Microbial Inputs in Coffee (*Coffea arabica* L.) Production Systems, Southwestern Ethiopia

Implications for Promotion of Biofertilizers and Biocontrol Agents

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Doctoral thesis
Swedish University of Agricultural Sciences/Uppsala 2007

Acta Universitatis Agriculturae Sueciae
2007:117

Cover: Mature Arabica coffee plant, phosphate solubilizing (clear zone on white back ground) and siderophore producing (yellow zones on blue back ground) rhizobacteria.

(Photo: Alemu Wondafirash (for coffee plant) and Harald Cederlund (for bacterial cultures).

ISSN 1652-6880
ISBN 978-91-85913-16-9

Tryck: SLU Service/Repro, Uppsala 2007

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Abstract

Arabica coffee is the key cash crop and top mainstay of the Ethiopian economy and requires sustainable production methods. Southwestern natural forests, the site of this study, are believed to be the centre of origin and diversity for *Coffea arabica* and still harbour wild Arabica coffee that may serve as an important gene pool for future breeding. Cost reductions, sustainability and quality improvement are now the major priorities in coffee production systems and require organic growing of coffee. Current developments in sustainability involve rational exploitation of soil microbial activities that positively affect plant growth and this study examines this possibility. The composition of coffee shade tree species and density of arbuscular mycorrhizal fungi (AMF) spores and coffee-associated rhizobacteria in different coffee production systems in southwestern Ethiopia were investigated. The main objectives were to: 1) systematically identify the dominant coffee shade tree species; 2) quantify and characterize AMF populations with respect to spatial distribution; 3) screen for beneficial rhizobacteria (microbial biofertilizers and biocontrol agents), particularly in the rhizosphere of coffee plants; and 4) characterize rhizobacterial isolates of particular interest using molecular tools (polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis and 16S rDNA gene sequencing). Sampling and determination of microbial functional characteristics followed standard methods. Nineteen dominant shade tree species belonging to 14 plant families were identified, with the tree legume (*Millettia ferruginea*) dominating. All soil samples contained AMF spores and members of the Glomeromycota, *Glomus* spp. dominating. AMF spore density was affected by sampling point, site, depth, shade tree species and shade tree/coffee plant age. Coffee-associated rhizobacterial isolates showed multiple beneficial traits (phosphate solubilization, production of organic acids, siderophores, indoleacetic acid, hydrogen cyanide, lytic enzymes and degradation of an ethylene precursor). Many isolates also revealed a potent inhibitory effect against emerging fungal coffee pathogens such as *Fusarium xylarioides*, *F. stilboides* and *F. oxysporum*. According to *in vitro* studies *Bacillus*, *Erwinia, Ochrobactrum, Pseudomonas,* and *Serratia* spp. were the most important isolates to act as potential biofertilizers, biocontrol agents or both. Thus, these indigenous isolates deserve particular attention and further greenhouse and field trials could ascertain their future applicability for inoculum development.

Keywords: ACC, fungal coffee pathogens, Glomeromycota, hydrogen cyanide, IAA, lytic enzymes, phosphobacteria, PGPR, siderophores, tree legumes

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Dedication

To my beloved brother Bayyeta Muleta, who had a great dream for my success in education but passed away at a very early age without seeing any of those long journeys.

"The expert at any thing was once a beginner."
-Hayes

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This thesis is based on the work contained in the following papers, referred to in the text by their Roman numerals:

I. Muleta, D., Assefa, F., Nemomissa, S. & Granhall, U. 2007. Composition of coffee shade tree species and density of indigenous arbuscular mycorrhizal fungi (AMF) spores in Bonga natural coffee forest, southwestern Ethiopia. *Forest Ecology & Management 241*, 145-154.

II. Muleta, D., Assefa, F., Nemomissa, S. & Granhall, U. Distribution of arbuscular mycorrhizal fungi spores in soils of smallholder agroforestry and monocultural coffee systems in southwestern Ethiopia. *Submitted*

III. Muleta, D., Assefa, F., Börjesson, E. & Granhall, U. Phosphate-solubilising rhizobacteria associated with *Coffea arabica* L. in natural coffee forests of southwestern Ethiopia. *Submitted*

IV. Muleta, D., Assefa, F. & Granhall, U. 2007. *In vitro* antagonism of rhizobacteria isolated from *Coffea arabica* L. against emerging fungal coffee pathogens. *Engineering in Life Sciences* 7, 1-11.

V. Muleta, D., Assefa, F., Hjort, K., Roos, S. & Granhall, U. Characterization of rhizobacteria isolated from wild *Coffea arabica L.* with emphasis on some plant growth promoting traits. *Submitted*

Abbreviations

AAU	Addis Ababa University
AMF	Arbuscular mycorrhizal fungi
ACC	1-aminocyclopropane-1-carboxylate
CN^-	Cyanide anion
GA	Gluconic acid
GDH	Glucose dehydrogenase
GHG	Greenhouse gases
HCN	Hydrogen cyanide
HAP	Hydroxyapatite
IAA	Indoleacetic acid
IBC	Institute for Biodiversity Conservation
ISP	International Science Programme
MPS	Mineral phosphate solubilization
MHB	Mycorrhiza helper bacteria
PHPR	Plant health promoting rhizobacteria
PI	Inorganic phosphate
PSB	Phosphate solubilizing bacteria
PSMs	Phosphate solubilizing microorganisms
SLU	Swedish University of Agricultural Sciences
L-TRP	L-tryptophan

Introduction

The studies presented in this thesis were carried out within the framework of a bilateral collaboration between the Swedish University of Agricultural Sciences (SLU) and Addis Ababa University (AAU), Ethiopia, with the main objectives of capacity building and research promotion in the agricultural sector in the country in order to stimulate cooperation and biotechnology development. The work was fully funded by the Swedish International Development Cooperation Agency (Sida), through its Department for Research Cooperation (SAREC), and the coordination role was performed by the International Science Programme (ISP), Uppsala University, Sweden. The programme phase dealt with the development of environmentally friendly technologies potentially leading to enhancement of production and productivity of coffee at its centre of origin, southwestern Ethiopia. The project was entitled 'Microbial Inputs in Coffee (*Coffea arabica* L.) Production Systems, Southwestern Ethiopia'. The long-term goals of the studies reported here were to initiate development of new biotechnologies such as the use of biofertilizers and biocontrol agents (microbial inputs) to improve plant growth, *i.e.* coffee production. Understanding of plant-microbial interactions in the coffee rhizosphere with special emphasis on shade trees and the understorey cash crop coffee (from the plant side) and arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria (from the microbial aspect) are vital for low-input sustainable production. Specific research tasks were to: (1) study the composition of coffee shade trees and arbuscular mycorrhizal fungi associated with wild coffee populations; and (2) isolate and characterize (traditional and molecular systematics) beneficial coffee-associated rhizobacteria. Future stages of the project will involve challenging coffee seedlings in greenhouse and field conditions with microbes shown in *in vitro* studies to possess useful attributes, in order to select pertinent bio-inoculants.

Arabica coffee has become a major global commodity. Its cultivation, processing, trading, transportation and marketing provide employment for millions of people. Coffee has for centuries played an important role in the Ethiopian economy and represents the main cash crop cultivated by small-scale farmers for social, economic, political and ecological sustainability (Mekuria *et al.*, 2004; Petit, 2007). Coffee production mainly involves agroforestry-based systems, although there are both natural coffee forests and monoculture plantations. The first two are well accredited in improving soil properties, where coffee grows beneath various shade trees (mainly tree legumes), and are well suited for sustainable production compared with conventional monocultural (unshaded) coffee systems (Cardoso *et al.*, 2003; Gole, 2003). In addition, the presence of wild Arabica coffee at the centre of its origin is of paramount importance for genetic conservation of this global commodity (Aga *et al.*, 2003; Gole, 2003).

The economic and ecological problems of today have re-invigorated the idea of using biofertilizers and biocontrol agents in order to reduce the application of costly and environmentally-polluting agrochemicals to a minimum (Hart & Trevors, 2005; Rodríguez *et al*., 2006). Agrochemicals (namely fertilizers and pesticides) have greatly influenced natural rhizosphere microbes in agrosystems (Matson *et al*., 1997). Plant beneficial microbial bioresources promise to replace or supplement many such destructive, high-intensity practices and support ecofriendly crop production (Hart & Trevors, 2005; Rodríguez *et al*., 2006). In particular, use of arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR) for the benefits of agriculture and ecosystem functions is gaining worldwide importance and acceptance (Vessey, 2003; Lucy *et al*., 2004; Hart & Trevors, 2005; Rodríguez *et al*., 2006). These are bioresources that may become potential tools for providing substantial benefits in agriculture, as they are key elements for plant establishment under nutrient-imbalance conditions. Beneficial soil microbes can help improve plant growth, nutrition and competitiveness and plant responses to external stress factors by an array of mechanisms (Vessey, 2003; Lucy *et al*., 2004; Rodríguez *et al*., 2006). They can also inhibit soil-borne plant pathogens and induce plant resistance to these (Leeman *et al*., 1996**;** Vessey, 2003; Lucy *et al*., 2004).

Mycorrhizal technology can be profitably applied in forestry and in agricultural and horticultural crops for better nutrient utilization (Jeffries *et al*., 2003). The contributions of AMF to coffee production systems in coffee growing regions of the world have been well recognized (Vaast *et al*., 1998; Habte & Bittenbender, 1999). The use of AMF and PGPR as natural fertilizers is reported to be advantageous for the development of sustainable agriculture in nutrient (particularly phosphorus) -deficient tropical soils (Rodríguez *et al*., 2006).

There is currently no published information on the use of AMF and PGPR in Ethiopian Arabica coffee production systems. However, several reports (Jiménez-Salgado *et al*., 1997; Sakiyama *et al*., 2001; Vega *et al*., 2005) reveal that putative agriculturally beneficial bacteria are associated with *Coffea arabica* L.

It therefore appeared worthwhile to quantify and screen indigenous beneficial microbial bioresources at sites where pathogens, antagonists or biofertilizers are expected to display wide abundance and biodiversity. The greatest microbial biodiversity is expected at the centre of origin of the plant species with which they are associated (K. Lindström, pers. comm.) and Requena *et al.* (1997) have verified that the utmost benefit to the plant host arises from native plant beneficial microbes such as AMF and PGPR compared with commercial or introduced forms. Consequently, the

potential biotechnological applications of native microbes in promotion of plant growth have been well accredited (Pandey *et al.*, 2006).

Management of microbes either through selection and inoculation of specific microbial strains or simply by promoting naturally existing microbes holds great promise for sustainable agriculture compared with artificial inputs (Hart & Trevors, 2005; Vassilev *et al.*, 2006). Synergistic interactions with AMF (Artursson *et al.*, 2006) are also of great importance for mycorrhizae-dependent Arabica coffee (Habte & Bittenbender, 1999). Therefore, the work presented in this thesis focused on the composition of coffee shade tree species and on rhizospheric microbes of Arabica coffee (from natural forest, agroforestry-based or monoculture plantations) that displayed biofertilizer or biocontrol agent attributes (Papers I-V), with the long-term aim of enhancing plant growth within sustainable agriculture in the future.

Role of coffee in the Ethiopian economy

The estimated coffee production area (2% of total cultivated land) in Ethiopia is in the range 320,000-700,000 ha (FAO, 1987), although there are a potential 6 million ha of cultivable land suitable for coffee production (Mekuria *et al.*, 2004). In general, all Ethiopian coffee cultivation systems appear to be under the same system of cultivation techniques. However, the major conventional production systems include: i) forest coffee (10%); ii) semi-forest coffee (35%); iii) garden coffee (50%); and iv) plantation coffee (5%) (Aga *et al.*, 2003; Mekuria *et al.*, 2004; Petit, 2007).

The economy of Ethiopia is based on agriculture, and coffee is the central agricultural export product. Historically, Ethiopia is the oldest exporter of coffee in the world and it is the largest coffee producer and exporter in Africa (ITC, 2002). Coffee is a means of subsistence for the rapidly growing population of the country as a complement or even sole source of income, and it plays a fundamental role in both the cultural and socio-economic life of the nation. LMC (2003) estimates that 15 million people are dependent on coffee for at least a significant part of their livelihood. Ethiopian coffee (Arabica coffee) ranks highly in intrinsic quality of the bean (Bhattacharya & Bagyaraj, 2002) and it is the principal economic species, contributing over 70% of the world's commercial coffee (Gole *et al.*, 2002). Ethiopian farmers normally produce nine spectra of the finest single-origin/speciality coffees (Jimma, Nekemte, Illubabor, Limu, Tepi, Bebeka, Yirga Chefe, Sidamo and Harar), which are now well diffused into the trade circuits of the coffee industry (Mekuria *et al.*, 2004).

Southwestern Ethiopia, the origin of wild Arabica coffee

More genetically diverse cultivars of *C. arabica* exist in Ethiopia than anywhere else in the world (Aga *et al.*, 2003), which has led botanists and scientists to agree that Ethiopia is the centre of origin (primary gene centre) for diversification and dissemination of the coffee plant (Fernie, 1966; Zeven & Zhukovsky 1975; Bayetta, 2001). Currently, natural coffee forests are limited mostly to the southwestern area of the country, where remnants of rainforest still exist on patchy areas (Taye, 2001; Gole *et al.*, 2002; Aga *et al.*, 2003; Gole, 2003). These contain the only wild populations of *Coffea arabica* in the world, which may serve as a gene pool for further international Arabica coffee breeding activities (Fernie, 1966; Zeven & Zhukovsky, 1975; Bayetta, 2001; Aga *et al.*, 2003; Gole, 2003). They are also highly important for *in situ/ex situ* conservation of Arabica coffee. It is well accepted that coffee seeds in general cannot be stored for long-term conservation in seed gene banks (Aga, 2005), and therefore the collections of coffee genetic resources are traditionally maintained as living trees or shrubs in field gene banks (Berthaud & Charrier, 1988). Thus, this southwestern area of Ethiopia is of particular value to the world as a whole, as it is the home and cradle of biodiversity of Arabica coffee seeds with the best inherent quality (Bhattacharya & Bagyaraj, 2002) and production potential (Zeven & Zhukovsky, 1975) due to the occurrence of wild coffee populations. In southwestern Ethiopia, agroforestry-based and monoculture coffee systems are also extensively cultivated. The potential of coffee production in this region is very high as a result of suitable altitude, ample rainfall, optimum temperature (Gemechu, 1977), suitable planting material (van der Vossen, 2001; Aga *et al.*, 2003) and good soil fertility (Höfner, 1987). Thus, because of the aforementioned facts, increased attention has been drawn to this region.

