Chapter 1

Nitrogen Fixation Outside and Inside Plant Tissues

C.P. Chanway, R. Anand and H. Yang

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1. Introduction

Nitrogen is one of the most important elements in biological systems, comprising the main building blocks of nucleic acids, enzymes and proteins among its multiple functions. In nature, it exists primarily in the gaseous form and constitutes approximately 78% of the atmosphere. Despite its abundance, nitrogen (N) is one of the most growth-limiting nutrients in terrestrial and aquatic ecosystems (Dalton & Krammer, 2006) because its gaseous form is inert and unusable by most living organisms except for nitrogen fixing microorgansisms. For it to become biologically available, atmospheric nitrogen must be transformed or "fixed" from its inert gaseous form (N_2) to ammonia (NH_3), which can then be assimilated into a variety of important biochemicals. This transformation, which requires a large amount of energy to break apart the triple-bonded N atoms that comprise gaseous N_2, is called 'nitrogen fixation' (NF). Nitrogen is fixed naturally through energy-releasing abiotic processes such as lightening, forest fires and volcanic activity. These processes produce oxides of N in the atmosphere that subsequently dissolve in rain and descend to the ground as NH_3 molecules. Approximately 12% of annual global NF is fixed in this way (Bezdicek & Kennedy, 1998). Fertilizer production using high temperatures and pressures in the Haber-Bosch process occurs widely and accounts for approximately 20% of annual global NF (Bezdicek & Kennedy, 1998). However, the process is fossil-fuel intensive and consumes 3-5% of the world's natural gas annually (Myrold & Bottomley, 2007). Alternatively, NF occurs through the normal metabolic activity of many prokaryotic microorganisms, known as diazotrophs, through a process commonly referred to as biological nitrogen fixation (BNF). This essentially "free" process is responsible for the addition of almost all biologically available N that enters terrestrial ecosystems, some 140 million metric tons per year (Bezdicek & Kennedy, 1998; Galloway et al., 2008). Biological nitrogen fixation is an ATP-demanding process that is catalyzed by the enzyme complex known as nitrogenase, which is found in many members of the Bacteria and Archaea (Galloway

et al., 2008). Terrestrial BNF occurs primarily in the soil, by either free-living diazotrophs or those associated to varying degrees with plants (see below).

2. Free-living bacteria

Free-living diazotrophic bacteria are those that do not associate with plants (*cf.* rhizosphere bacteria below) 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 (Reed et al., 2011). Since most soils are C and N limited, the amount of N_2 they fix in soil is restricted by access to energy sources, *i.e.,* substrates to generate adenosine triphosphate (ATP) and micronutrients required for the synthesis and functioning of nitrogenase (Reed et al., 2011). BNF by free-living diazotrophs is also limited by the severe oxygen sensitivity of nitrogenase (Postgate, 1998), which is a problem that has been at least partially overcome in different ways by diazotrophs participating in nitrogen-fixing symbioses (see below). Antagonistic microbial interactions such as parasitism and competition for nutrients (Cacciari et al.,1986; Bashan & Holguin, 1997; Bashan et al., 2004) further reduce the amount of N_2 they fix. While the general belief is that free-living diazotrophs do not contribute large quantities of fixed N to most terrestrial ecosystems, perhaps 3-5 kg/ha/yr (Postgate, 1998; Newton, 2007), their cumulative N contributions are thought to be important in some tropical and temperate forest ecosystems (Cleveland et al., 1999; Gehring et al., 2005; Reed et al., 2007; 2008).

