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MINI-REVIEW

Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges

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Abstract The potential of nitrogen-fixing (NF) bacteria to form a symbiotic relationship with leguminous plants and fix atmospheric nitrogen has been exploited in the field to meet the nitrogen requirement of the latter. This phenomenon provides an alternative to the use of the nitrogenous fertiliser whose excessive and imbalanced use over the decades has contributed to green house emission (N_2O) and underground water leaching. Recently, it was observed that non-leguminous plants like rice, sugarcane, wheat and maize form an extended niche for various species of NF bacteria. These bacteria thrive within the plant, successfully colonizing roots, stems and leaves. During the association, the invading bacteria benefit the acquired host with a marked increase in plant growth, vigor and yield. With increasing population, the demand of non-leguminous plant products is growing. In this regard, the richness of NF flora within non-leguminous plants and extent of their interaction with the host definitely shows a ray of hope in developing an ecofriendly alternative to the nitrogenous fertilisers. In this review, we have discussed the association of NF bacteria with various non-leguminous plants emphasizing on their potential to promote host plant growth and yield. In addition, plant growth-promoting traits observed in these NF bacteria and their mode of interaction with the host plant have been described briefly.

Keywords Biological nitrogen fixation (BNF) · Nitrogen-fixing (NF) bacteria · Endophyte · *Rhizobium* · Non-legume · Growth promotion

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Introduction

Non-leguminous plants like rice, maize and wheat belonging to the Poaceae family form staple food for the approximately 6.5 billion people around the world. An exponential rise in world population indicates the need for increased crop production. According to the Food and Agriculture Organization of the United Nations, world cereal production in 2008 is forecast to increase 2.6% to a record 2,164 million tons. This rise in crop production has been a result of the indiscriminate use of chemical fertilisers (N, P, K) in combination with advanced technology. Nitrogen fertilisation of non-leguminous crops is one of the most expensive inputs in agriculture. However, approximately 65% of the applied mineral nitrogen is lost from the plant–soil system through gaseous emissions, runoff, erosion and leaching. Environmental impact of this loss ranges from greenhouse effects, diminishing stratospheric ozone and acid rain to changes in the global N cycle and nitrate pollution of surface and ground water (Rejesus and Hornbaker 1999). With growing environment-related concerns, various alternatives are being harnessed to reduce the dependence on N fertiliser for plant nutrients. It is in this context that the use of the nitrogen-fixing (NF) bacteria in agricultural practices is gaining importance. A NF bacteri-

um can exist freely or in symbiosis and in either case entraps atmospheric nitrogen and converts the unreactive N_2 molecule to NH_3, a form that is readily utilised by plants. This process is termed as biological nitrogen fixation (BNF) and is catalysed by the oxygen-sensitive enzyme nitrogenase, present within the bacteria, by the following reaction:

$$N_2+8H+8e^-+16ATP \xrightarrow{\text{Nitrogenase}} 2NH_3+H_2+16ADP+16Pi$$

A rich flora of NF bacteria has been discovered within and around non-leguminous plants. The NF bacterial population thriving within the plant without causing any apparent disease is termed 'endophytes,' whereas those isolated from the rhizosphere of the plant are referred to 'rhizospheric bacteria.' It has been suggested that endophytes are placed in a more favourable environment than rhizospheric bacteria as they are less vulnerable to competition from other soil bacteria and are shielded from various biotic and abiotic stresses (Reinhold-Hurek and Hurek 1998). Additionally, endophytes enjoy direct provision of nutritional elements within the host and a low O_2 factor that assists optimal nitrogenase activity. In return, endophytes benefit the host plant's growth and development through BNF and growth-promoting substances (Sevilla and Kennedy 2000). Apparently, this intimate association led researchers to anticipate the use of these bacteria in developing a sustainable agriculture. In our review, based on developments in the last decade, we have discussed natural tendencies of NF bacteria (endophytic/rhizospheric) to coexist with non-host gramineous crops and benefit their growth and development. We have also elaborated various growth-promoting activities of the bacteria responsible for the growth promotion of the host plant during the interaction.

