

Beneficial Organisms for Nutrient Uptake

VFRC Report 2014/1



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VFRC Report 2014/1

Washington, D.C., USA

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Citation

Nina Koele, Thomas W. Kuyper and Prem S. Bindraban, 2014.
Beneficial organisms for nutrient uptake. VFRC Report 2014/1, Virtual Fertilizer Research Center, Washington, D.C. 63 pp.; 4 figs.; 330 ref.



Virtual Fertilizer Research Center

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List of acronyms and abbreviations

AM	Arbuscular mycorrhiza
AMF	Arbuscular mycorrhizal fungi
ATP	Adenosine triphosphate
B	Boron
BNF	Biological nitrogen fixation
Ca	Calcium
Co	Cobalt
Cu	Copper
DSE	Dark septate (root endophytic) fungi
Fe	Iron
IFDC	International Fertilizer Development Center
IITA	International Institute of Tropical Agriculture
INM	Integrated nutrient management
IRRI	International Rice Research Institute
K	Potassium
LMWOA	Low molecular weight organic acids or anions
MHB	Mycorrhiza helper bacteria
Mo	Molybdenum
N	Nitrogen
P	Phosphorus
PGPR	Plant growth-promoting rhizobacteria
PSB	Phosphate-solubilizing bacteria
PSF	Phosphate-solubilizing fungi
PSM	Phosphate-solubilizing microorganism
QTL	Quantitative trait locus
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
S	Sulfur
Se	Selenium
SRI	System of rice intensification
SRL	Specific root length
SSU	Small subunit
VFRC	Virtual Fertilizer Research Center
Zn	Zinc

1 Introduction

Plant roots in soil are surrounded by a multitude of soil organisms. The direct surrounding of the root is strongly affected by the root, and this environment is called the rhizosphere. The rhizosphere is characterized by higher amounts of carbon (due to exudates by living roots and also by dead root cells) and lower amounts of nutrients (due to plant uptake which is often fast, creating a so-called depletion zone around the root) compared to the bulk soil.

Soil microorganisms have a variety of impacts on the plants, ranging from highly beneficial to strongly antagonistic. Such effects are partly a property of the organisms, and therefore we often call organisms mutualists or parasites; partly effects are context-dependent, and we then classify behaviors as mutualistic or parasitic. The nature of the interaction can also vary, ranging from obligate for both partners (where neither plant nor microorganism can survive and reproduce independently under conditions of normal soil fertility) to facultative (where both partners could live and reproduce independently).

Among the beneficial interactions with plants, some microorganisms interact with pathogenic soil biota, thereby protecting the plant. Other microorganisms play a direct role in enhancing nutrient acquisition for the plant. These microorganisms are the subject of this review. The nature of the interaction ranges from obligate to facultative. Microorganisms play major roles in the acquisition of nitrogen (N) and phosphorus (P); they also play roles in the acquisition of micronutrients, a topic that has been less studied than the acquisition of macronutrients. In this review the various groups are dealt with separately. However, such groups interact in nature, so there is always a risk of studying one group of organisms in isolation. Because of such interactions, it is not easy to structure the report. For instance, should the additional P demand that N-fixing legumes have be discussed under the rhizobia or should they be discussed under the heading of arbuscular mycorrhizal fungi (AMF)? Should rhizobia, when not associated with legumes (where they form nodules and form a symbiosis) but with cereals where the association is more loose be dealt with under the general heading of rhizobia or under the topic of plant growth-promoting rhizobacteria? We have included several cross-references to point to such interactions in this fascinating world of microbial life that can contribute to nutrient uptake for plants and thereby contribute to sustainable agriculture that will enable us to feed the nine billion people in 2050.

2 Arbuscular mycorrhizal fungi

2.1 General review, specification in terms of ecosystem services

The symbiosis with AMF (phylum Glomeromycota) in crop plants (and wild plants, including the ancestors of cultivated plants) is widespread (Smith and Read, 2008; Smith and Smith, 2011). The overwhelming majority of cultivated plants form arbuscular mycorrhiza (AM). As has been stated “*in agricultural field conditions plants do not, strictly speaking, have roots, they have mycorrhizas.*” Growth of a host plant can be improved by mycorrhizal colonization provided that soil-available P (or another element where plant uptake is diffusion-limited, such as Zn or copper [Cu]) is a limiting factor for plant growth. The degree of growth improvement is affected by factors such as host plant species, fungal species, and soil conditions (Tawarayama, 2003). AMF account for 5-50% of the biomass of soil microbes (Olsson et al., 1999), and biomass of hyphae of AMF may amount to 54-900 kg ha⁻¹ (Zhu and Miller, 2003). Dead material of hyphae also makes a substantial contribution to the soil organic matter pool (Verbruggen et al., 2012a).

The mycorrhizal symbiosis promotes plant nutrition and environmental stability in natural ecosystems, and is of great importance in agriculture. Plants allocate considerable, but variable, quantities of carbon to mycorrhizal fungi, c. 10-20% of net primary productivity (Leake et al., 2004; Högberg and Read, 2006). These carbon fluxes to the fungus have often been described as the 'cost' of the mycorrhizal symbiosis and therefore the cost argument has been used to describe 'parasitic' behavior of mycorrhizal fungi. However, cost-benefit analysis of mycorrhiza (especially under agricultural conditions with the use of mineral fertilizer) is more complex (see section 2.6). In return, mycorrhizal fungi provide plants with the majority of nutrients (Leake et al., 2004). Again, calculation of the mycorrhizal fungal contribution to plant nutrient uptake is not straightforward, due to interactions between plant and fungal transporters and changes in nutrient gradients in the rhizosphere and mycorrhizosphere (see section 2.4). In summary, mycorrhizal fungi are the interface between plant roots and the soil matrix, and mediate ecosystem processes at the root-soil interface, including (but not restricted to) nutrient acquisition and uptake (Dickie et al., 2013).

AMF are especially known for their major role in P acquisition through improved scavenging (Lambers et al., 2008). Through the increased soil volume (extension of the depletion zone around roots due to the fact that the diffusion rate of P through soils is slower than uptake rate around roots and hyphae) accessed by the arbuscular mycorrhizal hyphae, the plant has greater access to orthophosphate, inorganic phosphate in the soil solution (Smith and Read, 2008). AMF assist largely in the uptake of P under P-limiting soil conditions. At P-sufficiency or even P-excess in the soil solution, the mycorrhizal contribution to P uptake diminishes, both due to plant regulation of the extent of the mycorrhizal symbiosis and direct negative effects of excess P on fungal performance. While attention has been more focused on the regulatory role by the plant on the extent of mycorrhizal root colonization and the extent of extraradical hyphae, separating the impact of a direct (soil-mediated) from an indirect (plant-mediated) effect of nutrient excess is likely important in case of foliar application of fertilizer (as under that condition only the indirect pathway is operative). It is likely that the importance of the direct pathway has been underestimated. Whereas the main mechanism for P uptake by AMF is through the larger soil volume exploited by the fungal hyphae, additional mechanisms for enhanced P acquisition have been suggested. Some studies suggested that exudation of phosphate-solubilizing enzymes, (acid) phosphate mono-esterase, phosphate di-esterase, and phytase allow mycorrhizal plants to acquire more phosphorus from organic sources (Tarafdar and Marschner, 1994). However, other studies showed that this mechanism was effective in the lab with well-defined phosphorus sources but were unable to demonstrate this effect with a mixture of organic P sources in a soil matrix (Cardoso et al., 2006). One major cause for the different perspective on a mycorrhizal role in the uptake of P from organic sources is that under many conditions it is still unclear whether P mobilization from organic sources is limited by enzyme availability or by availability of non-sorbed phosphorus (Tinker and Nye, 2000). Similarly, studies by Tawaraya et al. (2006) and Arocena et al. (2011) have suggested that mycorrhizal plants have access to mineral forms of phosphorus (sorbed P) through the excretion of low molecular weight organic acids or anions (LMWOA), especially under acidic conditions, such as citrate, oxalate or malate. However, recent experiments have provided no support for the ability of the fungi to produce these compounds and have indicated that the mycorrhizal symbiosis downregulates exudation of organic anions (Gao et al., 2008; Ryan et al., 2012) implying that other, still poorly characterized mechanisms are responsible for a faster uptake from the sorbed phosphorus pools (Cardoso et al., 2006).

Some studies have also shown uptake of N by AMF from organic substrates (Cheng et al., 2012; Veresoglou et al., 2012), but the extent to which arbuscular mycorrhizal plants benefit from increased access to these pools is still under debate. Hodge and Fitter (2010) suggested that AMF use organic N predominantly for their own nutrition and

do not transport it to the host plant. In general, nutrient immobilization by mycorrhizal fungi under nutrient-poor (especially nitrogen-deficient) conditions may be an important explanation for lack of positive plant responses.

AMF can take up N as ammonium or nitrate (Cardoso and Kuyper, 2006), but according to Smith and Smith (2011), it is unknown whether arbuscular mycorrhizal N uptake fulfills the host's N needs, and Hodge and Fitter (2010) argued that N taken up by AMF remains within the fungi and is not further transported to the host plant. However, plant uptake of N is affected indirectly by enhancing N-fixing microorganisms that can co-occur with AMF. The question whether such combinations of beneficial root-colonizing microorganisms result in synergies will be discussed below in sections on N-fixing microorganisms.

Colonization of plant roots by AMF not only affects plant mineral nutrition, but can also have numerous other beneficial effects under stress conditions (Pozo and Azcon-Aguilar, 2007; Miransari, 2010; Smith et al., 2010; Seguel et al., 2013). AMF can work like filters, binding heavy metals at the hyphal surface and/or in a glycoprotein called glomalin, and thereby protect their hosts from toxic metal concentrations in soil at high availability of heavy metals (Audet and Charest, 2007). Because of larger size of mycorrhizal plants, heavy metal concentrations could also be diluted in plant tissue. (At low availability of these metals, such as in Zn-deficient soils, the mycorrhizal symbiosis increases access to these essential metals, see Gao et al., 2007; Cavagnaro, 2008). Heavy metal stress alleviation by AMF was reviewed by Hildebrandt et al. (2007). AMF also can protect plants against conditions of suboptimal water potential (drought, flooding, salinity) (Miransari, 2010; Smith et al., 2010; Birhane et al., 2012). Auge (2001) reviewed the impact of AMF on plant-water relations and drought. AMF can increase plant water uptake by the increased soil volume that the hyphae scavenge, by improved hyphal-soil contact, by improving the plant nutrient status (P, K), by increasing photosynthesis through enhanced leaf stomatal conductance or by sink stimulation (Auge, 2001; Kaschuk et al., 2009). Worchel et al. (2013) and Kivlin et al. (2013) performed meta-analyses of fungal symbionts' effects on plant growth under global change and found that all fungal symbionts including AMF reduce negative effects of drought on plants. Protective effects against biotic stress (pathogenic bacteria, fungi or nematodes) have been described consistently for below-ground interactions (e.g., Pozo and Azcon-Aguilar, 2007; Singh and Vyas, 2009; Smith et al., 2010). Protective effects are explained by various mechanisms including priority of occupation of root cells, improved mineral nutrition of AM plants, priming of plant defense reactions by AMF and antagonistic effects on microorganisms (e.g., Singh and Vyas, 2009; Cameron et al., 2013). These latter authors also proposed the term mycorrhiza-induced resistance. The effectiveness of this symbiosis for pathogen control depends on early colonization of the mycorrhizal fungus compared to that of the pathogens. Pre-inoculation of banana plantlets by AMF could therefore be a major factor to reduce damage by parasitic nematodes. A specific protective effect has also been noted against the parasitic plants *Striga*, *Orobanche* and *Phelipanche*, parasitic plants that are especially damaging under nutrient-poor (nitrogen-deficient, phosphorus-deficient) conditions (Lendzemo et al., 2006). Negative responses by mycorrhizal fungi on other agricultural weeds have also been reported, but it is still unclear whether and how mycorrhizal management could contribute to weed control (Veiga et al., 2011; Rinaudo et al., 2010). Conversely, cases have been described whether mycorrhizal plants, including weeds during the fallow stage can help preventing long-fallow disorders in Australia (Thompson et al., 2013).

For almost all agricultural crops, the symbiosis with AMF is the natural, normal situation, and the non-mycorrhizal situation often used in comparative research is non-natural. Pictures of large mycorrhizal plants and small non-mycorrhizal plants should therefore not be interpreted as an argument for inoculation, but rather as a warning how much additional effort and costs are needed to achieve plants of the same size. Such experiments with non-

mycorrhizal controls are often executed under controlled conditions in pots or small containers, and this set up may create further problems when it comes to scaling up mycorrhizal experiments to field conditions.

While mycorrhizal fungi usually increase phosphorus uptake efficiency by the crop, it has also been observed that the phosphorus concentration of leaves of mycorrhizal plants are higher than those of non-mycorrhizal plants (Treseder, 2013). As the phosphorus concentration (mg P per gram C) is the inverse of nutrient use efficiency (gram C per mg P), the paradoxical situation exists that the mycorrhizal symbiosis *increases* nutrient uptake efficiency but *reduces* (although to a smaller extent) the nutrient use efficiency. This paradox has not been explained but could indicate that other nutrients can become limiting for mycorrhizal plants, possibly nitrogen because fungal nitrogen demand can be relatively high (Hodge and Fitter, 2010).

2.2 Associated plant species

As indicated above, almost all plants are mycorrhizal under conditions of normal soil fertility, and under such conditions, mycorrhizal plants usually show a higher fitness (better growth, higher seed production, enhanced stress resistance, etc.) than non-mycorrhizal plants. Differential performance of mycorrhizal and non-mycorrhizal plants is called mycorrhizal *responsiveness*. Mycorrhizal responsiveness is due to the interaction of plant species or plant genotype, mycorrhizal fungal species and soil conditions. Historically, responsiveness to mycorrhiza has been called plant dependency on mycorrhiza. However, Janos (2007) argued that dependency is a genetic trait of the plant only; and that dependency and responsiveness are not necessarily correlated.

Tawaraya (2003) listed arbuscular mycorrhizal responsiveness (called dependency by him) of different plant species and cultivars. He noted that on average cultivated plants showed lower responsiveness than their wild relatives and progenitors. This observation raises questions about the impact of plant breeding on mycorrhizal responsiveness (see below). Based on his list, an earlier list by Habte and Manjunath (1991), who tried to categorize different crops in terms of responsiveness (dependency), and meta-analyses by Lekberg and Koide (2005) and Hoeksema et al. (2010) we can conclude the following:

- The 10 major food crops of the world (maize, wheat, rice, potato, cassava, soybean, sweet potato, sorghum, yam, plantain) are all responsive to AM.
- The majority of all published studies show a positive response to AM. Only 10-20% of the studies did not show a response or showed a negative response. Cases of negative response ('parasitism') have received substantial attention in the agronomic literature and have given rise to the often-expressed opinion that in highly fertilized agricultural systems (especially with P fertilizers) mycorrhiza is not only not useful but often detrimental because costs (carbon use by the fungus) outweigh the benefits (increased access to nutrients and/or secondary benefits). We return to this issue in section 2.6.
- Mycorrhizal responsiveness can often be predicted from plant traits. Plants with relatively thick unbranched roots with few root hairs (e.g., species of *Allium* like onion or leek, or cassava) are much more responsive to mycorrhiza than species with thinner, more branched roots with many root hairs (most cereals). Nitrogen-fixing legumes, which have a higher P-demand than most other plant species, are also quite responsive to mycorrhiza.
- Some plants that have the ability to form mycorrhiza do not show them under conditions that are unfavourable for the fungi. Flooded rice for instance is poorly colonized by mycorrhizal fungi, but rice growing under more aerobic conditions (including rice grown under the system of rice intensification [SRI]) is often well-colonized and shows growth response to mycorrhiza (Watanarojanaporn et al., 2013). Also agricultural practices (intensive tillage, use of certain fungicides) can reduce mycorrhizal inoculum. It is likely that such agricultural practices over ecological and evolutionary time scales have adverse effects on plants and mycorrhizal fungi (Johnson, 1993; Kiers et al., 2002; Verbruggen and Kiers, 2010), especially in countries with highly

industrialized agriculture. A study in Kenya, on the other hand, showed a much smaller response in mycorrhizal fungal species composition due to tillage and fertilizer application (Muriithi-Muchane, 2013).

