Chapter 5

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

Akshit Puri, Kiran Preet Padda and Chris P. Chanway

Additional information is available at the end of the chapter

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

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}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

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 (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 (NO_3^-) and ammonium (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 5.06×10^7 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:

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi \quad (1)$$

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 α- and two β-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 Fe_4-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 Fe_4S_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}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 (H—C≡C—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}N is a widely used and accepted method. This method is based on the principle that soil has a noticeably different ^{15}N to ^{14}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}N to ^{14}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}N, they are expected to have an enhanced ratio as compared to the noninoculated ones (^{15}N incorporation method). When available soil N is labeled with ^{15}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}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
Pseudomonas aeruginosa PM389	Pearl millet (*Pennisetum glaucum*)	Wheat (*Triticum aestivum* L.)	Amplification of *nifH* genes; acetylene reduction assay	[104]
Azospirillum amazonense AR3122; *Burkholderia vietnamiensis* AR 1122;	Rice (*Oryza sativa* L.)	Rice (*Oryza sativa* L.)	Acetylene reduction assay	[97]
Paenibacillus kribbensis HS-R01, HS-R14; *Bacillus aryabhattai* HS-S05; *Bacillus megaterium* KW7-R08; *Klebsiella pneumoniae* KW7-S06, KW7-S22, KW7-S27, KW7-S33; *Bacillus subtilis* CB-R05; *Microbacterium binotii* CB-S18; *Microbacterium trichotecenolyticum* SW521-L21, SW521-L37;	Rice (*Oryza sativa* var. *Japonica*)	Rice (*Oryza sativa* var. *Japonica*)	Amplification of *nifH* genes	[106]
Bacillus subtilis EB-04; *Bacillus pumilus* EB-64, EB-169; *Paenibacillus* sp. EB-144	Banana tree cultivar 'Prata Anã' (*Musa acuminata* × *balbisiana*)	—	Amplification of *nifH* genes; acetylene reduction assay	[103]
Bacillus sp. CNPSo 2476, CNPSo 2477, CNPSo 2478; *Enterobacter* sp. CNPSo 2480	Corn (*Zea mays* L.)	Corn (*Zea mays* L.)	Amplification of *nifH* genes; acetylene reduction assay	[102]
Gluconacetobacter diazotrophicus Pal5T-BR11281; *Amazon Azospirillum* Cbamc-BR11145; *Herbaspirillum seropedicae* HRC54-BR11335; *Herbaspirillum rubrisubalbicans* HCC103-BR11504; *Burkholderia tropica* PPe8T-BR11366	Sugarcane (*Saccharum officinarum*)	Sugarcane (*Saccharum officinarum*)	Kjeldahl method; natural abundance of ^{15}N in leaf samples; isotopic ^{15}N dilution	[98]
Burkholderia spp.; *Klebsiella* spp.; *Novosphingobium* spp.; *Sphingomonas* spp.	Rice (*Oryza sativa*)	Rice (*Oryza sativa*)	Acetylene reduction assay	[105]
Paenibacillus polymyxa P2b-2R	Lodgepole pine (*Pinus contorta var. latifolia*)	Corn (*Zea mays*), canola (*Brassica napus* L.), tomato (*Solanum lycopersicum*)	Amplification of *nifH* genes; acetylene reduction assay; isotopic ^{15}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 biomarker. 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}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)	%Ndfa[a]		% growth promotion						References
				Foliar nitrogen concentration[b]		Seedling length[c]		Seedling biomass[d]		
		P2b-2R	P2b-2R*gfp*	P2b-2R	P2b-2R*gfp*	P2b-2R	P2b-2R*gfp*	P2b-2R	P2b-2R*gfp*	
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]

[a]Percent nitrogen derived from the atmosphere (%Ndfa).

[b]Percent increase in foliar nitrogen concentration by inoculation with *P. polymyxa* strains P2b-2R and P2b-2R*gfp*.

[c]Percent seedling length promoted by inoculation with *P. polymyxa* strains P2b-2R and P2b-2R*gfp*.

[d]Percent seedling biomass promoted by inoculation with *P. polymyxa* strains P2b-2R and P2b-2R*gfp*.

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-2R***gfp***, 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.