Extended niche for the nitrogen-fixing bacteria

The occurrences of Rhizobia (species of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium* and *Sinorhizobium*) as a natural endophyte in leguminous plants have been widely documented. However, the domain of endophytes is not restricted to the class Leguminoceae. In the last decade, a large population of endophytes ranging from 10^6 to 10^7 cells per gram of fresh tissue were isolated from various non-leguminous plants in different parts of the world (Yanni et al. 1997; Muthukumarswamy et al. 1999; Mirza et al. 2001). Unlike the legume–*Rhizobium* interaction, endophytes are not restricted to a specific compartment within the non-legumes but occur in the mainstream plant roots, stems and leaves. For isolating endophytes, plant surfaces (root, shoot) are systematically surface-sterilised using sodium hypochloride or mercuric chloride to remove contaminant surface bacteria. Reinhold-Hurek and Hurek (1998) have reviewed various surface sterilisation techniques and their efficiencies in isolating endophytes from non-legumes. The extract from the surface-sterilised plant part is then plated on nitrogen-free media. The competence of the isolated bacteria as an endophyte is verified by re-inoculation to sterile rice seedlings (Yanni et al. 1997; Prayitno et al. 1999). Based on the ability of the isolated endophyte to re-infect the host plant and fulfill 'Koch's postulate,' they are designated as 'true endophytes' (Reinhold-Hurek and Hurek 1998). Koch's postulate states the criteria to determine whether certain bacterium is the disease-causing agent. One of these criteria is to re-isolate the disease-causing bacterium from the patient. In case of the endophyte–plant interaction, the bacterium responsible for the growth promotion of a plant after inoculation should be re-isolated from infected plants. More recent approaches of isolation involve direct analysis of bacterial gene sequences obtained from the source plant tissue harbouring the endophyte (Engelhard et al. 2000; Hurek et al. 2002). By this technique, Conn and Franco (2004) established a larger diversity of endophytes in wheat as opposed to that obtained by culture-dependent method. Culture-dependent methods confer selective advantage to a certain group of bacteria and hence do not provide a complete overview of the endophytic population. Knauth et al. (2005) developed a messenger ribonucleic acid (mRNA)-based profiling of nitrogenase (*nifH*) genes and obtained a significant varietal difference in rice root associated *nifH*-expressing communities. Similarly, Zhang et al. (2007) used a *nifH* gene-based short oligonucleotide microarray to analyse NF diazotrophs in roots of wild rice in Namibia. Their results demonstrated that a small population of the total identified diazotroph was fixing nitrogen actively within the host.

Rhizobial inoculation enhances plant growth and development

The importance of isolated endophyte in agribusiness depends on their performance in promoting host plant growth and development in field. Various experiments conducted under gnotobiotic as well as pot and field conditions illustrate the biofertlisation properties of some of the isolated endophytes towards the acquired host (Yanni et al. 1997, 2001; Matthews et al. 2001; Muthukumaraswamy et al. 2005, 2007). Endophytes, used as inoculants for plants (non-legume), proved to be an efficient source of N that can partly substitute urea N in the cultivation of rice and other cereals (Yanni et al. 1997; Baldani et al. 2000; Govindarajan et al. 2007). Plant growth promotion by rhizobial inoculation involved rise in plant biomass,

nitrogen content, grain yield and carryover effect on straw and resulted in persistence of the inoculated strain in N-deficient and N-containing soil (Yanni et al. 1997, 2001; Biswas et al. 2000a, b; Peng et al. 2002). Table 1 shows the relative ability of different NF bacteria to benefit their cereal host under gnotobiotic, green house and field conditions.

Yanni et al. (1997) isolated 11 strains of the clover-nodulating bacteria, *Rhizobium leguminosarum* bv. *trifolii* from rice roots in Egypt where rice has been grown in rotation with clover for generations. Inoculation of *R. trifolii* E11 and E12 to rice variety Giza significantly increased its total N content (95% confidence) grain yield, grain N content and harvest index of rice (99% confidence) under gnotobiotic and field conditions (Yanni et al. 1997, 2001; Biswas et al. 2000a,b). The overall yield and N accumulation of the plant in field went up by 3.6 t h^{-1} and 19–28%, respectively (Yanni et al. 1997, 2001; Biswas et al. 2000a). It was observed that rhizobial inoculation enhanced stomatal conductance, thereby increasing the photosynthesis rates by 12% in rice varieties where 16% grain yield increase was noted. This indicated a positive correlation between increased grain yield and photosynthetic rate at zero N-level (Peng et al. 2002). Apparently, they suggested that certain strains of rhizobia can promote rice growth and yield through the mechanism that improve single leaf net photosynthetic rate. Alternately, some of the *Rhizobium* isolates inhibited rice seedling growth and development (Prayitno et al. 1999; Perrine et al. 2005). This inhibition occurred in presence of nitrate/nitrite supplied as the sole source of N in the media. Perrine-Walker et al. (2005, 2007a) hypothesised that the inhibitory effect observed in these strains were due to enzymes of nitrate metabolism encoded by genes in the pSymA plasmid of the bacteria. The activity of these enzymes led to the reduction of nitrate to nitrite and subsequent accumulation of nitric oxide (NO), which is inhibitory to the plant growth. Interestingly, plant growth-promoting rhizobacteria (PGPR) *R. trifolii* R4 is capable of further reducing NO to N_2 due to the presence of additional reductases (nitrous/NO; Perrine et al. 2007a).

In most of the trials, an initial inoculum density of 10^8–10^9 cells per millilitre was enough to obtain an optimum growth response in the inoculated plant (Yanni et al. 1997, 2001; Biswas et al. 2000a,b; Chaintruel et al. 2000). However, different techniques were adopted to inoculate the plant in green house or in fields. Commonly used methods include: dipping seed or seedling roots in broth culture before sowing, application of bacterial suspension directly to the soil, inoculating seedlings and setts of sugarcane, seed coating with inoculum strains and foliar spraying with the bacterial suspension (Yanni et al. 1997, 2001; Muthukumarasamy et al. 1999; Baldani et al. 2000; Biswas et al. 2000a,b; Gutierrez-Zamora and Martinez-Romero 2001; Matthews et al. 2001; Riggs et al. 2001; Feng et al. 2006). Unfortunately, it is not known which of these techniques deliver the bacteria most efficiently to the plant for maximum output in terms of growth and production.