- Only very few (annual) crops are non-mycorrhizal. Major non-mycorrhizal crops are members of the Brassicaceae (cabbage, canola) or Amaranthaceae (spinach, quinoa, sugar beet) families. Inclusion of these crops in rotations likely reduces mycorrhizal inoculum potential, having adverse effects on subsequent crops that are dependent on mycorrhiza. Especially with Brassicaceae, it is likely that reduction of mycorrhizal inoculum (also due to toxic compounds produced by those plants) can have large carryover effects.
- Tree crops are always mycorrhizal.
- As almost all plants are normally mycorrhizal, the presence of mycorrhiza should be assumed for an unknown plant.

2.3 Crop genetic variation in mycorrhizal responsiveness

Tawaraya (2003) noted that wild ancestors and relatives of cultivated plants often show a higher responsiveness to mycorrhiza than crops. Similar observations have been made for older crop cultivars compared to more recently released crop cultivars. Such observations have given rise to the hypothesis that plant breeding (under conditions of high soil fertility) have inadvertently selected against the ability of plants to associate with mycorrhizal fungi and to benefit from the mycorrhizal symbiosis, and that therefore ecological agriculture needs to reconsider the genetic basis of crops in order to breed them for higher mycorrhizal responsiveness under less nutrient-rich conditions.

Genetic variation in plant responsiveness has been studied for several crops (T.W. Kuyper et al., unpublished data). Crops that show genetic variation in responsiveness (tested by growing different cultivars under uniform soil with the same mycorrhizal fungus) include: onion, Welsh onion, peanut, oat, peach palm, chili pepper, papaya, chickpea, oil palm, strawberry, soybean, barley, tomato, plantain, tobacco, olive, rice, millet, sorghum, common bean, pea, wheat, cowpea, maize and breadfruit tree.

However, research on possibilities to breed plants for increased responsiveness has indicated several problems. First, assessment of responsiveness (a ratio of performance of mycorrhizal plants relative to non-mycorrhizal plants) is problematical, because high responsiveness of a cultivar can be both due to good performance in the mycorrhizal condition and also to poor performance in the non-mycorrhizal condition. Consequently, breeding for yield stability (including yield stability with variable amounts of mycorrhizal inoculum) can increase performance of non-mycorrhizal plants, which then translates into lower mycorrhizal responsiveness. Second, a search for molecular traits (quantitative trait loci [QTLs]) that correlate with mycorrhizal responsiveness did indicate that many of those QTLs are also indicative for root traits (specific root length [SRL], root hair length and frequency, root biomass, etc.) and that direct breeding for root traits may be more efficient than breeding for responsiveness. Third, it is likely that a comparison of different cultivars would show extensive genotype × soil, genotype × AM fungus, and genotype × soil × AM fungus interactions, making direct application of that knowledge for field management difficult. Critical reviews on the topic of the possibilities of plant breeding for increased mycorrhizal responsiveness have been provided by Sawers et al. (2010), Galván et al. (2011) and Scholten and Kuyper (2012). These papers also provide references to the earlier studies that formed the basis for the hypothesis that modern plant breeding has inadvertently selected against mycorrhizal responsiveness.

2.4 Uptake of other nutrients than N and P

Uptake of other nutrients is variable and affected by nutrient limitations (Lambert et al., 1979). Zn uptake is increased by AMF (Jansa et al., 2003; Gao et al., 2007), and also Ca, Cu, potassium (K), sulfur (S) as SO_4^{2-} uptake has been shown to be increased through the arbuscular mycorrhizal symbiosis (Marschner and Dell, 1994; Rhodes

and Gerdemann, 1980). The mycorrhizal contribution to enhanced uptake depends on whether the uptake of nutrients is mainly determined by mass flow or by diffusion: uptake of nutrients that react strongly with the solid soil phase and where diffusion is the main pathway for uptake is strongly enhanced by the mycorrhizal symbiosis (next to P, also Zn, Cu, to a smaller extent K and ammonium), whereas for nutrients where mass flow is the main pathway (nitrate, sulfate, calcium) the mycorrhizal contribution is more limited. The importance of mycorrhiza for Zn uptake is also important in cases where phosphorus deficiency is remedied by high P fertilization and where high P doses result in reduced mycorrhizal activity. In such cases, Zn-uptake could be reduced, which together with the dilution effect (the phosphorus gift results in larger plants), could create strong Zn-deficient plants (Lambert et al., 1979). Also Zn availability in the plant product (cereal grain) could be reduced due to zinc-phytic acid interactions. The mycorrhizal symbiosis can possibly alleviate such forms of Zn deficiency for human nutrition (Ryan et al., 2008). The importance of mycorrhizal fungi in uptake of these essential micronutrients has not received sufficient attention. As research is currently showing that crop performance is likely co-limited by these micronutrients (and not only limited by the macronutrients N and P and sometimes K), further research on the mycorrhizal role in micronutrient uptake is required.

Calculation of the mycorrhizal contribution to nutrient uptake is, however, not straightforward. The classical balance approach, where the mycorrhizal contribution is determined as the difference between nutrient content of the mycorrhizal and non-mycorrhizal plant, after subtracting seed nutrient content has been shown to be inadequate. Smith et al. (2003) showed, through a labeling approach, that the mycorrhizal contribution to phosphorus uptake can be 100%, even though the mycorrhizal plant did not take up more phosphorus than the mycorrhizal control. It has not been resolved whether this dominance of the mycorrhizal pathway is due to molecular (RNA) interference between fungal and plant transporters or is due to the spatial geometry in the rhizosphere and mycorrhizosphere, where the phosphorus concentration close to the root may be too low (and the internal concentration still sufficient) to allow expression of plant transporters. Furthermore, alleviation of nutrient limitation through mycorrhiza could result in larger plants with a more extensive root system through which it takes up higher amounts of other nutrients. It may, however, be incorrect to ascribe this nutrient uptake directly to the mycorrhizal symbiosis. Consequently, the data on the mycorrhizal contribution to uptake of other nutrients needs reassessment. Therefore, Smith and Smith (2011) correctly stated that it is still unresolved whether uptake of these elements provides a substantial proportion of plant needs.

2.5 Strains and functioning

Globally, more than 200 species of AMF have been described and named. However, current molecular ecological studies have indicated that the actual species number is higher. An rDNA (small subunit [SSU])-based phylogeny indicates that currently some 350 virtual taxonomic units ('species') are known, a number that is likely to increase as more regions and plants are being studied (Öpik et al., 2013).

Virtually all members of the phylum Glomeromycota (the group that constitutes the AMF) can associate with plants. Most species are able, under controlled conditions, to associate with a large number of plant species, and that has given rise to the statement that there is no specificity in the mycorrhizal symbiosis. However, field investigations have shown non-random associations between plants and fungal species. Also differential benefit from a mycorrhizal fungus by different plants has been shown. While that may result in a classification of fungi as relatively co-operative or non-cooperative (Kiers et al., 2011), it is not clear whether the underlying behavior is a genetic trait of the fungus or is context-dependent. An important study by Angelard et al. (2010) addressed this issue. These authors tried to generate genetic variation in the mycorrhizal fungal species *Glomus intraradices* and were able to increase plant size (of rice plants) fivefold after inoculation of the 'best' progeny of that fungus. However, the same

study did not show any genetic improvement of that fungus when inoculated with *Plantago lanceolata*. It remains unclear to what extent therefore fungal 'breeding' or 'selection' programs can contribute to a substantially higher mycorrhizal efficiency.

As indicated above, the mycorrhizal symbiosis has several benefits (the symbiosis is called multifunctional as it does not only contribute to enhanced nutrient uptake, but also provides protection against pathogens, contributes to soil structure improvement, etc. (see Newsham et al., 1995). Attempts have been made to link these differential benefits to the fungal phylogeny or to functional traits. It has been proposed that a major functional difference exists between species with relatively high intraradical colonization (supposed to make a major contribution to nutrient provision to the plant and pathogen protection) and species with relatively extra high extraradical colonization (with a larger role in improving soil structure) and that this distinction roughly separates to Glomeraceae from the Gigasporaceae (Hart et al., 2001). However, the generality of that proposal has not yet been established. Attempts to come up with a functional classification of fungal traits of the AMF are in their infancy. Chagnon et al. (2013) classified functional traits and suggested they result in the distinction of three fungal strategies that coincide with the three major plant strategies recognized (ruderals, stress tolerators, competitors). Another classification of fungal species along the r-K continuum (separating fungal species with an annual life cycle with prolific spore production and a large capacity to regenerate from hyphal disruption through disturbance from those with a perennial life cycle with a lower spore production) is potentially relevant in the debate about agriculture based on annual crops or forms of agriculture of perennials crops (including agricultural systems that include perennial plants like shrubs and trees) but needs more systematic testing (De Carvalho et al., 2010).

The relation between species (or functional) diversity and ecosystem functioning has also been debated for AMF (Kuyper and Giller, 2011). From the perspective of mycorrhizal application (or the use of microbial inoculants in general) the relevant implication is whether we should apply monospecific inoculum (of one best performing strain, e.g., the improved strain generated by Angelard et al. [2010]) or whether a more diverse mixture of different mycorrhizal fungi is likely to be more beneficial. The literature in that respect has not yet come to a common conclusion. It should also be kept in mind that there could be potential trade-offs between fungal traits that result in largest beneficial effects to the plant and traits that allow the fungus to be competitively superior. Finally, because the mycorrhizal symbiosis is multifunctional and different benefits (of intraradical and extraradical mycelium) are almost certainly the result of evolutionary trade-offs, a higher mycorrhizal fungal diversity is likely to be more beneficial, especially in systems of intercropping and crop rotations, because of fungal selectivity for certain plants and or specificity in fungal benefits to different plants (Kuyper and Giller, 2011). Verbruggen et al. (2012a) noted a further trade-off between agricultural sustainability and crop productivity, where AMF, and especially those from organically managed agricultural fields, did not improve maize yield, but reduced phosphorus leaching.

2.6 Host relationships

AMF receive (or take) carbon from the host plant in return for nutrients, water and protection against toxic metal levels and pathogens. However, despite the perceived mutualistic symbiosis of AMF and plants, negative growth responses of AMF on plants have been repeatedly reported (Johnson et al., 1997). A recent meta-analysis by Hoeksema et al. (2010) indicated that around 20% of all studies did not demonstrate an improvement of plant performance and a part of these even resulted in mycorrhizal plants being smaller than non-mycorrhizal plants. Such studies have given rise to the concept of carbon costs of mycorrhizal symbiosis. The underlying model assumes a carbon for nutrient trade; and with increasing nutrient availability and decreasing costs for the plant to acquire the nutrients, the final balance becomes negative (Grimoldi et al., 2006). This phenomenon, negative mycorrhizal responsiveness, has been called 'parasitism' (Johnson et al., 1997). Causes of negative

responsiveness are sometimes used as an argument why mycorrhizal associations are unimportant in intensive agriculture. However, carbon costs have seldom been quantified (through measuring photosynthesis and carbon allocation to the fungus). A meta-analysis by Kaschuk et al. (2009) indicated that at the same internal nutrient concentrations of nitrogen and phosphorus mycorrhizal plants have higher photosynthesis rates, a process called sink stimulation by microbial root symbionts. The same phenomenon has been demonstrated for rhizobia. The increases in carbon gain were calculated as roughly equivalent to the carbon used by the fungus (around 10% of photosynthesis), suggesting that the mycorrhizal symbiosis might often come 'for free.' The debate of the usefulness of the mutualism-parasitism continuum is still ongoing, with the co-authors of the earlier Johnson et al. (1997) paper now taking different positions (Johnson and Graham, 2013; Smith and Smith, 2013).

Negative growth effects may be due to other factors than carbon use by the fungus (Facelli et al., 2009; Grace et al., 2009; Smith et al., 2009). They include nitrogen immobilization by the fungus, suppression of root exudates, changes in hormonal balance, root architecture and root to shoot ratio. In the wheat belt of SE Australia, wheat (*Triticum aestivum*) varieties with low AMF colonization show higher production than varieties with higher colonization, and this may be related to the suppression of exudate production in these P-poor soils (Ryan et al., 2005). Finally, it has been suggested that the carbon invested by plants should not only be compared to the immediate return (of phosphate or other nutrients), but should also be looked at as long-term investment by the plant, ensuring a continuous, stable supply of mineral nutrients (Landis and Fraser, 2008).

2.7 Optimal conditions

In natural ecosystem soils, pH is the most important determinant of AMF community composition, showing that environmental factors shape the AMF composition (Dumbrell et al., 2010). At small spatial scale within the soil (e.g., the rhizosphere), secondary factors regulate AMF community composition such as root exudates (which also depend on the internal nutrient status of the plant). Similar studies in agricultural systems point to large roles for phosphorus availability and degree of disturbance as determinants of the community composition of AMF (Oehl et al., 2011). As indicated above, a larger species diversity is likely to be beneficial in cases of crop rotation or intercropping, because different plant species derive a larger benefit from different fungal species. Rotations may also prevent dominance of fungi that cause a lower benefit for the host plant (Johnson et al., 1992) through a process of negative feedback. Maintaining mycorrhizal diversity is also important because of the suite of other ecosystem services provided by the symbiosis (Gianinazzi et al., 2010; Fester and Sawers, 2011). More important than species composition is the presence of sufficient inoculum at the start of the growing season.

Smith and Smith (2011) stated that high density of AMF inoculum in soils is important to achieve crop colonization by AMF and subsequent increases in growth and yield. AMF inoculum of agricultural soils is encouraged by minimum or zero tillage and break crops that associate with AMF, avoidance of long periods of bare fallow, burning of crop residues or frequent cropping with non-host species such as members of the Brassicaceae or Chenopodiaceae (e.g., canola or beet) families (Abbott and Robson, 1991; McGonigle et al., 2011). Negative effects of soil disturbance on mycorrhizal fungi and hence early uptake of nutrients have been shown for maize in Canada (Miller, 2000) in the earliest growth phase, where disturbance resulted in early phosphorus deficiency despite adequate fertilizer application, and for legumes where disturbance resulted in lower colonization and lower amounts of nitrogen fixed by soybean (Goss and De Varennes, 2002). Negative impacts of long bare fallows on subsequent growth of flax in Australia were shown to be caused by reduced mycorrhizal potential (Thompson et al., 2013). Plowing also results in a strong reduction of species richness (Daniell et al., 2001) with only very few weedy species of the genus *Glomus* surviving. The negative impact of agricultural practices on fungal performance can be alleviated by using fungal inocula with low sensitivity for agricultural practices such as plowing, application of

fertilizers and use of pesticides. A number of such fungal strains have been described in recent years and might be used as the basis for further breeding approaches (Angelard et al., 2010), creating AMF strains resistant to agricultural practices and likely to remain active inoculum in agricultural fields. Nevertheless, even such relatively resistant strains are likely to be negatively affected by conditions such as the presence of large quantities of mineral fertilizer, especially of highly soluble phosphates, or the unavailability of host plants for prolonged periods.