Author details

Akshit Puri[1], Kiran Preet Padda[1] and Chris P. Chanway[1,2]*

*Address all correspondence to: chris.chanway@ubc.ca

1 Department of Soil Science, Faculty of Land and Food Systems, The University of British Columbia, Vancouver, Canada

2 Department of Forest and Conservation Sciences, Faculty of Forestry, The University of British Columbia, Vancouver, Canada

References

[1] Leghari SJ, Wahocho NA, Laghari GM, Laghari AH, Bhabhan GM, Talpur KA, Bhutto TA, Wahocho SA, Lashari AA. Role of nitrogen for plant growth and development: A review. Advances in Environmental Biology. 2016;**10**(9):209-219

[2] Greenwood NN, Earnshaw A. Chemistry of the Elements. 2nd ed. Oxford: Butterworth-Heinemann; 1997

[3] Galloway JN, Cowling EB. Reactive nitrogen and the world: 200 years of change. AMBIO: A Journal of the Human Environment. 2002;**31**(2):64-71. DOI: 10.1579/0044-7447-31.2.64

[4] Myrold DD, Bottomley PJ, Biological N. Inputs. In: Paul EA, editor. Soil Microbiology, Ecology and Biochemistry. Burlington: Elsevier Academic Press; 2007. pp. 365-388

[5] McCosh FWJ. The plant and nitrogen. Boussingault. Dordrecht: Springer Netherlands. 1984:123-138. DOI: 10.1007/978-94-009-6297-2_10

[6] Dance I. Elucidating the coordination chemistry and mechanism of biological nitrogen fixation. Chemistry - An Asian Journal. 2007;**2**(8):936-946. DOI: 10.1002/asia.200700131

[7] Kim J, Rees DC. Nitrogenase and biological nitrogen fixation. Biochemistry. 1994;**33**(2): 389-397. DOI: 10.1021/bi00168a001

[8] Rubio LM, Ludden PW. Biosynthesis of the iron—Molybdenum cofactor of nitrogenase. Annual Review of Microbiology. 2008;**62**:93-111. DOI: 10.1146/annurev.micro.62.081307.162737

[9] Seefeldt LC, Hoffman BM, Dean DR. Mechanism of Mo-dependent nitrogenase. Annual Review of Biochemistry. 2009;**78**:701-722. DOI: 10.1146/annurev.biochem.78.070907.103812

[10] Brill WJ. Biochemical genetics of nitrogen fixation. In: Ciferri O, Dure L, editors. Structure and Function of Plant Genomes. New York: Springer; 1983. pp. 231-237. DOI: 10.1007/978-1-4684-4538-1_23

[11] Dixon R, Eady RR, Espin G, Hill S, Laccarino M, Kahn D, Merrick M. Analysis of regulation of *Klebsiella pneumoniae* nitrogen fixation (*nif*) gene cluster with gene fusions. Nature. 1980;**286**(5769):128-132. DOI: 10.1038/286128a0

[12] Danso SKA. Assessment of biological nitrogen fixation. Nutrient Cycling in Agroecosystems. 1995;**42**(1-3):33-41. DOI: 10.1007/BF00750498

[13] McClure PR, Israel DW. Transport of nitrogen in the xylem of soybean plants. Plant Physiology. 1979;**64**(3):411-416. DOI: 10.1104/pp.64.3.411

[14] Herridge DF. Effects of nitrate and plant development on the abundance of nitrogenous solutes in root-bleeding and vacuum-extracted exudates of soybean. Crop Science. 1984;**24**(1):173-179. DOI: 10.2135/cropsci1984.0011183X002400010041x

[15] Kessel CV, Roskoski JP, Keane K. Ureide production by N_2-fixing and non-N_2-fixing leguminous trees. Soil Biology and Biochemistry. 1988;**20**(6):891-897. DOI: 10.1016/0038-0717(88)90100-9

[16] Hardy RWF, Holsten RD, Jackson EK, Burns RC. The acetylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. Plant Physiology. 1968;**43**(8):1185-1207. DOI: 10.1104/pp.43.8.1185

[17] Hardarson G, Danso SKA. Methods for measuring biological nitrogen fixation in grain legumes. Plant and Soil. 1993;**152**(1):19-23. DOI: 10.1007/BF00016330

[18] Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. Science. 2008;**320**(5878):889-892. DOI: 10.1126/science.1136674

[19] Young JPW. Phylogenetic classification of nitrogen-fixing organisms. In: Stacey G, Burris RH, Evans HJ, editors. Biological Nitrogen Fixation. New York: Chapman and Hall; 1992. pp. 43-86

[20] Reed SC, Cleveland CC, Townsend AR. Functional ecology of free-living nitrogen fixation: A contemporary perspective. Annual Review of Ecology, Evolution, and Systematics. 2011;**42**:489-512. DOI: 10.1146/annurev-ecolsys-102710-145034

[21] Döbereiner J, Pedrosa FO. Nitrogen-Fixing Bacteria in Non-Leguminous Crop Plants. Madison: Science Tech; 2011

[22] Lindow SE, Brandl MT. Microbiology of the phyllosphere. Applied and Environmental Microbiology. 2003;**69**(4):1875-1883. DOI: 10.1128/AEM.69.4.1875-1883.2003

[23] Postgate JR. Nitrogen Fixation. 3rd ed. Cambridge: Cambridge University Press; 1998