3. Rhizosphere bacteria

The rhizosphere is defined as soil that surrounds plant roots and is under their direct metabolic influence (Curl & Truelove, 1987). Proximity to plant roots is important for soil organisms as actively growing plant roots deposit approximately 20% of annual photosynthate in the rhizosphere (Nguyen, 2003), but depending on the type of plant and its growth stage, more than 50% of newly-fixed carbon may be deposited in the rhizosphere at any given time. The soluble carbon compounds that plants deposit through their root systems are known as root exudates. These comprise a wide range of carbon compounds (amino acids, peptides, proteins, enzymes, "growth factors", vitamins and phytohormones) (Grayston et al., 1997; Jones et al., 2004; Shi et al., 2012) and are released continuously during the growing season through a process known as root exudation (Jones et al., 2004). This process has been shown to significantly stimulate growth and population sizes of most soil microorganisms, but effects are particularly noticeable in soil bacteria and fungi. The degree of stimulation is significant: in comparison with bulk soil not under the influence of plant roots, the rhizosphere typically supports 5-100 x larger bacterial and fungal populations than non-rhizosphere or "bulk" soil (Warembourg, 1997; Dobelaere et al., 2003). Due to their ability to fix N_2, diazotrophs can have a competitive advantage over non-N_2 fixing bacteria in the rhizosphere and prevail in it

particularly when soil N is limited (Döbereiner & Pedrosa, 1987). In addition to stimulating their own growth, rhizosphere diazotrophs representing several genera (*e.g., Acetobacter, Azoarcus, Azospirillum, Azotobacter, Beijerinckia, Burkholderia, Enterobacter, Herbaspirillum, Klebsiella, Paenibacillus* and *Pseudomonas*) have been shown to enhance the growth of the plants that generate a suitable rhizosphere. These include agriculturally-important species such as rice, wheat, barley, potato and several vegetable crops (Dobelaere et al., 2003). While it is logical to assume diazotrophic rhizosphere bacteria enhance plant growth through BNF, these microorganisms are often capable of producing plant growth-enhancing phytohormones and pathogen-suppressing antibiotics (Chanway, 2002). Many are also able to enhance the availability of N, P and S in the rhizosphere enzymatically, rendering it difficult to conclude with certainty the mechanism by which plant growth is stimulated.

4. Phyllosphere

The leaf surface, or phyllosphere is another microsite known to be colonized by a wide range of microorganisms, including diazotrophic bacteria (Lindow & Brandl, 2003). While comparatively little work has been done on phyllosphere-colonizing diazotrophs, it is likely that their contribution to plant N nutrition is modest, owing to the energy constraints and problems associated with oxygen toxicity of nitrogenase such N_2 fixing microorganisms would experience. Nevertheless, phyllosphere-colonizing diazotrophs should be evaluated further for possible contributions to plant nutrition.

5. Cyanobacterial associations

Cyanobacteria are prokaryotes belonging to the domain *Bacteria* that are capable of fixing carbon, through oxygenic photosynthesis, as well as nitrogen through BNF. As a result, cyanobacteria are nutritionally independent to a large degree (Meeks, 1988) and occupy diverse habitats, ranging from freshwater and oceanic ecosystems, temperate soils to extreme environments such as hot springs and deserts (Herrero et al., 2001). To counter the adverse effects of oxygen evolution from photosynthesis on BNF, diazotrophic cyanobacteria have developed strategies such as temporal separation of oxygenic photosynthesis and micro-oxic NF (Cerveny et al., 2013) and formation of thick-walled, non-photosynthetic heterocysts with microaerobic interiors that are conducive for NF (Wolk et al., 1994).

Many diazotrophic cyanobacterial species enter into symbioses with eukaryotes including phytoplankton, fungi and terrestrial plants. Cyanobacterial symbionts (cyanobionts) in these associations may contribute a significant portion of N required for growth of both organisms through BNF in N-limited aquatic and terrestrial environments (Schell & Alexander, 1973; Hobara et al., 2006). In the ocean, they are frequently found in association with diatoms (Ferrario et al., 1995) and brown algae (Carpenter, 1972), while in fresh water, the cyanobiont *Anabaena azollae* forms a symbiotic association with water ferns belonging to the genus

Azolla (Talley et al., 1977). The *Azolla-Anabaena* symbiosis often occurs in rice culture, where it results in enhanced rice performance due to a N fertilizer effect from BNF (Yanni, 1992). Diazotrophic cyanobacteria have also been found in the coralloid roots of cycads (Gehringer et al., 2010), auricles of liverworts (Adams & Duggan, 2008), slime cavities of hornworts (Adams & Duggan, 2008), stem glands of *Gunnera* (Bergman et al., 1992), hyphae of lichens (Jayasinghearachchi & Seneviratne, 2004) and on the leaf surfaces of mosses (Solheim & Zielke, 2002), where they fix and transfer N to the non-N_2 fixing partner. To facilitate locating a suitable symbiotic partner, some cyanobacteria belonging to the order *Nostocales* can differentiate into motile segments termed hormogonia that travel chemotactically toward a potential partner (Campbell & Meeks, 1989). If the partner is suitable, hormogonia differentiate back into vegetative cells and enter into an active diazotrophic symbiosis where they fix and provide N for both partners.

