Chapter 9
Nitrogen-Fixing Endophytic Bacteria for Improved Plant Growth

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9.1 Introduction

Nitrogen is an essential element for life as we know it. It is a primary component of all the proteins and nucleic acids in living cells. Plants without sufficient nitrogen are stunted, have yellow chlorotic leaves, and produce low yields. Although the atmosphere contains nearly 80% dinitrogen gas, this form of nitrogen is not biologically available to most life. To produce enough food for a growing human population, global agriculture production has become more dependent on the use of chemical fertilizers at the cost of the environment, climate, and possibly human health. The Haber–Bosch process for producing ammonia for plant growth spurred the Green Revolution, allowing significant increases in crop yield. The process requires large amounts of natural gas, high pressure, and high temperature (Vance 2001). Developed at a time of inexpensive and plentiful fossil fuels, the benefits of increased crop yield at low cost were reaped without apparent thought to the environmental costs. With the rising prices of energy, the cost of fertilizer has also dramatically increased. There is, therefore, a need both financially and environmentally to improve crop growth without reliance on chemical fertilizers.

The use of synthetic nitrogen fertilizers has a number of negative effects on the environment (Cocking 2005). Less than half of the applied fertilizer is actually taken up by the crops; the remainder is lost to leaching, run-off into streams, or metabolism by soil microbes (Bhattacharjee et al. 2008). Elevated nitrogen run-off into water systems causes a rapid growth of algae from the input of nutrients, depleting the water of oxygen and resulting in widespread dead zones. The ammonia in the soil is metabolized by some bacteria into reactive nitrogen forms. These include nitric oxide (NO) and nitrogen dioxide (NO_2), collectively termed NOx.

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These reactive forms become ozone that is damaging to plants and animals, and is a significant greenhouse gas. Another form of reactive nitrogen is nitrous oxide (N_2O) that is 300 times more potent than carbon dioxide as a greenhouse gas. As a result, farmlands have a strong impact on air pollution, contributing 8.9 million tons of nitrogen annually (Peplow 2005). In addition to these environmental impacts of the use of chemical fertilizers, there may also be more direct effects on human health. A review by the US National Institutes of Health suggested that increased nitrate concentrations in drinking water could contribute to cancer, and nitrogen-related air pollution could be elevating the incidence of cardiopulmonary ailments (Townsend and Howarth 2010).

Prior to the use of chemical fertilizers, farmers relied more on biological nitrogen fixation (BNF) well known to occur in the legume–rhizobium symbiosis. In this complex interaction, leguminous plants such as peas and beans recruit specific rhizobial bacterial species, form nodules on the roots, and permit the infection of the nodules with nitrogen-fixing bacteria. The plant provides sugars that it synthesizes through photosynthesis to its symbionts as long as they provide usable nitrogen to the plant. These specific bacteria are able to biologically convert atmospheric dinitrogen gas into ammonia using the enzyme nitrogenase and high levels of ATP, the energy currency of living cells. The interaction between the legume and the rhizobium is highly regulated, and a great deal of communication occurs between the two organisms as the symbiosis is being established. The relationship benefits not only the plants and microbes but also the farmer, since good crop yields are obtained without the addition of fertilizer. Often, non-leguminous crops are rotated with legumes because there is sufficient fixed nitrogen remaining in the soil for another crop.

For decades, researchers studied the legume–rhizobium relationship with the goal of initiating such symbiosis between rhizobium and non-leguminous crop plants. Although spontaneous nodules sometimes developed, and rare infections occurred, none of the forced interactions yielded significant transfers of fixed nitrogen to the crop. A breakthrough was made in the 1980s when research into the microbes living within sugarcane, a non-leguminous tropical crop species, indicated that a significant amount of biologically fixed nitrogen was transferred to the plant from these internal symbionts. Since then, researchers began to focus more on this hitherto unstudied ecosystem within plants.

The interior of plants provides habitat for a wide range of bacteria and fungi that benefit the plant host by increasing nutrient acquisition, stress tolerance, pathogen resistance, and aiding in phytoremediation of environmental pollutants (Reis et al. 2000; Hirsch 2004; Cook et al. 1995; Siciliano et al. 2001; Vance 2001; Bhattacharjee et al. 2008). The term “endophyte” was coined by Dobereiner to describe these internal microbes that spend a significant part of their life cycle within plants and do not cause disease (Dobereiner 1992). Although endophytes can increase plant growth through a variety of mechanisms, an especially intriguing method of growth promotion is through providing fixed nitrogen. Only a subset of these endophytic microorganisms “fixes” nitrogen into the usable forms of ammonia and nitrate. Such microbes are termed diazotrophs, and have been

found in major food crops including rice, maize, sugar cane, sweet potato, and coffee. In some cases, the nitrogen-fixing bacteria from these crop plants associated not only with the plant species from which they were isolated but also with unrelated plants. Applications of nitrogen-fixing bacteria on non-leguminous crops could potentially increase growth and yield while reducing the need for chemical fertilizers (Bhattacharjee et al. 2008).