Fester and Sawers (2011) stated that the negative correlation between AMF diversity and anthropogenic soil disturbance is consistent enough that AMF diversity (or root colonization levels) has been proposed as a measure for soil quality (Baar, 2010). Jansa et al. (2009) proposed a standard assay to assess mycorrhizal colonization potential to determine the impacts of agricultural management on mycorrhizal fungi. However, whereas disturbance (plowing) has usually negative effects on species diversity, the mixing process itself could affect spatial structure. Verbruggen et al. (2012b) showed that with mixing (and hence reducing spatial structure) the relative success of more cooperative AM fungal species increases, refuting earlier claims that spatial structure is necessary to maintain the more beneficial mycorrhizal fungi (Bever et al., 2009).

Crop rotations with non-mycorrhizal crops have shown retarded growth of subsequent, mycorrhiza-responsive crops (McGonigle et al., 2011), although the effect of cabbage on subsequent growth of bunch onion was dependent on cabbage cultivar (T.W. Kuyper et al., unpublished data), with strong negative effects by some cabbage genotypes and no effects by others.

AMF colonization of the host plant is also impacted by soil fertility and often fertilizer addition (especially phosphorus addition) will reduce AMF colonization (e.g., Abbott and Robson, 1982; Johnson, 1993). Addition of phosphorus fertilizer impacts the mycorrhizal fungus both directly (via the soil) and indirectly (through the plant). While there is plant control over mycorrhizal colonization by the plant through the internal phosphorus status (and such control also regulates competition among different mycorrhizal fungi), it is likely that a major effect of phosphorus is due to the direct negative effect. It is likely that foliar application will have a less negative effect on the symbiosis, although the topic has not often been studied.

Not surprisingly therefore, a role for and the benefits of AMF symbiosis in fertilizer-intensive agriculture has been questioned (Ryan and Graham, 2002). These authors pointed out a larger role for mycorrhiza in forms of agriculture that rely more on organic amendments and less on inputs of mineral fertilizer, such as organic agriculture in the northern hemisphere (Oehl et al., 2004; but see Galván et al., 2009 for observation that conventional and organic onion fields did not differ in mycorrhizal fungal species diversity), but also forms of agriculture in developing countries where resource-constrained farmers are often organic-by-default. However, the relevant question is not whether mycorrhiza inoculum addition can increase yields above yield increases due to fertilizer, but whether similar yields can be obtained but with lower fertilizer amounts, because the mycorrhizal symbiosis increases the phosphorus uptake efficiency. From that perspective the negative effects of unbalanced fertilization (which can also cause secondary deficiencies in micronutrients such as zinc), disturbance and use of fungicides need attention. It has been claimed that these human practices select over ecological and evolutionary time scales for mycorrhizal fungi that are on average less beneficial for the plant and/or provide less other ecosystem services (Kiers et al., 2002; Verbruggen and Kiers, 2010). Such effects have indeed been shown in Europe, but far less so in a study in Kenya (Muriithi-Muchane, 2013). However, in European agricultural fields cases have been reported where the higher mycorrhizal inoculum from organic fields reduced nutrient losses, but this ecosystem service was traded off against crop productivity (Verbruggen et al., 2012a).

2.8 Examples of application and potential use for upscaling

As most crop plants associate with AMF naturally, the question is whether and under what conditions inoculum addition is necessary rather than management of the naturally occurring mycorrhizal fungi. As mentioned previously, to optimize soil inoculum of AMF for crops little soil disturbance is needed, which is usually not feasible in high-production agriculture, unless farmers switch to forms of conservation agriculture. Focused inoculation of the crop with specific AMF has been researched as alternative to natural inoculation via soil inoculum. Smith and Smith (2011) reviewed AMF optimization for agriculture and concluded that practical problems persist: (1) no universal 'elite' AMF exist to maximize growth of all AMF plants because of fungal selectivity and multifunctionality; (2) AMF are obligate symbionts so that production of high-quality inoculum is host-specific and thus expensive; and (3) large-scale inoculation in the field is expensive, and survival of the AMF inoculants is problematic as there could be a trade-off between competitive ability and plant benefit. In addition, Smith and Smith (2011) warn that as long as P fertilizer is in easy supply the AMF inoculation will be less favored as high P availability, due to application of easily soluble phosphorus fertilizer, diminishes the formation of AMF symbiosis with host plants (Abbott and Robson, 1982; Johnson, 1993).

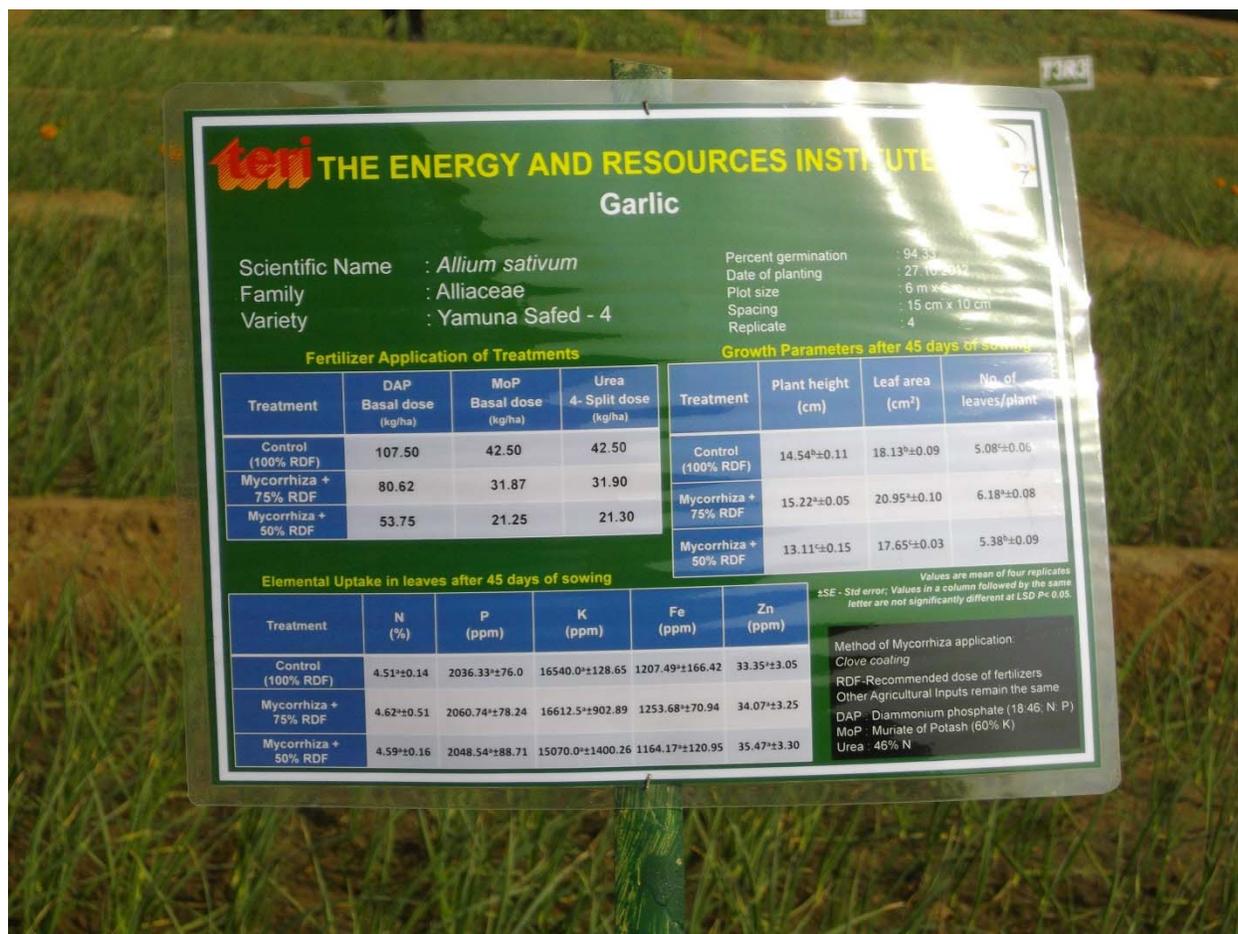


Photo 1. Large field experiments have shown the benefits of fertilizer reduction in the presence of inoculum addition. Photo by Thomas W. Kuyper.

Mycorrhizal inoculum is supplied by several companies. Sometimes the carrier material contains nutrients, so ascription of effects to the fungus is not always straightforward. Because of problems with inoculum quality, there

have been efforts to establish a system of accredited inoculum suppliers whose inoculum complies with specific standards (Vosatka et al., 2012). However, costs for maintaining a quality control system are non-negligible and inoculum itself is also bulky. There have been no published reports of seed coating by mycorrhizal fungi. This possibility was mentioned once and an illustration of coated seeds was also provided (Adholeya et al., 2005: figure 2, p. 318), however, further information on this product has not been published as far as we could ascertain. A case of commercial inoculum application was described for Nigeria (Salami and Osonubi, 2002). In India, TERI is making big progress in research for more effective inoculum production and application. Large field experiments have shown the benefits of fertilizer reduction in the presence of inoculum addition (see Photo 1). A novel development is the production of *in vitro* produced inoculum, which is claimed to be clean from other organisms and can be produced cheaply. Ceballos et al. (2013) reported substantially increased yield of cassava in Colombia when using *in vitro* produced inoculum, while at the same time reductions of P fertilizer could be achieved. However, the authors also noted that, despite the yield increases as a consequence of mycorrhizal fungal inoculum, return-on-investment was not larger than with conventional cassava cultivation.

2.9 Quantitative estimates of nutrients to be taken up, quantification in terms of ecosystem services

Quantitative estimates of AMF impact on plant nutrition are difficult to make because the non-mycorrhizal control in either laboratory or field studies is an unnatural status of the plant (Smith and Smith, 2011). Field experiments with different levels of mycorrhizal inoculum at the start of the growing season (through disturbance such as plowing, the use of selective fungicides or by pre-growing fields with non-mycorrhizal crops such as cabbage) have shown that also in the field the net benefits of AMF symbiosis on plant fitness outweigh (short-term) negative effects (Singh and Vyas, 2009).

The ecosystem services delivered by the mycorrhizal symbiosis has been discussed by Gianinazzi et al. (2010) and Fester and Sawers (2011). Attempts to quantify services are very rare. An early attempt was made by Miller et al. (1994), who described a methodology but did not provide quantitative data. Kuyper and Giller (2011) estimated phosphorus saving through increased nutrient uptake efficiency of possibly around 10%, and this amounted to US \$2 billion. Further quantifiable ecosystem services could be the contribution to nitrogen fixation in legumes (as these plants need higher amounts of phosphorus, they also depend on mycorrhiza). Assuming 10% yield increases of legumes due to mycorrhiza would result in an additional service with an annual value of 10% of the ecosystem service provided by nitrogen fixers. This would amount to another US \$9 billion annually. The ecosystem services related to enhanced drought tolerance, improved resistance against pests and pathogens and improved soil quality have not been quantified.

2.10 Suggestions for use in agricultural production

Inoculation of crops with AMF strains that are targeted at increasing crop yield, and that repress less mutualistic AMF strains that reduce yield, has shown some success according to meta-analyses by Lekberg and Koide (2005) and Hoeksema et al. (2010), but due to the obligate biotrophic nature of AM fungi, the development of cost-efficient large-scale production methods to obtain high-quality AM fungal inoculum is complicated, and their commercial exploitation is still in its infancy (Ijdo et al., 2011). Nevertheless, Angelard et al. (2010) cultivated (improved) AMF strains that increased the growth of rice (but not of a second plant!). Still, it is unlikely that selected AMF strains will be able to display the wide variety of benefits of a diverse AMF community (Fester and Sawers, 2011).

Furthermore, Ryan and Graham (2002) and Lekberg and Koide (2005) concluded that high soil phosphate levels as a result of application of highly soluble inorganic P fertilizers (e.g., superphosphate) limit the effectiveness of AMF inoculation, so that targeted inoculation of crops with AMF may only be effective in low-fertilizer input systems (Fester and Sawers, 2011), such as organic agriculture where P availability is low (Ryan and Graham, 2002), and only rock phosphate is used, which is less soluble and therefore more slowly released than conventional P fertilizers. In intensive agriculture it may be possible to reduce negative impacts on mycorrhiza by foliar application (because the direct soil effects of P on the symbiosis are stronger than the indirect, plant-mediated effects), by microdosing or other forms of selective placement around the growing plant. However, these topics have been rather underresearched.

There are also various reports about the use of arbuscular mycorrhizal inoculum in micropropagation systems and soilless production systems. Under those conditions mycorrhizal plants show better post-transplanting performance, probably through enhanced nutrient uptake, changed hormonal balances and pathogen protection (Kapoor et al., 2008). Possible fine-tuning of nutrient management with application of mycorrhizal inoculum needs further research.

However, in a holistic approach taking into account benefits of AMF not related to increased nutrient uptake (pathogen protection, increased soil stability, protection from toxic levels of metals), management shifts that contribute to establishing and maintaining a (functionally) diverse mycorrhizal fungal community while maintaining high productivity levels is almost certainly much more cost-effective (Jeffries et al., 2003; Cardoso and Kuyper, 2006; Gosling et al., 2006; Shennan, 2008). This holistic approach aims to improve soil microbial and rhizosphere communities in general, motivated by the possibility that diverse soil microbial communities provide ecosystem functions that cannot be generated by specific organisms, species or strains (Fester and Sawers, 2011). For instance Jansa et al. (2005) observed synergistic effects by combining fungal strains, and mycorrhizal fungi are known to enhance nitrogen fixation by symbiotic N fixers (Smith et al., 1979; Barea et al., 2002).

Mycorrhizal management could also have an impact on food quality. For instance Ryan et al. (2008) noted that mycorrhizal plants possessed lower amounts of phytate; consequently, a higher proportion of the zinc in the plant seed was available for humans during consumption. Similarly, effects of mycorrhizal fungi on increased production of secondary compounds have been reported (Zeng et al., 2013); for instance, the production of artemisin, a high-potential anti-malarial drug, was much higher in mycorrhizal than non-mycorrhizal *Artemisia annua* (Kapoor et al., 2007). Finally, Larsen et al. (2006) noted higher selenium (Se) uptake in mycorrhizal garlic, which should result in health effects of humans who consume garlic.