6. Legume x *Rhizobium* symbiosis

The legume x *Rhizobium* symbioses is a well-known mutualism involving plants from the angiosperm family *Leguminosae* (synonym *Fabaceae*) and bacteria belonging to the family *Rhizobiaceae* (Postgate 1998). In this symbiosis, diazotrophic soil bacteria belonging to the genus *Rhizobium* (or closely related genera) seek out and infect roots of suitable legume plant hosts using a complex chemical signaling system. Bacteria then colonize certain root cortex cells and initiate formation of a new plant organ, the root nodule. Bacteria proliferate within root nodule cells and then differentiate into a nitrogen fixing form called a bacteroid, to fix N_2. The plant vascular system is continuous with that of the root nodule, which enables newly fixed N to be rapidly translocated to other parts of the plant most in need of N. Energy for BNF is provided to bacteroids from plant photosynthesis and the oxygen concentration in root nodule cells is tightly regulated by an iron containing protein very similar in composition to hemoglobin called leghemoglobin. Bacteroids in this coadapted symbiosis are capable of high rates of BNF, *i.e.*, up to 600 kg/ha/yr, particularly when compared to BNF in the rhizosphere (15-25 kg/ha/yr) or by free-living diazotrophs (3-5 kg/ha/yr).

The legume x *Rhizobium* symbiosis has been studied widely from ecological, agronomic and molecular biological perspectives to not only enhance the nitrogen-fixing efficacy of existing symbioses but to determine if similar associations might also be developed with non-legume crops (Oldroyd et al., 2011, Udvardi & Poole, 2013). Notwithstanding impressive gains in our understanding of this symbiosis, its inherent complexity currently precludes our ability to extend effective nodulation or nitrogen fixing capacity to non-legume species (Beatty & Good, 2011). However, systemic infection of non-legumes with endophytic diazotrophic bacteria has been observed in several plant species and in some cases, appears to satisfy the majority of the "host" plant's N requirement with amounts of BNF similar to that of legume root nodules (see *Sugarcane and Other Crops* and *Nitrogen Fixation in Gymnosperms* below).

7. Actinorhizal symbiosis

The actinorhizal symbiosis refers to a root nodule-forming, nitrogen-fixing symbiotic relationship that is functionally analogous to the legume x *Rhizobium* symbiosis but distinct in most of the details. It is restricted to members of a small group of woody, non-legume pioneer species known as Actinorhizal plants and diazotrophs belonging to a single genus, *Frankia*, in a phylum of mostly filamentous soil bacteria, the Actinobacteria. Actinorhizal plants comprise approximately 200 plant species in 24 genera belonging to 8 plant families: *Casuarinaceae, Betulaceae, Myricaceae, Elaeagnaceae, Coriariaceae, Rhamnaceae, Datiscaceae* and *Rosaceae* (Huss-Danell, 1997). All but some members of *Datiscaceae* are shrubs or trees, and all are relatively shade-intolerant pioneering species that are able to colonize N-poor sites due to their ability to enter into the actinorhizal symbiosis (Crocker & Major, 1955; Chapin et al., 1994). *Frankia* spp. are notoriously difficult to grow in culture hence our understanding of these microorganisms and the actinorhizal symbioses lags far behind that of the well-characterized legume x *Rhizobium* associations. Owing to their recalcitrance to growth in culture, taxonomy of the genus *Frankia* is poorly developed, with only one species designation since a member of this genus was first reproducibly isolated over 30 years ago (Callaham et al., 1978). Numerous strains of *Frankia* have since been isolated from inside surface-sterilized root nodules and are designated as *Frankia* spp. followed by a strain number or name.

