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Nitrogen Fixing Biofertilizers; Mechanism and Growth Promotion: A Review

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Nitrogen is generally considered one of the major limiting nutrients in plant growth. The biological process responsible for reduction of molecular nitrogen into ammonia is referred to as nitrogen fixation. A wide diversity of nitrogen-fixing bacterial species belonging to most phyla of the Bacteria domain has the capacity to colonize the rhizosphere and to interact with plants. Leguminous and actinorhizal plants can obtain their nitrogen by association with rhizobia or Frankia via differentiation on their respective host plants of a specialized organ, the root nodule. Other symbiotic associations involve heterocystous cyanobacteria, while increasing numbers of nitrogen-fixing species have been identified as colonizing the root surface and, in some cases, the root interior (Nitrogen fixing endophytes) of a variety of cereal crops and pasture grasses. Bacterial mechanisms of plant growth promotion include biological nitrogen fixation (BNF), synthesis of phytohormones, environmental stress relief, inhibition of plant ethylene synthesis, as well as increasing availability of nutrients like iron through production of siderophores.

Key words: Biofertilizers, Nitrogen, Rhizobium, Actinorhizal plants, Siderophores.

Bio-fertilizers are products containing living cells of different types of microorganisms which when, applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promotes growth by converting nutritionally important elements (nitrogen, phosphorus) from unavailable to available form through biological process such as nitrogen fixation and solubilization of rock phosphate[1]. These potential biological fertilizers would play key role in productivity and sustainability of soil and also protect the environment as eco-friendly and cost effective inputs for the farmers[2].

Fixed nitrogen is a limiting nutrient in most environments, with the main reserve of nitrogen in the biosphere being molecular nitrogen from the atmosphere. Molecular nitrogen cannot be directly assimilated by plants, but it becomes available through the biological nitrogen fixation process that only prokaryotic cells have developed. For many years, a limited number of bacterial species were believed to be nitrogen fixers[3], but in the last 30 years nitrogen fixation has been shown to be a property with representatives in most of the phyla of Bacteria and also in methanogenic Archaea[4]. The property of symbiotically fixing nitrogen within nodules of vascular plants is found in two major groups of bacteria not phylogenetically related: rhizobia (Alpha-proteobacteria) that associate essentially with leguminous plants belonging to one superfamily of angiosperms (Fabaceae)[5], and Frankia (in Actinobacteria)that associate with a broader spectrum of plants from eight families[6]. Another important group of nitrogen-fixing bacteria is that of the cyanobacteria, found in association with a large variety of higher and lower plants, fungi and algae[7]. Associative symbiosis refers to a wide variety of nitrogen-

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fixing species that colonize the root surface of non-leguminous plants, without formation of differentiated structures[8]. Among these, the frequent isolation of bacteria from surface-sterilized root led to identification of a new category, nitrogenfixing endophytes[9].

The nitrogen fixation process

Enzymatic conversion of molecular nitrogen to ammonia is catalyzed by nitrogenase, an oxygen labile enzyme complex highly conserved in free living and symbiotic diazotrophs. The most common form of nitrogenase, referred to as Mo-nitrogenase or conventional nitrogenase, contains a prosthetic group with molybdenum, FeMoCo(Iron-Molybdenum-Cobalt group). Some bacteria, such as Azotobacter and several photosynthetic nitrogen fixers (including some cyanobacteria), carry additional forms of nitrogenase whose cofactor contains vanadium (V-nitrogenase) or only iron (Fe-nitrogenase)[10]. The nitrogenase enzyme, which has been purified from different sources, is composed of two metalloproteins. Component 1, also designated Mo-Fe protein, is a tetramer of 220,000 Da composed of two non-identical subunits α and β, while component 2, also designated Fe protein, is a dimer of 68,000 Da formed by identical subunits (Fig. 1). Two FeMoCo are bound to α subunits of the MoFe protein. In addition, there are two other prosthetic groups containing 4Fe- 4S clusters. 'P-clusters' are covalently bound to cysteine residues of MoFe protein bridging α and β subunits. The third type of Fe-S group is linked to the Fe protein[11]. Nitrogen reduction is a very complex mechanism not as yet fully elucidated. The result of net reduction of molecular nitrogen to ammonia is generally accounted for by the following equation:

$N_2 + 16\,Mg\text{-}ATP + 8\,e^- + 8H^+ = 2NH_3 + H_2 + 16\,Mg\text{-}ADP + 16\,Pi$

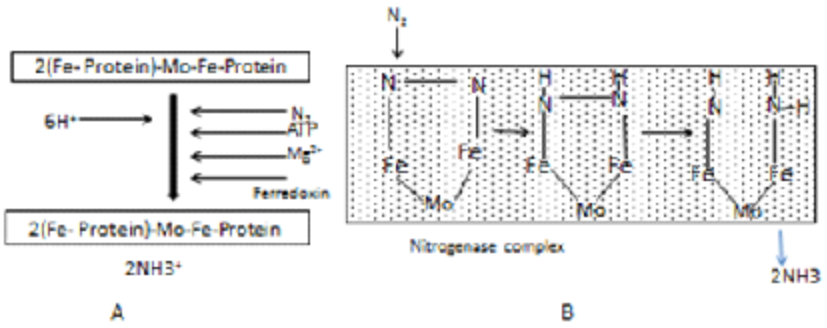

Fig. 1. Nitrogen fixation by Nitrogenase complex (A) and mechanism of N_2 conversion by component I (B)

Two metalloproteins *i.e.* larger Mo-Fe-protein and smaller Fe-protein components are involved in N_2 fixation. Fe-protein interacts with ATP and Mg^{++}, and receive electron from ferredoxin or flavodoxin when it is oxidized. Mo-Fe-protein of nitrogenase complex combines with the reducible substrates *i.e.* N_2 and yields two molecules of NH_3(Fig. 2). It appears that N_2 is reduced step-wise without breaking N-N bond until the final reduction and production of ammonia is accomplished. Finally two molecules of NH_3 are released from the enzyme. Finally, electron is transferred to oxidize Mo-Fe-protein which becomes reduced and Fe-protein is oxidized. It is the reduced form of Mo-Fe-protein which combines with N_2 and other substrates to result in NH_3 and other various products with respect to substrate. H_2 produced during this reaction is further utilized by some microorganisms which possess hydrogenase[12]. Reutilization of H_2 increases nitrogenase activity by protecting it from inhibition of H_2. Ammonia is further synthesized into a number of metabolic products in microbial cells, however, ammonia is not accumulated in the cell, although a few species may create it; rather it is incorporated into organic forms by combining with an organic acid (a -keto-glutaric acid) to give rise to amino acid *e.g.* glutamic acid. The ammonia may also combine with organic molecules to yield alanin or glutamine.

