



## Nitrogen Fixation with Non-Leguminous Plants

Said Muhammad<sup>1\*</sup>, Zaheer Abbas<sup>1</sup>, Samina Bashir<sup>2</sup>, Ghulam Qadir<sup>1</sup>, Salman Khan<sup>3</sup>

<sup>1</sup> Department of Botany, Hazara University Mansehra, Pakistan.

<sup>2</sup> Department of Biotechnology, Quaid-i-Azam University, Islamabad, Pakistan.

<sup>3</sup> Department of Genetics Hazara University Mansehra, Pakistan.

### \*Corresponding Author

**Abstract:** Nitrogen is generally considered one of the major limiting nutrients in plant growth. The biological process responsible for reduction of molecular nitrogen in ammonia called nitrogen fixation. An extensive diversity of nitrogen fixative bacterial species fit to most phyla of the bacteria domain has the ability to settle the rhizosphere and to communicate with plants. Leguminous plants can produce their nitrogen by association with differentiation on their particular host plants from a specialized organ, the root nodule. Other symbiotic relations contain heterocystous cyanobacteria, although a growing numbers of nitrogen fixative species have been identified such as *Azobactor*, *Azospirillum*, *Endophytes bacteria*, *Frankia* are colonizing on the root surface of cereal crops and in certain conditions, inside the roots of a variety of cereal crops tree, and pasture grasses. The Basic and advanced facets of these associations are discussed in this review.

**Keywords:** Non-leguminous plants, Nitrogen fixation, *Azobactor*, *Azospirillum*, *Endophytes bacteria*, actinorhizal association

### INTRODUCTION

The "green revolution" in agriculture in developing world, which led to a large increase in cereal production since 1960s, has been a result of the development of plant genotypes highly sensitive to chemical fertilizers, particularly nitrogen. It takes about 18.5 Mcal of fossil energy to produced 1 kilogram of fertilizer nitrogen and even however, unlike other fertilizers, these elements have unlimited supply in the air, it is more than 6 times the energy needed to produce phosphate or potassium fertilizers (Da Silva *et al.*, 1978). There is solid proof that non-leguminous field crops sometimes advantage from relations with diazotrophs. Significantly, the potential advantage from N<sub>2</sub> fixation usually comes from spontaneous associations that can rarely be managed in agricultural practice. Especially for dry land systems, these associations seem very unreliable to elevate the nitrogen status of plants. But, recent technical developments linking the induction of nodular structures on the roots of cereal crops, like as wheat and rice, offer the prospect that reliable symbioses with free-living diazotrophs, such as the *Azospirillum brasilense*, or with *rhizobia* could possibly be reached.

*Azospirillum brasilense* and other associative bacterial systems has been the subject of extensive research, using a variety of assessment techniques to understand the diastrophic rhizocoenosis.

These studies clearly indicate that these associations are ruled by many soils, water, nutrient, agrochemical, plant genotype and other biological factors. Considerable efforts have been made so far to select efficient bacterial strains as inoculants and identify host genotypes that support high level of nitrogenous activity in addition to other useful features of effective associative relationships.

The Knowledge so far about how the N<sub>2</sub>-fixation system in rice functions suggests the need for optimal management practices to ensure a greater contribution of plant-microbe associations. Nitrogen is a limiting nutrient in most environments, with the main nitrogen reserve in the biosphere being molecular nitrogen of the atmosphere. Molecular nitrogen cannot be directly adapted by plants, but becomes available through the process of biological nitrogen fixation that only prokaryotic cells have established. Proliferation of bacteria in soil adhering to the root surface was discovered in the last nineteenth century, together with the discovery of nitrogen fixation. The term “rhizosphere” was later invented by Hiltner in 1901 to refer to soil directly surrounding the roots under the influence of the plant (Rovira, 1991).