2.11 Ectomycorrhizal fungi for forestry

Ectomycorrhizal fungi associated with most trees are important for forestry production (e.g., Pinales, Fagales, Myrtaceae, Dipterocarpaceae) and are capable of nutrient uptake from both inorganic and organic recalcitrant sources (e.g., Read and Perez-Moreno, 2003; Hoffland et al., 2004). Trappe (1977) and Marx (1980) reviewed methods and ectomycorrhizal species for inoculation of plantation tree seedlings in nurseries, although it is noted that if enough inoculum is present in the plantation field soil, nursery inoculation may not be needed. Nursery inoculation is especially needed in the case of exotics if compatible inoculum is lacking. Plantations of pine in the southern hemisphere were initially established after soil with ectomycorrhizal inoculum was introduced from the northern hemisphere (Netherlands, Great Britain). Subsequent plantations were established with soil with inoculum from existing populations. Currently, however, with increased inoculum availability, pines behave as invasive plants in the southern hemisphere.

Virtually all plantation trees rely on ectomycorrhizal fungi for their nutrition and maintaining a good source of inoculum and high ectomycorrhizal diversity is vital for plantation production (Perry et al., 1987). Similarly as for agriculture, the diversity of ectomycorrhizal fungal communities in forest soils is affected by management practices such as harvesting, liming, fertilizing with nitrogen to enhance tree growth, choice of tree species and monoculture versus mixed species composition (Teste et al., 2012). Whereas AMF are sensitive to high phosphorus availability, ectomycorrhizal fungi are sensitive to high nitrogen availability. Both the direct pathway (negative effects of soil nitrogen on the symbiosis) and the indirect (tree-mediated) pathway regulate the fungal response to nitrogen. Like in AM the direct pathway is more important. A decline in ectomycorrhizal fungi has been noted in the last decades in forests saturated with N due to high anthropogenic N deposition over the past decades (Lilleskov et al., 2011; Bahr et al., 2013; Kuyper, 2013). Decline of ectomycorrhizal fungi and a poorer functioning of the symbioses results in poorer tree performance due to nutrient deficiencies and unbalances (especially the N:P ratios), increased sensitivity to drought and enhanced susceptibility to pathogens. Recovery of ectomycorrhiza under conditions of lowered nitrogen loads is a (very) slow process.

2.12 Dark septate root endophytic fungi

According to Jumpponen and Trappe (1998), dark septate root endophytes (DSE) have been reported for nearly 600 plant species representing about 320 genera and 100 families, occurring from the tropics to arctic and alpine habitats. The DSE belong to the Ascomycota and form a polyphyletic and taxonomically diverse group of fungi that functionally and ecologically overlap with soil fungi, saprotrophic rhizosphere-inhabiting fungi, obligate and facultative pathogenic fungi and mycorrhizal fungi. The nature of the interaction between DSE and their host plant is still a matter of debate (Grünig et al., 2008; Newsham, 2011); however, Newsham (2011) did not find negative records of DSE on plant performance in a meta-analysis of 18 controlled studies, and generally DSE had positive effects on plant biomass and N uptake, especially when N was provided in organic form. However, the study by Mayerhofer et al. (2013) provided more evidences of neutral to negative effects on plant biomass than positive effects. As for mycorrhizal fungi Mandyam and Jumpponen (2005) stress that DSE-plant symbioses should be considered multifunctional and not limited to nutrient acquisition and resultant positive host growth responses, but also in terms of stress tolerance and pathogen and herbivore deterrence. Due to the diversity of responses of individual fungal species and individual plants (crops) and lack of knowledge of the mechanisms through which they improve plant performance, DSE are currently not being managed in agriculture.

2.13 *Piriformospora indica*

This species is a member of the Sebaciales, a fungal group that can form root symbioses with a large range of plants that are capable of (also) forming ectomycorrhizal, ericoid mycorrhizal, orchoid mycorrhizal and arbuscular mycorrhizal symbioses. *Piriformospora indica* was originally isolated from the spore of an arbuscular mycorrhizal fungus found in the Thar Desert in India and root-colonizing abilities were shown (Verma et al., 1998). It is currently known from various (sub-)tropical regions, Asia, Australia, South America (Oelmüller et al., 2009). Plant growth-promoting effects were discovered for various hosts, and its application to plant production was proposed (Varma et al., 1999). The species initially has a biotrophic life style causing programmed cell death; subsequent specific host plant immune responses result in improved pathogen control (Qiang et al., 2012; Unnikumar et al., 2013). Consequently, plants colonized by this species are larger and have taken up more nutrients than plants that are not colonized. However, increased nutrient uptake is likely the consequence of increased plant size, not the cause of it. The mechanisms through which the species enhances plant fitness are currently being elucidated through molecular tools. Waller et al. (2005) found that barley plants colonized by *P. indica* were more resistant to pathogens

and more tolerant to salt stress and showed higher yield. Franken (2012) lists studies that show plant growth promoting effect of *P. indica*. In terms of increased plant mineral nutrient uptake it remains unsure whether the fungus is responsible directly for increased uptake, or through increased root growth (Franken, 2012). He concluded that the interaction of *P. indica* with plant roots has been intensively studied, and genome sequence and transformation systems are available, but that to use the root endophyte in agricultural practice, a product for commercial use must be established and registered. Currently no commercial inoculum of this species is available on the market. Knowledge about this fungus and its potential applications has recently been summarized by Varma et al. (2013).

2.14 Other fungal root endophytes

Other fungi colonizing roots that have beneficial effects on plant health have also been described. Examples are *Trichoderma viride* and non-pathogenic strains of *Fusarium oxysporum*. Such fungi reduce damage by pathogens resulting in more healthy and larger plants that consequently take up more nutrients. However, no direct role for such fungi in nutrient uptake has been reported. There is no symbiotic interface through which carbon (from the tree) and nutrients (from the fungus) are being exchanged.

2.15 Mycorrhiza helper bacteria – three-way interactions

Duponnois and Garbaye (1991) and Garbaye (1994) observed the effect of the bacterium *Pseudomonas fluorescens*, which significantly stimulated the formation of ectomycorrhiza by the fungus *Laccaria laccata* (later reclassified as *L. bicolor*), and termed these bacteria mycorrhiza helper bacteria (MHB). Frey-Klett et al. (2007) provided further information on the effects of MHB during the symbiosis, including improved conductivity of the soil and responsiveness of the roots to fungal recognition and establishment. Furthermore, Frey-Klett et al. (2007) reported that MHB promote survival, germination of propagules, and mycelial growth of ectomycorrhizal fungi. Moreover, they reported that both the fungus and root select the bacterial population in the rhizosphere soil, promote the growth of fungus, and determine the receptivity of the root to the fungus.

Most attention has been devoted to specific helper bacteria of the ectomycorrhizal symbiosis. However, specific bacteria also occur around and on the hyphae of AMF. Artursson et al. (2006) reviewed these interactions between AM fungi and rhizosphere bacteria, and concluded that the underlying mechanisms behind such tripartite associations were not well understood and more insight into these interactions is imperative for optimization of the effective use of AM fungi in combination with their bacterial partners as a tool for increasing crop yields. For that reason no commercial inocula of MHB are available on the market.

Other fungal-bacterial interactions are dealt with elsewhere in this report. These include interactions between AMF and the nitrogen-fixing rhizobia (Barea et al., 2002) and interactions between AMF and phosphate-solubilizing bacteria (PSB) that can release phosphorus from sparingly soluble P sources (Kucey et al., 1989; Bidondo et al., 2012). These three-way interactions are discussed in sections 3.1 and 3.11.

The release of Flavonoids and Strigolactones (Steinkellner et al., 2007) and physical contact between bacteria and mycorrhizal fungi are important for the establishment of these three-way interactions (Bonfante and Anca, 2009). This three-way interaction has been studied in terms of increased plant nutrition and growth (e.g., Calvaruso et al., 2007, Koele et al., 2009, Frey-Klett et al., 2011) from which it appeared that microbial niches and functioning are closely related, and the interactions are largely dependent on environmental factors. However, field studies need to confirm the symbiotic three-way interactions to have ecological benefits.

Several of these bacteria (e.g., *Pseudomonas fluorescens*) have been described as having beneficial effects on plant growth and are known as plant growth-promoting rhizobacteria (PGPR). They are dealt with in section 3.14.

3 Rhizobia

3.1 General review, specification in terms of ecosystem services

Rhizobia are a group of bacteria that induce and infect nodules on roots or stems of plants of most (but not all) members in the family Fabaceae (Leguminosae; also known as legumes) and fix nitrogen. Nitrogen fixation by root-inhabiting rhizobia has also evolved in the genus *Parasponia* (Ulmaceae), and this evolutionary novelty has given rise to optimism that the genetic machinery for associations with rhizobia and hence successful N-fixation can also be transferred to our major food crops. This optimism finds further support in the observation that the initial molecular dialogue of rhizobia and legumes is built upon the initial dialogue between AMF and plants, almost all of which forming AM (see previous chapter). However, no successful nodulation and subsequent N-fixation by rhizobia has been reported for crops other than legumes, so the potential of root nodule symbioses in cereals still remains unclear. Not all species of the Fabaceae form symbiosis with N-fixing rhizobia. The association between legumes and rhizobia shows various forms of host specificity or selectivity. Host specificity is determined by the nod genes (the genes that determine nodulation), not by the nif genes (the genes that determine N fixation). For that reason selectivity does not result in selection of the most effective rhizobia by legumes; legumes have therefore been described as 'incredibly naive' (Den Herder and Parniske, 2009). While the symbiosis from the plant perspective is almost obligatory (these legumes cannot survive without effective rhizobia under conditions of normal soil fertility), it is not obligatory from the bacterial point of view, as the bacteria can also survive in soil as saprotrophs. In soils with enough available N, bacteria and host plants remain unassociated (Bonilla and Bolanos, 2010). Nitrogen deficiency triggers the symbiotic interaction and the symbiosis develops completely only when low N status of the soil is maintained (Bonilla and Bolanos, 2010).

Nitrogen fixation is an energy expensive process exceeding 16-18 mol Adenosine triphosphate (ATP) per mol N₂ fixed (Bergersen, 1991). Nitrogen fixation is therefore more energy demanding than N uptake, which explains why under conditions of ample nitrogen supply N fixation is suppressed. However, like in the case of AMF, these costs are compensated by a process called sink stimulation of photosynthesis by mutualistic root symbionts (Kaschuk et al., 2009). Legumes associated with N-fixing rhizobia also require more P than other plants, both because of the additional energy demand (Cassman et al., 1981) but also because legumes have a lower nitrogen use efficiency than other plants (McKey, 1994) and phosphorus use efficiency is also lower because of N:P stoichiometry (Vitousek et al., 2002).

Tripartite associations of host plants with both rhizobia and AMF benefit the host plant by increased P uptake through the mycorrhizal association balancing the high input of N through rhizobial N-fixation (Barea et al., 2002; Bucher, 2007; Bonfante and Anca, 2009). The question to what extent this tripartite symbiosis has additive or synergistic effects is dealt with below. Wang et al. (2011) found that a deep-rooted soybean variety increased in yield when inoculated with both rhizobia and AMF in a field experiment under low N and P additions. Similarly for mung bean, Yasmeen et al. (2012) noted higher yield and N and P content upon dual inoculation with rhizobia and AMF under field conditions. Abd-Alla et al. (2013) noted that AMF inoculation in combination with rhizobia inoculation of faba bean in alkaline soils facilitated the mobilization of elements such as P, Fe, K and other minerals

involved in synthesis of nitrogenase and leghaemoglobin, increasing nodulation and yield. Highest yield of chickpea was observed under inoculation with AMF, rhizobia, whey addition and irrigation by Erman et al. (2011). Gray (2011) summarized that in most instances the tripartite interactions between legumes, AMF and rhizobia cause increases in legume productivity, and the N:P:C supply ratio as influenced by the tripartite symbiotic associations plays a fundamental role in controlling the legume's photosynthetic rate and biomass productivity. For cultivation of legumes, this relationship between rhizobia and mycorrhizal fungi is of great importance because it influences the infection rate and mineral nutrition as well as the physical and chemical conditions of the soil by adding organic matter and increasing the growth of these plants (Figueiredo et al., 2013).

Bucher (2007) described that several genes are induced both during mycorrhizal cell infection and rhizobial cell infection, supporting the hypothesis that during evolution of terrestrial plants, nature has added components of an ancient signaling pathway to optimize P nutrition with components that allow certain plants to acquire sufficient amounts of N (or that certain further microorganisms 'hijacked' and further expanded on that signaling cascade). In terms of ecosystem services, this means that optimal plant macronutrient use is obtained by symbioses with both rhizobia and mycorrhizal fungi under N and P poor soil conditions. Both rhizobia and mycorrhizal fungi are suppressed to form symbioses in soils rich in N and P, both through a direct pathway (through the soils) and through an indirect pathway (plant regulation in case of nutrient sufficiency). The combined occurrence on the same legume root of rhizobia and AMF means that by careful management of (or by inoculation with, in case effective symbionts are not available) rhizobia and AMF, N and P fertilizer input can (and should) be lowered, reducing fertilizer costs while maintaining a good yield. The input of nitrogen-rich litter can also reduce N-limitation for subsequent crops, but the degree to which legumes maintain a positive N balance (or still contribute to soil N mining) depends on the amount of N fixed and the amount of N harvested through fruits and leaves (Vanlauwe and Giller, 2006).

Contrary to mycorrhizal fungi, rhizobia can be called biofertilizers, because they transform atmospheric nitrogen into reactive nitrogen that is available for plant growth. AMF should not be called biofertilizers, because they do not increase the phosphorus pool, but only more successfully scavenge the soil for phosphorus.

3.2 Associated plant species

Symbiosis with rhizobia and nitrogen fixation has been demonstrated in around 20% of the around 13,000 leguminous species, belonging to around 60% of the legume genera (Corby, 1988; de Faria et al., 1989; Sprent, 2001; Sprent, 2007). However, a large number of legumes remain unresearched and the capacity to form symbiosis with rhizobia has not been demonstrated. It is therefore likely that the final number of N-fixing legumes is much higher, especially in the papilionoid groups. However, from the number of non-fixing genera, especially in the more primitive legume groups, but also in more advanced groups where the ability to fix N has been lost, it cannot be assumed that any legume can fix atmospheric N (Giller, 2001). (This may be another difference with AM, where it can almost be assumed that an unknown plant is very likely able to form AM.)

Important crops that associate with rhizobia are: groundnut (*Arachnis hypogaea*), chickpea (*Cicer arietinum*), soybean (*Glycine max*), pigeonpea (*Cajanus cajan*), New World pulses (*Phaseolus* species), Old World pulses (*Vigna* species), pea (*Pisum sativum*), lentil (*Lens culinaris*), and faba bean (*Vicia faba*). Other important legumes occur in pastures such as clover (*Trifolium* species) or are trees in agroforestry systems (*Leucaena leucocephala*, *Faidherbia albida*, *Acacia* species).