Shade coffee production for sustainable land use: Overview

Agroforestry systems can increase soil nutrient availability and accelerate phosphorus cycling due to the fact that the deeper tree roots remarkably improve soil conditions (Young, 1997). This kind of land use system is therefore of paramount importance, particularly in densely populated, sloping regions in the humid and sub-humid tropics, which includes the major coffee growing areas of Ethiopia.

Intensive methods of unshaded coffee production do not take into consideration the environmental and social consequences (Polzot, 2004).

Normally, sun-grown coffee displays a reduction in structural complexity and diversity and is associated with a number of negative by-products, ranging from reduced forest cover, increased soil erosion, chemical runoff and water contamination to consolidation of plantations into large agribusinesses. It has also been suggested that monoculture reduces the spectrum of beneficial fungal species found in the soil after several years of continuous cultivation or when natural ecosystems are transformed into agro-ecosystems (Sieverding, 1991). Such transformation is a common practice in southwestern Ethiopia, where the present studies were carried out (Paper II). The current instability in coffee prices on the world market can be attributed to transition from shade-grown to sun-grown coffee (Rice & McLean, 1999). However, recently a paradigm shift has begun to occur, where traditional production systems that were once considered unprofitable are being revisited (Polzot, 2004). Studies have revealed that the agroforestry coffee systems are more effective in promoting soil conservation than conventional monoculture (unshaded) coffee systems (Cardoso *et al.*, 2003).

Moreover, coffee has favourable characteristics for agroforestry practices. In its original habitat, coffee naturally occurs in native forests (Taye, 2001; Aga *et al.*, 2003; Gole, 2003; Paper I). The period of flowering, when coffee requires more light, coincides with the dry season, in which the agroforestry trees lose their leaves. A side effect of this is that coffee trees do not compete for water with other species (Polzot, 2004). Coffee production increases when grown in habitats suitable for sustaining pollinators, for instance, honey bees in shade-grown coffee (Roubik, 2002). Therefore, increasing tree cover in coffee production is a viable option for mitigating climate change that also provides social, economic and ecological benefits (Polzot, 2004). Like other agroforestry systems that employ a woody component, shade-grown coffee agroecosystems contribute to the removal of carbon from the atmosphere and its storage on land.

In Ethiopia, farmers traditionally grow coffee as an important cash crop under various types of shade trees, mainly dominated by leguminous tree species (Taye, 2001; Gole, 2003; Papers I & II). Wide use of tree legumes for providing shade has also been well documented in many coffee growing countries across the globe (Perfecto *et al.*, 1996; Albertin & Nair, 2004; Polzot, 2004). The list of well-known and dominant shade trees documented in Ethiopia increases from time to time but mainly encompasses *Albizia*, *Acacia*, *Bersama*, *Cordia*, *Croton*, *Dracaena, Entada, Ehretia, Erythrina, Ficus, Leucaena, Millettia, Olea, Pavetta, Prunus, Schefflera, Syzygium* and others (FAO, 1968; Teketay & Tegeneh, 1991; Taye, 2001; Gole, 2003; Papers I & II).

Smallholders represent 95% of total production in low input-low output systems, making shaded Ethiopian coffee production naturally 'organic' (Petit, 2007). Farmers usually do not apply agrochemicals and Ethiopia has the potential to produce certified organic high quality coffee due to favourable growing conditions and the high diversity of genetic resources in *Coffea arabica* (Aga *et al.*, 2003; Mekuria *et al.*, 2004). Thus, the present investigation placed special emphasis on this type of production system, which protects the environment and maintains biodiversity due to shade tree species (Perfecto *et al.*, 1996). The effect of shade trees on Arabica coffee production has been tested for a long time and the general belief is that the advantages outweigh the suggested negative impacts (Beer *et al.*, 1998; Muschler, 2001).

Improvement of coffee attributes

Evidence is increasing that better coffee attributes are generally produced by shaded systems, particularly those dominated by tree legumes (Muschler, 2001; Muleta *et al.*, unpubl.). More precisely, studies from Costa Rica (Muschler, 2001) have determined the main benefits of shading on coffee plants to be: (1) higher weight of fresh fruits; (2) larger beans; (3) higher visual appearance ratings for green and roasted beans; (4) higher acidity and body ratings; and (5) absence of off-flavours.

Climate regulation

The importance of overstorey trees in buffering temperature extremes (day/night) in coffee production systems is well documented (Beer *et al.*, 1998; Polzot, 2004). Shade is reported to reduce the effect of excessive heat on the coffee plants during the day and to reduce heat losses at night. Furthermore, Beer *et al.* (1998) have recorded the advantages of tree cover in reduction of wind speed, which in turn minimizes crop desiccation and soil erosion losses. Shade trees also make a great contribution in reduction of hail damage (Beer *et al.*, 1998; Muleta *et al.*, unpubl).

Organic matter contribution, nutrient cycling and maintenance of biodiversity

The roles of coffee agroecosystems in contributing massive leaf litter input, stimulating organic matter turnover and decreasing soil erosion have been well addressed (Beer *et al.*, 1998). Coffee agroecosystems store significant amounts of carbon in aboveground woody biomass of shade trees, the litter layer and soil organic matter compared with unshaded systems, and thus act

as potential carbon sinks (Polzot, 2004). Significant aboveground plant carbon pools contribute to reductions in greenhouse gas (GHG) emissions and the alleviation of GHG accumulation in the atmosphere. Beer *et al.* (1998) point out that coffee agroecosystems could prevent the release of up to 1000 t C ha^{-1}. Thus, the contributions shaded coffee plantations make to climate change mitigation can be quite significant (Polzot, 2004).

Tree legumes predominate as overstorey trees, both in natural coffee forests (Taye, 2001; Paper I) and agroforestry-based coffee systems (Paper II) in southwestern Ethiopia. Leguminous shade trees are acknowledged for their good capacity for fixing atmospheric nitrogen (Granhall, 1987; Beer *et al.*, 1998) by forming symbiotic associations with certain soil bacteria, rhizobia (Roskoski, 1982; Assefa & Kleiner, 1998; Grossman *et al.*, 2006). In Mexico, organic farmers claim that *Inga* (tree legume) shade improves coffee plant health (Grossman, 2003). Similarly, in Costa Rica (Albertin & Nair, 2004) and in Ethiopia (Muleta *et al.*, unpubl), the majority of farmers commonly mention legume shade trees as the first class tree species to include in their coffee fields. Altogether, native leguminous tree species are often used to supply all or a proportion of the N needs of coffee bushes and reduce the dependence on synthetic fertilizers (Soto-Pinto *et al.*, 2000; Sprent & Parsons, 2000; Grossman *et al.*, 2006), which is fundamental to low-input sustainable agricultural practices in most developing countries.

In Ethiopia, various types of shade trees in agroforestry-based coffee plantations (Asfaw, 2003) and afromontane forests (Wubet *et al.*, 2003, 2004) have been reported to form associations with certain beneficial soil fungi, *e.g.* arbuscular mycorrhizal fungi (AMF). More precisely, coffee bushes under some shade trees, mainly leguminous, in both natural coffee forest (Paper I) and agroforestry-based coffee (Paper II) are associated with higher numbers of AMF spores than those under non-leguminous trees. Beer *et al.* (1998) verified that nutrient turnover and the transfer of major bioelements N, P, K, Ca, and Mg to the soil are greater in shaded plantations due to excess litter from both trees and coffee bushes.

Increased shade density and complexity is reputedly highly beneficial for conservation of biodiversity (Perfecto *et al.*, 1996; Polzot, 2004). Perfecto *et al.* (1996) have reported that many traditional shaded coffee plantations resemble natural forests more than any other agricultural system in use, in terms of structure and ecology. Studies in Costa Rica indicate that shaded coffee systems can support greater numbers of animal populations (Hall, 2001) and can act as buffer zones to protected areas and serve as biological corridors, thus providing pathways for the migration of fauna between natural reserves (Polzot, 2004).

Weed suppression

Canopy cover may suppress the major weeds in coffee plantations, such as African couch grass (*Digitaria scalarum*), which in turn can minimize synthetic herbicide application and reduce labour inputs, giving rise to cheaper production (Beer *et al.*, 1998). In Bonga natural coffee forest, the lower stratum ($\leq$ 2 m) contained various plant species, mainly *Desmodium* (Paper I), which has been reported to be an efficient suppressor of aggressive and spontaneous weeds (Bradshaw & Lanini, 1995).

Reduction of disease and pest problems

Cool and wet weather in combination with increased shade can favour the incidence of some fungal diseases in shaded coffee systems. Nevertheless, shade has also been shown to minimize the occurrence of some fungal diseases that may pose serious problems in sun-grown crops (Polzot, 2004). In addition, Beer *et al.* (1998) indicate that shade trees may provide habitats for biological control agents due to their rich biodiversity, thus reducing the prevalence of disease and the dependence on pesticides in shaded coffee production systems.

Minimizing groundwater pollution risks

Groundwater can be contaminated during application of synthetic fertilizers in sun-grown coffee fields, often causing increased health risks. Beer *et al.* (1998) reported that groundwater contamination by nitrate and nitrite is more common under intensive coffee production with little or no shade compared with shaded coffee production systems.

Food production and other benefits

Other valuable benefits associated with shade trees involve fruits suitable as food (Peeters *et al.*, 2003). The inclusion of fruit-bearing trees as shade in coffee plantations provides farmers with access to additional foods, such as mangos, oranges, bananas and avocados (Polzot, 2004).

Apart from their contribution to understorey coffee bushes, farmers derive benefits from shade trees in terms of firewood and timber (Beer *et al.*, 1998; Peeters *et al.*, 2003, Muleta *et al.*,unpubl.). For instance, *Cordia africana*, the main timber tree in the country, universally provides shade to coffee plants in southwestern Ethiopia (FAO, 1968). Timber-producing shade trees have low management costs and can be considered "revenue storage" for farmers

that can be cashed during periods of low coffee prices or crop failure (Polzot, 2004). Other valuable benefits associated with shade trees involve honey production and other options for income (Hailu *et al.*, 2000, Muleta *et al.*, unpubl.). In Ethiopia, the most common shade tree species such as *Croton macrostachyus* (Giday, 2001), *Albizia gummifera* and *Syzygium guineense* (Geyid *et al.*, 2005) also play a vital role in traditional medicine to combat various infectious diseases.

Another added advantage of shaded coffee systems is the ever increasing demand and willingness of consumers to pay best prices for organic and fair-trade coffee (Wikström, 2003; van der Vossen, 2005). Premium prices may compensate for the possibly low yield but economically viable and sustainable returns of shaded coffee systems (Beer *et al.*, 1998).

Arbuscular mycorrhizal fungi (AMF)

AMF are soil-dwelling fungi that form associations with the roots of a plethora of terrestrial plants (angiosperms, gymnosperms and many pteridophytes and bryophytes) by forming distinct symbiotic structures (Fig. 1). The AM fungi were formerly included in the order Glomales in the Zygomycota (Redecker *et al.*, 2000), but they have recently been moved to a new phylum, the Glomeromycota (Schüßler *et al.*, 2001).This group of fungi is still an untapped resource for sustainable soil management. They are ubiquitous soil-borne microbial fungi, whose origin and divergence have been dated back more than 450 million years (Redecker *et al.*, 2000). AMF can be found in virtually almost all ecosystems in temperate, tropical and arctic regions, except under waterlogged conditions (Smith & Read, 1997). As a group, they may have the single largest effect on plant performance of any rhizosphere-associated microbe, functioning as an extension of the root system of the plant and increasing absorptive area (Leake *et al.*, 2004). Arbuscular mycorrhizal (AM) associations are of great importance in forest ecology, land rehabilitation, plant health and yield in low input systems of the tropics through key ecological processes (Sieverding, 1991).

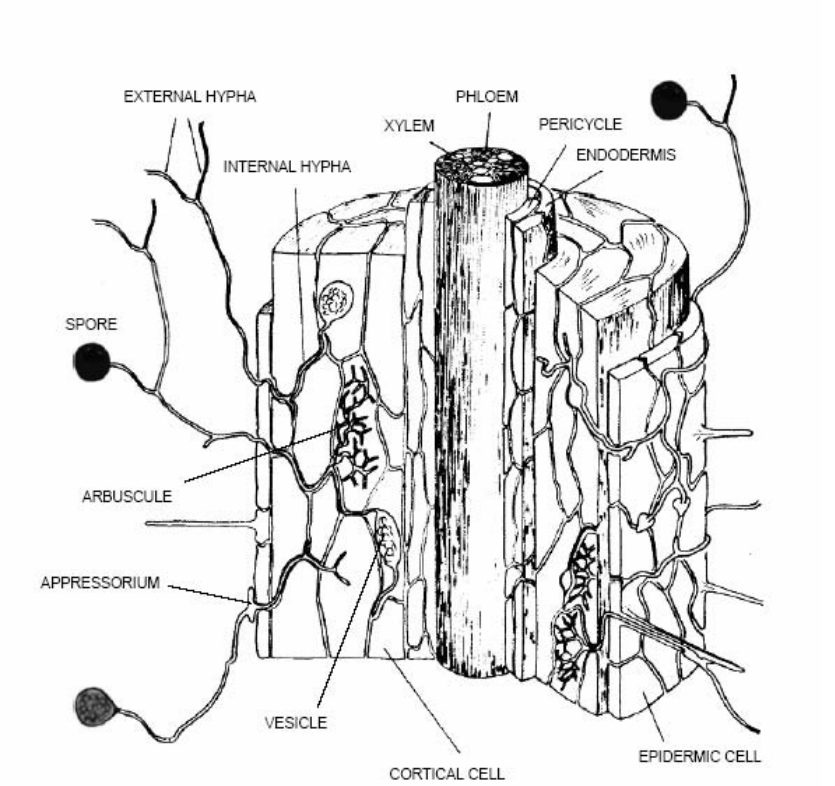


Fig. 1. Cross-section of a plant root with mycorrhizal features (Source: Azcón-Aguilar & Barea, 1980).

