soils contaminated with Cd, Zn and Ni Salts. Agrokém Talajt 47:277–288

Young CC, Chen C, Chaoi CC (1990) Effect of *Rhizobium*, VAM fungi band phosphate solubilizing bacteria on yield and nineral uptake of crops in subtropical soils. Trans Int Congr Soil Sci Kyoto, Japan, pp 55–60

Zaidi A, Khan MS, Amil M (2003) Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea (*Cicer arietinum* L). Eur J Agronom 19:15–21

Zak JCRM, Parkinson D (1982) Initial vesicular-arbuscular mycorrhizal development of wheat-grass on two amended mine spoils. Can J Bot 60:2241–2248

15 Bacteria Helping Mycorrhiza Development

Robin Duponnois

15.1 Introduction

Mycorrhizal fungi are an ubiquitous component of most ecosystems throughout the world and play an important role in soil processes (Bethlenfalvay and Linderman 1992; Hooker and Black 1995; Van der Hejden et al. 1998). It is well known that mycorrhizal plants transfer more assimilates to the roots than non-mycorrhizal plants. This effect is usually considered to be a result of the carbon demand of the fungus which may assimilate 10% of the carbon allocated to the roots (Fitter 1991) and of the higher respiration rate of mycorrhizal roots compared with non mycorrhizal roots (Kucey and Paul 1982). As the fungal symbiosis modifies plant physiology and root functions, the composition of root exudates is highly altered by the symbiotic process which induces a significant effect on the bacterial composition in the rhizosphere. This fungal effect is commonly named "Mycorrhizosphere Effect" (Linderman 1988). The microbial communities of the mycorrhized roots differ largely from those of the uninfected roots and of the surrounding soil (Katznelson et al. 1962; Garbaye and Bowen 1989; Garbaye 1991). Specific relationships occur between mycorrhizal fungi and mycorrhizosphere microflora and there is abundant literature which relates that mycorrhizal relationships are largely influenced by these micro-organisms (Rambelli 1973; Bowen 1980; De Oliveira 1988; De Oliveira and Garbaye 1989). These microbial effects could be competitive with the fungal symbiont but, in contrast, some others could be beneficial to the mycorrhizal infection process. In that case, it has been demonstrated that some bacteria isolated from mycorrhizas, sporocarps and mycorrhizosphere soil could enhance mycorrhizal development. These bacteria have been named "Mycorrhiza Helper Bacteria" (Duponnois and Garbaye 1991). The purpose of this review is to analyse some results where positive interactions occurred between mycorrhizal fungi and rhizobacteria (Mycorrhiza Helper Bacteria). The scope of this chapter will be largely limited to interactions

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with bacteria and ectomycorrhizal fungi on early mycorrhiza establishment where literature is more abundant.

15.2 Setting in Evidence Mycorrhiza Helper Bacteria (MHB)

The presence of such bacterial isolates, closely associated with mycorrhizas was suggested for the first time by Garbaye and Bowen (1989). These authors postulated that some bacteria could interact with the host fungus through the fungal mycelium or inside the mycorrhiza at the interface between the root and the fungal mantle. Ectomycorrhizas of *Rhizopogon luteolus*/*Pinus radiata* were surface-sterilized to isolate only bacteria located inside the ectomycorrhizas. The results showed that most of the bacterial isolates belonging to the fluorescent pseudomonads group and about 80% of the bacterial strains tested in this experiment stimulated mycorrhiza formation whereas 20% had neutral or negative effect. The same kind of experiment was followed by Garbaye et al. (1990), Duponnois and Garbaye (1991) and Duponnois (1992). These authors have isolated some bacteria from sporocarps of *Laccaria bicolor* and ectomycorrhizas of Douglas fir with *L. bicolor* collected from Douglas fir plantations in France. About 45 bacterial isolates were tested for the effect on the mycorrhizal formation between the Douglas fir and *L. bicolor* strain S238. The results showed that 14 bacterial strains have significantly stimulated the ectomycorrhizal colonization of the Douglas fir root systems after 4 months culture in glasshouse conditions. No specific relationships between the bacterial effect on mycorrhiza formation and the origin of the bacteria and their taxonomic position have been recorded. For example, a strain of *Bacillus subtilis* has increased the ectomycorrhizal rate (per cent of short roots mycorrhized with *L. bicolor*) to 97.3% vs 67.3%, recorded in the control treatment (inoculated fungus without bacteria) whereas a same positive effect has been measured with a strain of *Pseudomonas fluorescens*. This MHB effect has been also tested in different environmental conditions (glasshouse and nursery conditions) which indicated the relative competitiveness of these introduced bacteria against the indigenous microflora (Duponnois 1992). Moreover, the same stimulations of mycorrhiza formation have been observed when the ectomycorrhizal fungus and the MHB have been confronted in axenic conditions (Duponnois and Garbaye 1991) which proved that the stimulation is an intrinsic property of each bacterial strain and did not result from interactions within the microbial community in the rhizosphere.

Then these MHB, defined as telluric bacteria promoting the development of mycorrhizal symbiosis (Garbaye 1994), have been isolated from different

Table 15.1. Mycorrhization helper bacteria in mycorrhizal fungus/host plants associations

	Host plant	Fungal symbiont	References
Ectomycor.	*Fagus sylvatica*	*Hebeloma crustuliniforme*	De Oliveira (1988)
	Pinus radiata	*Rhizopogon luteolus*	Garbaye and Bowen (1989)
	P. menziesii	*L. laccata* S238 N	Duponnois and Garbaye (1991)
	Pinus sylvestris	*L. laccata* S238	Rozycki et al. (1994)
	Eucalyptus diversicolor	*L. fraterna*	Dunstan et al. (1998)
	Pinus sylvestris	*Lactarius rufus*	Poole et al. (2001)
	Picea abies	*Amphinema byssoides*	Geri et al. (2000)
Endomycor.	*Trifolium parviflorum*	*Glomus sp.*	Mosse (1962)
	Lycopersicon esculentum	*G. fasciculatus*	Bagyaraj and Menge (1978)
	Trifolium subterraneum	*Not determined*	Meyer and Linderman (1986)
	Ipomoea batata	*Glomus clarum*	Paula et al. (1992)
	Herbaceous species	Not determined	Von Alten et al. (1993)

plant-fungal combinations such as Arbuscular Mycorrhizal (AM) fungi (herbaceous plants) or ectomycorrhizal fungi (trees) (Table 15.1).

The effect of MHBs on ectomycorrhizal symbiosis has often been investigated with a few northern hemisphere tree species like *Pseudotsuga menziesii*, *Quercus robur* (Duponnois and Garbaye 1991; Garbaye et al. 1992) and a limited number of fungi, principally *L. bicolor* (Frey-Klett et al. 1997; Frey et al. 1997). However, it has also been demonstrated that some bacteria could help ectomycorrhizal formation in tropical conditions with Australian acacias. Fluorescent pseudomonad strains were isolated from the soil and along the root systems of *Acacia holosericea* (an Australian *Acacia* species) seedlings growing in a soil collected from an Australian *Acacia* plantation where fruit bodies of *Pisolithus* spp. have been recorded. After three months culture in glasshouse conditions, the cultural soil was highly colonised by the ectomycorrhizal fungal hyphae and could be considered as a soil compartment such as the mycorrhizosphere and the mycosphere soil. Then the isolated bacteria were tested for their effect on mycorrhiza formation between *A. holosericea* and *Pisolithus albus* strain COI007. Most of the bacterial strains isolated from the soil stimulated mycorrhiza formation whereas this positive effect was only recorded with a few bacterial strains coming from the roots. This stimulatory effect was significantly linked with the origin of the fluorescent pseudomonad strains. These results suggested that MHBs were not strictly rhizobacteria and were in accordance with those of Frey-Klett et al. (1997) who demonstrated that an

MHB (*Pseudomonas fluorescens* isolate BBc6) of the mycorrhizal symbiosis between *L. bicolor* and Douglas fir was associated with the mycelium of the fungus in the soil. Moreover, Sen et al. (1994) demonstrated that BBc6 was attached to the hyphal wall of *L. bicolor* in in vitro experiment.

15.3 Biological Properties of MHBs

The effect of MHBs is not restricted to their promoting influence on ectomycorrhizal establishment. Another property of these bacteria has been demonstrated through several experiments: the fungal specificity of MHBs. One bacterial isolate (*Pseudomonas fluorescens* BBc6), isolated from sporocarps of *Laccaria bicolor*, could stimulate mycorrhiza formation by *Laccaria* species but inhibit the symbiosis establishment by other fungi (Garbaye and Duponnois 1992). This result has been observed in a wide range of experimental conditions (Table 15.2). In contrast, there was a lack of bacterial specificity for host plants. This bacterial isolate BBc6 promoted mycorrhiza formation with *L. bicolor* S238 and host plants such as *Picea abies*, *Pinus nigra*, *Pinus sylvestris*, *Pseudotsuga menziesii* and an angiosperm *Quercus*

Table 15.2. Effect of an MHB (*P. fluorescens* BBc6) on mycorrhiza formation (% of mycorrhized short roots) of Douglas fir seedlings inoculated with ectomycorrhizal fungi in different experimental conditions

Experimental condition	Fungal isolate	Mycorrhiza formation (%) Control (without bacteria)	+ BBc6
In vitro	*Laccaria laccata* S238	12.0a[a]	30.5b
	Hebeloma cylindrosporum		
	Paxillus involutus		
	Coenococcum geophilum		
Glasshouse	*L. laccata* S238	32.1a	89.6b
	L. bicolor 993	78.6a	97.9b
	L. bicolor S3	5.2a	19.6a
	L. bicolor A4B3 × A1B2	21.7a	45.7b
	Telephora terrestris	18.0a	4.2b
Nursery	*L. laccata* S238	75.2a	93.6b
	L. bicolor D101	55.9	89.8b
	L. proxima	38.7a	43.4a
	H. cylindrosporum	89.6a	57.8b
	P. involutus	35.1a	14.9a

[a] Data in the same row followed by the same letter are not significantly different according to the Student "t" test ($P < 0.05$)

robur (Garbaye et al. 1992). It has been demonstrated that mycorrhiza formation by *Laccaria fraterna* with *Eucalyptus diversicolor* increased significantly by up to 296% in treatments co-inoculated with *P. fluorescens* BBc6 (Dunstan et al. 1998). However, in treatments co-inoculated with *L. laccata* (strain E766) and BBc6, mycorrhizal development was significantly inhibited.

However, these conclusions on the fungus specificity of MHBs are not in accordance with those of Duponnois and Plenchette (2003). The effects of a MHB, *Pseudomonas monteilii* strain HR 13 have been investigated on the mycorrhization of (i) an Australian *Acacia, A. holosericea,* by several ectomycorrhizal fungi or one endomycorrhizal fungus *Glomus intraradices*, and of (ii) several Australian *Acacia* species by *Pisolithus alba* strain IR 100 under glasshouse conditions. A stimulating effect of HR13 on the ectomycorrhizal establishment has been recorded with all the fungal isolates (strains of *Pisolithus* and of *Scleroderma*) (Fig. 15.1). The same effect of bacteria was recorded on frequency of endomycorrhizal colonization of *A. holosericea* seedlings by *G. intraradices* with vesicles and hyphae frequencies (Fig. 15.2). In conclusion, although *P. monteilii* HR13 was isolated from *P. alba*, it stimulated mycorrhiza development of

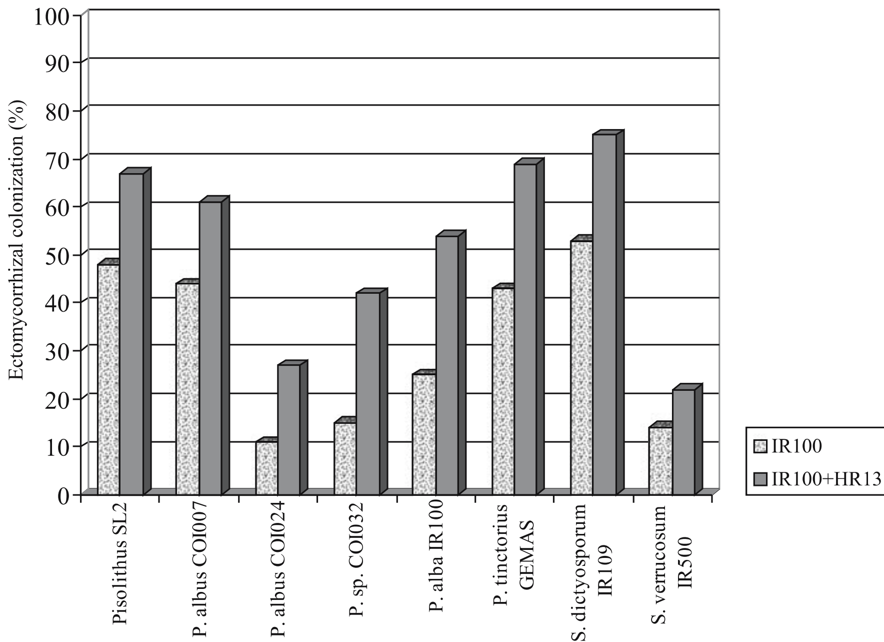

Fig. 15.1. Effect of *Pseudomonas monteilii* strain HR13 on the ectomycorrhizal formation between *Acacia holosericea* and *Pisolithus* or Scleroderma fungal isolates

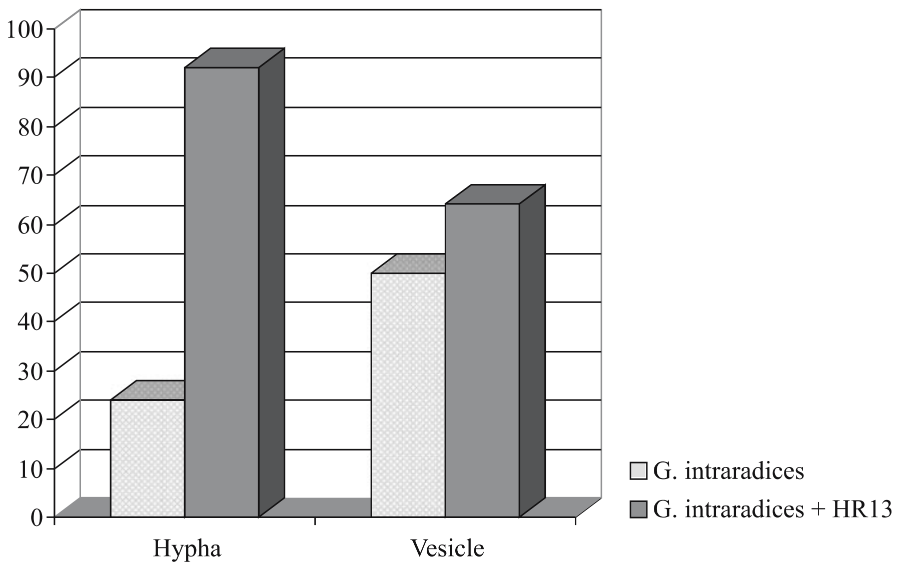

Fig. 15.2. Effect of *Pseudomonas monteilii* strain HR13 on the endomycorrhizal formation between *Acacia holosericea* and *Glomus intraradices*

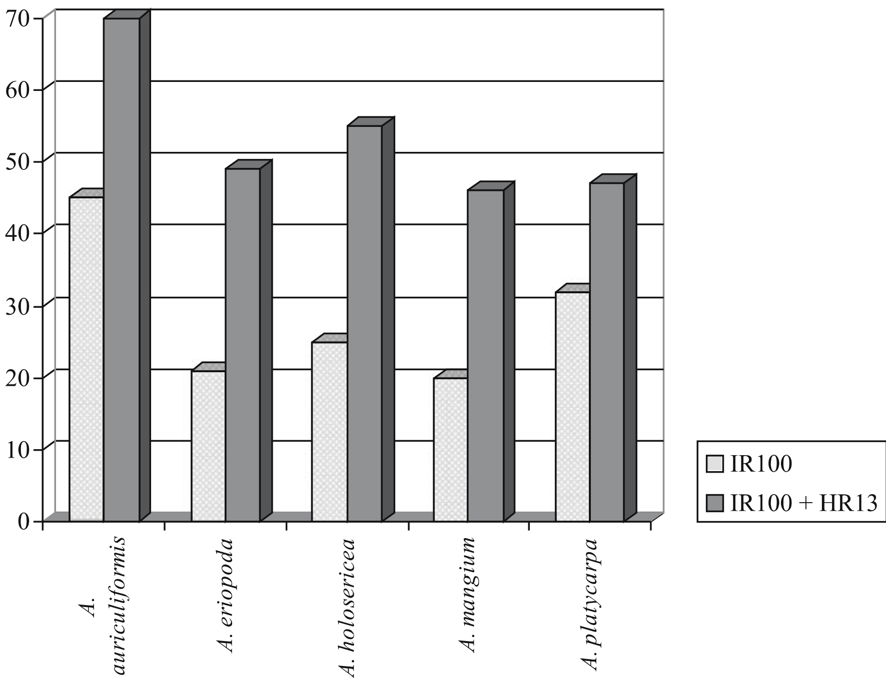

Fig. 15.3. Effect of *Pseudomonas monteilii* strain HR13 on the ectomycorrhizal formation between *Pisolithus albus* strain IR100 and several Australian Acacia species

A. holosericea with two species of *Scleroderma* and, more surprisingly, with the arbuscular mycorrhizal fungus *G. intraradices*. Bacterial inoculant HR13 has also significantly promoted the ectomycorrhizal colonization for all the Australian *Acacia* species tested in this experiment (*A. auriculiformis*, *A. eriopoda*, *A. holosericea*, *A. mangium* and *A. platycarpa*) (Fig. 15.3). The non-plant-specific effect of MHBs is demonstrated but the fungus-specificity of MHBs seems to be restricted to the system with *L. laccata* S238 and its helper bacteria.

