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REVIEW PAPER

Nitrogen signalling in plant interactions with associative and endophytic diazotrophic bacteria

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Abstract

Some beneficial plant-interacting bacteria can biologically fix N_2 to plant-available ammonium. Biological nitrogen fixation (BNF) is an important source of nitrogen (N) input in agriculture and represents a promising substitute for chemical N fertilizers. Diazotrophic bacteria have the ability to develop different types of root associations with different plant species. Among the highest rates of BNF are those measured in legumes nodulated by endosymbionts, an already very well documented model of plant–diazotrophic bacterial association. However, it has also been shown that economically important crops, especially monocots, can obtain a substantial part of their N needs from BNF by interacting with associative and endophytic diazotrophic bacteria, that either live near the root surface or endophytically colonize intercellular spaces and vascular tissues of host plants. One of the best reported outcomes of this association is the promotion of plant growth by direct and indirect mechanisms. Besides fixing N, these bacteria can also produce plant growth hormones, and some species are reported to improve nutrient uptake and increase plant tolerance against biotic and abiotic stresses. Thus, this particular type of plant–bacteria association consists of a natural beneficial system to be explored; however, the regulatory mechanisms involved are still not clear. Plant N status might act as a key signal, regulating and integrating various metabolic processes that occur during association with diazotrophic bacteria. This review will focus on the recent progress in understanding plant association with associative and endophytic diazotrophic bacteria, particularly on the knowledge of the N networks involved in BNF and in the promotion of plant growth.

Key words: Associative bacteria, biological nitrogen fixation, diazotrophic bacteria, endophytic bacteria, nitrogen signalling, plant growth promotion.

Introduction

Nitrogen (N) is one of the most important plant nutrients for development. In a wide range of agricultural crop systems, the limited natural N supply in soil restricts plant yields (Robertson and Vitousek, 2009); therefore, crop productivity relies heavily on N fertilization. The benefits of chemical N fertilizers added to cropping systems come with well-documented high energy costs and environmental damage. In this context, developing strategies for improving nitrogen use efficiency (NUE) is crucial for the establishment of a sustainable agriculture and represents an important challenge of this century.

A wide range of interactions occur between plants and microorganisms. These microorganisms could be beneficial, harmful, or neutral, according to their effects on plant development (Dobbelaere *et al.*, 2003). Among beneficial associations between plants and microorganisms, those of great interest are the ones related to the biological conversion of the N_2 in the air to plant-available ammonium, carried out by diazotrophic bacteria. This type of plant–bacteria interaction is another major source of N input in agriculture and represents a promising alternative to chemical N fertilizers.

Diazotrophic bacteria have the ability to develop different types of root associations with different plant species. The best studied symbiotic interaction between diazotrophic bacteria and plants are those that involve legumes and nitrogen-fixing bacteria of *Rhizobium* genera (Oldroyd, 2013). In *Rhizobium* associations, bacteria are endosymbionts, living inside differentiated structures formed in roots, called nodules. The predominant function of the nodule is to produce an environment that is conducive to bacterial N fixation, imposing restrictions on the free flow of oxygen, which otherwise limits N fixation (Oldroyd, 2013).

Some bacteria live in the rhizosphere and are called rhizobacteria (Kloepper and Beauchamp, 1992) (Fig. 1). Several of these are found on the root surface, where they are usually designated associative N-fixing bacteria (Elmerich and Newton, 2007) (Fig. 1). Also, there are some bacteria that can be detected inside surface-sterilized plants, called endophytic N-fixing bacteria, and one of their traits is that it is located inside the plant and do not cause any visible harmful effects (Reinhold-Hurek and Hurek, 1998; James and Olivares, 1998; Monteiro *et al.*, 2012) (Fig. 1). Nevertheless, sometimes it is difficult to distinguish between associative and endophytic plant colonization, as some associative bacteria can also be observed inside plant tissues, even though they are less abundant than the endophytic bacteria (Elmerich, 2007).

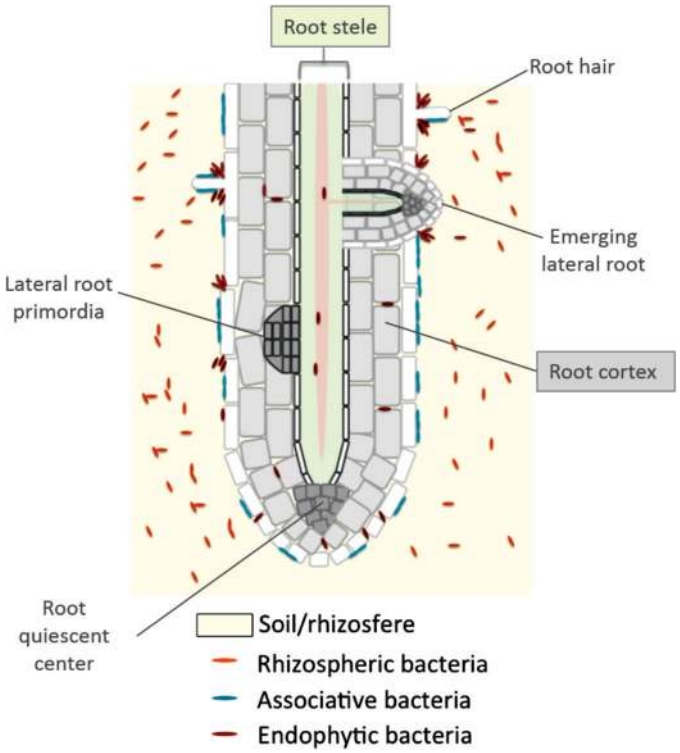

Fig. 1. Root niches for colonization by diazotrophic bacteria. Rhizospheric bacteria (orange cells) colonize the rhizosphere soil area without invading internal plant tissues. Associative bacteria (blue cells) are in close interaction with the plant surface and sometimes can be found within plant tissues. Endophytic bacteria (dark red cells) colonize any region within the epidermis of the plant root, and they can reside in apoplastic intercellular spaces and the xylem vessel apoplast. In general, the endophytes invade the internal plant tissues through sites of injury in the epidermis, root tips, and root cracks formed at the sites of lateral root emergence. Some endophytic bacteria can spread to distant plant organs (stem, leaves, seeds, and fruits).