Although the study of endophytes and their hosts is relatively young compared to the study of rhizobia and legumes, some similarities and differences are beginning to be elucidated (Fig. 9.1). In both cases, plant signal molecules (organic acids, carbohydrates, and amino acids) are released in the exudate coming from the roots, and these signals act as chemo-attractants to the microorganisms. The bacteria need to come into contact with the plant root. In the case of rhizobia and legumes, a complex interaction takes place with many variations in detail but generally leading to root hair curling, invasion via an infection thread, and ultimate colonization of the root nodule (Masson-Boivin et al. 2009). By contrast, endophytic associations including the details of how endophytes enter, pass the casparian strips in the plant root endoderm, and colonize the plant interior are not yet known. Since plant genotype is a key factor in determining how much nitrogen is obtained from endophytes (Reis et al. 2000) and because different plant species in the same environment can have different endophytic species compositions, it seems clear that there is specificity as in the legume–rhizobia symbiosis. In a microarray study of the plant genes induced by inoculation with endophytic bacteria, it was revealed that certain plant signaling genes (receptors and protein kinases) were expressed exclusively in the inoculated plants (Nogueira et al. 2001). More studies of this type

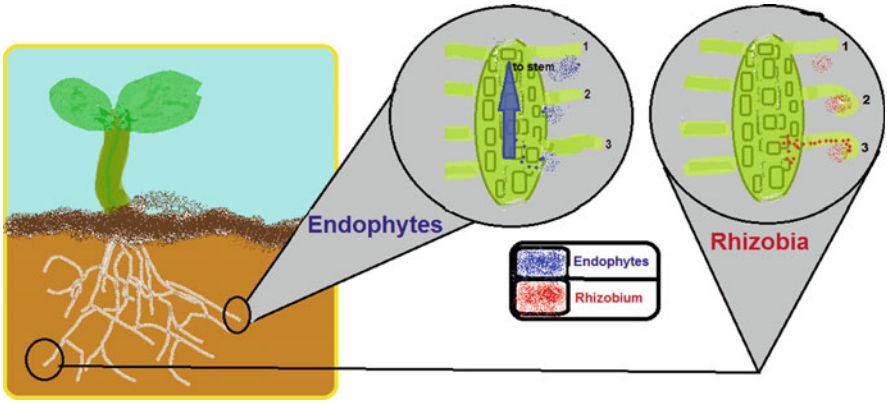

Fig. 9.1 Comparison of infection and colonization of rhizobia and endophytes. In step 1, both classes of bacteria move to the root area in response to plant signals. In step 2, the rhizobia initiate root hair curling, and then enter the plant in step 3 through an infection thread of plant origin. The rhizobia then colonize a specific region within the resulting root nodule. By contrast, endophytes generally enter at lateral root junctions (or at wound sites) in an opportunistic fashion in step 2. They colonize the plant interior and can move upward, in some cases spreading to the stems and leaves, in the vascular tissue and in intercellular spaces (Figure courtesy of Christina M. Doty)

must be conducted in order to determine which plant genes are involved generally in plant–endophyte interactions.

Unlike most rhizobial infections that enter in a plant-assisted direct manner, endophytes seem to enter more in an opportunistic way. Using fluorescently labeled bacteria, researchers have determined that the common entry points for endophytes are at lateral root junctions and at wound sites, similar to pathogen entry points. Low levels of cell wall degrading enzymes are probably released in order to enter without initiating the plant defense response. Upon successful entry, the endophytes appear to form microcolonies within the vascular tissue or in the spaces between plant cells. The initial colonization of the plant by an endophyte is largely driven by the chance opportunity that a competent endophyte that can persist within that plant (Hardoim et al. 2008) will happen to be near enough to perceive the plant signals, and be able to enter at either a wound, or at disturbed cells at lateral root junctions or root cap. Therefore, it is probably a strong advantage for plants growing in nutrient-poor environments to be able to propagate asexually through cuttings. In this way, the new plant already comes with a viable collection of appropriate endophytes (Stettler 2009).

Another difference between the rhizobium–legume and endophyte–plant partnership is in the protection of the nitrogenase enzyme from oxygen. In order for nitrogenase to reduce dinitrogen gas, the immediate environment of the enzyme must be anaerobic. Rhizobia within root nodules are in a low-oxygen environment, and leghemoglobin within the nodule scavenges any free oxygen. Although in most cases it is not known how diazotrophic endophytes protect nitrogenase from oxygen, many possibilities are available (Gallon 1992). In the case of the sugarcane endophyte, the ability of *Gluconacetobacter diazotrophicus* to fix nitrogen in the aerobic environment of the stem is attributed to "respiratory protection" whereby the extremely rapid respiration from metabolism of high levels of sucrose within the sugarcane stem leads to a microaerobic environment needed for the oxygen-sensitive nitrogenase enzyme (Flores-Encarnacion et al. 1999). Other endophytes may use physical barriers including exopolysaccharides to exclude oxygen or interior vesicles to compartmentalize the nitrogenase, or biochemical methods to scavenge interior oxygen (Gallon 1992).