[24] Oldroyd GED, Murray JD, Poole PS, Downie JA. The rules of engagement in the legume-rhizobial symbiosis. Annual Review of Genetics. 2011;**45**:119-144. DOI: 10.1146/annurev-genet-110410-132549

[25] Udvarte M, Poole PS. Transport and metabolism in legume-rhizobia symbioses. Annual Review of Plant Biology. 2013;**64**:781-805. DOI: 10.1146/annurev-arplant-050312-120235

[26] Huss-Danell K. Actinorhizal symbioses and their N_2 fixation. New Phytologist. 1997; **136**(3):375-405

[27] Schell DM, Alexander V. Nitrogen fixation in Arctic coastal tundra in relation to vegetation and micro-relief. Arctic. 1973;**26**:130-137

[28] Hobara S, McCalley C, Koba K, Giblin AE, Weiss MS, Gettel GM, Shaver GR. Nitrogen fixation in surface soils and vegetation in an Arctic tundra watershed: A key source of atmospheric nitrogen. Arctic, Antarctic, and Alpine Research. 2006;**38**(3):363-372. DOI: 10.1657/1523-0430(2006)38[363:NFISSA]2.0.CO;2

[29] Döbereiner J, Alvahydo R. Sóbre a influénciada canade-acucar na occoréncia de "*Beijerinckia*" no solo II. Influéncia das diversas partes do vegetal. Revista Brasileira de Biologia. 1959;**19**: 401-412

[30] Döbereiner J. Nitrogen fixing bacteria of the genus *Beijerinckia* Drex. in the rhizosphere of sugarcane. Plant and Soil. 1961;**15**(3):211-216. DOI: 10.1007/BF01400455

[31] Rennie RJ, de Freitas JR, Ruschel AP, Vose PB. Isolation and identification of nitrogen fixing bacteria associated with sugarcane (*Saccharum* sp.). Canadian Journal of Microbiology. 1982;**28**(5):462-467. DOI: 10.1139/m82-070

[32] Magalhaes FMM, Baldani JI, Souto SM, Kuykendal JR, Döbereiner JA. New acid tolerant *Azospirillum* species. Anais da Academia Brasileira de Ciências. 1983;**55**:417-430

[33] Seldin L, van Elsas JD, Penido EGC. *Bacillus azotofixans* sp. nov., a nitrogen-fixing species from Brazilian soils and grass roots. International Journal of Systematic Bacteriology. 1984;**34**:451-456. DOI: 10.1099/00207713-34-4-451

[34] Baldani JI, Baldani VLD, Seldin L, Döbereiner J. Characterization of *Herbaspirillum seropedicae* gen. Nov., sp. nov., a root associated nitrogen fixing bacterium. International Journal of Systematic Bacteriology. 1986;**36**:86-93. DOI: 10.1099/00207713-36-1-86

[35] Döbereiner J, Day JM, Dart PJ. Nitrogenase activity in the rhizosphere of sugarcane and some other tropical grasses. Plant and Soil. 1972;**37**(1):191-196. DOI: 10.1007/BF01578494

[36] Ruschel AP. Associative N_2-fixation by sugar cane. In: Vose PB, Ruschel AP, editors. Associative N_2-Fixation. Vol. 2. Boca Raton: CRC; 1981. pp. 81-90

[37] Cavalcante VA, Döbereiner J. A new acid tolerant nitrogen fixing bacterium associated with sugarcane. Plant and Soil. 1988;**108**(1):23-31. DOI: 10.1007/BF02370096

[38] Boddey RM, Urquiaga S, Reis V, Döbereiner J. Biological nitrogen fixation associated with sugar cane. Plant and Soil. 1991;**137**(1):111-117. DOI: 10.1007/BF02187441

[39] Stephan MP, Oliveira M, Teixeira KRS, Martinez-Drets G, Döbereiner J. Physiology and dinitrogen fixation of *Acetobacter diazotrophicus*. FEMS Microbiology Letters. 1991;**77**(1): 67-72. DOI: 10.1111/j.1574-6968.1991.tb04323.x

[40] de Bary A. Morphologie und Physiologie Pilze, Flechten, und myxomyceten. In: Hofmeister W, editor. Handbuch der Physiologischen Botanik. Zweiter Band. Leipzig: Wilhelm Engelmann; 1866. Available from: http://babel.hathitrust.org/cgi/pt?id=hvd.32044053007316 Accessed: 2017-10-02

[41] Chanway CP. Endophytes: They're not just fungi. Canadian Journal of Botany. 1996; **74**(3):321-322. DOI: 10.1139/b96-040

[42] Chanway CP, Anand R, Yang H. Nitrogen fixation outside and inside plant tissues. In: Ohyama T, editor. Advances in Biology and Ecology of Nitrogen Fixation. Croatia: InTech; 2014. pp. 3-23. DOI: 10.5772/57532