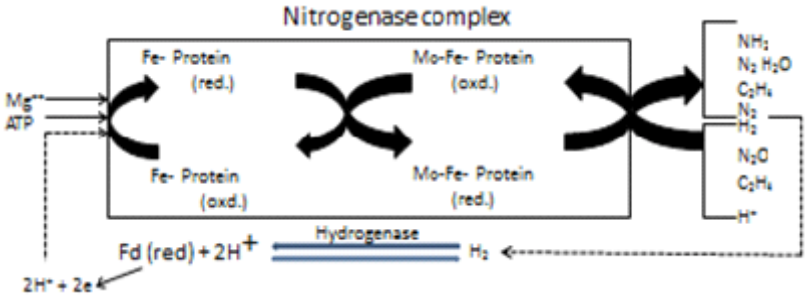

Fig. 2. Mechanism of electron flow during N_2 (and other substrates) reduction

Nitrogen fixing biofertilizers

Biological nitrogen fixation is one way of converting elemental nitrogen into plant usable form[14]. Nitrogen-fixing bacteria (NFB) that function transform inert atmospheric N_2 to organic compounds[15]. Nitrogen fixer or N fixers organism are used in biofertilizer as a living fertilizer composed of microbial inoculants or groups of microorganisms which are able to fix atmospheric nitrogen. They are grouped into free-living bacteria (*Azotobacter* and *Azospirillium*) and the blue green algae and symbionts such as *Rhizobium*, *Frankia* and *Azolla*[16]. The list of N_2-fixing bacteria associated with nonlegumes includes species of *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Acetobacter*, *Azomonas*, *Beijerinckia*, *Bacillus*, *Clostridium*, *Enterobacter*, *Erwinia*, *Derxia*, *Desulfovibrio*, *Corynebacterium*, *campylobacter*, *Herbaspirillum*, *Klebsiella*, *Lignobacter*, *Mycobacterium*, *Rhodospirillum*, *Rhodo-pseudomonas*, *Xanthobacter*, *Mycobacterium* and *Methylosinus*[17]. Although many genera and species of N_2-fixing bacteria are isolated from the rhizosphere of various cereals, mainly members of *Azotobacter* and *Azospirillum* genera have been widely tested to increase yield of cereals and legumes under field condition.

Nitrogen fixing biofertilizers

Rhizobium

Belongs to family *Rhizobiaceae*, symbiotic in nature, fix nitrogen 50-100 kg/ ha with legumes only. It colonizes the roots of specific legumes to form tumour like growths called root nodules, which acts as factories of ammonia production. *Rhizobium* has ability to fix atmospheric nitrogen in symbiotic association with legumes and certain non-legumes like *Parasponia*. Based on cross-inoculation studies, six species of *Rhizobium* are defined according to the legume host(s) which theynodulate (Table 1). A cross-inoculation group refers to a collection of leguminous species that develop nodules on any member of that particular plant group. Therefore, a single cross inoculation group ideally includes almost all host species which are infected by an individual bacterial strain[18].

Table 1. Species of *Rhizobium* and cross inoculation groups of host plants[18]

Rhizobium sp.	Host genera	Cross inoculation
R. japonicum	*Glycine*	Soybean *groups*
R. leguminosarum	*Pisum, Lathyrus, Vicia,* Lens	Pea group
R. lupini	*Lupinus, Ornithopus*	Lupin group
R. meliloti	*Melilotus, Medicago, Trigonella*	Alfalfa group
R. phaseolus	*Phaseolus*	Bean group
R. trifolii	*Trifolium*	Clover group
Other species of *Rhizobium*	*A rack is, Crotalaria, Vigna, Pueraria*	Cowpea group

Azospirillum

Belongs to family *Spirilaceae*, heterotrophic and associative in nature. In addition to their nitrogen fixing ability of about 20-40 kg/ ha, they also produce growth regulating substances. Although there are many species under

this genus like, *A.amazonense, A.halopraeferens, A.brasilense*, but, worldwide distribution and benefits of inoculation have been proved mainly with the *A.lipoferum and A.brasilense.* The *Azospirillum* form associative symbiosis with many plants particularly with those having the C_4-dicarboxyliac path way of photosynthesis (Hatch and Slack pathway), because they grow and fix nitrogen on salts of organic acids such as malic, aspartic acid. Thus it is mainly recommended for maize, sugarcane, sorghum, pearl millet etc. The Azotobacter colonizing the roots not only remains on the root surface but also a sizable proportion of them penetrates into the root tissues and lives in harmony with the plants. They do not, however, produce any visible nodules or out growth on root tissue.

Azotobacter

Belongs to family *Azotobacteriaceae*, aerobic, free living, and heterotrophic in nature. *Azotobacters* are present in neutral or alkaline soils and *A. chroococcum* is the most commonly occurring species in arable soils. *A. vinelandii, A. beijerinckii, A. insignis* and *A. macrocytogenes* are other reported species. The number of *Azotobacter* rarely exceeds of 104 to 105 g^{-1} of soil due to lack of organic matter and presence of antagonistic microorganisms in soil. The bacterium produces anti-fungal antibiotics which inhibits the growth of several pathogenic fungi in the root region thereby preventing seedling mortality to a certain extent. The occurrence of this organism has been reported from the rhizosphere of a number of crop plants such as rice, maize, sugarcane, bajra, vegetables and plantation crops.

Blue Green Algae (Cyanobacteria) and Azolla

These belongs to eight different families, phototrophic in nature and produce Auxin, Indole acetic acid and Gibberllic acid, fix 20-30 kg N/ha in submerged rice fields as they are abundant in paddy, so also referred as 'paddy organisms'. N is the key input required in large quantities for low land rice production. Soil N and BNF by associated organisms are major sources of N for low land rice. The 50-60% N requirement is met through the combination of mineralization of soil organic N and BNF by free living and rice plant associated bacteria[19]. To achieve food security through sustainable agriculture, the requirement for fixed nitrogen must be increasingly met by BNF rather than by industrial nitrogen fixation. Most N fixing BGA are filamentous, consisting of chain of vegetative cells including specialized cells called heterocyst which function as micro nodule for synthesis and N fixing machinery. BGA forms symbiotic association capable of fixing nitrogen with fungi, liverworts, ferns and flowering plants, but the most common symbiotic association has been found between a free floating aquatic fern, the Azolla and *Anabaena azollae* (BGA). The important factor in using *Azolla* as biofertilizer for rice crop is its quick decomposition in the soil and efficient availability of its nitrogen to rice plants. Besides N-fixation, these biofertilizers or biomanures also contribute significant amounts of P, K, S, Zn, Fe, Mb and other micronutrient. Azolla can be applied as green manure by incorporating in the fields prior to rice planting. The most common species occurring in India is *A. pinnata* and same can be propagated on commercial scale by vegetative means.