For many years, it was a limited number of bacterial species were nitrogen fixers (Postgate, 1981), but over the past 30 years, nitrogen fixation has been shown to be an active property in most of the phyla of Bacteria and also in methanogenic Archaea (Young, 1992). In addition to leguminosae, many plant families can also fix nitrogen globally. These are non-legumes because they are called somewhat negatively, fix as much nitrogen as legumes, but for various historical reasons, they have been relatively neglected by scientists. Many of these non-nitrogens fixing legumes are native to North America and are found mainly in rice. Sandy soils slow down nitrogen. The most common are alders (*Alnus* spp.) and *Myrica pensylvanica* in the North. While, On the west coast of North America, in various woodland and open coniferous forest, various species of *Ceanothus* and *Alnus* are nitrogen fixers other than legumes. In the arid mountains of the west, buffaloberry (*Shepherdia canadensis*), bitterbrush (*Purshia tridentata*), and the mountam mahogames (*Cercocarpus* spp) are important. A few Australian pine weeds (*Casuanna* spp.), is important for the stabilization of beaches and throughout the central west and East Coast, the autumn and Russian olives (*Elaeagnus umbellata* and *E. angustifolia*) were widely planted along the banks of the highway. All these plants thrive in poor soils where few other plants grow. Their ability to fix nitrogen is an important factor in their survival in hospitable conditions for ordinary plants.

In legumes and non-legumes, nitrogen fixation is achieved living within the roots of the plant. This is a classic example of mutually useful symbiosis: the plant delivers bacteria with sugars and a variety of minerals, and bacteria deliver the host with a usable source of nitrogen. The genus *Frankia* unlike the *Rhizobia* that exist as separate cells, actinomycetes develop into long chains of cells like fungal hyphae but much smaller. All plants infected with *Frankia*, except for trees and shrubs, while among legumes, annual herbs and trees can be infected with rhizobia. The legumes (genus *Parasponia*) which include about 200 plant species in 19 genera and 8 families are nodulated by nitrogen-fixing micro-organisms called *Frankia*.

The actinomycetal nature of *Frankia* was noticed using cytological techniques for many years when the Torrey's group isolated a strain of *Frankia* and cultivated, in-vitro for the first time haemoglobin were observed in the nodules of *Comptonia peregrina*, and *Elaeagnus angustifolia*, and trace amount were found in *Ceanothus americanus* and *Darirca gfoemerata* (Callaham *et al.*, 1978).

This review examines the nitrogen cycle in mountain farming where non-legume N<sub>2</sub>-fixation by legumes is likely to be important for crop growth. Evidence of associative uptake is provided by accumulation of N<sub>2</sub> in the top 15 cm soil under grasses, from Nitrogen budgets for crop production obtained from pot experiments and in field in tropical and temperate environments, measurements of nitrogenase (C<sub>2</sub>H<sub>2</sub> reduction) activity, uptake of <sup>15</sup>N<sub>2</sub> by plants and <sup>15</sup>N isotope dilution.

Factors influencing activity such as the plants contribution of carbon substrate and the effectiveness of its use by bacteria and responses of cultures to inoculation with *Azospirillum* are detailed. Decomposition of crop residues, particularly straw, can withstand high levels of N<sub>2</sub>-fixation. Cyanobacteria in the form of soil surface crusts also actively fix nitrogen in many environments.

Fixation by nodulated non-leguminous trees, *Casuarina* and *Parasponia*, has useful effects on some cropping systems in Asia. Actinorrhizal symbioses Strains of the bacteria *Frankia* infect a wide range of non-leguminous trees and shrubs. These (actinorrhizal) plants fix a large amount of nitrogen in forests, especially in poor soils and some can grow in dry or acidic conditions

These attributes create them excellent primary colonizers of post-glacial and post-mining soils. Even though nodules have been observed on actinorrhizal plants for more than 150 years, *Frankia* was not isolated in pure culture until 1978. Many different strains of the bacterium have since been isolated. Some have recently been grown on a scale sufficient for inoculant production. *Frankia* behave very well as a bacterium that infect legumes, but much less is known about the actinorrhizal symbioses. The bacterium multiplies rapidly in the root zone and there are signs of recognition among the *Frankia* and the plant. The bacterium invades the root and the plant responds to this invasion by the development of a nodule. In contrast to soy nodules, in which the oxygen concentration is somewhat low, actinorrhizal nodules contain oxygen at approximately atmospheric levels.

*Frankia* develops vesicles, or pockets, in the nodules of most host plants that apparently protect the nitrogen from oxygen and fix it. Another group contains some trees and nitrogen fixation in non-leguminous shrubs, whose roots become, infected with bacterium *Frankia*, e.g. alder tree.

