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# Nitrogen-Fixation by Endophytic Bacteria in Agricultural Crops: Recent Advances

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.71988>

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## Abstract

Endophytic bacteria represents a unique class of bacteria that can colonize interior tissues of plant and provide a range of benefits to the plant similar to those provided by the rhizospheric bacteria. Certain endophytic bacteria can provide nitrogen to the plants through biological nitrogen fixation, which is an important source of nitrogen input in agriculture and represents a promising substitute for chemical fertilizers, and are known as endophytic diazotrophic bacteria. Besides fixing nitrogen, endophytic bacteria can produce plant growth hormones like auxin and gibberellin, help in nutrient uptake, and increase the plant's tolerance to biotic and abiotic stresses. Various direct and indirect methods have been used to quantify the amount of nitrogen fixed by these bacteria, including the acetylene reduction assay, which is a quick but indirect method, and the  $^{15}\text{N}$  isotopic dilution assay, which is a robust and accurate method. Research on endophytic diazotrophic bacteria has come a long way, and in this chapter, we have briefly discussed the mechanisms of biological nitrogen fixation and methods to quantify the fixed nitrogen along with reviewing recent studies focused on evaluating the role of endophytic diazotrophic bacteria in promoting plant growth in both native and nonnative crop hosts.

**Keywords:** endophytic bacteria, diazotroph, biological nitrogen fixation, plant growth promotion, agricultural crops

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## 1. Introduction

Nitrogen (N) is an essential component of all proteins and enzymes, nucleic acids that make up DNA, and chlorophyll that enables the process of photosynthesis in plants [1]. It is a very common element in nature that is present in abundant amounts in atmosphere, lithosphere, and hydrosphere of the earth [2]. However, much of this N is in the form of dinitrogen ( $\text{N}_2$ ), which is inert and cannot be used by plants. In order for plants to use this dinitrogen, it has

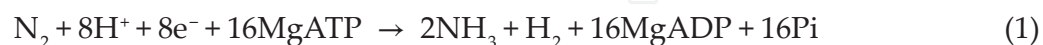
to be reduced/fixed into forms like nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ). N fixation, the process by which dinitrogen is reduced to plant-available forms, is, therefore, a vital process for the sustenance of life on earth. A major industrial process by which dinitrogen is converted into ammonia is known as the Haber-Bosch process. This artificial N-fixation process was established in 1913 and uses a catalyst (iron with a small amount of aluminum added) at high pressure (as much as  $5.06 \times 10^7$  Pa) and high temperature (600–800 K) consuming large amounts of fossil fuel. Ammonia produced through this highly expensive process is combined with other elements to produce nitrogenous fertilizers like urea and ammonium nitrate. Although the use of these fertilizers is inevitable in meeting rising food demand to sustain the growing global population, their indiscriminate use has set off very negative effects on the environment [3]. Naturally, N is commonly fixed by two processes. The first is atmospheric N fixation by lightning, in which the enormous amount of energy contained in lightning breaks dinitrogen molecules and enables their atoms to combine with oxygen in the air forming N oxides that dissolve in rain. These oxides of N then form nitrates that are carried to the earth in rainfall [4]. The second is biological N fixation (BNF), in which certain prokaryotic microorganisms, known as diazotrophs, fix N by breaking down the triple bond of dinitrogen using a highly specialized enzyme complex called nitrogenase enzyme and convert it to ammonia [4]. This chapter mainly focuses on diazotrophic bacteria that can fix N while living in the internal tissues of plants. In this chapter, only recent developments (from last 5 years) related to this subject have been discussed.

## 2. Biological nitrogen fixation (BNF)

Farmers since ancient Chinese and Roman civilizations practiced crop rotation with legumes to increase soil fertility and agricultural productivity. However, the science behind such practice was first revealed by Boussingault in 1838, who established that legumes can fix N. But it was not until 1886 when Hellriegel and Wilfarth provided a firm evidence that microbes are responsible for N fixation occurring in leguminous plants [5].