Previous investigations have now established that many of the endophytic inoculations to commercially important crops like rice, sugarcane and wheat can reduce N fertiliser input in cultivation (Yanni at el. 1997, Baldani et al. 2000, Matthews et al. 2001, Saleh et al. 2001; Govindarajan et al. 2006, 2007). Yanni at el. (1997) used one third of the recommended dose of N fertilisation in addition to *R. trifolii*, in a rice field to produce equivalent grain yield as obtained by the full-recommended dose of fertiliser (144 kg N ha^{-1}). Similarly, *Burkholderia* MG43 inoculation in sugarcane resulted in an effect greater than increasing the fertiliser from half to the full recommended rate, saving the cost of ~140 kg ha^{-1} N fertiliser (Govindarajan et al. 2006).

Herbaspirillum is a broad-host-range endophyte, which colonises sugarcane, rice, wheat, sorghum and other cereal. Baldani et al. (2000) tested eighty different strains of *H. seropedicae* originally isolated from rice, maize and sorghum in order to select inocula for rice. They observed that 12% of the tested strains led to a 100% increase in rice fresh weight over control. In successive experiments, only a few strains could maintain their performance. In a green-house, *Herbaspirillum* increased rice yield significantly (at 5% probability level) to 7.5 g per plant (Mirza et al. 2000). In addition, the N content of Al-tolerant rice varieties inoculated with *H. seropedicae* Z67 demonstrated a significant rise of 29–61% in roots and 37–85% in shoots (Gyaneshwar et al. 2002). Similarly, *Burkholderia* sp. is another endophyte, which has been widely studied in the field. Different forms of the bacteria (rhizospheric and endophytic) in field increased rice grain yield by 0.5–0.8 t ha^{-1} and plant biomass by 22 mg per plant (Baldani et al. 2000). This is equivalent to a 69% increase over the uninoculated control plant biomass. *Burkholderia* sp. strain PsJN required a gene similar to *nadC* to promote potato tube growth (Wang et al. 2006). The gene *nadC* encodes the enzyme quinolinate phosphoribosyltransferase (QAPRTase). The enzymatic activity of QAPRTase catalyses the de novo formation of nicotinamide dinucleotide forming nicotinic acid mononucleotide (NaMN) as the by-product. A nadC mutant was unable to synthesise the intermediary substrate NaMN and failed to promote the growth of the host plant. However, the growth-promoting activity of a PsJN mutant was restored by the in vitro supplementation of commercial NaMN (10–100 μM) to the media. In addition to growth promotion, *B. phytoformis* PsJN also offered the inoculated plant with cold tolerance compared to a non-bacterialized control (Barka et al. 2006). Under chilling conditions,

Table 1 Benefits of the association of NF bacteria and non-leguminous plants

Host plant	Endophyte/diazotroph inoculant	Colonisation	Condition of cultivation	Percent increase	Reference
BNF[a]					
Rice	*Azoarcus*	Roots of grasses	Gnotobiotic	16 (total dry weight)	Reinhold- Hurek and Hurek 1997; Engelhard et al. 2000
	Burkholderia	Roots	Greenhouse	68 (shoot biomass), 19 (seed biomass)	Baldani et al. 2000
	Photosynthetic *Bradyrhizobium*	Rhizosphere (survives as endophyte)	Gnotobiotic	20 (total plant biomass)	Chaintruel et al. 2000
	Gluconacetobacter diazotrophicus	Stem, roots	Gnotobiotic	30 (total dry weight)	Muthukumarasamy et al. 2005, 2007
	Herbaspirillum seropedicae	Roots	Gnotobiotic	38–54 (root biomass), 22–50 (shoot biomass), 37.6 (plant dry weight), 52–112, 71 (fresh and dry weight)	Elbeltagy et al. 2001, Gyaneshwar et al. 2002, James et al. 2002, Baldani et al. 2000
	Serratia marcescens	Roots, stem	Gnotobiotic	23 (total dry weight)	Gyaneshwar et al. 2001
Maize	*Burkholderia* sp.	Stems, roots, rhizosphere	Greenhouse, Field	36–48, 5.9–6.3 (yield)	Estrada et al. 2005, Riggs et al. 2001
	Azospirillum brasilense	Roots, stems	Greenhouse	13–25 (yield)	Riggs et al. 2001
Sugarcane	*Gluconacetobacter diazotrophicus*	Roots, stems	Pot trial	18.83–49.86 (plant biomass)	Suman et al. 2005, 2007
	H. seropedicae, *H. rubrisubalbicans*	–	Greenhouse	35 (dry matter)	Oliveira et al. 2002
PGPR[a]					
Rice	*Rhizobium leguminosarum* bv. *trifolii*	Roots	Greenhouse and field	15–22, 8–22 (grain yield)	Yanni et al. 1997, 2001; Biswas et al. 2000a,b
	B. vietnamiensis	Rhizosphere	Nursery pot trial, field	23 and 59 (shoot/root weight), 19 (yield), 13–22 (yield)	Trân Van et al. 2000
Wheat	*R. trifolii*	Roots	Pot trial	24 (wheat shoot dry matter and grain yield)	Hilali et al. 2001
	Cellulomonas sp.	Rhizosphere	Greenhouse, field	33 (root growth)	Egamberdiyeva and Höflich 2002
Maize	*R. trifolii*	Roots	Greenhouse, field	34 (yield), 11 (yield)	Riggs et al. 2001
	Sinorhizobium sp.	–	Greenhouse	49–82 (yield)	Riggs et al. 2001
	A. brasilense	Roots	Pot, field	50–90, 33 (grain yield)	Dobbelaere et al. 2001
	R. etli bv. *phaseoli*	Roots	Gnotobiotic	20–45 (total biomass)	Gutierrez-Zamora and Martinez-Romero, 2001
	H. seropedicae	Roots	Greenhouse, field	49–82 (yield), 19.5 (yield)	Riggs et al. 2001
	Pseudomonas sp.	Roots	Gnotobiotic	11.7 (total biomass)	Shaharoona et al. 2006
Sugarcane	*G. diazotrophicus*	Micro-propagated	Greenhouse	26 (plant dry weight)	Muñoz-Rojas and Caballero-Mellado (2003)
BNF+PGPR					
Rice	*Pantoea agglomerans*	Root and shoot tissue	Gnotobiotic	63.5 (total biomass)	Verma et al. 2001; Feng et al. 2006
	Combination of *G. diazotrophicus* LMG7603, *H. seropedicae* LMG6513, *A. lipoferum* 4B LMG4348, and *B. vietnamiensis* LMG10929	–	Pot, field	9.5, 23.6	Govindarajan et al. 2008
	B. vietnamiensis MGK3	Roots, shoots	Pot, field	5.6–12.16 (yield)	Govindarajan et al. 2008
Wheat	*H. seropedicae*	Seeds	Greenhouse	49–82 (total biomass)	Riggs et al. 2001