Nitrogen-fixing endophytes

An increasing number of reports describe the occurrence of nitrogen-fixing bacteria within plant tissues of a host plant that does not show diseases symptoms,with the most studied genera being Azoarcus sp, Gluconacetobacter and Herbaspirillum[20]. 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[21]. In the case of Azoarcus, type IV pili were found to be essential for that process and hydrolytic enzymes, or endoglucanases, are involved in tissue penetration[22]. The concentration of bacteria recovered 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 the case of sugar cane infection with *G. diazotrophicus*[23] and in the case of infection of the C_4- gramineous plant *Miscanthus sinensis* by *H. frisingense*[24].

Rhizobium-legume symbiosis

Host plants

Many leguminous plant species can enter into a symbiotic relationship with root-nodule bacteria, collectively referred to as rhizobia. Traditionally, three main subfamilies (family Leguminosae) are distinguished: Caesalpinoidae, Mimosoidae and Papilionidae. The Caesalpinoidae has very few nodulating members, whereas most of the important agricultural crops are members of the Papilionidae. Mimosoidae has recently received attention, since, in many cases, bacteria recovered from their nodules belong to the beta subclass of Proteobacteria[25]. Only one non-legume, the woody plant Parasponia sp., can be nodulated by rhizobia and utilize nitrogen fixed by the bacteria[26]. The rhizobia are gram-negative and belong to the large and important Proteobacteria division. The alpha-proteobacterial genera Agrobacterium, Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium, Devosia, Methylobacterium, Ochrobactrum and Phyllobacterium all harbor nodule-forming bacteria, and so do the beta-proteobacterial Burkholderia and Cupriavidus[27]. The taxonomic classification of rhizobia follows standard procedures and is based on the phylogeny of housekeeping genes and whole-genome similarities[28]. Since nodulation functions did not evolve until long after bacterial housekeeping properties, it is thus not always possible to distinguish nodule formers by their names.

Nodulation genes and nod factors

A common genetic determinant for rhizobia is the presence of genes encoding nodulation and nitrogen fixation functions (nod, nol, noe, nif and fix genes). These genes are often carried on plasmids or other accessory elements, such as symbiotic islands, and properties encoded by them can be easily lost or gained[29]. The nod, nol and noe gene products are involved in production of a nodulation signal, the Nod factor, which is a lipo-chitooligosaccharide. Initiation of nodule formation on compatible host plants results from a molecular dialogue between the host and the bacteria (Fig. 3)[30]. The host plants produce flavonoids (and related secondary metabolites) in the rhizosphere. These signals can be perceived by a specific bacterial receptor, NodD, which acts as a transcriptional activator of other nodulation genes. The core of the Nod factor molecule is encoded by canonical nodA, nodB and nodC whereas, for example, nodFE are involved in polyunsaturation of the fatty acyl group attached to the core molecule[31]. Other nodulation genes encode enzymes which add a variety of substituents to the core, as in the case of Nod factors produced by *Azorhizobium caulinodans*[32]. The Nod factor acts as an elicitor of root nodule formation by the plant by triggering a developmental program leading to construction of the root nodule and entry of rhizobia into the nodule[33]. It is an important host specificity determinant[30] (Spaink,2000). Recently, the Nod factor paradigm was challenged by[34], who discovered that certain photosynthetic, stem- and root-nodulating bradyrhizobia do not possess canonical nodABC genes but use other mechanisms for signalling to the plant. Their experiments led them to hypothesize that a purine derivative might play a role in triggering nodule formation instead of the Nod factor. This points to the complexity of the symbiotic system and shows that bacteria have employed diverse strategies to gain entry into the roots.

The infection process and nodule organogenesis

The rhizobial infection occurs via plant root hairs which, prior to the infection process, respond to the presence of compatible rhizobia by deformation (shepherd's crooks, cauliflower structures, *etc*.). At the deformation stage, the plant perceives the rhizobial signal and initiates a developmental program aimed at formation of symbiotically nitrogen-fixing nodules[35]. A set of plant genes, initially called nodulins, is specifically activated in response to nodulation factor perception[36]. Calcium spiking is observed in the early steps of root hair infection suggesting that calcium plays a role of secondary messenger in the infection process. Further steps in the signalling cascade lead to induction of cortical cell division[37]. A nodule meristem is thus formed within the root while the rhizobia enter through a plant-derived infection thread—a tube formed to facilitate rhizobia entry to the deeper layers. The infection threads grow transcellularly and finally, rhizobia wrapped into a plant derived membrane, now called symbiosome membrane, are delivered

into plant cells (Fig. 3). Nodules are either of an indeterminate type with an apical meristem, or they are determinate, meaning that the peripherally located meristem stops functioning after nodule completion[38]. In some nodules, all plant cells are infected with rhizobia, whereas in other nodule types, there are interstitial cells without symbiosomes. All mimosoid legumes and over half of the papilionid legumes represent this latter infection and nodulation type[26]. In their interesting and speculative review, however, caesalpinoid legumes, which are seldom nodulated, display symbiosis in which the bacteria are retained within infection threads throughout symbiosis. Another evolutionarily interesting nodulation mode is present in many agronomically important crop plants as well, namely, infection through cracks in the root ("crack entry"). This type is represented in *e.g.* Lupinus and Arachis. Interestingly, in the case of the aquatic legumes (e.g. *Sesbania rostrata*) infection occurred through root hair curling except under flooding conditions where the mode of infection was by crack entry[39].

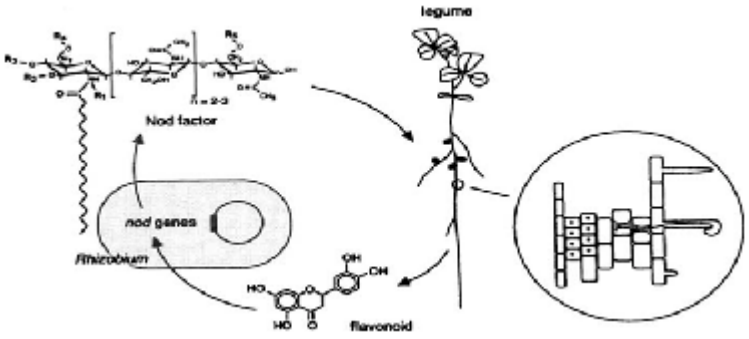

Fig. 3. Diagrammatic Represent of Nodule Formation in a Legume,And the Life cycle of the Root nodule Bacteria (*Rhizobium sp*.)