Endophytic bacteria invade plant tissues but they differ from endosymbionts, as they do not reside intracellularly in living plant cells and their colonization does not induce the formation of any differentiated plant structure. In bacterial biological nitrogen fixation (BNF) associations, endosymbionts and endophytic bacteria may have an advantage over associative diazotrophic bacteria and rhizobacteria, since they live within plant tissues, establishing themselves in less competitive niches that present better conditions for N fixation and assimilation of fixed N by the plant (Reinhold-Hurek and Hurek, 1998, 2011).

Calculation of global BNF rates indicated an estimate of 50–70 Tg of N fixed biologically per year in agricultural systems worldwide (Herridge *et al.*, 2008). Among the highest rates of BNF are those measured in legumes nodulated by endosymbionts. In Brazilian soybean culture, adaptation and selection of genotypes was carried out with zero N added, which resulted in the choice of the most efficient BNF varieties (Döbereiner, 1997). Consequently, Brazil became the only country in the world to obtain, with absolutely no N applications, high yields of soybean, which became the country's largest export product (Hungria *et al.*, 2006). Other economically important crops, especially monocots such as Poaceae, can obtain a substantial part of their N from BNF associations with endophytic and associative diazotrophic bacteria. Although the amount of fixed N is not as large as that measured in endosymbiosis, large increases in yield have been reported in the field (Dobbelaere *et al.*, 2003; Vessey, 2003; Bhattacharyya and Jha, 2012). Thus, this particular type of plant–bacteria association consists of a natural beneficial system to be explored.

This review will focus on the recent progress in the understanding of plant association with associative and endophytic N-fixing bacteria, particularly on the knowledge of N networks involved in BNF and promotion of plant growth.

Associative and endophytic diazotrophic bacteria

Apart from their common ability to fix N_2, associative and endophytic diazotrophic bacteria are genetically diverse. They have been identified in several genera of alpha-, beta-, and gamma-Proteobacteria including *Azospirillum*, *Azorhizobium*, *Azoarcus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas*, and *Rhizobium* (Vessey, 2003; Kennedy *et al.*, 2004; Magnani *et al.*, 2010; Santi *et al.*, 2013). Several methods have been used to assess the occurrence and location of these diazotrophic bacteria, including the immunological detection of bacteria, fluorescence tags, electron microscopy, confocal laser scanning microscopy, and specific oligonucleotide probes (Rosenblueth and Martínez-Romero, 2006; Verma *et al.*, 2010).

Associative diazotrophic bacteria, such as *Azospirillum lipoferum* and *Azotobacter* sp., live in close association with

the root's surface, particularly in the root hair and elongation zones (James, 2000; Rosenblueth and Martínez-Romero, 2006) (Fig. 1). On the other hand endophytes such as *Gluconacetobacter diazotrophicus*, *Azoarcus* spp., *Herbaspirillum* spp., and some strains of *Azospirillum brasilense* (James, 2000; Rosenblueth and Martínez-Romero, 2006; Reinhold-Hurek and Hurek, 2011) do not survive well in the soil, though they colonize the root cortex and stele (Fig. 1). Although these bacteria can be found in leaf mesophyll cells (Dong *et al.*, 1994, 1995, 1997; James and Olivares, 1998; James *et al.*, 2001), roots normally have higher numbers of endophytes compared with above-ground tissues (Rosenblueth and Martínez-Romero, 2006). Endophytic colonization occurs in intercellular spaces, xylem vessels, and lignified xylem parenchyma, as well as in dead cells, such as those found on lysigenous aerenchyma (James, 2000).

Several studies have described in detail all the steps of plant invasion and colonization by associative and endophytic diazotrophic bacteria (Reinhold-Hurek and Hurek, 1998; Rosenblueth and Martínez-Romero, 2006; Compant *et al.*, 2010). In brief, plant–bacterial interaction starts in the rhizosphere and is induced by root exudates that attract diazotrophic bacteria. Chemotaxis mechanisms involved in the bacterial migration towards plant roots include the presence of flagella that allow bacteria to come into contact with roots, together with type IV pili and twitching motility. Root colonization also depends on the adhesion and anchoring of the bacteria onto the root system, as well as microbial proliferation and the formation of biofilm structures at the root surface. Bacterial surface exopolysaccharides and lipopolysaccharides (LPSs) are involved in the adhesion and colonization of roots (Rosenblueth and Martínez-Romero, 2006; Reinhold-Hurek and Hurek, 2011) (Fig. 1). For endophytic colonization, the emergence points of lateral roots and, to some extent, differentiation and elongation zones near the root tip, where slightly disrupted or not completely differentiated tissues may facilitate penetration, are considered sites for primary colonization into roots (James and Olivares, 1998; Reinhold-Hurek and Hurek, 1998, 2011). Root intercellular spaces in the epidermal and cortical regions and lysed plant cells are major sites of colonization, but vascular tissue and xylem cells may also be invaded, an occurrence which is likely to allow systemic spreading into the shoots (Reinhold-Hurek and Hurek, 1998; Rosenblueth and Martínez-Romero, 2006; Compant *et al.*, 2010).