The study of the partners of these symbioses and their connections offers new perspectives and unique opportunities to obtain genetic information that has parallels in legumes. A hemoglobin-like complex, capable of binding and releasing oxygen easily, has recently been identified in the nodules of some actinorrhizal plants. It could be similar in function to legemoglobin in legumes—maintenance of low *Azospirillum* of oxygen flux. Bacteria of the genus *Azospirillum* are nitrogen fixing association rhizobacteria that are found in close association with plant roots.

They can have helpful effects on plant growth and the yield of many agronomic crops under various environmental and soil conditions. In the case of rice, *A. lipoferum* and *A. brasilense* have been isolated from the roots and stems<sup>11</sup> and *A. amazonense* has been isolated from the roots<sup>12</sup>. Microscopical evidence as to the endophytic nature of *Azospirillum* in rice has been presented<sup>13</sup> and the colonization of 2,4-D-induced para-nodules by an ammonium-excreting mutant of *A. brasilense* has been reported<sup>14</sup>. An ammonium-excreting mutant of *A. brasilense* (Wa3) promoted better growth of wheat plants compared to wild type<sup>15</sup> and another strain (C3) was able to transfer the nitrogen fixed directly to the (S. P. Saikia, Vanita Jain., 2007). N<sub>2</sub> is available to rice even in fields that have been planted for many years without fertilizer application. (Sen., 1928) have reported the presence of heterotrophic N<sub>2</sub>-fixing bacteria in the rice root.

However, the importance of his suggestion was deserted until (Yoshida and Ancajas., 1971) found that some N<sub>2</sub>-binding activity was associated with wetland rice root. Nitrogen-fixing bacteria constitute a significant percentage of the total microflora of the rhizosphere of lowland rice. Using the acetylene reduction method, (Ishizawa *et al.*, 1970) and (Yoshida and Ancajas., 1971) found high nitrogenous activity in roots of low land rice.

Submergence seems to provide suitable conditions for N<sub>2</sub>-fixation on rice roots grown under low land conditions. *Acetobacter diazotrophicus* was shown to be the major supplier of stable nitrogen to sugarcane, which accounted for the capacity of certain varieties to be continuously cropped for many years in the same soil in the total absence of added nitrogen fertilizer. Besides fixing nitrogen, *A.*

*diazotrophicus* produces plant hormones like as auxin. Therefore, the bacteria could enhance plant growth by nitrogen fixation and hormone production. The endophyte is passed from one crop to the next through the standard vegetative reproduction by stem piece. nitrogen fixing did not occur at rates sufficient to facilitate high yields of sugarcane, (Cavalcante & Döbereiner., 1988) searched for microorganisms potentially involved in sugarcane tissues and isolated a diazotrophic bacterium, *Glucono acetobacter diazotrophicus*, before called *Acetobacter diazotrophicus*. Several extra N<sub>2</sub>-contributing diazotrophic endophytes were then associate with sugarcane including two *Herbaspirillum* species (Cavalcante & Döbereiner., 1988; Baldani *et al.*, 1992; 2005), *Azoarcus* spp. (Reinhold *et al.*, 1993) and *Azospirillum brasilense* (Bellone and Bellone., 2006).

The hope of increased crop yields soil inoculation with N<sub>2</sub>-relating bacteria goes back almost a century to Caron in Germany. *Azotobacter* was one of the organisms involved in early research. Favorable results were reported in Russia by (Kostychev *et al.*, 1926), although the results in Russia and elsewhere were generally inconsistent and variable (Mishustin and Shil'Nikova., 1971).

The problem of inconsistency has not been solved by using well authenticated inoculum strains. One of the problems with *Azotobacter* is its poor performance of colonizing of the rhizosphere, except for the *Azotobacter paspali* – *Paspalumnotatum* association (Dobereiner., 1974). Associative and entophytic nitrogen fixers, Nitrogen-fixing plant growth-promoting rhizobacteria. Nitrogen-fixing bacteria that contribute to plant growth or the prevention and suppression of diseases are called plant growth promoting rhizobacteria (PGPR).

Their isolation from the roots of forage grasses and cereal crops and many other plants, in both natural and cultivated ecosystems, extensively performed (Döbereiner and Pedrosa., 1987; Baldani and Baldani., 2005). This led to identifying two groups with respect to the degree of association with the hostplant: rhizospheric and endophytic colonizers.