Table 1 (continued)

Host plant	Endophyte/diazotroph inoculant	Colonisation	Condition of cultivation	Percent increase	Reference
Sugarcane	*B. vietnamiensis*	Rhizosphere, stem, roots	Field	19.5 (yield)	Govindarajan et al. 2006
	G. diazotrophicus	Roots, stems	Field	13–16 (yield)	Govindarajan et al. 2006
	H. seropedeceae	Roots, stems	Field	5–12 (yield)	Govindarajan et al. 2006
	Enterobacter	Roots	Gnotobiotic	55 and 70 (root and shoot biomass)	Mirza et al. 2001
	Kleibsiella sp. GR9	Roots, stems	Field	13–19.5 (biomass)	Govindarajan et al. 2007

[a] Mechanism of growth promotion: BNF refers to biological nitrogen fixation and PGPR refers to plant growth-promoting rhizobacterial activities

B. phytoformis PsJN characteristically improved the photosynthetic activity and starch accumulation ($P<0.05$) in *Vitis vinifera* when compared to the uninoculated plant. The increased resistance was a result of the rise in proline and phenolic content of the plant due to bacterial colonisation, which plays an important role in the adaptation to stress (Barka et al. 2006). The elevation in phenolics is a kind of stress response in the host plant due to bacterial invasion, also observed in rice–endophyte interaction (Mishra et al. 2006).

Endophytic bacteria can be used discretely or as a mixture for inoculating plants in pots or fields. A mixture of bacterial isolates used as an inoculum gave a synergistic result in terms of plant growth and development (Govindarajan et al. 2008). Govindarajan et al. (2008) used a mixture of *H. seropedicae* LMG6513, *Azospirillum lipoferum* 4B LMG4348, *Gluconacetobacter diazotrophicus* LMG7603 and *B. vietnamiensis* LMG10929 at a concentration of 10^8 cfu ml^{-1} to inoculate 5-day-old uninfected rice seedlings. Among all of the assays, the mixed inoculant performed best, producing an increase of 14.4% in yield compared to an average of 6.2% obtained from individual strains (Govindarajan et al. 2007). However, in using a mixed inoculum, the compatibility of each strain with each other in the mixture determined the overall performance of the inoculum in enhancing plant growth. A mixture of *G. diazotrophicus* LMG7603, *A. amazonense* and *Burkholderia* sp. when applied to sugarcane gave a comparatively lower yield than individual inoculation of *B. vietnamiensis* MG43 and *G. diazotrophicus* LMG7603 (Oliveira et al. 2002; Govindarajan et al. 2006). However, a mixed inoculum of *G. diazotrophicus* LMG7603, *B. vietnamiensis* LMG10929, *H. seropedicae* LMG6513 and *A. lipoferum* 4B LMG4348 when compared to the above mixture performed with a higher growth response in rice (Govindarajan et al. 2007). Although the performance of the mixture used by Govindrajan et al. (2007) has not been assessed in sugarcane, these studies emphasise the importance of strain selection in a mixed inoculum for obtaining higher performance in the plant.

Realizing the potential of endophyte in agribusiness, countries like Brazil have already adopted the practice of use of plant growth-promoting bacteria in non-legume cultivation. With the huge number of bacteria isolated and identified to have a positive effect on the growth and development of non-legumes like rice, wheat, maize and sugarcane, the prospect of developing effective microbial biofertilisers for these plants appears to be bright. Nevertheless, the widespread adoption of this practice would require a critical analysis of the production variability that was observed at different sites and in different crop rotations.