As plant colonization is established, one of the best-reported outcomes of association is the promotion of plant growth by direct and indirect mechanisms. Besides fixing N, associative and endophytic diazotrophic bacteria produce plant growth hormones, such as auxin and gibberellin (Baca and Elmerich, 2007; Spaepen *et al.*, 2007), and several of them are also reported to improve nutrient uptake (Sturz and Nowak, 2000; Richardson *et al.*, 2009; Saha *et al.*, 2013). In addition, various experiments demonstrated that associative and endophytic bacteria may indirectly benefit plant development by increasing the plant's tolerance to biotic and abiotic stresses (Arencibia *et al.*, 2006; Rosenblueth and Martínez-Romero, 2006; Yasuda *et al.*, 2009). Beneficial results of these associations include a significant increase in the plant's height and biomass, root length, dry matter production, and grain yield, which are summarized in Table 1. A positive synergistic effect from the co-inoculation of associative diazotrophic bacteria and rhizobia on legume nodulation and yield has also been reported (Hungria *et al.*, 2013).

Regulation of biological nitrogen fixation during association

The advent of biochemical and genomic technologies has allowed a great advance in the comprehension of BNF mechanisms used by associative and endophytic bacteria (Dixon and Kahn, 2004). The basic machinery of N fixation and regulation is very similar to those already well characterized in *Rhizobium* species (Burris and Roberts, 1993; Mylona *et al.*, 1995; Steenhoudt and Vanderleyden, 2000; Monteiro *et al.*, 2012), as their genomes have an *nifHDK* operon, encoding both nitrogenase components: the dinitrogenase protein (MoFe protein, *NifDK*), which contains a molybdenum–iron cofactor that is the site of N_2 reduction; and the dinitrogenase reductase protein (Fe protein, *NifH*) that transfers electrons from an electron donor to the nitrogenase protein (Burris and Roberts, 1993; Steenhoudt and Vanderleyden, 2000; Monteiro *et al.*, 2012).

An important issue concerning associative and endophytic N-fixing bacteria is whether they contribute directly with fixed N to the plant. There have been discussions on whether death and subsequent mineralization of diazotrophic bacteria could indirectly release significant amounts of fixed N (Lethbridge and Davidson, 1983; Lee *et al.*, 1994). However, as described in legume nodules, mineralization is inefficient and delayed when compared with active release of immediate products of BNF by living bacteria (Mylona *et al.*, 1995).

The ability of associative and endophytic diazotrophic bacteria to fix atmospheric N within a host has been proven using different biochemical approaches such as ^{15}N isotope dilution experiments, $^{15}N_2$ reduction assays, or ^{15}N natural abundance assays. There are still problems with these techniques depending on the plant species and management, particularly for field assessments and for measuring a variation in N-fixing levels; nevertheless, important technical adjustments have been made (James, 2000; Boddey *et al.*, 2001). Nowadays, the ^{15}N isotope techniques are considered as the most appropriate to quantify the contribution of BNF associated with non-legumes, and include the contribution of BNF in a complete crop cycle. For plants of the Poaceae family, capable of interacting with endophytic and associative diazotrophic bacteria, the contribution of BNF is usually much lower than in associations with endosymbionts, with values on average of <10% of N derived from BNF (Herridge *et al.*, 2008). Nevertheless, BNF quantification experiments have conclusively shown that associative and endophytic bacteria can fix N in plant tissues with higher efficiency. An increase in N content of rice inoculated with *Herbaspirillum* sp., *Burkholderia* sp., or *Azospirillum* sp. was demonstrated, reaching up to 31% of the N derived from BNF (Baldani *et al.*, 2000; Elbeltagy *et al.*,

Table 1. *Examples of plant growth promotion benefits of the interaction of associative and endophytic bacteria with plants*

Host plant	Bacteria	Effect on growth promotion	References
Rice	*Azoarcus* sp.	Dry weight	Hurek *et al.* (1994)
	Burkholderia sp.	Shoot and shoot biomass; grain yield	Baldani *et al.* (2000); Oliveira *et al.* (2002)
	Gluconacetobacter diazotrophicus	Dry weight	Muthukumarasamy *et al.* (2007)
	Herbaspirillum seropedicae	Root and shoot biomass; yield	Elbeltagy *et al.* (2001); Baldani *et al.* (2000); Riggs *et al.* (2001); Mirza *et al*. (2000)
	Azobacter sp.	Root length	Alam *et al.* (2001)
	Enterobacter sp.	Root length; dry matter yield, grain yield	Alam *et al.* (2001)
	Rhizobium leguminosarum bv. *trifolli*	Grain yield	Yanni *et al*. (1997, 2001)
Maize	*Burkholderia* sp.	Yield	Riggs *et al.* (2001)
	Azospirillum brasilense	Yield	Riggs *et al.* (2001); Dobbelaere *et al.* (2001); Fallik *et al.* (1994)
	Herbaspirillum seropedicae	Yield	Riggs *et al.* (2001)
Sugarcane	*Gluconacetobacter diazotrophicus*	Plant biomass; yield	Suman *et al.* (2005); Sevilla *et al.* (2001); Oliveira *et al.* (2002)
	Herbaspirillum seropedicae	Dry matter; yield	Oliveira *et al.* (2002)
	Herbaspirillum rubrisubalbicans	Dry matter	Oliveira *et al.* (2002)
	Enterobacter sp.	Root biomass and shoot	Mirza *et al.* (2001)
	Klebsiella sp.	Biomass	Iniguez *et al.* (2004)
Sorghum	*Azospirillum brasilense*	Lateral root number; root weight; root length	Sarig *et al.* (1992); Dobbelaere *et al.* (2001)
Wheat	*Azospirillum brasilense*	Yield	Dobbelaere *et al.* (2001)
	Herbaspirillum seropedicae	Plant biomass	Riggs *et al.* (2001)
Pearl millet	*Azospirillum brasilense*	Yield; lateral root number, root hairs	Tien *et al.* (1979)
Soybean	*Azospirillum brasilense*	Root length	Molla *et al.* (2001)
Poa pratensis	*Enterobacter cloacae*	Root hairs	Haahtela *et al*. (1999)
	Klebsiella pneumonia	Root hairs	Haahtela *et al*. (1999)