Classical microbiological techniques involving cultivation of bacteria identified soil bacteria belonging to genera such as *Azospirillum*, *Azotobacter*, *Alcaligenes*, *Bacillus* *Beijerinckia*, *Campylobacter*, *Derxia*, several members of Enterobacteriaceae (*Klebsiella*, *Pantoea*) and *Pseudomonas stutzeri* (Rennie *et al.*, 1982; Balandreau., 1983; Elmerich *et al.*, 1994) As most of these strains were isolated from surface-sterilized root samples, this suggests that proportions of these cells are protected from sterilizing agents, but it may also reflect some colonization of the root tissues. Other isolates, such as those of *Azoarcus* (Hurek and Reinhold-Hurek., 2002), *Burkholderia* (Caballero-Mellado *et al.*, 2004), *Herbaspirillum* and *Glucon acetobacter* (Baldani and Baldani., 2005), or *K. pneumoniae strain 342* (Chelius and Triplett., 2000), happened to belong to endophytes. The complete genome of *Azoarcus* and *P.stutzeri* A1501 was established genomes projects of *Azospirillum*, *Azotobacter*, *Herbaspirillum* and *Gluconacetobacter* are under completion.

Colonization of the root system, *Azospirillum* as a model bacterium. Colonization of the root surface has been best studied in *Azospirillum*. Spirillum-like bacteria were first isolated by (Beijerinckin., 1923), they were rediscovered by (Johanna Döbereiner., 1974), (Von Bülow and Döbereiner., 1975). The *Azospirillum* genus was described by (Tarrant *et al.*, (1978), it belongs to the Alpha proteobacteria (Schmid and Hartmann., 2007). *A. brasilense*, *A. lipoferum*, *A. amazonense*, *A. irakense*, *A. halopraeferens*, *A. largimobile* and *A. doebereineriae*. *Azospirillum* species display an extremely wide ecological distribution and are associated in nature with a wide diversity of plants, including those of agronomic importance such as wheat, rice, sorghum and maize and several non-gramineous species (Döbereiner and Pedrosa., 1987).

These bacteria are aerobic non-fermentative chemo organotrophs, vibrioid to S-shaped, containing poly hydroxy alkanate granules (PHA). In liquid medium, motility is ensured by a polar flagellum. In some species (*A.brasilense*, *A. lipoferum*, *A. amazonense*), later flagellation allows swarming on a

solid surface.

Another important property of azospirilla is the ability to differentiate resistant forms that are non-motile ovoid cyst-like cells, much larger than vegetative cells and surrounded by a thick capsule (Lamm and Neyra.,1981). Cyst formation is concomitant with cell aggregation into macroscopic flocks occurring in some culture conditions. Encapsulated *azospirilla* show greater resistance to desiccation and heat. Cyst formation and production of siderophores and bacteriocins (Tapia-Hernández *et al.*,1990) are likely to play a role in survival of these bacteria under unfavorable conditions and in competition with other members of the soil microflora. Bacterial motility as well as chemotactic responses towards root exudates are involved in the initial step of the root colonization process (Vande Broek *et al.*,1998).

Attachment to the root system is mediated by the polar flagellum and is followed by irreversible anchoring of the bacteria (Steenhoudt and Vanderleyden., 2000). The polar flagellum is glycosylated and binds wheat root, whereas the lateral flagella are not essential during the adsorption phase. As *rpoN* controls flagellar biogenesis, *rpoN* mutants are impaired in colonization. An operon carrying chemotaxis genes (*che*) was known in *A.brasilense* (Hauwaerts *et al.*, 2002). However, *cheB* and *cheR* mutants were only partially impaired in their chemotactic response, suggesting multiple chemotaxis systems in this bacterium (Stephens *et al.* 2006). Several genes governing motility in *A. brasilense* Sp7 have been mapped onto a 90 MDa plasmid, while other genes are located on the chromosome.

The complete nucleotide sequencing of this plasmid was established (Vanbleu *et al.*, 2005). The plasmid also carries several genes governing surface polysaccharides that might play some role in the colonization process. The structural gene for major outer membrane protein, *omaA*, was characterized and found to encode an adhesion with high affinity to roots (Burdman *et al.*, 2001).