Agronomic and ecological roles of AMF

Most of the root systems of agricultural/horticultural plants and crops are colonized by AMF (Sieverding, 1991). The most prominent effect of the fungus is improved phosphorus nutrition of the host plant in soils with low phosphorus levels due to the large surface area of their hyphae and their high affinity P uptake mechanisms (Koide, 1991). There are also reports of

production by AMF of organic acids that could solubilize the insoluble mineral phosphates (Lapeyrie, 1988), an added advantage in terms of improvement of P uptake by host plants. AMF mycelia have also been shown to increase uptake of many other nutrients, including N, S, B, Cu, K, Zn, Ca, Mg, Na, Mn, Fe, Al, and Si (Clark & Zeto, 2000). In some cases, AMF may be responsible for acquiring 100% of host nutrients (*e.g.* P; Smith *et al.,* 2004). Marschner (1998) and Hodge & Campbell (2001) have indicated that the improved plant nutrition is due to (i) increased root surface through extraradical hyphae, which can extend beyond root depletion zone, (ii) degradation of organic material and (iii) alteration of the microbial composition in the rhizosphere.

New research suggests that AMF have multiple ecosystem functions and are ideal tools for any field where plants and their communities are manipulated, including sustainable agriculture, landscape restoration and horticulture, among others (Fig. 2; Hart & Trevors, 2005). This multifunctional nature of AMF encompasses mineralization of organic nutrients, seedling establishment, increased pathogen resistance, herbivore tolerance and pollination, and soil stability, heavy metal tolerance/bioremediation, drought (hydraulic stresses)/chilling resistance and alleviation of desertification among others (Fig. 2; Jeffries *et al*., 2003; Hart & Trevors, 2005).

The roles of AMF to their hosts in a given environment, however, are largely dependent on the nutrient status of the soil, particularly P. Highly fertile soils generally exhibit lower mycorrhizal fungal populations. It is known that the AM fungi are not able to colonize plant roots strongly under P-sufficient conditions (Koide & Schreiner, 1992). In certain cases, the growth rates of plants can be reduced by AM colonization in the presence of available P (Peng *et al*., 1993).

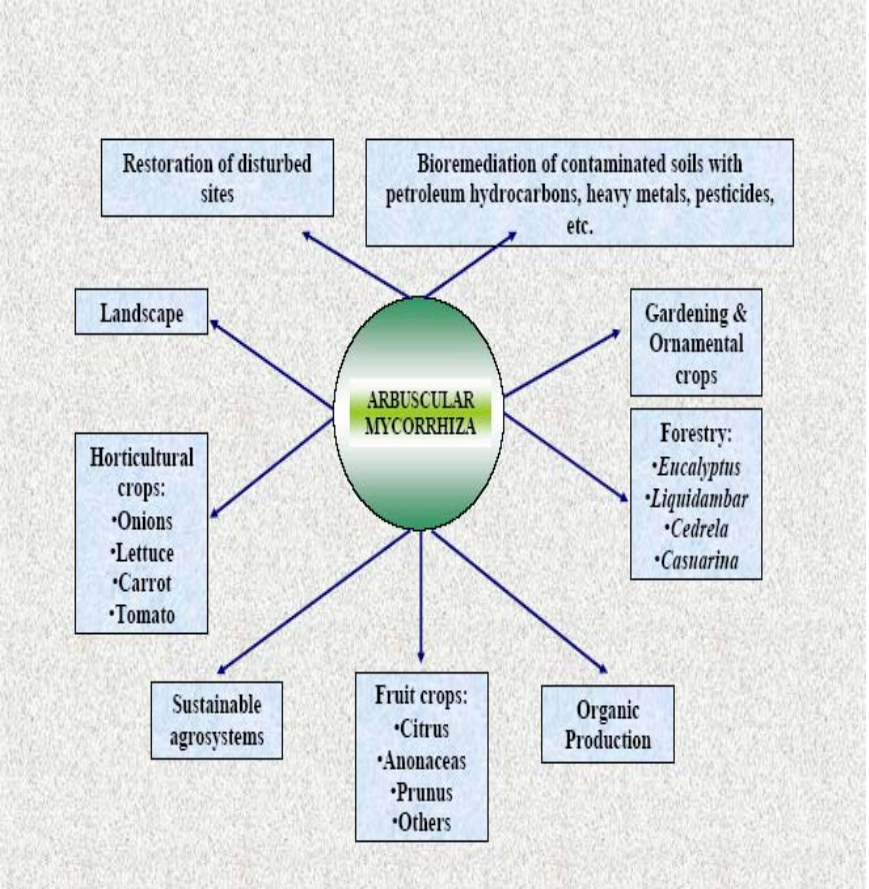


Fig. 2. Wider applications of AMF in production systems and ecosystems (Source: http://aggie-horticulture.tamu.edu/faculty/davies/students/alarcon/AMFApplications.pdf; 10-Jul-2007)

AMF and horticultural crop production (e.g. *Coffea arabica* L.)

Agricultural land carrying low input production systems is a natural mycorrhizal habitat, with a high diversity of AMF (up to 40 species per site; Vandenkoornhuyse *et al.*, 2002). Most horticultural and crop plants are symbiotic with arbuscular mycorrhizal fungi and drive great benefits from these particular associations. Coffee plants (*Coffea arabica*) are usually associated with arbuscular mycorrhizal (AM) fungi and highly dependent on these particular associations (Habte & Bittenbender, 1999; Miyasaka & Habte, 2001). A total of 22 species of AM fungi that are important in

Arabica coffee plantations in central Sao Paulo State, Brazil, have been identified, with predominance of *Glomus*, *Acaulospora* and other genera (Lopes *et al.*, 1983). Cardoso *et al.* (2003) have demonstrated differences in the distribution of mycorrhizal fungal spores in soils under agroforestry and monocultural coffee systems in Brazil, with higher AMF spore density under the former production system, in keeping with the results from Ethiopia (Paper II). Arabica coffee rhizospheres in both natural forest (Paper I) and agroforestry-based coffee production systems (Paper II) in southwestern Ethiopia contain AMF propagules, with predominance of *Glomus*. Various types of shade trees in forests (Wubet *et al.*, 2003, 2004), including medicinal and nitrogen-fixing species, have also been found to be associated with AMF in Ethiopia. Furthermore, investigations in natural forests (Muleta *et al.*, unpubl.) indicate that wild Arabica coffee seedlings show a reasonable level of root colonization (30%) as observed elsewhere (Lopes *et al.*, 1983).

The benefits that coffee plants obtain from AMF associations include improved growth, nutrition, water relations and tolerance to pathogens and/or parasitic nematodes. Vaast & Zasoski (1992) evaluated the effects of AMF and nitrogen sources on rhizosphere soil characteristics, growth and nutrient acquisition of Arabica coffee seedlings and showed that mycorrhizal plants grew better and accumulated more N, Ca and Mg than non-mycorrhizal plants. Furthermore, Fernández-Martín *et al.* (2005) investigated the effects of AM and a soil-earthworm mixture on the growth of coffee plants and revealed that leaf area increased by 6-140% with AM application and that mass of the endophytic mycorrhizal fungi was inversely dependent on soil fertility.

Vaast *et al.* (1997) investigated the effects of a root-lesion nematode (*Pratylenchus coffeae*), AM fungi and timing of inoculation on the growth and nutrition of a nematode-susceptible Arabica coffee cultivar. The results indicated that in the presence of *P. coffeae*, early AM-inoculated plants remained P sufficient and their biomass was 75-80% of that of nematode-free controls.

The benefits that AMF impart to their hosts vary depending on specific time of application. The best results are often obtained when plants are inoculated during propagation (micropropagation, cuttings and seedlings). For instance, AMF inoculation showed a significant positive effect (P-sufficient) on *in vitro* propagated Arabica coffee microcuttings compared with control plants (Vaast *et al.*, 1997).

Plant growth promoting rhizobacteria

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. In the rhizosphere, very important and intensive interactions take place between the plant, soil, microorganisms and soil microfauna, influenced by compounds exuded by the root and by microorganisms feeding on these compounds (Antoun & Prévost, 2006). All this activity makes the rhizosphere the most dynamic environment in the soil. Gobat *et al*. (2004) have distinguished three rhizosphere fractions: 1) the endorhizosphere (interior of the root); 2) the rhizoplane (surface of the root); and 3) the rhizospheric soil that adheres to the root when the root system is shaken manually. The volume of the soil that is not influenced by the root is defined as non-rhizospheric soil or bulk soil.

The rhizosphere is the front-line between plant roots and soil-borne pests. Therefore it seems logical that microorganisms that colonize the same niche could be ideal candidates for sustainable agriculture (Weller, 1988). In the rhizosphere, bacteria are the most abundant microorganisms (Antoun & Prévost, 2006). Rhizobacteria are rhizosphere-competent bacteria that aggressively colonize plant roots; they are able to multiply and colonize all the ecological niches found on the roots at all stages of plant growth, in the presence of a competing microflora (Antoun & Kloepper, 2001). Rhizobacteria can have a neutral, detrimental or beneficial effect on plant growth. Deleterious rhizobacteria are presumed to adversely affect plant growth and development through the production of undesirable metabolites (phytotoxins) or through competition for nutrients or inhibition of the beneficial effects of mycorrhizae (Sturz & Christie, 2003).

Beneficial rhizobacteria are termed either plant growth promoting rhizobacteria (PGPR) or plant health promoting rhizobacteria (PHPR) according to their mode of action (Sikora, 1992). The term PGPR was first used by Kloepper & Schroth (1978) and investigations on PGPR have been escalating at an ever increasing rate since then.

The PGPR are defined by three intrinsic characteristics (Barea *et al*., 2005): (i) they must be able to colonize the root, (ii) they must survive and multiply in microhabitats associated with the root surface, in competition with other microbiota, at least for the time needed to express their plant promotion/protection activities, and (iii) they must promote plant growth. The PGPR are known to participate in many important ecosystem processes. They were first used for agricultural purposes in the former Soviet Union and India and are now being tested worldwide (Lucy *et al*., 2004). These authors have also summarized the benefits of PGPR for plant growth,

which include increases in: germination rate, root growth, yield (including grain), leaf area, biocontrol, chlorophyll content, hydraulic activity, tolerance to drought, shoot and root weights.

Mechanisms of action: Overview

A wide array of beneficial rhizosphere bacteria have been categorized as PGPR including mainly diazotrophs, bacilli, pseudomonads and rhizobia (Antoun & Prévost, 2006). PGPR may induce plant growth promotion through different direct or indirect modes of action (Glick *et al.*, 1999; Antoun & Prévost, 2006). Direct mechanisms include improvement of plant nutrient status (liberation of phosphates and micronutrients from insoluble sources; non-symbiotic nitrogen fixation), iron sequestration by siderophores, the production of bacterial volatiles and phytohormones and lowering of the ethylene level in the plant. The indirect effects can be exerted by antibiotic production, depletion of iron from the rhizosphere, induced systemic resistance, synthesis of antifungal metabolites, production of fungal cell wall lysing enzymes, competition for sites on the root, stimulation of other beneficial symbioses and degradation of xenobiotics in inhibitor-contaminated soils. Somers *et al.* (2004) have classified PGPR into the following functional groups depending on their inherent activities as: i) biofertilizers (increasing the availability of nutrients to the plant), ii) phytostimulators (plant growth promoting, usually by the production of phytohormones: auxin, cytokinin, gibberelin), iii) rhizoremediators (degrading organic pollutants), and iv) biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites).

Phosphate solubilizing bacteria (PSB)

Theoretical estimates have suggested that the accumulated phosphorus (P) in agricultural soils due to fixation is sufficient to sustain maximum crop yields world-wide for about 100 years (Goldstein *et al.*, 1993). However, although P is abundant in soils in both inorganic form (originating mainly from applied P fertilizer) and organic form (derived from microorganisms, animals and plants) (Paul & Clark, 1989), it is still one of the major plant growth-limiting nutrients. On average, most nutrients in the soil solution are present in millimolar amounts, but phosphorus is present only in micromolar or lesser quantities (Ozanne, 1980). These low levels of P are due to the high reactivity of soluble P with calcium (Ca), iron (Fe) or aluminium (Al), which leads to P precipitation (Fig. 3). Inorganic P in acidic soils is associated with Al and Fe compounds, whereas calcium phosphates are the predominant form of inorganic phosphates in calcareous soils.