2001). Sugarcane field trials demonstrated that 170 kg of N ha^{-1} $year^{-1}$ came from BNF. Inoculation experiments with different strains of diazotrophic bacteria (*G. diazotrophicus*, *H. seropedicae*, *H. rubrisubalbicans, A. amazonense*, *Burkholderia* sp., and *Enterobacter* sp.) reported an ~30% contribution of BNF, while a maximum increase of 39% in total biomass was obtained (Mirza *et al.*, 2001; Oliveira *et al.*, 2002). In other crops, such as sorghum and maize, inoculated with *Azospirillum* sp., BNF contributions up to 58% were also demonstrated (Boddey and Knowles, 1987; Garcia de Salamone *et al.*, 1996). The use of bacterial mutants was also helpful in demonstrating that associative and endophytic bacteria contribute with fixed N to plant. Analyses of ammonium-excreting mutants of *Azospirillum* sp. have demonstrated that wild-type bacteria are beneficial to wheat and rice plants, presumably by fixing N_2 and rapidly transferring the fixed product to plants (Christiansen-Weniger *et al.*, 1992; Kennedy *et al.*, 1997). It has also been demonstrated that *G. diazotrophicus* co-cultured with an amylolytic yeast can release up to 48% of its fixed N and make it available to the yeast, suggesting that a similar process might occur during association with plants (Cojho *et al.*, 1993). Wheat plants grown in N-deficient media and inoculated with the *nifH* mutant of *Klebsiella pneumoniae* showed severe signs of N deficiency in contrast to the wild-type *K. pneumonia*-inoculated plants (Iniguez *et al.*, 2004). The *G. diazotrophius* as well as the *Azoarcus* sp. *nif*− mutant strains were significantly less effective in increasing plant growth during experiments with sugarcane and rice plants, respectively (Hurek *et al.*, 1994; Sevilla *et al.*, 2001). In addition, transcriptional fusions with *gusA* and *gfp* have been successfully used to demonstrate *nif* expression within plant tissues (Egener *et al.*, 1999; Roncato-Maccari *et al.*, 2003). Together with BNF quantitative analysis, these results indicate that associative and endophytic bacteria do fix N in plant tissues, which can be an important trait for plant growth promotion.

In a last step, plants assimilate and metabolize ammonium provided by diazotrophs. They have two major pathways for assimilating ammonium into amino acids: by the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle; and by the enzyme glutamate dehydrogenase (GDH) (Masclaux-Daubresse *et al.*, 2010). In *Rhizobium*–legume association, it was demonstrated that GS has a central role in the plant ammonium assimilation within fixing nodules (Schubert, 1986; Udvardi and Day, 1997). Several other genes involved in N assimilation were specifically induced in fixing nodules, indicating the importance of this metabolism when BNF is actively occurring (Barnett *et al.*, 2004; Colebatch *et al.*, 2004). In sugarcane, expression of N assimilation genes in response to endophytic colonization with *G. diazotrophicus* and *H. rubrisubalbicans* has been studied (Nogueira *et al.*, 2001, 2005). Five members of the GS family were identified in sugarcane, and three of those encoded cytosolic GS (*scGS1.a*, *scGS1.b*, and *scGS1.c*). Expression analyses suggested that

scGS1.b can be important for N assimilation in sugarcane, including not only N provided by BNF, but also N supplied by the soil and by remobilization (Nogueira *et al.*, 2005).

Nitrogen uptake regulation during association

In addition to contributing with fixed N to plants, it has been reported that inoculation of associative and endophytic bacteria is correlated with improved N uptake from soil. *Azospirillum brasilense* inoculation of wheat and sorghum plants, as well as maize seeds enhanced uptake of nitrate and other nutrients (Lin *et al.*, 1983). Sugarcane inoculation with *G. diazotrophicus* also resulted in improved N uptake (Suman *et al.*, 2005). *In vivo* inoculation of rice with 10 different associative and endophytic diazotrophic bacteria, including *Paenibacillus* sp., *Bacillus* sp., *Burkholderia* sp., *Herbaspirillum* sp., and *Azorhizobium* sp., indicated that bacterial inoculation had a significant positive impact on N uptake and on the shoot and root growth (Islam *et al.*, 2009).