Plant growth promotion by endophytes: proposed mechanism

Beneficial effects savoured by the host plant in an endophyte–plant interaction have been speculated to be the result of (1) BNF by the colonizing bacteria and (2) plant growth-promoting substances produced by the rhizobacteria. In some cases, a cumulative participation of both the above mechanisms was observed. Table 1 summarises the different mechanism by which endophytes has been proposed to participate in the host plant growth promotion.

Nitrogen accumulation

Nitrogen is the most significant yield-limiting element in many agricultural production systems. It is known that in legumes, BNF by symbiotic bacteria provides a substantial amount of nitrogen required by the plant. When NF bacterium co-exists as an endophyte within non-legumes, the plant's total nitrogen content rises uniformly. Nitrogen accumulation in inoculated non-legumes may be the result of: BNF (Boddey et al. 1995; Elbeltagy et al. 2001; Oliveira et al. 2002) or the increase in nitrogen uptake from the soil (Yanni et al. 1997; Prayitno et al. 1999). Systematic study by various workers in Brazil over the years led to the observation that some sugarcane varieties grown for

decades or even a century do not show any decline in the soil N reserve or yield despite the supply deficit of N (Boddey et al. 1995). In some varieties of sugarcane, grown in well-irrigated and fertilised tank (with proper supply of K and P) without N, yield increase was in the range of 170 to 230 t ha^{-1} in the first year. In sugarcane varieties CB45-3, SP70-1143 and Krakatau, the trend of yield increase continued for 3 subsequent years. In these varieties, 60–80% of the nitrogen accumulated was a result of BNF (Boddey et al. 1995).

The ability of an endophyte to fix atmospheric nitrogen within a host has been proved using different approaches: acetylene reduction assay, ^{15}N isotope dilution experiments, $^{15}N_2$ reduction assays or ^{15}N natural abundance assays. Dalton and Kramer (2006) have discussed the experimental details and shortcomings of these assays. These experiments have conclusively shown that an increase in the host-plant N content as high as 30–45 mg of N per plant (6-week-old seedlings) in rice to 170 kg of N per hectare per year in sugarcane was a result of BNF (Boddey et al. 1995; Iniguez et al. 2004). In the wild rice variety *Oryza officinalis*, acetylene reduction and $^{15}N_2$ gas incorporation were deployed to determine the in planta nitrogen fixation after inoculation with endophytic *Herbaspirillum* sp. strain B501. The percentage of $\frac{15}{\delta}N_2$ incorporation was 381 as compared to 0.4 of the uninoculated plant, which proved the role of nitrogen fixation by *Herbaspirillum* sp. strain B501 in rice (Elbeltagy et al. 2001). Another instance is the growth-promoting endophyte *Burkholderia* colonizing rice, where an estimated 31% (372 μg N per plant) of rice plant nitrogen were derived by BNF (Baldani et al. 2000). Commercially important rice Basmati and Super Basmati are also known to benefit from inoculation with *Herbaspirillum* and *Azospirillum* (Mirza et al. 2000). Under greenhouse trials, these rice varieties derived 19% and 47% of their nitrogen requirement from the atmosphere. In a separate experiment, Oliveira et al. (2002) inoculated micropropagated sugarcane with 2×10^5 cells per millilitre of five different strains of NF bacteria (*G. diazotrophicus*, *H. seropedicae*, *H. rubrisubalbicans*, *A. amazonense* and *Burkholderia* sp.) originally isolated from sugarcane. These strains were used together in various combinations. After acclimatisation for 45 days in a greenhouse, plantlets were transferred to pots containing N^{15} for assessment of nitrogen fixation by the N^{15} isotope dilution technique. The bacterial inoculation documented a maximum rise of 39% in total biomass (645 g per plant, 400 days after inoculation) over the uninoculated control. In the process, the inoculated bacteria assimilated 30% nitrogen by BNF (Oliveira et al. 2002). Similarly, a phytohormone-producing diazotroph *Enterobacter* of sugarcane inoculated to roots of micropropagated sugarcane assimilated 29% of nitrogen by atmospheric fixation (Mirza et al. 2001). In all the above cases, the bacteria that colonised and invaded the plant upon inoculation contributed the fixed nitrogen (Boddey et al. 1995; Oliveira et al. 2002). Even in grasses, nitrogen fixation by colonizing bacteria was documented, although the amount of nitrogen fixed was lower compared to rice or sugarcane (Iniguez et al. 2004). Plants inoculated with wild *Azoarcus* had higher dry weights, lower N^{15} and 1.4 mg more N than plants inoculated with the *nif* K^- mutant strain BHNKD4 (non-NF; Hurek et al. 2002). They speculated using a N balance study that the difference in N content was not due to N uptake from potting media, as the soil in which the experiments were carried out was not fertilised with N for more than 8 months.