15.4 Mechanisms Involved in the MHB Effect

Garbaye (1994) has reviewed the main hypothesis which could explain the MHB effect. According to the literature, the promoting influence of MHBs on mycorrhiza formation could be result of a bacterial effect on (i) the events of the mycorrhizal symbiosis before the root infection and the mycorrhiza formation and/or (ii) the mechanisms of recognition between the fungal symbiont and the host plant roots (Fig. 15.4).

The pre-symbiotic phase of the ectomycorrhizal symbiosis includes the germination of fungal propagules and the saprophytic development of the fungal mycelium in the soil. MHBs could accelerate the germination of spores, sclerotia and other fungal propagules which ensure the conservation and dissemination of the fungus in the soil. It has been reported that yeasts

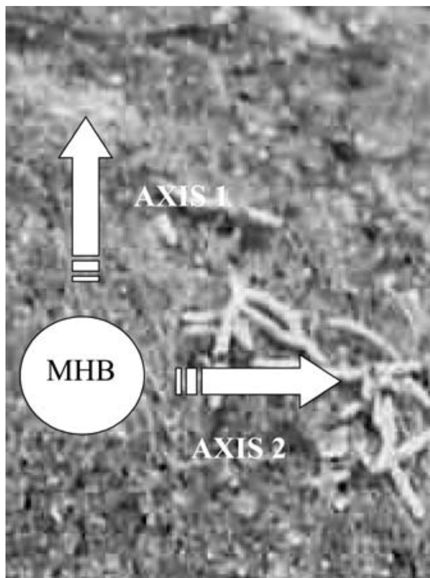

Fig. 15.4. Main hypothesis concerning the mechanisms underlying the MHB effect. *Axis 1*: MHB effect on the saprophytic fungal growth in the soil. *Axis 2*: MHB effect on the mechanisms of recognition between the fungal symbiont and the host plant root

(*Rhodotorulla* species) and bacteria could stimulate basidiospores germination (Fries 1987). The same promoting effects have been reported with corynebacteria and *Pseudomonas stutzeri* on the germination of basidiomycete spores (Ali and Jackson 1989). Whereas data concerning bacterial effects on the germination of ectomycorrhizal propagules are rather scarce, this subject has been better studied with arbuscular mycorrhizal fungi. It has been shown that rhizosphere bacteria could increase the germination of chlamydospore of *Glomus mosseae* and *Glomus versiforme* (Mosse 1962; Azcon 1987; Mayo et al. 1986). More recently, some research works showed that Bacterium-Like Organisms (BLOs) are observed in many arbuscular mycorrhizal fungi (Scannerini and Bonfante 1991) such as *Geosiphon pyrifome* (Schussler et al. 1994), *Endogone flammicorona* (Bonfante-Fasolo and Scannerini 1977) or *Gigaspora margarita* (Bianciotto et al. 1996). In this last study, the BLO was identified as a member of the genus *Burkholderia*. All these bacteria were intimately associated with their host fungus but their ecological significance is actually unknown. Another experiment has been performed with spores of *Pisolithus albus* and *Acacia mangium* (Duponnois and Lesueur, unpublished data) using a disinfected soil in glasshouse conditions. After three months culture, no ectomycorrhizas were detected along the root systems of the spores inoculated plants but a large population of fluorescent pseudomonads was recorded from the cultural soil. After six months culture, ectomycorrhizas were observed but no fluorescent pseudomonads population had totally declined. As fluorescent pseudomonads have not been detected in the spores inoculum, the main explanation for their presence in soil after three months is that these bacteria could be present inside or over the cell wall of ectomycorrhizal spores. Moreover, Frey-Klett et al. (1997) have observed that the population of fluorescent pseudomonads decline during the first four months of culture and hypothesize that MHBs had an early effect on the presymbiotic growth of the fungus. In our experiment, fluorescent pseudomonads population followed the same pattern. It disappeared after three months culture and some isolates can stimulate fungal growth. It suggests that these bacteria act significantly on the establishment of ectomycorrhizal symbiosis and have to be considered in the development and functioning of this fungal symbiosis. Further research has to be done to test their effect on the ectomycorrhizal symbiosis and to demonstrate their MHB status.

To date, most of the data concern the interactions between MHBs and the saprophytic growth of the fungal symbiont. The MHB could help the growth of the fungus in its saprophytic stage in the rhizosphere soil and on the root surface. Duponnois (1992) showed that bacteria adhered to the hyphae suggested close relationships between both microorganisms. Using a test with a poor medium to simulate through an in vitro system the environmental conditions which are encountered by the fungus

in the soil, Duponnois (1992) found a highly significant correlation between the ability of the bacteria to promote or inhibit fungal growth and their influence on mycorrhiza formation. Duponnois and Garbaye (1990) found that in nutrient-limiting conditions, MHB could act by at least two mechanisms: (i) direct trophic effect and (ii) detoxication of the medium enriched in fungal metabolites. Bacteria can produce some organic acids (i.e. citric and malic acids) metabolised by the fungal isolates but they can also break down some toxic molecules (i.e. polyphenol compounds which are toxic for mycorrhizal fungi as *Paxillus involutus*). Moreover MHB can excrete some volatile compounds which inhibit or stimulate fungal growth (Garbaye and Duponnois 1992). Carbon dioxide could play a significant effect in these interactions. It has been previously shown that, depending on the CO_2 concentration, it can inhibit or promote the growth of different fungi (Imolehin and Grogan 1980; Le Tacon et al. 1983). Other compounds could be involved such as ethylene, ammonia, amines, alcohols, sulphur compounds or low-molecular weight fatty acids (Duponnois 1992).

The main proposed mechanism for the MHB effect was that MHB stimulate hyphal growth in the rhizosphere. However, Duponnois and Plenchette (2003) have observed that, in an in vitro experiment, no positive MHB effect has been recorded with *Scleroderma* isolates whereas this MHB (*P. menzielii*, strain HR13), promoted mycorrhiza formation between *A. holosericea* and *Scleroderma* fungal strains. The same conclusion has been arrived by Founoune et al. (2002) with fluorescent pseudomonad strains and *A. holosericea*/*Pisolithus albus* ectomycorrhizal symbiosis.

Garbaye (1994) has listed other hypotheses such as the effect of MHBs on the receptivity of the root. MHB could stimulate the production of phenolic compounds such as hypaphorine and increase the aggressiveness of the fungal symbiont. For example, it has been demonstrated that hypaphorine, the betain of tryptophan, is over-accumulated in *P. tinctorius* tissues during the first steps of the mycorrhizal establishment. Moreover, it has been shown that the induction of root chitinase activities is related to the fungal strain aggressiveness (Albrecht et al. 1994). MHBs could be implicated in these enzymatic processes.

15.5 Application of MHBs

According to the MHB properties which have been previously listed, the main benefits which could be expected by using MHBs in controlled mycorrhization in forest nurseries, are: (i) optimize the mycorrhizal establishment with the inoculated fungal isolate and (ii) reduce the quantity of fungal inoculum mixed to the soil. To date, the main results have been obtained

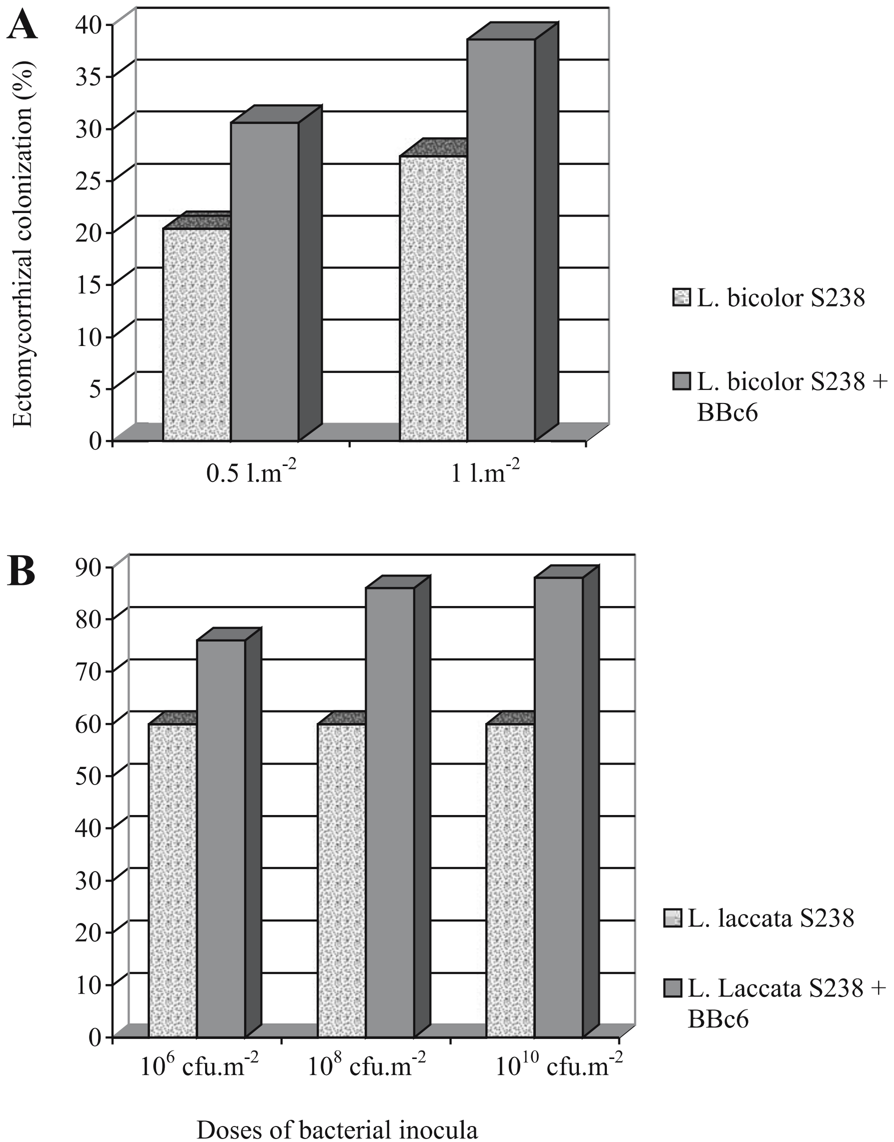

Fig. 15.5. Effect of a MHB (*P. fluorescens* isolate BBc6) on the ectomycorrhization of Douglas fir seedlings with Laccaria bicolor S238 N in bare root forest nurseries: **A** with different doses of fungal inoculum (peat vermiculite fungal inoculum) (0.5 l m^{-2} and 1 l m^{-2}); **B** with different doses of bacterial inocula (10^6 cfu m^{-2}, 10^8 cfu m^{-2} and 10^{10} cfu m^{-2})

with the Douglas fir – *Laccaria bicolor* S238N symbiotic system using the MHB *Pseudomonas fluorescens* BBc6. This bacterial isolate promoted mycorrhiza formation from around 60% (per cent of short roots mycorrhized with *L. bicolor* S238 N) to 87%. This experiment was carried out with a peat-vermiculite fungal inoculum. The same positive effect was recorded when this bacterial strain was entrapped together with the fungus in alginate beads (Duponnois 1992). After four months culture, BBc6 increased ectomycorrhizal infection from 42% to 75%. The MHB effect was also observed with different bacterial inoculation doses from 10^5 to 10^9 ufc m^{-2}. Moreover, it has been demonstrated that, with these two kinds of fungal inocula (peat-vermiculite or alginate beads), BBc6 inoculation reduced the quantity of fungal inoculum mixed to the soil ($1\,l\,m^{-2}$ to $0.5\,l\,m^{-2}$) while ensuring the same mycorrhizal index. All these results are summarized in Fig. 15.5.

15.6 Conclusions

In conclusion, it appears that some bacteria can help the establishment of the ectomycorrhizal symbiosis in temperate and tropical conditions. The main mechanisms involved in this phenomenon concern the interactions between the MHB and the fungal symbiont. However, other mechanisms may be implied such as the stimulating effect of MHBs on the production of phenolic compounds such as hypaphorine which could increase the aggressiveness of the fungal symbiont. Further research must be undertaken to (i) identify the compounds responsible for the promotion of the fungal growth and the signal molecules implied in the recognition process between the host and the fungus, (ii) determine the influence of MHBs on the dynamics of mycorrhizal symbiosis associated with a host plant and (iii) describe the impact of MHBS on the structure of fungal communities and their functions.

From a practical point of view, the use of MHBs could facilitate the introduction of controlled mycorrhization in nursery and forestry practices, especially in tropical areas where trees can potentially improve soil characteristics through a number of processes, such as nitrogen fixation, maintenance of soil organic matter, etc.

References

Albrecht C, Asselin A, Piché Y, Lapeyrie F (1994) Chitinase activities are induced in *Eucalyptus globulus* roots by ectomycorrhizal or pathogenic fungi, during early colonization. Physiol Plant 91:104–110

Ali AN, Jackson RM (1989) Stimulation of germination of spores of some ectomycorrhizal fungi by other microorganisms. Mycol Res 93:182–186

Azcon R (1987) Germination and hyphal growth of Glomus mosseae in vitro: effect of rhizosphere bacteria and cell free culture media. Soil Biol Biochem 19:417–419

Bagyaraj JM, Menge JA (1978) Interactions between a VA mycorrhiza and *Azotobacter* and their effects on rhizosphere microflora and plant growth. New Phytol 80:567–573

Bethlenfalvay GJ, Linderman RG (1992) Mycorrhizae in sustainable agriculture. ASA Special Publication No 54, Madison, Wisconsin, USA

Bianciotto V, Bandi C, Minerdi D, Sironi M, Tichy HV, Bonfante P (1996) An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. Appl Environ Microbiol 62:3005–3010

Bonfante-Fasolo P, Scannerini S (1977) Cytological observations on the mycorrhiza *Endogone flammicorona – Pinus strobus*. Allionia 22:23–34

Bowen GD (1980) Misconceptions, concepts and approaches in rhizosphere biology. In: Ellwood DC, Hedger JN, Latham MJ, Lynch JM, Slater JM (eds), Contempary microbial ecology. Academic Press, London, pp 283–304

De Oliveira VL (1988) Interactions chez les microorganismes du sol et l'établissement de la symbiose ectomycorhizienne chez le hêtre (*Fagus silvatica* L.) avec *Hebeloma crustuliniforme* et *Paxillus involutus*. Ph D thesis, University of Nancy 1. 118 p

De Oliveira VL, Garbaye J (1989) Les microorganismes auxiliaires de l'établissement des symbioses ectomycorhiziennes. Eur J Pathol 19:54–64

Dunstan WA, Malajczuk N, Dell B (1998) Effects of bacteria on mycorrhizal development and growth of container grown *Eucalyptus diversicolor* F. Muell. seedlings. Plant Soil 201:241–249

Duponnois R (1992) Les bactéries auxiliaires de la mycorhization du Douglas (*Pseudotsuga menziesii* (Mirb.) Franco) par *Laccaria laccata* souche S238. Thèse de l'Université Nancy 1. 245

Duponnois R, Garbaye J (1990) Some mechanisms involved in growth stimulation of ectomycorrhizal fungi by bacteria. Can J Bot 68:2148–2152

