# Biological Nitrogen Fixation: Importance, Associated Diversity, and Estimates

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# Chapter 10
# Biological Nitrogen Fixation: Importance, Associated Diversity, and Estimates

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**Abstract** Several processes mediated by soil microorganisms play an important role in nutrient cycling. One such process is biological nitrogen fixation (BNF) by representatives of various bacterial phylogenetic groups, which are called diazotrophs. These bacteria can be free-living, associate with plant species, or even establish symbiosis with legumes. Studies with diazotrophic organisms are of great importance due to their contribution to the nitrogen supply in different ecosystems, including natural and managed systems. It is estimated that global BNF adds 122 Tg of N yearly with cultivated agricultural systems fixing from 33 to 43 Tg, which occurs mostly by legume-rhizobia symbiosis. There is a large potential of BNF contribution by associative systems with tropical grasses, but there is uncertainty in these estimates due to several assumptions in the estimation process and fewer studies with this system when compared to the legume-rhizobia symbiosis. Recent progress in the understanding of diversity, colonization ability, action mechanisms, formulation, and application of these biological systems should facilitate their development as reliable components in the management of sustainable agricultural systems. Several efforts have been made to develop commercial inoculants using these organisms. The current progress in using microorganisms that fix nitrogen in a variety of applications is summarized and discussed herein.

## Introduction

Microorganisms that carry out biological nitrogen fixation (BNF) have great importance because this element is an essential component of proteins, nucleic acids, and other nitrogen compounds. Therefore, nitrogen is an essential component of life for all living beings (Döbereiner 1997). The process of BNF performed by symbiotic nitrogen-fixing bacteria with legume species, which are commonly known as alpha and beta rhizobia, provides high sustainability for ecosystems (Bomfeti et al. 2011). These microorganisms can help promote plant growth not only by supplying nitrogen but also by other mechanisms, such as production of siderophores, exopolysaccharides (EPS), and phytohormones; phosphate solubilization; and protection against phytopathogenic fungus (Dakora 2003; Figueiredo et al. 2008; Moreira et al. 2010).

Diazotrophs are found in a wide variety of habitats: free-living in soil and water, associative symbioses with grasses, symbiotic association in termite guts, actinorhizal association with woody plants, cyanobacterial symbioses with various plants, and root-nodule symbioses with legumes (Dixon and Kahn 2004). The two most important types of symbioses are $N_2$ fixation and acquisition of P and other nutrients by mycorrhizae (Rengel 2002; Bonfante and Anca 2009). For cultivation of legumes, relationship of rhizobia and mycorrhiza is of great importance because these bacteria influence the infection rate and mineral nutrition as well as the physical and chemical conditions of the soil by adding organic waste and increasing the growth of these plants (Andrade et al. 2000; Parniske 2008).

Symbiotic or even mutualistic relationships involving rhizobia depend on chemical signals between the two organisms. These signals define the rhizobia host specificity in the relationship. Selecting for the optimal combination of the rhizobia and the host generally results in more effective symbiosis and better growth of the host plant (Rengel 2002; Araújo et al. 2012).

Biofertilizers that can cater to the different needs of growing plants act as a consortium in addition to other microorganisms in the rhizosphere. Understanding the interaction between the consortium of microbial inoculants and plant systems will lay the foundation for harnessing more benefits from microbial inoculants for improving plant growth and yield (Raja et al. 2006). Single inoculants and combinations of plant growth-promoting bacteria (PGPB)/plant growth-promoting rhizobacteria (PGPR) are common inoculants, and their use is increasing in agricultural practices (Díaz-Zorita and Fernández-Canigia 2009). PGPB affect plants through a multitude of mechanisms. Several comprehensive and critical reviews describing the operational mechanisms of PGPB/PGPR have been published in recent years (Bashan et al. 2011; de-Bashan et al. 2012).

The formulation step is a crucial aspect of producing microbial inoculants, and it determines the success of a biological agent (Brahmaprakash and Sahu 2012). In recent years, the strong potential of biopolymers to be used as inoculants has been studied (Borschiver et al. 2008; Silva et al. 2009). Biopolymers have demonstrated potential as bacterial carriers for microbial inoculants. Another recent possibility for development of new inoculants or biofertilizers is the use of biofilm (Seneviratne et al. 2009). Furthermore, the role of these compounds in stress adaptation may be an important criterion for the selection of inoculant strains to raise plant productivity by BNF under different soil and climatic conditions (Bomfeti et al. 2011).

## BNF as the Key for Ecological Success

Nitrogen is the most abundant element in the atmosphere, and it is mainly present in the diatomic form ($N_2$). Nitrogen is an essential macronutrient for plant species. Some bacteria have enzymes with the ability to reduce $N_2$ and turn it into ammonia. Subsequently, ammonia is used in the synthesis of essential elements, which is a process known as BNF (Hungria et al. 2007; Di Ciocco et al. 2008). BNF can be symbiotic when there are mutualistic associations between plant species and fixing microorganisms (mainly rhizobia) or can be asymbiotic when it is carried out by free-living fixing microorganisms, such as species of the genera *Azotobacter* and *Beijerinckia* (Freitas 2007).

Rhizobia are distributed in different taxonomic groups according to their morphological, physiological, genetic, and phylogenetic characteristics (Lindström et al. 2006). Currently, they are classified into α- and β-rhizobia (Bomfeti et al. 2011). The genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium*, and *Sinorhizobium* belong to the group of α-proteobacteria, and bacteria of the genera

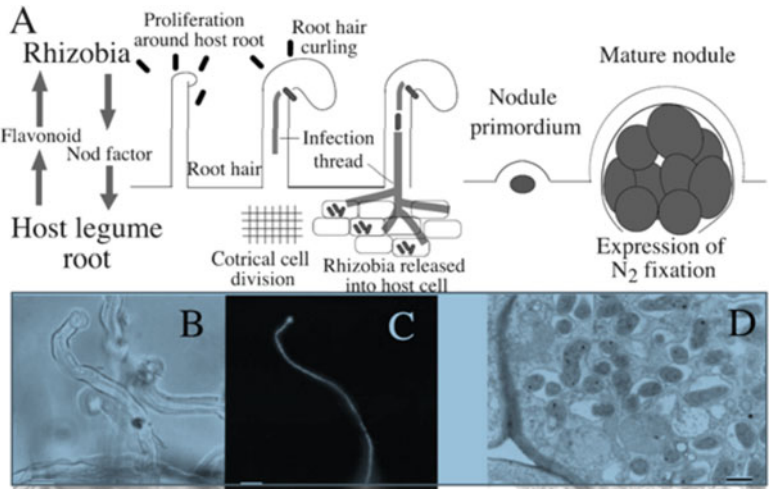


**Fig. 10.1** Nodulation by rhizobia. (**a**) Scheme of chemical signal exchanges and infection processes involving rhizobia. (**b**) Root-hair curing in *Lotus japonicus*. (**c**) *Mesorhizobium loti* cells tagged with constitutive *gfp* gene in an infection thread in the root hair shown in *panel* (**b**). (**d**) *M. loti* bacteroids in infected cells of a mature *L. japonicus* nodule. *Bar* indicates 10 **μ**m (**b**, **c**) and 1 **μ**m (**d**) (Adapted from Okasaki et al. 2004)

*Burkholderia*, *Cupriavidus*, and *Herbaspirillum* belong to the β-proteobacteria group (Weir 2011).

The effectiveness of the legume-rhizobia symbiotic system and the development of nodules result from the exchange of molecular chemical signals between plant and its symbiont (Okasaki et al. 2004; Zilli et al. 2009, 2011) (Fig. 10.1). Although legumes form root nodules mainly in response to Nod factors, the plant perception of endogenous signals, particularly plant hormones, is also thought to be important for the establishment of proper symbiotic interactions between rhizobia and legumes (Caetano-Anolles and Gresshoff 1991). The native species of nitrogen-fixing bacteria perform BNF at a low degree of efficiency. Therefore, it is necessary to obtain elite strains of rhizobia for efficient BNF (Figueiredo et al. 2008; Zilli et al. 2011).

Microorganisms that fix nitrogen require 16 mol of adenosine triphosphate (ATP) to reduce each mole of nitrogen (Hubbell and Kidder 2009). These organisms obtain this energy by oxidizing organic molecules. Non-photosynthetic free-living microorganisms must obtain these molecules from other organisms, and photosynthetic microorganisms, such as cyanobacteria, use sugars produced by photosynthesis to obtain these molecules. Associative and symbiotic nitrogen-fixing microorganisms obtain these compounds from the rhizosphere of their host plants (National Research Council 1994; Hubbell and Kidder 2009). Different $N_2$-fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems are shown in Fig. 10.2.