Nodule physiology

During nodule formation, host tissues develop to form a specialized tissue that maintains an environment in which nitrogen fixation can occur[40]. In the nodule, specialized organelle-like forms of bacteria called bacteroids are engulfed in plant-derived membranes, forming symbiosomes. The reduction in dinitrogen inside the nodule requires energy, which is provided by the plant. Photosynthate in the form of sucrose is transported to the nodule, whereas dicarboxylic acids further provide the bacteroids with carbon and energy through the symbiosome membrane. For generation of energy through respiration, a high flux but a low internal concentration of oxygen is achieved with the aid of leghemoglobin. Ammonia produced in the bacteroid needs to be transported to the plant through the symbiosome membrane. In addition to ammonia, alanine is transported. An amino acid flux back through the symbiosome membrane has also been proposed to be involved in the transport mechanism[41]. Ammonia is further assimilated into glutamine or asparagine in the plant cytosol. In determinate nodules, these are further converted into ureides in uninfected cells adjacent to the infected ones. In indeterminate nodules, this does not occur, and all plant cells are normally infected. The study of symbiosome biochemistry is impaired by technical difficulties involved when intact but isolated symbiosomes are used.

Actinorhizalsymbiosis

Host plants

Actinorhizal plants represent about 200 species distributed among 24 genera in eight angiosperm families[42]. Almost all genera are nodulated by Frankia in the Casuarinaceae, Coriariaceae, Eleagnaceae, Datisticaceae and Myricaceae families, whereas nodulation occurs occasionally in Betulaceae, Rhamnaceae and Rosaceae[43]. All actinorhizal plants are woody trees or shrubs except for Datisca, a genus of flowering plants. The genus Frankia comprises high mol% G+C Grampositive genera belonging to the family Frankiaceae in the order Actinomycetales[44]. Frankia is a filamentous bacterium forming

hyphal colonies without an aerial mycelium and characterized by a slow growth rate. One striking feature is its ability to differentiate two unique developmental structures that are critical to its survival: vesicles and spores (Fig. 4)[45]. Vesicles are the site for actinorhizal nitrogen fixation, while spores contained in multilocular sporangia are the reproductive structures of Frankia. Compared to that in rhizobia, the development of molecular genetic tools in Frankia has been difficult to implement mainly due to the relatively slow growth rate of filamentous hyphae; in most cases, genetic transformation, mutagenesis and functional complementation failed to provide conclusive results[46]. It was hypothesized that the absence of DNA-mediated transformation could result either from lack of gene expression, DNA restriction or the use of an inappropriate replicon. Hence, for some time, genetic analysis of Frankia has been mainly based on gene cloning via hybridization to genes from other organisms, phylogenetic analyses of selected gene sequences and isolation and characterization of plasmids[47].

Infection process

Two modes of infection of actinorhizal plants by Frankia have been described: intracellular root hair infection and intercellular root invasion[48]. Similarly to the situation encountered in rhizobia, the mode of infection depends on the host plant. Intracellular infection via root hairs (e.g. of Casuarina, Alnus, Myrica) starts with root hair curling following signal exchange between Frankia and the host plant. The signalling molecule pathway has not yet been identified, despite investigations in several laboratories[49]. However, preliminary characterization of a Frankia molecule capable of inducing root hair curling in host plants indicates that it differs from Nod factors in rhizobia[50], consistent with the absence of nod genes in Frankia genome. After invagination of growing filaments of Frankia in the curled root hairs, infection proceeds intracellularly in the root cortex. Frankia hyphae become encapsulated by a cell wall deposit that is believed to consist of xylans, cellulose and pectins of host origin[51]. At the same time, limited cell divisions occur in the cortex near the invading root hair, leading to formation of a small external protuberance called the prenodule[52]. Infection threads consist of lines of encapsulated Frankia hyphae progressing intracellularly toward the mitotically active zone and finally invading most cells of the prenodule. As the prenodule develops, cell divisions are induced in the pericycle located opposite the protoxylem pole, giving rise to another nodule primordium. In fact, actinorhizal prenodules do not evolve into nodules and the distantly induced primordium constitutes the nodule. The actual function of the prenodule was investigated in *Casuarina glauca*. A study of symbiosis- related gene expression coupled with cellular modification (cell wall lignification) indicated that prenodules displayed the same characteristics as nodules and hence could be considered very simple symbiotic organs[53]. Thus, sequential differentiation of prenodules and then nodules constitutes a major difference from the situation in legumes, where cortical cell divisions lead to formation of a unique nodule primordium evolving into a mature nodule. The prenodule might thus be a parallel symbiotic organ of its own or theremaining form of a common nodule ancestor forlegumes and actinorhizal plants. Prenodule formation does not occur in the intercellular root invasion process (*e.g*. Discaria, Ceanothus, Elaeagnus, Hyppophae). Frankia hyphae penetrate between two adjacent rhizoderm cells and progress apoplastically through cortical cells within an electron-dense matrix secreted into the intercellular spaces[48]. More recently, a second level of compartmentalization was described in *Casuarina glauca* nodules based on accumulation of flavans, which occurs in uninfected cells in the endodermis and cortex. These cells form layers that delimit Frankia-infected compartments in the nodule lobe and may play a role in restricting bacterial infection to certain zones of the nodule[54]. Molecular biology and actinorhizal nodule and plant gene expression. During differentiation of the actinorhizal nodule, a set of genes called actinorhizal genes is activated in the developing nodule. Heterologous probing and differential screening of nodule cDNA libraries with root and nodule-specific cDNA resulted in isolation and characterization of more than 25 nodule-specific or nodule-enhanced plant genes in several actinorhizal plants, including Alnus, Datisca, Eleagnus and Casuarina[55]. One of the earliest symbiotic genes characterized thus

far is cg12, which encodes a subtilisin-like protease expressed in Frankiainfected root hairs of *C. glauca*[56]. A homologue of the receptor-like kinase gene SymRK found in legumes was also recently shown to be necessary for actinorhizal nodule formation in the tree *Casuarina glauca*[57]. Recently emerging tools should contribute to increasing our knowledge of the molecular mechanisms of actinorhizal symbiosis over the next few years. One is the development of the first genomic platform for the study of plant gene expression in actinorhizal symbiosis[58]. This was recently applied to a *C. glauca* gene nodule library and it revealed increased expression of genes involved in primary metabolism, protein synthesis, cell division and defense. The second tool is the use of hairpin RNA to achieve post-transcriptional gene silencing in *C. glauca*, providing a versatile approach to assessing gene function during the nodulation process induced by Frankia[59]. Expressed sequence tags (ESTs) that exhibit homology with the early symbiotic genes DM_{I2} and DM_{I3}[60] from legumes involved in the Nod factor transduction pathway are currently being characterized using this RNA interference approach.