A transcriptional regulator of the LuxR-UhpA family, *flcA*, controlling differentiation into cysts and flocculation, was also described as playing a role in surface colonization (Pereg-Gerk *et al.*, 1998). Electron micrographs of colonized roots revealed that *azospirilla* was anchored to roots by fibrillar material, probably similar to the fibrillar material produced through flocculation. The Bacteria colonize the rhizoplane and are found in large numbers in the emergence of lateral roots and also near the root cap (De Oliveira Pinheiro *et al.*, 2002). The degree of invasion of plant tissues differs from strain to strain. In an early report, using fluorescent antibody staining techniques, it was found that *Azospirillum* colonize the intercellular spaces between the epidermis and the cortex of the root (Schank *et al.*, 1979).

Other techniques involving bacteria carrying *gus* and *lac Z* on the root surface, but also for assaying *nif* gene expression during the colonization process (Vandebroek *et al.*, 1993; Arsène *et al.*, 1994). The fluorescent in situ hybridization (FISH) technique developed for confocal laser scanning microscopy (CLSM), enabled in situ localization of bacteria on the root. It established that endophytic colonization of some *Azospirillum* strain such as *A. brasilense* Sp245 was found in the intercellular spaces of the root epidermis (Rothballer *et al.*, 2003). Attempts to identify genes expressed in the early stages of host plants interaction are under way. Nitrogen-fixing endophytes. An increasing number of reports describe the presence of nitrogen-fixing bacteria in plant tissues of a host plant with no diseases symptoms; *Gluconacetobacter* and *Herbaspirillum* (Hurek and Reinhold-Hurek., 2002, Lery *et al.*, 2008). Endophytes multiply and spread within plant tissues without causing damage.

Early steps in infection may be similar to those reported with rhizospheric bacteria, initially involving surface colonization at the site of emergence of root hairs (Hurek and Reinhold-Hurek, 2002). In the case of *Azoarcus*, type IV pili have been shown to be essential for that process and hydrolytic enzymes, or endoglucanases, participate in tissue penetration. The concentration of bacteria improved after sterilization of the root system can reach up to 108 CFU per g of dry weight.

Another characteristic is systemic spreading of bacteria, which can be found in plant xylem vessels and in shoots, as described in case of sugarcane infection with *G. diazotrophicus* (James and Olivares.,1998) and in case of infection of the C4-gramineous plant *Miscanthussinensis* by *H. frisingense* (Rothballer *et al.*, 2007). Bacteria are located mostly in intercellular spaces, but intracellular location is also seen in dead cells (Hurek and Reinhold-Hurek., 2002).

The main difference from rhizospheric bacteria is the fact that endophytic do not persist in the soil. Therefore, the frontier between rhizospheric and endophytic systems is not.

#### **Future Prospective:**

Brazilian sugar cane varieties are known to can obtain very considerable contributions of biologically fixed N under field conditions. Recent data suggest that water supply is dangerous to the maintenance of high BNF activity. A recent trial (16 areas totaling 900 ha) at a sugar cane plantation in Campos (NE Riode Janeiro State) showed that where year-round irrigation was used there was no response of ratoon cane to 200 kg ha<sup>-1</sup> of urea fertilizer and yields of sugarcane averaged 95 t ha<sup>-1</sup>. Because of this trial the plantation managers abandoned N fertilization on 4000 ha of irrigated cane making an annual economy of US \$ 250,000 (Boddey, 1995). All attempts to isolate *Acetobacter diazotrophicus* from sugar cane from anywhere the world have been successful except where high N fertilizer additions have been made. Apart from Brazil no data are yet available for the occurrence of *Herbaspirillum* spp. in this crop. The complete absence of *A. diazotrophicus* in soil and the restricted occurrence of *Herbaspirillum* spp. Suggest that once selected (or even genetically manipulated) strains of these bacteria are established in can plants in the field, the chances are slight that wild type strains will contaminate the plants to compete with them. For phyto sanitary reasons the use of direct planting of monoxenic micro propagated cane plantlets is now being tested at several cane plantations in S- oPaulo state and this may soon offer an economically viable opportunity to propagate cane plants infected by superior strains of endophytic diazotrophs. With regard to wetland rice it is evident that for BNF to contribute to high rice yields a great improvement in its efficiency is required.