Advances in agricultural sustainability will require an increase in the use of BNF as a major source of nitrogen for plants. Long-term sustainability of agricultural systems must rely on the use and effective management of internal resources. The process of BNF offers an economically attractive and ecologically sound means of

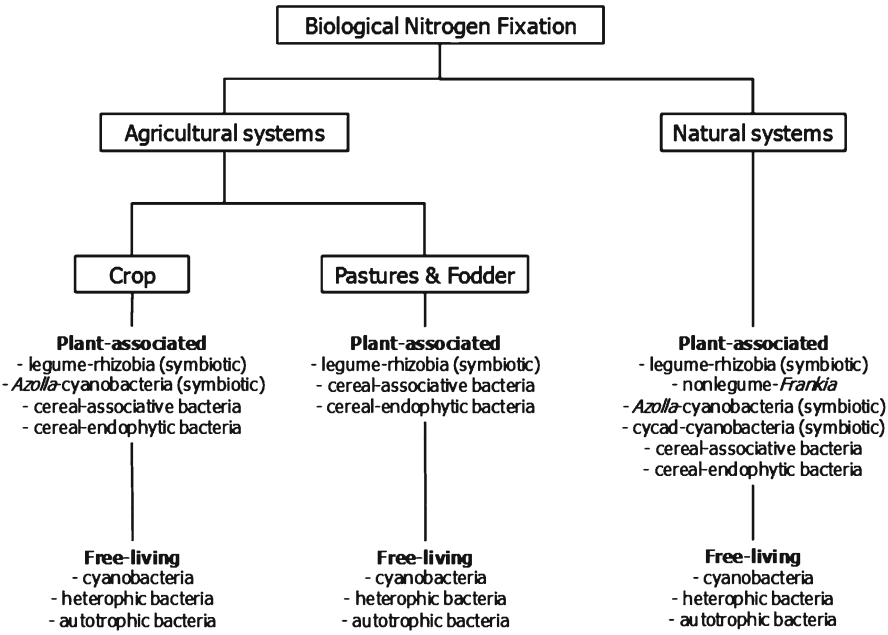


**Fig. 10.2** Biological $N_2$-fixing agents in agricultural and terrestrial natural systems (Adapted from Herridge et al. 2008)

reducing external nitrogen input and improving the quality and quantity of internal resources. Clearly, it is unreasonable to consider sustainable agriculture on a broad scale without BNF. Further research is needed to optimize the contribution of BNF to sustainable agriculture (Saikia and Jain 2007). The study of efficient use of N yields multiple advantages, such as the reduction of fertilizer doses to maintain productive levels and even genetic improvements to adapt plants to nitrogen-poor soils. The study of N acquisition and use should be linked to the understanding of the absorption, assimilation, and redistribution of this nutrient in cells as well as its balance between storage and use (Majerowicz et al. 2002).

Currently, new methods designed to increase nitrogen use efficiency are being intensely studied, especially through the recognition of biochemical and molecular pathways of absorption and assimilation in plants. Agroecological methods, such as BNF, are proposed to allow the sustainable use of this nutrient without production loss (Herridge et al. 2008).

## Diversity of BNF Systems

The high genetic variability of diazotrophs enables the occurrence of BNF in different systems (Franche et al. 2009), and this variability is important for the study of phylogenetic relationships. It is important for the study of phylogenetic

relationships, and diversity of bacterial genes is based not only on the taxonomic position but also on the need to fully exploit the potential of biotechnology (Woese 1994; Meitanis et al. 2008; Vale et al. 2008). According to Van Elsas and Boersma (2011), the study of microbial populations that inhabit the natural environment is essential in understanding the functioning of ecosystems. Microbial diversity is generally considered to be the number of individuals of different taxa and their distribution among taxa, and genetic diversity is the variation of genes and genotypes within groups (Lynch et al. 2004).

In the past, the study of microbial diversity was based on techniques that were dependent on cultivation, providing limited information due to lack of culture media which accurately reproduce the different ecological niches in a laboratory environment. Therefore, only a small fraction of microbial diversity existing in environmental systems can be cultivated in vitro, which causes an underestimation of the natural diversity (Tyson and Banfield 2005). Currently, microbial diversity can be assessed more broadly through the use of molecular biology techniques (both dependent on and independent of microorganism culture) enabling the detection of nucleic acids (Andreote et al. 2009; Roesch et al. 2010).

The diversity of the composition of ribosomal genes has been greatly discussed, especially for phylogenetic studies. In prokaryotes, such as diazotrophs, the 16S rRNA gene is the most widely used and is highly conserved. This gene is considered to be the most suitable for studies of microbial ecology and phylogeny and allows identification at the level of genus and species. 16S rRNA even allows for the analysis and correlation between genotype and the studied niche (Chéneby et al. 2000; Gribaldo and Brochier 2009). Diversity studies have identified different groups of diazotrophic bacteria: rhizobia (α-proteobacteria); Frankia (in Actinobacteria); cyanobacteria; bacteria belonging to various bacterial genera, such as *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Methylobacterium*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, and *Stenotrophomonas*, which colonize the surface of plant tissues without formation of differentiated structures; and endophytes (Kuklinsky-Sobral et al. 2004; Franche et al. 2009; Ribeiro et al. 2009; Lindstrom et al. 2010; Tripp et al. 2010; Monteiro et al. 2012). BOX A1R-based repetitive extragenic palindromic (BOX) polymerase chain reaction (PCR) is among several existing techniques in molecular biology that depend on the isolation and cultivation of bacterial communities in the laboratory. BOX PCR uses the same technique of repetitive extragenic palindromic sequence (REP) PCR, which is based on finding and amplifying repetitive regions of the bacterial genome. In this technique, repetitive and highly conserved regions of the bacterial genome are amplified, including Box elements, which are divided into three groups: BoxA, BoxB, and BoxC with BoxA being the most common. The BOX A1R primer allows a more detailed characterization of isolates, and it produces robust fragments of fingerprints with a complex pattern. Therefore, the BOX A1R primer is generally used to differentiate bacterial strains (Marques et al. 2008; Lee and Wong 2009). Torres et al. (2008) found a large diversity among endophytic bacteria isolated from root nodules formed by the symbiosis between rhizobia and bean (*Phaseolus vulgaris* L.).

**Table 10.1** Examples of molecular techniques for the study of the *nif* H gene

| Application | Molecular technique | Reference |
|---|---|---|
| Diversity analysis | Terminal restriction fragment length polymorphism analysis (T-RFLP) | Bannert et al. (2011), Beneduzi et al. (2008) |
| | Denaturing gradient gel electrophoresis (DGGE) | Li et al. (2012), Dias et al. (2012), Coelho et al. (2009), Martensson et al. (2009) |
| Gene quantification | Real-time PCR (qPCR) | Dias et al. (2012), Bannert et al. (2011), Coelho et al. (2009), Martensson et al. (2009) |
| Gene expression | Oligonucleotide microarray | Duc et al. (2009) |
| | Cloning and RT-PCR (reverse transcription) analysis | Honga et al. (2012), Thaweenut et al. (2011) |

Diazotrophs have the nitrogenase enzyme complex that is responsible for BNF. This enzyme system is composed of three subunits, and it is regulated by a complex system with multiple genes. Nitrogenase 1 (classic) is dependent on iron and molybdenum, and it is encoded by the *nif* gene. Nitrogenase 2 is dependent on vanadium, and it is encoded by the *vnf* gene. Nitrogenase 3 is dependent on iron, and it is encoded by the *anf* gene (Franche et al. 2009; Canfield et al. 2010). In free-living and associative microorganisms, the *nif* genes are responsible for encoding highly conserved subunits (Zehr et al. 2003; Falkowski et al. 2008; Franche et al. 2009). Because of the high conservation of these genes, phylogenetic studies based on these genes have shown similar results to those obtained using 16S rRNA. Thus, the *nif* gene has been used to characterize the genetic diversity of diazotrophs (Zehr et al. 2003).

The *nif* genes include *nif* D, *nif* H, and *nif* K, which all encode proteins of the nitrogenase enzyme complex. The *nif* H functional gene, which encodes the Fe-protein of nitrogenase, is well preserved and well studied as compared to other *nif* genes, which have been used for phylogenetic analysis of the diazotrophic bacterial community (Zehr et al. 2003; Franche et al. 2009). However, Gaby and Buckley (2011) assessed the global diversity of nitrogen-fixing microorganisms through the construction and analysis of an aligned database of 16,989 *nif* H sequences. They concluded that the diversity of diazotrophs is still poorly described and that many organisms remain to be discovered.

The techniques that evaluate bacterial diversity using the isolation and cultivation of bacteria in a laboratory followed by DNA extraction do not allow the study of uncultured microorganisms present in the sample environment, thereby restricting the diversity found. However, the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique allows access to the diversity of bacterial communities directly from their habitat without cultivation (Andreote et al. 2009). Therefore, various molecular techniques are being used to study the diversity, quantification, and analysis of *nif* H gene expression (Table 10.1).

## BNF Inputs to Agricultural Systems

While the Haber-Bosch nitrogen fertilizer production system is considered to have saved untold millions of people throughout the world, it is not without the following major concerns: the severalfold increase in reactive nitrogen cycling throughout the ecosphere; the relative lack of efficiency of reactive nitrogen under agricultural use, which may lead to major ecological issues of water contamination and eutrophication; and the demand for fossil fuel, which is generally demanded as natural gas (Rockström et al. 2009; Good and Beatty 2011; Kim et al. 2011; Sutton et al. 2011).