Duponnois R, Garbaye J (1991) Mycorrhization helper bacteria associated with the Douglas fir – *Laccaria laccata* symbiosis: effects in aseptic and in glasshouse conditions. Ann Sci For 48:239–251

Duponnois R, Plenchette C (2003) A mycorrhiza helper bacterium (MHB) enhances ectomycorrhizal and endomycorrhizal symbiosis of Australian *Acacia* species. Mycorrhiza 13:85–91

Fitter AH (1991) Costs and benefits of mycorrhizas: implications for functioning under natural conditions. Experimentia 47:350–355

Founoune H, Duponnois R, Meyer JM, Thioulouse J, Masse D, Chotte JL, Neyra M (2002) Interactions between ectomycorrhizal symbiosis and fluorescent pseudomonads on *Acacia holosericea*: isolation of mycorrhiza helper bacteria (MHB) from a soudano-Sahelian soil. FEMS Microbiol Ecol 41:37–46

Frey P, Frey-Klett P, Garbaye J, Berge O, Heulin T (1997) Metabolic and genotypic fingerprinting of fluorescent pseudomonads associated with the Douglas fir *Laccaria bicolor* Mycorrhizosphere. Appl Environ Microbiol 63:1852–1860

Frey-Klett P, Pierrat JC, Garbaye J (1997) Location and survival of mycorrhiza helper Pseudomonas fluorescens during establishment of ectomycorrhizal symbiosis between *Laccaria bicolour* and Douglas fir. Appl Environ Microbiol 63:139–144

Fries N (1987) Ecological and evolutionary aspects of spore germination in the higher basidiomycetes. Trans Br Mycol Soc 88:1–7

Garbaye J (1991) Biological interactions in the rhizosphere. Experientia 47:370–375

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

Garbaye J, Bowen GD (1989) Stimulation of ectomycorrhizal infection of *Pinus radiata* by some microorganisms associated with the mantle of ectomycorrhizas. New Phytol 112:383–388

Garbaye J, Duponnois R (1992) Application des BAM (Bactéries Auxiliaires de la Mycorhization) à l'inoculation du Douglas par *Laccaria laccata* S238 en pépinière forestière. Rev For Fr 44:491–500

Garbaye J, Duponnois R, Wahl JL (1990) The bacteria associated with Laccaria laccata ectomycorrhizas or sporocarps: effect on symbiosis establishment on Douglas fir. Symbiosis 9: 267–273

Garbaye J, Churin JL, Duponnois R (1992) Effects of substrate sterilization, fungicide treatment, and mycorrhization helper bacteria on ectomycorrhizal formation of pendunculate oak (*Quercus robur*) inoculated with *Laccaria laccata* in two peat bare-root nurseries. Biol Fert Soils 13:55–57

Geri B, Rupnik M, Kraigher H (2000) Isolation and identification of mycorrhization helper bacteria in Norway spruce, *Picea abies* (L.) Karst. Ann Rei Bot 40:65–70

Hooker JE, Black KE (1995) Arbuscular mycorrhizal fungi as components of sustainable soil-plant systems. Crit Rev Biotechnol 15:201–212

Imolehin ED, Grogan RG (1980) Effect of oxygen, carbon dioxide and ethylene on growth, sclerotial production, germination and infection by *Sclerotinia minor*. Phytopathology 70:1158–1161

Katznelson H, Rouatt JW, Peterson EA (1962) The rhizosphere effect of mycorrhizal and non mycorrhizal roots of yellow birch seedlings. Can J Bot 40:377–382

Kucey RMN, Paul EA (1982) Carbon flow, photosynthesis, and N_2 fixation in mycorrhizal and nodulated faba beans (*Vicia faba* L.). Soil Biol Biochem 14:407–412

Le Tacon F, Skinner FA, Mosse B (1983) Spore germination and hyphal growth of a vesicular-arbuscular mycorrhizal fungus, *Glomus mosseae* (Gerdeman and Trappe), under decreased oxygen and increased carbon dioxide concentrations. Can J Microbiol 29:1280–1285

Linderman RG (1988) Mycorrhizal interactions with the rhizosphere microflora: the mycorrhizosphere effect. Phytopathology 78:366–371

Mayo K, Davis R, Motta J (1986) Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. Mycologia 78:426–431

Meyer JR, Linderman RG (1986) Response to subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth promoting bacterium, *Pseudomonas putida*. Soil Biol Biochem 18:185–190

Mosse B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Paula MA, Urquiaga S, Siqueira JO, Dobereiner J (1992) Synergistic effects of vesicular-arbuscular mycorrhizal fungi and diazotrophic bacteria on nutrition and growth of sweet potato (*Ipomoea batatas*). Biol Fert Soil 14:61–68

Poole EJ, Bending GD, Whipps JM, Read DJ (2001) Bacteria associated with *Pinus sylvestris-Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation in vitro. New Phytol 151:743–751

Rambelli A (1973) The rhizosphere of mycorrhizae. In: Marks GC, Kozlowski TT (eds) Ectomycorrhizae, their ecology and physiology, Academic Press, New York, pp 299–349

Rozycki H, Kampert M, Strzelczyk E, Ly CY, Perry DA (1994) Effect of different soil bacteria on mycorrhizae formation in Scots pine (*Pinus sylvestris* L.) *in vitro* studies. Folia For Polon 36:92–102

Scannerini S, Bonfante P (1991) Bacteria and bacteria-like objects in endomycorrhizal fungi (Glomaceae) In: Margulis L, Fester R (eds) Symbiosis as a source of evolutionary innovation: speciation and morphogenesis. MIT Press, Cambridge, Mass, pp 273–287

Schussler A, Mollenhauer D, Schnepf E, Kluge M (1994) *Geosiphon pyriforme*, an endosymbiotic association of fungus and cyanobacteria: the spore structure resembles that of arbuscular mycorrhizal (AM) fungi. Bot Acta 107:36–45

Sen R, Nurmiaho-Lassila EL, Haahtela K, Korhonen T (1994) Attachment of *Pseudomonas fluorescens* strains to the cell walls of ectomycorrhizal fungi. In: Azcon-Aguilar C, Barea JM (eds) Mycorrhizas in integrated systems from genes to plant development. EUR 16728 EN, European Commission, Luxembourg, pp 661–664

Van der Hejden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity ecosystem variability and productivity. Nature 396:69–72

Von Alten H, Linderman A, Schönbeck F (1993) Stimulation of vesicular-arbuscular mycorrhiza by fungicides or rhizosphere bacteria. Mycorrhiza 2:167–173

16 Rhizosphere Regulation of Preinfection Behavior of Oomycete Plant Pathogens

Eric B. Nelson

16.1 Introduction

Limited carbon in many soils restricts or prevents active growth and metabolism of seed- and root-infecting plant pathogens (Lockwood 1977). As a result these organisms spend the vast majority of their development in a quiescent state. The earliest encounters of pathogens with plants occur in the spermosphere and developing rhizosphere. As seeds germinate and seedling roots grow, they release large quantities and varied types of exudate molecules, some of which awaken propagules from quiescence and trigger a series of developmental changes that result in the infection of the plant (Nelson 2004). These developmental responses occur in a strict temporal manner, dependent on the release of molecules that elicit and regulate these behaviors.

The emergence from quiescence to active seed or root infection represents a critical stage of pathogenic development, characterized by at least five distinct developmental phases: (i) propagule activation and germination, (ii) taxis or tropic growth toward the host, (iii) ectotrophic growth on host surfaces, (iv) the development of infection structures, and (v) host penetration. Each stage is highly regulated by biochemical and microbiological features of spermosphere and rhizosphere habitats. Consequently, seed- and root-infecting pathogens must possess efficient strategies and mechanisms for carbon acquisition and effective ways of competing with the multitude of organisms active in the spermosphere and rhizosphere.

To illustrate the regulatory impact of the rhizosphere environment on pathogen behavior, I will highlight examples from selected oomycete pathogens. These pathogens are characterized by a diversity of inoculum types and complex pathogenic development. They also serve to illustrate the breadth of responses and the types of spermosphere and rhizosphere molecules that regulate host responses.

Although the biochemical nature of spermosphere and rhizosphere habitats is important to our understanding of plant-associated microbial behavior, I will not cover these details in the current review. There are a number

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of excellent reviews that cover this subject in considerable detail (Curl and Truelove 1986; Lynch 1990; Bowen and Rovira 1999; Kuzyakov and Domanski 2000; Toal et al. 2000; Bertin et al. 2003; Nguyen 2003; Walker et al. 2003; Nelson 2004). The microbiology of the rhizosphere and its regulation of pathogen behavior will also not be covered here but the reader is referred to recent comprehensive reviews on this subject (Bowen and Rovira 1999; Whipps 2001; Kent and Triplett 2002).

16.2 Exudate Elicitors of Pathogen Responses to Plants

Much of our current understanding of exudate elicitors of pathogen development in the rhizosphere comes from studies with water-soluble exudates collected from a few days to several weeks after seed germination with no attempt to link the temporal exudation of the molecule to the temporal development of the pathogen in the spermosphere or rhizosphere. For example, propagules of *Pythium* spp. typically germinate in response to germinating seeds within 2 h of exposure, indicating that elicitors are released during the first 2 h after sowing. Nonetheless, the activity of exudates released within 2 h of the initiation of imbibition has rarely been investigated, making it impossible to make inferences about the molecules that are involved in this response. Furthermore, chemical analysis of in vitro collected seed or root exudates and the demonstration that these exudates stimulate germination of culture-produced propagules is equally insufficient in establishing the elicitors of pathogen responses to plants due to changes in behavior that are observed with propagules produced on plants (Nelson and Craft 1989; Nelson and Hsu 1994).

In an early review of the impacts of roots on soilborne pathogens, Mitchell (1976) argued that the significance of exudates in regulating soilborne pathogen activity can only be determined when specific temporal behavioral patterns of the pathogen can be related to the time and site of production of specific exudate components. Unfortunately, this vision has been largely ignored since we have no knowledge of the temporal behavior of soilborne pathogens in response to plants and only in a few instances has research definitively linked the exudation or lack of exudation of specific molecules to specific pathogen behavior. Consequently, solid conclusions about the relationships of seed or root exudate components to the regulation of pathogen behavior and the initiation of pathogenic interactions are difficult to make. Because of this, predicting the behavior and activity of seed- and root-infecting pathogens has not been entirely possible.

Despite these deficiencies, our knowledge of the important regulatory role of the spermosphere and rhizosphere on seed- and root-infecting

pathogens is growing. This is particularly true for oomycete pathogens. Under ideal situations, a comprehensive knowledge of such phenomena generally grows out of focused research efforts on model systems. Unfortunately, much of our knowledge in the area of rhizosphere regulation of oomycete and fungal pathogen behavior has come from a rather disconnected group of studies, comprising different species, hosts, and environmental conditions and spanning many decades. Therefore, the emerging picture of rhizosphere regulation of seed- and root-infecting pathogens represents only a composite picture, with details that may change depending on the exact pathosystem in question. An additional gap in our understanding is that, in many cases, temporal aspects of rhizosphere regulation are unknown, further complicating the inferences that can be made from such a collection of studies. My hope is that the synthesis that follows will stimulate research in this area so that a more comprehensive picture of pre-infection behavior can be developed.

16.3 Oomycete Pathogens

Oomycete pathogens in the genera *Aphanomyces, Phytophthora*, and *Pythium* possess complex developmental processes that exploit exogenous cues to detect, locate, and infect host tissues. They serve as illustrative models for revealing behavioral responses to plant seeds, roots, and exudate molecules. Several developmental stages are involved in plant pathogenesis. These include important propagules such as sporangia, zoospores, zoospore cysts, and oospores. In some species, chlamydospores or hyphal swellings may also be involved. Oospores are believed to serve as primary inoculum for most species of plant pathogenic oomycetes (Dick 2001). However, in some species of *Pythium* and *Phytophthora*, chlamydospores, sporangia, or hyphal swellings may also survive for extended periods (Erwin and Ribeiro 1996; Martin and Loper 1999). These propagules may germinate directly through the production of germ tubes, which grow tropically toward host tissues (Johnson and Arroyo 1983). Alternatively, oospores and sporangia may also germinate indirectly by producing zoospores, which swim chemotactically or electrotactically toward host tissues (Deacon 1996; Heungens and Parke 2000; van West et al. 2002).

One of the hallmarks of all oomycetes is the zoospore: an amazingly complex and short-lived motile propagule, arising from the germinated sporangium or oospore, and believed to represent the major infective unit of plant pathogenic species (Dick 2001). Our understanding of zoospore behavior in rhizosphere habitats comes almost solely from studies using artificial inoculations and, in most cases, in the absence of soil (Deacon

1988). Once released from the sporangium, zoospores respond to plants by proceeding through a distinct series of homing events. These developmental stages are characterized by the following: (i) sensing of an attractant, (ii) taxis of the zoospore toward the attractant, (iii) zoospore encystment and adhesion to the plant surface, (iv) cyst germination, and (v) tropic growth of cyst germ tubes toward the plant surface. These events, which occur within a 30- to 40 min period after zoospore release, are common among oomycete pathogens and are generally required for subsequent infections to occur (Deacon 1988, 1996).

16.3.1 *Aphanomyces* Species

Species of *Aphanomyces* are economically important root-rotting pathogens found worldwide in temperate climates. Among the plant pathogenic species are *A. euteiches, A. cochlioides, A. brassicae, A. raphani, A. iridis, A. cladogamus*, and *A. campostylus*, causing problems on various plants, especially those within the families Leguminosae, Chenopodiaceae, and Brassicaceae (Grünwald 2003). Although species such as *A. euteiches* have a limited host range, species such as *A. iridis* and *A. campostylus* are even more restricted in the hosts they infect.

The development of *Aphanomyces* in the rhizosphere gives rise to a series of life stages, each under the control of plant exudates and each playing important roles in plant pathogenesis. These stages include the development of oospores, sporangia, primary non-motile zoospores, secondary motile zoospores, and zoospore cysts. Currently, the bulk of our knowledge comes from studies of *A. euteiches* and *A. cochlioides*.

Aphanomyces euteiches

Germination of Oospores. Oospores of *Aphanomyces euteiches* persist in soil and in plant debris and serve as primary inoculum (Jones and Drechsler 1925; Scharen 1960). Germination may occur either by germ tubes or germ sporangia that subsequently give rise to zoospores. Light may also be needed to induce high levels of germination (Yokosawa and Kuninaga 1983). The mode of oospore germination in the rhizosphere is not clear but is believed to occur by way of zoospores (Scharen 1960). However, it is known that the nutritive environment can dictate which mode of germination prevails; high nutrient levels favor direct germination whereas lower nutrient levels favor the production of germ sporangia. Intuitively, oospores in the rhizosphere should germinate directly through the formation of germ tubes. However, few details of the developmental biology of *A. euteiches* in association with plants are available, with few studies since the original

descriptions by Jones and Drechsler (1925) where many aspects of the developmental biology of *A. euteiches* were outlined.

Oospores embedded in particles of plant debris recovered from *A. euteiches*-infested soil were shown to germinate at relatively high percentages (33–40%) in the rhizospheres of pea, bean, soybean, and corn with little or no germination in nonrhizosphere soil (Scharen 1960). Olofsson (1968) later observed maximal oospore germination between pH values of 4.5 and 4.8, particularly from decomposed root particles recovered from infested soil. While the mode of germination (direct or indirect) in the rhizosphere was not described, germination from plant debris occurred by way of zoospores.

Recently, Shang et al. (2000) found that oospores of *A. euteiches* germinated at much higher levels when placed directly on plant roots than when exposed to root exudates, similar to earlier observations with *A. raphani* (Ghafoor 1964). Greater germination was observed on roots of pea, bean, and oats than on alfalfa, corn, and tomato roots. Greater germination was observed on lateral roots of pea and bean than on taproots. Differences were also observed among *A. euteiches* pathotypes (Pfender and Hagedorn 1982; Malvick et al. 1998), with significantly greater germination of bean pathotypes occurring on bean roots than on pea roots. Similar trends with host preference were observed for pea pathotypes (Shang et al. 2000). Again, the mode of germination was not described but presumably occurred by way of germ tubes.

Germination of Sporangia. Sporangia of *A. euteiches* develop from germinating oospores in as little as 24–48 h after placing roots under flooded conditions and at temperatures between 14 and 28 °C (Jones and Drechsler 1925; Scharen 1960). In the presence of particular cations, especially Ca^{++}, and in a carbon depleted environment, sporangia and zoospores develop rapidly (Llanos and Lockwood 1960; Mitchell and Yang 1966). Primary zoospores emerge within 24 h and encyst upon release. After 2–3 h, secondary motile zoospores emerge from the primary zoospore cysts (Scharen 1960). Beyond the early gross observations of calcium and carbon leaching inducing the development of sporangia and zoospores, little is known of the regulatory mechanisms that control this important process.