In contrast to the easily cultured, gram negative rod shaped cells that typify *Rhizobium* (and relatives) species, *Frankia* spp. strains possess gram-variable cells of three distinct types: filamentous vegetative hyphae, reproductive spores and N_2-fixing vesicles (Benson & Silvester, 1993). The infection of plant roots and subsequent formation of root nodules bear some similarity to these processes in *Rhizobium*-infected legumes, but the origin and composition of *Frankia*-induced root nodules differs significantly (Wall & Berry, 2008). Unlike the *de novo* structure of a legume root nodule, actinorhizal root nodules are comprised of numerous tightly or loosely packed lobes, each of which originates from the pericycle indicating that it is a modified lateral root. Unlike *Rhizobium* bacteroids, the filamentous *Frankia* hyphae differentiate into an active nitrogen fixing form by developing numerous vesicles inside the root nodule or they may differentiate into spores and contribute nothing to the N economy of the plant. The tendency to sporulate in the nodule appears to be a strain specific characteristic that is influenced by the identity of plant host species. The oxygen concentration in root nodules is regulated primarily by varying the number and thickness of nitrogen fixing vesicle wall layers, however, the elegant heme protein leghemoglobin, which facilitates oxygen diffusion and supply in legume root nodules, has also been detected in nodules of some actinorhizal plants. Physiological and structural differences between actinorhizal and legume root nodules suggest that actinorhizal symbioses are less evolved and less efficacious in NF compared to legume x *Rhizobium* symbioses, but an effective actinorhizal symbiosis can fix several hundred kilograms of N per hectare annually, an amount which is similar to an effective legume symbiosis.

8. Bacterial endophytes

The presence of microorganisms in plant tissues might reasonably be considered an indication that a disease state is imminent, however this is not necessarily the case. Several decades ago, Trevet and Hollis (1948) reported the occurrence of bacteria within tissues of healthy potato plants and several studies have since demonstrated that internal tissues of healthy plants are colonized by bacteria. The term 'bacterial endophytes' has been used to describe bacteria that reside within living plant tissues without causing disease (Wilson, 1995; van der Lelie et al., 2009), however it does not differentiate whether such bacteria are (i) truly harmless, (ii) latent pathogens (Sinclair & Cerkauskas, 1996) or (iii) able to elicit production of symbiotic structures such as root nodules on the host. We use the term 'endophyte' in this paper to describe bacteria that 'can be detected at a particular moment within the tissue of apparently healthy plant hosts' (Schultz & Boyle, 2005) without inducing disease or organogenesis (Iniguez et al., 2005). In contrast to free-living, rhizosphere or phyllosphere microorganisms, bacterial endophytes 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 (Loper et al., 1985; Cocking, 2003; Rosenblueth & Martinez-Romero, 2006). In addition, bacterial endophytes colonize niches that are more conducive to forming mutualistic relationships with plants through NF, for example, as suggested in sugarcane and other crops (see below) (Richardson et al., 2009).

8.1. Bacterial endophytes of sugarcane and other agricultural crops

In the 1980's, Brazilian researchers were perplexed by the consistently high yields of field-grown sugarcane, an N-demanding crop, without exogenous N fertilizer application and looked for a microbiological explanation for this apparently anomalous observation. After it was determined that rhizospheric NF did not occur at sufficient rates to facilitate high sugarcane yields, Cavalcante & Döbereiner (1988) looked for microorganisms within sugarcane tissues that might be involved and isolated a diazotrophic bacterium, *Gluconoacetobacter diazotrophicus*, previously known as *Acetobacter diazotrophicus*. Its ability to establish high endophytic populations and to fix N_2 in high sucrose concentrations (Boddey et al., 1991) at low pH (Boddey et al., 1991; Stephan et al., 1991), conditions which typify sugarcane tissues, led to the suggestion that this diazotrophic bacterium could satisfy almost all of the sugarcane N requirements. Several other N-contributing diazotrophic endophytes were subsequently found to associate with sugarcane including two *Herbaspirillum* species (Cavalcante & Döbereiner, 1988; Baldani et al., 1992; 2002), *Azoarcus* spp. (Reinhold et al., 1993) and *Azospirillum brasilense* (de Bellone and Bellone, 2006). However, more recent studies indicate that the primary source of diazotrophy may involve a consortium of bacteria, possibly including uncultured strains, that live on or inside plant tissues (Burbano et al., 2011; Taulé et al., 2012). Since the discovery of diazotrophic endophytes in sugarcane (*Saccharum officinarum* L.) (Ruschel et al., 1975), several other agriculturally important crop species including rice (*Oryza sativa*) (Shrestha & Ladha, 1996), maize (*Zea mays* L.) (Montañez et al., 2009) and kallar grass