[43] Trevet IW, Hollis JP. Bacteria in storage organs of healthy plants. Phytopathology. 1948; **38**:960-967

[44] Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW. Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology. 1997;**43**(10):895-914. DOI: 10.1139/m97-131

[45] Kobayashi D, Palumbo J. Bacterial endophytes and their effects on plants and uses in agriculture. In: Bacon CW, White JF, editors. Microbial Endophytes. New York: Marcel Dekker; 2000. pp. 199-233

[46] Sturz AV, Christie BR, Nowak J. Bacterial endophytes: Potential role in developing sustainable systems of crop production. Critical Reviews in Plant Sciences. 2000;**19**(1):1-30

[47] Suman A, Yadav AN, Verma P. Endophytic microbes in crops: Diversity and beneficial impact for sustainable agriculture. In: Singh DP, Abhilash PC, Prabha R, editors. Microbial Inoculants in Sustainable Agricultural Productivity. New Delhi: Springer; 2016. pp. 117-143. DOI: 10.1007/978-81-322-2647-5_7

[48] Loper JE, Haack C, Schroth MN. Population dynamics of soil pseudomonads in the rhizosphere of potato (*Solanum tuberosum* L.). Applied and Environmental Microbiology. 1985;**49**(2):416-422

[49] Cocking E. Endophytic colonization of plant roots by N-fixing bacteria. Plant and Soil. 2003;**252**(1):169-175. DOI: 10.1023/A:1024106605806

[50] Rosenblueth M, Martínez-Romero E. Bacterial endophytes and their interaction with hosts. Molecular Plant-Microbe Interactions. 2006;**19**(8):827-837. DOI: 10.1094/MPMI-19-0827

[51] Richardson A, Barea J-M, McNeill A, Prigent-Combaret C. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant and Soil. 2009;**321**(1-2):305-339. DOI: 10.1007/s11104-009-9895-2

[52] Reinhold-Hurek B, Hurek T. Interactions of gramineous plants with *Azoarcus* spp. and other diazotrophs: Identification, localization, and perspectives to study their function. Critical Reviews in Plant Sciences. 1998;**17**(1):29-54

[53] Reinhold-Hurek B, Hurek T. Life in grasses: Diazotrophic endophytes. Trends in Microbiology. 1998b;**6**(4):139-144. DOI: 10.1016/S0966-842X(98)01229-3

[54] Santi C, Bogusz D, Franche C. Biological nitrogen fixation in non-legume plants. Annals of Botany. 2013;**111**(5):743-767. DOI: 10.1093/aob/mct048

[55] James EK, Gyaneshwar P, Mathan N, Barraquio WL, Reddy PM, Iannetta PPM, Olivares FL, Ladha JK. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Molecular Plant-Microbe Interactions. 2002; **15**(9):894-906. DOI: 10.1094/MPMI.2002.15.9.894

[56] Sessitsch A, Reiter B, Pfeifer U, Wilhelm E. Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and Actinomycetes-specific PCR of 16S rRNA genes. FEMS Microbiology Ecology. 2002;**39**(1):23-32. DOI: 10.1111/j.1574-6941.2002.tb00903.x

[57] Berg G, Krechel A, Ditz M, Sikora RA, Ulrich A, Hallmann J. Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. FEMS Microbiology Ecology. 2005;**51**(2):215-229. DOI: 10.1016/j.femsec.2004.08.006

[58] Okunishi S, Sako K, Mano H, Imamura A, Morisaki H. Bacterial flora of endophytes in the maturing seed of cultivated rice (*Oryza sativa*). Microbes and Environments. 2005;**20**(3):168-177. DOI: 10.1264/jsme2.20.168

[59] Compant S, Mitter B, Colli-Mull JG, Gangl H, Sessitsch A. Endophytes of grapevine flowers, berries, and seeds: Identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microbial Ecology. 2011;**62**(1):188-197. DOI: 10.1007/s00248-011-9883-y

[60] de Melo Pereira GV, Magalhaes KT, Lorenzetii ER, Souza TP, Schwan RF. A multiphasic approach for the identification of endophytic bacterial in strawberry fruit and their potential for plant growth promotion. Microbial Ecology. 2012;**63**(2):405-417. DOI: 10.1007/s00248-011-9919-3

[61] Trognitz F, Piller K, Nagel M, Borner A, Bacher C-F, Rechlik M, Mayrhofer H, Sessitsch A. Isolation and characterization of endophytes isolated from seeds of different plants and the application to increase juvenile development. In: Tagung Zukünftiges Saatgut—Produktion, Vermarktung, Nutzung und Konzervierung. Future Seed—Production, Marketing, Use and Conservation; 24-26 November 2014; Austria. Irdning: Höhere Bundeslehr- und Forschungsanstalt für Landwirtschaft Raumberg-Gumpenstein; 2014. pp. 25-28