Zoospore Chemotaxis. Once secondary motile zoospores are released from primary zoospores, they progress through a series of developmental stages including chemotaxis, attachment, encystment, and germination, each of which is believed to be pre-programmed since in other oomycetes, no nutrient uptake, protein synthesis, or nucleic acid synthesis occurs until after germ tubes are evident (Penington et al. 1989). Root exudates serve only as modulators of such development and regulate chemotactic and germination responses (Deacon 1996; Deacon and Saxena 1998). Within

seconds of release in the rhizosphere and exposure to root exudates, secondary zoospores of *A. euteiches* swim chemotactically toward plant roots (Cunningham and Hagedorn 1962b; Deacon and Saxena 1998). Similar responses occur in response to seed exudates (Kraft and Boge 1996; Heungens and Parke 2000). Zoospores of *A. euteiches* are particularly attracted to pea roots but also to other legumes and corn. The region immediately behind the root tip represents the site of greatest zoospore attraction. However, in pasteurized soils artificially infested with *A. euteiches* oospores, the taproot 1–2 cm below the seed appeared to be the most susceptible region of the root and not the zone immediately behind the root tip (Williams-Woodward et al. 1998). It is likely in these studies that the oospores used as inoculum germinated directly and not through the production of zoospores.

A number of isoflavones closely related to prunetin that are released into the rhizosphere from zoospore-attracting regions of the root are believed to serve as the principle chemoattractants for *A. euteiches* zoospores (Yokosawa et al. 1986; Sekizaki and Yokosawa 1988; Sekizaki et al. 1993). Other species of *Aphanomyces* with different host preferences respond to different chemoattractive compounds released from host hypocotyls (Yokosawa and Kuninaga 1979; Yokosawa et al. 1986; Horio et al. 1992; Kikuchi et al. 1995). Most of the zoospores within a population of *A. euteiches* can remain motile for at least 24 h in soil with a small percentage of the population remaining motile for up to five days (Yokosawa and Kuninaga 1977). Although zoospores may lose their motility, they can remain viable and infective for several weeks (Yokosawa and Kuninaga 1977).

Zoospore Encystment and Cyst Germination. Once zoospores reach the surface of a root, they aggregate along mature regions as well as around the root cap region, forming massive clumps of zoospores (Cunningham and Hagedorn 1962a). Presumably these sites represent areas of diffusible attractants. Encystment follows within 10 min of reaching the root, stimulated at least in part by exudates released from the root and possibly through the recognition of root surface components (Deacon and Saxena 1998). By 20 min after encystment, zoospore cysts begin to swell and germinate. Zoospore cysts may germinate by forming one or more germ tubes per cyst (Scharen 1960). High levels of germination can occur within 30–40 min after encystment, stimulated in part by Ca^{++} (Kraft and Boge 1996; Deacon and Saxena 1998).

Once germinated, germ tubes penetrate between root epidermal cells within 2 h (Cunningham and Hagedorn 1962b). Cunningham observed that, by 24 h, hyphae had colonized the endodermis. Around 60 h after penetration, oogonia and oospores were observed in pea root tissues. While penetration did not appear to differ between resistant and susceptible pea lines, more recent data indicate that fewer oospores form four days post penetration in roots of resistant pea lines than in more susceptible pea lines

(Kraft and Boge 1996). A new "crop" of oospores develop in roots by 14 days post infection (Kjoller and Rosendahl 1998).

Aphanomyces cochlioides

Germination of Oospores. *A. cochlioides* is an important damping-off and root rot pathogen of sugar beets, spinach, and other species within the Chenopodiaceae (Drechsler 1929; Papavizas and Ayers 1974). *A. cochlioides* spends a large proportion of its life history in soil as oospores (Dyer and Windels 2003), germinating upon exposure to exudates from germinating seeds and developing seedlings (Papavizas and Ayers 1974; Rush and Vaughn 1993; Dyer and Windels 2003). Although no direct evidence exists for the germination of *A. cochlioides* oospores in the rhizosphere of spinach or sugar beet, indirect evidence, based on dose response experiments using oospores as inoculum, indicates that mature oospores do indeed germinate in the rhizosphere (Paternoster and Burns 1996; Dyer and Windels 2003). Nonetheless, the timing of such germination events and the oospore conditions required for germination have not been described. Furthermore, the mode of germination (i.e. direct or indirect) in the rhizosphere is also unknown, but it has been assumed that germination occurs by way of zoospores.

Germination of Sporangia. The formation and behavior of sporangia of *A. cochlioides* in the rhizosphere is also unknown and no descriptions of *A. cochlioides* sporangia in the rhizosphere exist. Drechsler (1929) described sporangia developing on infected sugar beet seedlings where he observed the formation of sporangia within hours of placing infected seedlings in water. Sporangia may release a few to nearly 300 non-motile primary zoospores per sporangium. Within 2–3 h, secondary motile zoospores emerge, following developmental sequences commonly described for *A. euteiches* (Scharen 1960).

Zoospore Chemotaxis. In contrast to the lack of studies on oospore and sporangium behavior, zoospores of *A. cochlioides* have been studied in considerable detail, particularly with regard to their chemotactic response to host tissues following their release from sporangia. The biology of these responses has been recently reviewed (Islam and Tahara 2001a) and only a few general points will be discussed here. While zoospores of *A. euteiches* are commonly attracted to seedling root tissues of pea, *A. cochlioides* is most strongly attracted to underground portions of seedling hypocotyls of sugar beets or seedling roots of spinach where zoospores attach and form clumped masses of zoospore cysts in an apparent random pattern over the plant surface (Rai and Strobel 1966; Yokosawa et al. 1988). Similar attraction to hypocotyls and clumping on cruciferous hosts has been observed with

zoospores of *A. raphani* (Yokosawa et al. 1974; Yokosawa and Kuninaga 1979). As with *A. euteiches*, zoospores of *A. cochlioides* lose their motility within 24 h (Yokosawa et al. 1988) yet they remain viable and infective for an impressively long time. For example, Yokosawa and Kuninaga (1977) found zoospores to survive in soils and remain infective for up to 35 days, depending on soil moisture levels; cysts remained more infective in wet soils than in dryer soils.

Within minutes of exposure to root tips or cotyledons, zoospores can be seen aggregating around plant tissues (Islam et al. 2001, 2002). Zoospores are attracted to spinach and sugar beet roots by the flavone cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone), active at concentrations of 10^{-9} to 10^{-10} mol l^{-1} (Takahashi et al. 1987; Horio et al. 1992; Takayama et al. 1998), to *Chenopodium album* roots by the phenolic amide *N-trans*-feruloyl-4-*O*-methyldopamine, active at concentrations of 10^{-8} mol l^{-1} (Horio et al. 1993), and to various nitrates and chlorides from sugar beet hypocotyls (Yokosawa et al. 1988). In addition to these attractants, 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylene dioxyflavone has also been isolated from spinach leaves, where it is active in attracting *A. cochlioides* zoospores at concentrations of 10^{-6} mol l^{-1} (Tahara et al. 2001). Earlier studies have also shown *A. cochlioides* zoospores to be attracted to gluconic acid from sugar beet seedling roots (Rai and Strobel 1966).

It is believed that zoospore attractants serve as a means of establishing host specificity of *A. cochlioides*. However, non-host plants may release compounds into the rhizosphere that act as attractants and/or stimulants whereas others act solely as repellants (Mizutani et al. 1998; Tahara et al. 1999). In *Portulaca oleracea* roots, a non-host plant for *A. cochlioides*, both an attractant, *N-trans*-feruloyltyramine, and a repellant, 1-linoleoyl-2-lysophosphatidic acid monomethyl ester, can be isolated. This suggests that perhaps the repellant activity and not the attractant activity may be responsible for establishing compatible interactions of zoospores with plant hosts. Other potentially inhibitory compounds include those with estrogenic activity (Islam and Tahara 2001b).

Zoospore Encystment and Germination. Once zoospores reach the root or hypocotyl surface, they rapidly encyst (swimming ceases within 3–5 min and flagellae retract within 15–30 min), germinate (within 40 min), form appressoria (within 50–60 min), and subsequently infect the plant (Islam et al. 2002). The infections that occur within the first 2 h of arriving at the root have perhaps the most significant impacts on disease development (Macwithey 1961). Many of the developmental changes that take place during encystment and infection are mediated by some of the same compounds (e.g. cochliophilin A) that serve as zoospore attractants (Islam et al. 2003).

16.3.2 *Pythium* Species

As with *Aphanomyces* species, the germination of oospores, sporangia, and zoospore cysts of *Pythium* species represents essential and critical steps in pathogenesis. Currently over 125 species of *Pythium* have been described (Dick 2001), many of which are pathogens of plants whereas others are either saprophytes or parasites of fungi, other oomycetes, algae, and even mammals. Most plant pathogenic species have rather broad host ranges (e.g. *Pythium ultimum* and *P. aphanidermatum*). However, some species are restricted to particular plant families (e.g. *P. graminicola* and *P. arrhenomanes*). These economically important pathogens largely infect seeds, seedlings and young developing roots, although fruit rots and foliar blights are not uncommon (Martin and Loper 1999).

Developmentally, *Pythium* species, are complex, producing a variety of propagules under regulatory control of plant exudates. These propagules include oospores, sporangia, motile primary zoospores, zoospore cysts, and hyphal swellings. Some species produce all of these developmental stages whereas others may produce only one or two propagule types (van der Plaats-Niterink 1981).

Oospores are considered to be the primary survival propagule of most *Pythium* species (Hoppe 1966). However, the sporangia of species may also persist for extended periods in soil in the absence of a host (Hoppe 1966; Stanghellini and Hancock 1971; Hancock 1981). While oospore behavior of *Pythium* species had been observed and described in vitro for many decades, it wasn't until 1957 that the first direct evidence of their germination in the rhizosphere was described by Barton (1957). He observed that oospores of *P. mamillatum* germinated by the formation of a germ tube in close proximity to young turnip seedlings and further demonstrated that seedling root exudates contained the stimulatory factors. Since that time, relatively few studies have been conducted to verify these responses with oospores of *P. mamillatum* or other *Pythium* species. With the exception of *P. ultimum* and *P. aphanidermatum*, even less is know about the behavior of other developmental stages (e.g. sporangia, hyphal swellings, and zoospores) in the rhizosphere.

Pythium ultimum

Germination of Oospores. Oospores of *Pythium ultimum* form abundantly and rapidly in infected plant tissues (Mellano and Munnecke 1970; Dow and Lumsden 1975) and serve as important survival propagules as well as primary inoculum. Oospore germination can occur either directly by the formation of a germ tube (e.g. *P. ultimum* var. *ultimum*), or indirectly

through the formation of a zoosporangium, followed by the release of zoospores (e.g., *P. ultimum* var. *sporangiferum* = *P. debaryanum*) (Trow 1901; Drechsler 1946, 1952). A key requisite step in the germination of *P. ultimum* oospores is a thinning of the oospore wall (Ayers and Lumsden 1975; Lumsden and Ayers 1975; Johnson and Arroyo 1983). This process may take up to 10 weeks when incubated in soil or soil extracts (Lumsden and Ayers 1975; Johnson and Arroyo 1983) but a high level of conversion can occur within 15 days (depending on the age of the oospore). The conversion of oospores to thin walls may be enhanced in the presence of oxygen and at pH levels above 6.5 (Johnson 1988). Furthermore, thinning is also enhanced with increasing soil moisture levels and at temperatures at or above 25 °C (Lumsden and Ayers 1975; Lifshitz and Hancock 1984). Although high soil moisture levels tend to favor oospore wall thinning, no thinning occurs in saturated soils (Johnson et al. 1990).

Once converted, oospores may germinate within 2 h by the formation of a germ tube (Lumsden and Ayers 1975). Johnson and Arroyo (1983) observed direct germination of oospores of *P. ultimum* in the rhizosphere of cotton. Maximum levels of germination occurred within 1.5 mm of the root tip or root hair region where germ tubes grew tropically toward the root surface. Similar to the observations with *Aphanomyces*, the highest levels of germination occurred with oospores in direct root contact. Indirect evidence indicates that germination also occurs in the spermosphere (Howell and Stipanovic 1980; Stasz and Harman 1980a,b) and on bean hypocotyls (Dow and Lumsden 1975), but direct temporal and developmental details are lacking.

Rarely has indirect germination of *P. ultimum* oospores been described. Drechsler (1952) provided a detailed description of the indirect germination process, yet no descriptions of germination in association with plants subsequent to this report are known.

Germination of Sporangia/Hyphal Swellings. Much more is known about the behavior of *P. ultimum* sporangia (used here to refer to both zoosporangia and hyphal swellings) than of oospores in the spermosphere and rhizosphere and a considerable body of literature exists on the responses of sporangia to plant exudates (Nelson 1990). In the spermosphere, sporangia of *P. ultimum* have been shown in many studies to germinate directly within 1–1.5 h, maximum germination occurring 3–4 h after exposure to seeds (Nelson 2004). Subsequent germ tube growth may exceed 300 μm h^{-1} (Stanghellini and Hancock 1971). Because of this rapid germination in response to a host, there has been much interest over the years in trying to determine the eliciting factors.

Unsaturated fatty acids present in seed exudates are believed to be the primary elicitors of *Pythium ultimum* sporangium germination (Ruttledge

and Nelson 1997). Saturated fatty acids as well as sugars, amino acids, and other organic acids have not shown any stimulatory activity (Ruttledge and Nelson 1997). Among the most abundant fatty acids are linoleic acid (C18:2), and oleic acid (C18:1) which are believed to be the two most important exudate molecules eliciting germination of *P. ultimum* sporangia.

Microbial interference with this signaling results in reduced germination and subsequent infection of seeds by *P. ultimum* sporangia (van Dijk and Nelson 1998, 2000; Kageyama and Nelson 2003; McKellar and Nelson 2003). Furthermore, removal of these stimulatory exudate components early in the seed germination process by pre-germinating seeds dramatically reduces sporangium germination and seed infection (Nelson 2004). Both lines of investigation point to the importance of these germination stimulants to the pathogenesis of *P. ultimum*. Similar responses occur with roots and root exudates (Liu et al. 1997).

Zoospore Chemotaxis, Encystment, and Germination. The release of zoospores from sporangia of *P. ultimum* (i.e. *P. ultimum* var. *sporangiferum* = *P. debaryanum*) has not been studied in any detail in spermosphere or rhizosphere habitats since Drechsler's first descriptions of the phenomenon (Drechsler 1946, 1952). In a few limited studies zoospores of *P. ultimum* var. *sporangiferum* were shown to be attracted to roots of a number of plant species (Deacon and Mitchell 1985; Mitchell and Deacon 1986b). Accumulation occurs typically in the root hair region and the zone of cell elongation just behind the root cap (Spencer and Cooper 1967). Presumably zoospores are attracted to these sites because of the elevated levels of glutamic acid that are believed to be released (Spencer and Cooper 1967). Zoospores accumulate rapidly on roots within 1–2 min (Deacon and Mitchell 1985), encyst within 10–15 min (Spencer and Cooper 1967), and germinate within 40–45 min (Deacon and Mitchell 1985). Few differences between the proportion of swimming and encysted zoospores were seen across a range of plant species (Mitchell and Deacon 1986b).

Observations of zoospore cysts on artificially-inoculated pea roots suggest that the spatial distribution of cysts across the root surface can change with inoculum density (Dandurand et al. 1995). At low and intermediate densities cysts were either randomly or uniformly distributed over the root surface whereas at high inoculum densities, cysts aggregated over the root surface. Such aggregation has been described previously in other oomycetes and in other species of *Pythium* (Reid et al. 1995). Although the reasons for the aggregation are not entirely clear, it is believed to induce chemotropic growth of germ tubes emerging from zoospore cysts, enhance zoospore accumulation on root surfaces and thereby increase inoculum potential for infection, and enhance zoospore survival.

Ectotrophic Growth, Penetration and Infection. Once propagules reach the plant surface and germinate, infection proceeds rapidly, regardless of whether the infection court is a seed or a root. Seeds may be colonized by *P. ultimum* as early as 2–4 h after planting, with nearly 100% seed colonization occurring within 12–24 h of planting and high levels of embryo infection by 48 h (Nelson 2004). Seed exudates are required for this rapid seed colonization to occur and if early seed colonization is prevented, seeds do not become infected. On root surfaces, appressoria form after encystment and germination of zoospore cysts (Mellano and Munnecke 1970). Infection of seedling radicles can occur within 2–8 h (Spencer and Cooper 1967; Mellano and Munnecke 1970) followed by the inter- and intracellular colonization by mycelia. Oospores then develop in roots four to six days after infection (Mellano and Munnecke 1970).