In contrast, even with all of these caveats, the growing population and its desirable increase in income will only demand higher levels of food production, particularly of meat and dairy products (Godfray et al. 2010). Meeting these demands will be impossible without a reliable nitrogen source. While chemically fixed fertilizer will necessarily be a part of the various options deployed by agricultural and soil scientists throughout the world, an increased reliance on BNF is one of the major alternatives for both maintaining and/or increasing agricultural yield and reducing both environmental and economical concerns linked to nitrogen fertilizer use (Doane et al. 2009; Hvistendahl 2010). One point to keep in mind is that these alternatives are not either/or solution sets and should not be thought as such. Both alternatives are simply tools to increase agricultural yield to allow human resources needs to be fulfilled in such a way that future generations will have access to at least the same pool of natural resources as previous ones did.

Unfortunately, even though obtaining global nitrogen fertilizer estimates is relatively easy, obtaining estimates for biologically fixed nitrogen is not easy (Herridge et al. 2008; Peoples et al. 2009); this factor may be a major constraint on an increased dependency on this source. One of the first reasons for this difficulty is the sheer number of possible biological systems, which all have different BNF capabilities, natural ranges, cultivated ranges, areas, and potential yields (Fig. 10.3). Burris (2008) has been quoted by Herridge et al. (2008) as having said that "potential authors could use a variety of methods to fill in the values in the N cycle, from gazing at crystal balls, consulting sages to cranking out computer-generated random numbers." However, the most common method is a literature review, and choosing the numbers thought to be a more logical approach. This difficulty in estimating global BNF can be roughly divided into several different reasons:

1. Methodological problems in field-scale BNF estimation
2. Highly variable BNF rates, which are strongly affected by environmental and agricultural concerns
3. Difficulty in estimating individual cropping systems, worldwide distribution, and cultivated areas

The first and second reasons intermingle with the high variation in BNF rates and lead to highly variable estimates, for example, for soybeans (*Glycine max*), which range from 0 to 450 kg shoot $N.ha^{-1}$ according to different sources cited by Peoples

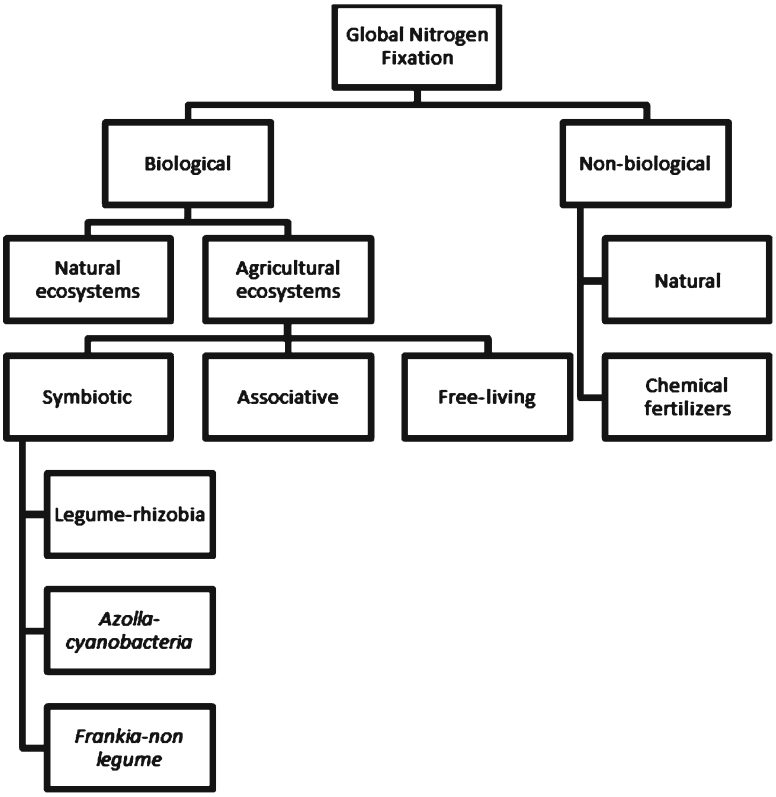


**Fig. 10.3** Main sources of biologically available nitrogen, not including mining the soil organic matter reserve

et al. (2009). In addition, another major problem is that the root system is routinely not included in the BNF estimates, which may lead to soybean going from a net exporter (Di Ciocco et al. 2011) to a net fixer of soil N (Singh et al. 2004). When considering the importance and number of agricultural systems in which soybean participates, this change may have significant effects on overall N balance.

Even with all of these caveats, it is still highly important to achieve an overall estimate, which has been well executed in several recent reports on a global or national scale (Herridge et al. 2008; Peoples et al. 2009; Yang et al. 2010). One common approach has been to obtain an estimate for the average BNF rate per hectare and then to multiply by another estimate of total cultivated area of the specific system. This approach may also be performed with some type of subdivision as exemplified by the recognition of different BNF rates for Argentinean-, Brazilian-, Chinese-, and North American-grown soybeans (Gan et al. 2002; Nicol et al. 2002; Okogun et al. 2005; Hungria et al. 2006; Oberson et al. 2007; Schipanski et al. 2010; Di Ciocco et al. 2011).

As indicated by the previous examples, a further point to consider is that BNF estimation is much more common for the legume-rhizobia symbiosis than

for other systems, such as grass-endophyte associations or *Azolla*-cyanobacteria symbiosis, which results from a better knowledge of the system, a much more precise estimate of occurrence, a vastly higher number of punctual experiments from which to derive raw data, and the sheer importance of the legume-rhizobia symbiosis in world agriculture.

Most estimates for global BNF are 120 Tg of N per year (estimations summarized by Herridge et al. (2008)) with the crop legume-rhizobia symbiosis accounting for approximately a sixth of that estimation (according to several authors summarized by Peoples et al. (2009)). These values are less than those estimated for the nitrogen fertilizer industry of *ca*. 140 Tg of N per year (Canfield et al. 2010). In contrast, while most estimates indicate that approximately 1–2 % of global energy consumption is directly linked to N fertilizer production and that *ca*. 300 Tg of fossil C is derived just from its production (not including the large fossil C cost of its distribution), there is close to zero fossil fuel use linked to BNF, which does not mean that there is not any C emission linked to BNF. There should also be a lower $NO_x$ emission derived from BNF, and thus lower glasshouse effects, because all of the N is in an organic form by definition and, thus, should not be available for denitrification most of the time (Jensen et al. 2012). This consideration is also important when considering that there is an international tendency to demand a more sustainable agriculture with less resource consumption for a given yield level, which could be maximized through further use of BNF as a rule in agricultural systems (Wilkins 2008), and if non-BNF advantages of legume inclusion are considered, such as the reduction in disease incidence or nutrient mining of deeper layers allowed by their greater root system (Sileshi et al. 2008; Köpke and Nemecek 2010; Fornara 2011).

All of these advantages require a greater need for management knowledge, which is one of the primary reasons for the greater reliance on N fertilizer as an alternative. The greater need for management knowledge is due to the highly localized effects of environment and cropping systems, which demand a high level of knowledge of the farmer to maximize their efficiency as exemplified by the variable effects of species and cultivars of legumes seeded with barley, the effect of a fertility gradient on BNF from legume-grass mixtures (Schipanski and Drinkwater 2012), or the impact of cutting management and *Desmodium* species in a mixture with corn (Kifuko-Koech et al. 2012).

## Mycorrhizal Infection of Legume Roots to Stimulate Nodulation

A mycorrhiza (Greek; mycos = fungus and rhiza = root) is a symbiotic association between certain soil fungi and plant roots (Bonfante and Anca 2009). Based on morphoanatomy of colonized roots, mycorrhizae are grouped into ectomycorrhiza, ectendomycorrhiza, and endomycorrhiza (Azcón 2000). Ectomycorrhizae are characterized by the formation of a mycelial mantle on the root with only intercellular penetration of the cortex by fungal mycelium and the formation of a "Hartig

net" (Bonfante 2001). Ectendomycorrhizae are generally ectomycorrhiza with intracellular penetration but with anatomical differences according to the host plant. Endomycorrhizae are the most common type of mycorrhiza formed by arbuscular mycorrhizal fungi (AMF) that penetrate intercellularly and intracellularly in the host root cortex, currently belonging to the phylum Glomeromycota with 3 classes (*Archaeosporomycetes*, *Glomeromycetes*, and *Paraglomeromycetes*) composed of 5 orders (*Archaeosporales*, *Diversisporales*, *Gigasporales*, *Glomerales*, and *Paraglomerales*), 14 families, 29 genera, and approximately 230 described species (Oehl et al. 2011) (Fig. 10.4). These fungi are distributed in most ecosystems representing a broader association between plants and fungi, and they are present on more than 80 % of plant species (Smith and Read 2008), including almost all species of agronomic interest and pastoral and tropical forest (Moreira and Siqueira 2002; Bonfante and Genre 2008).