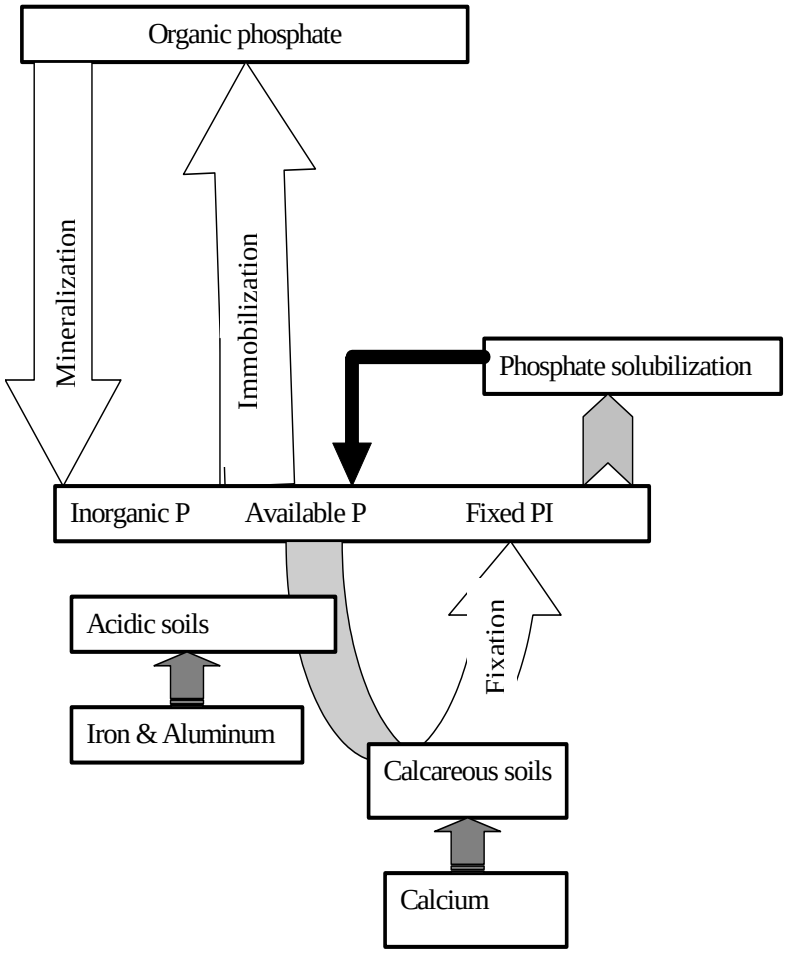


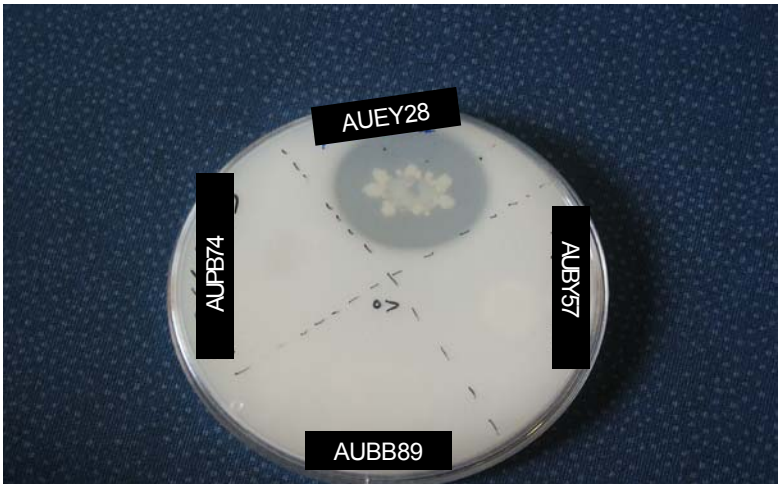
Fig. 3. Phosphorus channels in soil. (Source: modified from Bagyaraj *et al.*, 2000).

Organic P may also make up a large fraction of soluble P, as much as 50% in soils with high organic matter content (Barber, 1984). Phytate, a hexaphosphate salt of inositol, is the major form of P in organic matter, contributing between 50 and 80% of the total organic P (Alexander, 1977). Although microorganisms are known to produce phytases that can hydrolyze phytate, phytate tends to accumulate in virgin soils because it is rendered insoluble as a result of forming complex molecules with Fe, Al and Ca (Alexander, 1977). Phospholipids and nucleic acids form a mother pool of labile P in soil that is easily available to most of the organisms present (Molla & Chowdary, 1984).

To circumvent the problem of P deficiency, the addition of phosphate fertilizers has become a common practice in modern agriculture. The production of chemical phosphate fertilizers is a highly energy-intensive process, requiring energy worth US$4 billion per annum in order to meet the global needs (Goldstein *et al.*, 1993). The situation is further compounded by the fact that almost 75-90% of added P fertilizer is precipitated by Fe, Al and Ca complexes present in the soils, creating a demand for suitable alternatives to mobilize this fixed fraction of the important bioelement (Stevenson, 1986). Soil microorganisms are able to mobilize insoluble mineral phosphate in a more environmentally friendly and sustainable manner.

The involvement of microorganisms in solubilization of inorganic phosphates was known as early as 1903 (Kucey *et al.*, 1989). It is estimated that P solubilizing microorganisms may constitute 20 to 40% of the culturable population of soil microorganisms and that a significant proportion of these can be isolated from rhizosphere soil (Kucey, 1983; Chabot *et al.*, 1993). Most PSB are isolated from the rhizosphere of various plants and are known to be metabolically more active than those isolated from sources other than rhizosphere (Baya *et al.*, 1981). In the present study, over 72% of the rhizobacteria (both Gram-negative and Gram-positive) associated with wild Arabica coffee rhizospheres were shown to be able to solubilize mineral P (Paper III). Important phosphate solubilizing microorganisms (PSMs) including bacteria and fungi have been well reviewed (Rodríguez & Fraga, 1999). In general, P solubilizing bacteria commonly outnumber P solubilizing fungi 2-150 fold (Kucey, 1983; Kucey *et al.*, 1989). However, fungal isolates exhibit greater P solubilizing ability than bacteria in both liquid and solid media (Kucey, 1983). In addition, the P solubilizing ability in bacteria (Fig. 4; Paper III) may be lost upon repeated sub-culturing but no such loss has been observed in the case of P solubilizing fungi (Kucey, 1983). The majority of the phosphate solubilizing microorganisms (PSMs) mobilize Ca-P complexes and only a few can solubilize Fe-P and Al-P complexes (Kucey *et al.*, 1989).

(a)



(b)

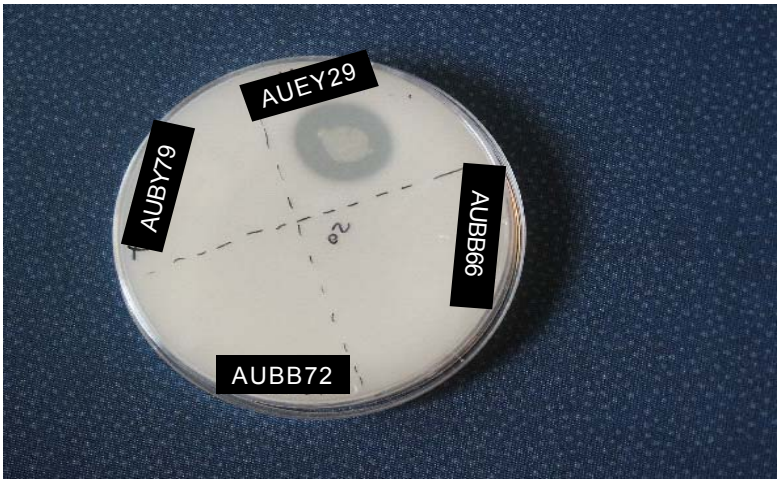


Fig. 4. Insoluble phosphate solubilization studies on Pikovskaya's agar (PA): (a) and (b) show two consistent and efficient phosphate solubilizing isolates (large haloes), whereas six others lost their activity (no visible halo) during repeated subculturing on PA (Paper III).

Phosphorus biofertilizers in the form of microorganisms can help in increasing the availability of fixed phosphates for plant growth by

solubilization (Goldstein, 1986; Kucey *et al.*, 1989). PSMs also exhibit other traits beneficial to plants, such as production of phytohormones, antibiotics, siderophores, vitamins, antifungal substances and hydrogen cyanide (Kloepper *et al.*, 1989; Rodríguez & Fraga, 1999; Papers IV & V). In addition to being better scavengers of soluble P (P biofertilizers), the microorganisms involved in P solubilization can also enhance plant growth by increasing the efficiency of biological nitrogen fixation, enhancing the availability of trace elements such as Fe, Zn, *etc.* (Fig. 5; Kucey *et al.*, 1989; Rodríguez & Fraga, 1999). It is well established that every aspect of the process of formation of the N_2 fixing nodule is limited by the availability of P and legumes show a high positive response to P supplementation (Deng *et al.*, 1998). This most likely has significant positive implications for the dominant legume shade trees in the current study areas (Papers I & II).

At the molecular genetics level, the precise mechanism used by different PSMs still remains mostly unidentified (Rodríguez *et al.*, 2006). Nevertheless, it is generally believed that the production of organic acids, added to a steep drop in pH, is the main driving force for mobilization of mineral phosphates (Illmer *et al.*, 1995; Goldstein, 1996; Rodríguez & Fraga, 1999; Paper III). Moreover, Goldstein (1996) proposed direct glucose oxidation to gluconic acid (GA) as a major mechanism for mineral phosphate solubilization (MPS) in Gram-negative bacteria. As a result of acidification of the surrounding medium, soluble orthophosphate ions ($H_2PO_4^{-1}$ and HPO_4^{-2}) can be readily released. The PSMs produce a range of low molecular weight organic acids such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, glycolate, *etc.* (Goldstein, 1986; Kim *et al.*, 1998; Paper III). More precisely, the organic acids secreted can either directly dissolve the mineral phosphate as a result of anion exchange of PO_4^{-3} by acid anion or can chelate both Fe and Al ions associated with phosphate (Moghimi *et al.*, 1978). Strong support for this suggested mechanism has been provided by evidence that addition of NaOH abolishes the P solubilization process, indicating that pH reduction of the system is responsible for the P solubilizing abilities of PSMs.

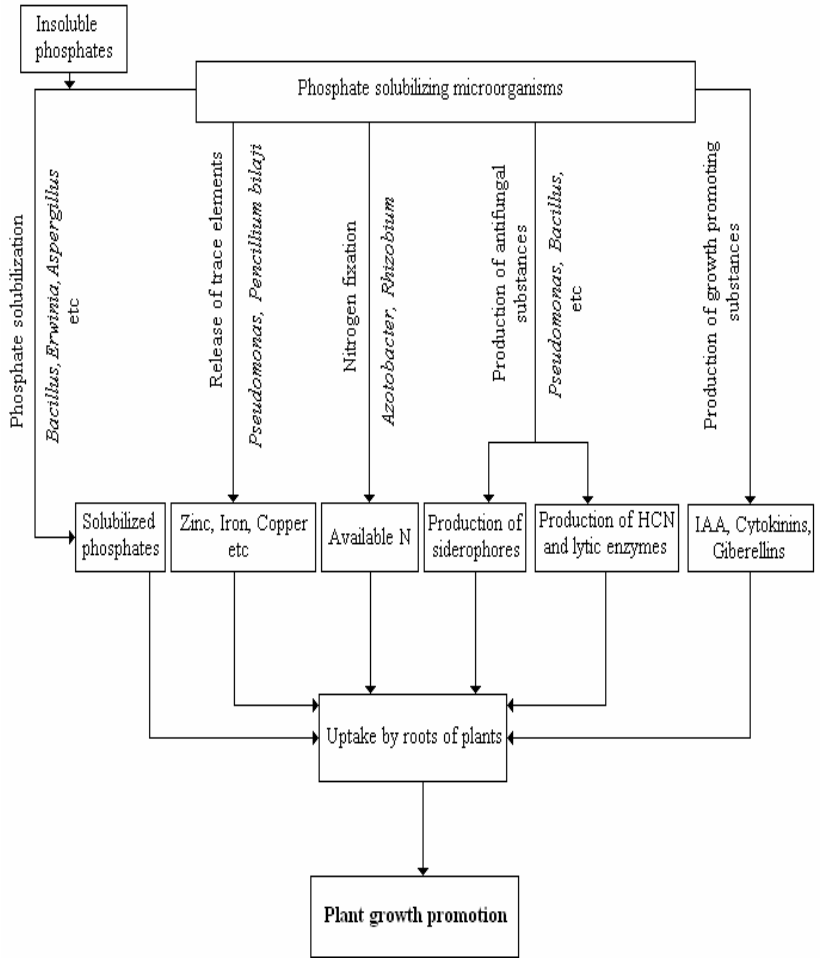


Fig. 5. Mechanisms of plant growth promotion by PSMs. (Source: Modified from Khan *et al.*, 2006; Papers III, IV, V).

However, acidification does not seem to be the only mechanism of P solubilization, as the ability to reduce the pH in some cases does not correlate with the ability to solubilize mineral phosphates (Subba Rao, 1982). For instance, a genomic DNA fragment from *Enterobacter agglomerans* showed mineral phosphate solubilization activity in *E. coli* JM109, although the pH of the medium was not altered (Kim *et al.*, 1997). Similarly, Kucey (1988) has demonstrated that the chelating property of the organic acids is also important, as it has been shown that the addition of 0.05M ethylene diamine tetraacetic acid (EDTA) to the medium has the same solubilizing effect as inoculation with a phosphate solubilizing organism. In addition, under some circumstances phosphate solubilization has been observed at

only slightly acidic or alkaline pH values (Altomare *et al.*, 1999). On the other hand, mineral phosphate solubilization has been reported in the absence of detectable chelating agents or organic acids, merely by acidifying the medium (Illmer *et al.*, 1995). Overall, the exact mechanisms utilized by PSMs remain to be discovered (Rodríguez *et al.*, 2006).

Microorganisms also rely on various forms of enzymes (Garcia *et al.*, 1992; Rodríguez *et al.*, 2006) in order to mobilize organic phosphate sources. These include: (1) non-specific phosphatases, which perform dephosphorylation of phospho-ester or phosphoanhydride bonds in organic matter; (2) phytases, which specifically cause P release from phytic acid; and (3) phosphonatases and C-P lyases, enzymes that perform C-P cleavage in organophosphonates. The main activity apparently corresponds to the work of acid phosphatases and phytases because of the predominant presence of their substrates in soil. The overall plant and microbial mechanisms to increase P availability in the rhizosphere excluding mycorrhizal association are presented in Fig. 6.