### 2.1. Chemistry and genetics of BNF

The overall chemical reaction of BNF catalyzed by the nitrogenase enzyme is represented below:



Nitrogenase is a complex enzyme comprised of two metalloproteins: the Mo-Fe protein, also called dinitrogenase protein, and the Fe protein, also called dinitrogenase reductase protein. The dinitrogenase protein is a heterotetramer composed of two  $\alpha$ - and two  $\beta$ -subunits with an overall molecular weight of 240kDa. This protein contains two types of metal centers, the FeMo-cofactor and the P-cluster pair, of which the FeMo cofactor is the active site where dinitrogen binds, whereas the P-cluster mediates electron transfer between the Fe protein

and the FeMo cofactor. The dinitrogenase reductase protein is a homodimer of two identical subunits, with an overall molecular mass of ~60 kDa. It contains two ATP/ADP molecules and one  $\text{Fe}_4\text{-S}_4$  cluster [6, 7].

The overall functioning of nitrogenase can be summarized as a key biochemical cycle that involves five steps [6, 7]: (i) the reduction of Fe protein by electron carriers such as flavodoxin or ferredoxin; (ii) association of the reduced Fe protein (including two MgATP complexes) with the Mo-Fe protein in preparation for electron transfer; (iii) hydrolysis of MgATP, which enables transfer of one electron to the Mo-Fe protein (via  $\text{Fe}_4\text{S}_4$  and the P-cluster); (iv) electron transfer to dinitrogen and thus its reduction, while it is bound to the active site within the Mo-Fe protein; and (v) dissociation of the two protein molecules, exchange of ATP back into the Fe protein, and rereduction of the Fe protein.

The structure and function of nitrogenase enzyme are encoded by ~20 genes, known as N-fixation genes (*nif* genes), organized in 7 operons (*nif* cluster) spanning over 24 kb. These genes fall into three categories, structural, regulatory, and supplementary, and can be housed either in genomic DNA or on plasmids. The Fe protein is encoded by the *nifH* gene and the Mo-Fe protein is encoded by *nifD* and *nifK* genes [8, 9]. The *nifD*, *nifH*, and *nifK* genes are recognized as structural *nif* genes since they are responsible for encoding the aforementioned structural subunits [10]. The *nif* cluster of the free-living bacterium *Klebsiella pneumoniae* is the most studied of *nif* genes and serves as a model for understanding the regulation, synthesis, and assembly of nitrogenase enzyme [11].

## 2.2. Quantification of biologically fixed N

BNF can be measured using various methods, the most common being: N balance method, xylem solute analysis, acetylene reduction assay, and stable isotope ( $^{15}\text{N}$ ) method [12]. In the N balance method, the amount of N fixed is estimated by calculating the difference between total N content of plants inoculated by diazotrophs and those that are not inoculated. In this method, it is assumed that both inoculated and noninoculated plants absorb equal amounts of N from the soil, which is hard to justify as there are differences in root morphology and physiological attributes [12]. In the xylem solute analysis, the composition of N compounds flowing through the xylem sap to the shoot of the plant is determined. The N absorbed by plants from the soil is predominantly nitrate, whereas the fixed N is primarily in the form of amides and ureides [13]. This difference in composition of N compounds is used to make quantitative measurements of N fixation [14]. However, its major disadvantage is that only a very small proportion of N-fixing plants export fixed N in the form of ureides [15]. The acetylene reduction assay is a popular technique used to indirectly measure BNF by estimating the nitrogenase enzyme activity. It is based on the ability of nitrogenase to reduce acetylene ( $\text{H}-\text{C}\equiv\text{C}-\text{H}$ ) to ethylene by breaking the triple bond between carbon atoms. Samples are incubated in a gas-tight chamber and a portion of the head space is injected with acetylene. After incubation, gas samples are collected from the chamber and analyzed for ethylene production using gas chromatography [16]. It is a simple, low cost, and sensitive assay that can measure BNF in bacterial cultures, detached nodules, plant parts, or even whole plants.

The major disadvantage is the short-term nature of the assay and the autoinhibition of acetylene conversion to ethylene [17]. The stable isotope method using  $^{15}\text{N}$  is a widely used and accepted method. This method is based on the principle that soil has a noticeably different  $^{15}\text{N}$  to  $^{14}\text{N}$  ratio as compared to the atmosphere, which has a constant ratio (0.3663%). Therefore, plants absorbing fixed N from the atmosphere will have a different  $^{15}\text{N}$  to  $^{14}\text{N}$  ratio as compared to the ones absorbing N only from the soil. When plants inoculated with diazotrophs are grown in air labeled with  $^{15}\text{N}$ , they are expected to have an enhanced ratio as compared to the noninoculated ones ( $^{15}\text{N}$  incorporation method). When available soil N is labeled with  $^{15}\text{N}$ , a reduction in the ratio is expected since the inoculated plants tend to incorporate fixed N from the air as compared to the noninoculated plants, which take up labeled N from the soil ( $^{15}\text{N}$  isotope dilution method) [17].