*Leguminosae* is the third largest family of angiosperms and is the second most economically important in the world only behind *Poaceae*. With three subfamilies (*Mimosoideae*, *Caesalpinioideae*, and *Papilionoideae*), 727 genera, and 19,325 species, this family has a cosmopolitan distribution (Lewis et al. 2005). In Brazil, there are approximately 210 genera and 2,694 species of *Leguminosae* (Lima et al. 2010). Legumes play a key role in the balance of nitrogen in ecosystems due to their ability to fix nitrogen and improve soil quality in agroforestry, silvopastoral, and forestry (Foelkel 2012). Legumes are also ecologically important because they are well adapted to the first colonization and exploitation of different environments due, in part, to their association with nitrogen-fixing bacteria or mycorrhizal fungi (Silva and Tozzi 2011).

The rhizobia-legume symbiosis is responsible for producing annual levels of at least 35 million tons of nitrogen (Freire 1992). Nitrogen-fixing bacteria, including rhizobia and mycorrhizal fungi, form mutualistic symbiotic associations with legumes. In this association, which is known as tripartite (Bonfante and Anca 2009; Vega et al. 2010), the mycorrhizal mycelia through the network may increase the absorption and solubilization of phosphorus by translocating phosphorus in the soil to rhizobia located on plant nodules. Rhizobia fix nitrogen and provide it in the form of ammonia to the plant, which, in turn, provides carbohydrate to microsymbionts (Silveira et al. 2001; Gross et al. 2004). For cultivation of legumes, this relationship between rhizobia and mycorrhiza 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 waste and increasing the growth of these plants (Andrade et al. 2000).

Under conditions of phosphorus deficiency, legumes have low nodulation and nitrogen fixation unless their roots are colonized by mycorrhizas or if the soil is fertilized with high phosphorus levels. Moreover, the mycorrhizal condition influences the efficient competition among strains of rhizobia to occupy the nodules in the roots of the host (Miranda and Miranda 2002; Garg and Manchanda 2008). Kaschuk et al. (2010) studied the AMF-rhizobia symbiosis in 12 legume species, and they reported an increase in the photosynthetic rate and grain yield of legumes. This result is important because this rate compensates for the transfer of plant

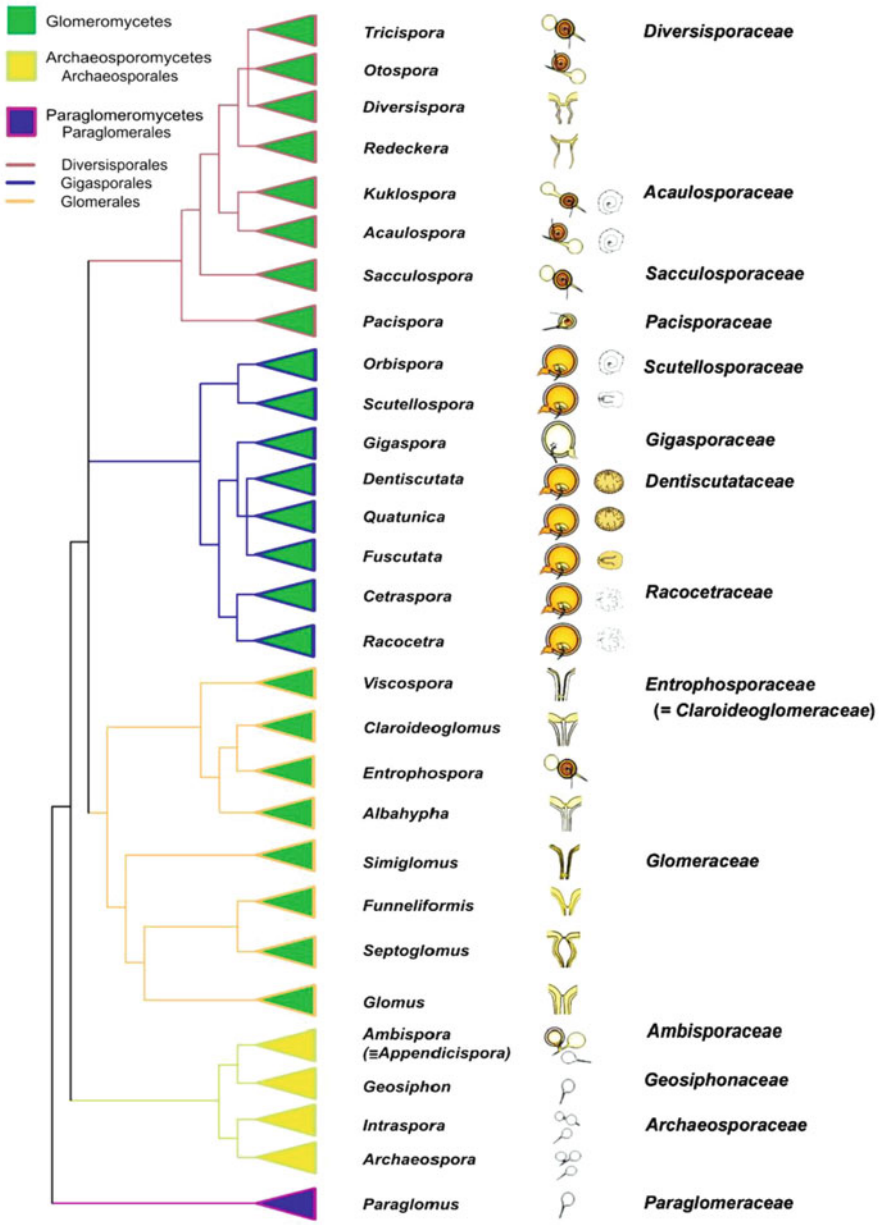


**Fig. 10.4** Representative tree of the phylum Glomeromycota based on molecular and morphological analyses (*Source*: Oehl et al. 2011)

photosynthates for microorganisms. Burity et al. (2000) studied thrush (*Mimosa caesalpiniaefolia*) inoculated with *Glomus etunicatum*, *Acaulospora morrowae*, *A. longula*, and *Rhizobium* sp., and they observed a larger increase in nodulation and root colonization by mycorrhiza. Jesus et al. (2005) studied two species of legumes

(*Piptadenia gonoacantha* (Mart.) Macbr. and *Piptadenia paniculata* Bentham) and found that these two species depend on mycorrhiza for satisfactory growth and nodulation with rhizobia. The synergy between the mycorrhizal fungi and rhizobia microsymbionts on legumes has been well documented in several studies (Mergulhão et al. 2001; Diniz et al. 2002; Jesus et al. 2005; Kaschuk et al. 2010; Lima et al. 2011; Mendes Filho et al. 2011). However, according to Scotti (1997), the benefit of these microorganisms to the host plant depends on the compatibility between the strain of rhizobia and mycorrhizal fungi inoculated.

Some strains of bacteria can positively influence and establish symbiosis with mycorrhizal fungi (Garbaye 1994; Frey-Klett et al. 2007), and these synergistically effective are called "mycorrhiza helper bacteria" (MHB) (Duponnois and Garbaye 1991; Garbaye 1994). Importantly, MHB have specificity for fungi but do not have specificity for plants (Garbaye 1994; Duponnois and Plenchette 2003). Duponnois and Garbaye (1991) were the first to observe the effect of bacteria *Pseudomonas fluorescens* to significantly stimulate the formation of ectomycorrhizal fungus *Laccaria laccata*. After the review on the effects observed in MHB work by Garbaye (1994), Frey-Klett et al. (2007) provided further information on MHB functionality during symbiosis. Frey-Klett et al. (2007) reported that MHB improve the conductivity of soil and responsiveness to root fungus by plant-fungus recognition and establishment of symbiosis, and they also reported that MHB promote survival, germination of propagules, and mycelial growth of 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. The strains of MHB identified to date belong to gram-negative Proteobacteria (*Agrobacterium*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Rhizobium*, and *Klebsiella*), gram-positive Firmicutes (*Bacillus*, *Brevibacillus*, and *Paenibacillus*), and gram-positive Actinomycetes (*Rhodococcus*, *Streptomyces*, and *Arthrobacter*) (Frey-Klett et al. 2007). This demonstrates the diversity of bacteria having potential use in biotechnological processes. Rigamonte et al. (2010) suggested that the first effect of MHB on ectomycorrhiza is related in stimulating the growth of these fungi. Another feature of MHB in mycorrhizal plants is acting as the stimulus for the formation of lateral roots in addition to growth of the fungus, which may lead to an increased number of sites of interaction between plant and fungus (Schrey et al. 2005), thereby increasing mycorrhizal colonization on the host plant (Rigamonte et al. 2010). Moreover, MHB may improve the nutritional status of mycorrhiza by providing nitrogen and contributing to solubilization of soil minerals. Some strains of MHB are able to compete with bacteria that inhibit mycorrhiza (Garbaye 1994); thus, these strains reduce the concentration of antifungal metabolites in the plant rhizosphere, which favors the release of fungi exudates that serve as nutrients for the bacteria (Rigamonte et al. 2010). According to Frey-Klett et al. (2007), MHB enhance spore germination and mycelial growth by producing growth factors, inhibiting competitors, inhibiting antagonists, and promoting detoxification of antagonistic substances. According to Bonfante and Anca (2009), the release of bioactive molecules and physical contact between bacteria and mycorrhizal fungi are important in the establishment of their interactions, which are the main

signaling mechanisms observed in the tripartite rhizobia/mycorrhiza/legume association (Oldroyd and Downie 2008). Legumes have an exceptional ability to form a symbiotic association with rhizobia and mycorrhizal fungi, and the positive effects of the symbiosis depend on the combination of interactions among the host plant, symbionts, and environmental factors. Thus, additional research is needed to better understand the functions and mechanisms of the interaction between fungi and bacteria in and on the roots of these host plants (Kannan et al. 2011).