9.2 Methods of Analysis

Most studies of diazotrophic endophytes begin with the isolation of microbes from within the plant that are capable of growing on nitrogen-free medium (outlined in Fig. 9.2). First, the outer surface of the plant tissue is sterilized using a bleach solution, ethanol, or mercuric chloride in order to eliminate contaminating microorganisms on the outside of the plant that may or may not be involved in any symbiosis with the plant. Adequate surface sterilization can be assessed by either placing the uncut plant tissue on medium and monitoring for bacterial growth, plating the last of the rinse solutions, or by using microscopy

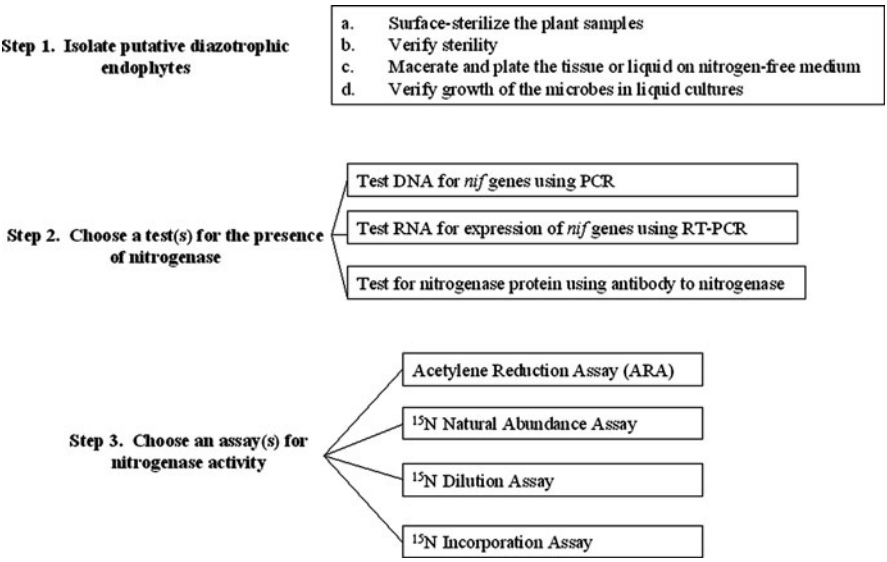

Fig. 9.2 Flowchart of the methods for analysis of diazotrophic (N-fixing) endophytes

(Dong et al. 2003). To isolate the bacteria within the plant, the tissue is macerated and then either directly placed on medium supporting the growth of most nitrogen-fixing bacteria or suspended in a solution that is then spread on the plates. Although this is the standard method for isolating endophytes, a recent paper demonstrated that hundreds of times more colonies could be counted when using the ELISA technique (Silva-Froufe et al. 2009). The standard technique assumes that the endophytes are not tightly adhering to the plant cells and that they do not aggregate, but neither assumption is probably correct. Using this fluorescent tagging method allows more accurate quantification of the number of endophytes within the plant. However, the ELISA technique requires nitrogenase antibody that can recognize a broad range of nitrogenases, so this method will need to be used mostly for quantifying specific endophyte species.

Development of appropriate methods for quantifying nitrogen fixation by microbes within plants has been challenging. Unlike the situation with rhizobia and legumes where all the nitrogen fixation is occurring in a concentrated area in root nodules, diazotrophic endophytes can fix nitrogen throughout the plant body. Furthermore, since the bacteria are within the plant, it is difficult to establish whether the fixed nitrogen is being transferred to the plant as in the rhizobium symbiosis or whether the plant is obtaining the nitrogen only after the microbial cells die and lyse open. Each of the methods described below has advantages and disadvantages, so the use of a combination of methods provides the most convincing evidence for nitrogen fixation by the endophytes.