Pythium aphanidermatum

Germination of Oospores. Oospores of *P. aphanidermatum* are generally more germinable than those of *P. ultimum* (Burr and Stanghellini 1973; Stanghellini and Burr 1973b; Stanghellini and Russell 1973). High percentages of *P. aphanidermatum* oospores germinate rapidly when produced under high lipid environments (Ruben et al. 1980) and when provided an appropriate stimulus (Stanghellini and Burr 1973b; Suave and Mitchell 1977; Ruben et al. 1980; Tedla and Stanghellini 1992), high soil moisture and temperature (Adams 1971; Stanghellini and Burr 1973a; Stanghellini and Stowell 1983; Tedla and Stanghellini 1992). Although germination increases with increasing exposure of oospores to soil and to desiccation (Ayers and Lumsden 1975; Ruben et al. 1980; Lumsden 1981; Lin et al. 1992), germinability and virulence decline rapidly after about 20–30 days in the absence of host plants (Mondal et al. 1995, 1996; Mondal and Hyakumachi 2000). This occurs when carbon lost from oospores, either through respiration or exudation, cannot be replenished by seed or root exudates.

Oospores germinate readily in the rhizospheres of a variety of host species (Elad and Chet 1987; Tedla and Stanghellini 1992), on plant hypocotyls (Dow and Lumsden 1975), and in soil in response to plant residues (Trujillo and Hine 1965). In rhizosphere soil from sugar beet (*Beta vulgaris* L.), significant oospore germination was observed within 4 h after exposing oospores to rhizosphere soil (Tedla and Stanghellini 1992). Maximum germination occurred after 12 h. Germ tube growth rates of > 630 μm h^{-1} were observed. Subsequent colonization of sugar beet baits occurred within 1 h, reaching a maximum by 72 h. Germinating oospores that resulted in successful infections were located within 1 mm of the root surface (Stanghellini and Stowell 1983).

In response to bean seed exudate added to soil, oospores germinated within 1.5 h by the formation of one to three germ tubes/oospore (Stanghellini and Burr 1973b). When placed adjacent to bean seeds, sugarbeet seeds, or two-week-old sugarbeet seedlings, maximum direct oospore germination was observed within 6–10 h. In the presence of host plants or plant exudates only direct germination was observed. However, in saturated soils indirect germination occurred at low levels. As with sporangia of *P. ultimum*, the germination behavior of *P. aphanidermatum* oospores is strongly influenced by other microorganisms in the rhizosphere (Elad and Chet 1987; Tedla and Stanghellini 1992).

The molecules from seed or root exudates that elicit oospore germination responses are unknown. Germination is not stimulated by sugars (Trujillo and Hine 1965) unless oospores have been passed through snails (Stanghellini and Russell 1973) or allowed to incubate in dried soils (Stanghellini and Burr 1973a). Germination appears to be stimulated by complex lipids (Ruben et al. 1980) and, under some conditions, asparagine (Stanghellini and Burr 1973b; Lumsden 1981; Elad and Chet 1987; Tedla and Stanghellini 1992).

Germination of Sporangia. The germination of sporangia of lobulate sporangial species such as *P. aphanidermatum* in the rhizosphere has not been extensively studied. A difficulty in studying these sporangia in natural habitats is their ephemeral nature. Culture-produced sporangia of *P. aphanidermatum* did not survive in an air dried-soil for more than two days (Stanghellini and Burr 1973a). Nevertheless, long-term survival of *P. aphanidermatum* sporangia in soil has been reported to occur for as long as six weeks (Peethambaran and Singh 1977), and viable sporangia similar to *P. aphanidermatum* were detected in an air-dried soil planted to cotton (Devay and Garber 1982).

Under artificial and flooded conditions, *P. aphanidermatum* sporangia that formed in infected bean tissue germinated by releasing zoospores within 6 h after flooding (Dow and Lumsden 1975). However, no direct evidence for zoospore release in the rhizosphere exists. Within 1.5 h of amending soils with bean seed exudates, sporangia germinated directly by producing one to three germ tubes, even when soils were saturated (Stanghellinia and Burr 1973a). However, in the absence of seed exudate, 90% of the sporangia released zoospores in saturated soils. Similarly, Tedla and Stanghellini (1992) also observed only direct germination of sporangia in the rhizosphere. Since there is no direct evidence for zoospore release from sporangia of *P. aphanidermatum* or any other *Pythium* species in the rhizosphere, we can only infer possible regulatory factors from what is commonly observed in vitro and that zoospore release occurs under flooded low nutrient conditions. This would suggest that zoospore release

may not be common in nutrient rich habitats such as the rhizosphere and that direct germination may predominate. However, directed studies are needed to verify such speculation.

Zoospore Chemotaxis. Once released from sporangia, it is clear that zoospores of *P. aphanidermatum* are attracted both to seeds (Heungens and Parke 2000) and to roots, primarily in the root hair region and at wound sites (Royle and Hickman 1964a,b; Hickman and Ho 1966; Kraft and Endo 1967; Chang-Ho 1970; Kraft 1974; Singh and Pavgi 1977; Tripathi and Grover 1978; Deacon and Mitchell 1985; Mitchell and Deacon 1986b, 1987; Goldberg et al. 1989; Jones et al. 1991; Zhou and Paulitz 1993; Wulff et al. 1998; Zheng et al. 2000). Molecules released as exudates from roots into soil or deposited on the root surface regulate these homing responses (Royle and Hickman 1964b; Jones et al. 1991; Donaldson and Deacon 1993c) and control developmental changes of the zoospore once it reaches the plant surface (Donaldson and Deacon 1992, 1993b,c). While the nature of chemoattractants from seeds or roots are unknown, a variety of sugars and amino acids appear to be likely candidates (Jones et al. 1991; Donaldson and Deacon 1993c). Of particular importance, not only to *P. aphanidermatum* but also to *P. catenulatum* and *P. dissotocum*, are L-aspartate, L-asparagine, L-glutamate, L-glutamine, and D-mannose. D-Fructose, sucrose, maltose, and L-alanine are also attractive but only to *P. aphanidermatum* zoospores. L-Glutamic acid concentrations as low as 1 mmol l^{-1} were attractive, whereas it blocked chemotaxis to L-asparagine, L-glutamine, and L-alanine but not to L-aspartic acid (Donaldson and Deacon 1993c).

Some non-chemoattractive amino acids were shown to inhibit chemotaxis (Donaldson and Deacon 1993c), suggesting that the balance between chemoattractive and chemoinhibitory amino acids may control host range to some degree. This hypothesis is strengthened by the studies of Tripanthi and Grover (1978), who found that plants resistant to *P. aphanideratum*(= *P. butleri* in their study) contained arabinose and arginine, both of which reduce zoospore attraction and encystment, whereas they were absent in root exudates of susceptible plants. Furthermore, if these amino acids were added to the rhizosphere of susceptible plants, disease severity was reduced.

What is not clear from all the above studies on chemotaxis is the ecological relevance of the concentrations of chemoattractants tested. Mixtures of amino acids applied at ecologically relevant concentrations to ryegrass roots did not induce a chemotactic response whereas 10× concentrations induce rapid encystment and prevented zoospores from accumulating on wound sites where zoospores naturally accumulate (van West et al. 2002). Since few studies have been directed toward the identification of chemoattractants to *P. aphanidermatum* and other oomycete species, considerably

more work is needed to understand the role of such exudate elicitors in chemotactic responses to roots of various plant species.

As zoospores swim they are fueled largely by lipids stored in the zoospore prior to release from the sporangium. This allows zoospores to swim for several hours in the absence of a host seed or root. Zoospores swim in a helical fashion at speeds up to 100–150 μm s^{-1} (Donaldson and Deacon 1993a). However, in the presence of an attractant adjacent to a host, swimming behavior is altered, characterized by more frequent turning or more circular swimming with the ventral groove of the zoospore aligned next to the host (Royle and Hickman 1964a; Jones et al. 1991). Zoospores appear to probe the root surface, revisiting the eventual docking site several times (Jones et al. 1991). These swimming behaviors and subsequent germination are regulated by Ca^{++} (Donaldson and Deacon 1992, 1993a) and by rhizosphere bacteria (Paulitz et al. 1992; Zhou and Paulitz 1993; Heungens and Parke 2000).

Reducing the availability of Ca^{++} through the addition of calcium chelators can eliminate helical swimming behavior of *P. aphanidermatum* zoospores. Addition of Ca^{++}, other divalent cations, or calcium channel blockers also alters swimming behavior. Furthermore, calmodulin antagonists can dramatically reduce zoospore swimming speed (Donaldson and Deacon 1993a). When perturbed in this way, zoospores are not responsive to amino acid attractants.

Zoospore Electrotaxis. Despite the evidence that chemoattractant molecules might be involved in zoospore homing responses to plant roots, recent evidence suggests that electrical currents, and not exudate amino acids are involved in this chemotactic response (van West et al. 2002). Plant roots generate weak electrical fields in the rhizosphere that may stimulate electrotactic swimming of oomycete zoospores. The root cap, meristematic, and elongation zones are characteristically anodic (outward flow of positive current) whereas the root hair zone and wound sites are characteristically cathodic (inward flow of current) (van West et al. 2002). Currents may commonly be in the range of 7–24 mV cm^{-1} (van West et al. 2002). Zoospores of *P. aphanidermatum* display cathodotaxis (Morris and Gow 1993), whereas other *Pythium* and *Phytophthora* species are commonly anodotactic. *P. aphanidermatum* zoospores may even be repelled by anodic regions of the root (Deacon and Donaldson 1993). Responses to these weak electrical fields can be modulated by reagents that affect calcium-ion transport or calmodulin function (Morris and Gow 1993) and may also influence encystment and docking since the posterior flagellum of *P. aphanidermatum* used in the docking process is negatively charged whereas the anterior flagellum is relatively electropositive (Morris and Gow 1993).

Zoospore Encystment and Germination. Once reaching the root surface, zoospores of *P. aphanidermatum* appear to dock to the root surface by way of their flagellae (Jones et al. 1991). Zoospores orient themselves in a very specific manner relative to the root with the water expulsion vacuole adjacent to the root surface. The adherence of zoospores to roots appears to be pH dependent, being reduced dramatically below pH 4.8 as compared to pH 6.0 (Huang and Tu 1998). Adherence of zoospores to roots is further facilitated by the secretion of glycoproteins from zoospores to form an adhesive (Estrada-Garcia et al. 1990a,b). Encystment of large masses of zoospores then follows very rapidly around wound sites and around root hairs (Kraft and Endo 1967). This is accompanied by the rapid synthesis of a cell wall (Grove and Bracker 1978).

Encystment appears to be a very complicated process mediated to some degree by exudate amino acids as well as root surface mucilage (Grove and Bracker 1978; Jones et al. 1991; Zheng et al. 2000) along with Ca^{++} signaling pathways (Donaldson and Deacon 1992). In contrast to earlier conclusions of fucosyl residues of root mucilage contributing to encystment (Longman and Callow 1987; Estrada-Garcia et al. 1990b), it is believed that encystment is induced more specifically by polyuronates and perhaps arabinoxylans present in root mucilage (Jones et al. 1991; Deacon and Donaldson 1993; Donaldson and Deacon 1993b). These encystment responses may account for the host specialization observed with some *Pythium* species (Mitchell and Deacon 1986b, 1987). Other studies have shown that concentrations of L-glutamic acid and L-aspartic acid as low as 5 mmol l^{-1} and 6 mmol l^{-1}, respectively can enhance zoospore encystment (Donaldson and Deacon 1993c).

Germination of zoospore cysts of *P. aphanidermatum* typically occurs 9 to 15 min after docking at the root surface (Jones et al. 1991) in response to components of root exudates (Chang-Ho 1970). Amino acids such as L-glutamic acid, L-asparagine, and L-aspartic acid enhance germination of *P. aphanidermatum* zoospore cysts (Donaldson and Deacon 1993c). Glucose, sucrose, and maltose were also stimulatory (Donaldson and Deacon 1993c). In the absence of Ca^{++}, these stimulants were ineffective, suggesting again a pivotal role of Ca^{++} in mediating pathogenesis-related developmental responses in the rhizosphere.

Emerging germ tubes may exhibit a chemotropic response to the source of the germination stimulant (Royle and Hickman 1964a; Mitchell and Deacon 1986a; Jones et al. 1991). Effective chemotropic stimulants include a variety of amino acids, alcohols, and aldehydes (Jones et al. 1991). This response, like other developmental aspects of the zoospore homing response, is modulated by divalent cations, especially Ca^{++} (Donaldson and Deacon 1992, 1993a).

The emergence of the germ tube from the zoospore cyst appears to be predetermined and arises from the site of the original water expulsion vacuole (Jones et al. 1991). Docking of zoospores with the water expulsion vacuole adjacent to the root surface increases the likelihood of rapid host penetration. Host penetration then occurs usually by the direct penetration of germ tubes without the production of appressoria (Gold and Stanghellini 1985) followed by rapid invasion of root tissues (Spencer and Cooper 1967; Dow and Lumsden 1975; Rey et al. 1996).

16.3.3 *Phytophthora* Species

Root rotting species of *Phytophthora* are worldwide in distribution and among the most important soilborne pathogens affecting a range of plant species (Erwin and Ribeiro 1996). While certain aspects of the rhizosphere behavior of root-rotting *Phytophthora* species are known, other aspects have rarely been studied. For example, we have a considerable understanding of zoospore behavior in the rhizosphere, particularly of *P. cinnamomi*, *P. palmivora*, and *P. sojae* (Erwin and Ribeiro 1996) but virtually no understanding of sporangium and oospore development and germination in the rhizosphere.

To illustrate the developmental aspects of *Phytophthora* pathogenesis in the rhizosphere, I will highlight two homothallic species, *P. sojae* and *P. medicaginis*. Both *P. sojae* and *P. medicaginis* are important root rot and damping-off pathogens of soybeans and alfalfa, respectively worldwide and particularly in the United States (Wrather et al. 2001). Host ranges of each are rather restricted (Kuan and Erwin 1980a); isolates of *P. medicaginis* do not infect soybean whereas isolates of *P. sojae* do not infect alfalfa. The behavior of both species represents the range of responses typical of most *Phytophthora* species; much of this being confirmed with a number of other *Phytophthora* species, particularly the heterothallic species *P. cinnamomi* and *P. palmivora*.

Phytophthora sojae and *P. medicaginis*

Germination of Oospores. Oospores of *P. sojae* and *P. medicaginis* form abundantly in roots of their respective hosts (Slusher and Sinclair 1973; Beagle Ristaino and Rissler 1983; Erwin and Li 1986) and serve as the major long-term survival structure for these species (Stack and Millar 1985). When exposed to seeds or plant roots, germination subsequently ensues. Factors that influence the rate and magnitude of oospore germination in *Phytophthora sojae* and *P. medicaginis* have been investigated (Erwin and

Mccormick 1971; Sneh et al. 1981; Förster et al. 1983; Sutherland and Cohen 1983; El-Hamalawi and Erwin 1986a,b; Jiang et al. 1989; Jiang and Erwin 1990). However, as with many other root-infecting oomycetes, few studies have examined oospore germination in the presence of plants or plant exudates thus limiting our understanding of the rhizosphere factors that regulate this stage of pathogenesis.

Jimenez and Lockwood (1982) were the first to study the germination behavior of *P. sojae* oospores in natural soil and in the presence of plant exudates. They observed high rates of germination in response to soybean exudates in natural soil (50–60%) compared with responses in deionized water (~10%). Oospore wall thinning, a prerequisite for germination, occurred optimally at 20–24 °C. Whereas soybean seedlings and other plant tissues stimulated a rapid and high level of oospore germination, glucose (0.5 mg ml^{-1}) and high levels of other nutrients tend to inhibit germination (Long et al. 1975, Jimenez and Lockwood 1982, Schechter and Gray 1987). Similarly, high levels of nutrients such as glucose, sucrose, oleic acid, and linoleic acid inhibit the germination of *P. medicaginis* oospores (Förster et al. 1983).

In a companion study, Sneh et al. (1981) observed greater germination of *P. sojae* oospores in soil or in aqueous soil extracts than in distilled water or autoclaved soil extracts. Oospores germinated by forming sporangia more commonly in darkness when in soil suspensions or extracts. Furthermore, soybean seedling disease incidence in naturally infested soil artificially infested with oospores was higher in darkness than under continuous fluorescent light.