(*Leptochloa fusca* L.) (Malik et al., 1997) have been postulated to receive significant amounts of fixed N_2 in this way.

Despite sugarcane's apparent potential to derive much of its N from BNF, it has not been proven that *G. diazotrophicus* or any endophytic diazotroph is the primary causal agent of N accumulation by this plant in the field. Non-culturable endophytes with plant growth-promoting potential have been detected in sugarcane tissues (Hallmann et al., 1997; Mendes et al., 2007). In addition, all known culturable bacterial endophytes including *G. diazotrophicus* colonize the rhizosphere as well as internal plant tissues, rendering it difficult if not impossible to distinguish endophytic from non-endophytic BNF. There is also no clear evidence that N is transferred directly from endophytic diazotrophs to the host plant, unlike other symbiotic N_2 fixing systems where specific nitrogenous compounds such as ureides in soybean (Herridge, 1982) and citrulline in alder (Leaf et al., 1958) are synthesized to transport fixed N from the site of NF to other host tissues. It is generally accepted that the N transfer to the host from rhizospheric NF results from the release of mineralized N from dead bacterial cells (Dobbelaere et al., 2003; Momose et al., 2009; Mia & Shamsuddin, 2010). Whether or not a similar mechanism operates with endophytic diazotrophs remains unanswered.

If endophytic diazotrophs are ultimately proven to be the primary cause of BNF and growth promotion of their host plants, such a plant x microbe association would represent another type of mutualistic symbiosis where the plant provides photosynthate and a competition-free, microaerobic environment for effective N_2 fixation (Hallman et al., 1997; Reinhold-Hurek & Hurek, 1998a,b; Santi et al., 2013) for microorganisms in return for plant growth-promoting amounts of N from BNF. In contrast to the legume x *Rhizobium* and actinorhizal symbioses, no symbiosis-specific structures (*e.g.,* nodules or chambers housing bacteria) have been detected in infected host, though Anand & Chanway (2013 a)observed occasional plant cells filled with diazotrophic *Paenibacillus polymyxa* in inoculated lodgepole pine tissues (Fig. 1b).

8.2. Nitrogen fixation in gymnosperms: Effects on seedling growth and N content

Lodgepole pine (*Pinus contorta* var. *latifolia* (Dougl.) Engelm.), a commercially important gymnosperm species indigenous to western North America, is capable of growing in very rocky substrates and is notable for its ability to thrive on nutrient poor, N-limited soils (Weetman et al., 1988; Chapman & Paul, 2012). Based on earlier work with lodgepole pine suggesting that rhizospheric BNF contributed only small amounts of N to seedlings (Chanway & Holl, 1991) as well as reports that BNF in sugarcane was endophytic, we searched for endophytic diazotrophs in lodgepole pine as a possible explanation for the ability of this species to grow on N-deficient substrates. We successfully isolated several *Paenibacillus* strains that possessed significant acetylene reduction activity from extracts of surface-sterilized lodgepole pine seedling and tree tissues (Bal et al., 2012) and tested them for BNF with lodgepole pine and western red cedar seedlings in a ^{15}N soil dilution assay (Bal & Chanway, 2012a,b). When pine was reintroduced to one of the strains, *Paenibacillus polymyxa* strain P2b-2R, and grown in a very N-limited soil, seedlings were found to derive more than half (66%) of their foliar N from BNF, but their growth was *inhibited* compared to non-inoculated

controls 9 months after planting (Bal & Chanway, 2012a). Similar effects, *i.e.,* BNF and seedling growth inhibition, were observed with western red cedar (Bal & Chanway, 2012b).