At the molecular level, BNF in host–endophyte interaction was ascertained using *nif* mutants of the non-legume-colonizing endophyte (Iniguez et al. 2004; Hurek et al. 2002; de Campos et al 2006). Rice plants grown in nitrogen-deficient media and inoculated with non-NF *nifH* mutant of *Klebsiella pneumoniae* 324 showed severe signs of nitrogen deficiency in contrast to the wild *K. pneumoniae*-inoculated batch (Iniguez et al. 2004). The wild *K. pneumoniae*-inoculated plants assimilated 42% and 41% of the plant's nitrogen from the atmosphere. Contrary to the previous case, rice plants inoculated with a mutant strain of *A. brasilense* Sp7:: Tn5-33 with enhanced in vitro nitrogen fixation accumulated 351 mg per plant dry matter (de Campos et al. 2006). This accumulation is equivalent to the control plant treated with an additional 5 mM NH_4NO_3. Observations from these workers highlighted a correlation between nitrogen accumulations in plants by the NF bacteria and growth promotion. Subsequently, Hurek et al. (2002) isolated 85-nucleotide-long *nifH* poly(A) mRNA from roots of inoculated Kallar grass, showing that *Azoarcus* sp. BH72 was metabolically active in expressing nitrogenase gene within the plant. From the same sample, isolated from roots of inoculated grass, *nifH* genes could be amplified, whereas *nifH* mRNA levels in control plants were not sufficiently high to allow detection. The expression of BH72 nifH in test plants but not in control plants confirmed that the source of plant nitrogen in inoculated plants was from N_2 fixation by *Azoarcus*. In spite of the fact that gramineous plants do not posses in vivo specialised features that make a conducive environment for the functioning of enzymes involved in the BNF pathway, endophytic bacteria successfully express nitrogen fixation structural *nif* genes within the host. The expression of the gene encoding iron protein of nitrogenase (*nifH*) was detected in epidermal cells, the intercellular region of root cortex and vascular tissue of wheat, maize, sorghum and rice roots (Hurek et al. 2002; Egener et al. 1999; Roncato-Maccari et al. 2003). Indeed, in 7-day-old seedlings of *O. officinalis*, the *nifH* transcription of the colonizing *Herbaspirillum* sp. B501gfp followed a circadian rhythm

(You et al. 2005). During the light phase, the transcription level of the *nifH* gene reached 100 times the level during the dark phase. This is contradictory and goes against the so called 'oxygen paradox' as the light phase generates an aerobic condition (21% O_2). At this stage, it is not known how the enzyme activity is protected under such circumstances. However, You et al. (2005) suggested that this might be an adaptation of the endophyte directed to derive maximum benefit of the photosynthate generated during the light phase.

Other PGPR activities of endophytes

Some workers observed that the overall growth promotion and nitrogen assimilation in a plant inoculated with bacteria is not solely due to BNF by the endophyte. During extensive greenhouse and field experiments using non-sterilised soils, Riggs et al. (2001) observed that when maize seeds are inoculated with *H. seropedicae* under greenhouse conditions, the yield increased by 49–82% with applied fertilizer N, whereas without fertilisation, the increase was only 16%. This indicated the participation of factors other than BNF, which improved the maize plant's proficiency to use the available fertiliser N (Table 1). Similarly, Sevilla et al. (2001) also suggested the participation of other growth-promoting factors in addition to N fixation as both wild and *nifH*$^-$ mutants of *A. diazotrophicus* promoted growth of sugarcane in the presence of nitrogen. Further, in the association of the NF *R. trifolii* or Bradyrhizobia and rice, there was no evidence of in planta nitrogen fixation by the bacteria (Yanni et al. 1997, 2001; Chaintruel et al. 2000).

Most endophytes with plant growth-enhancing properties are producer of phytohormones: indolacetic acid, gibberellins and cytokinins (Biswas et al. 2000a, b; Yanni et al. 2001, Verma et al. 2001), iron-sequestering siderophores (Yanni et al. 2001; Verma et al. 2001), phosphate-solubilising enzymes (Verma et al. 2001) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Khalid et al. 2005). Growth hormones produced by the bacteria enhanced the development of lateral roots, improving the plant's nutrient uptake from the rhizosohere (Yanni et al. 2001). ACC deaminase producing *Pseudomonas* spp. brought significant increases in plant height, root weight and total biomass in the presence of nitrogen in maize plants (Shaharoona et al. 2006). ACC deaminase production by PGPR lowered ACC, the immediate precursor of ethylene, thereby lowering the level of ethylene. Lower levels of ethylene in and around roots in turn promoted growth and elongation of roots (Glick 1995). Release of auxins and ACC deaminase in vitro by the rhizobacteria was linearly correlated with the host plant growth promotion (Khalid et al. 2005). Subsequently, indole-3-acetic acid and ACC deaminase production is being deployed as tool for identification and screening of endophytes (Khalid et al. 2005; Shaharoona et al. 2006).

Non legume–*Rhizobium* interaction

Despite the widespread occurrence of endophyte in non-leguminous plants, there is only limited data on the mechanism involved in the endophyte–host interaction. Infection and colonisation of a non-legume by the NF bacteria differ significantly from *Rhizobium*–legume symbiosis.