The mechanisms involved in increasing nutrient uptake are still not clear, and could be indirectly related to the effects of these bacteria on plant development. In wheat inoculated with *Azospirillum* sp., it was determined that rates of nitrate ion uptake have improved because of a general increase in root surface area, and not because of a specific nitrate uptake rate (Kapulnik *et al.*, 1985). A bacterial-mediated increase in the root weight, as well as the root length and root surface area, is a common response to associative and endophytic diazotrophic bacterial inoculation, leading to an increase in the volume of soil explored by the plant (Galleguillos *et al.*, 2000; Bertrand *et al.*, 2001; Holguin and Glick, 2001; Vessey, 2003) (Table 1). Fallik *et al.* (1994) reported that inoculation of maize with *A. brasilense* resulted in a proliferation of root hairs, which could have a dramatic effect on increasing the root surface area. Likewise, evaluation of pearl millet root morphology after *A. brasilense* inoculation demonstrated an increase in the lateral root numbers, and in the root hair density covering the lateral roots (Tien *et al.*, 1979). An increase in root dry weight was observed after sugarcane inoculation with *G. diazotrophicus* (Sevilla *et al.*, 2001; Oliveira *et al.*, 2002). Treatment of soybean with *A. brasilense* caused an increase in the total root length (Molla *et al.*, 2001). Other authors also reported the effects of different associative *Azospirillum* sp. on the root surface area in sorghum, wheat, and maize, those effects being mainly an increased number of lateral roots (Sarig *et al.*, 1992; Dobbelaere *et al.*, 2001). Inoculation with *Enterobacter cloacae* and *K. pneumonia* significantly increased root hair number of *Poa pratensis* (Haahtela *et al.*, 1990).

Despite a positive correlation between N uptake and root architecture having already been demonstrated (Coque *et al.*, 2008), it is still not clear if this is the only mechanism involved in promoting N uptake during associative and endophytic diazotrophic associations. Murty and Ladha (1988) demonstrated that seedlings of rice inoculated with *A. lipoferum* significantly enhanced ammonium uptake in a hydroponic system without a concomitant increase in the surface area of the roots. Other mechanisms might possibly be involved, depending on the plant and diazotrophic bacteria species that established the interaction; further analyses are still necessary to elucidate this correlation.

Phytohormone regulation during association

Production of plant growth hormones by associative and endophytic bacteria is considered an important and, eventually, the major mechanism promoting host growth (Baca and Elmerich, 2007; Spaepen *et al.*, 2007). Auxin, cytokinin, and gibberellin production has been reported in several associative and endophytic diazotrophic bacteria such as *Azospirillum* sp., *Klebsiella* sp., *G. diazotrophicus*, *Azoarcus* sp., *Herbaspirillum* sp., *Enterobacter* sp., and *Azobacter* sp. (Baca and Elmerich, 2007). Auxin and cytokinin are important regulators of plant development, regulating processes involved in determination of the root architecture (Kramer and Bennett, 2006). Gibberellin production plays an important role in the early stages of plant development by enhancing shoot and root growth and increasing root hair density (Izumo *et al.*, 1996; Richards *et al.*, 2001). Some strains of *Azospirillum* can produce ethylene, depending on the presence of methionine and different carbon sources (Strzelczyk *et al.*, 1994). Nevertheless, several associative and endophytic diazotrophic bacteria produce the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) (Baca and Elmerich, 2007), and its activity can divert ACC from the ethylene biosynthesis pathway (Blaha *et al.*, 2006; Desbrosses *et al.*, 2009). It has been proposed that they reduce the accumulation of ethylene and re-establish a healthy root system (Santi *et al.*, 2013), but the mechanisms involved are still not clear. A model proposed by Glick *et al.* (1998) suggests that ACC is exuded from seeds or plant roots, and it is metabolized by bacteria expressing ACC deaminase activity. This would stimulate plant ACC efflux, decreasing the root ACC concentration and root ethylene accumulation that would promote root growth.

Genome sequence approaches for different associative and diazotrophic bacteria revealed several genes involved in phytohormone biosynthesis, corroborating biochemical data (Krause *et al.*, 2006; Fouts *et al.*, 2008; Bertalan *et al.*, 2009; Kaneko *et al.*, 2010; Pedrosa *et al.*, 2011; Weilharter *et al.*, 2011). Genes involved in auxin biosynthesis have been reported for *Klebsiella* sp., *G. diazotrophicus*, *H. seropedicae*, *Burkholderia* sp., *Enterobacter* sp., and *Azospirillum* sp. Gibberellin biosynthesis-related genes were reported in *G. diazotrophicus*, and the *ACC* gene was described in *Azospirillum* sp. and *H. seropedicae*.

Mutants in bacterial hormone biosynthesis and production have been described, and their use is helping to better understand the role of phytohormones during association. In *Azospirillum* sp., IAA (indole-3-acetic acid) might be synthesized by at least two biosynthetic pathways; therefore, mutants that completely lack IAA production could not be generated

(Steenhoudt and Vanderleyden, 2000). Transcriptome analysis of *A. brasilense* mutant in the *ipdC* gene, that encodes an indole-3-pyruvate decarboxylase involved in IAA biosynthesis, revealed broad transcriptional changes in the mutant, suggesting that IAA production can have a role on bacterial physiology and that it can possibly act as an important signalling molecule in this association (Van Puyvelde *et al.*, 2011). Analyses of *A. brasilense* expressing an *ipdC* promoter–*gusA* fusion suggested that the end-product of the biosynthetic pathway (IAA) could be involved in a positive feedback regulation responsible for increasing *ipdC* transcription levels (Lambrecht *et al.*, 1999; Vande Broek *et al.*, 1999). Evidence that auxin levels and/or remobilization increase within plant tissues came from the analysis of bacteria-inoculated plants expressing the auxin-inducible reporter *DR5-GUS* (Ulmasov *et al.*, 1997). *DR5-GUS* expression was up-regulated in rice plants inoculated with *Burkholderia kururiensis* as compared with non-inoculated plantlets, suggesting that auxin production is modulated during association (Mattos *et al.*, 2008).