Based on these results, we hypothesized that soil N depletion would eventually restrict the growth rate of control seedlings to a point where they would be outperformed by N_2-fixing seedlings, and set up longer term growth experiments to evaluate this possibility. After a 13-month growth period, pine seedlings treated with P2b-2R were observed to derive most of their foliar N (79%) from BNF (Anand et al., 2013), which was confirmation of the ability of lodgepole pine to fix N after colonization by *P. polymyxa*. When compared with previous results, we found that inoculated seedlings grown for 7, 9 and 13 months derived 30%, 66% (Bal & Chanway, 2012a) and 79% (Anand et al., 2013), respectively, of their foliar N from the atmosphere. The progressive increase in the proportion of N derived from BNF *i.e.,* %Ndfa, with seedling age suggests that BNF is an important component of N nutrition of pine in N-limited soil. Urquiaga et al. (1992) also observed an increasing reliance on BNF with seedling age, and concomitant decreasing soil N, in sugarcane cv. Krakatau. The %Ndfa rose from 6% to 55% during the interval 100 - 250 days after emergence. In addition to high %Ndfa, 13-month old seedlings treated with P2b-2R also accumulated significantly more biomass (78%) than controls, and had overcome the growth inhibition observed in seedlings of younger ages. While *P. polymyxa* possesses several characteristics that can result in plant growth promotion (Chanway, 2002), the enhanced performance of 13-month old seedlings was likely caused by an increase in the amount of N derived from BNF because the proportion of foliar N from BNF (68%-79%) (Anand et al., 2013) in older seedlings was only marginally higher than that of nine-month old seedlings (64%-66%) (Bal & Chanway, 2012a), but the concentration of foliar N (2.35%) (Anand et al., 2013) was 5-fold greater than nine-month old seedlings. The difference in total foliar N content, *i.e.,* foliar N concentration x foliar biomass, was even greater and represented an elevation of foliar N concentration from a level considered to be very severely N deficient in all nine-month old seedlings (Bal & Chanway, 2012a) and the 13-month old controls (Anand et al., 2013) to one that is adequate for healthy lodgepole pine (Ballard & Carter, 1986). The comparatively early onset of BNF and delayed seedling growth response suggest that development of a fully effective N_2-fixing bacterial population, able to enhance foliar N concentration in pine, is not a rapid process. Establishment of fully effective BNF in pine does not appear to depend on the population size of endophytic diazotrophs because P2b-2R colonization of root, stem and needle tissues in older seedlings did not differ significantly from younger seedlings (Anand et al., 2013), though it is possible that external root colonization was quantitatively related to BNF. However, if BNF is endophytic and requires physiological modifications of bacteria, *e.g.,* differentiation of *Paenibacillus* into a N_2-fixing form similar to *Rhizobium* bacteroids (Postgate, 1998), or plants, *e.g.,* the establishment of specialized sites of BNF within plant tissues or cells, to be fully effective, some time may be required for them to complete. Our observations of intracellular colonization by P2b-2R*gfp* (Figure 1; Anand & Chanway, 2013a) are consistent with this idea but further research is required to evaluate the physiology of *P. polymyxa* in association with pine as well as the relationship of bacterial population size to BNF.