Oospores of *P. sojae*, *P. medicaginis*, and other species contain prominent nuclei (earlier described as pellucid bodies or pellucid spots) (Erwin and Mccormick 1971; Jiang et al. 1989). After oospores are produced, a key aspect of the maturation phase is the migration and fusion of nuclei. Once karyogamy occurs, germination is allowed to proceed (Jiang et al. 1989). Although not studied in any detail, it is likely that rhizosphere components may accelerate karyogamy in oospores of *P. sojae* and *P. medicaginis* since a greater percentage of oospores germinate in root extracts or adjacent to seedlings (Förster et al. 1983; El-Hamalawi and Erwin 1986a,b; Jiang et al. 1989; Jiang and Erwin 1990).

The mode (germ tubes or germ sporangia) by which oospores germinate in the rhizosphere remains somewhat of a mystery since no direct observations of germination in the rhizosphere have been made. Jimenez and Lockwood (1982) observed a greater percentage of germinated oospores with germ sporangia when incubated in the dark and adjacent to soybean seedlings than when incubated in the light or in the absence of seedlings, suggesting that the formation of germ sporangia is the likely mode of germination in the rhizosphere. Furthermore, in *P. medicaginis*,

most oospores germinated in alfalfa root exudate and root extract by the formation of germ sporangia (Förster et al. 1983; El-Hamalawi and Erwin 1986a,b). However, at low concentrations of root exudate, the proportion of oospores germinating by forming germ tubes exceed that of oospores germinating by germ sporangia. At high concentrations of root exudate, just the opposite occurred (El-Hamalawi and Erwin 1986b). El-Hamalawi and Erwin (1986a) were able to associate the enhanced oospore germination in alfalfa root exudates with particular amino acids. Yet, some combinations of amino acids stimulated germination predominantly by germ tubes whereas other combinations stimulated germination by germ sporangia.

Without knowing the concentrations of amino acids and other exudate components in the rhizosphere, predictions about the mode of germination in nature are difficult. In contrast to these results, studies with oospore inoculum of *P. sojae* (Kittle and Gray 1979a,b) in which oospore density-disease incidence relationships were modeled point to the possibility that oospores germinate directly in the rhizosphere by the formation of germ tubes and not by the formation of germ sporangia. Resolving these developmental responses will be key in understanding the nature of pathogenesis in *P. sojae* and other species such as *P. medicaginis*.

Germination of Sporangia. It is often assumed that zoosporangia giving rise to zoospores represent the primary mode of seed and root infection by plant pathogenic *Phytophthora* species. Yet, surprisingly, few studies have examined sporangium formation and germination in association with plants. While the evidence for zoosporangium production in the rhizosphere during oospore germination is lacking, it is clear that sporangia of both *P. sojae* and *P. medicaginis* form on roots of susceptible plants in soil after infection and colonization of roots (Slusher and Sinclair 1973; Pfender et al. 1977). Sporangia of *P. sojae* and *P. medicaginis* germinate indirectly (by releasing zoospores) at temperatures below the growth optimum or directly (by the formation of germ tubes) at temperatures above the growth optimum and at soil water potentials approaching saturation (Ho 1970; Pfender et al. 1977; Eye et al. 1978; Macdonald and Duniway 1978a,b,c). As a result, the role of zoospores in the overall pre-infection phase of pathogenesis is unclear and will be determined by conditions that influence the formation of sporangia but also the temperatures and water potentials under which sporangium germination occurs.

Jimenez and Lockwood (1982) observed that sporangia of *P. sojae* produced in natural soils seldom germinated or released zoospores. Interestingly, however, they found that light was inhibitory to sporangium production in vitro but this inhibition could be reversed in the presence of soybean seedlings. Similarly, flavonoid and isolflavonoid compounds

present in seed and root exudates of soybean and stimulatory to other developmental stages of *P. sojae*, appear to be inhibitory to sporangium formation and germination (Rivera-Vargas et al. 1993; Vedenyapina et al. 1996).

The formation of *P. medicaginis* sporangia in alfalfa radicles was influenced not only by temperature but also water potential (Pfender et al. 1977). For example, optimum temperatures for production of sporangia were between 12 and 16 °C, but only in flooded soil. At water potentials of –0.6 bar, few formed and none formed at –2.8 bar. Furthermore, no sporangia were formed at a temperature of 28 °C. Under optimal conditions of cool temperatures and flooded soils, sporangia form rapidly (within 4 h) with zoospore release occurring 6–8 h later. Zoospore release occurs over an extended period in soil, being 95% complete by 72 h (Pfender et al. 1977). However, maximum release will only occur at matric potentials between 0 and –1 mb (Macdonald and Duniway 1978b). This can occur even though sporangia dry down short of zoospore release (Macdonald and Duniway 1978c).

Zoospore Chemotaxis. The behavior of zoospores of *P. sojae* and *P. medicaginis*, once released from the sporangium, is reasonably well understood and numerous studies have been conducted to elucidate the subsequent developmental responses following zoospore release. One of the prominent features of *Phytophthora* zoospores, and in particular *P. sojae* and *P. medicaginis* zoospores, is their strong attraction to roots of host plants. This occurs either through chemotactic responses to root exudate molecules (Zentmyer 1961; Ho and Hickman 1967a,b; Khew and Zentmyer 1973; Kuan and Erwin 1980b) or through electrotactic responses (van West et al. 2002).

Once released from the sporangium, zoospores of *P. sojae* may remain motile for up to 24 h (Mehrotra 1972) whereas zoospores of *P. medicaginis* lose most of their motility within 12 h depending on soil texture (Macdonald and Duniway 1978a). During this time *P. sojae* zoospores are strongly attracted to seeds, roots, and plant exudates of soybean (Ho and Hickman 1967a,b). The attractiveness of soybean seeds and roots appears to be rather specific, involving the responses to isoflavones such as daidzein and genistein released from soybean roots into the rhizosphere (Morris and Ward 1992; Tyler et al. 1996; Morris et al. 1998) and released from germinating seeds within hours of sowing (Graham 1991). *P. sojae* zoospores are attracted to these compounds at concentrations as low as 0.1 nmol l^{-1} whereas other *Phytophthora* species are not (Morris and Ward 1992). Considerable genetic variation in response to these isolflavones exists, with minimal attractive concentrations among strains ranging from 0.25 to 10 nmol l^{-1} (Tyler et al. 1996). These isoflavones result in increased levels of Ca^{++} in

zoospores, affecting whether the subsequent cysts germinate by forming germ tubes or by releasing secondary zoospores (Xu and Morris 1998; Connolly et al. 1999) and also establishing the specificity of their interaction with soybean roots. A wide variety of flavones are not attractive to zoospores and isoflavones released by most legumes other than soybean have been show to repulse zoospore attraction (Tyler et al. 1996), indicating that this homing response may involve complicated chemoreception to identify suitable hosts for infection.

Zoospore Electrotaxis. In addition to chemotactic responses, zoospores of *P. sojae* may also respond to electrical fields that develop around roots (Khew and Zentmyer 1974), a phenomenon displayed by a variety of *Phytophthora* species (Morris and Gow 1993; van West et al. 2002). Electrical currents up to 1.2 mV cm^{-1} (5 μA) exerted a positive attraction of *P. sojae* zoospores whereas currents > 1.2 mV cm^{-1} gave rise to repulsion responses (Khew and Zentmyer 1974). Under extreme conditions of flooding stress, roots may generate electrical fields of up to 500 mV cm^{-1} but commonly generate fields < 15 mV cm^{-1} (van West et al. 2002). Such zoosporic responses may provide a means of zoospores findings rapidly growing regions of the root or wound sites for subsequent infections.

Zoospore Encystment and Germination. Zoospores of *P. sojae* and *P. medicaginis* arrive at the root surface generally within 30 min of release and accumulate at regions of high exudation or at wound sites where they cluster at regions just behind the root tip (Ho and Hickman 1967b; Kuan and Erwin 1980b) and rapidly encyst (Enkerli et al. 1997). Zoospore encystment involves resorption or shedding of the flagella, transforming to a globose shape, and rapid extrusion of a temporary cell wall; all complete within 10 min of arriving at the root surface (Carlile 1983). Such a rapid encystment can be induced by high concentrations (1 μmol l^{-1}) of the soybean isoflavones daidzein and genistein. These isoflavones not only stimulate encystment, but also stimulate cyst germination and chemotropic responses of emerging germ tubes toward host roots (Tyler et al. 1996). In the event that zoospores encyst before arriving at the root or in the absence of a host, they generally survive no more than three weeks (Macdonald and Duniway 1979).

Within minutes of encysting, cysts germinate either by forming a germ tube or through the release of a secondary zoospore. In high nutrient environments such as on the root surface, cysts germinate by forming germ tubes (Ho and Hickman 1967b), reaching nearly 100% germination in as little 4 h (Ho and Hickman 1967a). Repeated germination to form secondary zoospores is suppressed by soybean root extracts (Ho and Hickman 1967a) and, as mentioned above, is regulated by external Ca^{++} (von Broembsen and Deacon 1996, 1997).

Germ tubes from *P. sojae* zoospore cysts appear to prefer to infect roots at the junctions of the anticlinal walls of the epidermal cells (Morris et al. 1998). Similar observations have been made with *P. medicaginis* zoospore cysts (Kuan and Erwin 1980b). This response does not appear to be regulated by isoflavones but rather by the surface contours of the root epidermal cells since *P. sojae* germ tubes growing on the surface of a porous membrane grew through the pores they encountered. Once the germ tube had penetrated the pore, they proliferated in the underlying medium (Morris et al. 1998). A similar response has been observed with *P. palmivora* (Bircher and Hohl 1997).

16.4 Conclusions

It should be apparent that our understanding of the behavioral regulation of particular oomycete species in the spermosphere and rhizosphere has emerged from a rather scattered collection of studies on many different hosts under varied soil and environmental conditions and spanning many decades. Incongruencies from study to study have made a comprehensive synthesis difficult. Furthermore, significant gaps exist in our understanding of specific stages of development in some species. In some cases, details of some developmental aspects have not been investigated or verified since the original reports and description of the species. In contrast, considerable in depth studies have been conducted on specific pre-infection developmental stages of some oomycete species on certain hosts. Due to this fragmentation, the validity of data in one pathosystem for making inferences on developmental regulation in other pathosystems is questionable.

What inferences can be made about the regulatory role of plant roots and root exudations on the preinfection behavior of oomycete plant pathogens? It is clear that many species require specific sets of chemical and physical factors associated with specific hosts for controlling the type and magnitude of developmental changes in the rhizosphere and their subsequent impacts on plant infection. Nonetheless, a number of generalized trends follow.

The physical properties of soils supporting root-pathogen interactions are extremely important in determining the developmental changes that take place and the success or failure of seed or root infections. Of particular importance are soil moisture, temperature, and pH, each of which serves as a critical modulator of oomycete behavior in the rhizosphere. For example, nearly all oomycete pathogens require high soil moisture and cool temperatures (i.e. temperatures cooler than the pathogen optimum) for maximum plant infection. This occurs, in part, because of the specific

developmental changes (i.e. indirect germination of propagules) that take place under these sets of conditions. Under drier and warmer conditions, zoospores are not released and sporangia and oospores germinate directly by the formation of germ tubes. These sets of physical properties then set the direction for subsequent pathogen developmental changes that are under the regulation of rhizosphere molecules.

Some generalizations may also be made about the molecules that frequently play regulatory roles in oomycete development in the rhizosphere. It is clear that amino acids, flavonoids, and lipids play important roles in oomycete development. Amino acids serve as chemoattractive and chemorepellent molecules for zoospores and as germination stimulatory molecules for oospores. Fatty acids and some complex lipids are important stimulants of sporangium and perhaps oospore germination and clearly have an important influence on the developmental biology of oomycetes in general (Hendrix 1970: Berg and Patterson 1986: Nes 1987: Kerwin and Duddles 1989: Jee and Ko 1997). Flavonoids too are becoming increasingly important as regulators of zoospore behavior and asexual reproduction in *Aphanomyces* and *Phytophthora*. Further work, linking the temporal release of these molecules to specific developmental changes in the rhizosphere, is needed.

The levels of such molecules not only determine the magnitude of such responses but may also determine the type of response at a given developmental stage. For example, with all of the oomycete species highlighted in this review, high nutrient levels suppressed zoospore release in favor of direct germination of sporangia and oospores. It is often assumed that zoospores are the primary infective propagule in the rhizosphere. However, it is possible that under the high nutrient levels present in the rhizosphere at certain times in the course of plant development that zoospores may not be as prevalent as previously believed, although they may serve as important infective units at other stages of plant development. This is a significant hypothesis that is in need of solid experimentation. This again, emphasizes the importance of looking at such responses in an ecologically relevant time frame.

It has also become clear with oomycetes that divalent cations, particularly calcium, have important regulatory roles on oomycete development. Yet, the distribution and dynamics of calcium release in rhizospheres is not known. The emphasis in nearly all rhizosphere studies has been with organic compounds with few studies on the availability and biological impacts of inorganic compounds. Such studies will be important in furthering our understanding of rhizosphere regulation of oomycete behavior.

As studies on the rhizosphere regulation of oomycetes, fungi, and other organisms move forward, it will be important to recognize the diversity

of microhabitats within the rhizosphere and their impacts of oomycete development. For example, certain species are attracted to or congregate at certain regions of a root whereas other species are attracted to other regions. For example, *P. aphanidermatum* is largely attracted to the root hair region of a root whereas most *Phytophthora* species are attracted to regions just behind the root tip. These apparently subtle preferences may have dramatic impacts on their developmental ecology due to the types of molecules and the dynamics of their release in these to regions.

The rhizosphere regulation of oomycete behavior is under the influence of a strong temporal element, driven largely by the plant. This temporal factor is particularly evident with seed-infecting pathogens, but is also of importance to root-infecting pathogens, particularly in oomycete pathosystems where developmental changes occur rapidly. The nature of the molecules that are deposited in the rhizosphere change with time and are strongly controlled by the course of plant development. Similarly, the developmental stage of the pathogen present at any point in time is determined largely by how the organism is developmentally programmed but also by the modulating effect of soil physical factors. The synchronization of pathogen development with plant development leads to successful root infections, largely because specific developmental stages are present in the rhizosphere at times when specific regulatory molecules are released.

In the future, the synchronization of pathogen development with that of the plant will be key to unraveling the complexities of pathogen developmental ecology in the rhizosphere. Many gaps in our understanding of the rhizosphere ecology of oomycetes, fungi, and bacteria exist. As new technologies become available and analytical techniques become more powerful, our opportunities to explore this final frontier in pathogen ecology will be enhanced and our abilities to predict and manipulate pathogen activities in the rhizosphere will come closer to reality.