### 2.3. N-fixing organisms

The ability to fix N, in other words, the presence of nitrogenase enzyme, is only limited to certain bacteria and archaea [18]. Within these groups, it is quite widely distributed revealing considerable phylogenetic diversity among diazotrophs. A comprehensive list of N-fixing bacteria and archaea, under 12 broad phylogenetic groups based on 16S rDNA phylogeny was prepared by Young [19]. Diazotrophs are also widely distributed ecologically. They can be found living in soils and water freely, in the rhizosphere and phyllosphere and inside the plant tissues, in symbiotic association with legumes and actinorhizal association with woody plants, and in cyanobacterial symbiosis with phytoplankton, fungi, and terrestrial plants [19]. Free-living diazotrophs are those that do not associate with plants and are found in soils that are free from the direct influence of plant roots. These microorganisms are ubiquitous in terrestrial and aquatic environments and are physiologically very diverse [20]. Many diazotrophs can be found dwelling in the rhizosphere of a plant. Due to their ability to fix N, diazotrophs can have a competitive advantage over other microbes in the rhizosphere. They prevail in the rhizosphere particularly when soil N is limited [21]. The phyllosphere (leaf surface) is another microsite known to be colonized by diazotrophs [22]. The symbiotic association between legume and *Rhizobium* is a well-known mutualistic relationship involving *Leguminosae* plants and *Rhizobiaceae* bacteria [23]. This symbiosis has been studied widely from ecological, agronomic, and molecular biological perspectives not only to enhance the N-fixing efficacy of existing symbioses but also to determine if similar associations might be developed with nonleguminous plants [24, 25]. The actinorhizal association is functionally analogous to the legume and *Rhizobium* association but is restricted between a small group of woody plant species known as Actinorhizal plants and diazotrophs belonging to a genus, *Frankia* [26]. Many diazotrophic cyanobacteria also form symbiotic association with eukaryotes and are known to contribute a significant portion of N required for growth of both organisms through BNF in N-limited aquatic and terrestrial environments [27, 28].

The presence of diazotrophs in nonleguminous plants was first detected by Brazilian researchers in the rhizosphere and rhizoplane of sugarcane (*Saccharum officinarum*) [29, 30]. In subsequent studies, various diazotrophs like *Azospirillum lipoferum*, *Azospirillum amazonense*, *Bacillus azotofixans*, *Enterobacter cloacae*, *Erwinia herbicola*, and *Bacillus polymyxa* [31–34] were isolated from the rhizosphere of sugarcane. Initially, it was postulated that nitrogenase



activity only occurs in the rhizosphere soil but not in roots [35, 36]. However, later it was determined that rhizospheric N fixation does not occur at sufficient rates to facilitate high sugarcane yields. Cavalcante and Döbereiner [37] were the first to report the isolation of a diazotroph (*Gluconacetobacter diazotrophicus*) from internal tissues of a nonleguminous plant (stem and root tissues of sugarcane) and postulated that this bacterium might be involved in fixing high amounts of N biologically. This bacterium was able to multiply considerably and fix N at high sucrose concentrations [38] and in low pH conditions typically found in internal tissues of sugarcane [38, 39]. This led to the postulation that it can satisfy almost all of the sugarcane N requirements while living inside their tissues. Such bacteria that were able to multiply inside the tissues of a live plant and promote its growth through one or more mechanisms had already been discovered many years ago and are known as 'endophytic bacteria.'