3.3 Uptake of other nutrients than N and P

Due to increased uptake of N through N fixation, other nutrients are usually limiting. Several elements are in higher demand by legumes than by other plants, and these include phosphorus (P), boron (B), calcium (Ca), cobalt (Co), molybdenum (Mo) and iron (Fe). These macro- and micronutrients play important roles in energy generation, nodulation and N-fixation (Redondo-Nieto et al., 2003). P is needed for generation of ATP (and also because legumes have a low P use efficiency due to N:P stoichiometry, see above). B is needed for the maintenance of nodule cell wall and membrane structure, requirement for B has been reported for rhizobial infection and the nodule invasion process, and is also essential for symbiosome development and bacteroid maturation (Bolaños et al., 2001), and for early events in plant-bacteria signaling (Redondo-Nieto et al., 2001). Ca in plants is involved in the structure and function of cell wall and membrane, and cytosolic free Ca^{2+} is important as a second messenger in the signaling mechanism of many plant responses (Bush, 1995). A high Ca^{2+} supply increased the number of nodules (Lowther and Loneragan, 1968), and Munns (1970) reported that Ca^{2+} is especially required for early infection events. Richardson et al. (1988) demonstrated that high Ca^{2+} increased the amount of nod-gene inducing compounds in root exudates. Furthermore Mo, Co and Fe are crucial for N-fixation as constituents of several enzymes such as nitrate reductase and nitrogenase (Giller, 2001). Some, but not all, legumes have a large demand for K. It is therefore possible that legumes are more prone to (micro-)nutrient co-limitation, the more so as availability of these micronutrients is pH dependent. At low pH, Mo deficiency is more likely to occur, whereas Fe deficiency is more likely in alkaline soils. However, the importance of micronutrient deficiency has likely been underestimated. Current attempts to apply N-fixation (such as in the project N2Africa) provide improved seeds, rhizobial inoculants and starter fertilizer that contains phosphorus and also these micronutrients. The best way to apply some of these micronutrients (microdosing close to the developing root; or foliar sprays) needs further research. We did not find recent information about the importance of foliar sprays for rhizobial functioning.

3.4 Strains and functioning

The classification of nitrogen-fixing bacteria (formerly and colloquially known as rhizobia – defined as N-fixing bacteria that form root nodules on legumes and possess so-called nod [nodulation] and nif [nitrogenase] genes) is undergoing rapid and major modifications (Giller, 2001). It has been accepted for a long time that these rhizobia were all closely related and belong to the alpha-proteobacteria. A simple subdivision split those bacteria in fast-growing and slow-growing bacteria. It is now clear that there are several other bacteria, also belonging to the beta-proteobacteria, that possess the same ability to nodulate and to fix nitrogen. The genes that are responsible for these processes are often located on plasmids or symbiotic islands that can be transferred laterally to other bacteria, giving rise to new nitrogen-fixing bacteria. The consequence of horizontal gene transfer is that a bacterial phylogeny (based on ribosomal genes) is not congruent with a phylogeny based on the genes for nodulation nor with genes for nitrogen fixation. A comparison of phylogenies based on rDNA, nod genes and nif genes has shown that the nodulating *Methylobacterium* species have derived the nif genes through horizontal gene transfer from *Gluconacetobacter diazotrophicus*, an endophyte that can fix N in association with sugarcane. Species of *Burkholderia* (beta-proteobacteria) with N-fixation capacity have derived these genes from rhizobia from the alpha-proteobacteria (Rivas et al., 2009). Some rhizobia are therefore more closely related to free-living or associative nitrogen-fixers than to true rhizobia. Phylogenetic studies have also shown that certain bacteria that cannot fix nitrogen are still closely related to rhizobia. A famous example is *Rhizobium radiobacter* (formerly known as *Agrobacterium tumefaciens*), a bacterium that forms gall-like swellings on roots of many plant species. Current taxonomies list 88 species of rhizobia (Rivas et al., 2009) or 98 species (Weir, 2012) in two classes, three orders, seven families and 13 genera (Table 1). The number of rhizobia is likely to grow in the coming years. Some other

bacteria have been isolated from legume-nodules but are more likely opportunistic colonizers of nodules as they do not possess nod genes.

Table 1. Classification of rhizobia. After Weir (2012). Species number indicates number of rhizobia, between brackets the number of non-rhizobial species of that genus

Class	Genus	Species number
Alpha-proteobacteria	<i>Rhizobium</i>	30 (+11)
	<i>Mesorhizobium</i>	21 (+1)
	<i>Ensifer</i> (formerly <i>Sinorhizobium</i>)	17
	<i>Bradyrhizobium</i>	9 (+1)
	<i>Phyllobacterium</i>	3
	<i>Microvirga</i>	3
	<i>Azorhizobium</i>	2
	<i>Ochrobactrum</i>	2
	<i>Methylobacterium</i>	1
	<i>Devosia</i>	1 (+1?)
	<i>Shinella</i>	1
Beta-proteobacteria	<i>Burkholderia</i>	7 (+?)
	<i>Cupriavidus</i>	1 (+?)

Other free-living bacteria also have the ability to fix nitrogen (carrying *nif*, but not *nod* genes). Species with that ability, when having been mentioned as being of particular agronomic relevance, are discussed elsewhere in this report (see section 4).

Nod and *nif* genes, while located relatively closely together on plasmids, show the ability for independent evolution. One interesting consequence is that partner choice of rhizobia by legumes is disconnected from their ability to fix nitrogen. Legumes have therefore been described as ‘unbearably naive’ (Den Herder and Parniske, 2009). Some rhizobial strains have reduced or even lost the ability to fix nitrogen; such strains are known in the literature as ‘cheaters.’

Some rhizobia have been described from non-leguminous plants (e.g., *R. oryzae* – originally described from rice plants but subsequently shown to be able to nodulate various legumes). As rhizobia contain both a free-living and a symbiotic stage, rhizobia could also have beneficial effects in the rhizosphere of non-leguminous plants. Such effects are often due to hormonal effects, making these bacteria part of what is known as PGPR. These effects are discussed elsewhere in the report (see section 3.14).

3.5 Host relationships

The symbiosis between legume host plant and rhizobia shows several degrees of selectivity, implying that only certain strains of rhizobia can associate with (or nodulate) a given legume species, and there must be recognition between the host legume and the rhizobia (Giller, 2001). Most soils contain indigenous bacteria that are able to form nodules in specific legume plants, however in agriculture inoculation of the soil with specific strains occurs as well (see below). The legume host plant exudes flavonoid and isoflavonoid molecules that stimulate the *nod* genes of the rhizobia. In turn the rhizobia secretes a Nod factor into the rhizosphere to confer specificity of recognition

between rhizobia and host plant (Giller, 2001). Upon recognition the plant will start to form nodules that are then invaded by the rhizobia who will start fixing N (Giller, 2001). Maximum N-fixation is only reached when the plant is sufficiently nodulated, which only occurs under continuous N limitation of the soil (Bonilla and Bolanos, 2010). The initial molecular dialogue between rhizobia and legumes is based on the molecular dialogue between AMF and plants (Bucher, 2007).

3.6 Genetic variation among species, genetic variation among and between plant species that allows optimizing use through targeted plant breeding

Similar to the arbuscular mycorrhizal symbiosis, there exists genetic variation among plant varieties, cultivars and landraces for their ability to associate with rhizobia and fix nitrogen. This genetic variation has been extensively researched in cowpea (*Vigna unguiculata* – Belane and Dakora, 2010) and soybean (*Glycine max*). Especially the latter species has been subject to breeding efforts that took the ability to nodulate and fix nitrogen into account. Studies by Gwata et al. (2005) have suggested that promiscuity is the result of the presence of both recessive alleles at two loci. For other species efforts to breed for increased N-fixation are uncommon, because many plant characteristics contribute to N-fixation and breeding for increased fixation would need to be environment specific (Giller, 2001). Soybean, however, is being bred according to two alternatives: promiscuity and specificity. Soybean is in its origin quite selective in symbiosis, so its relatively recent introduction to new continents such as North and South America and Africa meant inoculation with rhizobia was needed because a lack of indigenous strains that were able to nodulate soybean or because strains were relatively ineffective (Peoples et al., 2009). In Brazil and North America, soybean was bred for an even more restricted host selectivity such that the plant cannot or hardly any longer associate with the naturally occurring ineffective strains (Herridge and Rose, 2000; Hungria et al., 2006; Peoples et al., 2009). Such breeding inevitably implies the use of inoculation (often through seed coating) that needs to be regularly repeated in order to maintain a sufficient inoculum density. The practice entails higher costs, and these may not be in reach of resource-poor farmers. An alternative soybean breeding strategy was therefore applied at the International Institute of Tropical Agriculture (IITA) in Nigeria, where they developed soybean genotypes with reduced nodulation specificity that can nodulate effectively with indigenous *Bradyrhizobium* strains populations in Nigeria. While these indigenous strains fix lower amounts of N, they guarantee N fixation under a wide range of agro-ecologies and hence avoid the problems of scarcity or absence of highly effective inoculum (Mpepereki et al., 2000; Abaidoo et al., 2007). According to Thuita et al. (2012), promiscuous soybean selected in Nigeria and introduced in Kenya are all nodulated by indigenous rhizobia. Similarly Musiyiwa et al. (2005) showed nodulation of a different promiscuous soybean in Zimbabwe. Promiscuous soybean can still fix higher amounts of N and become more productive if inoculated with more specific strains of *Bradyrhizobium* (Thuita et al., 2012). Also in the case of soybean, formation of nodules is no measure for effectiveness of N-fixation and increased growth, as shown by Zengeni and Giller (2007).

Soybean is likely exceptional because of the limited range of rhizobia with which it associates in its natural range. (It should be noted, however, that there are possibly other legumes, currently still of minor economic importance that also show relatively high levels of selectivity and where targeted breeding activities are likely successful. However, we know much more of soybean in this regard than of most other legumes.) Other legumes are less selective, and this has allowed successful introduction of South American bean species (*Phaseolus* species) into Africa (Ojiem et al., 2006). Plant introductions have in some cases incidentally introduced rhizobial inoculum into new continents. These exotic inocula may have subsequently switched to indigenous vegetation where they outcompeted native inoculum. At the same time they were less effective in N-fixation with these native plants, thereby shifting the competitive balance between indigenous and exotic legumes allowing the exotic legumes to

cause what has been described as invasional meltdown (Rodriguez-Echevarria, 2010; Rodriguez-Echevarria et al., 2012). This switching has contributed to the invasions of Australian *Acacia* species around the globe.

Commercial inoculants show large variability in their effectiveness. In continents where new legumes were introduced for agriculture such as the Americas and Australia inoculant manufacturing has developed to account for the lack of indigenous rhizobia forming symbioses with the introduced legumes (Herridge, 2008). According to Herridge (2008) farmers in these regions probably use inoculants as insurance, where the cost of the inoculant is measured against the potential loss of crop because of insufficient N-fixation and resultant poor growth. However, in regions such as Asia and Africa, where legumes have been used in agriculture for centuries, inoculation with specific strains has not shown a consistent increase in yield, and farmers are reluctant to invest in inoculants. Because the indigenous soil rhizobia are probably also more effective in Old World than in New World soils, due to a longer history of cropping of legumes, developing promiscuous legume breeds is preferred over breeding for high specificity (Herridge, 2008). However, Thuita et al. (2012) inoculated a promiscuous and a specific soybean with commercial inoculants and found higher yield of the promiscuous soybean upon inoculation. The results of Thuita et al. (2012) show that promiscuous soybean varieties respond to inoculation, and that nodulation, N-fixation and biomass yield are improved if the strain is infective and effective. Thus commercial products produced elsewhere can be an important source of effective strains for use in areas where soybean is being introduced or where low populations of indigenous rhizobia hinder biological N-fixation (Thuita et al., 2012). Nevertheless, Thuita et al. (2012) found no benefits of the commercial products Twin-N and Leguspirflo, which contain endophytic bacteria targeted to increased nodulation of legumes as well as enhance N-fixation in non-legumes. (See also section 4.4 for an evaluation of inoculants for N-fixation in non-leguminous plants.)

Kiers et al. (2007) have suggested that breeding programs for soybean, which took place in field that were well fertilized, may inadvertently have been selected for reduced rhizobial benefit, in a process that is analogous to the decreased mycorrhizal benefit due to evolutionary changes in AMF.

For legumes other than soybean, much less research has been carried out, and it remains uncertain under what conditions inoculation with rhizobia may enhance N-fixation and crop yield compared to management of locally existing rhizobial inoculum through good agronomic practice (Giller, 2001).

3.7 Optimal conditions

The infection rate and effectiveness of rhizobia on legumes are influenced primarily by the N status of the soil, and N-poor conditions are needed for full nodulation and N-fixation (Bonilla and Bolanos, 2010). However, other nutrients should not be limiting, especially P, which can be best prevented by associations with AMF (Bucher, 2007; Bonfante and Anca, 2009). Under highly acidic conditions (tropical oxisols and ultisols) both P-limitation and aluminum toxicity may hamper successful N-fixation (Hungria and Vargas, 2000). Saint Macary et al. (1992) furthermore noted that the right bacterial strains need to be present for the cropped legume and that enough of inoculum or indigenous population is applied or present. Peoples and Herridge (2002) remarked that basic improvements in crop agronomy are probably most promising to enhance crop yield: nutrition (both a balanced availability of N and P, and sufficient K and Ca; but also including trace elements such as Mo, Fe, Co, B), weed control, diseases and pests, and cropping sequence and intensity need to be managed.

3.8 Examples of application and potential use for upscaling

As discussed above, N-fixing rhizobia are commonly used in agriculture in the cultivation of legumes. Vitousek et al. (2013) reviewed the importance of pre-industrial nitrogen fixation, and the subsequent increases due to human planting of legumes. Current human use of legumes in cropping systems (around 50 Tg N fixed annually) is probably in the same order of magnitude as the amount fixed in natural vegetation. Research on N fixation has been focused especially on soybean and has been extensively studied to breed for specificity or promiscuity for rhizobia. For other legumes less research and commercial efforts are undertaken to breed plants or isolate rhizobia to specifically increase yields, as most other legumes are less specific or selective in the association with rhizobia species than soybean but environmental factors confound breeding: no general response to rhizobium inoculations under different environmental conditions has been found (Giller, 2001). It is thus dependent on environment, legume species and rhizobium strain whether the legume yield can be increased, and due to this context-dependency or idiosyncrasy it is usually not commercially viable to invest in small-scale legume breeding or rhizobium inoculation. However, in cases with poorly functioning fertilizer markets as in large parts of Sub-Saharan Africa, there may be a niche for rhizobial inoculants, although a large number of institutional impediments need to be removed before this strategy can be successful (Chianu et al., 2011).