A meeting held at IRRI (Philippines) in 1992 was dedicated solely to this subject. Three possible strategies to increase BNF contributions to wetland rice were discussed (Bennett and Ladha., 1992). Induction of "nodulation" of rice using hydrolytic enzymes (AI-Mallah *et al.*, 1989), 2,4-D (Kennedy and Tchan., 1992) or other means (Rolfe and Bender.,1990) and subsequent infection with *Rhizobium*, *Azospirillum* or other diazotrophs. Huge progress has been made over past 10 years toward understanding nitrogen fixation at the molecular level. Transfer to plants of nitrogen-fixation genes is an intriguing consideration.

First look at the process of biological nitrogen fixation, then the more recent developments in molecular genetics that can make such gene transfer possible in the years to come. We have successfully attempted to construction in vitro cellulo-biochemical model of N<sub>2</sub> fixing symbiosis between wheat seedlings and *Azospirillum*, there are many challenges to be negotiated before this model system can be considered as a true symbiosis.

At this stage the main questions that need to be asked about the model are: i) is there direct transmission of fixed nitrogen to host plant, or is any N<sub>2</sub> fixation simply bound to the growth of *Azospirillum*? If the latter is true, it is unlikely that sufficient plant. It is surprising, considering the potential importance that has attributed to *Azospirillum*, that little is known of any mechanism of transfer of fixed nitrogen to a host plant. Indeed, no evidence exists that a direct transfer occurs at all (Danneberg, Zimmer, & Bothe, 1989) approaches integrating technical developments and goals in biological sciences could lead to crop improvement.

Research on extending nitrogen-fixing symbiosis to rice using molecular and genetic approaches is under way. I conclude that non-legume N<sub>2</sub>-fixation contributes significantly to the production of

some major cereal crops in temperate and tropical environments and open the way for further research to increase the yield of cereal crops without disturbing the environment.

Cost of crop decrease up to 60 percent to develop such crop and requirement of nitrogen fertilizer also decrease due to such crops.

## References

1. Al-Mallah, M. K. K, Davey MR and Cocking EC 1989 Formation of nodular structures on rice seedlings by rhizobia. *J. Exp. Bot*, 40, 473-478.
2. Arsene, F., Katupitiya, S., Kennedy, I. R., & Elmerich, C. (1994). Use of lacZ fusions to study the expression of nif genes of *Azospirillum brasilense* in association with plants. *Molecular plant-microbe interactions: MPMI (USA)*.
3. Balandreau, J. (1983). Microbiology of the association. *Canadian journal of microbiology*, 29(8), 851-859.
4. Baldani, J. I., & Baldani, V. L. (2005). History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience. *Anais da Academia Brasileira de Ciências*, 77(3), 549-579.
5. Baldani, V. L. D., Baldani, J. I., Olivares, F., & Döbereiner, J. (1992). Identification and ecology of *Herbaspirillum seropedicae* and the closely related *Pseudomonas rubrisubalbicans*. *Symbiosis-Rehovot*, 12, 65-65.
6. Bennett, J., & Ladha, J. K. (1992). Introduction: feasibility of nodulation and nitrogen fixation in rice.
7. Boddey, R. M. (1995). Biological nitrogen fixation in sugar cane: a key to energetically viable biofuel production. *Critical Reviews in Plant Sciences*, 14(3), 263-279.
8. Broek, A. V., Michiels, J., Van Gool, A., & Vanderleyden, J. (1993). Spatial-temporal colonization patterns of *Azospirillum brasilense* on the wheat root surface and expression of the bacterial nifH gene during association. *Mol. Plant-Microbe Interact.*, 6, 592-600.
9. Burdman, S., Dulguerova, G., Okon, Y., & Jurkevitch, E. (2001). Purification of the major outer membrane protein of *Azospirillum brasilense*, its affinity to plant roots, and its involvement in cell aggregation. *Molecular plant-microbe interactions*, 14(4), 555-561.
10. Caballero-Mellado, J., Martínez-Aguilar, L., Paredes-Valdez, G., & Estrada-De Los Santos, P. (2004). *Burkholderia unamae* sp. nov., an N<sub>2</sub>-fixing rhizospheric and endophytic species. *International Journal of Systematic and Evolutionary Microbiology*, 54(4), 1165-1172.
11. Callaham, D., Deltredici, P., & Torrey, J. G. (1978). Isolation and cultivation in vitro of the actinomycete causing root nodulation in *Comptonia*. *Science*, 199(4331), 899-902.
12. Cavalcante, V. A., & Döbereiner, J. (1988). A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant and soil*, 108(1), 23-31.
13. Chelius, M. K., & Triplett, E. W. (2000). Immunolocalization of Dinitrogenase Reductase Produced by *Klebsiella pneumoniae* in Association with *Zea mays* L. *Applied and environmental microbiology*, 66(2), 783-787.
14. Da Silva, J. G., Serra, G. E., Moreira, J. R., Conçaves, J. C., & Goldemberg, J. (1978). Energy balance for ethyl alcohol production from crops. *Science*, 201(4359), 903-906.
15. Danneberg, G., Zimmer, W., & Bothe, H. (1989). Energy transduction efficiencies in nitrogenous oxide respirations of *Azospirillum brasilense* Sp7. *Archives of microbiology*, 151(5), 445-453.
16. de Bellone, S. C., & Bellone, C. H. (2006). Presence of endophytic diazotrophs in sugarcane juice. *World Journal of Microbiology and Biotechnology*, 22(10), 1065-1068.