Production of phytohormones (particularly IAA)

Phytohormones, also called plant growth regulators, are well known for their regulatory role in plant growth and development and work at extremely low concentrations. The most common, best characterized and physiologically most active auxin in plants is indole-3-acetic acid (IAA). L-tryptophan (L-TRP), an amino acid, serves as a physiological precursor for biosynthesis of auxins in higher plants and in microbes (Frankenberger & Arshad, 1995). Root exudates are natural sources of TRP for the rhizosphere microflora, which may enhance auxin biosynthesis in the rhizosphere (Martens & Frankenberger, 1994).

Indoleacetic acid is known to stimulate both a rapid response (*e.g.* increased cell elongation) and a long-term response (*e.g.* cell division and differentiation) in plants (Cleland, 1990). More specifically, IAA is a phytohormone that is known to be involved in root initiation, cell division and cell enlargement (Salisbury, 1994). A significant activity of PGPR is the production of auxin-type phytohormones that affect root morphology and thereby improve nutrient uptake from soil (Barea *et al.*, 2005). Lucy *et al.* (2004) have shown that IAA-producing PGPR increase root growth and root length, resulting in greater root surface area, which enables the plant to access more nutrients from soil.

The capacity to synthesize IAA is widespread among soil- and plant-associated bacteria.

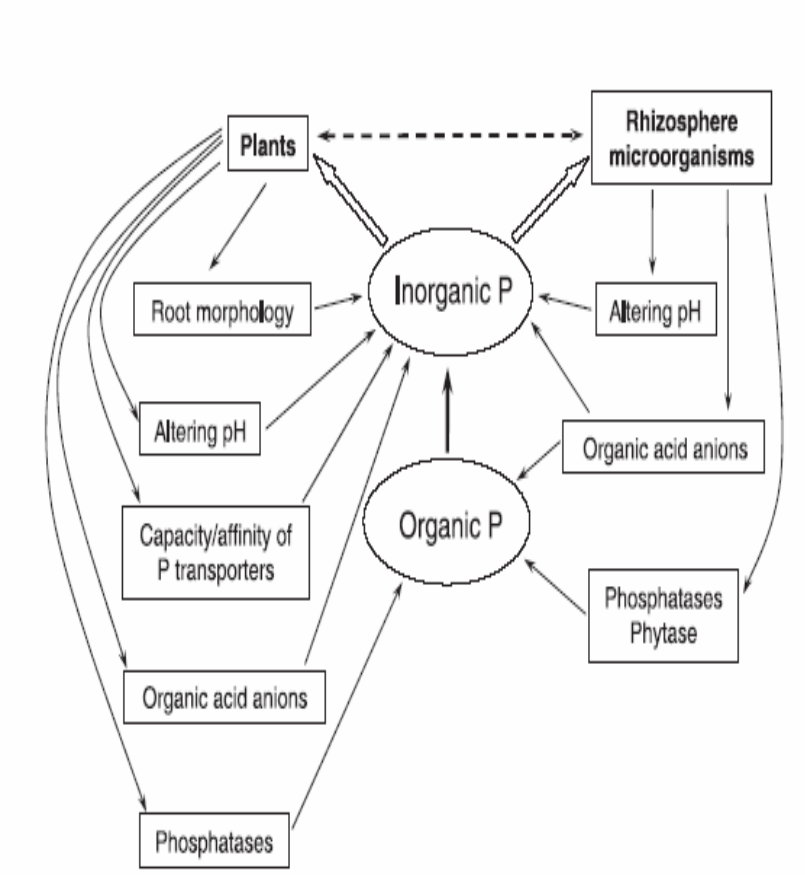


Fig. 6. Plant and microbial mechanisms increasing phosphorus (P) availability in the rhizosphere (mycorrhizal colonization not considered). Plants and microorganisms can increase the availability of inorganic P by altering rhizosphere pH and exuding organic acid anions. Plants can also increase the capacity to take up P by increasing the root surface area via (i) growing long and thin roots with numerous thin root hairs, and (ii) changing the capacity and/or affinity of plasma membrane-embedded P transporters. Plants and microorganisms can mobilize P from organic pools and convert it to available inorganic forms by phosphatases. The phytase enzyme exuded by microorganisms is capable of converting phytate into P esters that phosphatases can break down to inorganic P. The outline arrows indicate P uptake. (Source: Rengel & Marschner, 2005).

By and large, microorganisms isolated from the rhizosphere and rhizoplane of various crops are more active in producing auxins than those from root-

free soil because of rich supplies of substrates exuded from roots compared with non-rhizosphere soil (Strzelczyk & Pokojska-Burdzeij, 1984). A 3-fold higher IAA content was found in the rhizosphere compared with non-rhizosphere environments (Narayanaswami & Veerraju, 1969). It has been estimated that 80% of bacteria isolated from the rhizosphere can produce IAA (Patten & Glick, 1996; Ahmad *et al.*, 2006). Similarly, over 66% of wild Arabica coffee-associated rhizobacteria secreted IAA (Paper V).

A survey of the IAA biosynthesis pathways utilized by plant-associated bacteria reveals that pathogenic bacteria such as *Pseudomonas syringae*, *Agrobacterium tumefaciens* and *Erwinia herbicola* synthesize IAA predominantly via the indole-3-acetamide (IAM) pathway. Synthesis by this route is generally constitutive. PGPR such as *Rhizobium*, *Bradyrhizobium* and *Azospirillum* species synthesize IAA, mainly via the indole-3-pyruvic acid (IPyA) pathway, which may be subject to more stringent regulation by plant metabolites (Patten & Glick, 1996). Other rhizobacteria may produce cytokinins (Timmusk *et al.*, 1999) and gibberellins (Khan *et al.*, 2006).

Lowering of ethylene production

The term 'stress ethylene' was coined by Abeles (1973) to describe the acceleration of ethylene biosynthesis by plants in response to biological and environmental stresses. Ethylene stimulates senescence and leaf and fruit abscission, inhibits plant growth (*i.e.* roots) and triggers cell death near infection sites (Bashan & de-Bashan, 2005). In agriculture it is important to control ethylene levels, often by lowering them in order to prevent economic losses.

1-aminocyclopropane-1-carboxylate (ACC) is the immediate direct physiological precursor of ethylene. Several soil microorganisms, mainly *Pseudomonas* spp. synthesize the enzyme ACC deaminase (reviewed by Glick *et al.*, 1999) which degrades ACC, thus preventing plant production losses by inhibitory levels of ethylene. In the present study, over 27% of rhizobacteria (all *Pseudomonas* spp.) isolated from wild *Coffea arabica* rhizospheres were able to degrade ACC (Paper V). Glick *et al.* (1998) put forward the theory that the mode of action of some PGPR was the production of ACC deaminase. Those authors suggested that ACC deaminase activity would decrease ethylene production in the roots of host plants and result in root lengthening. In some cases, the growth promotion effects of ACC deaminase-producing PGPR appear to be best expressed in stressful situations (Grichko & Glick, 2001).

Biocontrol of fungal plant diseases (particularly coffee diseases)

Phytopathogenic microbes have an immense impact on agricultural productivity, greatly reducing crop yields and sometimes causing total crop loss (Antoun & Prévost, 2006). Major pathogens induce well-known root or vascular diseases with obvious symptoms (Weller, 1988). Pathogenic fungi in general and *Fusarium* spp. in particular are highly destructive pathogens of both greenhouse and field-grown major crops under favourable conditions for disease development. The disease caused by this fungus is characterized by yellowing of the older leaves, browning of the vascular system, wilting in a later stage and finally death of the whole plant. Chlamydiospores of the pathogen remain in infested soils for several years and invasion occurs through wounds on the root surface.

At present, emerging serious fungal wilt diseases are one of the biggest challenges confronting African coffee growers, with noticeable yield losses (Adugna *et al.*, 2001; Geiser *et al.*, 2005; Serani *et al.*, 2007). Coffee wilt disease or tracheomycosis caused by *Fusarium xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* Heim and Saccas) is becoming an important major coffee disease of both Robusta and Arabica coffee in coffee growing regions of Africa (Adugna *et al.*, 2001; Geiser *et al.*, 2005; Silva *et al.*, 2006). The incidence of coffee vascular disease (tracheomycosis) in Ethiopia is reported to be 60%, with significant yield losses due to very severe damage and ultimate death of millions of coffee bushes (Adugna *et al.*, 2001). Other important coffee pathogens reported from Ethiopia include *Fusarium stilboides* Wollenw (telemorph: *Gibberella stilboides*) (Silva *et al.*, 2006) and *Fusarium oxysporum* Schlechtend.: Fr. (Wellman, 1954). However, studies reveal that *F. xylarioides* causes more deaths of young coffee plants than any other *Fusarium* spp. (Serani *et al.*, 2007).

Currently, control of plant disease is a pressing need for agriculture across the globe, particularly in economically disadvantaged countries. Existing practices for controlling plant disease are fundamentally based on genetic resistance in the host plant, management of the plant and its environment, and synthetic chemicals (Strange, 1993). The high cost of pesticides, the emergence of fungicide-resistant pathogen biotypes and other social and health-related impacts of conventional agriculture on the environment have increased interest in agricultural sustainability and biodiversity conservation (van der Vossen, 2005). Moreover, many of the synthetic chemicals may lose their usefulness due to revised safety regulations and concern over non-target effects (Guy *et al.*, 1989).

Thus, there is a need for new solutions to plant disease problems that provide effective control while minimizing cost and negative consequences

for human health and the environment (Cook *et al.*, 1996). In most systems, the biological elements are the primary factors in disease suppression and the topic of 'biological control of plant pathogens' has gained feasibility in the context of sustainable issues (Weller *et al.*, 2002). The rich diversity of the microbial world provides a seemingly endless resource for this purpose. Biological control is also likely to be more robust than disease control that is based on synthetic chemicals. The complexity of the organism interactions, the involvement of numerous mechanisms of disease suppression by a single microorganism, and the adaptedness of most biocontrol agents to the environment in which they are used all contribute to the belief that biocontrol will be more durable than synthetic chemicals (Cook, 1993). Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defence for roots against attack by pathogens (Weller, 1988). The groups of soil microorganisms with antagonistic properties towards plant pathogens are diverse, including plant-associated prokaryotes and eukaryotes (Barea *et al.*, 2005). Increased plant productivity by biocontrol mechanisms is indirect and results from the suppression of deleterious microorganisms and soil-borne pathogens, by PGPR in particular (Schippers *et al.*, 1987).

Bacillus/Paenibacillus spp. have been tested on a wide variety of plant species for their ability to control diseases. They are appealing candidates for biocontrol because they produce endospores that are tolerant to heat and desiccation (Weller, 1988). Currently, *Pseudomonas* spp. are also receiving much attention as biocontrol agents due to their remarkable potential for rhizosphere competence (Bashan & de-Bashan, 2005). The world-wide interest in these groups of bacteria was sparked by studies initiated for sustainable production systems. The fluorescent pseudomonads (De Freitas & Germida, 1990) and *Bacillus* spp. (Landa *et al.*, 1997) are the main candidates for the biological control of diseases induced by fungal pathogens and they have been applied successfully to suppress fusarium wilts of various plant species. Similarly, among wild Arabica coffee rhizosphere isolates, *Bacillus* and *Pseudomonas* spp. in particular showed remarkable inhibition against *Fusarium xylarioides*, *F. stilboides* and *F. oxysporum* under *in vitro* conditions (Fig. 7, Paper IV).

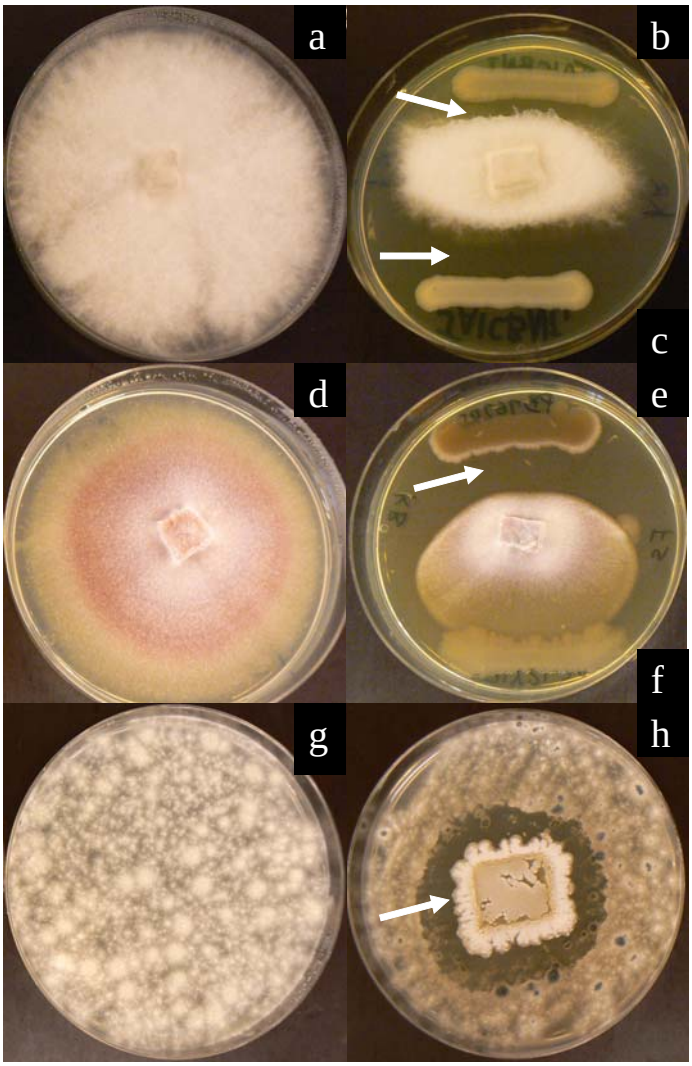


Fig. 7. Control plates (left row) and dual culture media showing some rhizobacteria and coffee pathogen interactions: a) *F. oxysporum*, b) *P. chlororaphis* (AUPB23) vs *F. oxysporum*, c) *P. chlororaphis* (AUPB24) vs *F. oxysporum*, d) *F. stilboides,* e) *Pseudomonas* sp.(AUPB15) vs *F. stilboides*, f) *Bacillus* sp. (AUBY95) vs *F. stilboides* (no inhibition), g) *F. xylarioides*, h) *B. subtilis* vs *F. xylarioides*. Arrows indicate the zones of inhibition (Paper IV).