### 3. Endophytic bacteria

The term 'endophyte' was first coined more than 150 years ago by de Bary [40] for pathogenic fungi entering the internal tissues of leaves. Since then, many authors have redefined this term, but each has its own restrictions. Taken literally, the word endophyte means 'in the plant' (endon = within; phyton = plant) [41]. Since our main focus in this chapter is on 'endophytic bacteria,' we would like to reiterate the definition notated by Chanway et al. [42]: "bacteria that can be detected at a particular moment within the tissue of apparently healthy plant hosts without inducing disease or organogenesis are known as endophytic bacteria." The occurrence of endophytic bacteria in internal tissues was first reported inside a healthy potato plant [43]. Since then, many scientific studies have been focused on isolating the endophytic bacteria from a variety of plant species and evaluating their benefits for agricultural plants [44–47]. In contrast to free-living, rhizosphere or phyllosphere microorganisms, endophytic bacteria are better protected from abiotic stresses such as extreme variations in temperature, pH, nutrient, and water availability as well as biotic stresses such as competition [48–50]. In addition, endophytic bacteria colonize niches that are more conducive to forming mutualistic relationships with plants [51], for example, providing fixed N to the plant and getting photosynthate in return [52–54]. Following the rhizospheric colonization, endophytic bacteria can colonize various plant organs such as roots, stem, leaves, flowers, fruits, and seeds [55–61], indicating different capacities of endophytic bacteria to colonize various plant compartments. They can even colonize legume nodules [62] and tubercles of mycorrhizal fungi [63]. The endophytic bacterial population is extremely variable in different plant organs and tissues and have been shown to vary from as low as hundreds to as high as  $10^9$  cfu per gram plant tissue [64–67].

Localization of endophytic bacteria within plant tissues requires techniques that facilitate observation on a tiny spatial scale. Various methods have been used to locate bacteria *in planta* and visualize them at their sites of colonization, but each one has its own limitations. Most methods require either chemical or physical treatment of plant tissues for *in situ* detection and visualization of endophytic bacteria [68]. However, the use of autofluorescent proteins in conjunction with confocal laser scanning microscopy (CLSM) eliminates the need for any

chemical treatment of plant tissues and requires minimal physical preparation of plant tissue samples before microscopic visualization. The green fluorescent protein (GFP) gene found in the jellyfish *Aequorea aequorea* is the most popular autofluorescent protein used for localization of endophytic bacteria. GFP is a useful biomarker because it does not require any substrate or cofactor in order to fluoresce. GFP cassettes can be integrated into the bacterial

Endophytic diazotrophic bacteria	Isolated from	Colonized into	Method used to confirm N-fixing ability	References
<i>Pseudomonas aeruginosa</i> PM389	Pearl millet ( <i>Pennisetum glaucum</i> )	Wheat ( <i>Triticum aestivum</i> L.)	Amplification of <i>nifH</i> genes; acetylene reduction assay	[104]
<i>Azospirillum amazonense</i> AR3122; <i>Burkholderia vietnamiensis</i> AR 1122;	Rice ( <i>Oryza sativa</i> L.)	Rice ( <i>Oryza sativa</i> L.)	Acetylene reduction assay	[97]
<i>Paenibacillus kribbensis</i> HS-R01, HS-R14; <i>Bacillus aryabhattai</i> HS-S05; <i>Bacillus megaterium</i> KW7-R08; <i>Klebsiella pneumoniae</i> KW7-S06, KW7-S22, KW7-S27, KW7-S33; <i>Bacillus subtilis</i> CB-R05; <i>Microbacterium binotii</i> CB-S18; <i>Microbacterium trichotecenolyticum</i> SW521-L21, SW521-L37;	Rice ( <i>Oryza sativa</i> var. Japonica)	Rice ( <i>Oryza sativa</i> var. Japonica)	Amplification of <i>nifH</i> genes	[106]
<i>Bacillus subtilis</i> EB-04; <i>Bacillus pumilus</i> EB-64, EB-169; <i>Paenibacillus</i> sp. EB-144	Banana tree cultivar 'Prata Anã' ( <i>Musa acuminata</i> × <i>balbisiana</i> )	—	Amplification of <i>nifH</i> genes; acetylene reduction assay	[103]
<i>Bacillus</i> sp. CNPSo 2476, CNPSo 2477, CNPSo 2478; <i>Enterobacter</i> sp. CNPSo 2480	Corn ( <i>Zea mays</i> L.)	Corn ( <i>Zea mays</i> L.)	Amplification of <i>nifH</i> genes; acetylene reduction assay	[102]
<i>Gluconacetobacter diazotrophicus</i> Pal5T-BR11281; <i>Amazon</i> <i>Azospirillum</i> Cbamc-BR11145; <i>Herbaspirillum seropedicae</i> HRC54-BR11335; <i>Herbaspirillum rubrisubalbicans</i> HCC103-BR11504; <i>Burkholderia tropica</i> PPe8T-BR11366	Sugarcane ( <i>Saccharum officinarum</i> )	Sugarcane ( <i>Saccharum officinarum</i> )	Kjeldahl method; natural abundance of <sup>15</sup> N in leaf samples; isotopic <sup>15</sup> N dilution	[98]
<i>Burkholderia</i> spp.; <i>Klebsiella</i> spp.; <i>Novosphingobium</i> spp.; <i>Sphingomonas</i> spp.	Rice ( <i>Oryza sativa</i> )	Rice ( <i>Oryza sativa</i> )	Acetylene reduction assay	[105]
<i>Paenibacillus polymyxa</i> P2b-2R	Lodgepole pine ( <i>Pinus contorta</i> var. <i>latifolia</i> )	Corn ( <i>Zea mays</i> ), canola ( <i>Brassica napus</i> L.), tomato ( <i>Solanum lycopersicum</i> )	Amplification of <i>nifH</i> genes; acetylene reduction assay; isotopic <sup>15</sup> N dilution	[109, 113, 117, 119]