The most common second stage of analysis following verification of growth in nitrogen-free medium is to determine if the bacterium contains the genes encoding

nitrogenase. This enzyme is required for the conversion of dinitrogen gas to ammonia, and is conserved in all nitrogen-fixing organisms including the Achaea, a separate Domain from the Bacteria. Genomic DNA is isolated from the endophyte and is subjected to PCR with primers that are general enough (degenerate) to amplify most nitrogenase sequences. Nested PCR using primers that bind within the area amplified by the first set of primers is performed to eliminate non-specific DNA bands. The resulting PCR product is then cloned and sequenced to verify that the amplified DNA does correspond to the nitrogenase gene. The nitrogenase enzyme is encoded by three genes, *nifH*, *nifD*, and *nifK*; however, most researchers test only for one of the genes, usually *nifH*. One problem with using the presence of the *nif* gene as evidence for nitrogen-fixing ability is that often the gene is not actually expressed while the endophyte is in the plant. A *nifH*-based microarray was developed to study the diazotrophs of Kallar grass (Zhang et al. 2007). The study revealed that, although there were many different species of diazotrophic endophytes present, only one species, *Azoarcus*, was the dominant species that was expressing the nitrogenase gene *in planta*. In addition to examining the endophytes for the nitrogenase genes (DNA) or expression (RNA) of those genes, is to directly assay for the presence of the nitrogenase enzyme. However, as mentioned for the ELISA study described above, antibodies against the nitrogenase enzyme are usually too specific and would work mostly for detecting the type of nitrogenase from which the antibody was raised.

A common method of testing if an endophyte has a functional nitrogenase enzyme is the acetylene reduction assay (ARA). This assay takes advantage of the fact that acetylene, like dinitrogen gas, has a triple bond that is reduced by nitrogenase. Reduction of acetylene results in the formation of ethylene gas which is easily quantified using gas chromatography. This assay is highly effective in rhizobium–legume studies since the bacteria fix nitrogen while concentrated in small root nodules. It is more difficult to do similar studies with endophytes that are unevenly distributed within the plant. Furthermore, wounded plant tissue releases ethylene, a plant stress hormone, thus interfering with the quantification of ethylene produced from acetylene reduction. To solve this dilemma, cultures of the bacteria are often tested independently of the plant.

The high percentage of endophytic strains that test negative for the ARA yet have the nitrogenase gene and are able to grow on nitrogen-free medium indicates that this assay may not be an accurate measure of diazotrophy for this class of organisms. It has been shown in studies of plant growth-promoting rhizospheric bacteria that there seems to be selection for microbes that can decrease the amount of ethylene produced by the host plant (Hardoim et al. 2008). Using the enzyme ACC deaminase, the bacteria break up the precursor for ethylene from the plant, causing the plant to not over-react to stress. But some plant-associated bacteria may also have a mechanism for degrading the ethylene directly. Work in the 1970s revealed that some soil bacteria can metabolize ethylene (de Bont and Albers 1976). Perhaps some of the putative diazotrophic endophytes that tested negative for ARA were degrading the ethylene they produced from acetylene.

The stable isotope of nitrogen, ^{15}N, is used in three different approaches for quantifying nitrogen fixation. The natural abundance assay relies on more of the lighter ^{14}N being in the atmosphere and more of the heavier ^{15}N being in soil, such that an organism that received most of its nitrogen via nitrogen fixation would have a ratio of more ^{14}N than ^{15}N. Another approach is to deliberately label the soil with ^{15}N using labeled ammonium sulfate, and then testing for a dilution of the isotopic signature with ^{14}N from the atmosphere. A possible problem with this assay is that it requires the addition of ammonium to the soil that may inhibit nitrogenase gene expression that is tightly regulated to the amount of available nitrogen. This method has the advantage of being easier to detect than natural abundance, and it is less expensive than the $^{15}N_2$ incorporation assay which is the most direct method for studying nitrogen fixation. In this assay, the plant is placed in a sealed chamber with some of the air spiked with the labeled nitrogen gas. If the plant is receiving any nitrogen through nitrogen fixation from its endophytes, the plant tissue will become labeled. Highly pure $^{15}N_2$ gas is available to avoid complication with contaminating ^{15}N-labeled ammonia gas. The best study would be to use *nifH* mutants to determine if the plant tissue was labeled only when inoculated with the wild-type strain and not with the mutant (Sevilla et al. 2001) and to verify that the label was incorporated into plant proteins (Iniguez et al. 2004).

9.3 Diazotrophic Endophytes

As described in introductory section, diazotrophic endophytes refer to nitrogen-fixing microorganisms within plants. Following is a review of the current research on these diazotrophs from different groups of crop plants.

9.3.1 Diazotrophic Endophytes from Monocot Crops: Sugarcane, Rice, and Maize

Although this chapter is focused on crop plants, the reader is strongly encouraged to read the reviews on diazotrophic endophytes of the forage grass, Kallar grass (*Leptochloa fusca*) that have been well studied (Reinhold-Hurek and Hurek 1998; Elmerich et al. 1997; Hurek et al. 2002), and other grasses (Reis et al. 2000; Dalton et al. 2004).

The best example of a monocot crop species with nitrogen-fixing endophytes is sugarcane (*Saccharum* spp.). This crop species was grown successfully for many decades without addition of fertilizer. ^{15}N experiments conducted in the early 1990s suggested that a few sugarcane varieties received large amounts of their nitrogen from BNF (Urquiga et al. 1992). Experiments by Boddey et al. demonstrated that 80% of the total nitrogen for three sugarcane varieties could be attributed to BNF

(Boddey 1995). The vegetative propagation of sugarcane by nodal sections and the traditional farming methods without fertilizer may have selected for superior diazotrophs.