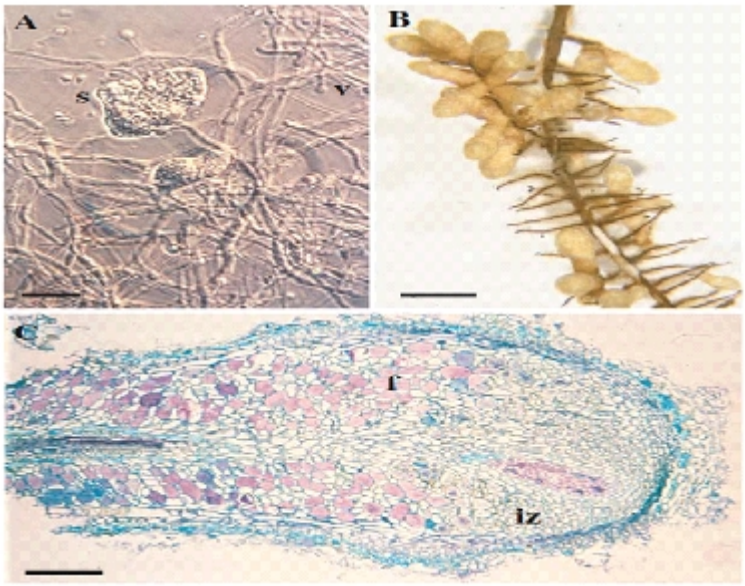

Fig. 4. Frankia and actinorhizal nodules A. Frankia in pure culture; nitrogen-fixing vesicles (v) and sporangia (s) can be observed. B. Actinorhizal multilobed nodules on the root system of the actinorhizal plant *Allocasuarina verticillata*. C Pseudolongitudinal section of a nodular lobe from *A. verticillata*; the nitrogen-fixing zone contains large cells filled with Frankia (f), and the infection zone (iz) is located in the apex of the nodular lobe

Cyanobacteria and symbiosis

Cyanobacteria are widely distributed in aquatic and terrestrial environments. Long regarded as algae because they performed oxygenic photosynthesis, they are now classified into the domain of Bacteria, in five Sections based mostly on morphological criteria[61]. Indeed, cyanobacteria constitute the largest and most diverse group of Gram-negative prokaryotes. While nitrogen fixation is found both in unicellular and filamentous species, associations with plants are essentially limited to heterocystous cyanobacteria Nostocales, primarily of the genus Nostoc and Anabaena. Besides vascular plants, there exist a wide variety of non-vascular lower plant belonging to bryophytes, including liverworts and hornworts, algae and fungi, which develop associations with cyanobacteria, as well as many marine eukaryotes[62]. We shall limit this review to associations with gymnosperms (Cycads), angiosperms (Gunnera) and pteridophytes (Azolla).

Differentiation of heterocysts, nitrogen-fixing specialized cells

Some filamentous cyanobacteria are able to differentiate specialized cells called heterocysts under nitrogen limitation conditions (Fig. 5). An anaerobic environment compatible with the functioning of nitrogenase in heterocysts is linked to formation of multilayered envelopes external to the outer membrane, elimination of a functional oxygen-producing photosystem II, and additional changes in their physiology not detailed here[63]. Nitrogen fixed by heterocysts is exported to vegetative cells of the filaments; in return, vegetative cells provide heterocysts with carbohydrates derived from their photosynthetic activity. This interdependence ensures filament growth under conditions of nitrogen limitation. Initial information on heterocyst differentiation came essentially from the study of Anabaena sp. strain PCC 7120, a strain not known to associate with plants[63]. Heterocysts develop within about 24 h from vegetative cells located at semi-regular intervals in the filaments (Fig. 5). The signalling pathway that leads to initiation of heterocyst differentiation at a particular location in the filament is complex, and the number of genes identified as being involved in the developmental process is regularly increasing[64]. Two of them, hetR and ntcA, have a critical function in initial steps of heterocyst differentiation[65], while patS is involved in heterocyst spacing[66]. HetR is a transcriptional regulator with autoprotease activity, which functions as a master switch in heterocyst differentiation. NtcA is a global nitrogen regulator that belongs to the CRP (cAMP receptor proteins) superfamily and which acts as a sensor of nitrogen deprivation in response to internal concentrations of 2-oxoglutarate. NtcA plays a role in control of hetR expression under N deprivation consistent with the fact that *ntcA* mutants cannot form heterocysts. PatS is a small diffusible peptide inhibitor of heterocyst differentiation, probably by inhibiting the hetR transcription activation function[67]. Thus, the semiregular pattern of heterocyst formation may derive from the autoregulatory activity of HetR and diffusion of the PatS peptide68. HetN, a protein similar to ketoacyl reductase, is also thought to downregulate expression of hetR. The percentage of heterocysts in filaments grown in the free-living state is in the range of 5 to 10% of cells, whereas it reaches 30 to 40% of cells within the filaments hosted by the plant and, in the particular case of Gunnera, up to 60–80%[68]. This reflects a direct correlation between the efficiency of nitrogen fixation and heterocyst frequency. Whereas Anabaena azollae appears to be an obligate symbiont, other symbiotic cyanobacteria can be grown in free-living culture and retain their ability to infect their host plant. Thus, properties of mutants impaired in heterocyst differentiation can be assayed in the host plant. Mutants of ntcA, hetR and hetF have been obtained in N. punctiforme strain PCC 92293, an isolate from Gunnera, which can also infect *Anthoceros punctatus*. None could differentiate heterocysts, similarly to what was found with corresponding mutants of non-symbiotic strain PCC 7120[69]. Both hetF and hetR mutants can infect *A. punctatus* with a frequency similar to that of the wild type, but are unable to support growth of the plant because of their inability to develop heterocysts and fix dinitrogen[70]. The ntcA mutant failed to infect Anthoceros because it is also impaired in formation of hormogonia, which is the "infection unit".