**Table 1.** List of endophytic diazotrophic bacteria recently isolated and associated with agricultural crops.

chromosome and expressed through an inducible or constitutive promoter of indigenous or exogenous origin [69–72]. Alternatively, a plasmid-borne GFP gene can be introduced into bacterial cells of interest [73–75]. Bacterial cells expressing GFP can be visualized by epifluorescence microscopy or CLSM [76, 77]. This technique has been used with various agricultural crops including wheat (*Triticum* spp.) [78], rice (*Oryza sativa*) [78–80], corn (*Zea mays*) [78, 81], tomato (*Solanum lycopersicum*) [82], ryegrass (*Lolium multiflorum*) [83], creeping bentgrass (*Agrostis stolonifera*) [84], and grapevine (*Vitis vinifera*) [72].

### 3.1. Endophytic diazotrophic bacteria

A few years after the discovery of diazotrophs by Cavalcante and Döbereiner [37] in the stem and root tissues of sugarcane plant, Döbereiner [85] coined the term “endophytic diazotrophic bacteria” to designate all diazotrophs able to colonize primarily the root interior of graminaceous plants, survive very poorly in soil and fix N in association with these plants [86]. Since the discovery of endophytic diazotrophic bacteria in sugarcane, other agronomically important crop species like rice [87–89], corn [90–93], wheat [94], canola (*Brassica napus* L.) [95], and Kallar grass (*Leptochloa fusca* L.) [96] have been postulated to receive significant amounts of fixed N in this way. In the following section, recent studies (from last 5 years) about endophytic diazotrophic bacteria and their role in promoting the growth of agricultural crops primarily by providing N nutrition as a result of BNF and secondarily through other plant growth–promotion (PGP) mechanisms have been discussed in detail (listed in **Table 1** as well).

## 4. Recent studies highlighting the role of endophytic diazotrophic bacteria in agricultural crops

Rice is a major staple crop in many countries around the world. It is a highly N-demanding crop; thus, it becomes extremely important to find alternatives to reduce the use of chemical N fertilizers applied to rice without decreasing the productivity. Endophytic diazotrophic strains were isolated from root, culm, and leaf tissues of traditional rice varieties (Zebu Branco and Manteiga) cultivated traditionally by the local farmers in the Maranhão state, Brazil [97]. Ten strains showing consistent acetylene reduction activity and capable of producing indole-3-acetic acid (IAA) were identified as belonging to the genera *Azospirillum*, *Sphingomonas*, and *Burkholderia*. These endophytic diazotrophic strains were inoculated into 10 different traditional varieties of rice to select the best strain/rice variety interaction by growing them in gnotobiotic, greenhouse, and field conditions. Although a strain belonging to the genus *Azospirillum* showed highest biomass enhancement (48%) under gnotobiotic conditions, *Burkholderia vietnamiensis* strain AR1122 inoculated into a traditional variety Arroz 70 showed best results as compared to other strain/variety combinations when grown under greenhouse and field conditions. The grain yield of Arroz 70 variety was also significantly enhanced when inoculated with the strain AR1122 in comparison to a control treatment that was provided with sufficient amounts of N fertilizer. These results clearly indicate that *Burkholderia vietnamiensis* strain AR1122 is a candidate biofertilizer for traditional rice varieties in Brazil and