[62] Benhizia Y, Benhizia H, Benguedouar A, Muresu R, Giacomini A, Squartini A. Gamma proteobacteria can nodulate legumes of the genus *Hedysarum*. Systematic and Applied Microbiology. 2004;**27**(4):462-468. DOI: 10.1078/0723202041438527

[63] Paul LR, Chapman WK, Chanway CP. Diazotrophic bacteria reside inside *Suillus tomentosus*/*Pinus contorta* tuberculate ectomycorrhizae. Botany. 2013;**91**(1):48-52. DOI: 10.1139/cjb-2012-0191

[64] Jacobs MJ, Bugbee WM, Gabrielson DA. Enumeration, location, and characterization of endophytic bacteria within sugar beet roots. Canadian Journal of Botany. 1985;**63**(7): 1262-1265. DOI: 10.1139/b85-174

[65] Misaghi IJ, Donndelinger CR. Endophytic bacteria in symptom-free cotton plants. Phytopathology. 1990;**80**(9):8080-8811

[66] Sturz AV, Christie BR, Matheson BG, Nowak J. Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. Biology and Fertility of Soils. 1997;**25**(1):13-19. DOI: 10.1007/s003740050273

[67] Chi F, Shen S, Cheng H, Jing Y, Yanni YG, Dazzo FB. Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. Applied and Environmental Microbiology. 2005;**71**(11):7271-7278. DOI: 10.1128/AEM.71.11.7271-7278.2005

[68] Anand R, Paul L, Chanway C. Research on endophytic bacteria: Recent advances with forest trees. In: Schulz B, Boyle C, Sieber TN, editors. Microbial Root Endophytes. Berlin: Springer Heidelberg; 2006. pp. 89-106. DOI: 10.1007/3-540-33526-9_6

[69] Tombolini R, Unge A, Davey ME, Bruijn FJ, Jansson JK. Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. FEMS Microbiology Ecology. 1997;**22**(1):17-28. DOI: 10.1111/j.1574-6941.1997.tb00352.x

[70] Tombolini R, Jansson JK. Monitoring of GFP tagged bacterial cells. In: La Rossa R, editor. Bioluminescence Methods and Protocols. Totowa: Humana Press; 1998. pp. 285-298. DOI: 10.1385/0-89603-520-4:285

[71] Xi C, Lambrecht M, Vanderleyden J, Michiels J. Bi-functional *gfp*-and *gus*A-containing mini-*Tn*5 transposon derivatives for combined gene expression and bacterial localization studies. Journal of Microbiological Methods. 1999;**35**(1):85-92. DOI: 10.1016/S0167-7012(98)00103-1

[72] Compant S, Reiter B, Sessitsch A, Nowak J, Clement C, Ait Barka E. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Applied and Environmental Microbiology. 2005;**71**(4):1685-1693. DOI: 10.1128/AEM.71.4.1685-1693.2005

[73] Timmusk S, Grantcharova N, Wagner EGH. *Paenibacillus polymyxa* invades plant roots and forms biofilms. Applied and Environmental Microbiology. 2005;**71**(11):7292-7300. DOI: 10.1128/AEM.71.11.7292-7300.2005

[74] Chelius MK, Triplett EW. *Dyadobacter fermentans* gen. Nov., sp. nov., a novel gram-negative bacterium isolated from surface-sterilized *Zea mays* stems. International Journal of Systematic and Evolutionary Microbiology. 2000;**50**:751-758. DOI: 10.1099/00207713-50-2-751

[75] Anand R, Chanway CP. Detection of GFP-labeled *Paenibacillus polymyxa* in auto fluorescing pine seedling tissues. Biology and Fertility of Soils. 2013;**49**(1):111-118. DOI: 10.1007/s00374-012-0727-9

[76] Villacieros M, Power B, Sanchez-Contreras M, Lloret J, Oruezabal RI, Martin M, Fernandez-Pinas F, Bonilla I. Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. Plant and Soil. 2003;**251**(1): 47-54. DOI: 10.1023/A:1022943708794

[77] Germaine K, Keogh E, Garcia-Cabellos G, Borremans B, Lelie D, Barac T, Oeyen L, Vangronsveld J, Moore FP, Moore ERB, Campbell CD, Ryan D, Dowling DN. Colonisation of poplar trees by gfp expressing bacterial endophytes. FEMS Microbiology Ecology. 2004;**48**(1):109-118. DOI: 10.1016/j.femsec.2003.12.009

[78] Sevilla M, Kennedy C. Colonization of rice and other cereals by *Acetobacter diazotrophicus*, an endophyte of sugarcane. In: Ladha JK, Reddy PM, editors. The Quest for Nitrogen Fixation in Rice. Makati: International Rice Research Institute; 2000. pp. 151-165