Mechanisms used by biocontrol PGPR

Pathogen suppression by antagonistic microorganisms can result from one or more mechanisms depending on the particular antagonist involved (Barea *et al.*, 2005). An effective biocontrol agent often acts through a combination of several different mechanisms (Whipps, 2001).

Siderophore production

Living organisms require iron as a component of proteins involved in important life processes such as respiration, photosynthesis and nitrogen fixation. Iron is one of the major elements in the earth's crust but soil organisms such as plants and microbes have difficulty in obtaining sufficient iron to support their growth because of formation under aerobic conditions of ferric oxides, which cannot be readily transported into cells. Under such iron starvation, bacteria, fungi and plants secrete small, specialized efficient iron (III) chelator molecules commonly known as siderophores (Drechsel & Jung 1998). After the iron-siderophore complexes have formed, these now soluble complexes are internalized via active transport into the cells by specific membrane receptors (Glick *et al.*, 1999). Following either cleavage or reduction to the ferrous state, the iron is released from the siderophore and used by a cell (Glick *et al.*, 1999).

Lankford (1973) coined the term siderophore to describe low molecular weight (approximately 600 to 1500 daltons) molecules that bind ferric iron with an extremely high affinity. Siderophore was derived from a Greek term meaning iron carrier (Ishimaru, 1993). The dominant iron-binding ligands of siderophores are hydroxamates and catecholates (phenolates), but carboxylate, oxazoline, α-hydroxy carboxylate and keto hydroxyl bidentate siderophores have also been found (Essén *et al.*, 2006). In addition, hybrid siderophores with more than one type of ligand group exist (Neilands, 1981). Each functional group presents two atoms of oxygen, or less commonly, nitrogen, that bind to iron (III). While bacterial siderophores are structurally diverse, fungal siderophores are dominated by hydroxamate siderophores (Drechsel & Jung, 1998). On the other hand, plant siderophores are linear hydroxy- and amino-substituted iminocarboxylic acids, such as mugineic and avenic acids (Sugiura *et al.*, 1981).

Many bacteria are capable of producing more than one type of siderophore or have more than one iron-uptake system to take up multiple siderophores (Neilands, 1981). A considerable number of wild Arabica coffee-associated rhizobacteria (67%) produce siderophores (Paper IV). Wide arrays of beneficial plant-associated bacterial genera, *e.g. Pseudomonas, Azotobacter, Bacillus*, *Enterobacter*, *Serratia*, *Azospirillum* and *Rhizobium* secrete various types of siderophores (Glick *et al.*, 1999; Loper & Henkels 1999; Paper IV).

Siderophores function mainly in the solubilization, transport and storage of iron (Stephan *et al.*, 1993). Some other important mechanisms by which siderophore-producing bacteria contribute to the promotion of plant growth are described briefly below.

Siderophores produced by certain strains of fluorescent *Pseudomonas* spp. have been linked to suppression of soil-borne plant diseases. It has been suggested that siderophores act antagonistically by sequestering iron from the environment, restricting growth of the pathogen (Bashan & de-Bashan, 2005). Convincing evidence for the involvement of siderophores in disease suppression is readily available (Bashan & de-Bashan, 2005). For example, a mutant strain of *P. putida* that overproduces siderophores has been shown to be more effective than the wild bacterium in controlling the pathogenic fungus *Fusarium oxysporum* in tomato. Many wild strains that lose their siderophore trait also lose biological control activity. The extent of disease suppression as a consequence of bacterial siderophore production is affected by several factors (Bashan & de-Bashan, 2005), including the specific pathogen, the species of biocontrol PGPR, the soil type, the crop and the affinity of the siderophore for iron. For instance, siderophore-mediated suppression should be greater in neutral and alkaline soils than in acid soils (Baker *et al.*, 1986). Thus, disease suppression under controlled laboratory conditions is only an indication of the efficacy of the biocontrol agent in the field.

Pathogens are thought to be sensitive to suppression by siderophores for several reasons: (a) they produce no siderophores of their own; (b) they are unable to use siderophores produced by the antagonists or by other microorganisms in their immediate environment; (c) they produce too few siderophores or biocontrol PGPR produce siderophores that have a higher affinity for iron than those produced by fungal pathogens, allowing the former microbes to scavenge most of the available iron, and thereby prevent proliferation of fungal pathogens; or (d) they produce siderophores that can be used by the antagonist, but they are unable to use the antagonist's siderophores (Weller, 1988; Bashan & de-Bashan, 2005).

Bashan & de-Bashan (2005) have reported that depletion of iron from the rhizosphere normally does not affect plant growth, as plants can thrive on less iron than can microorganisms. However, some plants can bind and release iron from bacterial iron-siderophore complexes, and use the iron for growth. Thus, these plants benefit in two ways: from the suppression of pathogens and from enhanced iron nutrition, resulting in increased plant growth.

Pseudomonas siderophores have also been implicated in inducing systemic resistance (ISR) in plants (Leeman *et al.*, 1996), *i.e.* enhancement of the

defence capacity of the plant against a broad spectrum of pathogens. Exposure to pathogens, non-pathogens, PGPR and microbial metabolites stimulates the plant's natural self-defence mechanisms before a pathogenic infection can be established, effectively `immunizing' the plant against fungal, viral and bacterial infections (Bashan & de-Bashan, 2005). Protection occurs by accumulation of compounds such as salicylic acid, which plays a central protective role in acquired systemic resistance, or by enhancement of the oxidative enzymes of the plant. While acquired systemic resistance is induced upon pathogen infection, induced systemic resistance can be stimulated by other agents, such as PGPR inoculants. The feasibility of protecting plants by induced systemic resistance has been demonstrated for several plant diseases. For instance, plants inoculated with the biocontrol PGPR *P. putida* and *Serratia marcescens* were protected against the cucumber pathogen *P. syringae pv. Lachrymans* (Bashan & de-Bashan, 2005).

Hydrogen cyanide (HCN) production

Considerable numbers of free-living rhizospheric bacterial communities, mainly *Pseudomonas* spp. (Faramarzi *et al.*, 2004; Ahmad *et al.*, 2006; Faramarzi & Brand, 2006; Paper IV), are capable of generating HCN by oxidative decarboxylation from direct precursors such as glycine, glutamate, or methionine (Castric, 1977). Other rhizobacterial genera reported to produce HCN include *Bacillus* (Ahmad *et al.*, 2006; Faramarzi & Brand, 2006) and *Chromobacterium* (Faramarzi & Brand, 2006; Paper IV). However, hydrogen cyanide has not been detected in cultures of *Pseudomonas aeruginosa*, *Serratia marcescens*, *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (Michaels & Corpe, 1965).

In general, cyanide is formed during the early stationary growth phase (Knowles & Bunch, 1986). Cyanide occurs in solution as free cyanide, which includes the cyanide anion (CN^-) and the non-dissociated HCN. It does not take part in growth, energy storage or primary metabolism, but is generally considered to be a secondary metabolite that has an ecological role and confers a selective advantage on the producer strains (Vining, 1990). Cyanide is a phytotoxic agent capable of inhibiting enzymes involved in major metabolic processes and is considered one of the typical features of deleterious rhizobacterial isolates (Bakker & Schippers, 1987). Nevertheless, at present its applications in areas of biocontrol methods (see below) are increasing (Voisard *et al.* 1989; Devi *et al.*, 2007).

Cyanogenesis in bacteria accounts in part for the biocontrol capacity of the strains that suppress fungal diseases of some economically important plants (Voisard *et al.*, 1989). For instance, for many pseudomonads, production of metabolites such as hydrogen cyanide (HCN) is the primary mechanism in the suppression of root fungal pathogens. Cyanogenic bacterial species have

also been found to be effective in killing the subterranean termite *Odontotermes obesus,* an important pest of major agricultural crops and forest plantation trees, under *in vitro* conditions (Devi *et al*., 2007), in addition to suppression of plant parasitic nematodes (Siddiqui *et al*., 2006). Hydrogen cyanide (HCN) effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. However, producer microbes, mainly pseudomonads, are reported to be resistant (Bashan & de-Bashan, 2005).

Production of lytic enzymes

A large array of other microbial substances is involved in the suppression of phytopathogenic growth and subsequent reduction in damage to plants. These substances include lytic enzymes such as chitinase, ß-1,3-glucanase, protease and lipase (Bashan & de-Bashan, 2005). Many *Pseudomonas* and *Bacillus* species are capable of producing some of these hydrolytic enzymes (Paper IV). For example, *Pseudomonas stutzeri* produces extracellular chitinase and ß-1,3-glucanase, which lyse the pathogen *Fusarium* sp. (Bashan & de-Bashan, 2005). *Cladosporium werneckii* and *B. cepacia* can hydrolyze fusaric acid (produced by *Fusarium*), which causes severe damage to plants (Bashan & de-Bashan, 2005). Direct evidence for the role of cell-wall degrading enzymes in biocontrol *in vivo* comes from studies utilizing mutant strains overexpressing or lacking a particular enzyme, or transgenic plants expressing these enzymes (Pozo *et al*., 2004).

Antibiotics

Many organisms operative in pathogen suppression also act via antibiosis (Mazzola, 2002). Antibiotic production by biocontrol PGPR is perhaps the most powerful mechanism against phytopathogens (Bashan & de-Bashan, 2005). Indeed, the first clear-cut experimental demonstration that a bacteria-produced antibiotic could suppress plant disease in an ecosystem was made by Tomashow & Weller (1988). Fluorescent pseudomonads (Paper IV) have been shown to produce a range of antibiotics, *e.g.* 2,4-diacetylphloroglucinol, which suppress the growth of various soil-borne fungal phytopathogens (Mazzola, 2002).

Competition

Competition for nutrients and suitable niches is another key mechanism among pathogens and biocontrol PGPR in biocontrol of some plant diseases (Bashan & de-Bashan, 2005). Members of the pseudomonads are highly efficient in competition for root resources among rhizobacterial communities (Barea *et al*., 2005). On plant surfaces, host-supplied nutrients include exudates, leachates, waste products of other organisms or senesced

tissue (Pal & Gardener, 2006). To successfully colonize the phytosphere, a microbe must effectively compete for the available nutrients. Biocontrol rhizosphere bacteria have the ability to multiply and spread in the rhizosphere environment, to colonize potential infection sites on the root and to act by direct contact with the pathogens (Insunza *et al.*, 2002). Although difficult to prove directly, much indirect evidence suggests that competition between pathogens and non-pathogens for nutrient resources is important for limiting disease incidence and severity (Bashan & de-Bashan, 2005; Pal & Gardener, 2006). The degree of the susceptibility of soil-borne pathogens to the prevailing competition remarkably varies among microbes. In general, soil-borne phytopathogens such as species of *Fusarium* and *Pythium* that infect through mycelial contact are more susceptible to competition from other soil- and plant-associated microbes than those pathogens that germinate directly on plant surfaces and infect through appressoria and infection pegs (Pal & Gardener, 2006).

Studies have often revealed multiple modes of action of the population of putative PGPR inhabiting the rhizosphere (Weller, 1988; Haas & Keel, 2003). It is important to remember that in a given biological agent more than one mechanism may operate to suppress a pathogen, and the relative importance of a particular mechanism may vary with the physical or chemical conditions in the rhizosphere (Weller, 1988). In addition, *Pseudomonas* spp. produce several metabolites with antimicrobial activity towards other bacteria, fungi and even nematodes (Haas & Keel, 2003). Several reports also show the potential of combining different biocontrol agents with different disease-suppressive mechanisms in the field (de Boer *et al.*, 2003) and the combined inoculation of selected rhizosphere microorganisms has been recommended for maximising plant growth and nutrition (Probanza *et al.*, 2001).

Interactions between AMF and rhizobacteria

Despite the difficulty in selecting a multifunctional microbial inoculum, appropriate microbial combinations can be recommended for a given biotechnological input related to improvement of plant performance. Beneficial plant-microbe interactions in the rhizosphere are primary determinants of plant health and soil fertility (Jeffries *et al.*, 2003). The rhizosphere of mycorrhizal plants (mycorrhizosphere) harbours a great array of microbial activities responsible for several key ecosystem processes (Barea *et al.*, 2002). A typical beneficial effect is that exerted by the 'mycorrhiza-helper-bacteria' (MHB), a term coined by Garbaye (1994) for those bacteria known to stimulate mycelial growth of mycorrhizal fungi and/or enhance mycorrhizal formation. Within the mycorrhizosphere, AMF interact positively with various types of rhizobacterial communities that have proven agronomic and/or ecological significance, including symbiotic/free living

N_2-fixing bacteria, phosphate solubilizing bacteria, heavy metal detoxifying bacteria, microbial biocontrol agents and microbes that are involved in soil aggregate formation (Barea *et al.*, 2005). Certain rhizobacteria are known to produce compounds such as phytohormones that increase the rates of root exudation (Azcón-Aguilar & Barea, 1992). Consequently these rhizosphere microorganisms may be able to affect the presymbiotic stages of AM development, such as spore germination rate and mycelial growth for root colonization (Azcón-Aguilar & Barea, 1995). Once the arbuscular symbiosis has developed, AM hyphae influence the surrounding soil, *i.e.* the mycorrhizosphere (Linderman, 1988), resulting in the development of distinct microbial communities relative to the rhizosphere and bulk soil (Andrade *et al.*, 1997). Mycorrhiza formation in its turn changes several aspects of plant physiology and some nutritional and physical properties of the rhizospheric soil (Barea *et al.*, 2002) and consequently results in alteration of the microbial composition in the rhizosphere (Marschner, 1998; Hodge & Campbell, 2001).