Using various techniques like fluorescent-tagged endophytes, antibodies, fluorescent microscopy, scanning and transmission electron microscopy, the bacterial route of entry into the host plant has been tracked and scored in many cases (Prayitno et al. 1999; Chaintreuil et al. 2000; James et al. 2001; Verma et al. 2004; Perrie-Walker et al. 2007b). Within 90–120 min of inoculation of a non-legume, green fluorescent protein (GFP)-labelled *Rhizobium* strains ANU843, E4 and R4 were observed on the main root surface (Prayitno et al. 1999; Perrie-Walker et al. 2007b). Nevertheless, a 24-h time period was required for complete adherence of the bacteria to the root surface, which thereafter remained stable for a further 2 weeks (Prayitno et al. 1999; Gyaneshwar et al. 2001; Chi et al. 2005; Perrie-Walker et al. 2007b). Endophytic bacteria are a more aggressive coloniser and are capable of outcompeting others in the surroundings (Verma et al. 2004). *R. trifolii* occurred at a density of $\sim 1.7 \times 10^6$ per gram of rhizosphere soil surrounding rice roots (Yanni et al. 1997). In some regions of a protruding lateral root, curled root hairs enclosing high numbers of GFP-labelled bacteria were observed with *R. trifolii* R4-treated rice (Perrie-Walker et al. 2007b). In addition, infection thread-like structures were also evident within inoculated plants. Subsequently, the bacteria are propagated to the next generation through seeds or vegetative means (Verma et al. 2001). Successful entry into the host plant by endophytes is made through: root tips, lateral root cracks at the point of emergence of lateral roots, injured sites on root epidermis and damaged stomata (Chaintreuil et al. 2000; James et al 2002; Sevilla et al. 2001; Chi et al. 2005; Perrine-Walker et al. 2007b). In *Arabidopsis thaliana*, 100% of the inoculated plants were colonised at the point of emergence of lateral roots with *H. seropedicae* (James et al. 2002). After a successful infiltration, *Rhizobium* is disseminated throughout the host plant interior without evoking an observable defense reaction in the plant. The intercellular and cortex regions of the root formed a preferred site of initial colonisation of the endophytic bacteria (Chaintruel et al. 2000; Verma et al. 2004). Colonisation further extended to the intercellular space of the root cortex to the xylem vessels to further

intercellular spaces in leaf mesophylls (Gyaneshwar et al. 2002; Roncato-Maccari et al. 2003). For the mechanism of bacterial dissemination in aerial parts, many workers proposed the theory of 'xylem translocation' (James et al. 2002; Chi et al. 2005). It was observed that a large population of *G. diazotrophicus* occurred in the xylem vessel and parenchyma (possibly the phloem) of inoculated sugarcane plantlets and greenhouse-grown plants (Fuentes-Ramirez et al. 1999; James et al. 2001). Further, Chi et al. (2005) observed that following an endophytic colonisation by GFP-tagged *Sinorhizobium meliloti* and *Azorhizobium caulinodans* ORS57, the bacteria ascended into the stem base, leaf sheaths and leaves reaching a population of 9×10^{10} rhizobia per cubic centimetre of infected tissue. Once the bacteria penetrate the plant, cell wall-hydrolysing enzymes, like CMCase, polygalacturonase, pectinolyase produced by *R. leguminosarum* bv. *trifolii*, cellulase and pectinase produced by *Pantoea agglomerans*, *H. seropedicae* Z67 and *H. rubrisubalbicans* assisted the process of invasion and dissemination of the bacteria within the host (Yanni et al. 2001; Verma et al. 2001; James et al. 2002). The use of cell wall-degrading enzymes endogluconase and polygalacturonase in the infection of *V. vinifera* by *Burkholderia* sp. was also emphasised in the work of Compant et al. (2005).

An intriguing question in such interactions is how the plant identifies a beneficial microorganism. At present, not much is known about rhizobial factors that help the endophyte to suppress or avoid host defence responses. Nevertheless, it is likely that some kind of 'quorum-sensing' mechanism as in *Rhizobium*–legume symbiosis might also exist in this case which helps in the establishment of a successful relationship. Rhizobial inoculation to rice plants is associated with an increased accumulation of phenolics such as gallic, tannic, ferulic and cinnamic acids in the plant leaves (Mirza et al. 2001). Such increases in phenolic acid are a pathogenic stress-related phenomenon in plants (Pieterse et al. 2002). Defense reactions triggered in response to rhizobial invasion is termed as rhizobacteria-mediated 'induced systemic resistance' (ISR). ISR is controlled by a signalling pathway in which jasmonic acid and ethylene play key roles; in contrast, pathogen-induced 'systemic acquired response' is regulated by salicylic acid (reviewed by Pieterse et al. 2002). Ethylene signalling is triggered by a family of receptors in sugarcane in response to *G. diazotrophicus* and *Herbaspirillum* sp. inoculation. In contrast to pathogenic interaction, putative ethylene receptors expression was up-regulated during invasion by beneficial bacteria (Cavalcante et al. 2007). They speculated that the up-regulation of one such receptor SCER1 might reduce ethylene sensitivity and therefore plant defence against the diazotrophic endophytes. Another receptor-like kinase, SHR5, identified from sugarcane, was repressed during an endophytic association. This is an exclusive phenomenon observed in an endophytic interaction and could not be seen in a pathogenic interaction. Although not well understood, the product of this kinase has a role to play in the signal transduction process involved in the establishment of a successful endophytic interaction. An ISR developed in response to PGPR did not alter the establishment of an interaction but rather enhanced the invading plant's growth and development (Mirza et al. 2000). It was observed that the specific tolerance of sugarcane towards *A. diazotrophicus* was due to certain glycoproteins of the host which binds to cells of *A. diazotrophicus*. The glycosidic moiety is composed of fructose units linked by β-(1-2) bonds and adheres more effectively to *A. diazotrophicus* compared to *Leuconostoc mesenteroides*, an epiphytic bacterium which lives on sugarcane leaf surfaces (Legaz et al. 2000). In a recent study by Ormeño-Orrillo et al. (2008), the role of rhizobial lipopolysaccharide (LPS) in maize rhizosphere and root colonisation has been emphasised. They observed that three transposon mutants of *R. tropici* defective in LPS biosynthesis were significantly impaired in competitive root colonisation with the parental strain when co-inoculated in a 1:1 ratio. In addition, LPS provided a protective coat against many hydrophobic and lipophilic antimicrobial compounds produced by plants as the mutants were more susceptible than the wild.