[79] Rouws LF, Meneses CH, Guedes HV, Vidal MS, Baldani JI, Schwab S. Monitoring the colonization of sugarcane and rice plants by the endophytic diazotrophic bacterium *Gluconacetobacter diazotrophicus* marked with *gfp* and *gusA* reporter genes. Letters in Applied Microbiology. 2010;**51**(3):325-330. DOI: 10.1111/j.1472-765X.2010.02899.x

[80] Alquéres S, Meneses C, Rouws L, Rothballer M, Baldani I, Schmid M, Hartmann A. The bacterial superoxide dismutase and glutathione reductase are crucial for endophytic colonization of rice roots by *Gluconacetobacter diazotrophicus* PAL5. Molecular Plant-Microbe Interactions. 2013;**26**(8):937-945. DOI: 10.1094/MPMI-12-12-0286-R

[81] Mousa WK, Shearer CR, Limay-Rios V, Zhou T, Raizada MN. Bacterial endophytes from wild maize suppress *Fusarium graminearum* in modern maize and inhibit mycotoxin accumulation. Frontiers in Plant Science. 2015;**6**:805. DOI: 10.3389/fpls.2015.00805

[82] Kumar A, Munder A, Aravind R, Eapen SJ, Tümmler B, Raaijmakers JM. Friend or foe: Genetic and functional characterization of plant endophytic *Pseudomonas aeruginosa*. Environmental Microbiology. 2013;**15**(3):764-779. DOI: 10.1111/1462-2920.12031

[83] Sun K, Liu J, Gao Y, Jin L, Gu Y, Wang W. Isolation, plant colonization potential, and phenanthrene degradation performance of the endophytic bacterium *Pseudomonas* sp. Ph6-*gfp*. Scientific Reports. 2014;**4**:5462. DOI: 10.1038/srep05462

[84] Shehata HR, Lyons EM, Jordan KS, Raizada MN. Bacterial endophytes from wild and ancient maize are able to suppress the fungal pathogen *Sclerotinia homoeocarpa*. Journal of Applied Microbiology. 2016;**120**(3):756-769. DOI: 10.1111/jam.13050

[85] Döbereiner J. Recent changes in concepts of plant bacteria interactions: Endophytic N_2 fixing bacteria. Ciência e Cultura. 1992;**44**(5):310-313

[86] Baldani JI, Olivares FL, Hemerly AS, Reis FB Jr, Oliveira ALM, Baldani VLD, Goi SR, Reis VM, Döbereiner J. Nitrogen-fixing endophytes: Recent advances in the association with graminaceous plants grown in the tropics. In: Elmerich EC, editor. Biological Nitrogen Fixation for the 21st Century. Dordrecht: Springer Netherlands; 1998. pp. 203-206. DOI: 10.1007/978-94-011-5159-7_90

[87] Baldani VLD, Baldani JI, Döbereiner J. Inoculation of rice plants with the endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* spp. Biology and Fertility of Soils. 2000;**30**(5-6):485-491. DOI: 10.1007/s003740050027

[88] Gyaneshwar P, James EK, Mathan N, Reddy PM, Reinhold-Hurek B, Ladha JK. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. Journal of Bacteriology. 2001;**183**(8):2634-2645. DOI: 10.1128/JB.183.8.2634-2645.2001

[89] Hurek T, Handley LL, Reinhold-Hurek B, Piche Y. *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Molecular Plant-Microbe Interactions. 2002;**15**(3):233-242. DOI: 10.1094/MPMI.2002.15.3.233

[90] Olivares FL, Baldani VLD, Reis VM, Baldani JI, Döbereiner J. Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems, and leaves, predominantly of Gramineae. Biology Fertility of Soils. 1996;**21**(3):197-200. DOI: 10.1007/BF00335935

[91] Riggs PJ, Chelius MK, Iniguez AL, Kaeppler SM, Triplett EW. Enhanced maize productivity by inoculation with diazotrophic bacteria. Australian Journal of Plant Physiology. 2001;**28**(9):829-836. DOI: 10.1071/PP01045

[92] Roesch LFW, Camargo FAO, Bento FM, Triplett EW. Biodiversity of diazotrophs within the soil, root and stem of field grown maize. Plant and Soil. 2008;**302**(1-2):91-104. DOI: 10.1007/s11104-007-9458-3

[93] Montañez A, Abreu C, Gill PR, Hardarson G, Sicardi M. Biological nitrogen fixation in maize (*Zea mays* L.) by ^{15}N isotope-dilution and identification of associated culturable diazotrophs. Biology and Fertility of Soils. 2009;**45**(3):253-263. DOI: 10.1007/s00374-008-0322-2

[94] Sabry RS, Saleh SA, Batchelor CA, Jones J, Jotham J, Webster G, Kothari SL, Davey MR, Cocking EC. Endophytic establishment of *Azorhizobium caulinodans* in wheat. Proceedings of the Royal Society of London B: Biological Sciences. 1997;**264**(1380):341-346. DOI: 10.1098/rspb.1997.0049