Muthukumar *et al.* (2001) have indicated that microorganisms act synergistically when inoculated simultaneously. Many biocontrol agents, both Gram-negative (Barea *et al.*, 1998; Barea *et al.*, 2005) and Gram-positive (Budi *et al.*, 1999) strains, at least (cf. above) do not have inhibitory effects on AM formation. None of the *Pseudomonas* strains tested to date affect: (i) the numbers or diversity of the native AM fungal population; (ii) the percentage of root length that becomes mycorrhizal; or (iii) AM performance (Barea *et al.*, 2005). On the other hand, the antifungal activities of certain *Pseudomonas* spp. may improve plant growth and nutrient (N and P) acquisition by the mycorrhizal plants (Barea *et al.*, 1998). Among Gram-positives, a *Paenibacillus* sp. isolated from the mycorrhizosphere of sorghum shows antagonistic activity against soil-borne fungal pathogens and stimulates mycorrhization (Budi *et al.*, 1999). The same applies to certain *P. polymyxa* strains associated with wheat (Artursson *et al.*, unpubl.).

Ratti *et al.* (2001) found that a combination of the arbuscular mycorrhizal fungus *Glomus aggregatum* and the PGPR *Paenibacillus polymyxa* and *Azospirillum brasilense* maximized biomass and P content of the host plant *Cymbopogon martinii* when grown with an insoluble source of inorganic phosphate. Similarly, both *Enterobacter* sp. and *Bacillus subtilis* were found to promote the establishment of the AM *Glomus intraradices* and to increase plant biomass and tissue N and P contents (Toro *et al.*, 1997). Kim *et al.* (1998) also found that P content increased with inoculation with either the AM *Glomus etunicatum* or the phosphate solubilizing PGPR *Enterobacter agglomerans*; however, the highest N and P uptake was observed when tomatoes were inoculated with both organisms. It is interesting that in each of the above reports, one or more of the helper bacteria are known to have P solubilizing capabilities and this clearly suggests that the bacteria are acting

in concert with the AM to improve P acquisition of the host plant. AM inoculation *per se* improves the establishment of both inoculated and indigenous phosphate solubilizing rhizobacteria acting as MHB (Toro *et al.*, 1997; Barea *et al.*, 2002). In the mycorrhizosphere, AMF also interact with various soil-borne fungal phytopathogens such as agents of *Fusarium* wilt. A growing body of evidence reveals that inoculation with AMF significantly suppresses disease development and incidence induced by *Fusarium* spp. (Harrier & Watson, 2004). The potential biotechnological applications of native free-living microbes with multiple beneficial traits (Vassilev *et al.*, 2006) and synergistic interactions (Babana & Antoun, 2006) in promotion of plant growth have been well addressed.

Biofertilizers for sustainable agriculture

Sustainable farming systems strive to minimize the use of costly and environmentally unfriendly synthetic pesticides/agrochemicals and to optimize the use of alternative management strategies to improve soil fertility and control soil-borne pathogens (Harrier & Watson, 2004). A more sustainable agriculture that is 'ecologically sound, economically viable, socially just and humane' (Gips, 1987) should aim to recycle minerals in the soil with no or few external inputs, maintain a high biodiversity in agro-ecosystems, favour mechanical and biological weed control, and better exploit soil-plant-microbe interactions for plant nutrition and protection against pests (Edwards *et al.*, 1990). An answer to this is the biofertilizer, an environmentally friendly fertilizer now used in many countries. During the last couple of decades, the use of biofertilizers-PGPR for sustainable agriculture has increased tremendously in various parts of the world. Vessey (2003) defined biofertilizer as a substance that contains living microorganisms which, when applied to seed, plant surfaces or soil, colonize the rhizosphere or the interior of the plant and promote growth by increasing the supply or availability of primary nutrients to the host plant. The term is not synonymous with organic/biological fertilizer or biopesticide. The main sources of biofertilizers are PGPR, beneficial rhizospheric fungi such as arbuscular mycorrhizae and *Penicillium bilaii* and cyanobacteria (blue-green algae) that are long known to have plant growth promoting effects via increasing the nutrient status of host plants (Vessey, 2003). Various studies have demonstrated a positive influence of biofertilization on horticultural plant growth, development and yield (Rodríguez Sr., 2006). Significant increases in growth and yield of agronomically important crops in response to inoculation with biofertilizers have been reported (Asghar *et al.*, 2002). Moreover, AM products are now commercially available as biofertilizers in Europe, Asia and the U.S.A (Narutaki & Miyamoto, 1996; Talavera *et al.*, 2001).

The mode of action by which biofertilizers enhance the nutrient status of host plants (cf. above) can be categorized into some important areas (Vessey, 2003): (1) biological N_2 fixation; (2) increasing the availability of nutrients in the rhizosphere (*e.g.* solubilization of phosphorus); (3) inducing increases in root surface area; (4) enhancing other beneficial symbioses of the host such as arbuscular mycorrhizae and phytohormone production; 5) production of enzymes that decrease phytohormone production by the host, induction of the host to produce signal substances to other symbionts (*e.g.* flavonoids); and (6) combination of modes of action. Recorded important benefits from biofertilizers include: 1) Increasing crop yield by 20-30%; 2) replacing chemical nitrogen and phosphorus by 25%; 3) activating the soil biologically; 4) restoring natural soil fertility; and 5) providing protection against drought and some soil-borne diseases (http://www.vasat.org/learning_resources/OrganicFAQs/biofertilizer.htm; 21-Aug-2007). In addition, some PGPR appear to promote growth by acting as both biofertilizer and biopesticide. For instance, strains of *Burkholderia cepacia* have been shown to have biocontrol characteristics to *Fusarium* spp., but also to stimulate growth of maize under iron-poor conditions via siderophore production (Bevivino *et al.*, 1998). The overall simplified methods of using biofertilizers are presented in Fig. 8.

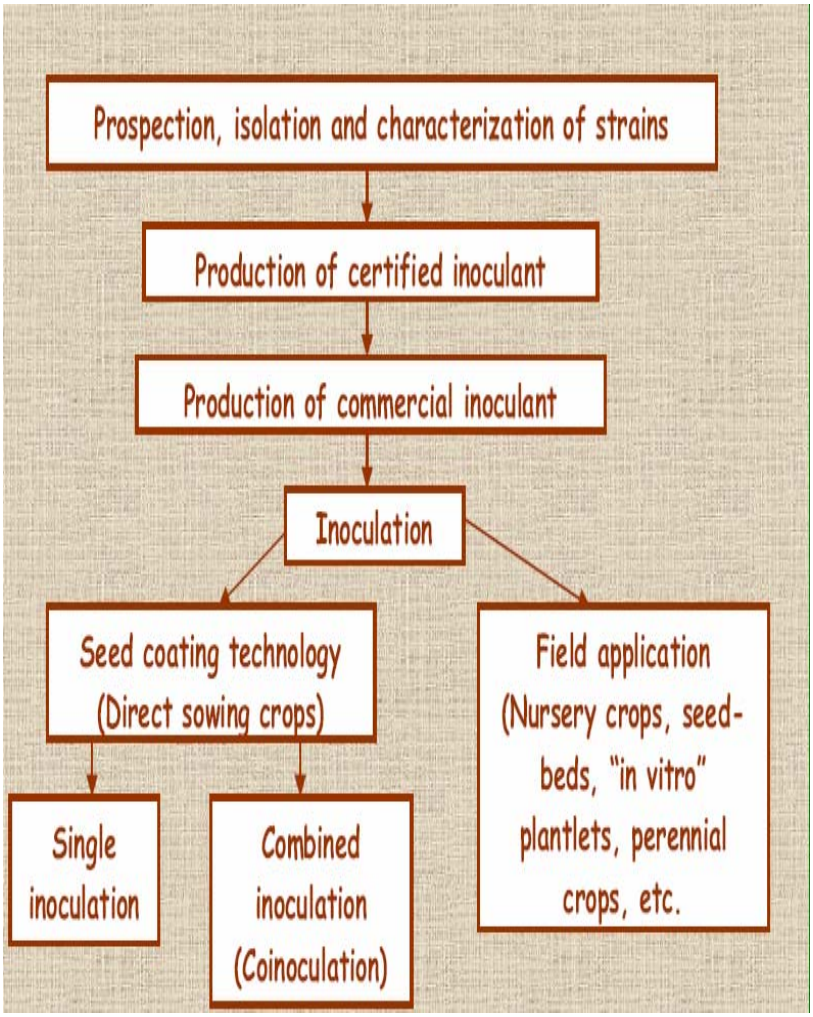


Fig. 8. General methodology for obtaining and using biofertilizers. Source: (http://www.pugwash.org/reports/ees/cuba2004/02%20Pugwash/07_Ondina.pdf.; 21-Aug-2007)

Conclusions

The main findings of this thesis can be summarized as follows:

A number of the shade trees studied, particularly the tree legumes, are ideal for agroforestry systems because most coffee rhizospheres under them presented higher AMF spore counts and greater diversity, even in deep soil layers, than unshaded coffee plants (Papers I and II). Canopy bases and topsoil layers harboured higher mean spore densities of AMF (Paper II). Overall, members of Glomeromycota were dominated by *Glomus* and *Acaulospora* (Papers I and II). The presence of these native AMF genera in particular in the study areas is highly vital for the establishment and growth of wild Arabica coffee seedlings.

Phosphate solubilizing rhizobacterial isolates from wild coffee plants were screened for P solubilization efficiency (Paper III). In all cases, pH and mobilized P values had an inverse relationship. By and large, Gram-negative phosphobacteria showed remarkable superior activities over the *Bacillus* group in terms of lowering the pH and releasing P into the growth medium. 2-ketogluconic and gluconic acids were the principal organic acids exuded by all Gram-negative wild Arabica coffee-associated rhizobacteria and caused steep declines in pH values. The production of these organic acids can be suggested to be the main mechanism used by these rhizobacteria to mobilize insoluble P sources. Higher concentrations of 2-ketogluconic acid were measured in HAP medium (the most insoluble P source), indicating enhanced induction of glucose dehydrogenase (GDH) as a result of phosphate starvation. Isolates AUEY28 and AUEY29 (both *Erwinia* sp.) showed remarkable P solubilizing abilities, making them the most promising candidates for a bioinoculant development programme.

Potent inhibitory effects were exhibited by several coffee-associated rhizobacterial isolates against deleterious coffee wilt diseases caused by *Fusarium* spp. (Paper IV). Wild Arabica coffee-associated antagonists showed more prominent inhibitory activity against *F. xylarioides* and *F. stilboides* than against *F. oxysporum*. The highest percentage inhibition against the target fungal pathogens was caused by the isolate AUPB24 (*P. chlororaphis*). The antagonists were found to produce various inhibitory substances as possible mechanisms of inhibition of the coffee fungal pathogens.

PCR-RFLP and 16S rRNA gene analyses revealed a limited number of rhizobacteria, mainly *Pseudomonas* and *Bacillus* spp., but this study does provide first-hand information on the presence of some strains closely related to rhizobacteria of proven importance for plant growth promotion (Paper V). Several members of the pseudomonads showed some direct phytobeneficial traits, *e.g.* production of IAA and utilization of ACC.

Overall, the rhizobacterial isolates showed multiples of beneficial traits that can qualify them either as potential biofertilizers or biocontrol agents (Papers III-V). The natural coffee forests of southwestern Ethiopia are therefore ideal focal sites not only for *in situ* coffee genetic resources and biodiversity conservation but also for isolation of rhizobacteria with biocontrol and biofertilizer capacities for the promotion of organically grown coffee.

Future trends

Given that this investigation is the first of its kind in coffee growing areas of Ethiopia and that studies on the wild Arabica coffee-associated AMF and rhizobacteria are generally lacking, there is much opportunity for further research in this field, both in Ethiopia and elsewhere. Field-collected AMF spores and identification based on morphotypes (as in this study) provide only a static picture of the AMF community. A fuller understanding of the AMF community composition in natural coffee forests can be obtained by using trapping and molecular methods that directly involve plant roots and/or spores in combination with the conventional techniques. It is also recommended that further studies be conducted to determine microbial communities by involving both culture and culture-independent techniques (extraction and analysis of total soil DNA) to reveal the real picture of rhizobacteria diversity associated with wild Arabica coffee. The current *in vitro* study verified the presence of many indigenous beneficial rhizobacteria of wild Arabica coffee plants that can function both as potent biofertilizers and biocontrol agents. The development of better screening procedures and understanding of the genetic basis of phosphate solubilization and rhizospheric competence will help in developing novel PSMs that could be studied in greenhouse and field trials to ascertain their future applicability for inoculum development. In general, the availability of new and powerful technologies for studying co-operative microbial interactions in the rhizosphere guarantees a greater understanding of these processes, which will facilitate their successful applications in biotechnology. Further studies may address the consequences of the co-operation between microbes in the rhizosphere under field conditions to assess their ecological impacts and biotechnological potential. As our understanding of the mechanisms used by PGPR advances, it becomes feasible to enhance their capacity to stimulate plant growth by modifying promising traits in both areas of biofertilizers and biocontrol agents, e.g., by introducing genes responsible for the biosynthesis of desirable metabolites that can extend the range of their abilities to improve sustainable plant productivity, while maintaining environmental quality.

Thus, future research in rhizosphere biology which relies on the development of molecular and biotechnological approaches should increase our knowledge of coffee rhizospheres and make it possible to achieve integrated management of soil microbial populations.

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Acknowledgements

First and foremost I praise the only Almighty God (Jesus) for his miraculous help to me in undertaking my work and covering this lengthy journey peacefully. Of all sections in my thesis, I found the Acknowledgements the most unwieldy part that took me some months to complete! Since thesis work represents the inputs of a huge number of people and organizations/institutions, unavoidably, there is a risk that someone or something is overlooked. Anyway, I am highly thankful to all people who contributed to the successful completion of my work in one way or another.