17. de Oliveira, P. R., Boddey, L. H., James, E. K., Sprent, J. I., & Boddey, R. M. (2002). Adsorption and anchoring of *Azospirillum* strains to roots of wheat seedlings. *Plant and Soil*, 246(2), 151-166.
18. Dobereiner, J. (1974). Nitrogen-fixing bacteria in the rhizosphere. *The biology of nitrogen fixation*, 86-120.
19. Dobereiner, J., & Pedrosa, F. O. (1987). Nitrogen-fixing bacteria in nonleguminous crop plants. Science Tech Publishers.
20. Hauwaerts, D., Alexandre, G., Das, S. K., Vanderleyden, J., & Zhulin, I. B. (2002). A major chemotaxis gene cluster in *Azospirillum brasilense* and relationships between chemotaxis operons in  $\alpha$ -proteobacteria. *FEMS microbiology letters*, 208(1), 61-67.
21. Hurek, T., Handley, L. L., Reinhold-Hurek, B., & Piché, Y. (2002). *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. *Molecular Plant-Microbe Interactions*, 15(3), 233-242.
22. Ishizawa, M., & Endo, H. (1970). Mutagenic effect of a carcinogen, 4-nitroquinoline 1-oxide, in bacteriophage T4. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 9(1), 134-137.
23. James, E. K., & Olivares, F. L. (1998). Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. *Critical Reviews in Plant Sciences*, 17(1), 77-119.
24. Kennedy, I. R., & Tchan, Y. T. (1992). Biological nitrogen fixation in non-leguminous field crops: Recent advances. *Plant and Soil*, 141(1-2), 93-118.
25. Kostychev, S., Sheloumova, A., & Shul'gina, O. (1926). Nitrogen content of soils of the southern coast of Crimea. *Sovetskii Agronomy*, 1.
26. Lamm, R. B., & Neyra, C. A. (1981). Characterization and cyst production of *azospirilla* isolated from selected grasses growing in New Jersey and New York. *Canadian Journal of Microbiology*, 27(12), 1320-1325.
27. Lery, L. M., Coelho, A., von Kruger, W. M., Gonçalves, M. S., Santos, M. F., Valente, R. H., ... & Teixeira, K. R. (2008). Protein expression profile of *Gluconacetobacter diazotrophicus* PAL5, a sugarcane endophytic plant growth-promoting bacterium. *Proteomics*, 8(8), 1631-1644.
28. Mishustin, E. N., & Shil'nikova, V. K. (1971). Nitrogen fixation by blue-green algae. *Biological fixation of atmospheric nitrogen*. MacMillan Press. London. Raising blue-green algae is easy. *Intensive Agric*, 16(2), 14-15.
29. Pereg-Gerk, L., Paquelin, A., Gounon, P., Kennedy, I. R., & Elmerich, C. (1998). A transcriptional regulator of the LuxR-UhpA family, FlcA, controls flocculation and wheat root surface colonization by *Azospirillum brasilense* Sp7. *Molecular plant-microbe interactions*, 11(3), 177-187.
30. Postgate, J. (1981). Microbiology of the free-living nitrogen-fixing bacteria, excluding Cyanobacteria. In 4. International Symposium on Nitrogen Fixation. Canberra, ACT (Australia). 1 Dec 1980.
31. Reinhold-Hurek, B., Hurek, T., Gillis, M., Hoste, B., Vancanneyt, M., Kersters, K., & De Ley, J. (1993). *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigenus* sp. nov. and *Azoarcus communis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 43(3), 574-584.