should be investigated with other genotypes of rice for a sustainable rice crop production. In Brazil, sugarcane has been one of the fastest growing crops, reaching new frontiers and decisively influencing the economic, social, and cultural development. However, similar to rice, it is also one of the most N-demanding crops that makes it crucial to invest in research on alternatives other than chemical N fertilizers like biofertilizers with diazotrophs, so as to ensure a competitive and sustainable development of sugarcane industry. A study conducted in 2014 reported the effects of inoculating the sugarcane plants with a consortium of five different endophytic diazotrophic bacteria of *Gluconacetobacter diazotrophicus*, *Herbaspirillum*, and *Burkholderia* [98]. In this study, the consortium was evaluated with regard to the agronomic performance and N nutrition of sugarcane in field against chemical N fertilizer and it was found that the consortium of inoculant increased the stalk yield of sugarcane similar to the chemical fertilization. However, authors did not find any evidence of BNF in sugarcane by the consortium of diazotrophic strains, which indicates that the diazotrophic strains used in this study may possess other PGP characteristics that could have resulted in increased yields of sugarcane. In another study, *Gluconacetobacter diazotrophicus* strain PAL 5, which has been studied extensively for its N-fixing and PGP abilities [99], and a strain belonging to the genus *Herbaspirillum* were inoculated into sugarcane plants to evaluate their drought stress recovery [100]. After being subjected to 21 days of drought stress, bacteria-inoculated plants had significantly higher shoot and root dry weight (50 and 70%, respectively) and total N content in leaves (77%). Authors also reported that these diazotrophic strains induce preservation of leaf water potential and relative water content by closing stomata efficiently resulting in plant water preservation during the drought, which highlights the ability of these endophytic diazotrophic bacteria to protect the plant from abiotic stresses. Another type of abiotic stress, that is, salinity, has been recently reported to stimulate the population and diversity of endophytic diazotrophic bacteria in forage cactus (*Opuntia stricta*) [101]. In this study, the population density of endophytic diazotrophic strains in root tissues was evaluated by using the most probable number method (MPN) and strains were characterized phenotypically to evaluate the diversity. Authors reported that the forage cactus plants that received the highest amount of saline water had the highest population density of putative endophytic diazotrophic bacteria with high phenotypic diversity. These findings indicate that endophytic diazotrophic bacteria thrive when conditions are adverse by assisting the host plant through direct or indirect mechanisms to flourish in poor conditions.

Corn is an agriculturally important crop that is extensively grown and consumed by a large population around the world. Szilagyi-Zecchin et al. [102] isolated and identified six endophytic strains from roots of corn growing in the southern Brazilian region of Campo Largo, PR. Out of these six endophytic isolates, four were able to grow on N-free media, consistently reducing acetylene, and were found positive for the presence of *nifH* gene. Apart from showing positive results for N-fixing activity, two out of these four strains (identified as *Bacillus* sp.) also showed other PGP characteristics, like production of IAA, siderophores, and lytic enzymes and antagonism against the common pathogenic fungi. When all endophytic isolates were reinoculated into corn to check for *in vivo* plant growth promotion, another endophytic diazotrophic strain belonging to the genus *Enterobacter* significantly enhanced seed

germination by 47% and root volume by 44% [102]. In yet another study conducted in Brazil, 40 endophytic strains were isolated from roots of banana (*Musa* L.) tree cultivar 'Prata Anã' [103]. Banana is a very common edible fruit (botanically a berry), produced primarily in the tropics but consumed all around the world. Banana trees grow rapidly and require substantial amount of nutrients in the soil for their development and fruit production. Out of the 40 strains isolated in that study, 20 strains were able to grow on N-free media, but only four isolates showed positive results for N-fixing activity when analyzed using acetylene reduction assay and Kjeldahl method. All four isolates were identified as belonging to the genus *Bacillus* and were also tested positive for *in vitro* phosphate solubilization and IAA production, thus, indicating their potential to be used as growth-promoting microbial inoculants for banana trees pending *in vivo* greenhouse or field experiments.