In addition to phytohormone production by bacteria, plants might modulate their endogenous biosynthesis of these growth regulators, and others, in response to association with microorganisms. Plant gene expression profiling studies are helping to understand and integrate plant phytohormone biosynthesis and responses during association. The transcript profile of *in vitro* grown sugarcane plants inoculated with *G. diazotrophicus* and *H. rubrisubalbicans* revealed differentially expressed genes related to auxin, gibberellin, and ethylene classes of growth hormones (Nogueira *et al.*, 2001; Souza *et al.*, 2001; Vargas *et al.*, 2003). Two ethylene receptors and one transcription factor have opposite patterns of expression in response to beneficial diazotrophs and pathogenic bacteria, and in two sugarcane genotypes with contrasting BNF efficiency (Cavalcante *et al.*, 2007). The involvement of ethylene signalling in other beneficial endophytic rhizobacteria associations was also described (Iniguez *et al.*, 2005; Léon-Kloosterziel *et al.*, 2005). Evidence indicates that increases in ethylene receptor levels reduce plant defence responses in plant–microorganism interactions (Ciardi *et al.*, 2000; Nukui *et al.*, 2004). The expression profile of inoculated sugarcane plants suggested that specific components of the ethylene signalling pathway might identify a beneficial association, switching off some ethylene responses to allow bacterial colonization and the establishment of an endophytic type of interaction (Cavalcante *et al.*, 2007). The transcriptional profile of rice plants inoculated with *H. seropedicae* identified expressed sequence tags (ESTs) involved in auxin and ethylene pathways that are regulated during association (Brusamarello-Santos *et al.*, 2012). Expression analyses revealed that two repressors of auxin response—*IAA18-like* and *IAA11-like*—are down-regulated in plants inoculated with *H. seropedicae*; and one transcription factor involved in ethylene response—*ERF2-like*—is repressed upon inoculation with *H. seropedicae*, corroborating the expression pattern observed in the inoculated sugarcane plants (Brusamarello-Santos *et al.*, 2012)

Taken together, the data demonstrate that plant and bacterial phytohormone biosynthesis and plant phytohormone signalling are regulated during association. This regulation may result in improved plant growth, and root growth promotion might indirectly increase N uptake. Besides improving plant nutrition, promotion of root development might bring benefits to bacteria since root tissues are also the main habitat for associative and endophytic bacteria; however, it is still unclear how determinant it is for the success of bacterial colonization.

Nitrogen regulation during association

One interesting question to be addressed is how N metabolism modulates plant interaction with beneficial associative and endophytic diazotrophic bacteria. Besides regulating plant hormonal levels, N forms such as ammonium, nitrate, and organic compounds are reported to signal and regulate various other metabolic processes, in both plants and bacteria (Dixon and Kahn, 2004; Gutiérrez *et al.*, 2007; Krouk *et al.*, 2011). Together with hormones, N is an important modulator of root architecture. Thus, N could participate in and possibly integrate different steps involved in the establishment of a beneficial and successful association, playing a key role in determining the efficiency of the interaction.

Although BNF can contribute with large amounts towards the total N needs of plants, crops colonized with associative and endophytic diazotrophic bacteria still depend on N fertilizers. One of the factors involved in the efficiency of the BNF process is the nutritional profile of the soil. When grown in soils with different levels of fertilization, sugarcane plants inoculated with a mixture of associative and endophytic diazotrophic bacteria obtain a higher level of BNF contribution in soils with a low N content (Oliveira *et al.*, 2003).

Low contributions from BNF observed in high N content soils could be a consequence, at least in part, of N control over nitrogenase activity. The nitrogenase complex of diazotrophic bacteria fixes N_2 only under microaerobic N-limiting conditions. The main mechanism for the regulation of nitrogenase activity by ammonium involves reversible inactivation of the nitrogenase reductase subunit by ADP-ribosylation (Hartman, 1989; Fu *et al.*, 1990). Two key genes are involved in this post-translational regulatory process: *draT* and *draG* (Fu *et al.*, 1990; Zhang *et al.*, 1992, 1993). Nevertheless, the endophytic diazotroph *H. seropedicae* does not harbuor *draT* and *draG* genes in its genome, possibly because it uses an alternative mechanism for the regulation of its nitrogenase in response to ammonium. Presumably, modulation involves the reallocation of electrons and ATP from nitrogenase in order to metabolize the ammonium (Fu *et al.*, 1990; Zhang *et al.*, 1992, 1993). In this case, the molecular signalling pathway involved in nitrogenase inhibition in response to ammonium includes GlnK and AmtB proteins, a signal transduction protein of the PII family and a putative ammonium channel, respectively (Chubatsu *et al.*, 2011).

In addition to nitrogenase activity control, there is evidence that the N content in soil can regulate bacterial colonization. It was observed that the number of endophytic diazotrophic bacteria isolated from sugarcane tissues decreased in plants that were fertilized with high doses of N

compared with the number of bacteria in plants that received small doses of N fertilizer (Oliveira *et al.*, 2003). In agreement with this, Fuentes-Ramirez and Mart (1999) reported that sugarcane colonization by *G. diazotrophicus* was inhibited at high concentrations of N fertilizer in the form of ammonium nitrate. In addition, the type of N source seems to determine the effect on growth inhibition of *G. diazotrophicus*. N, especially in the form of ammonium, appears to suppress growth and colonization while nitrate does not appear to inhibit it so markedly (Fuentes-Ramirez and Mart, 1999; Oliveira *et al.*, 2003). Furthermore, pleomorphic forms of diazotrophic bacteria were observed after treatment with high levels of N, especially when the source was ammonium (Muthukumarasamy *et al.*, 2002). Moreover, it seems that different bacterial species respond in different ways to N. Berger *et al.* (2013) observed that the endophytic diazotrophic bacteria *Enterobacter radicincitans* colonize tomato plants better at high N concentrations. Association with diazotrophic endosymbionts is also regulated by N content in soil. In soybean inoculated with *Rhizobium japonicum*, high nitrate levels decreased the mass of the nodules, the number of nodules per plant, and its nitrogenase activity (Carroll *et al.*, 1985).