32. Rennie, R. J., Freitas, J. D., Ruschel, A. P., & Vose, P. B. (1982). Isolation and identification of N<sub>2</sub>-fixing bacteria associated with sugar cane (*Saccharum* sp.). *Canadian Journal of Microbiology*, 28(5), 462-467.
33. Rolfe, B. G., & Bender, G. L. (1990). Evolving a *Rhizobium* for non-legume nodulation. In *Nitrogen Fixation* (pp. 779-780). Springer, Boston, MA.
34. Rothballer, M., Schmid, M., & Hartmann, A. (2003). In situ localization and PGPR-effect of *Azospirillum brasilense* strains colonizing roots of different wheat varieties. *Symbiosis*, 34(3), 261-279.
35. Rothballer, M., Schmid, M., & Hartmann, A. (2007). Diazotrophic bacterial endophytes in Gramineae and other plants. In *Prokaryotic symbionts in plants* (pp. 273-302). Springer, Berlin, Heidelberg.
36. Rovira, A. D. (1991). Rhizosphere research-85 years of progress and frustration. In *The rhizosphere and plant growth* (pp. 3-13). Springer, Dordrecht.
37. Saikia, S. P., & Jain, V. (2007). Biological nitrogen fixation with non-legumes: An achievable target or a dogma? *Current science*, 317-322.
38. Schenk, M. K., & Barber, S. A. (1979). Phosphate Uptake by Corn as Affected by Soil Characteristics and Root Morphology 1. *Soil Science Society of America Journal*, 43(5), 880-883.
39. Schmid, M., & Hartmann, A. (2007). Molecular phylogeny and ecology of root associated diazotrophic  $\alpha$ - and  $\beta$ -proteobacteria. In *Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations* (pp. 21-40). Springer, Dordrecht.
40. Sen, J. (1929). Is bacterial association a factor in nitrogen assimilation by rice plants. *Agric. J. India*, 24, 229-231.
41. Steenhoudt, O., & Vanderleyden, J. (2000). *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS microbiology reviews*, 24(4), 487-506.
42. Stephens, M., Sloan, J. S., Robertson, P. D., Scheet, P., & Nickerson, D. A. (2006). Automating sequence-based detection and genotyping of SNPs from diploid samples. *Nature genetics*, 38(3), 375.
43. Tapia-Hernández, A., Mascarua-Esparza, M. A., & Caballero-Mellado, J. (1990). Production of bacteriocins and siderophore-like activity by *Azospirillum brasilense*. *Microbios*, 64(259), 73-83.
44. Tarrand, J. J., & Krieg, N. R. Dö bereiner, J. (1978). A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can J Microbiol*, 24, 967-980.
45. Van de Broek, A., Lambrecht, M., & Vanderleyden, J. (1998). Bacterial chemotactic motility is important for the initiation of wheat root colonization by *Azospirillum brasilense*. *Microbiology*, 144(9), 2599-2606.
46. Vanbleu, E., Choudhury, B. P., Carlson, R. W., & Vanderleyden, J. (2005). The nodPQ genes in *Azospirillum brasilense* Sp7 are involved in sulfation of lipopolysaccharides. *Environmental microbiology*, 7(11), 1769-1774.
47. Vandebroek, B. C., & Keane, A. R. (1993). U.S. Patent No. 5,249,141. Washington, DC: U.S. Patent and Trademark Office.
48. Von Bülow, J. F., & Döbereiner, J. (1975). Potential for nitrogen fixation in maize genotypes in Brazil. *Proceedings of the National Academy of Sciences*, 72(6), 2389-2393.

49. Yoshida, T., & Ancajas, R. R. (1971). Nitrogen Fixation by Bacteria in the Root Zone of Rice  
1. Soil Science Society of America Journal, 35(1), 156-158.
50. Young, J. P. W. (1992). Phylogenetic classification of nitrogen-fixing organisms. Biological  
nitrogen fixation, 1544, 43-86.