The dominant diazotrophic endophyte of sugarcane is *G. diazotrophicus* (previously termed *Acetobacter diazotrophicus*). It grows best in very high levels of sucrose (100 g/L), is acid-tolerant, and exists in high numbers within plants, but it survives poorly in soil. This bacterium is capable of secreting nearly half of its fixed nitrogen in a form that the plant can utilize (Cohjo et al. 1993). A variety of different methods has been used to demonstrate that *G. diazotrophicus* fixes nitrogen and transfers that nitrogen to the host plant. Since inoculation of plants with endophytes could result in increased mass because of other factors besides fixed nitrogen, inoculation with a mutant strain that no longer fixes nitrogen makes an excellent experiment. Inoculation with *nif* mutants (non-nitrogen fixing) of this organism resulted in reduced sugarcane growth under nutrient-limited conditions compared with inoculation with the wild-type strain, suggesting that fixed nitrogen is transferred to the plant under normal symbiotic conditions (Sevilla et al. 2001). The plants inoculated with the wild-type (nitrogen-fixing) strain had more tillers, leaves, and height, and this improved growth was sustained during the 4-month field study. For example, there was an increase of 40% more fresh cane weight in the plants inoculated with the wild-type compared with the mutant. The colonization of the plants by the wild-type and mutant strains was verified and shown to be the same; therefore, the effect was specific to the ability to fix nitrogen. Immunogold labeling of nitrogenase demonstrated that the enzyme was expressed in the endophytic microbes. The ^{15}N incorporation test was also conducted. The shoots and roots of the plants inoculated with the wild-type strain were labeled with ^{15}N to a small extent, further demonstrating that nitrogen fixation was occurring within the plant. When nitrogen was not a limiting nutrient; however, the microbe still enhanced plant growth, suggesting that it helps the plant in multiple ways, probably from the production of plant hormones (IAA and gibberellins).

Although *G. diazotrophicus* was shown to fix nitrogen within the sugarcane, it seems that other endophytes must also contribute substantial nitrogen. A larger study that used the ^{15}N dilution method and seven different combinations of five endophyte species demonstrated that the best BNF growth effect occurred when all five strains were inoculated onto the plants (Oliveira et al. 2002). The endophytes were from the genera *Azospirillum*, *Burkholderia*, *Herbaspirillum*, and *Gluconacetobacter*. A doubling of the BNF contribution was seen between inoculation with the whole consortia of five strains compared to inoculation with only one strain. The dry weights were increased substantially after 400 days between the uninoculated controls and the multi-species inoculated plants. The improvements were substantial despite the fact that the sterility of the uninoculated controls was not maintained, and these control plants did get colonized during the experiment. The growth effects seen in this study demonstrate that inoculating micropropagated plants with a consortia of nitrogen-fixing endophytes has long-term yield enhancements even under normal conditions of competition with other microbes.

Burkholderia and *Herbaspirillum* species are important contributors to the growth enhancements of sugarcane. *B. vietnamiensis* colonizes sugarcane in large numbers (115,000 colony-forming units per gram of plant tissue), it reduces acetylene, the indirect assay for nitrogen fixation, and it contains the nitrogenase genes (Govindarajan et al. 2006). Growth experiments in the field demonstrated that inoculation with the strain MG43 resulted in a 20% increase in growth. In a study comparing *B. tropici* and *Herbaspirillum* using fluorescent in situ hybridization (FISH), it was demonstrated that both microbes colonized the sugarcane within 12 h after inoculation but in different sites (Oliveira et al. 2009).

As rice is the staple food in the diet for over 40% of the world's population, it is critical to develop sustainable methods to increase yields. Using traditional agriculture, 4 tons of rice per hectare can be grown with 50 kg of nitrogen per hectare coming from BNF (Stoltzfus et al. 1997). Using modern agriculture, a yield of 5–8 tons per hectare can be achieved but at the cost of 60–100 kg of nitrogen added through chemical fertilizer per hectare. In order to increase yields without chemical fertilizers, research into diazotrophic endophytes of rice began. By screening 133 rice endophytes, 13 isolates of diverse genera were identified that were positive for both the *nifD* gene and the ARA (Stoltzfus et al. 1997). In a more recent study of rice plants in Vietnam, 13 diazotrophic endophytes were identified using the same criteria but all of these were *Burkholderia* species (Govindarajan et al. 2008). The most active in the ARA was named *B. vietnamiensis* strain MGK3. By marking the strain with *gusA*, it was determined that the strain entered at lateral root junctions and the root tip, and colonized intercellular spaces of the root cortex after 15 days. Inoculation of seedlings with this strain resulted in increases of rice yield of 5–12%. When mixed with other diazotrophic endophyte species including *G. diazotrophicus*, *Azospirillum lipoferum*, and *H. seropedicae*, the combination resulted in a yield increase of 9–23%. ^{15}N dilution assays both in pots and in the field demonstrated nitrogen fixation, with up to 42% of the N derived from nitrogen fixation. Inoculation of rice with diazotrophic endophytes increased yield, root biomass, and tiller number.