Another application of legumes and rhizobia is that intercropping or crop rotation with legumes usually increases yields of non-legumes (Peoples et al., 2009). (Intercropping may also increase legume yields, in cases of Fe-limitation – Zuo et al., 2000.) Legumes can increase soil fertility and hence crop productivity by (1) increasing inputs of carbon and nitrogen to the soil, especially through below-ground inputs but also from leaf litter or stover (Evans et al., 2001; Schwenke et al., 2002) and (2) producing litter that because of its usually high-quality (low C:N, low amounts of lignin and polyphenols) rapidly releases (mineralizes) nutrients (Wani et al., 1995) and thereby increases concentrations of mineral N in the soil (Herridge et al., 1995; Rochester and Peoples, 2005). In case of intercropping there can also be direct N transfer from legumes to cereals through root exudates and via the mycorrhizal network (He et al., 2003). These benefits are usually related to the belowground additions of legumes to the soil through root biomass, which according to Peoples et al. (2009) is usually not well quantified. (Many balance calculations assume that below-ground C and N inputs are 30% of above-ground production.) Furthermore there are non-N related benefits of legumes: some legumes (e.g., white lupin, pigeon pea, chickpea) can mobilize fixed forms of soil phosphorus by the secretion of organic acids or anions such as citrate and malate (and other compounds) from their roots (Hocking, 2001). N-fixation also causes rhizosphere acidification and this local acidification could improve acquisition of (micro-)nutrients. Legumes can improve soil structure and organic matter content, which is also a major contributor to physical soil quality. Rochester et al. (2001) suggest that faba bean, soybean, field pea and lablab change the soil strength to allow better root system development of cotton. Aslam et al. (2003) showed that chickpea increased C contents of soil, and Shah et al. (2003) showed that retention of mung bean and lentil crop residues increased soil N and C for the following non-legume crop. Furthermore legumes can reduce pathogens: Stevenson and van Kessel (1996) found that 91% of the wheat yield benefit from a preceding pea crop came from reduced leaf disease and weed infestation, and lupins and field peas may suppress diseases (Kirkegaard et al., 2008). Specific suppression of the parasitic weed *Striga* by lupin (Weisskopf et al., 2009) or *Desmodium* (the push-pull system: Khan et al., 2002) has also been described. Legumes also enhance activity and diversity of beneficial microbial communities, mycorrhizal fungi and meso- and macrofauna such as earthworms (Lupwayi and Kennedy, 2007). However, the benefits are not generally present, and especially the increased N content of the soil after legumes is an item of debate. First, the amount of N harvested (grains, leaves, etc.) can be higher than the amount of N-fixed, and the legumes also contribute to soil nitrogen mining (Vanlauwe and Giller, 2006). Furthermore, care must be taken that belowground biomass and N content of legumes are being assessed and incorporated in calculations (Giller, 2001).

3.9 Quantitative estimates of nutrients to be taken up, quantification in terms of ecosystem services

Kuyper and Giller (2011) tried to quantify in monetary terms the ecosystem services delivered by rhizobia through N savings. That calculation suggested a monetary value of around US \$90 billion. (In this report, 10% of that value is allocated to AMF because of the increased P demand that is essential for efficient N fixation, see section 2.9) Carryover effects (legacy effects) of increased N inputs in soils and higher productivity of subsequent crops and over-yielding of cereals in intercropping systems were not included. The non-N benefits of intercropping or rotations with legumes as discussed above (increased organic matter and hence potential mitigation of climate change (Jensen et al., 2012), decreased pathogens, increased soil microbial activity and diversity) are important ecosystem services that have also not been quantified, as they cannot easily be attributed to rhizobia. Finally, as pointed out by Jensen et al. (2012) and Figueiredo et al. (2013), there is close to zero fossil fuel use linked to the use of rhizobia, in contrast with the use of fertilizers, and also lower NO_x emissions.

However, quantification of the amount of N fixed by rhizobia remains difficult (Herridge, 2008; Peoples et al., 2009). Figueiredo et al. (2013) divided the difficulty in estimating N-fixation in three categories:

1. Methodological problems in field-scale N-fixation estimation.
2. Highly variable N-fixation rates, which are strongly affected by environmental and agricultural concerns.
3. Difficulty in estimating individual cropping systems, worldwide distribution, and cultivated areas. Yet, as there are global estimates of the amount of reactive N that is added annually to the earth system through legumes that have been planted and managed by humans (Vitousek et al., 2013), a global estimate can still be attempted.

Table 2 provides estimates of amounts of N fixed by different legumes under optimal conditions.

Table 2. Fractions and amounts of N fixed (kg N ha⁻¹.yr⁻¹) by grain legumes. Data from Giller (2001)

Legume species	Fraction of N fixed	Amount of N fixed
Peanut	16-92	21-206
Pigeonpea	0-100	0-166
Chickpea	0-96	0-124
Soybean	12-100	14-188
Lentil	9-91	4-83
Bean	0-73	0-125
Cowpea	32-76	9-201

In addition, as noted before, the root system is usually not included in estimations of N-fixation (Giller, 2001; Figueiredo et al., 2013); at best, a constant value (around 30% of above-ground productivity) is often applied.

Nevertheless, Giller (2001) argues that many cases where small N-fixation rates were observed seem largely due to environmental constraints such as drought, low pH and low soil fertility and are less likely to be caused by insufficiency of amount or quality of rhizobial inoculum (soybean being a notable exception, as noted above.)

3.10 Suggestions for use in agricultural production

Peoples et al. (2009) stated: “Strategies are available to improve N-fixation beyond what is currently being achieved. Provided that a legume crop is abundantly nodulated and effectively fixing N, enormous benefits in terms of crop production and N fixed can be derived from the application of good agronomic principles. However, in many parts of the world, the relevant technologies are either not in the hands of the farmers, or they cannot be adopted because of lack of knowledge or infrastructure, economic constraints, or operational imperatives.”

A major program that tries to increase agricultural productivity by resource-constrained farmers in Africa through the use of legumes is N2Africa (<http://www.n2africa.org/>). Farmers currently receive improved seeds, rhizobium inoculated and a small package of fertilizer (without N, but with micronutrients). Yield increases have been achieved, but still less than was originally envisaged. It is still unclear to what extent insufficient quality or quantity of rhizobial inoculum plays a role in explaining this yield gap.

3.11 Legumes, rhizobia and arbuscular mycorrhiza – a case of synergy?

The fact that legumes associate both with rhizobia (resulting in enhanced N) and AMF (resulting in enhanced P) has given rise to the question of in what way these organisms interact. Experiments under controlled conditions have shown that combinations of rhizobia and AM fungi are usually more productive than plants that are only infected by rhizobia or AM fungi. This higher yielding when both microbial symbionts are present has led authors to assume that the interactions between both groups of mutualistic symbionts have *synergistic* effects, a term that, however, is poorly defined at best. It is probably best to try to define synergy in such a way that a difference can be made between additive effects and synergistic effects. We define synergy if, in an experimental setting where both rhizobia and AM fungi are separate factors, the interaction terms rhizobia × AM fungi are significant. Synergy can then be both positive (when the yield is higher than the additive effect) or negative (when the yield is higher than would be predicted under an additive model). In case there is no significant interaction term, the yield is additive. Based on that criterion, Larimer et al. (2010) tested the effect of combinations of rhizobia and AM fungi, and concluded that effects were not synergistic but additive.

3.12 Actinobacteria for agroforestry and rehabilitation of degraded land

Actinobacteria *Frankia* associate with woody plants, and many of the trees associating with actinobacteria can grow rapidly on poor soils (see Giller, 2001 for a list of plant species that can form actinorrhizal symbioses). These plant species are phylogenetically part of the so-called N₂-fixing clade (Doyle, 2011). Therefore trees associating with actinobacteria are used extensively in agroforestry systems and in rehabilitation and remediation of degraded or contaminated soils. The genera *Alnus* and *Casuarina* are predominantly used in temperate and tropical areas respectively for fuelwood and timber production while slowing down soil erosion and degradation and ameliorating soil fertility (Giller, 2001). Afforestation with N-fixing *Alnus* has been shown to increase soil N, C and P (Binkley, 2003; Uri et al., 2011), and ligno-cellulose degradation and the mineralization of organic nitrogen, phosphate, and sulfate compounds is increased (Selmants et al., 2005). However, in some sites N-fixation is largely beyond plant demand leading to N excess, nitrification, nitrate leaching and simultaneous acidification.

Karthikeyan et al. (2009) showed that *Casuarina equisetifolia* inoculated with AMF, *Frankia* and phosphate-solubilizing bacteria had much larger survival on bauxite mine spoil compared to uninoculated seedlings and that nutrient uptake and biomass were increased as well.

3.13 Rhizobia and cereals

Indigenous rhizobia can colonize the rhizosphere of cereals and in some cases provide benefits to growth (Chaintreuil et al., 2000; Yanni et al., 2011). Thus inoculation of non-legumes with rhizobia has attracted interest as biofertilizer (Mia and Shamsuddin, 2010). Matiru and Dakora (2004) showed that roots of sorghum and millet landraces from Africa were easily infected by rhizobial isolates from five unrelated legume genera, and that with sorghum in particular, plant growth and phosphorus uptake were significantly increased by rhizobial inoculation, although it is not clear whether the bacteria increased P uptake or stimulated the plant to take up more P. Matiru and Dakora (2004) suggested that field selection of suitable rhizobia/cereal combinations could increase cereal yields through direct benefits by the cereals from the legumes-rhizobia mutualism. Similarly, Yanni et al. (2011) pursued the use of rhizobia associated with clover for increased productivity of rice in Egypt. Biswas et al. (2000) found increased uptake of N, P, K and Fe in rice inoculated with rhizobia and other growth-promoting bacteria, even though rice does not commonly associate with rhizobia and it does not form nodules. They argue that the rhizobia and other rhizosphere bacteria altered root morphology (through hormonal effects) including more root hairs, increasing the volume of soil for nutrient uptake. In this case, rhizobia function as PGPR, a topic dealt with below.

However, Yanni et al. (2001) and Vargas et al. (2010) warn that the effects of rhizobia on cereal growth and yield are not consistent or even unambiguously demonstrated, and that direct effects (increased N uptake through N-fixation) and indirect effects (e.g., increased N uptake from mineralized organic N in soil, protection against pathogens) are hard to assess separately.

3.14 Plant growth-promoting rhizobacteria (PGPR)

Under the name of PGPR, a diverse group of microorganisms (mainly bacteria, but also fungi) is included. Many of these improve plant performance and these larger plants take up more nutrients. However, larger uptake may be the consequence of larger plant size (through other mechanisms) than the cause. With these microorganisms there is no symbiotic interface where carbon and nutrients are exchanged. The microorganisms are best considered as saprotrophs that live on dead organic material in the rhizosphere. Molecular-ecological investigations have shown that each plant species (and likely even individual varieties or cultivar) have to some extent their unique microbial assemblage in the rhizosphere. A major effect of these bacteria is that these change the hormonal balance of the plant, which can result in improved root growth and hence nutrient uptake. Rhizobia in combination with grasses likely act through the production of phytohormones (Machado et al., 2013). A rhizobium that was associated with rice not only colonized roots but also migrated to above-ground plant parts as stem, leaf sheath and leaves (Chi et al., 2005). A species of *Bradyrhizobium* was found to increase N-uptake in sweet potato, although the mechanism was not fully elucidated (Terakado-Tonooka et al., 2013). PGPR can also act as natural enemies of pathogens and pests in the rhizosphere. Important reviews on this topic are: Vessey (2003), Lugtenberg and Kamilova (2009) and Richardson et al. (2009).

Currently, it is unlikely that such microbial consortia can be manipulated, controlled, and used to increase nutrient uptake or use efficiency in predictable ways. Because of the high context-specificity, it is unlikely that there will be general products that work under a diversity of cropping systems in a wide range of ecological conditions. Possibly quality control issues (in case some products are introduced on the market) will be a further constraint.

In the framework of this report we will discuss only one specific group of PGPR, called phosphate-solubilizing bacteria (PSB) or fungi (PSF; however, that acronym is less often used than PSB). Several fungi have the same

capacity. These organisms can specifically mine soils for sources of P that are otherwise not or hardly available to plants.

3.15 Phosphate-solubilizing fungi and bacteria, controlled versus field

Phosphorus fertilizers are a finite resource that is calculated to run out within the next few decades (Cordell et al., 2009). Current fertilizer use efficiency is often quite low (estimated 10-20% in many soils) because the strength of the soil sink (through the presence of metal oxides and metal hydroxides) very largely surpasses the capacity of the plant to quickly take up this phosphorus. The phosphorus not taken up by plants enters in pools that are quite stable and where the exchange rate with soil solution phosphorus is far too slow to impact plant growth. However, phosphate accumulation in these stable pools could ultimately build up a phosphate store in soil that would gradually reduce the need for phosphorus fertilizer (see Sattari et al., 2012) although the contribution that this would make towards solving the problem of peak phosphorus is still very difficult to estimate. Also estimating the rate at which phosphorus runs out is beset with several difficulties – because of variable estimates of P stocks that can be economically mined, the quality of these additional P reserves (as sources of rock phosphate also contain cadmium and uranium) and future changes in phosphorus recycling in industrialized countries (Van Kauwenbergh, 2010). But irrespective of the exact estimate, inefficient use of phosphorus fertilizers is a problem for sustainable agriculture and for the challenge to feed the world and raising use efficiency will be needed.

Raising fertilizer phosphorus use efficiency can be achieved through two mechanisms – increasing scavenging soils for available phosphorus through mycorrhizal associations (see section 2.1) or through plant breeding that results in improved root systems and increasing mining where sparingly soluble phosphorus sources are made available. Phosphate-solubilizing fungi and bacteria could enhance P nutrition of plants through P mining and hence sustainable P recycling within soils. Certain microorganisms have the ability to solubilize P from these metal (hydr-)oxides, making P plant available. Richardson and Simpson (2011) summarized three mechanisms by which microorganisms can increase P for plant uptake: (1) increased root growth (as a general property of PGPR, often due to hormonal effects; this being part of the scavenging mechanism); (2) alteration of sorption equilibria in the soil, rendering soil P plant available; and (3) induction of metabolic processes that are effective in directly solubilizing and mineralizing P from sparingly available forms of soil inorganic and organic P, such as exudation of organic acids or anions, siderophores or enzymes (phosphatases such as acid or alkaline phosphate mono-esterase and phytase).

Research on PSB and fungi with similar capabilities has often remained in the stage of experimental investigations under controlled conditions, and scaling up to field level has turned out to be difficult. Khan et al. (2007) noted that solubilization of phosphate compounds by naturally abundant phosphate-solubilizing microorganisms (PSMs) is very common under *in vitro* conditions, but that the performance of PSM *in situ* has been contradictory. The widespread application of P-solubilizing bacteria and fungi therefore remains limited by a poor understanding of microbial ecology and population dynamics in soil, and by inconsistent performance over a range of environments (Whitelaw, 2000; Khan et al., 2007). Problems encountered in this respect are that the production of organic anions is carbon demanding. In the lab this problem is solved by adding easily available C sources to a microbial culture and then assessing dissolution of P from sparingly soluble minerals. However, adding such compounds in the field may result in competition with other (rhizosphere) microorganisms that may be competitively superior (they convert that carbon into microbial biomass rather than into organic acids or anions), and the organic anions themselves may also be degraded by other rhizosphere organisms. Addition of carbon sources and subsequent microbial growth could also result in microbial nitrogen immobilization. While phosphatases could release phosphate molecules from organic compounds, it is likely that the rate-limiting step in soils (from the perspective of increasing

P availability) is not the quantity of phosphatases but the availability of organic phosphate, as these compounds are to a very large extent also strongly adsorbed to metal oxides and hydroxides (Tinker and Nye, 2000).

One could try to achieve P dissolution through PSM by adding these organisms to sources of sparingly soluble P (rock P), adding organic sources (e.g., organic waste) and then adding the soluble P as fertilizer. However, the then easily available P added to soil will still be subject to the same kind of (strong) adsorption and occlusion reactions as easily soluble mineral fertilizer P. This issue is further dealt with in a separate report commissioned by the Virtual Fertilizer Research Center (VFRC).