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a staple cereal crop of the hottest and driest areas of tropics and subtropics. Pearl millet is commonly grown in Rajasthan, India, which has an arid climate and uncertain and erratic rainfall season. In a study reported in 2013, endophytic diazotrophic strains were isolated from pearl millet plants growing in a field with a nutrient-deficient sandy clay loam soil located in Rajasthan [104]. *Pseudomonas aeruginosa* strain PM389 was the most dominant diazotrophic strain in pearl millet plants harvested from this field, whose upward migration and establishment in the stem tissues were later tracked by using enterobacterial repetitive intergenic consensus sequences-PCR (ERIC-PCR) as a bio-marker. Efficient reduction of acetylene during the acetylene reduction assay and presence of *nifH* gene indicated the N-fixing potential of the strain PM389. As reported in the study, this strain possesses other PGP characteristics as well, like mineral phosphate solubilization, siderophore production, and antagonistic activity against many pathogenic bacterial and fungal species. In addition, when inoculated into a nonnative plant species (wheat), strain PM389 significantly increased seed germination rate, root and shoot length, and vigor index, which highlights its ability to infect other crop hosts and promote their growth [104]. Local cultivars that have been grown traditionally for many years could serve as a source for potential endophytic diazotrophic bacteria that could be applied to modern commercial varieties as biofertilizers. This theory was proved by scientists from Thailand, who isolated 396 potential endophytic diazotrophic strains from 6 different landraces of rice growing in Chiang Mai, Thailand [105]. Based on the results of acetylene reduction assay, authors chose 21 isolates that were further screened to 10 on the basis of tests conducted for other PGP characteristics. These strains belonged to genera *Burkholderia*, *Klebsiella*, *Novosphingobium*, and *Sphingomonas* and were able to recolonize the tissues of a commercial rice cultivar Khao Dawk Mali 105 along with increasing the N content in the seedlings and promoting seedling length and dry weight. Korean rice cultivars have also been evaluated for the presence of endophytic diazotrophic bacteria [106]. Twelve potential endophytic diazotrophic strains were isolated and identified as belonging to the genera *Paenibacillus* [107], *Bacillus*, *Microbacterium*, and *Klebsiella* and were tested positive for the presence of *nifH* gene. When reinoculated into rice plants, these strains improved plant growth, increased height and dry weight, and showed antagonistic effects against fungal pathogens, thus, establishing their potential role as biofertilizer and biocontrol agents for Korean rice cultivars.

Our lab group has been working with endophytic diazotrophic bacteria from many years and has published several reports regarding the role of these bacteria in fixing N and promoting plant growth in both agricultural and forest ecosystems [108]. In 2012, our lab discovered an endophytic diazotrophic bacterium, *P. polymyxa* P2b-2R, from stem tissues of lodgepole pine (*Pinus contorta* var. *latifolia*) trees naturally regenerating at a site located near Williams Lake, BC, Canada [109]. Strain P2b-2R was able to grow on N-free media and consistently reduced significant amounts of acetylene in the acetylene reduction assay [109]. This bacterial strain was able to fix significant amounts of atmospheric N (up to 79%) when reinoculated into lodgepole pine and evaluated using foliar  $^{15}\text{N}$  isotope dilution method [110–112]. It was also observed that strain P2b-2R possesses *nif* genes required to encode the nitrogenase enzyme, thus confirming the N-fixing ability of this strain [113].

Host plant	Harvest (days)	%Ndffa <sup>a</sup>		% growth promotion						References
				Foliar nitrogen concentration <sup>b</sup>		Seedling length <sup>c</sup>		Seedling biomass <sup>d</sup>		
		P2b-2R	P2b-2Rgfp	P2b-2R	P2b-2Rgfp	P2b-2R	P2b-2Rgfp	P2b-2R	P2b-2Rgfp	
Corn	10	6.65	—	5.42	—	10.0	—	20.9	—	[117]
	20	10.8	10.9	13.6	25.0	13.8	41.3	26.1	34.0	[117, 121]
	30	19.6	14.1	14.2	22.6	35.3	36.3	30.9	55.5	[117, 121]
	40	15.7	18.0	17.1	27.6	24.7	27.6	28.4	48.9	[121]
	90	30.2	32.2	27.3	31.8	51.9	68.4	52.7	66.9	[122]
Canola	20	8.08	13.0	28.7	37.8	17.8	37.4	57.0	91.6	[119]
	30	12.9	15.1	18.0	36.1	20.5	48.7	53.7	93.5	[119]
	40	16.2	22.1	23.4	40.8	28.4	69.4	37.1	108	[119]
	60	21.8	—	40.3	—	24.9	—	30.1	—	[118]
	90	27.1	35.1	11.7	25.0	70.7	102.5	100.8	159.1	[120]
Tomato	20	10.0	8.32	33.3	25.5	40.6	48.4	56.1	44.1	[119]
	30	12.3	11.2	30.6	23.2	36.5	37.5	69.0	61.4	[119]
	40	18.1	16.7	30.0	22.5	24.9	28.3	93.0	82.9	[119]

<sup>a</sup>Percent nitrogen derived from the atmosphere (%Ndfa).