## Bacterial Biofertilizers

Plant yield is dependent on nutrients, such as nitrogen (N), and farmers usually need to apply at least 100 kg of N per hectare (Deaker et al. 2004). However, N fertilizers are expensive, and chemical fertilization may promote soil pollution. In contrast, biofertilizers are gaining importance in sustainable agriculture. The term "biofertilizer" specifies that the fertilizer meets the nutritional requirements of a crop through microbiological means. In several countries, biofertilizers are synonymous with bacterial inoculants (Brahmaprakash and Sahu 2012).

A bacterial inoculant is a formulation that contains one or more beneficial bacterial strains or species in an easy-to-use and economical carrier material. Inoculants are the "vehicle" to transport living bacteria from the factory to living plants to produce the desired effects on plant growth (Bashan 1998). For legume plants, BNF can be used by inoculating legume seeds with rhizobia (Deaker et al. 2004). In Brazil, the use of inoculants for legume plants began in the 1950s (Freire et al. 1968), and the inoculation process currently saves approximately 11 billion dollars per year when considering only soybean crops (Hungria 2012). Although Brazil has a long tradition in research and production of inoculants for legumes, the studies of nitrogen fixation in *Azospirillum*-grass associations have only begun recently; thus, only a few commercial inoculants are available in the market (Bashan 1998).

Peat is the most frequently used carrier for the rhizobial inoculant industry because it has high water-holding capacity and large surface area, which support rhizobial growth and survival in large numbers (Smith 1992). However, a peat-based inoculant requires a significant amount of processing, such as mining, drying, milling, and neutralizing, before its use in a commercial production system. The formulation step is a crucial aspect for producing microbial inoculants and determines the success of a biological agent. Formulation typically consists of establishing viable bacteria in a suitable carrier together with additives that aid in the stabilization and protection of microbial cells during storage and transport and at the target (Brahmaprakash and Sahu 2012). The formulation should also be easy to handle and apply so that it is delivered to the target in the most appropriate manner and form and should also protect bacteria from harmful environmental factors and maintain or enhance the activity of the organisms in the field (Xavier et al. 2004).

A good quality inoculant should be composed of a superior carrier material. Smith (1992) suggested that the following features are characteristics of a superior

quality carrier material for microbial inoculants: high water-holding capacity, high water retention capacity, no heat production from wetting, nearly sterile, chemically uniform, physically uniform, nontoxic in nature, easily biodegradable, nonpolluting, nearly neutral pH (or easily adjustable pH), and supports bacterial growth and survival. Inoculants come in four basic dispersal forms (powders, slurries, granules, and liquids). The use of each type of inoculant depends upon market availability, farmers' choice, cost, and the need of a particular crop under specific environmental conditions (Arora et al. 2010).

These characteristics have prompted researchers to find new carrier materials, including clays, inorganic soils (Chao and Alexander 1984), compost (Iswaran et al. 1972), wheat bran (Jackson et al. 1991), spent agricultural waste material (Sadasivam et al. 1986), and spent mushroom compost (Bahl and Jauhri 1986). Apart from these materials, many other synthetic and inert materials, such as vermiculite (Sparrow and Ham 1983), perlite, ground rock phosphate, calcium sulfate, polyacrylamide gels (Dommergues et al. 1979), and alginate (Bashan 1986), have also been evaluated.

Liquid inoculant formulations are one solution to the problems associated with processing solid carriers. Liquid inoculant formulations may use various broth cultures amended with agents that promote cell survival in the package and after application to seeds or soil. Additives to liquid inoculant formulations should have a role in protecting the cells on seeds at high temperatures and during desiccation. Many types of biopolymers have been used for inoculant production due to their ability to limit heat transfer, good rheological properties, and high water activities (Mugnier and Jung 1985).

In recent years, the strong potential of biopolymers as inoculants has been studied (Freitas et al. 2003; Borschiver et al. 2008; Silva et al. 2009; Abd Elgadir et al. 2012). Biopolymers may be defined as polymers (proteins, nucleic acids, or polysaccharides) that are produced by living organisms (Borschiver et al. 2008). These polymers have demonstrated potential as bacterial carriers for microbial inoculants. These formulations encapsulate living cells, protect microorganisms against many environmental stresses, and gradually release the cells into soil in large quantities where the polymers are degraded by soil microorganisms.

Another recent possibility for the development of new inoculants or biofertilizers is the use of biofilm. Biofilm consists of microbial cells (algal, fungal, bacterial, and/or other microbial cells) in addition to an extracellular biopolymer (known as an extracellular polymeric substance) (EPS) produced by the cells, which provides structure and protection to the community. The formation of fungal-bacterial biofilms (FBBs) by bacterial colonization on biotic fungal surfaces provides enhanced metabolic activities as compared to monocultures. Incorporation of a nitrogen ($N_2$)-fixing rhizobial strain to FBBs to form fungal-rhizobial biofilms (FRBs) has been shown to improve potential biofilm applications in N-deficient settings and in the production of biofilm inoculants for biofertilizers and biocontrol in plants (Seneviratne et al. 2007). Biofilms attached to the plant roots of some crops help in the cycling of nutrients as well as in the biocontrol of pests and diseases, resulting in improved agricultural productivity (Seneviratne 2003). A developed FRB inoculant

has been shown to significantly increase $N_2$ fixation in soybean by approximately 30 % as compared to a conventional inoculant consisting only of rhizobia (monoculture inoculant) (Jayasinghearachchi and Seneviratne 2004). Reports have indicated that these symbiotic bacteria may have the potential to be used as PGPR with nonlegumes. Seneviratne et al. (2009) observed the heavy colonization of FBBs/FRBs on root hairs of rice (*Oryza sativa*), tea (*Camellia sinensis*), anthurium (*Anthurium andraeanum*), and wheat (*Triticum aestivum*). Such FRBs may act as "pseudonodules" by fixing $N_2$ biologically on the roots of nonlegumes.

## Concluding Remarks

Microorganisms are potential tools for sustainable agriculture and are the trend for the future. The BNF process offers an economically attractive and ecologically sound means of reducing external nitrogen input and improving the quality and quantity of internal resources. There is an urgent need for research to clearly define what bacterial traits are useful and necessary for different environmental conditions and plants so that optimal bacterial strains can be selected. However, field experiments are needed to provide a better understanding of the biological efficacy for increased yields in crop systems. The availability of complete genome sequences and functional genomics of symbiotic microorganisms (bacteria and mycorrhizal fungi) will enhance the understanding of symbiosis in the plant family, and obtaining this knowledge is a major challenge for future research. Increased use of BNF is one of the major pathways to maintain or increase yield and to reduce the environmental footprint of agriculture, which may be used to address the current challenge of meeting the fast-growing worldwide demand for agricultural products.

## References

Abd Elgadir M, Akanda MJH, Ferdosh F, Mehrnoush A, Karim AA, Noda T, Sarker MZI (2012) Mixed biopolymer systems based on starch. Molecules 17:584–597

Andrade AG, Costa GS, Faria SM (2000) Deposição e decomposição da serapilheira em povoamentos de *Mimosa caesalpiniifolia*, *Acacia mangium* e *Acacia holosericea* com quatro anos de idade em planossolo. Rev Bras Ciênc Solo 24:777–785

Andreote FD, Azevedo JL, Araújo WL (2009) Assessing the diversity of bacterial communities associated with plants. Braz J Microbiol 40:417–432

Araújo ASF, Leite LFC, Iwata BF, Lira Junior MA, Xavier GR, Figueiredo MVB (2012) Microbiological process in agroforestry systems. A review. Agron Sustain Dev 32:215–226

Arora NK, Khare E, Maheshwari DK (2010) Plant growth promoting rhizobacteria: constraints in bioformulation, commercialization, and future strategies. In: Maheshwari DK (ed) Plant growth and health promoting bacteria, vol 18, Microbiology monographs. Springer, Berlin

Azcón R (2000) Papel de la simbiosis micorrízica y su interacción com otros microorganismos rizosféricos en el crecimiento vegetal y sostenibilidad agrícola. In: Alarcón A, Ferrera-Cerrato R

(eds) Ecología fisiología y biotecnología de la micorriza arbuscular. MundiPrensa-Colegio de Postgraduados, Montecillo

Bahl N, Jauhri S (1986) Spent compost as a carrier for bacterial inoculant production. In: Wuest PJ, Royse DJ, Beelman RB (eds) Proceedings of the international symposium on scientific and technological aspects of cultivating edible fungi. The Pennsylvania State University, University Park, pp 63–68