As maize is also a staple crop and, like sugarcane, is now being used for biofuels, it is another crop for which sustainable agricultural practices need to be developed that also meet the growing demand. Studies in Mexico revealed that *B. unamae* was a prevalent endophyte in maize that colonized roots and stems, reduced acetylene, and had the *nif* gene cluster (Caballero-Mellado et al. 2004). In another study, *B. tropici* was identified from both maize and its ancestor, teosinte, and grew on nitrogen-free medium and was ARA-positive (Reis et al. 2004). In a more recent study, 178 endophytes were isolated from maize in Uruguay that could grow on nitrogen-free medium (Montanez et al. 2009). Only 11 of these were found to be ARA(+), and these included species from the familiar genera *Pantoea*, *Pseudomonas*, *Rhanella*, *Herbaspirillum*, *Azospirillum*, and *Rhizobium*. The maize cultivars used in this study were tested by the ^{15}N dilution assay and shown to be using BNF.

The diazotrophic maize endophyte, *Klebsiella pneumoniae*, was able to fix nitrogen not only in its original host but also in the major food crop, wheat (*Triticum aestivum*). In an excellent model paper, it was proven that fixed nitrogen

was transferred from the endophyte to the wheat cultivar, Trenton (Iniguez et al. 2004). Addition of the wild-type endophyte relieved nitrogen-deficiency symptoms and increased total nitrogen of the plant, whereas inoculation with a nitrogenase mutant or a killed control did not yield this effect. After 6 weeks without fertilizer, the control plants were stunted and chlorotic while the plants inoculated with the wild-type strain had more chlorophyll, 50% more dry weight, 285% more total N in shoots, and 654% more N in roots. ^{15}N dilution experiments verified that not only was nitrogen fixation occurring within the plant, the fixed nitrogen was utilized by the plant since the chlorophyll also had the isotopic signature. Unfortunately, these positive results were limited to one cultivar of wheat; there was no effect for two other wheat cultivars, a rice line, two barley lines, and several lines of maize.

9.3.2 *Diazotrophic Endophytes from Dicot Crops: Sweet Potato and Coffee*

Compared to the amount of research on diazotrophic endophytes of monocots, there is much less known about them in dicot crops. The nitrogen-fixing microbes, *Azospirillum* (Hill et al. 1983) and *Gluconacetobacter* (Paula et al. 1991), were isolated from sweet potato plants (*Ipomoea batatas*), the fifth most important food crop in developing countries. Nitrogen fixation was observed in sweet potato using the ^{15}N natural abundance assay (Yoneyama et al. 1998). A survey of *nif*H-containing microbes as conducted using sweet potato from farms in Uganda and Kenya that did not use chemical fertilizers (Reiter et al. 2003). This culture-independent assay revealed that there were 17 different microbial groups, as separated by RFLP analysis. About 50% of the species were *Rhizobium* sp. Sweet potato plants in Japan were also analyzed (Terakado-Tonooka et al. 2008). Using the ^{15}N natural abundance assay, it was determined that 40% of the N was from BNF, an amount of 103–135 kg N per hectare. Rather than looking for the presence of *nifH* as in the African study, they used RT-PCR to look for active expression of the nitrogenase gene in endophytes of the stems and storage roots. As with the African study, most of the sequences were of *Rhizobium* species, but there were also *H. seropedicae*, *B. vietnamiensis*, *B. unamae*, *Pelomonas saccharophila*, *Cyanobacteria*, and *Azohydromonas australica* sequences. Since there were no *nifH* sequences in the sprouts prior to transplanting, all of these microbes colonized the plants from the soil environment. By studying active *nif*H expression, the authors were able to see a seasonal effect. The highest *nifH* expression was in summer and not in autumn, correlating with the highest photosynthetic rate of the plant.

In a small study of American sweet potato endophytes, it was found that 4 out of 11 isolates produced the plant hormone, auxin, and 1 isolate was a putative nitrogen-fixing endophyte (Khan and Doty 2009). This isolate (SP-A) grew in nitrogen-free medium and had the nitrogenase gene. Although the *nifH* fragment

was 99% identical to that of *Azotobacter vinelandii*, the 16S rDNA sequence was 100% identical to *Stenotrophomonas maltophilia*. It is interesting to note that most studies do not report the sequence of both the 16S rDNA and the *nif* gene, so it is unknown how common it might be that endophytes harbor nitrogenase sequences from unrelated soil bacteria. Recent studies involving endophyte-assisted phytoremediation of pollutants have demonstrated that bacteria can conjugate *in planta*, transferring plasmids from poor colonizers to effective endophytes of the roots, and upward to endophytes of the stem (Taghavi et al. 2005; Weyens et al. 2009). It may be that plasmids containing nitrogenase genes or other symbiosis-related genes are also transferred among endophytes.