The genus Azolla includes seven species that have been grouped into two sections, Euazolla and Rhizosperma, based on the structure of their sporocarps. A view of *Azollapinnata* is shown in Fig. 5c. Symbiosis between the aquatic fern Azolla and *A. azollae* is of particular interest because it is the only plant-prokaryote symbiosis known to persist throughout the reproductive cycle of the host plant[71]. During vegetative growth, the symbiont is located in a distinct leaf cavity at the base of the dorsal lobe of the leaves (Fig. 5). Vegetative maintenance of the association depends on retention of *A. azollae* filaments, morphologically similar to hormogonia, at the apical meristem of fronds. The directed movement of the cyanobacterium within the host is accomplished by specialized plant epidermal trichomes. It has been hypothesized that the specific surface properties of hormogonia, which differ from those of vegetative filaments, enable recognition by trichomes, so that only generative hormogonium cells serve as inocula for new cavities developing at the apex of the frond. The

leaf cavity of Azolla can also host other bacteria together with the symbiotic Anabaena; the function of these bacteria remains unknown. The cavity is surrounded by mucilage and completely lined by an envelope. As leaf maturation occurs, the non-heterocystous hormogonium of the youngest leaves develop into heterocystous filaments located at the periphery of the cavity. Finally, the symbiotic cavities respond to the presence of cyanobacteria by elaborating long, finger-like cells that may serve to increase the surface area for nutrient exchange. Nitrogen is released from the cyanobiont almost exclusively as NH_4^+. During sexual reproduction, the cyanobiont colony survives in the indusium cap of the megaspore. In Azolla, deoxyanthocyanins produced by the aquatic fern also contribute to induction of hrmA expression[72].

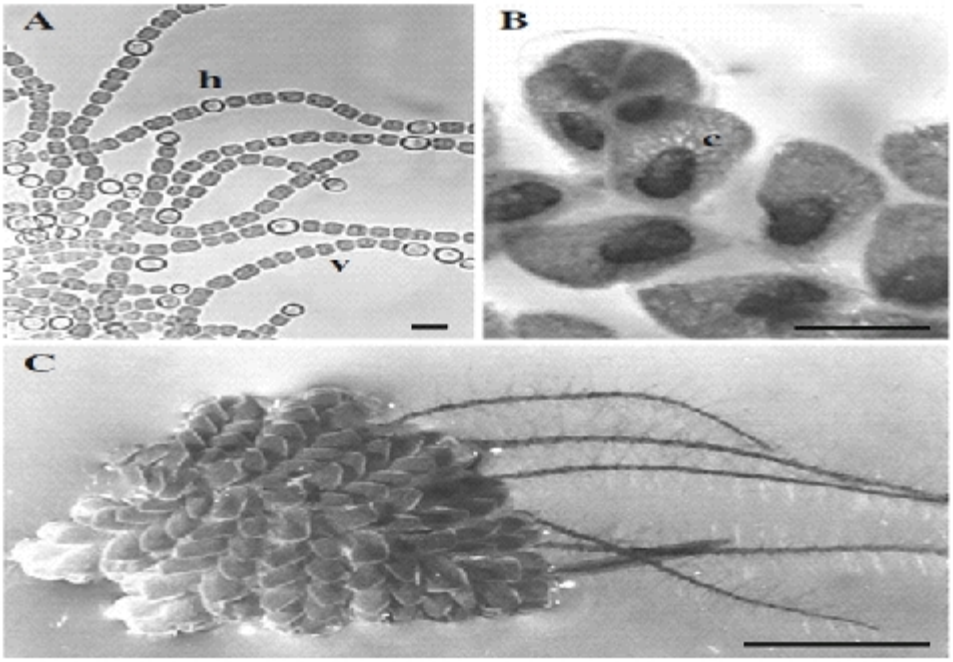

Fig. 5. Free-living Anabaena and Azolla A Free-living Anabaena strain cultured in medium deprived of nitrogen; heterocysts (h) can be observed among vegetative cells (v). B Frond of *Azolla pinnata* digested by cellulose and pectinase; cavities (c) filled with symbiotic *Anabaena azollae* are visible. C Frond of *A. pinnata*.

Mechanisms for plant growth promotion

Phytohormones

Plant growth regulators participate in the growth and development of cells, tissues, organs, and in fact the entire plant. These compounds are active in plants in very minute amounts and their synthesis is extremely regulated. Plants not only produce phytohormones but also, numerous plant associated bacteria both beneficial and harmful, produce one or more of these substances[73]. Among the PGPR species, *Azospirillum* is well known for its ability to excrete phytohormones such as gibberellins[74], cytokinins [75] and auxins[76]. Many studies suggest the involvement of indole-3-acetic acid (IAA), produced by *Azospirillum*, in morphological and physiological changes of the inoculated plant roots (Tien *et al.*, 1979). It is noteworthy that bacterial plant dependent response induces IAA synthesis by *Pantoea agglomerans*[77], and also, greater auxin production by rhizospheric strains of *P. polymyxa* than by non-rhizospheric isolates. Differential behavior of the isolates in relation to the proximity to plant tissues could be linked to a great competitiveness of the more actively phytohormone-synthesizing strains. Inoculation experiments of single, or mixtures of strains, previously isolated from different distances from roots, could help in determining this issue. Also, it would be exciting to determine if the rhizosphere gradient of plant

exudates participates in determining a differential response in the bacterial synthesis and release of phytohormones. The phytohormone-mediated roles of bacterial ephyphitic communities on plants are yet not clear. The future of biofertilizers based on hormone-producing bacteria seems very promising. Large numbers of experiments have shown that bacterial participation raises the phytohormone levels in plants. This may be via bacterial synthesis or through bacterial induction of plant hormone synthesis but both offer economical and ecological advantages.

ACC deaminase activity

Ethylene exposition induces different observable changes in plants, including reduction in the growth rate[78]. This is especially true in stressed dicot plants, since monocots are less sensitive to ethylene[79]. It has been proposed that PGPR may enhance plant growth by lowering the plant ethylene levels[80]. In these cases, the immediate precursor of ethylene is 1-aminocyclopropane-1-carboxylate (ACC). This compound is hydrolyzed by bacteria-expressing ACC-deaminase activity. Ammonia and ±-ketobutyrate, products of this hydrolysis, are used by the ACC-degrading bacterium as nitrogen and carbon sources[81]. Bacteria belonging to phylogenetically distant genera such as *Alcaligenes* sp., *Bacillus pumilus*, *Pseudomonas* sp. and *Variovorax paradoxus*[82]as well as, *Azoarcus*, *Azorhizobium caulinodans*, *Azospirillum* spp., *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., *Burkholderia vietnamiensis* and others[74] were identified by their ability to grow on minimal media containing ACC as sole nitrogen source.