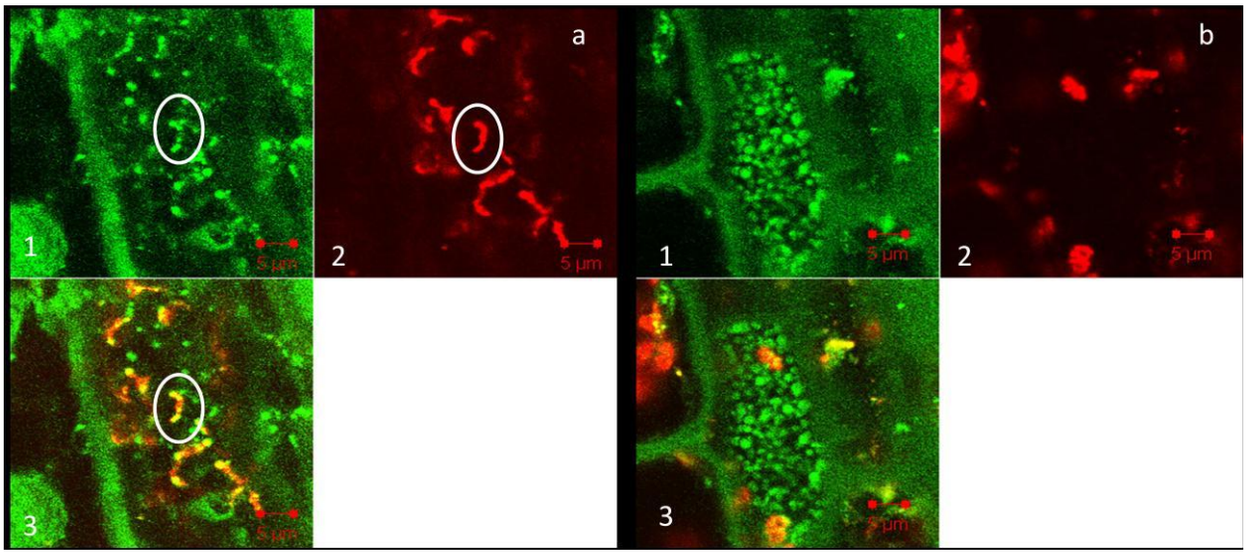

Figure 1. Colonization of cortex cells from a pine stem by green fluorescent protein (GFP) labeled *Paenibacillus polymyxa* strain P2b-2R*gfp* using confocal laser scanning microscropy. Stem sections were viewed using green channel light (Figure 1a & b, panel 1) to visualize bright green GFP-labeled bacteria or red channel light (Figure 1a & b, panel 2) to visualize chloroplasts within pine cells. Using green channel light, GFP-labeled bacteria appear as green dots in a single pine cell (Figure 1a & b, panel 1). Under red light, chloroplasts appear as larger red organelles in the same single pine cells (Figure 1a & b, panel 2). Cells viewed using green and red channel light simultaneously (Figure 1a & b, panel 3) show GFP-labeled bacteria and chloroplasts within a pine cell. The white circle in Figure 1a, panel 3 encloses (i) several GFP-labeled bacterial cells, (ii) a single chloroplast in Figure 1a, panel 2 and GFP-labeled bacteria associated very closely with the chloroplast in Figure 1a, panel 3. Complete methodology is described in Anand and Chanway (2013a).

8.3. Nitrogen fixation in gymnosperms: Colonization of seedlings

The ability of *P. polymyxa* to colonize gymnosperm seedlings internally and externally has been evaluated in several greenhouse experiments (Bent & Chanway, 2002; Bal & Chanway, 2012a,b; Anand et al., 2013), but the ability of a green fluorescent protein-tagged derivative of P2b-2R to colonize internal pine tissues (Anand & Chanway, 2013a) provided convincing evidence that P2b-2R is endophytic, notwithstanding possible differences in behavior of GFP-tagged and wild type cells (van der Lelie et al., 2009). The population densities of P2b-2R inside pine tissues were observed to be comparable to densities reported for endophytic diazotrophs of crop plants such as rice (*Oryza sativa* L.), 10^4 - 10^5 cfu/g tissue (Elbeltagy et al., 2001), surgarcane, 10^5 - 10^7 (Sevilla et al., 2001) and grape (*Vitis vinifera* L.), 10^5 -10^9 (Compant et al., 2005) as well as hybrid spruce (*Picea glauca* x *P. engelmannii*), 10^3 – 10^5 (Shishido et al., 1999) and other tree species, 10^1 – 10^7 (Izumi, 2011). Recovery of P2b-2R from stem and needle tissues suggests bacteria migrate from the roots and soil to aerial plant organs: the observation that root and stem populations decreased while needle populations increased (Anand et al., 2013) supports this idea. Compant et al. (2005) observed a similar trend in grape plantlets inoculated with *Burkholderia sp*. PsJN and suggested that the stem acts as a transportation corridor for bacteria to reach leaves, which they considered a sink for endophytes. However, it is also possible that primordial pine stem and needle tissues were colonized at germination by P2b-2R residing on the seed coat and in

the spermosphere, resulting in growth of bacterial populations *in situ* after shoots expanded, but the mechanism and patterns by which P2b-2R colonizes aerial pine tissues cannot be affirmed without further study.