References

Adams PB (1971) *Pythium aphanidermatum* oospore germination as affected by time, temperature, and pH. Phytopathology 61:1149–1151

Ayers WA, Lumsden RD (1975) Factors affecting production and germination of oospores of three *Pythium* species. Phytopathology 65:1094–1100

Barton R (1957) Germination of oospores of *Pythuim mamillatum* in response to exudates from living seedlings. Nature 180:613–614

Beagle Ristaino JE, Rissler JF (1983) Histopathology of susceptible and resistant soybean *Glycine max* roots inoculated with zoospores of *Phytophthora megasperma* f.sp. *glycinea*. Phytopathology 73:590–595

Berg LR, Patterson GW (1986) Phylogenetic implications of sterol biosynthesis in the oomycetes. Exp Mycol 10:175–183

Bertin C, Yang XH, Weston LA (2003) The role of root exudates and allelochemicals in the rhizosphere. Plant Soil 256:67–83

Bircher U, Hohl HR (1997) Environmental signalling during induction of appressorium formation in *Phytophthora*. Mycol Res 101:395–402

Bowen GD, Rovira AD (1999) The rhizosphere and its management to improve plant growth. Adv Agron 66:1–102

Burr TJ, Stanghellini ME (1973) Propagule nature and density of *Pythium aphanidermatum* in field soil. Phytopathology 63:1499–1501

Carlile MJ (1983) Motility, taxis, and tropism in *Phytophthora*. In: Erwin DC, Bartnicki-Garcia S, Tsao PH (eds) *Phytophthora*: its biology, taxonomy, ecology and pathology. APS Press, St Paul, Minn, pp 95–107

Chang-Ho Y (1970) The effect of pea root exudate on the germination of *Pythium aphanidermatum* zoospore cysts. Can J Bot 48:1501–1514

Connolly MS, Williams N, Heckman CA, Morris PF (1999) Soybean isoflavones trigger a calcium influx in *Phytophthora sojae*. Fung Gen Biol 28:6–11

Cunningham JL, Hagedorn DJ (1962a) Attraction of *Aphanomyces euteiches* zoospores to pea and other plant roots. Phytopathology 52:616–618

Cunningham JL, Hagedorn DJ (1962b) Penetration and infection of pea roots by zoospores of *Aphanomyces euteiches*. Phytopathology 52:827–834

Curl EA, Truelove B (1986) The rhizosphere. Springer, Berlin Heidelberg New York

Dandurand LM, Knudsen GR, Schotzko DJ (1995) Quantification of *Pythium ultimum* var *sporangiiferum* zoospore encystment patterns using geostatistics. Phytopathology 85:186–190

Deacon JW (1988) Behavioral responses of fungal zoospores. Microbiol Sci 5:249–252

Deacon JW (1996) Ecological implications of recognition events in the pre-infection stages of root pathogens. New Phytol 133:135–145

Deacon JW, Donaldson SP (1993) Molecular recognition in the homing responses of zoosporic fungi, with special reference to *Pythium* and *Phytophthora*. Mycol Res 97:1153–1171

Deacon JW, Mitchell RT (1985) Toxicity of oat roots, oat root extracts, and saponins to zoosores of *Pythium* spp. and other fungi. Trans Br Mycol Soc 84:479–487

Deacon JW, Saxena G (1998) Germination triggers of zoospore cysts of *Aphanomyces euteiches* and *Phytophthora parasitica*. Mycol Res 102:33–41

Devay JE, Garber RH (1982) Role of *Pythium* species in the seedling disease complex of cotton in California. Plant Dis 66:151–154

Dick MW (2001) Straminipilous fungi. Kluwer Academic Publishers, Dordrecht

Donaldson SP, Deacon JW (1992) Role of calcium in adhesion and germination of zoospore cysts of *Pythium*: a model to explain infection of host plants. J Gen Microbiol 138:2051–2059

Donaldson SP, Deacon JW (1993a) Changes in motility of *Pythium* zoospores induced by calcium and calcium-modulating drugs. Mycol Res 97:877–883

Donaldson SP, Deacon JW (1993b) Differential encystment of zoospores of *Pythium* species by saccharides in relation to establishment on roots. Physiol Mol Plant Pathol 42: 177–184

Donaldson SP, Deacon JW (1993c) Effects of amino acids and sugars on zoospore taxis, encystment and cyst germination in *Pythium aphanidermatum* (Edson) Fitzp., *P. catenulatum* Matthews and *P. dissotocum* Drechs. New Phytol 123:289–295

Dow RL, Lumsden RD (1975) Histopathology of infection of bean with *Pythium myriotylum* compared with infection with other *Pythium* species. Can J Bot 53:1786–1795

Drechsler C (1929) The beet water mold and several related root parasites. J Agric Res 38:309–361

Drechsler C (1946) Zoospore development from oospores of *Pythium ultimum* and *Pythium debaryanum* and its relation to rootlet-tip discoloration. Plant Dis Rep 30: 226–227

Drechsler C (1952) Production of zoospores from germinating oospores of *Pythium ultimum* and *Pythium debaryanum*. Bull Torrey Bot Club 79:431–450

Dyer AT, Windels CE (2003) Viability and maturation of *Aphanomyces cochlioides* oospores. Mycologia 95:321–326

Elad Y, Chet I (1987) Possible role of competition for nutrients in biocontrol of Pythium damping-off by bacteria. Phytopathology 77:190–195

El-Hamalawi ZA, Erwin DC (1986a) Components in alfalfa root extract and root exudate that increase oospore germination of *Phytophthora megasperma* f. sp. *medicaginis*. Phytopathology 76:508–513

El-Hamalawi ZA, Erwin DC (1986b) Physical, enzymic, and chemical factors affecting viability and germination of oospores of *Phytophthora megasperma* f.sp. *medicaginis*. Phytopathology 76:503–507

Enkerli K, Hahn MG, Mims CW (1997) Ultrastructure of compatible and incompatible interactions of soybean roots infected with the plant pathogenic oomycete *Phytophthora sojae*. Can J Bot 75:1493–1508

Erwin DC, Li XH (1986) Variability in cultural type, oospore formation and virulence on alfalfa of single zoospore and oospore isolates of *Phytophthora megasperma* f.sp. *medicaginis* (*Pmm*). Phytopathology 76:1102

Erwin DC, Mccormick WH (1971) Germination of oospores produced by *Phytophthora megasperma* var. *sojae*. Mycologia 63:972–977

Erwin DC, Ribeiro OK (1996) Phytophthora diseases worldwide. APS Press, St Paul, Minn

Estrada-Garcia MT, Callow JA, Green JR (1990a) Monoclonal antibodies to the adhesive cell coat secreted by *Pythium aphanidermatum* zoospores recognize 200×10^3 M_r glycproteins stored within large peripheral vesicles. J Cell Sci 95:199–206

Estrada-Garcia T, Ray TC, Green JR, Callow JA, Kennedy JF (1990b) Encystment of *Pythium aphanidermatum* zoospores is induced by root mucilage polysaccharides, pectin and a monoclonal antibody to a surface antigen. J Exp Bot 41:693–699

Eye LL, Sneh B, Lockwood JL (1978) Factors affecting zoospore production by *Phytophthora megasperma* var *sojae*. Phytopathology 68:1766–1768

Förster H, Ribeiro OK, Erwin DC (1983) Factors affecting oospore germination of *Phytophthora megasperma* f.sp. *medicaginis*. Phytopathology 73:442–448

Ghafoor A (1964) Radish black root rot: host range, nutrition, and oospore production and germination. Phytopathology 54:1167–1171

Gold SE, Stanghellini ME (1985) Effects of temperature on Pythium root rot of spinach grown under hydroponic conditions. Phytopathology 75:333–337

Goldberg N, Hawes MC, Stanghellini ME (1989) Specific attraction to and infection of cotton root cap cells by zoospores of *Pythium dissotocum*. Can J Bot 67:1760–1767

Graham TL (1991) Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiol 95:594–603

Grove SN, Bracker CE (1978) Protoplasmic changes during zoospore encystment and cyst germination in *Pythium aphanidermatum*. Exp Mycol 2:51–98

Grünwald NJ (2003) The biology of the genus *Aphanomyces*. In: Grünwald NJ, Coyne C (eds) Proceedings of the 2nd International *Aphanomyces* Workshop, June 17–18, 2003, USDA, ARS, Pasco, WA, USA, pp 9–14

Hancock JG (1981) Longevity of *Pythium ultimum* in moist soils. Phytopathology 71:1033–1037

Hendrix JW (1970) Sterols in growth and reproduction of fungi. Annu Rev Phytopathol 8:111–130

Heungens K, Parke JL (2000) Zoospore homing and infection events: effects of the biocontrol bacterium *Burkholderia cepacia* AMMDR1 on two oomycete pathogens of pea (*Pisum sativum* L.). Appl Environ Microbiol 66:5192–5200

Hickman CJ, Ho HH (1966) Behavior of zoospores in plant pathogenic phycomycetes. Annu Rev Phytopathol 4:195–220

Ho HH (1970) A study on the growth and sporulation of *Phytophthora megasperma* var. *sojae*. Sydowia 24:51–58

Ho HH, Hickman CJ (1967a) Asexual reproduction and behavior of zoospores of *Phytophthora megasperma* var *sojae*. Can J Bot 45:1963–1981

Ho HH, Hickman CJ (1967b) Factors governing zoospore responses of *Phytophthora megasperma* var *sojae* to plant roots. Can J Bot 45:1983–1994

Hoppe PE (1966) *Pythium* species still viable after 12 years in airdried muck soil. Phytopathology 56:1411

Horio T, Kawabata Y, Takayama T, Tahara S, Kawabata J, Fukushi Y, Nishimura H, Mizutani J (1992) A potent attractant of zoospores of *Aphanomyces cochlioides* isolated from its host, *Spinacia oleracea*. Experentia 48:410–414

Horio T, Yoshida K, Kikuchi H, Kawabata J, Mizutani J (1993) A phenolic amide from roots of *Chenopodium album*. Phytochemistry 33:807–808

Howell CR, Stipanovic RD (1980) Suppression of *Pythium ultimum* induced damping-off of cotton seedlings by *Pseudomanas fluorescens* and its antiobiotic pyoluteorin. Phytopathology 70:712–715

Huang R, Tu JC (1998) Effects of hydrogen ion concentration and temperature on attachment of zoospores of *Pythium aphanidermatum* to tomato roots. Mededelingen-Fac Landbouwk Univ Gent 63:855–859

Islam MT, Tahara S (2001a) Chemotaxis of fungal zoospores, with special reference to *Aphanomyces cochlioides*. Biosci Biotechnol Biochem 65:1933–1948

Islam MT, Tahara S (2001b) Repellent activity of estrogenic compounds toward zoospores of the phytopathogenic fungus *Aphanomyces cochlioides*. Z Naturforsch (C) 56: 253–261

Islam MT, Ito T, Tahara S (2001) Morphological studies on zoospores of *Aphanomyces cochlioides* and changes during interaction with host materials. J Gen Plant Pathol 67:255–261

Islam MT, Ito T, Tahara S (2002) Microscopic studies on attachment and differentiation of zoospores of the phytopathogenic fungus *Aphanomyces cochlioides*. J Gen Plant Pathol 68:111–117

Islam MT, Ito T, Tahara S (2003) Host-specific plant signal and G-protein activator, mastoparan, trigger differentiation of zoospores of the phytopathogenic oomycete *Aphanomyces cochlioides*. Plant Soil 255:131–142

Jee HJ, Ko WH (1997) Stimulation of sexual reproduction in *Phytophthora cactorum* and *P. parasitica* by fatty acids and related compounds. Mycol Res 101:1140–1144

Jiang J, Erwin DC (1990) Morphology, plasmolysis, and tetrazolium bromide stain as criteria for determining viability of *Phytophthora* oospores. Mycologia 82:107–113

Jiang J, Stephenson LW, Erwin DC, Leary JV (1989) Nuclear changes in *Phytophthora* during oospore maturation and germination. Mycol Res 92:463–469

Jimenez B, Lockwood JL (1982) Germination of oospores of *Phytophthora megasperma* f.sp. *glycinea* in the presence of soil. Phytopathology 72:662–666

Johnson LF (1988) Effects of atmospheric gases and light on changes in thickness of oospore walls and on germinability of oospores of *Pythium ultimum*. Phytopathology 78: 435–439

Johnson LF, Arroyo T (1983) Germination of oospores of *Pythium ultimum* in the cotton rhizosphere. Phytopathology 73:1620–1624

Johnson LF, Qian P, Ferriss RS (1990) Soil matric potential effects on changes in wall morphology, germination, and lysis of oospores of *Pythium ultimum*. Phytopathology 80:1357–1361

Jones FR, Drechsler C (1925) Root rot of peas in the United States caused by *Aphanomyces euteiches* (n.sp.). J Agric Res 30:293–325

Jones SW, Donaldson SP, Deacon JW (1991) Behavior of zoospores and zoospore cysts in relation to root Infection by *Pythium aphanidermatum*. New Phytol 117:289–301

Kageyama K, Nelson EB (2003) Differential inactivation of seed exudate stimulants of *Pythium ultimum* sporangium germination by *Enterobacter cloacae* influences biological control efficacy on different plant species. Appl Environ Microbiol 69:1114–1120

Kent AD, Triplett EW (2002) Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu Rev Microbiol 56:211–236

Kerwin JL, Duddles ND (1989) Reassessment of the role of phospholipids in sexual reproduction by sterol-auxotrophic fungi. J Bacteriol 171:3831–3839

Khew KL, Zentmyer GA (1973) Chemotactic response of zoospores of 5 species of *Phytophthora*. Phytopathology 63:1511–1517

Khew KL, Zentmyer GA (1974) Electrotactic response of zoospores of 7 species of *Phytophthora*. Phytopathology 64:500–507

Kikuchi H, Horio T, Kawabata J, Koyama N, Fukushi Y, Mizutani J, Tahara S (1995) Activity of host-derived attractants and their related compounds toward the zoospores of phytopathogenic *Aphanomyces cochlioides*. Biosci Biotechnol Biochem 59:2033–2035

Kittle DR, Gray LE (1979a) Production and pathogenicity of oospores of *Phytophthora megasperma* var *sojae*. Plant Dis Rep 63:628–631

Kittle DR, Gray LE (1979b) Storage and use of *Phytophthora megasperma* var *sojae* oospores as inoculum. Phytopathology 69:821–823

Kjoller R, Rosendahl S (1998) Enzymatic activity of the mycelium compared with oospore development during infection of pea roots by *Aphanomyces euteiches*. Phytopathology 88:992–996

Kraft JM (1974) The influence of seedling exudates on the resistance of peas to Fusarium and Pythium root rot. Phytopathology 64:190–194

Kraft JM, Boge WL (1996) Identification of characteristics associated with resistance to root rot caused by *Aphanomyces euteiches* in pea. Plant Dis 80:1383–1386

Kraft JM, Endo RM (1967) Infection of primary roots of bentgrass by zoospores of *Pythium aphanidermatum*. Phytopathology 57:86–90

Kuan TL, Erwin DC (1980a) Formae speciales differentiation of *Phytophthora megasperma* isolates from soybean and alfalfa. Phytopathology 70:333–338

Kuan TL, Erwin DC (1980b) Predisposition effect of water saturation of soil on Phytophthora root rot of alfalfa. Phytopathology 70:981–986

Kuzyakov Y, Domanski G (2000) Carbon input by plants into the soil. Rev J Plant Nutr Soil Sci 163:421–431

Lifshitz R, Hancock JG (1984) Environmental influences on the passive survival of *Pythium ultimum* in soil. Phytopathology 74:128–132

Lin YS, Lo CT, Hwang CH (1992) Oospore dormancy, germination, and survival of *Pythium aphanidermatum*. Plant Prot Bull Taichung 34:235–245

Liu L, Punja ZK, Rahe JE (1997) Altered root exudation and suppression of induced lignification as mechanisms of predisposition by glyphosate of bean roots (Phaseolus vulgaris L.) to colonization by *Pythium* spp. Physiol Mol Plant Pathol 51:111–127

Llanos C, Lockwood JL (1960) Factors affecting zoospore production by *Aphanomyces euteiches*. Phytopathology 50:826–830

Lockwood JL (1977) Fungistasis in soils. Biol Rev 52:1–43

Long M, Keen NT, Ribeiro OK, Leary JV, Erwin DC, Zentmyer GA (1975) *Phytophthora megasperma* var. *sojae*: development of wild-type strains for genetic research. Phytopathology 65:592–597

Longman D, Callow JA (1987) Specific saccharide residues are involved in the recognition of plant root surfaces by zoospores of *Pythium aphanidermatum*. Physiol Mol Plant Pathol 30:139–150

Lumsden RD (1981) A nylon fabric technique for studying the ecology of *Pythium aphanidermatum* and other fungi in soil. Phytopathology 71:282–285

Lumsden RD, Ayers WA (1975) Influence of soil environment on the germinability of constitutively dormant oospores of *Pythium ultimum*. Phytopathology 65:1101–1107

Lynch JM (1990) The rhizosphere. Wiley, New York

Macdonald JD, Duniway JM (1978a) Influence of soil texture and temperature on the motility of *Phytophthora cryptogea* and *P. megasperma* zoospores. Phytopathology 68:1627–1630

Macdonald JD, Duniway JM (1978b) Influence of the matric and osmotic components of water potential on zoospore discharge in *Phytophthora*. Phytopathology 68:751–757

Macdonald JD, Duniway JM (1978c) Temperature and water stress effects on sporangium viability and zoospore discharge in *Phytophthora cryptogea* and *P. megasperma*. Phytopathology 68:1449–1455

Macdonald JD, Duniway JM (1979) Use of fluorescent antibodies to study the survival of *Phytophthora megasperma* and *Phytophthora cinnamomi* zoospores in soil. Phytopathology 69:436–441

Macwithey HS (1961) In vitro inoculation of sugar beet seedlings with *Aphanomyces cochlioides* Drechs. J Am Soc Sugar Beet Technol 11:309–312

Malvick DK, Grau CR, Percich JA (1998) Characterization of *Aphanomyces euteiches* strains based on pathogenicity tests and random amplified polymorphic DNA analyses. Mycol Res 102:465–475