Bannert A, Kleineidam K, Wissing L, Mueller-Niggemann C, Vogelsang V, Welzl G, Cao Z, Schloter M (2011) Changes in diversity and functional gene abundances of microbial communities involved in nitrogen fixation, nitrification and denitrification in a tidal wetland versus paddy soils cultivated for different time periods. Appl Environ Microbiol 77:6109–6116

Bashan Y (1986) Alginate beads as synthetic inoculant carriers for the slow release of bacteria that affect plant growth. Appl Environ Microbiol 51:1089–1098

Bashan Y (1998) Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol Adv 16:729–770

Bashan Y, Trejo A, de-Bashan LE (2011) Development of two culture media for mass cultivation of *Azospirillum* spp. and for production of inoculants to enhance plant growth. Biol Fertil Soils 47:963–969

Beneduzi A, Peres D, Costa PB, Zanettini MHB, Passaglia LMP (2008) Genetic and phenotypic diversity of plant-growth-promoting bacilli isolated from wheat fields in southern Brazil. Res Microbiol 159:244–250

Bomfeti CA, Florentino LA, Guimarães AP, Cardoso PG, Guerreiro MC, Moreira FMS (2011) Exopolysaccharides produced by the symbiotic nitrogen-fixing bacteria of Leguminosae. Rev Bras Ciênc Solo 35:657–671

Bonfante P (2001) At the interface between mycorrhizal fungi and plants: the structural organization of cell wall, plasma membrane and cytoskeleton. In: Hock B (ed) Mycota, vol IX, Fungal associations. Springer, Berlin

Bonfante P, Anca IA (2009) Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annu Rev Microbiol 63:363–383

Bonfante P, Genre A (2008) Plants and arbuscular mycorrhizal fungi: an evolutionary-developmental perspective. Trends Plant Sci 13:492–498

Borschiver S, Almeida LFM, Roitman T (2008) Monitoramento Tecnológico e Mercadológico de Biopolímeros. Polím: Ciênc Tecnol 18:256–261

Brahmaprakash GP, Sahu PK (2012) Biofertilizers for sustainability. J Indian Inst Sci 92:37–69

Burity HA, Lyra MCCP, Souza ES, Mergulhão ACES, Silva MLRB (2000) Efetividade da inoculação com rizóbio e fungos micorrízicos arbusculares em mudas de sabiá submetidas a diferentes níveis de fósforo. Pesqui Agropecu Bras 35:801–807

Caetano-Anolles G, Gresshoff M (1991) Plant genetic control of nodulation. Annu Rev Microbiol 45:345–382

Canfield D, Glazer AN, Falkowski PD (2010) The evolution and future of earth's nitrogen cycle. Science 330:192–196

Chao WL, Alexander M (1984) Mineral soils as carriers for *Rhizobium* inoculants. Appl Environ Microbiol 47:94–97

Chéneby D, Philippot L, Hartmant A, Henalt C (2000) 16 S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. FEMS Microbiol Ecol 34:121–128

Coelho MRR, Marriel IE, Jenkins SN, Lanyon CV, Seldin L, O'Donnell AG (2009) Molecular detection and quantification of nifH gene sequences in the rhizosphere of sorghum (*Sorghum bicolor*) sown with two levels of nitrogen fertilizer. Appl Soil Ecol 42:48–53

Council NR (1994) Biological nitrogen fixation: research challenges. National Academy Press, Washington, DC

Dakora FD (2003) Defining new roles for plant and rhizobial molecules in sole and mixed plant cultures involving symbiotic legumes. New Phytol 158:39–49

Deaker R, Roughley RJ, Kennedy IR (2004) Legume seed inoculation technology – a review. Soil Biol Biochem 36:1275–1288

De-Bashan LE, Hernandez JP, Bashan Y (2012) The potential contribution of plant growth-promoting bacteria to reduce environmental degradation – a comprehensive evaluation. Appl Soil Ecol 61:171–189

Di Ciocco C, Coviella C, Penón E, Díaz-Zorita M, López S (2008) Biological fixation of nitrogen and N balance in soybean crops in the pampas region. Span J Agric Res 6(1):114–119

Di Ciocco C, Penón E, Coviella C, López S, Díaz-Zorita M, Momo F, Álvarez R (2011) Nitrogen fixation by soybean in the Pampas: relationship between yield and soil nitrogen balance. Agrochimica (SI) 55:305–313

Dias ACF, Silva MCP, Cotta RS, Dini-Andreote F, Soares FL Jr, Salles JF, Azevedo JL, van Elsas JD, Andreote FD (2012) Abundance and genetic diversity of *nifH* gene sequences in anthropogenically affected Brazilian mangrove sediments. Appl Environ Microbiol 78:7960–7967

Díaz-Zorita M, Fernández-Canigia MV (2009) Field performance of a liquid formulation of *Azospirillum brasilense* on dryland wheat productivity. Eur J Soil Biol 45:3–11

Diniz MCNM, Burity HA, Figueiredo MVB (2002) Development and regrowth of cunhã (*Clitoria ternatea* L.) under water stress, in association with mycorrhizal fungi-*Bradyrhizobium*. Agrochimica 48:108–115

Dixon R, Kahn D (2004) Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol 2:621–631

Doane TA, Horwath WR, Mitchell JP, Jackson J, Miyao G, Brittan K (2009) Nitrogen supply from fertilizer and legume cover crop in the transition to no-tillage for irrigated row crops. Nutr Cycl Agroecosyst [SI] 85:253–262

Döbereiner J (1997) Importância da fixação biológica de nitrogênio para a agricultura sustentável. Biotecnol Ciênc Desenvolv 1:2–3 (Encarte Especial)

Dommergues YR, Diem HG, Divies C (1979) Polyacrylamide entrapped *Rhizobium* as an inoculant for legumes. Appl Environ Microbiol 37:779–981

Duc L, Neuenschwander S, Rehrauer H, Wagner U, Sobek J, Schlapbach R, Zeyer J (2009) Development and experimental validation of a *nifH* oligonucleotide microarray to study diazotrophic communities in a glacier forefield. Environ Microbiol 11:2179–2189

Duponnois R, Garbaye J (1991) Effect of dual inoculation of Douglas fir with the ectomycorrhizal fungus *Laccaria laccata* and mycorrhization helper bacteria (MHB) in two bare root forest nurseries. Plant Soil 138:169–176

Duponnois R, Plenchette C (2003) A mycorrhiza helper bacterium enhances ectomycorrhizal and endomycorrhizal symbiosis of Australian *Acacia* species. Mycorrhiza 13:85–91

Falkowski PG, Fenchel T, Delong E (2008) The microbial engines that drive Earth's biogeochemical cycles. Science 320:1034–1038

Figueiredo MVB, Burity HA, Martinez CR, Chanway CP (2008) Alleviation of water stress effects in common bean (*Phaseolus vulgaris* L.) by co-inoculation *Paenibacillus* × *Rhizobium tropici*. Appl Soil Ecol 40:182–188

Foelkel C (2012) Os eucaliptos e as leguminosas: parte 3: *Acacia mangium*. ABTCP, São Paulo

Fornara D (2011) Symbiotic nitrogen fixation and the delivery of multiple ecosystem services: a global change perspective. CAB reviews: perspectives in agriculture, veterinary science. Nutr Nat Res (SI) 6:1–8

Franche C, Lindström K, Elmerich C (2009) Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil 321(1/2):35–59

Freire JRJ (1992) Fixação do nitrogênio pela simbiose rizóbio/leguminosas. In: Cardoso EJN, Tsai SNM, Neves MC (eds) Microbiologia do solo. Sociedade Brasileira de Ciência do Solo, Campinas

Freire JRJ, Rhor TG, Oliveira PJ, Xavier GR, Rumjanek NG (1968) Trabalhos em rizobiologia no Rio Grande do Sul. In: Reunião Latino Americana sobre Inoculantes para Leguminosas, Porto Alegre. Anais. Porto Alegre: Secretaria de Agricultura 4:19–24

Freitas SS (2007) Rizobactérias promotoras de crescimento de plantas. In: Silveira APD, Freitas SS (eds) Microbiota do solo e qualidade ambiental. Instituto Agronômico de Campinas, Campinas, pp 1–20