We also observed intact cells from different pine stem sections that were colonized internally by GFP-labeled bacteria (Anand & Chanway, 2013a). In some cases, GFP-labeled bacteria were observed in pine cells in close proximity to chloroplasts (Fig. 1a), which raises the possibility that bacteria colonized microsites near these energy-generating organelles. GFP labelling were also observed tightly packed within other pine cells (Fig. 1b). Whether bacteria in either of these pine cells (Fig. 1) were fixing N cannot be determined, but these unique endophytic colonization patterns warrant further study. Endophytic bacteria have also been observed inside cells of grape (Compant et al., 2005), grasses (Hurek et al., 1994), sugarcane (James & Olivares, 1998) and poplar (*Populus deltoides* x *P. nigra*) (van der Lelie et al., 2009). Intracellular colonization of the pine stem cortex was rare and we were unable to inspect needle tissues for P2b-2R*gfp* so it is unclear if colonized pine cells are of biological importance. GFP-labeled bacteria may simply have detected micro-colonies of bacteria feeding on dead or dying plant cells in the stem cortex. Alternatively, it is tempting to hypothesize a possible role for intracellular bacteria in BNF considering the foliar ^{15}N dilution observed in colonized seedlings (Bal and Chanway, 2012a,b; Anand et al., 2013; Anand & Chanway, 2013b). Could these cells be specialized seats of N_2-fixation, perhaps similar to *Rhizobium* bacteroids inside cells of legume root nodules? You et al. (1991) reported root cortex cells of rice, a non-leguminous plant, contained diazotrophic bacteria and some species of algae have been found to harbor endophytic bacteria near their chloroplasts (Colombo, 1978; Preisig & Hibberd, 1984). While this is a very interesting possibility, it is premature to focus on intracellular BNF in pine without more convincing supporting data.

Results with lodgepole pine strongly suggest that seedlings can fix N_2 when colonized by *P. polymyxa* and demonstrate that N_2-fixing seedlings develop healthy foliar N levels and enhanced growth in very N-limited soil. Western red cedar may also benefit from treatment with this bacterium (Bal & Chanway, 2012b; Anand & Chanway, 2013b), but amount of BNF and plant growth promotion were reduced compared to pine. Because *P. polymyxa* P2b-2R originated from pine seedling tissue, it is tempting to speculate that some degree of plant x microbe specificity may exist in this type of association. Research with pine and cedar raises several other questions regarding the ecological importance of endophytic diazotrophs in gymnosperms. For example, is *P. polymyxa* unique in its ability to enter into this type of interaction with gymnosperms or is this a comparatively common trait in conifer forests? If *P. polymyxa* is unique, how widespread is its distribution? How long must pine and diazotroph coexist before effective N fixation occurs and is BNF restricted to the seedling stage? What are the effects of soil N on colonization and BNF by *P. polymyxa*? It is possible that endophytic diazotrophs play a key role in natural regeneration of gymnosperms at N-poor sites but whether this is true and their importance in forest ecosystems can only be ascertained through future studies.

9. Conclusions and future research

Biological nitrogen fixation is a free and environmentally benign process through which biologically useful N can be generated for plant growth. Because nitrogen fixation is most effective when bacteria associate with plants to some degree, future research should focus on the various known plant associative and symbiotic nitrogen fixing systems. However, owing to the complexity of root-nodule based symbioses, colonization of plants by endophytic diazotrophs seems to hold the greatest potential for expansion of BNF to plant species that do not normally fix N_2. Initial steps might include mass screening of known endophytic diazotrophs with commercially important plant species while continuing studies of effective nitrogen fixing associations involving plants that are colonized by endophytic diazotrophs. Such studies will help to elucidate the molecular, physiological and ecological details of this potentially useful plant x microbe interaction.

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Author details

C.P. Chanway[1], R. Anand[2] and H. Yang[1]

1 Dept of Forest and Conservation Sciences, University of British Columbia, Vancouver, Canada

2 Children's Hospital, Vancouver, Canada

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