Environmental stress relief

Several associations between plants and beneficial bacteria show a protective response under restrictive environmental conditions *e.g* Wheat andfababeans subjected to saline stress showed greater growth when inoculated with *Azospirillum*, compared to non-inoculated plants[83]. This favourable effect may be attributable directly to bacteria or indirectly to the effect on plant physiology. The production of microbial metabolites like polysaccharides modifies the soil structure, and has a positive effect on plants grown in water stress. Growth parameters of sunflower plants under water stress inoculated with an exopolysaccharide (EPS)-producing *Rhizobium* sp. were greater than in uninoculated plants[84]. Promotion effect in wheat plants was also observed after inoculation with an EPS-producing *Pantoea agglomerans* isolate[85]. In wheat plants inoculated with *Paenibacillus polymyxa*, the aggregation of rhizospheric soil depended on a bacterial polysaccharide that enlarged the amount of soil adhering to roots[86]. Bacteria can also stimulate the plant to turn on particular metabolic activity like increasing its exudates, and consequently, improve rhizospheric soil qualities[87]. In the same way, inoculation of *Arabidopsis* with *P. polymyxa* the water-stress gene ERD15 is switched on[88]. Inoculated plants show improved response against pathogenic colonization and drought stress in comparison to control plants. Hence it seems that inoculation induces protection against biotic agents, and also against abiotic ones. Overall, PGPR can protect a plant, against aggressive environmental and particularly hostile soil conditions through the bacterial release of soil structure-improving substances, and by inducing the plant to activate stress responsive mechanisms. In hostile soils, the uses of bacteria that allow plants to thrive are probably the best option to obtain good yields at lesser ecological costs.

Enhancing phosphorus availability for plant growth

Phosphorus (P) is an essential plant nutrient with low availability in many agricultural soils. Today many agricultural soils have a high total P content due to the application of P fertilizers over long periods of time. On the other hand, much of this P is in mineral forms and is only slowly available to plants[89]. Most of the insoluble P forms are present as aluminum and iron phosphates in acid soils[90] and calcium phosphates in alkaline soils[91]. The ability of rhizosphere bacteria to solubilize insoluble P minerals has been attributed to their capacity to reduce pH by the excretion of organic acids (e.g. gluconate, citrate, lactate and succionate) and protons (during the assimilation of $NH4^{+}$) [90]. These bacteria have been characterized as members of the *Bacillus*,*Burkholderia*, *Enterobacter*, *Klebsiella*, *Kluyvera*, *Streptomyces*, *Pantoea* and *Pseudomonas* genera[92] in various studies of P solubilizing

bacteria from different rhizospheric soils. These microorganisms grow in media with tricalcium phosphate or similar insoluble materials as the only phosphate source and not only assimilate the element, but also solubilize quantities in excess of their nutritional demands, thereby making it available for plants[93].

Siderophore production

Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition. Under iron-limiting conditions PGPB produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion. Siderophores (Greek: "iron carrier") are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi and grasses. Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by active transport mechanisms[94]. Many siderophores are non-ribosomal peptides, although several are biosynthesised independently. Siderophores are also important for some pathogenic bacteria for their acquisition of iron. Siderophores are amongst the strongest binders to Fe^{3+} known, with enterobactin being one of the strongest of these. Distribution of siderophoreproducing isolates according to amplified ribosomal DNA restriction analysis (ARDRA) groups, reveals that most of the isolates belong to Gram- negative bacteria corresponding to the *Pseudomonas* and *Enterobacter* genera, and *Bacillus* and *Rhodococcus* genera are the Gram-positive bacteria found to produce siderophores

CONCLUSIONS

Nitrogenous biofertilizer help in increasing crop productivity by way of increased BNF, increased availability or uptake of nutrients or increased absorption and stimulation of plant growth through hormonal action or antibiosis, or by decomposition of organic residues. Furthermore, nitrogenous biofertilizers as to replace part of the use of chemical N-fertilizers reduces amount and cost of chemical N-fertilizers and thus prevents the environment pollution from extensive application of these fertilizers. With using the biological and organic fertilizers, a low input system can be carried out, and it can help in achieving sustainability of farms.

REFRENCES

1. Rokhzadi A., Asgharzadeh A., Darvish F., Nourmohammadi G. and Majidi E. Influence of plant growth-promoting rhizobacteria on dry matter accumulation and yield of chickpea (*Cicer arietinum* L.) under field condition. Am-Euras.*J. Agric. Environ. Sci*.,2008; **3**(2): 253-257.
2. El-Yazeid A.A., Abou-Aly H.A., Mady M.A. and Moussa S.A.M.. Enhancing growth, productivity and quality of squash plants using phosphate dissolving microorganisms (bio phosphor) combined with boron foliar spray. *Res. J. Agric. Biol. Sci.*2007; **3**(4): 274-286.
3. Postgate, J. Microbiology of the free-living nitrogenfixing bacteria, excluding cyanobacteria. In: Gibson AH, Newton WE (eds) Current perspectives in nitrogen fixation. *Elsevier/North-Holland Biomedical, Amsterdam*, 1981; pp 217–228.
4. Young,P..Phylogenetic classification of nitrogen-fixing organisms. In: *Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation*. Chapman and Hall Inc, New York, 1992; pp 43–86.
5. Sprent, J. I. Nodulation in legumes. Royal BotanicGardens, Kew, UK Sprent JI, James EK (2007) Legume evolution: where do nodules and mycorrhizas fit in? *Plant Physiol*, 2001;**144**:575–581.
6. Huss-Danell, K. Actinorhizal symbioses and their N2 fixation. *New Phytol*.1997;**136**:375–405.
7. Meeks JC, Elhai, J. Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plantassociated symbiotic growth states. Microbiol Mol Biol Rev, 2002; **66**:94–121.
8. Elmerich C, Newton WE Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations. Springer, The Netherlands Elmerich C, Zimmer W, Vieille C (1992) Associative nitrogenfixing bacteria. In*: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation*. Chapman and Hall Inc, New York,2007; pp 212–258.
9. Baldani JI, Baldani VLD History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience. *An Acad Bras Cienc*, 2005;**77**:549–579.
10. Newton WE Physiology, biochemistry and molecular biology of nitrogen fixation. In: *Bothe H, Ferguson SJ, Newton WE (eds) Biology of*

the nitrogen cycle. Elsevier, Amsterdam,2007; pp 109–130.

11 .Hu Y, Fay AW, Lee CC, Ribbe MW P-cluster maturation on nitrogenase MoFe protein. *Proc Nat Acad Sci USA*,2007;**104**:10424–10429.

12. Arnold W, Rump A, Klipp W, Priefer UB, Pühler A Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of Klebsiella pneumoniae. *J Mol Biol*,1988; **203**:715–738.

13. Merrick MJ, Edwards RA Nitrogen control in bacteria. *Microbiol Rev*,1995;**59**:604–622.

14. Gothwal R.K., Nigam V.K., Mohan M.K., Sasmal D. and Ghosh P. Screening of nitrogen fixers from rhizospheric bacterial isolates associated with important desert plants. *Appl. Ecol. Environ. Res*,2007;**6(2)**: 101-109.