Challenges and future perspectives

In the last decade, numerous studies were undertaken to optimise conditions and reap maximum benefit from various endophyte–non-legume interactions. However, most of the experiments to test the performance of endophytes were conducted under controlled conditions. A general decrease in performance was observed when the pot-grown inoculated plants are shifted to the field (Riggs et al. 2001; Gyaneshwar et al. 2002). Some of the factors that may affect the performance of an endophyte are: nitrogen content of the soil (Muthukumarasamy et al. 1999, 2002), soil type (de Oliveira et al. 2006) and host plant age and variety (Yanni et al. 1997; Muñoz-Rojas and Caballero-Mellado 2003; de Oliveira et al. 2006). In numerous investigations, the use of NF bacteria in combination with the N fertiliser subsequently reduced the amount of external supply of fertiliser being applied to the plant (Yanni et al. 1997; Saleh et al. 2001). The challenge, however, lies in optimizing the amount of applied fertiliser to obtain a good survival rate of NF bacteria in the rhizosphere. High-nitrogen fertilised soil (ammonia) reduced colonisation of sugarcane by both *G. diazotrophicus* and *H. seropedicae* (Fuentes-Ramırez et al. 1999; Bueno dos Reis Junior et al.

2000; Muthukumarasamy et al. 1999, 2002). Even the presence of any concentration of Ca^{2+} and $(PO_4)^{3-}$ above 50 mM in the media had a derogatory effect on the rate of *Azospirillum* adsorption on wheat root surface (Pinheiro et al. 2002). Consequently, Alfisol soil type (low soil fertility) supported a better performance of the endophytic inoculant in terms of BNF contribution and stem yield without N fertilisation for 3 consecutive years (de Oliviera et al. 2006). Muthukumaraswamy et al. (2002) speculated that high concentration of nitrogen sources especially ammonia (25 mM NH_4NO_3) in media led to morphological changes in the bacteria which might play a negative role in their survival. Nevertheless, the use of compost as the nitrogen source was found to counteract the derogatory effect of N fertiliser on bacterial colonisation and boost the number of colonizing bacteria (Muthukumaraswamy et al. 2007). Selection of plant genotype and age also influences the consistency of performance of the bacteria to contribute to host plant growth enhancement (Muñoz-Rojas and Caballero-Mellado 2003; de Oliveira et al. 2006). Muñoz-Rojas and Caballero-Mellado (2003) observed a drastic decrease in the *G. diazotrophicus* population with the age of the plant and the genotype. In some sugarcane varieties, apparently, the persistence of the endophyte was for a longer period and in higher numbers. In addition, environmental factors like the soil hydric stress and seasonal changes also contribute to the observed variation in diazotrophic bacteria number (Bueno dos Reis Junior et al. 2000). More field trials are therefore required to optimise these parameters including time and way of application of the inoculant and environmental factors (de Oliveira et al. 2006).

In this study, the documented ability of *Rhizobium* to interact naturally with rice or other gramineous plants flashes light on the use endophytic/rhizospheric bacteria for improving plant performance. However, to maximise benefit from these endophytes either through BNF or plant growth-promoting activities, a better understanding of endophyte ecology and mechanism of interaction at molecular level will be required. With complete genome sequencing of various endophytes and using transcriptomics/proteomics, various genes that are induced or repressed during colonisation can be identified. This insight in the mechanism will be promising in developing a more efficient plant–bacteria interaction to promote sustainable production of biomass in the field.

Conclusions

From the numerous literature available today, it is clear that the "NF bacteria–non-legume" interaction is a natural phenomenon. With the progressive understanding of the benefits conferred to the host by this association, we are one step closer to developing an ecofriendly nutrient source for cereal crops. Despite the recent advances, commercialisation of this technology demands extensive optimisation and comprehensive study of the aftereffects of the application. The prospects of this technology is far reaching keeping in consideration the rising cost and declining reserves of fossil fuels. Given that the research succeeds, the prodigious ramifications would mitigate environmental concerns arising from the use of nitrogenous fertiliser and its costs to poor farmers.

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