Control of the number of diazotrophic bacteria by high N is another interesting aspect of the effect of N on the regulation of a plant's association with diazotrophic bacteria: the fact that endogenous N status can regulate plant defence mechanisms (Wang *et al.*, 2002; Divon and Fluhr, 2007; Liu *et al.*, 2010). Some genes that regulate N and amino acid metabolism or transport have a strong regulatory function in plant–pathogen interactions (Snoeijers *et al.*, 2000). It is known that an increase in N compounds and amino acids, such as phenylalanine and hydroxyproline, is required for the activation of plant defence responses (Snoeijers *et al.*, 2000). Amino acid transporters, which are also regulated by N status, can also affect plant defence (Liu *et al.*, 2010; Hwang *et al.*, 2011; Seifi *et al.*, 2013). Apart from genes involved in N primary metabolism, genes that regulate the C/N ratio, which determines plant growth, were also reported to induce plant resistance (Maekawa *et al.*, 2012). The gene expression profile of rice roots supplied with high levels of nitrate showed an up-regulation of N uptake, N assimilation, hormone metabolism, and plant resistance genes, suggesting an integrated response of these pathways to high nitrate (Wang *et al.*, 2002). Nitric oxide (NO) is another N compound involved in plant defence, in a cross-talk signalling with salicylic acid and/or jasmonic acid (Wendehenne *et al.*, 2004). In plant interactions with diazotrophic bacteria, the promotion of lateral root development by *A. brasilense* in tomato seedlings is dependent on the formation of NO (Creus *et al.*, 2005). Thus NO could also act as a signalling molecule coordinating defence responses and growth promotion during plant association with associative and endophytic diazotrophic bacteria.

Therefore, plant genes involved in N metabolism could regulate the endogenous N status, and in this way they could indirectly participate, together with other factors, in signalling plant defence responses to allow, or to impede, colonization by associative and endophytic bacteria. Expression analyses of roots of tomato plants inoculated with *E. radicincitans* showed an increase in the levels of genes involved in N transport and assimilation for plants growing in a low N concentration and a decrease for plants growing in a high N concentration (Berger *et al.*, 2013). The same study found that key hormones in pathways related to plant defence, such as jasmonate and ethylene, were up-regulated in high N concentrations (Berger *et al.*, 2013). As already discussed, gene expression profile analysis in sugarcane revealed that genes involved in N metabolism and assimilation are regulated during association with endophytic diazotrophic bacteria (Nogueira *et al.*, 2001, 2005). Expression analyses of sugarcane plantlets inoculated with *G. diazotrophicus* and *H. rubrisubalbicans* revealed that 42% of putative defence-related genes were not expressed in inoculated plants (Lambais, 2001). The differential expression of the defence-related genes might be important in establishing a compatible interaction between sugarcane and diazotrophic endophytes. Members of the salicylic acid, ethylene, and jasmonic acid pathways were also regulated in sugarcane-inoculated plants (Nogueira *et al.*, 2001; Souza *et al.*, 2001; Vargas *et al.*, 2003).

We can thus speculate that N status could regulate the efficiency of the plant interaction with beneficial associative and endophytic diazotrophic bacteria by balancing the levels of bacterial colonization through modulation of plant defences, as well as by the control of the BNF process itself. Ammonium, glutamine, nitrate, and nitrite have all been shown to repress N_2 fixation, which means that the N status of the soil, as well as the endogenous plant and bacterial N content, could be regulating the BNF rates and bacterial colonization. One question that remains to be answered is whether the endogenous N status would act as a sensor for plants, allowing a successful colonization by associative and endophytic diazotrophic bacteria only in conditions where plants need N nutrition.

How specific mechanisms involved in the regulation of the efficiency of the association interact with N metabolism and signalling to take advantage of this interaction is still unknown. A proposed model for various levels of regulation that might take place within bacteria and plants during the association is presented in Fig. 2. The beneficial diazotrophic bacteria are able to fix and transfer N to the plant, raising the endogenous N status in plant cells. In parallel, this process is autoregulated by a feedback control, being negatively regulated by high levels of ammonium, which can be provided both by the BNF and by the assimilation of N from soil. Phytohormone production is another bacterial growth promotion trait, and plant phytohormone biosynthesis and signalling are regulated during the association. This hormonal regulation increases root growth and root surface area, providing more sites for bacteria to invade, colonize, and fix N, and more area for N uptake, which results in an increase in endogenous N levels. Moreover, N as nitrate, ammonium, or organic N forms can control endogenous hormonal balance, as well as bacterial recognition, colonization, and BNF processes. A perfect balance in all these mechanisms is important for the establishment of a beneficial and successful association, with positive effects on plant growth.