I would like to express my sincere gratitude to my Swedish supervisor, **Associate Prof. Ulf Granhall** for accepting me to work in your laboratory, the excellent guidance and wholehearted assistance. Your intelligent coordination and thought-provoking discussions made my work highly successful. Above all, I appreciate your high competence, patience and punctuality in reading, giving critical constructive suggestions on both the manuscripts and the thesis, as well as responding to all my queries at your highest priority (by email/in person), no matter how busy you were. Your initiation and realization of the idea of a joint degree is also highly acknowledged. You have also made an inestimable contribution to my future career by putting an end to those dull moments at the beginning of this study. I would also like to thank you and your wife, **Luba**, for inviting me to your home on several occasions and offering me excellent dinners.

My deepest thanks go to my Ethiopian supervisor **Dr. Fassil Assefa** for your great role in establishing collaborative study programme with Swedish Institutions and choosing me to be involved in this study programme. Your constant and unfailing material support and encouragement during the course of the study period, as well as valuable comments on the Paper I were quite impressive. Your pioneering project on microbial biofertilizers and biocontrol agents in Ethiopian coffee production systems is also highly commendable. I would also like to express my heartfelt gratitude to you and your wife, **Membere**, for inviting me to your home and offering me flavoursome suppers.

My earnest gratitude also goes to my Ethiopian co-supervisor **Dr. Sileshi Nemomissa** for invariable and consistent guidance, generous material supply and enthusiastic encouragement during the course of the study period and helpful comments on Papers I and II. I appreciate all the assistance I received from you throughout this challenging period of life. Your valuable advice on my future career and research is also highly appreciated. Once again, thank you very much for being an open-minded realist.

I am immeasurably grateful to all the staff members of the Department of Microbiology at Genetic Centre, Uppsala, Sweden, for their contribution to the success of this work and creation of a conducive environment during my stay particularly: **Prof. Johan Schnürer**, Head of the Department of Microbiology, SLU. You are one of a few people whom I frequently met during the weekends throughout my experiments. Your pleasing smile and constant encouragement was my special provision. I appreciate also your kind willingness in considering me as a PhD student at the Department of Microbiology (SLU) and facilitating the necessary processes to realize my objectives; **Associate Prof**. **Hans Jonsson** for encouragement and kind help during registration at SLU; **Associate Prof. Mikael Pell** and **Dr. Thomas H Eberhard** for unswerving help in solving my computer problems without any preconditions and retrieving data during computer theft; **Associate Prof. Volkmar Passoth** for wholehearted support in providing me with chemicals and kind guidance on how to obtain them; **Associate Prof**. **John Stenström,** thank you very much for being a good boss for our group. Thank you very much also for the latest interesting and stimulating scientific discussions during our group meeting, added to your regular pleasurable smile and laughter that always creates happy moments for the group in general and for me in particular. Thanks also to **Prof. Janet Jansson** for unfailing encouragement and constructive criticism on data interpretation; **Dr**. **Maria del Pilar Castillo** for encouragement and pleasant presents during trips, as well as good instructions on use of spectrophotometry; **Dr. Veronica Arthurson** for constant encouragement and help in taking pictures of stained coffee roots - your kind invitation to your PhD disputation party also always enlivens me; **Lotta Jäderlund** for your positive attitude to my requests about some laboratory techniques; **Cecilia Berglund** for your academic and informal assistance in acquainting me with Uppsala city. Your constant empathy for me and my family back home is highly appreciated; **Associate Prof. Stefan Roos** for your encouragement and high competence in construction of phylogenetic trees, as well as collaboration in Paper V; **Sanja Zulcic** and **Lotta Levén,** you are so kind and highly cooperative. Thank you very much for always being the same. **Sanja,** thank you also for being a gregarious and generous roommate. Thank you **Ann-Cristine Lundquist**, **Susanne Broqvist** and **Sture Larsson** for your kind help with respect to administrative activities. **Susanne,** thank you for your valuable advice and excellent recording of my educational documents at the Department. **Ingemar Baselius,** for your punctual and positive responses to all of my requests regarding laboratory equipment. **Evi Marklund**, you have been highly supportive of me from my first arrival in Sweden in 2003 to the present, both in social and scientific matters. I will never forget your frequent invitations to mouth-watering Swedish traditional foods. **Dr**. **Karin Hjort**, for your patience in coaching me on various molecular techniques during characterization of

rhizobacteria and for the collaboration on Paper V. **Elisabet Börjesson** and **Maria Hellman** for your assistance in ordering chemicals instantly, stable encouragement and creating a conducive environment for my work. I have a high regard for your bottomless concern for me and my new-born baby, as well as other family members. **Elisabet,** your kind collaboration in Paper III is also appreciated. **Karin Önneby,** your presence in the laboratory and providing all necessary apparatus and other consumables highly enhanced my work. It has been a privilege to work with you in the laboratory, since you were a greatly helpful laboratory mate. **Maria Erikson,** for your kindness in providing me with sufficient small jars which made phosphate solubilization studies easy and manageable. **Solveig Geidnert,** for your kind help in lending me some laboratory equipment during critical times. **Dr. Harald Cederlund**, for being such a nice roommate. I cannot express in words all your unreserved assistance in taking pictures of my cultures, critical reading of manuscripts and the thesis as well as allround support during my stay in Sweden. Your positive and wholehearted response with smiling face to all my requests is cherished. **Dr. Leticia Pizzul,** thank you for your cooperation. Your deep concern for me and my family back home, especially during the pregnancy of my lady, will always be remembered. Sharing a room with you and Harald gave me a golden opportunity to exploit your wealth of knowledge in all aspects by answering my never-ending questions including the formatting of this thesis. Both of you were also my living Swedish Dictionary. **Dr**. **Kristin Steger,** for your encouragement and nice disputation party as well as stimulating conversations on matters related to my home country.

My special thanks go to the Swedish family Prof**. Ingemar Ahlgren** and his wife **Associate Prof. Gunnel Ahlgren** for your kindness in introducing me to Uppsala and skating techniques, as well as delightful Swedish culture and other assistance. Your regular invitations to various occasions at both your home and restaurants are also worthy of mention. Due thanks go to the Swedish families of Ethiopian origin **Drs. Ararso Ittana** and his family, **Hedata Gudata** and **Prof. Girma Gebresenbet** for your trustworthy encouragement and support during my stay in Uppsala.

I would like to thank my previous and current fellow PhD students at Alnarp, Lund, Stockholm and Uppsala: **Girma Tilahun**, **Seyoum Leta**, **Abdella Gure**, **Mashilla Dejene**, **Jemal Demma**, **Teshome Leta**, **Jema Haji**, **Fikre Lobago**, **Adey Feleke**, **Zeleke W/Tensay**, **Dereje Beyene**, **Sissay Menkir**, **Mengistu Urge**, **Esayas Aga**, **Tarekegn Berhanu**, **Mulatu Geleta**, **Tileye Feyissa**, **Yitbarek Woldehawarat** and **Faris Hailu** for your stimulating scientific discussions. **Seyoum,** you are my factual friend to be committed to memory all the way through my life. I will never forget those important good moments and inspiring discussions we had at Stockholm and back home. **Girma**, it was very nice sharing a room

with you at Addis Ababa, where you have expressed your genuine commiseration during my difficulties and happiness at some point in my elation. **Jemal**, thank you for your appetizing traditional hot spiced butter and other foods, as well as your frequent company for shopping and cooking. **Teshome**, thank you for being good mechanic in fixing problems related to my bike and provocative conversations we had at Flogsta using that apparatus, BB.

I would like to thank the Department of Biology, Addis Ababa University, Ethiopia, for provision of laboratory facilities and other support for my work. **Drs. Kifle Dagne, Dawit Abate (**then Head of Department**)**, **Gurja Belay (**current Head of Department**)**, **Mrs. Mulalem Tamire** and **Banchu Mekonnen** for your kind assistance in issues related to administrative matters and continuous encouragement. **Getachew Tay**e for driving me to my study sites in southwestern Ethiopia for sample collection. I would like to thank **Drs. Abebe Getahun, Amare Gessesse, Tesfaye Alemu, Demeke Kifle**, **Fisseha Ittana, Emiru Seyoum** and **Bekele Jembere** for encouragement; **Prof. Mogessie Ashenafi** for rewarding encouragement and being a model researcher; **Prof. Legesse Negash** for excellent guidance on measurement of light penetration (light conditions); **Dr. Ensermu Kelbessa** and **Prof. Sebesebe Demisew** for kind material support; **Habte Jebessa**, **Challa Regassa**, **Drs. Tadesse Woldemariam** and **Teshome Soromsa** for material support and unfailing encouragement; **Merid Negash** for useful statistical advice and encouragement. I am thankful to **Hirut Teshome** and **Tigist Mengesha** for careful technical assistance during data collection; **Awel Assefa** and **Yohannes Negash** for kind support in fixing apparatus and supplying laboratory equipment; and **Mulunesh Ayele** and **Zenebech Aytenew** for great help in autoclaving and storing culture media. I would like to express my heartfelt gratitude to staff members of the Finance Office of the Science Faculty, Addis Ababa University for your kind assistance and excellent work.

My special thanks also go to the Institute of Biodiversity Conservation of Ethiopia for allowing me to take bacterial isolates abroad for the purposes of this study. **Adugna Abdi,** your contribution was quite immense and supportive in getting the required permission from aforementioned organization at the appropriate time.

I would like to thank Jimma Agricultural Research Centre, particularly **Million Abebe**, the Director of the Centre, **Dr. Girma Adugna**, Head of the Plant Pathology unit and his technical staff members for kind provision of facilities in the greenhouse and taking care of the pot plants. I am also thankful for the assistance I received from the coffee farmers and agronomists in my study areas at Bonga and Yayu districts.

I am grateful to Jimma University for sponsoring my salary. I am highly indebted to **Duguma Adugna** and **Osman Rahmatto** for kind provision of office facilities (Internet, printer and other services) during compilation of my thesis. Thank you very much also for your dependable support to my family. **Duguma,** I appreciate your hearty willingness to listen and give prompt solutions to all my queries.

Due thanks go to my friends **Drs. Kaba Urgessa**, **Fikre Lemessa** and **Amsalu Ayana**, for supporting me and sharing my good and bad tempers. **Drs. Kaba and Amsalu**, your efforts for betterment of my future are worth mentioning. Due thanks also go to **Melkamu Dumessa**, **Ketema Bacha**, **Balcha Abera**, **Gezahegn Barecha**, **Adugna Debela**, **Wondewossen Habtamu**, **Gedefa Abera**, **Debela Hirko**, **Legesse Adane**, **Afework Waktola**, **Desta Buli**, **Tsige Ketema, Sirashwork Assefa, Bezawit Temesgen and Alemu Wondafirash,** for provision of constant encouragement and support to me and my family during my absence. **Melkamu**, I will never forget your allround support throughout my study period to me and my family. **Legesse**, thank you very much for your support and for keeping in touch, both abroad and in Ethiopia. **Ketema**, I am highly grateful to you for your boundless help and for the inspiring conversations about everything between heaven and earth (***Irraa sirbuu***). **Ketema**, above all, I will never forget the secret behind ***something*** inside the freezer and about the metal vessel with white contents which ultimately turned black on one occasion. Your night company after late laboratory work between Qabana and Arat Killo during those harsh moments was also praiseworthy. Did we go mad to do that?? **Beza**, you kindheartedly played an active role in keeping me in touch with my family via the Internet in 2003/2004, for which you deserve special appreciation. I am grateful to **Temesgen Bakare** and his family for frequent calls, material support and encouragement to both my family and me. I am genuinely grateful to my friend fellow PhD student in USA **Kebede Alemu** for constant encouragement and keeping in touch. I am also grateful to JUCA community for your unlimited support to me and my family.

I would like to express my deepest gratitude to **Prof. Mitiku Habte** for excellent guidance on phosphate solubilization studies; **Prof. Joseph B. Morton** for kind provision of the INVAM colour chart and invaluable information; **Dr. David Geiser** for kind provision of fungal isolates; **Dr. Tesfaye Wubet** for material support and excellent guidance on trapping of AMF; **Dr. Andy Taylor** for admirable guidance on preparation of roots for staining; **Prof. Tomas Bryngelsson** for kind provision of coffee seeds; **Drs. H. Dahm** and **W. Wrótniak** for marvellous instructions on preparation of the siderophore assay medium and **Novartis International AG,** Switzerland for generously providing siderophore desferal. Many

thanks also to **Mary McAfee,** for being very helpful with correcting the English in manuscripts and thesis.

I would like to express my heartfelt thanks to **SIDA/SAREC**, Sweden, for financially supporting my studies through the administration of **International Science Programme** (**ISP**), Uppsala University. My inestimable thanks go to **Associate Prof. Malin Akerblom**, then Director for ISP for excellent administration of the programme. You put the brightest light on the ghastly shadows in front of me at the initial phase of my work. My sincere thanks also go to **Associate Prof. Peter Sundin,** the current Director of ISP, for wonderful administration of the programme and prompt responses to all my enquiries; **Hossein Aminaey** for kind help in fixing issues related to my accommodation, plane tickets and other supports; and **Linnéa Sjöblom** for kindly purchasing some chemicals and other laboratory equipment, including soil sieves.

Finally, I would like to express my earnest thanks to my wife **Elfinesh Tolera**. **Elfu**, your patience, unvarying encouragement, love, commitment and determination to take care of our children alone deserve special admiration. I can imagine the burden you have been shouldering! You are really my dependable pillar in all aspects. I am pleased to thank my children, **Ayana**, **Gamachis** and **Abdena Diriba**, for endurance and good manners during this lengthy study period. I am thankful to **Asfaw Tolessa** and **Tarike Gadissa** for your thoughtfulness in taking care of our children and provision of excellent support to family as a whole. **Thank you all!**