4 Endophytic N-fixing bacteria in grasses

4.1 General review, specification in terms of ecosystem services

Endophytic bacteria are found within plant tissue, either inter- or intracellularly, and they occur either facultative or obligate. Endophytic bacteria capable of N-fixing in commercially important grasses have received considerable research focus and strong suggestions or evidence have been provided that some tropical grasses, especially sugarcane (*Saccharum sp.*), wetland rice (*Oryza sativa*), elephant grass (*Pennisetum purpureum*) and kallar grass (*Leptochloa fusca*) can obtain part of their N needs via N-fixation of endophytic bacteria (James, 2000; De Morais et al., 2012). As will be discussed below, demonstration of the presence of *nif* genes in these bacteria has been straightforward, but assessing the amount of N fixed through an N balance has turned out to be much more difficult (Giller and Merckx, 2003).

Whereas many endophytic bacteria have been found in a wide range of grass species, interest has focused on *Acetobacter diazotrophicus* (currently known as *Gluconacetobacter diazotrophicus*) in association with sugarcane (Sevilla and Kennedy, 2000). ¹⁵N isotope dilution and ¹⁵N natural abundance studies have shown that sugarcane can fix substantial amounts of atmospheric N (Urquiaga et al., 1992; Boddey et al., 1995, 1999; Yoneyama et al., 1997; Taulé et al., 2012; Urquiaga et al., 2012), but the amount of N fixed is highly variable and dependent on plant genotype and environmental conditions. For instance Lima et al. (1987) in pot experiments showed N fixation of different sugarcane varieties to vary between 15% and 73% (based on N balance analyses), Urquiaga et al. (1992) estimated N fixation of sugarcane varieties in concrete tanks between 13% and 39% based on ¹⁵N isotope dilution analyses, and more recently Taulé et al. (2012) found N fixation in sugarcane shoots were between 41.3 and 58.8% at 10 mg N kg⁻¹ added to the soil and 34.8 and 49.7% at 50 mg N kg⁻¹ added to the soil (analyzed by ¹⁵N isotope dilution). However, in Australia, commercially grown sugarcane varieties showed no significant N fixation although quantification was not possible (Biggs et al., 2002). Similarly in South Africa a sugarcane cultivar showed no uptake of N through N-fixing endophytes, and endophytic bacterial strains isolated from the sugarcane cultivar did not show N-fixing abilities (Hoefsloot et al., 2005). Most studies on N-fixation by endophytic bacteria are performed in growth chambers or tanks and upscaling to field situations is not always possible. According to Ruschel (1981) and Boddey et al. (1995) the high endophytic N-fixation in sugarcane may have been due to selection of varieties with low response to mineral N fertilizer and high N-fixing ability, as sugarcane has been grown in low N input systems in Brazil. N-fixation by sugarcane in Brazil also seems to depend largely on optimizing conditions such as water supply (but see below for the problem if minute quantities of nitrate are present in irrigation water), P, K and micronutrient availability, especially molybdenum (Urquiaga et al., 1992). ¹⁵N studies are needed to determine whether in other

sugarcane producing countries, with high levels of mineral N fertilizer, N-fixation can reach similar levels to those in Brazil (Ruschel, 1981; Boddey et al., 1995; Giller, 2001).

Compared to sugarcane, greenhouse and field studies with other grasses have given less promising and highly variable results. In several publications it is also impossible to attribute increased N content of the grass to N-fixation rather than to other effects of bacteria on plant growth promotion through hormonal effects. Wheat has shown contradictory results of inoculation with endophytic N-fixers with some authors showing that inoculation resulted in no significant increase in total plant N, although isotopic N data showed that N₂ fixation did occur, but other authors find no evidence of N fixation in wheat. It may be concluded that the endophytic N-fixing bacteria probably only transferred fixed N to the plant when they died and were decomposed, and microbial nitrogen was mineralized (Lethbridge and Davidson, 1983; James, 2000). Few studies have looked at maize and sorghum, and those studies suggested that N-fixation by endophytic bacteria may be significant, but is highly variable among plant genotypes. Salomone and Dobereiner (1996) found negative, insignificant and positive effects of 100 kg N ha⁻¹ fixed on inoculation of maize genotypes with N-fixing endophytes. Hungria et al. (2010) noted positive effects on growth and nutrient uptake of maize and wheat inoculated with selected strains of *Azospirillum brasilense* and *A. lipoferum*, stating that rather than N fixation, plant growth promoting effects explain the observed positive growth effects by these bacteria.

Nevertheless greenhouse studies at the International Rice Research Institute (IRRI) showed that certain rice genotypes can obtain significant amounts of N from N-fixation, ranging from 1.5% to 21% N fixed measured by ¹⁵N isotope dilution in 70 rice genotypes (Shrestha and Ladha, 1996). James (2000) concluded that under low mineral N inputs endophytic N-fixation of rice can contribute to a large amount of N needs, although highly variable. Estrada et al. (2013) showed in a pot experiment that inoculation of rice with an N-fixing bacterial strain (*Burkholderia kururiensis*) also increases soluble P (hence rather acting as PSB), potentially lowering mineral N and P fertilizer inputs.

Finally, although studies have shown significant N-fixation in some grass species such as sugarcane, according to James (2000) these studies have not provided conclusive evidence that these plants are engaging in a symbiotic relationship with endophytic bacteria, as the techniques used do not distinguish between endophytic symbiosis fixation and N-fixation from free living heterotrophs and cyanobacteria. Furthermore, as mentioned by Hungria et al. (2010) other plant growth promoting factors than N fixation alone (for instance, hormonal effects, as commonly described for PGPR) could explain better yields of grains inoculated with endophytes.

It may therefore be worthwhile to reflect on the question why claims of N fixation by cereals (and estimates of the fractional of N fixed) are apparently so much more contentious than in the case of legumes with rhizobia. Giller and Merckx (2003) reflected on that issue. They concluded that several methods used are inadequate (for instance only demonstrating the presence of nif genes) and that a simple short-term N balance is potentially misleading, as increased N uptake could be due to other mechanisms (for instance hormonal effects resulting in changes in root architecture as a consequence of which nutrient uptake is improved as described more generally for PGPR). Finally, they noted problems that are inherent from field measurements. Considering the high productivity and high water use of sugarcane, they calculated that a nitrate concentration of 2 mg N l⁻¹ (which is very low) could result in around 30-40 kg in sugarcane, not too far off some statements about amounts of N fixed.

4.2 Associated plant species

Several plant species (both monocots and dicots) are regularly found in association with potentially N-fixing PGPR. The best known of these is sugarcane in Brazil. Ever since its introduction, there have been attempts to improve yield, and these attempts took place with absent or low fertilizer additions. This may be one reason why this plant is, more than other cereals, reported to fix substantial amounts of N (Baldani et al., 2002).

Table 3 provides an overview of published estimates of N fixation (or possibly additional N acquired) by endophytic bacteria associated with grasses. Other claims have been made on websites, but these claims have not always been backed up in the literature. They are discussed in this section.

Table 3. Range of N fixed by different grasses according to literature sources

Plant	Range of N fixed (% N from N fixation)	Notes	References
Sugarcane	0-80	Genotype dependent	Lima et al., 1987; Urquiaga et al., 1992; Taulé et al., 2012; Biggs et al., 2002; Hoefsloot et al., 2005; Urquiaga et al., 2012
Rice	1.5-21	Genotype dependent	Shrestha and Ladha, 1996
Sorghum	0-60	Low repeatability	Smith et al., 1984; Lee et al., 1994
Millet	0-77	Low repeatability	Smith et al., 1984
Wheat	0-32	Genotype dependent	Rennie et al., 1983
Maize	0-58	Genotype dependent	Garcia de Salamone and Dobereiner, 1996

4.3 Uptake of other nutrients than N and P

Saravanan et al. (2008) suggest that the endophytic N-fixing bacterium *Gluconacetobacter diazotrophicus* can also increase sugarcane Zn uptake, and assist in antagonistic activity and phytopathogen biocontrol. This claim is consistent with the general listing of such bacteria as PGPR.

4.4 Strains and functioning

James (2000) mentions as most likely candidates for biological nitrogen fixation (BNF) in grasses: *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. in sugarcane (Dobereiner et al., 1995; Boddey et al., 1995, 1999; Baldani et al., 1997), *Azoarcus* spp. in kallar grass (Reinhold-Hurek and Hurek, 1998a,b), and species of *Alcaligenes*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas* and *Rhizobium*, in rice and maize (Patriquin et al., 1983; Boddey et al., 1995; Triplett, 1996; Malik et al., 1997; Stoltzfus et al., 1997; Yanni et al., 1997; James et al., 2000). Santi et al. (2013) provide an updated list of cereals whose productivity increased after inoculation with nitrogen-fixing PGPR. These include species *Azoarcus* (rice), *Burkholderia* (rice, maize), *Gluconacetobacter* (maize – note that it is consistently misspelled in their paper as *Glucenobacter*), *Herbaspirillum* (rice, maize, wheat), *Serratia* (rice), *Azospirillum* (maize, wheat), *Azotobacter* (maize, wheat). Sugarcane was not discussed in their review. Bacteria belong both to the alpha- and beta-proteobacteria. Some

are tightly associated with plants and cannot well survive in soils (*Azoarcus*, *Herbaspirillum seropedicae*, *Gluconacetobacter diazotrophicus*) whereas most of these bacteria are normally free living and soil inhabiting.

4.5 Host relationships

The endophytic bacterium *Gluconacetobacter diazotrophicus* was found to establish better in plants when the plants were also associated with AMF, implicating that the bacteria gained access to the plant via the fungal entry points (Paula et al., 1991). Endophytes can be found in the roots, stems and leaves of plants. Giller (2001) remarked that, although the roots provide most available energy sources to endophytic bacteria, the bacteria are nevertheless found in all tissues.

4.6 Genetic variation among species, genetic variation among and between plant species that allows optimizing use through targeted plant breeding

Specific plant genotypes and bacterial strains are known that optimize N-fixing in sugarcane especially and rice (Salamone and Döbereiner, 1996; Garcia de Salamone et al., 1996; Shrestha and Ladha, 1996; Urquiaga et al., 1992). However, the use of endophytic bacteria is still largely restricted by lack of knowledge on specific strains and plant genotypes that enhance N fixation. Gaiero et al. (2013) commented that while high throughput sequencing (e.g., 454-pyrosequencing) is very useful in investigating endophyte communities, the important question regarding plant growth promotion by endophytes is whether the success of the plant-endophyte interaction can be predicted by genetic analyses.

4.7 Optimal conditions

With sugarcane, it was found that in low N conditions endophytic bacterial colonization is higher. However, in most studies regarding endophytic N fixation in sugarcane, all other conditions, water, temperature, other nutrients, were optimal. As with rhizobia, molybdenum is an important nutrient for establishment of endophytic bacteria, as well as a good water supply (Boddey et al., 2003).

4.8 Examples of application and potential use for upscaling

Although endophytic bacteria for N fixation are widely used in the sugarcane industry in Brazil (Lee et al., 2002), it remains unknown how much of plant N these bacteria fix, and upscaling to other continents and other species has not taken place. If genetic analyses succeed in predicting the success of specific strains for N fixation or other beneficial traits (P and other nutrient uptake, pathogen protection and other effects of PGPR), these strains may potentially be targeted at improving N fixation or N acquisition (depending on the mechanisms involved) in grass crops worldwide while decreasing the use of mineral N fertilizers. However, apart from genetic screening, combinations of strains and plant genotypes will have to be tested in the field under different environmental conditions and this will take considerable research efforts. Hungria et al. (2010) identified the first *Azospirillum* strains authorized for the production of commercial inoculants in Brazil.

4.9 Quantitative estimates of nutrients to be taken up, quantification in terms of ecosystem services

Urquiaga et al. (1992) calculated, based on ¹⁵N dilution studies, that 60-80% of sugarcane plant nitrogen (equivalent to 200 kg N/ha) is derived from BNF, and that *Gluconacetobacter diazotrophicus* was the principal contributor.

Govindarajan et al. (2008) reported rice grain yield increases of 6-24% when inoculated with various endophytic diazotrophic bacteria, the most effective being *Burkholderia vietnamensis*. Other BNF in rice are discussed by Choudhury and Kennedy (2004). Okon and Labandera-Gonzalez (1994) reported a 30% yield increase in wheat inoculated with *A. brasilense*. Based on 20 years of worldwide field application data, Okon and Labandera-Gonzales (1994) concluded that *Azospirillum* can increase crop growth and yield by 5 to 30% depending on soil and climatic conditions. However, although these yield increases can probably in part be attributed to nitrogen fixation, it remains unsure exactly how much N is fixed by endophytic bacteria inoculated in these grain species as opposed to indirect effects of the bacteria on plant growth, as it is difficult to attribute plant N to specific uptake sources (Giller, 2001, Giller and Merckx, 2003). These latter authors suggested that over long-term ecosystem development the contribution of N-fixing endophytes could be significant, even when measured amounts of annual N fixation in wild and crop plants (and that refers to the immediate contribution to sustainable agriculture) are low. In addition endophytic N-fixing has other beneficial attributes as PGPR, so that their value should perhaps not only be quantified in percentages of N fixed but also in their ability to maintain plant health and functioning, even under less favorable circumstances. Sessitsch et al. (2012) suggested from endophyte metagenome sequences that endophytes can also possess a high potential for plant-growth promotion, improvement of plant stress resistance, biocontrol against pathogens, and bioremediation. In particular, the fact that these bacteria seem stimulated by low N environments suggests that they can potentially lower the need for N fertilizers while maintaining high productivity. Gaiero et al. (2013) mentioned that endophytes interact within plant tissue, and that inoculation with a competitive strain may unbalance these interactions, reducing plant growth. From an ecological point of view, it is therefore important to maintain beneficial interactions.

4.10 Suggestions for use in agricultural production

As mentioned before, much work needs to be done in order to claim success with inoculation of many crops with endophytic N-fixing bacteria. Boddey et al. (2003) mentioned that until it is known which endophyte is responsible for N fixation in a given plant and under what conditions N fixation is optimal, serious development of commercial inoculants of N-fixing endophytes is hindered. In particular, upscaling from greenhouse experiments to field sites worldwide is missing (Lucy et al., 2004; Mallik and Williams, 2008; Figueiredo et al., 2013).

4.11 Claims on benefits provided by endophytic N-fixing bacteria

Although claims on the benefits of inoculation with endophytic N-fixing bacteria led to some commercial products, scientific data do not support the commercial claims, often found on websites, that their products can be used on a wide variety of crops. Recent literature reviews (Boddey et al., 2003; Mallik and Williams, 2008; Figueiredo et al., 2013) suggest that especially the specificity of bacterial strains on the one hand and plant species on the other hand, in combination with different environmental conditions, need to be clarified in field trials before inoculants of plants with specific bacterial strains can be of commercial value.

4.12 Alternative hypotheses

Because of the (somewhat) contentious nature of the claims of substantial fixation by grasses, including the major cereals, alternative hypotheses that explain plant benefits of endophytic bacteria have been proposed. As described above, the contentious nature is due to the fact that demonstration of *nif* genes in such bacteria is straightforward, as are measurements of larger biomass of plants (and higher N uptake by such plants), but that an accurate N-balance that unequivocally shows fixation of atmospheric N₂ by those bacteria to be the main mechanism is extremely difficult (Giller and Merckx, 2003). Alternative hypotheses are summarized as follows:

- Benefits to the plant other than increased N fixing, such as plant growth promoting hormone production (Okon and Labandera-Gonzalez, 1994).
- Benefits for plant protection against pathogens (Saravanan et al., 2008).
- Benefits in interactions with other (endophytic) plant symbionts (Paula et al., 1991).