<sup>b</sup>Percent increase in foliar nitrogen concentration by inoculation with *P. polymyxa* strains P2b-2R and P2b-2Rgfp.

<sup>c</sup>Percent seedling length promoted by inoculation with *P. polymyxa* strains P2b-2R and P2b-2Rgfp.

<sup>d</sup>Percent seedling biomass promoted by inoculation with *P. polymyxa* strains P2b-2R and P2b-2Rgfp.

These parameters were calculated using the formulas described in Puri et al. [122].

**Table 2.** Plant growth promotion and biological nitrogen fixation by *Paenibacillus polymyxa* strain P2b-2R and its GFP-tagged derivative, P2b-2Rgfp, when inoculated into agricultural crops, namely, corn, canola, and tomato.

Endophytic colonization of lodgepole pine by P2b-2R strain was confirmed by constructing a GFP-tagged derivative of P2b-2R and visualizing the sites of colonization using CLSM [75]. It was found that this strain can colonize both intercellular and intracellular spaces of lodgepole pine interior tissues possibly by degrading major cell wall components [75, 114]. Strain P2b-2R was able to colonize internal tissues of another gymnosperm tree species, western red cedar (*Thuja plicata*), and fix considerable amounts of N from the atmosphere along with enhancing seedling length and biomass of cedar [115, 116]. Subsequently, Puri et al. [117] hypothesized that strain P2b-2R could provide similar benefits to angiosperms, specifically agricultural crop species, by colonizing them endophytically. They tested this hypothesis by inoculating strain P2b-2R into agriculturally important crops, namely corn, canola, and tomato, and found that P2b-2R was able to colonize internal tissues of these crop species, fix substantial amounts of atmospheric N, and increase seedling length and biomass (see **Table 2**) [117–119]. These reports indicate the ability of strain P2b-2R to symbiotically associate with a broad range of hosts and promote their growth primarily by fixing atmospheric N. An interesting observation with the GFP-tagged P2b-2R strain (P2b-2R*gfp*) was reported recently where P2b-2R*gfp* inoculation significantly enhanced corn and canola seedling length and biomass as compared to the wild-type P2b-2R inoculation [119–122]. In addition, strain P2b-2R*gfp* fixed significantly higher amounts of N as compared to the wild-type strain. Subsequently, similar results were reported when both strains were inoculated into their original host, that is, lodgepole pine [123]. To the best of our knowledge, these were the very first *in planta* studies in literature reporting that GFP tagging of a bacterial strain could significantly enhance its ability to promote plant growth. Enhancement of these abilities *in vitro* after GFP-tagging were reported previously in *Azospirillum brasilense* [124]. A plausible reason for increased N fixing and plant growth-promoting efficacy of P2b-2R after GFP tagging could be the overexpression of structural *nif* genes (*nifH*, *nifD*, and *nifK*), which play an important role in the N-fixation process [121]. However, it is still unclear how GFP tagging affects the expression of structural *nif* genes of strain P2b-2R. Also, other plausible reasons behind the increased plant growth-promoting efficacy after GFP tagging need to be investigated.

## 5. Conclusions

Since their discovery in sugarcane tissues decades ago, endophytic diazotrophic bacteria have been characterized for their role in performing BNF. Studies have suggested that these bacteria can act as N biofertilizer for highly N-demanding crops like sugarcane, corn, and rice. Most recent studies have also focused their attention on testing the PGP characteristics of isolated endophytic diazotrophic strains other than N fixation, which indicates the growing concern of agricultural scientists to develop bacterial inoculants that can enhance plant growth through a variety of mechanisms, so as to decrease the dependence on chemical fertilizers. Endophytic diazotrophic strains like *P. polymyxa* P2b-2R that are able to colonize nonnative host and fix atmospheric N and promote their growth have great potential as biofertilizers for sustainable crop production.



## Acknowledgements

Authors would like to dedicate this work to Late Mr. Darshan K. Puri (1956–2014). You were, are and always will be an inspirational figure for us.

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