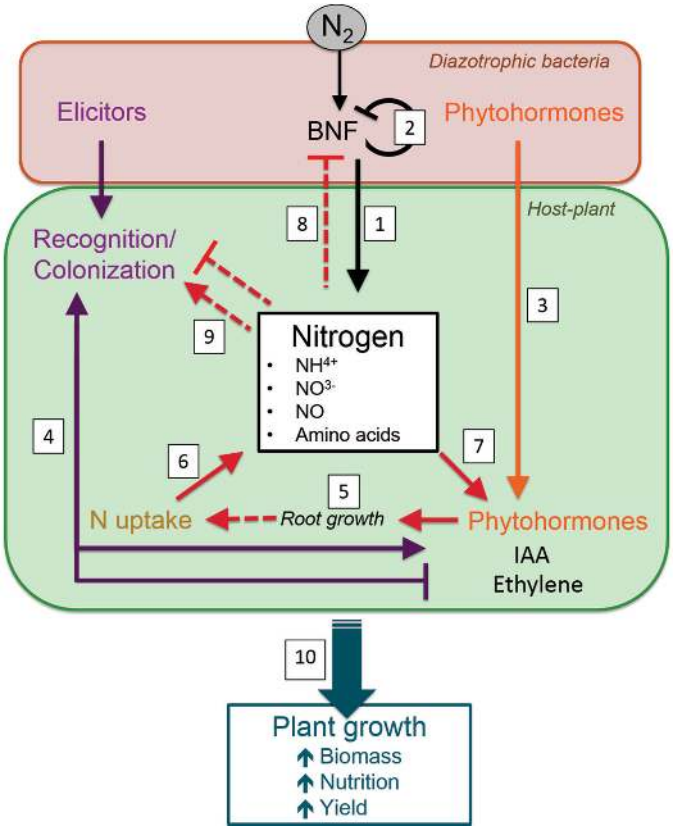

Fig. 2. Schematic representation of a proposed model for levels of regulation that might be operating within bacteria and non-legume plants during association. Levels of N status could act as a key signal regulating and integrating various metabolic processes that occur during plant association with endophytic and associative diazotrophic bacteria. In the scheme, red and green rectangles represent bacteria and plant cells, respectively. Steps of modulation already described are shown by solid lines and those that need to be proven are shown by dashed lines. Regulatory mechanisms can be activating (↓) and/or inhibiting (⊥) metabolic processes. (1) Diazotrophic bacteria associate with the plant, raising the endogenous N status in plant cells. (2) This process is autoregulated by a feedback control, being negatively regulated by high levels of ammonium. (3) Diazotrophic bacteria also produce phytohormones such as IAA and release them to the plant. (4) Plant biosynthesis and signalling of various phytohormones are modulated during the association, as they can be activated or inhibited. After bacterial recognition/colonization, the auxin pathway is induced while the ethylene pathway is inhibited. The switch off of some ethylene and defence responses could help bacterial colonization. (5) Hormonal regulation can promote root growth and an increased root surface area, which could improve N uptake. (6) Together with BNF, enhancement of N uptake contributes to increase endogenous N levels. As an important signalling molecule in plant cells, N could control different aspects of plant physiology. (7) N content could modulate the endogenous hormonal balance by regulating hormone metabolism. (8) An increase in plant N levels could regulate BNF efficiency. (9) Depending on N form and on its levels, by regulating defence responses, the effect on the recognition/colonization process could be positive or negative. (10) Regulation and integration of various metabolic processes by N status and the proper balance in all these mechanisms is important for the establishment of a beneficial and successful association, with positive effects on plant growth.

Future prospects and challenges

A big challenge in this century is to develop technologies leading to a sustainable agriculture. The use of chemical fertilizers cannot be eliminated without drastically decreasing food production. At the same time, there is an urgent need to lower the adverse environmental impacts of agricultural fertilizers. Different initiatives are in progress aiming to improve N nutrition and NUE in plants, such as the manipulation of plant N metabolism. BNF is a promising alternative to improve N nutrition, as the use of inoculants of diazotrophic bacteria in agriculture has been proven to enhance N availability and uptake, to promote plant growth, to increase biomass, and to keep the plants healthy (Kloepper *et al.*, 1999; Vessey, 2003; Adesemoye and Kloepper, 2009). The associative and endophytic diazotrophic bacteria naturally colonize and contribute with fixed N to several economically important plant species, comprising a natural system to be explored. However, the mechanisms regulating this particular type of plant–bacteria association are still not clear; thus, a better understanding of the mechanisms is necessary to allow improvement and manipulation of this association, and possibly an extension of it to non-natural hosts.

Quantitative analyses of BNF and plant growth promotion demonstrated that plant and bacterial genotypes are important factors in controlling the efficiency of the association (Carvalho *et al.*, 2011). In this context, one challenge in this area is the determination of the best combination of diazotrophic bacteria and plant varieties to obtain the maximum benefit from this association in agriculture. A huge effort should be made to understand the molecular and genetic factors controlling all steps of the association: recognition, colonization, N fixation, and plant growth promotion. Several advances came from genomic approaches, and integrative gene expression maps are being generated for some plant species colonized with associative and endophytic diazotrophic bacteria. Possible regulatory mechanisms involved were identified, and functional analyses are now necessary. Also, it is important to determine common regulatory pathways governing a successful association with diazotrophic bacteria, as well as those specific to particular plant–bacteria genotypes.

N status might act as a key signal regulating and integrating various metabolic processes that occur during association with diazotrophic bacteria. Besides directly providing ammonium to plants, the associative and endophytic diazotrophic bacteria enhance N uptake of inoculated plants, an effect that could be important for enhancing NUE. Nevertheless, high N levels inside plants seem to signal a feedback control, negatively regulating BNF and bacterial colonization. High N in soil could contribute to an increase in N levels in plants, activating this negative feedback control. Therefore, a clear understanding of the mechanisms in N regulation during plant interaction with associative and endophytic diazotrophic bacteria could provide tools to maximize the benefits for crop production.

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