Freitas RA, Gorin PAJ, Neves J, Sierakowski MR (2003) A rheological description of mixtures of a galactoxyloglucan with high amylose and waxy corn starches. Carbohydr Polym 51:25–32
Frey-Klett P, Garbaye J, Tarkka M (2007) The mycorrhiza helper bacteria revisited. New Phytol 176:22–36
Gaby JB, Buckley DH (2011) A global census of nitrogenase diversity. Environ Microbiol 13:1790–1799
Gan YB, Posthumus F, Van Keulen H, Kuiper P (2002) Effects of N management on growth, N-2 fixation and yield of soybean. Nutr Cycl Agroecosyst (SI) 62:163–174
Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210
Garg N, Manchanda G (2008) Effect of arbuscular mycorrhizal inoculation on salt-induced nodule senescence in *Cajanus cajan* (Pigeonpea). J Plant Growth Regul 27:115–124
Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C (2010) Food security: the challenge of feeding billion people. Science 327:812–818
Good AG, Beatty PH (2011) Fertilizing nature: a tragedy of excess in the commons. PLoS Biol 9:100–124
Gribaldo S, Brochier C (2009) Phylogeny of prokaryotes: does it exist and why should we care? Res Microbiol 160:513–521
Gross E, Cordeiro L, Caetano FH (2004) *Nodulação e micorrização* Anadenanthera peregrina *var.* falcata *em solo de cerrado autoclavado e não autoclavado*. Rev Bras Ciênc Solo 28:95–101
Herridge DF, Peoples MB, Boddey RM (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311:1–18
Honga Y, Maa Y, Wua L, Makib M, Qinb W, Chen S (2012) Characterization and analysis of *nif*H genes from *Paenibacillus sabinae* T27. Microbiol Res 167:596–601
Hubbell DH, Kidder G (2009) Biological nitrogen fixation. University of Florida IFAS Extension Publication SL16, pp 1–4
Hungria M (2012) Fixação biológica do N2 na agricultura de baixo carbono. In: Anais do FertBio 2012. UFAL, Maceió
Hungria M, Franchini JC, Campo RJ, Crispino CC, Moraes JZ, Sibaldelli RNR, Mendes IC, Arihara J (2006) Nitrogen nutrition of soybean in Brazil: contributions of biological $N_2$ fixation and N fertilizer to grain yield. Can J Plant Sci 86:927–939
Hungria M, Campo RJ, Mendes IC (2007) A importância do processo de fixação biológica do nitrogênio para a cultura da soja: componente essencial para a competitividade do produto brasileiro. Documentos 283-Embrapa Soja, Londrina
Hvistendahl M (2010) China's push to add by subtracting fertilizer. Science [SI] 327:801. doi:10.1126/science.327.5967.801
Iswaran V, Sen A, Apte R (1972) Plant compost as a substitute for peat for legume inoculants. Curr Sci 41:299
Jackson AM, Whipps JM, Lynch JM (1991) Production, delivery systems, and survival in soil of four fungi with disease biocontrol potential. Enzyme Microbiol Technol 13:636–642
Jayasinghearachchi HS, Seneviratne G (2004) A Bradyrhizobial-*Penicillium* spp. biofilm with nitrogenase activity improves $N_2$ fixing symbiosis of soybean. Biol Fertil Soils 40:432–434
Jensen ES, Peoples MB, Boddey RM, Gresshoff PM, Henrik HN, Alves BJR, Morrison MJ (2012) Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. Agron Sustain Dev 32:329–364
Jesus EC, Schiavo JA, Faria SM (2005) Dependência de micorrizas para a nodulação de leguminosas arbóreas tropicais. Rev Árvore 29:545–552
Kannan VR, Suganya S, Solomon EK, Balasubramanian V, Ramesh N, Rajesh P (2011) Analysis of interaction on between arbuscular mycorrhizal fungi and their helper bacteria by MILPA model. Res Plant Biol 1:48–62
Kaschuk G, Leffelaar PA, Giller KE, Alberton O, Hungria M, Kuyper TW (2010) Responses of legumes to rhizobia and arbuscular mycorrhizal fungi: a meta-analysis of potential photosynthate limitation of symbioses. Soil Biol Biochem 42:125–128

Kifuko-Koech M, Pypers P, Okalebo JR, Othieno CO, Khan ZR, Pickett JA, Kipkoech AK, Vanlauwe B (2012) The impact of *Desmodium* spp. and cutting regimes on the agronomic and economic performance of *Desmodium*–maize intercropping system in western Kenya. Field Crop Res 137:97–107

Kim T-W, Lee K, Najjar RG, Jeong H-D, Jeong HJ (2011) Increasing N abundance in the Northwestern Pacific Ocean due to atmospheric nitrogen deposition. Science 334:505–509

Köpke U, Nemecek T (2010) Ecological services of faba bean. Field Crop Res 115:217–233

Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL (2004) Isolation and characterization of soybean associated bacteria and their potential for plant growth promotion. Environ Microbiol 6:1244–1251

Lee A, Wong E (2009) Optimization and the robustness of BOX A1R PCR for DNA fingerprinting using trout lake *E. coli* isolates. J Exp Microbiol Immunol 13:104–113

Lewis GP, Schrire B, Mackinder B, Lock M (2005) Legumes of the world. Royal Botanic Gardens, Kew

Li X, Penttinen P, Gu Y, Zhang X (2012) Diversity of *nif* H gene in rhizosphere and non-rhizosphere soil of tobacco in Panzhihua, China. Ann Microbiol 62:995–1001

Lima HC, Queiroz LP, Morim MP et al. (orgs) (2010) Catálogo das Plantas e Fungos do Brasil. Jardim Botânico do Rio de Janeiro, Rio de Janeiro 2:989–1102

Lima AST, Xavier TF, Lima CEP, Oliveira JP, Mergulhão ACES, Figueiredo MVB (2011) Triple inoculation with *Bradyrhizobium*, *Glomus* and *Paenibacillus* on cowpea (*Vigna unguiculata* [L.] Walp.) development. Braz J Microbiol 42:919–926

Lindström K, Kokko-Gonzales P, Terefework Z, Räsänen LA (2006) Differentiation of nitrogen-fixing legume root nodule bacteria (Rhizobia). In: Cooper JE, Rao JR (eds) Molecular approaches to soil, rhizosphere and plant microorganism analysis. CABI, Wallingford/ Cambridge, pp 236–258

Lindstrom K, Murwira M, Willems A, Altier N (2010) The biodiversity of beneficial microbe-host mutualism: the case of rhizobia. Res Microbiol 161:453–463

Lynch JM, Benedetti A, Insam H, Nuti MP, Smalla K, Torsvik V, Nannipieri P (2004) Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms. Biol Fertil Soils 40:363–385

Majerowicz N, Pereira JMS, Médici LO, Bizon O, Pereira MB, Santos Júnior U (2002) Estudo da eficiência de uso do nitrogênio em variedades locais e melhoradas de milho. Rev Bras Bot 25:129–136

Marques ASA, Marchaison A, Gardan L, Samson R (2008) BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae – P. viridiflava* group. Genet Mol Biol 31:106–115

Martensson L, Díez B, Wartiainen I, Zheng W, El-Shehawy R, Rasmussen U (2009) Diazotrophic diversity, *nif*H gene expression and nitrogenase activity in a rice paddy field in Fujian, China. Plant Soil 325:207–218

Meitanis C, Chalkou KI, Kormas KA, Lymperopoulou DS, Katsifas EA, Hatzinikolaou DG, Karagouni AD (2008) Application of *rpoB* sequence similarity analysis, REP-PCR and BOX-PCR for the differentiation of species within the genus *Geobacillus*. Lett Appl Microbiol 46:395–401

Mendes Filho PF, Vasconcellos RLF, Cardoso EJBN (2011) Growth and development of jack-bean and pigeon-pea in cassiterirte mine spoil. J Soil Sci Environ Manag 2:74–79

Mergulhão ACES, Silva MLRB, Burity HA, Stamford NP (2001) Influência da dupla inoculação rizóbio e fungos micorrízicos arbusculares em plantas de sabiá sob solos de diferentes texturas. Rev Ecossistema 26:35–39

Miranda JCC, Miranda LN (2002) Importância da micorriza arbuscular para o cultivo da soja na região do cerrado. Comunicado Técnico 75- EMBRAPA-CPAC, Planaltina

Monteiro RA, Balsanelli E, Wassem R, Marin AM, Brusamarello-Santos LCC, Schmidt MA, Tadra-Sfeir MZ, Pankievicz VCS, Cruz LM, Chubatsu LS, Pedrosa FO, Souza EM (2012) *Herbaspirillum*-plant interactions: microscopical, histological and molecular aspects. Plant Soil 356:175–196