In coffee plants of Mexico, there are some diazotrophic plant-associated bacteria, namely *Gluconacetobacter* species. *G. diazotrophicus*, *G. johannae*, and *G. azotocaptans* were identified from the coffee tissues (Jimenez-Salgado et al. 1997) and in the rhizosphere (Fuentes-Ramirez et al. 2001). More studies have yet to be carried out to determine if the coffee plants are directly benefitting from these microbes, however.

More evidence of beneficial impact on a dicot species from diazotrophs has been documented for alfalfa (*Medicago* sp.). The *K. pneumoniae* strain Kp342 isolated from maize was inoculated onto the dicots, alfalfa and *Arabidopsis*, and the monocots, wheat and rice (Dong et al. 2003). This strain was a highly effective colonizer of monocots, requiring an inoculum amount of only one colony-forming unit to yield a high colonization. By contrast, the type strain for this species, a clinical isolate, required a 1,000-fold higher inoculum concentration and still colonized to a 100-fold lower level. Because of this strain difference, it is clear that the endophyte is an active participant in the colonization process. There were also differences in the colonization of monocots and dicots. The monocots were colonized to a level 100× higher than the dicots; however, the dicot species in the study were not the natural hosts for this maize endophyte.

9.3.3 Diazotrophic Endophytes from Bioenergy Crops: Poplar and Willow

In most of the previous examples of diazotrophic endophytes isolated from plants, the crop species were tropical. Only recently research has begun on diazotrophic endophytic microbes of the temperate climate, high biomass plants, poplar (*Populus* sp.) and willow (*Salix* sp.). Endophytic *Rhizobium tropici* was isolated from hybrid cottonwood (Doty et al. 2005). Although this species is known for its ability to nodulate an unusually wide diversity of legumes, its endophytic nature in non-legumes had not been previously reported.

In their native habitat, poplar and willow grow vigorously alongside rivers in rocky substrates with no organic matter (Fig. 9.3). Analysis of the stem tissues of poplar and willow in such an environment revealed large numbers of putative

Fig. 9.3 Poplar (*Populus trichocarpa*) and willow (*Salix sitchensis*) grow in nutrient-limited, rocky substrates in their native habitat in Western Washington state, USA. A variety of putative N-fixing endophytes were isolated from the stems (Doty et al. 2009)

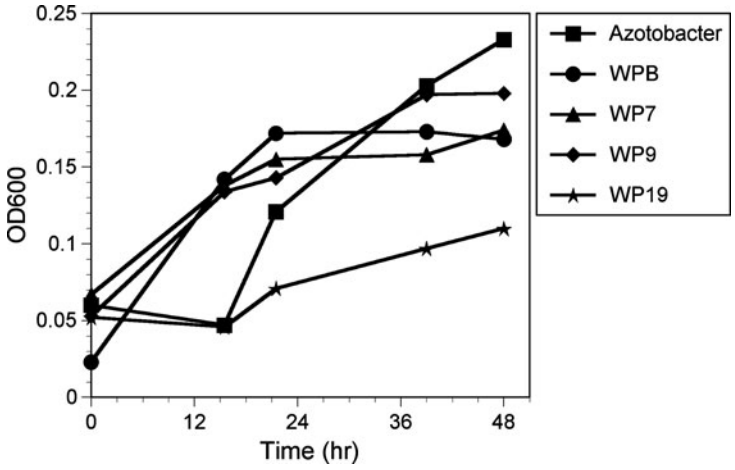

Fig. 9.4 Growth of wild poplar (WP) endophytes in nitrogen-free medium. *Azotobacter vinelandii* was included as a positive control. Although several of the endophytes had similar growth, only strain WPB was positive in the acetylene reduction assay (Doty et al. 2009)

diazotrophic endophytic bacteria (Doty et al. 2009). The bacterial strains belonged to the genera *Burkholderia*, *Rahnella*, *Herbaspirillum*, *Enterobacter*, *Acinetobacter*, *Sphingomonas*, and *Pseudomonas*. The strains grew well in nitrogen-free medium, and some examples are shown in Fig. 9.4. Of 14 isolates that grew on nitrogen-free medium that were analyzed further, only 5 were positive in the ARA, and of these, only 1 had strong activity. This isolate, *B. vietnamiensis*

strain WPB, was chosen for further study (Xin et al. 2009). The *nif*HDK cluster was cloned and sequenced, and showed a high identity with other *B. vietnamiensis* strains. The ^{15}N incorporation assay as well as the ARA demonstrated that strain WPB was fixing nitrogen aerobically. In addition, WPB produced high levels of the phytohormone, indole-3-acetic acid. When Kentucky bluegrass (Xin et al. 2009), corn, and rice (Redman and Doty, unpublished) were inoculated with WPB, the growth of the plants was increased. Inoculated bluegrass had 42% higher overall biomass, and 37% higher nitrogen content. The effectiveness of WPB for growth enhancement of poplar is currently being studied at the levels of greenhouse growth and in the field at sites with different levels of nitrogen using both wild-type and *nif* H mutant strains.