Martin FN, Loper JE (1999) Soilborne plant diseases caused by *Pythium* spp.: ecology, epidemiology, and prospects for biological control. Crit Rev Plant Sci 18:111–181

McKellar ME, Nelson EB (2003) Compost-induced suppression of Pythium damping-off is mediated by fatty acid metabolizing seed colonizing microbial communities. Appl Environ Microbiol 69:452–460

Mehrotra RS (1972) Behavior of zoospores of *Phytophthora megasperma* var *sojae* and *P. drechsleri* in soil. Can J Bot 50:2125–2130

Mellano HM, Munnecke DE (1970) Relationship of seedling age to development of *Pythium ultimum* on roots of *Antirrhinum majus*. Phytopathology 60:935–942

Mitchell JE (1976) The effect of roots on the activity of soil-borne plant pathogens. In: Heitefuss R, Williams PH (eds) Physiological plant pathology. Springer, Berlin Heidelberg New York, pp 104–128

Mitchell JE, Yang CY (1966) Factors affecting growth and development of *Aphanomyces euteiches*. Phytopathology 56:917–922

Mitchell RT, Deacon JW (1986a) Chemotropism of germ tubes from zoospore cysts of *Pythium* spp. Trans Br Mycol Soc 86:233–237

Mitchell RT, Deacon JW (1986b) Differential (host-specific) accumulation of zoospores of *Pythium* on roots of graminaceous and non-graminaceous plants. New Phytol 102:113–122

Mitchell RT, Deacon JW (1987) Differential adhesion of zoospore cysts of *Pythium* on roots of graminaceous and non-graminaceous plants. Trans Br Mycol Soc 88:401–403

Mizutani M, Hashidoko Y, Tahara S (1998) Factors responsible for inhibiting the motility of zoospores of the phytopathogenic fungus *Aphanomyces cochlioides* isolated from the non-host plant *Portulaca oleracea*. FEBS Lett 438:236–240

Mondal SN, Hyakumachi M (2000) Soil factors affecting carbon loss and pathogenecity of oospores of *Pythium aphanidermatum*. Soil Biol Biochem 32:111–118

Mondal SN, Kageyama K, Hyakumachi M (1995) Influence of soil matric potential on the debilitation of oospores of *Pythium aphanidermatum*. Soil Biol Biochem 27:1319–1324

Mondal SN, Kageyama K, Hyakumachi M (1996) Decreased germinability and virulence of oospores of *Pythium aphanidermatum* in relation to loss of endogenous carbon during incubation on soil. Soil Biol Biochem 28:545–553

Morris BM, Gow NAR (1993) Mechanism of electrotaxis of zoospores of phytopathogenic fungi. Phytopathology 83:877–882

Morris PF, Ward WB (1992) Chemoattraction of zoospores of the soybean pathogen *Phytophthora sojae* by isoflavones. Physiol Mol Plant Pathol 40:17–22

Morris PF, Bone E, Tyler BM (1998) Chemotropic and contact responses of *Phytophthora sojae* hyphae to soybean isoflavonoids and artificial substrates. Plant Physiol 117:1171–1178

Nelson EB (1990) Exudate molecules initiating fungal responses to seeds and roots. Plant Soil 129:61–73

Nelson EB (2004) Microbial dynamics and interactions in the spermosphere. Annu Rev Phytopathol 42:271–309

Nelson EB, Craft CM (1989) Comparative germination of culture-produced and plant-produced sporangia of *Pythium ultimum* in response to soluble seed exudates and exudate components. Phytopathology 79:1009–1013

Nelson EB, Hsu JST (1994) Nutritional factors affecting responses of sporangia of *Pythium ultimum* to germination stimulants. Phytopathology 84:677–683

Nes WD (1987) Biosynthesis and requirement for sterols in the growth and reproduction of Oomycetes. In: Fuller G, Nes WD (eds) Ecology and metabolism of plant lipids. American Chemical Society, Washington, DC, pp 304–328

Nguyen C (2003) Rhizodeposition of organic C by plants: mechanisms and controls. Agronomie 23:375–396

Olofsson J (1968) Influence of hydrogen-ion concentration on germination of naturally produced oospores of *Aphanomyces euteiches*. Plant Dis Rep 52:264–267

Papavizas GC, Ayers WA (1974) *Aphanomyces* species and their root diseases in pea and sugarbeet. In: US Dep Agric Res Serv Tech Bull 1485, Washington, DC

Paternoster MP, Burns RG (1996) A novel medium for the oosporogenesis of *Aphanomyces cochlioides*. Mycol Res 100:936–938

Paulitz TC, Zhou T, Rankin L (1992) Selection of rhizosphere bacteria for biological control of *Pythium aphanidermatum* on hydroponically grown cucumber. Biol Control 2: 226–237

Peethambaran CK, Singh RS (1977) Survival of different structures of *Pythium* spp. in soil. Indian Phytopathol 30:347–352

Penington CJ, Iser JR, Grant BR, Gayler KR (1989) Role of RNA and protein synthesis in stimulated germination of zoospores of the pathogenic fungus *Phytophthora palmivora*. Exp Mycol 13:158–168

Pfender WF, Hagedorn DJ (1982) *Aphanomyces euteiches* f.sp. *phaseoli*, a causal agent of bean root and hypocotyl rot. Phytopathology 72:306–310

Pfender WF, Hine RB, Stanghellini ME (1977) Production of sporangia and release of zoospores by *Phytophthora megasperma* in soil. Phytopathology 67:657–663

Rai PV, Strobel GA (1966) Chemotaxis of zoospores of *Aphanomyces cochlioides* to sugar beet seedlings. Phytopathology 56:1365–1369

Reid B, Morris BM, Gow NAR (1995) Calcium-dependent, genus-specific, autoaggregation of zoospores of phytopathogenic fungi. Exp Mycol 19:202–213

Rey P, Benhamou N, Tirilly Y (1996) Ultrastructural and cytochemical studies of cucumber roots infected by two *Pythium* species with different modes of pathogenicity. Physiol Mol Plant Pathol 49:213–231

Rivera-Vargas LI, Schmitthenner AF, Graham TL (1993) Soybean flavonoid effects on and metabolism by *Phytophthora sojae*. Phytochemistry 32:851–857

Royle DJ, Hickman CJ (1964a) Analysis of factors governing in vitro accumulation of zoospores of *Pythium aphanidermatum* on roots I. Behavior of zoospores. Can J Microbiol 10:151–162

Royle DJ, Hickman CJ (1964b) Analysis of factors governing in vitro accumulation of zoospores of *Pythium aphanidermatum* on roots II. Substances causing response. Can J Microbiol 10:201–219

Ruben DM, Frank ZR, Chet I (1980) Factors affecting behavior and developmental synchrony of germinating oospores of *Pythium aphanidermatum*. Phytopathology 70: 54–59

Rush CM, Vaughn KM (1993) Effect of irrigation, soil matric potential, and seed priming on sugar beet seed germination and damping-off caused by *Aphanomyces cochlioides*. Phytopathology 83:202–206

Ruttledge TR, Nelson EB (1997) Extracted fatty acids from *Gossypium hirsutum* stimulatory to the seed-rotting fungus, *Pythium ultimum*. Phytochemistry 46:77–82

Scharen AL (1960) Germination of oospores of *Aphanomyces euteiches* embedded in plant debris. Phytopathology 50:274–277

Schechter SE, Gray LE (1987) Oospore germination in *Phytophthora megasperma* f.sp. *glycinea*. Can J Bot 65:1465–1467

Sekizaki H, Yokosawa R (1988) Studies on zoospore attracting activity. 1. Synthesis of isoflavones and their attracting activity to *Aphanomyces euteiches* zoospores. Chem Pharm Bull 36:4876–4880

Sekizaki H, Yokosawa R, Chinen C, Adachi H, Yamane Y (1993) Studies on zoospore attracting activity 2. Synthesis of isoflavones and their attracting activity to *Aphanomyces euteiches* zoospores. Biol Pharm Bull 16:698–701

Shang H, Grau CR, Peters RD (2000) Oospore germination of *Aphanomyces euteiches* in root exudates and on the rhizoplanes of crop plants. Plant Dis 84:994–998

Singh SL, Pavgi MS (1977) Attraction of *Pythium butleri* zoospores to cauliflower roots. Phytopathol Med 16:126–128

Slusher RL, Sinclair JB (1973) Development of *Phytophthora megasperma* var *sojae* in soybean roots. Phytopathology 63:1168–1171

Sneh B, Eye L, Lockwood JL (1981) Factors affecting germination of oospores of *Phytophthora megasperma* var. *sojae*. J Phytopathol 101:314–322

Spencer JA, Cooper WE (1967) Pathogenesis of cotton (*Gossypium hirsutum*) by *Pythium* species: zoospore and mycelium attraction and infectivity. Phytopathology 57:1332–1338

Stack JP, Millar RL (1985) Relative survival potential of propagules of *Phytophthora megasperma* f.sp. *medicaginis*. Phytopathology 75:1398–1404

Stanghellini ME, Burr TJ (1973a) Effect of soil water potential on disease incidence and oospore germination of *Pythium aphanidermatum*. Phytopathology 63:1496–1498

Stanghellini ME, Burr TJ (1973b) Germination in vivo of *Pythium aphanidermatum* in oospores and sporangia. Phytopathology 63:1493–1496

Stanghellini ME, Hancock JG (1971) The sporangium of *Pythium ultimum* as a survival structure in soil. Phytopathology 61:157–164

Stanghellini ME, Russell JD (1973) Germination in vitro of *Pythium aphanidermatum* oospores. Phytopathology 63:133–137

Stanghellini ME, Stowell LS (1983) Distribution of *Pythium aphanidermatum* in rhizosphere soil and factors affecting expression of the absolute inoculum potential. Phytopathology 73:1463–1466

Stasz TE, Harman GE (1980a) Interactions of *Pythium ultimum* with germinating resistant or susceptible pea seeds. Phytopathology 70:27–31

Stasz TE, Harman GE (1980b) Time and site of infection of resistant and susceptible germinating pea seeds by *Pythium ultimum*. Phytopathology 70:730–733

Suave RJ, Mitchell DJ (1977) An evaluation of methods for obtaining mycelium-free oospores of *Pythium aphanidermatum* and *P. myriotylum*. Can J Bot 23:643–648

Sutherland ED, Cohen SD (1983) Evaluation of tetrazolium bromide as a vital stain for fungal oospores. Phytopathology 73:1532–1535

Tahara S, Mizutani M, Takayama T, Ohkawa K (1999) Plant secondary metabolites regulating behaviour of zoospores of the phytopathogenic fungus *Aphanomyces cochlioides*. Pest Sci 55:209–211

Tahara S, Ohkawa K, Takayama T, Ogawa Y (2001) The third naturally occurring attractant toward zoospores of phytopathogenic *Aphanomyces cochlioides* from the *Spinacia oleracea* host plant. Biosci Biotechnol Biochem 65:1755–1760

Takahashi H, Sasaki T, Ito M (1987) New flavonoids isolated from infected sugar beet roots. Bull Chem Soc Jpn 60:2261–2262

Takayama T, Mizutani J, Tahara S (1998) Drop method as a quantitative bioassay method of chemotaxis of *Aphanomyces cochlioides* zoospores. Ann Phytopathol Soc Jpn 64:175–178

Tedla T, Stanghellini ME (1992) Bacterial population dynamics and interactions with *Pythium aphanidermatum* in intact rhizosphere soil. Phytopathology 82:652–656

Toal ME, Yeomans C, Killham K, Meharg AA (2000) A review of rhizosphere carbon flow modelling. Plant Soil 222:263–281

Tripathi NN, Grover RK (1978) Participation of root exudates of susceptible and resistant plants in pathogenesis of diseases caused by *Pythium butleri*. Z Pflanzenkr Pflanzenschutz 85:15–21

Trow AH (1901) Observations on the biology and cytology of *Pythium ultimum* n.sp. Ann Bot 15:269–311

Trujillo E, Hine RB (1965) The role of papaya residues in papaya root rot caused by *Pythium aphanidermatum* and *Phytophthora parasitica*. Phytopathology 55:1293–1298

Tyler BM, Wu MH, Wang JM, Cheung W, Morris PF (1996) Chemotactic preferences and strain variation in the response of *Phytophthora sojae* zoospores to host isoflavones. Appl Environ Microbiol 62:2811–2817

van der Plaats-Niterink AJ (1981) Monograph of the genus *Pythium*. CVS, Baarn

van Dijk K, Nelson EB (1998) Inactivation of seed exudate stimulants of *Pythium ultimum* sporangium germination by biocontrol strains of *Enterobacter cloacae* and other seed-associated bacteria. Soil Biol Biochem 30:183–192

van Dijk K, Nelson EB (2000) Fatty acid competition as a mechanism by which *Enterobacter cloacae* suppresses *Pythium ultimum* sporangium germination and damping-off. Appl Environ Microbiol 66:5340–5347

van West P, Morris BM, Reid B, Appiah AA, Osborne MC, Campbell TA, Shepherd SJ, Gow NAR (2002) Oomycete plant pathogens use electric fields to target roots. Mol Plant-Microbe Interact 15:790–798

Vedenyapina EG, Safir GR, Niemira BA, Chase TE (1996) Low concentrations of the isoflavone genistein influence in vitro asexual reproduction and growth of *Phytophthora sojae*. Phytopathology 86:144–148

von Broembsen SL, Deacon JW (1996) Effects of calcium on germination and further zoospore release from zoospore cysts of *Phytophthora parasitica*. Mycol Res 100:1498–1504

von Broembsen SL, Deacon JW (1997) Calcium interference with zoospore biology and infectivity of *Phytophthora parasitica* in nutrient irrigation solutions. Phytopathology 87:522–528

Walker TS, Bais HP, Grotewold E, Vivanco JM (2003) Root exudation and rhizosphere biology. Plant Physiol 132:44–51

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52:487–511

Williams-Woodward JL, Pfleger FL, Allmaras RR, Fritz VA (1998) *Aphanomyces euteiches* inoculum potential: a rolled-towel bioassay suitable for fine-textured soils. Plant Dis 82:386–390

Wrather JA, Stienstra WC, Koenning SR (2001) Soybean disease loss estimates for the United States from 1996 to 1998. Can J Plant Pathol 23:122–131

Wulff EG, Pham ATH, Cherif M, Rey P, Tirilly Y, Hockenhull J (1998) Inoculation of cucumber roots with zoospores of mycoparasitic and plant pathogenic *Pythium* species: Differential zoospore accumulation, colonization ability and plant growth response. Eur J Plant Pathol 104:69–76

Xu C, Morris PF (1998) External calcium controls the developmental strategy of *Phytophthora sojae* cysts. Mycologia 90:269–275

Yokosawa R, Kuninaga S (1977) Longevity and infectivity of *Aphanomyces euteiches* and *A. cochlioides* zoospores in soil. Ann Phytopath Soc Jpn 43:501–507

Yokosawa R, Kuninaga S (1979) *Aphanomyces raphani* zoospore attractant isolated from cabbage: indole-3-aldehyde. Ann Phytopath Soc Jpn 45:339–343

Yokosawa R, Kuninaga S (1983) Effects of light on oospore germination of *Aphanomyces euteiches* isolated from pea root rot field soil in Furano, Hokkarido and from pea field soil of Miyakejima, Japan. Ann Phytopath Soc Jpn 49:56–62

Yokosawa R, Ogoshi A, Sakai R (1974) Taxis of *Aphanomyces raphani* to hypocotyl of host plant and role of exudates from hypocotyl. Ann Phytopath Soc Jpn 40:46–51

Yokosawa R, Kuninaga S, Sekizaki H (1986) *Aphanomyces euteiches* zoospore attractant isolated from pea root: prunetin. Ann Phytopath Soc Jpn 52:809–816

Yokosawa R, Kuninaga S, Sekizaki H (1988) Attractants of *Aphanomyces cochlioides* zoospores contained in sugar beet seedlings. Ann Phytopath Soc Jpn 54:133–140

Zentmyer GA (1961) Chemotaxis of zoospores for root exudates. Science 133:1595–1596

Zheng J, Sutton JC, Yu H (2000) Interactions among *Pythium aphanidermatum*, roots, root mucilage, and microbial agents in hydroponic cucumbers. Can J Plant Pathol 22:368–379

Zhou T, Paulitz TC (1993) In vitro and in vivo effects of *Pseudomonas* spp on *Pythium aphanidermatum* – zoospore behavior in exudates and on the rhizoplane of bacteria-treated cucumber roots. Phytopathology 83:872–876

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