Note that these alternative hypotheses are not mutually exclusive and that such effects are consistent with the general concept of PGPR. Most research efforts are focused on N fixation of endophytic bacteria, whereas it might be more worthwhile to explore other plant growth-promoting mechanisms, in combination with interactions with other beneficial microorganisms (Sessitsch et al., 2012).

5 Conclusions/vision

From the review of the literature on AMF, rhizobia and endophytic N-fixing bacteria, four main conclusions can be drawn:

- Low fertilizer inputs stimulate plant-beneficial microorganism interactions. Alternatively, high inputs of fertilizer negative impact on such microbes, especially through direct, soil-mediated effects.
- *Interactions* between different microorganisms and plants are important; however, *true synergies* should not automatically be assumed.
- Most mutualistic microbial symbiosis are multifunctional, and an exclusive emphasis on their ability to deliver N and P to the plants overlooks other ecosystem services provided by them.
- Upscaling the commercial use of microbial inoculants is still difficult and not widely applied, except for a few cases (soybean in Brazil, possibly sugarcane in Brazil). There are also issues with the commercial use of indigenous inoculum under current patent laws, as it is unclear to what extent such microbes (or the genetic information) belong to global commons can be transformed into private property (Kothamasi et al., 2011).

The following paragraphs will provide thoughts to make use of the characteristics of beneficial microorganisms for high-production sustainable agriculture.

5.1 Low fertilizer inputs stimulate plant-beneficial microorganism interactions

Beneficial microorganisms are currently mostly employed in countries or continents where conventional fertilizers are too expensive. However, this usually involves soil management in sustainable ways, not the acquisition of inocula of these microorganisms, as they often are also expensive. While high fertilizer input has a negative impact on these beneficial microorganisms, it should not be concluded that the use of microbial inocula and mineral fertilizer is always incompatible. The negative impact is stronger through the direct (soil-mediated) pathway than through the indirect (plant-mediated) pathway, so cases of foliar application, where possible, could be a potential alternative to benefit from both applications. The extent to which microdosing (localized application of fertilizer) affects the mycorrhizal symbiosis also needs further research. Furthermore, timing of fertilizer use, in relation to both dynamics of microbes and changing demands by plants over their growth cycle, could contribute to making combinations of both possible. Finally, in many cases it has also been shown that unbalanced nutrient applications, such as unbalances between N and P (Grman and Robinson, 2013) or high P gifts without regard for interactions between P and Zn acquisition (Lambert et al., 1979; Ryan et al., 2008), are much more problematic than balanced nutrient gifts. Therefore, an increased focus on the benefits provided by those microorganisms must not be considered as

an absolute obstruction to conventional fertilizer use. Both in the developed world and in developing countries (e.g., Africa; Babalola and Glick, 2012), there is therefore potential for microbial inoculants.

Mineral fertilizers, especially N, P and K, are applied widely but are not used to full efficiency and cause environmental problems when they accumulate in soil or groundwater, leach into surface waters and are washed out into the oceans, or leave from the ecosystem in the gaseous form, thereby contributing to climate change (N₂O) or to negative effects of natural ecosystems (atmospheric N deposition). Attempts are being made to increase the efficiency of fertilizers, and in the case of the mycorrhizal symbiosis, this increased phosphorus uptake efficiency (and hence the substantial P saving) may be very important. Ultimately, in such cases the effect of beneficial symbioses is increased plant production, and unless the microorganisms add nutrients to the soil (like with N-fixing rhizobia), larger biomass results in larger amounts of nutrients removed that must ultimately be replenished. In that respect AMF cannot be called biofertilizers – they scavenge the soil for P. PSMs (fungi and bacteria) mine the soil for P, but even in such cases, P needs to be ultimately replenished. Rhizobia can be called biofertilizers (adding atmospheric N), whereas the qualification is contentious in the case of N-fixing endophytes, depending on whether their main effect is through N-fixation or whether they act as PGPR, increasing plant health, plant size and uptake from the soil pool.

However, studies discussed in this report have shown that with lower dosages of fertilizers and the appropriate microorganisms, the same biomass and also quality (nutrition) of crop plants can be reached (Wang et al., 2011; Dodd and Ruiz-Lozano, 2012), because the microorganisms provide higher efficiency of nutrient uptake. Considering the need to reduce fertilizer use per unit biomass produced (or to increase fertilizer use efficiency), beneficial microbes will gain increasing importance in the process of sustainable or ecological intensification of agriculture (including forms of biological or organic agriculture). It could also be possible that in the case of non-responsive soils (very low-productive soils that respond hardly to mineral fertilizers, Vanlauwe et al., 2010) such beneficial microbes could play a role in alleviating that constraint.

5.2 Interactions between different microorganisms and plants

The beneficial microorganisms discussed here, AMF, rhizobia and endophytic N-fixing bacteria, interact with each other (Azcón et al., 2009; Wang et al., 2011; Bucher, 2007; Bonfante and Anca, 2009). Studies of one group in isolation may therefore give a biased picture.

In fact, one could draw a parallel with human social networks, where plants can be linked through mycorrhizal networks; where some plants (legumes) expand the networks with additional beneficial microorganisms (rhizobia) and other plants (Brassicaceae) try to unlink themselves from such networks (Venkateshwaran et al., 2013). The dependence on that social network is probably the explanation why over an evolutionary history of more than hundreds of millions of years plants have been unable to genetically integrate the genome of these symbionts into their genomes (Raven, 2010).

Furthermore, beneficial microorganisms interact with pathogenic microorganisms, and improve plant health and functioning, partly by excluding them from the interplant network (Saravanan et al., 2008; Sessitsch et al., 2012). These interactions are partially known, and double or triple inoculations of crop plants with microorganisms are being studied. However, these studies do not yield consistent results and are likely dependent on local environmental factors and the crop used. While some generalities are likely (e.g., there is no synergism between rhizobia and AMF, nutrient effects being rather additive; there is likely synergy between effects of mutualistic and pathogenic organisms on C acquisition and hence nutrient uptake, due to their protective function), application of

that knowledge under field conditions is still very difficult (Larimer et al., 2010). It also remains unclear whether and how introduced microorganisms via inoculation survive and if they survive, how they affect the local microbial populations of both pathogenic and beneficial microorganisms (Verbruggen et al., 2013). As also discussed below, development of plant growth promoting inocula from indigenous microorganisms has the potential to be better adapted to the local microbial community than exotic inocula.

5.3 Most mutualistic microbial symbioses are multifunctional

This review has focused on the role of mutualistic symbioses in the uptake of nutrients and reflected on the options to reduce inputs of mineral fertilizers in sustainable or ecological agricultural intensification, which is needed to feed around 9 billion people on the globe in 2050. However, such a perspective is too restrictive from the perspective of the ecosystem services provided by these microorganisms. Other effects on plant health (increased drought tolerance, better ability to withstand toxic amounts of heavy metals or aluminum, hormonal changes in the plant impacting on the root:shoot ratio, protection against above-ground and below-ground pathogens and herbivores) and soil health (contribution to soil structure, regulation of population dynamics of other organisms) are also important to assess the role of these beneficial microorganisms in agriculture. It is therefore too simplistic to consider such beneficial root symbionts as simply an extension of the plant root system; rather, they should be considered and investigated as key elements of soil quality and as major providers of a suite of ecosystem services (Hamel and Strullu, 2006).

Multifunctionality is also context-dependent. The relative roles of the mycorrhizal symbiosis in enhanced nutrient uptake depend on fungal traits and strategies (trade-offs between extraradical and intraradical mycelium), plant traits and strategies (roots with low SRL depend more on nutrient uptake, roots with high SRL more on pathogen protection; Newsham et al., 1996; Wehner et al., 2010) and possible soil properties (amounts of organic matter, pH, nutrient availability). It is therefore imperative that studies are needed for a wide range of cropping systems under different agro-ecological conditions. Especially studies that involve the role of mutualistic symbioses in intercropping and crop rotations are difficult to generalize. In such cases, attention should also shift from a focus on a supposed superior strain to a focus on functional and trait diversity of these microbes.

The implication of this context-dependency should be evident – it is extremely unlikely that specific superior strains (or even superior mixtures) are applicable to all major crops or cropping systems under all agro-ecological conditions. Whereas some inoculum sellers seem to come close to making that suggestion, the scientific literature provides no support for such claims.

5.4 Upscaling the commercial use of microbial inoculants

There is likely great potential for the use and management of beneficial microorganisms, in combination with other agricultural practices. According to Berg (2009) several advantages of microbial inoculants over chemical pesticides and fertilizers: (1) are more safe; (2) show reduced environmental damage and potentially smaller risk to human health; (3) show much more targeted activity; (4) are effective in small quantities; (5) multiply themselves but are controlled by the plant as well as by the indigenous microbial populations; (6) decompose more quickly than conventional chemical pesticides; (7) resistance development is reduced due to several mechanisms; and (8) can be also used in conventional or integrated pest management systems.

Berg (2009) mentions two alternatives to manage beneficial soil microorganisms: the management of the indigenous communities and the additions of autochthonous microorganisms.

Concerning the management of indigenous microbial communities' crop rotation, tillage, weed management and inputs of mineral fertilizers and organic amendments are important factors to consider (Raaijmakers et al., 2009; Verbruggen and Kiers, 2010). Crop rotations are needed to minimize the buildup of pathogens that are specific to one crop plant. (It is also possible that monocropping selects for less beneficial microbes [Johnson, 1993], a topic that demands further investigation.) However, some crops will also reduce beneficial microbial communities such as members of Brassicaceae or Chenopodiaceae (e.g., canola or beet) that reduce arbuscular mycorrhizal fungal inoculum in soil (Abbott and Robson, 1991; McGonigle et al., 2011). These latter authors observed increased uptake of Cu (increases of 50-80% for P, Cu and Zn if flax was grown in rotation after maize compared to flax after canola). Tillage has given conflicting results on pathogen suppression, as it is usually combined with other practices such as fertilizer application (Bailey and Lazarovits, 2003). For instance, tillage breaks mycorrhizal fungal hyphae, making it hard for them to survive (Bailey and Lazarovits, 2003). But tillage combined with introduction of organic amendments and crop residues lead to a reduction in soil pathogens and an increase in beneficial microorganisms over time (Bailey and Lazarovits, 2003). Leaving fields bare will reduce the amounts of microorganisms (Abbott and Robson, 1991; Thompson et al., 2013), and it may be more favorable to leave weeds in the non-growing season and only remove weeds shortly before planting of the crop.

Additions of autochthonous microorganisms have been of varying success and the technology is not generally ripe as an on-the-shelf set of practices. Sturz and Nowak (2000) describe failures of introducing rhizosphere bacteria because of competition with indigenous bacterial communities. However, Azcón et al. (2009) showed that amendment with organic residues and autochthonous AMF and bacteria increased plant growth and phosphorus nutrition as well as the indigenous microbial community. The study of Azcón et al. (2009) shows that integrated nutrient management (INM) can reduce the need for mineral fertilizers by reusing organic waste and increasing its nutrient availability with the help of beneficial microorganisms. Wu et al. (2005) showed in a greenhouse trial that maize biomass increased most with addition of beneficial microorganisms, a mix of AMF and N-fixing and P- and K-solubilizing bacteria, compared to mineral fertilizer or organic amendment additions. Wu et al. (2005) also showed that low soil N and P status increased the infection rates of AMF and N-fixing bacteria. The interactions between beneficial microorganisms are promising for further upscaling. Adesemoye and Kloepper (2009) conclude that to maintain high production agriculture the use of microorganisms to increase fertilizer use efficiency is an important step for INM. Vosátka et al. (2012) see potential for the development of large-scale AMF inoculum for agriculture, but the missing components are appropriate, cheap, highly reproducible and effective methods for inocula purity testing and quality control: often the increased nutrient uptake after AMF inoculation is not due to mycorrhizal colonization by commercial products, but through the added nutrients in the inoculant mix. The commercial production of AMF and other microbial inoculants faces other problems as well: the shelf-life of inoculants are problematic for some organisms, and the registration procedure is often expensive and time consuming (Ehlers, 2006), especially in cases that such products are also claimed as having biocontrol potential. And plant-associated bacteria, especially those from the rhizosphere, can be opportunistic human pathogens for which screening needs to be developed (Berg et al., 2005). Finally, Vosátka et al. (2012) stress that even with inoculation by autochthonous microorganisms, the indigenous community should be managed well, as the native microbial community is likely also to have positive effects on plant growth and nutrition under the local environmental conditions.

5.5 Inocula from native fungal and bacterial strains

Further research on the development of inocula needs to focus on large scale application procedures such as seed coating or mixing with irrigation (Vosátka et al., 2012), on quality of the inocula and claims from the producers, and on local environmental conditions under which inoculations will work. The COST Action 8.38 Management

Committee proposed a framework for EU regulations of quality control of AMF inocula (COST Action 8.38, 1999), and also the International Mycorrhiza Society strives to “define objective standards of quality for industrial inoculums.” Similar quality control procedures should be in place for all microbial inoculants, especially when inoculants are crossing borders and contaminations with exotic species becoming a hazard. Cases of inoculum introduction by soil attached to exotic plants have been described for rhizobia and ectomycorrhizal fungi, but not yet for AMF. In such cases, exotic beneficial microorganisms could contribute to what has been called invasional meltdown and this has been observed with exotic rhizobia (Rodríguez-Echevarría, 2010; Rodríguez-Echevarría et al., 2012).

Lower efficiency of commercial inoculum compared to resident (autochthonous) inoculum was reported in an agricultural soil (Janouskova et al., 2013) and in a situation of land restoration (Paluch et al., 2013). However, the opposite result (persistence of an inoculated AM fungus and yield increase of a legume) was observed by Pellegrino et al. (2012).

Mulas et al. (2013), however, proposed the strategy of using native strains as so-called biofertilizers and gave some examples of successful trials. For instance, Rodríguez-Blanco et al. (2010) show that it may be possible to isolate efficient native rhizobial strains to be used as inoculants for clover pastures in Uruguay. Roesti et al. (2006) used native PGPR and AMF strains to inoculate wheat in India and found similar or increased yield and nutrient content compared to no inoculations, and they argue that native strains are adapted to local soil conditions, microclimate and plant genotypes. Also in India Mäder et al. (2011) inoculated wheat, rice and black gram with different AMF inocula and wheat PGPR: they found high increases in wheat yield and nutrient uptake, but less so for rice and black gram, suggesting that the inocula need to be isolated from the target crop to be most effective. Mostasso et al. (2002) tested several strains isolated from Brazil cerrado to inoculate bean and superior performance of five strains was confirmed under field conditions and re-inoculation in the second year increased bean yield. Mulas et al. (2011) isolated rhizobial strains in Spain and found that inoculation of common bean with native rhizobial strains could completely replace the fertilization with mineral N fertilizers. Furthermore, Mulas et al. (2011) found that the beneficial effect of the inoculum was valid for the whole agro-ecological area, regardless of the specific properties of each soil and microclimatic conditions. These studies show that instead of focusing on microbial strains for commercial sales worldwide (monodominant superstrains), it might be more feasible to selectively screen for native strains of beneficial microorganisms and produce regional inocula. Management of such native strains and assemblages is likely also less complicated.

6 References

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