Given the natural habitat of poplar and willow alongside rivers that flood regularly, an advantage of diazotrophic endophytes over diazotrophs requiring root nodulation seems clear (Doty et al. 2009). While any investment into the development of root nodules would be lost after each flooding event that brings in additional silt and rocks, investments into symbioses with endophytes within the stems would be unaffected. In the areas from which the poplar and willow endophytes were isolated, alder trees with their root nodules of nitrogen-fixing *Frankia* were also present, but much further from the banks of the river, outside the flood zone. It remains to be seen if other colonizing plant species subjected to regular flooding are more likely to have symbioses with diazotrophic endophytes rather than with nitrogen-fixing bacteria requiring root nodules.

9.4 Conclusions

The use of nitrogen-fixing endophytes to enhance the growth of agricultural crops and bioenergy crops could become an effective strategy for more environmentally sustainable production. With the growing need for increased food and bioenergy biomass but with a greater understanding of the implications of conventional-intensive agriculture, the time is right for a greater emphasis on biological mechanisms for improved plant growth. Adding beneficial microbes to only the rhizosphere of agricultural crops has not been very successful (Sturz and Nowak 2000). Endophytes may have an advantage over this approach since there would be less competition as there is when adding soil bacteria to the established rhizosphere communities. In some of the cases described in this review, it is clear that diazotrophic endophytes can provide a substantial amount of fixed nitrogen to the plants. The strain and cultivar specificities seen in some of the examples point to the necessity of further research to understand the plant–microbe communications necessary for effective nitrogen fixation in non-legumes.

Some caution must be exercised before widespread application of this technology. In many of the studies, *Burkholderia* isolates were found to perform best in the nitrogen fixation assays and strongly increased plant growth. However, some isolates of *Burkholderia* species are associated with human lung diseases

(Parke 2005). Species in the Burkholderia cepacia complex (Bcc) have enormous potential as biocontrol agents, as degraders of harmful environmental pollutants, and as plant growth promoters. They are naturally abundant in soil, water, and plants. Some isolates are registered for use as microbial pesticides. But on the other side, some Bcc strains are opportunistic human pathogens, and some can cause fatal lung infection in the patients with cystic fibrosis (CF). Persons with this genetic disorder have an abnormally thick mucus in the lungs that can be colonized by some bacteria. At this time, it is not clear what marks certain strains as being pathogenic. In a study of several isolates of *B. cenocepacia* from clinical and environmental settings, it was determined that the environmental samples were not invasive according to an in vitro assay (Pirone et al. 2008). However, all the isolates were able to persist in lungs of infected mice, and all had the virulence-associated genes (*opc*I, *cci*L, and *ami*L). *B. vietnamiensis* was one of the species with strong plant growth-promoting activity. This species is present in 5.9% of the patients with CF, but there are no reports of it being associated with Cepacia syndrome, decreased survival, or transmissibility (Chiarini et al. 2006). In 2003, the US Environmental Protection Agency ruled that Bcc strains can be used only for bioremediation (Chiarini et al. 2006). However, since Bcc strains have high identity in the 16S rDNA genes, but only 30–60% DNA hybridization, it seems that an all-encompassing ban on Bcc strains is not appropriate given the wide diversity in this genus. It has been suggested that a revised ruling be made such that non-pathogenic Bcc strains can be used in agriculture (Chiarini et al. 2006).

Considering the enormous potential impact of the use of nitrogen-fixing endophytes on global crop production, funding for research in this area is limited. Many of the papers described in this review cited only partial support, institutional support, or individual fellowships as the funding sources for the research. There needs to be a greater public awareness of this environmentally-friendly, biologically based method for increasing plant growth. The study of diazotrophic endophytes impacts both basic and applied science. The discovery of diazotrophic endophytes in poplar and willow helps explain the biology of these and maybe other colonizing tree species. It may be that many non-leguminous plants in nutrient-poor environments have symbiotic relationships with nitrogen-fixing endophytes. It is essential that sustainable agricultural practices are developed that use resources more efficiently, maintain environmental health, and yet increase the food supply. With more study of the impacts of diazotrophic endophytes on plants, we can better understand the role of endophytes in natural systems as well as utilize that information for a new revolution in agriculture that is better for the environment.

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