[95] Germida J, de Freitas J. Nitrogen fixing rhizobacteria as biofertilizers for canola. In: Saskatchewan Canola Development Commission (Project code: CARP 9513). 1998. Available from: http://www.saskcanola.com/quadrant/System/research/reports/report-Germida-nitrogenfixing-long.pdf Accessed: 2017-10-02

[96] Malik KA, Bilal R, Mehnaz S, Rasul G, Mirza MS, Ali S. Association of nitrogen-fixing, plant-growth-promoting rhizobacteria (PGPR) with kallar grass and rice. Plant and Soil. 1997;**194**(1-2):37-44. DOI: 10.1023/A:1004295714181

[97] Araújo AES, Baldani VLD, Galisa PS, Pereira JA, Baldani JI. Response of traditional upland rice varieties to inoculation with selected diazotrophic bacteria isolated from rice cropped at the northeast region of Brazil. Applied Soil Ecology. 2013;**64**:49-55. DOI: 10.1016/j.apsoil.2012.10.004

[98] Schultz N, da Silva JA, Sousa JS, Monteiro RC, Oliveira RP, Chaves VA, Pereira W, da Silva MF, Baldani JI, Boddey RM, Reis VM, Urquiaga S. Inoculation of sugarcane with diazotrophic bacteria. Revista Brasileira de Ciência do Solo. 2014;**38**(2):407-414. DOI: 10.1590/S0100-06832014000200005

[99] Puri A, Padda KP, Chanway CP. Plant growth promotion by endophytic bacteria in nonnative crop hosts. In: Maheshwari DK, Annapurna K, editors. Endophytes: Crop Productivity and Protection. Switzerland: Springer International Publishing; 2017. pp. 11-45. DOI: 10.1007/978-3-319-66544-3_2

[100] Aguiar NO, Medici LO, Olivares FL, Dobbss LB, Torres-Netto A, Silva SF, Novotny EH, Canellas LP. Metabolic profile and antioxidant responses during drought stress recovery in sugarcane treated with humic acids and endophytic diazotrophic bacteria. Annals of Applied Biology. 2016;**168**(2):203-213. DOI: 10.1111/aab.12256

[101] dos Reis AG, da Silva TR, Carvalho BR, Neiva JNM, de Araújo GGL, Júnior PIF. Quantification and characterization of putative diazotrophic bacteria from forage palm under saline water irrigation. Revista Geama. 2017;**3**(4):261-268

[102] Szilagyi-Zecchin VJ, Ikeda AC, Hungria M, Adamoski D, Kava-Cordeiro V, Glienke C, Galli-Terasawa LV. Identification and characterization of endophytic bacteria from corn (*Zea mays* L) roots with biotechnological potential in agriculture. AMB Express. 2014; **4**:26. DOI: 10.1186/s13568-014-0026-y

[103] Andrade LF, De Souza GL, Nietsche S, Xavier AA, Costa MR, Cardoso AM, Pereira MC, Pereira DF. Analysis of the abilities of endophytic bacteria associated with banana tree roots to promote plant growth. The Journal of Microbiology. 2013;**52**(1):27-34. DOI: 10.1007/s12275-014-3019-2

[104] Gupta G, Panwar J, Jha PN. Natural occurrence of *Pseudomonas aeruginosa*, a dominant cultivable diazotrophic endophytic bacterium colonizing *Pennisetum glaucum* (L.) R. Br. Applied Soil Ecology. 2013;**64**:252-261. DOI: 10.1016/j.apsoil.2012.12.016

[105] Rangjaroen C, Rerkasem B, Teaumroong N, Noisangiam R, Lumyong S. Promoting plant growth in a commercial rice cultivar by endophytic diazotrophic bacteria isolated from rice landraces. Annals of Microbiology. 2015;**65**(1):253-266. DOI: 10.1007/s13213-014-0857-4

[106] Ji SH, Gururani MA, Chun SC. Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiological Research. 2014;**169**(1):83-98. DOI: 10.1016/j.micres.2013.06.003

[107] Padda KP, Puri A, Chanway CP. *Paenibacillus polymyxa*—A prominent biofertilizer and biocontrol agent for sustainable agriculture. In: Meena VS, Mishra P, Thakuria D, Bisht J, Pattanayak A, editors. Agriculturally Important Microbes for Sustainable Agriculture. Singapore: Springer; 2017. pp. 165-191. DOI: 10.1007/978-981-10-5343-6_6

[108] Puri A, Padda KP, Chanway CP. Beneficial effects of bacterial endophytes on forest tree species. In: Maheshwari DK, Annapurna K, editors. Endophytes: Crop Productivity

and Protection. Switzerland: Springer International Publishing; 2017. pp. 111-132. DOI: 10.1007/978-3-319-66544-3_6