Moreira FMS, Siqueira JO (2002) Microbiologia e Bioquímica do Solo. Editora UFLA, Lavras
Moreira FMS, Silva K, Nóbrega RSA, Carvalho F (2010) Diazotrophic associative bacteria: diversity, ecology and potential applications. Comput Sci 1(2):74–99
Mugnier J, Jung G (1985) Survival of bacteria and fungi in relation to water activity and the solvent properties of water in biopolymer. Appl Environ Microbiol 50:108–114
Nicol SMF, Arias CAA, Hungria M (2002) Genetics of nodulation and nitrogen fixation in Brazilian soybean cultivars. Biol Fertil Soil (SI) 36:109–117
Oberson A, Nanzer S, Bosshard C, Dubois D, Mäder P, FrossarD E (2007) Symbiotic N2 fixation by soybean in organic and conventional cropping systems estimated by 15N dilution and 15N natural abundance. Plant Soil (SI) 290:69–83
Oehl F, Sieverding E, Palenzuela J, Ineichen K, Silva GA (2011) Advances in *Glomeromycota* taxonomy and classification. IMA Fungus 2:191–199
Okasaki S, Nukui N, Sugawara M, Minamisawa K (2004) Rhizobial strategies to enhance symbiotic interactions: rhizobiotoxine and 1-aminocyclopropane-1-carboxylate deaminase. Microb Environ 19:99–111
Okogun JA, Sanginga N, Abaidoo R, Dashiell KE, Diels J (2005) On-farm evaluation of biological nitrogen fixation potential and grain yield of Lablab and two soybean varieties in the northern Guinea savanna of Nigeria. Nutr Cycl Agroecosyst 73:267–275
Oldroyd GE, Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 59:519–546
Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol 6:763–775
Peoples MB, Brockwell J, Herridge DF, Rochester IJ, Alves BJR, Urquiaga S, Boddey RM, Dakora FD, Bhattarai S, Maskey SL, Sampet C, Rerkasem B, Khan DF, Hauggaard-Nielsen H, Jensen ES (2009) The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. Symbiosis 48:1–17
Raja P, Una S, Gopal H, Govindarajan K (2006) Impact of bioinoculants consortium on rice root exudates, biological nitrogen fixation and plant growth. J Biol Sci 6:815–823
Rengel Z (2002) Breeding for better symbiosis. Plant Soil 245:147–162
Ribeiro RA, Barcellos FG, Thompson FL, Hungria M (2009) Multilocus sequence analysis of Brazilian *Rhizobium* microsymbionts of common bean (*Phaseolus vulgaris* L.) reveals unexpected taxonomic diversity. Res Microbiol 160:297–306
Rigamonte TA, Pylro VS, Duarte GF (2010) The role of mycorrhization helper bacteria in the establishment and action of ectomycorrhizae associations. Braz J Microbiol 41:832–840
Rockström J, Steffen W, Noone K, Persson A, Chapin FS III, Lambin EF, Lenton TM, Scheffer M, Folke C, Schellnhuber HJ, Nykvist B, de Wit CA, Hughes T, Van der Leeuw S, Rodhe H, Sörlin S, Snyder PK, Constanza R, Svedin U, Falkenmark M, Karlberg L, Corell RW, Fabry VJ, Hansen J, Walker B, Liverman D, Richardson K, Crutzen P, Foley JA (2009) A safe operating space for humanity. Nature [SI] 461:472–475
Roesch LFW, Fulthorpe RR, Jaccques RJS (2010) Biogeography of diazotrophic bacteria is soils. World J Microb Biotechnol 26:1503–1508
Sadasivam KV, Tyagi RK, Ramarethinam S (1986) Evaluation of some agricultural wastes as carriers for bacterial inoculants. Agric Wastes 17:301–306
Saikia SP, Jain V (2007) Biological nitrogen fixation with non-legumes: an achievable target or a dogma? Curr Sci 92(3):317–322
Schipanski ME, Drinkwater LE (2012) Nitrogen fixation in annual and perennial legume-grass mixtures across a fertility gradient. Plant Soil 357:147–159
Schipanski ME, Drinkwater LE, Russelle MP (2010) Understanding the variability in soybean nitrogen fixation across agroecosystems. Plant Soil 329:379–397
Schrey SD, Schellhammer M, Ecke M, Hampp R, Tarkka MT (2005) Mycorrhiza helper bacterium *Streptomyces* AcH505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria*. New Phytol 168:205–216
Scotti MRMML (1997) Fixação biológica do nitrogênio por espécies arbóreas. In: Vargas MAT, Hungria M (eds) Biologia dos solos dos cerrados. EMBRAPA-CPAC, Planaltina

Seneviratne G (2003) Development of eco-friendly, beneficial microbial biofilms. Curr Sci 85:1395–1396
Seneviratne G, Zavahir JS, Bandara WMMS, Weerasekara MLMAW (2007) Fungal-bacterial biofilms: their development for novel biotechnological applications. World J Microb Biotechnol 24:739–743
Seneviratne G, Thilakaratne RMMS, Jayasekara APDA, Seneviratne KACN, Padmathilake KRE, De silva MSDL (2009) Developing beneficial microbial biofilms on roots of non-legumes: a novel biofertilizing technique. In: Khan MS, Zaid A, Musarrat J (eds) Microbial strategy for crop improvement. Springer, Berlin
Sileshi G, Mafongoya PL, Chintu R, Akinnifesi FK (2008) Mixed-species legume fallows affect faunal abundance and richness and N cycling compared to single species in maize-fallow rotations. Soil Biol Biochem 40:3065–3075
Silva ED, Tozzi AMGA (2011) Leguminosae na Floresta Ombrófila Densa do Núcleo Picinguaba, Parque Estadual da Serra do Mar, São Paulo, Brasil. Biota Neotrop 11:299–325
Silva MF, Oliveira PJ, Xavier GR, Rumjanek NG, Reis VM (2009) Inoculantes formulados com polímeros e bactérias endofíticas para a cultura da cana-de-açúcar. Pesqui Agropecu Bras 44:1437–1443
Silveira JAG, Costa RCL, Oliveira JTA (2001) Drought-induced effects and recovery of nitrate assimilation and nodule activity in cowpea plants inoculated with *Bradyrhizobium* spp. under moderate nitrate level. Braz J Microbiol 32:187–194
Singh M, Kundu S, Biswas AK, Saha JK, Tripathi AK, Acharya CL (2004) Quantification of $N_2$ fixation and annual N benefit from $N_2$ fixation in soybean accrued to the soil under soybean-wheat continuous rotation (2004). J Plant Nutr Soil Sci-Z Pflanzenernahr Bodenkd 167:577–583
Smith RS (1992) Legume inoculant formulation and application. Can J Microbiol 38:485–492
Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, Boston
Sparrow SD, Ham GE (1983) Survival of *Rhizobium phaseoli* in six carrier materials. Agron J 75:181–184
Sutton MA, Oenema O, Erisman JW, Leip A, Van Grinsven H, Winiwarter W (2011) Too much of a good thing. Nature 472:159–161
Thaweenut N, Hachisuka Y, Ando S, Yanagisawa S, Yoneyama T (2011) Two seasons' study on *nif*H gene expression and nitrogen fixation by diazotrophic endophytes in sugarcane (*Saccharum* spp. hybrids): expression of *nif*H genes similar to those of rhizobia. Plant Soil 338:435–449
Torres AR, Araújo WL, Cursino L, Hungria M, Plotegher F, Mostasso FL, Azevedo JL (2008) Diversity of endophytic enterobacteria associated with different host plants. J Microbiol 46:373–379
Tripp HJ, Bench SR, Turk KA, Foster RA, Desany BA, Niazi F, Affourtit JP, Zehr JP (2010) Metabolic streamlining in an open-ocean nitrogen-fixing cyanobacterium. Nature 464:90–94
Tyson GW, Banfield JF (2005) Cultivating the uncultivated: a community genomics perspectives. Trends Microbiol 13:411–415
Vale FF, Encarnacao P, Vítor JMB (2008) A new algorithm for cluster analysis of genomic methylation: the *Helicobacter pylori* case. Bioinformatics 24(3):383–388
Van Elsas JD, Boersma FGH (2011) A review of molecular methods to study the microbiota of soil and the mycosphere. Eur J Soil Biol 47:77–87
Vega C, Arista M, Ortiz PL, Talavera S (2010) Anatomical relations among endophytic holoparasitic angiosperms, autotrophic host plants and mycorrhizal fungi: a novel tripartite interaction. Am J Bot 97:730–737
Weir BS (2011) The current taxonomy of rhizobia. New Zealand rhizobia website. disponível em: http://www.rhizobia.co.nz/taxonomy/rhizobia.html. Acesso em: 08 março, 2012
Wilkins RJ (2008) Eco-efficient approaches to land management: a case for increased integration of crop and animal production systems. Philos Trans R Soc B Biol Sci 363:517–525
Woese CR (1994) There must be a prokaryote somewhere – microbiologists search for itself. Microbiol Rev 58:1–9

Xavier IJ, Holloway G, Leggett M (2004) Development of rhizobial inoculant formulations. Online Crop Manage. Available at: http://www.plantmanagementnetwork.org/pub/cm/review/2004/develop/

Yang JY, Drury CF, Yang XM, de Jong R, Huffman EC, Campbell CA, Kirkwood V (2010) Estimating biological $N_2$ fixation in Canadian agricultural land using legume yields. Agric Ecosyst Environ 137:192–201

Zehr JP, Jenkins BD, Short SM, Steward GF (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Appl Environ Microbiol 5:539–554

Zilli JE, Xavier GR, Moreira FMS, Freitas ACR, Oliveira LA (2009) Fixação biológica de nitrogênio. In: Zilli JE, Vilarinho AA, Alves JMA (eds) A cultura do feijão-caupi na Amazônia Brasileira. Embrapa Roraima, Boa Vista

Zilli JE, Silva Neto ML, França Júnior I, Perin L, Melo AR (2011) Resposta do feijão-caupi à inoculação com estirpes de *Bradyrhizobium* recomendadas para a soja. Rev Bras Ciênc Solo 35:739–742