[109] Bal A, Anand R, Berge O, Chanway C. Isolation and identification of diazotrophic bacteria from internal tissues of *Pinus contorta* and *Thuja plicata*. Canadian Journal of Forest Research. 2012;**42**(4):807-813. DOI: 10.1139/x2012-023

[110] Bal A, Chanway CP. Evidence of nitrogen fixation in lodgepole pine inoculated with diazotrophic *Paenibacillus polymyxa*. Botany. 2012;**90**(9):891-896. DOI: 10.1139/b2012-044

[111] Anand R, Grayston S, Chanway CP. N_2-fixation and seedling growth promotion of lodgepole pine by endophytic *Paenibacillus polymyxa*. Microbial Ecology. 2013;**66**(2):369-374. DOI: 10.1007/s00248-013-0196-1

[112] Yang H, Puri A, Padda KP, Chanway CP. Effects of *Paenibacillus polymyxa* inoculation and different soil nitrogen treatments on lodgepole pine seedling growth. Canadian Journal of Forest Research. 2016;**46**(6):816-821. DOI: 10.1139/cjfr-2015-0456

[113] Anand R, Chanway CP. *nif* gene sequence and arrangement in the endophytic diazotroph *Paenibacillus polymyxa* strain P2b-2R. Biology and Fertility of Soils. 2013;**49**(7):965-970. DOI: 10.1007/s00374-013-0793-7

[114] Yang H, Puri A, Padda KP, Chanway CP. Substrate utilization by endophytic *Paenibacillus polymyxa* that may facilitate bacterial entrance and survival inside various host plants. FACETS. 2017;**2**:120-130. DOI: 10.1139/facets-2016-0031

[115] Bal A, Chanway CP. ^{15}N foliar dilution of western red cedar in response to seed inoculation with diazotrophic *Paenibacillus polymyxa*. Biology and Fertility of Soils. 2012;**48**(8):967-971. DOI: 10.1007/s00374-012-0699-9

[116] Anand R, Chanway C. N_2-fixation and growth promotion in cedar colonized by an endophytic strain of *Paenibacillus polymyxa*. Biology and Fertility of Soils. 2013;**49**(2):235-239. DOI: 10.1007/s00374-012-0735-9

[117] Puri A, Padda KP, Chanway CP. Can a diazotrophic endophyte originally isolated from lodgepole pine colonize an agricultural crop (corn) and promote its growth? Soil Biology and Biochemistry. 2015;**89**:210-216. DOI: 10.1016/j.soilbio.2015.07.012

[118] Puri A, Padda KP, Chanway CP. Evidence of nitrogen fixation and growth promotion in canola (*Brassica napus* L.) by an endophytic diazotroph *Paenibacillus polymyxa* P2b-2R. Biology and Fertility of Soils. 2016;**52**(1):119-125. DOI: 10.1007/s00374-015-1051-y

[119] Padda KP, Puri A, Chanway CP. Effect of GFP tagging of *Paenibacillus polymyxa* P2b-2R on its ability to promote growth of canola and tomato seedlings. Biology and Fertility of Soils. 2016;**52**(3):377-387. DOI: 10.1007/s00374-015-1083-3

[120] Padda KP, Puri A, Chanway CP. Plant growth promotion and nitrogen fixation in canola by an endophytic strain of *Paenibacillus polymyxa* and its GFP-tagged derivative in a long-term study. Botany. 2016;**94**(12):1209-1217. DOI: 10.1139/cjb-2016-0075

[121] Padda KP, Puri A, Zeng Q, Chanway CP, Wu X. Effect of GFP-tagging on nitrogen fixation and plant growth promotion of an endophytic diazotrophic strain of *Paenibacillus polymyxa*. Botany. 2017;**95**(9):933-942. DOI: 10.1139/cjb-2017-0056

[122] Puri A, Padda KP, Chanway CP. Seedling growth promotion and nitrogen fixation by a bacterial endophyte *Paenibacillus polymyxa* P2b-2R and its GFP derivative in corn in a long-term trial. Symbiosis. 2016;**69**(2):123-129. DOI: 10.1007/s13199-016-0385-z

[123] Tang Q, Puri A, Padda KP, Chanway CP. Biological nitrogen fixation and plant growth promotion of lodgepole pine by an endophytic diazotroph *Paenibacillus polymyxa* and its GFP-tagged derivative. Botany. 2017;**95**(6):611-619. DOI: 10.1139/cjb-2016-0300

[124] Rodriguez H, Mendoza A, Cruz MA, Holguin G, Glick BR, Bashan Y. Pleiotropic physiological effects in the plant growth-promoting bacterium *Azospirillum brasilense* following chromosomal labeling in the clpX gene. FEMS Microbiology Ecology. 2006;**57**(2): 217-225. DOI: 10.1111/j.1574-6941.2006.00111.x