15. Bakulin M.K., Grudtsyna A.S. and Pletneva A. Biological fixation of nitrogen and growth of bacteria of the genus Azotobacter in liquid media in the presence of Perfluoro carbons. *Appl. Biochem. Microbiol*,2007;**4**: 399-402.

16. Gupta A.K. 2004. The complete technology book on biofertilizers and organic farming. National Institute of Industrial Research Press. India.

17. Wani S.P. Inoculation with associative nitrogenfixing bacteria: Role in cereal grain production improvement. *Indian J. Microbiol*,1990;**30**: 363-393.

18. Claudine, F., Kristina, L. and Claudine, E. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil, 2009; **321**:35–59

19. Mutch LA, Young JPW Diversity and specificity of Rhizobium leguminosarum biovar viciae on wild and cultivated legumes. *Mol Ecol*,2004;**13**:2435–2444

20. Lery LMS, von Krüger WMA, Viana FC, Teixeira KRS, Bisch PM (2008) a comparative proteomic analysis of Gluconacetobacter diazotrophicus PAL5 at exponential and stationary phases of cultures in the presence of high and low levels of inorganic nitrogen compound. Biochim Biophys Acta Limpens E, Bisseling T (2003) Signaling in symbiosis. Curr Opin Plant Biol 6:343–350.

21. Tan, X. Y., Hurek, T., and Reinhold-Hurek, B., Effect of N-fertilization, plant genotype and environmental conditions on *nifH* pools in roots of rice, *Environ. Microbiol*,2003;**5:**1009- 1015.

22. Krause A, Ramakumar A, Bartels D, Battistoni F, Bekel T, Boch J, Böhm M, Friedrich F, Hurek T, Krause L, Linke B, McHardy AC, Sarkar A, Schneiker S, Syed AA, Thauer R, Vorhölter FJ, Weidner S, Pühler A, Reinhold-Hurek B, Kaiser O, Goesmann A Complete genome of the mutualistic, N(2)-fixing grass endophyte Azoarcus sp. strain BH72. *Nat Biotechnol*,2006;**24**:1385–1391.

23. James EK, Olivares FL Infection and colonisation of sugarcane and other graminaceous plants by endophytic diazotrophs. *Crit Rev Plant Sci*,1998;**17**:77–119.

24. Rothballer M, Eckert B, Schmid M, Fekete A, Scholter M, Lehner A, Pollmann S, Hartmann A Endophytic root colonization of gramineous plants by Herbaspirillum frisingense. *FEMS Microbiol Ecol*,2008;**66**:85–95.

25. Chen WM, Moulin L, Bontemps C, Vandamme P, Béna G, Boivin-Masson C Legumes symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. *J Bacteriol*,2003;**185**:7266–7272.

26. Sprent JI, James EK Legume evolution: where do nodules and mycorrhizas fit in? *Plant Physiol*, 2007; **144**:575– 581.

27. Lindström K, Martínez-Romero E International committee on systematics of prokaryotes subcommittee on the taxonomy of Agrobacterium and Rhizobium: minutes of the meeting, *Int J Syst Evol Microbiol*,2007;**57**:1365–1366.

28. Lindström K, Kokko-Gonzales P, Terefework Z, Räsänen LA Differentiation of nitrogen-fixing legume root nodule bacteria. In: *Cooper JE, Rao JR (eds) Molecular techniques for soil and rhizosphere microorganisms*. CABI Publishing, Wallingford,2006; pp 236–258.

29. MacLean AM, Finan TM, Sadowsky MJ Genomes of the symbiotic nitrogen-fixing bacteria of legumes., 2007; **144**:615–622

30. Spaink HP Root nodulation and infection factors produced by rhizobial bacteria. *Annu Rev Microbiol*,2000;**54**:257–288

31. Yang GP, Debellé F, Savagnac A, Ferro M, Schiltz O, Maillet F, Promé D, Treilhou M, Vialas C, Lindström K, Dénarié J, Promé JC Structure of the Mesorhizobium huakuii and Rhizobium galegae Nod factors: a cluster of phylogenetically related legumes are nodulated by rhizobia producing Nod factors with alpha,beta-unsaturated N-acyl substitutions. *Mol Microbiol*,1999;**34**:227–237.

32. Mergaert P, Van Montagu M, Promé JC, Holsters M Three unusual modifications, a D-arabinosyl, an N-methyl, and a carbamoyl group, are present on the Nod factors of Azorhizobium caulinodans strain ORS571. *Proc Natl Acad Sci USA*,1993;**90**:1551–1555.

33. Gage DJ Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev*,2004;**68**:280–300.

34. Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn

E, Avarre JC, Jaubert M, Simon D, Cartieaux F, Prin Y, Bena G, Hannibal L, Fardoux J, Kojadinovic M, Vuillet L, Lajus A, Cruveiller S, Rouy Z, Mangenot S, Segurens B, Dossat C, Franck WL, Chang WS, Saunders E, Bruce D, Richardson P, Normand P, Dreyfus B, Pignol D, Stacey G, Emerich D, Verméglio A, Médigue C, Sadowsky M Legumes symbioses: absence of nod genes in photosynthetic bradyrhizobia. *Science*,2007;**316**:1307–1312.

35. Dénarié J, Debellé F, Promé JC Rhizobium lipochitooligosaccharide nodulation factors: signalling moleculaes mediating regcognition and morphogenosis. *Annu Rev Biochem*, 1996; **65**:503–535.
36. Geurts R, Bisseling T Rhizobium nod factor perception and signalling. *Plant Cell*,2002;**14**(Suppl):S239–S249.
37. Oldroyd GED, Downie JA Coordinating nodule morphogenesis with rhizobial infection in Legumes. *Annu Rev Plant Biol*,2008;**59**:519–546.
38. Foucher C, Kondorosi E Cell cycle regulation in the course of nodule organogenesis in Medicago. *Plant Mol Biol*,2000;**43**:773–786.
39. Goormachtig S, Capoen W, Holsters M Rhizobium infection : lessons from the versatile nodulation behaviours of water tolerant legumes. *Trends Plant Sci*,2004;**9**:518– 522.
40. White J, Prell J, James EK, Poole P Nutrient sharing between symbionts. *Plant Physiol*, 2007; **144**:604–614.
41. Prell J, Poole P Metabolic changes of rhizobia in legume nodules. *Trends Microbiol*,2006;**14**:161–168.
42. Huss-Danell K Actinorhizal symbioses and their N2 fixation. *New Phytol*,1997;**136**:375–405.
43. Benson DR, Clawson ML (2000) Evolution of the actinorhizal plant symbioses. In: Triplett EW (ed) Prokaryotic nitrogen 52 Plant Soil (2009) 321:35–59 fixation: A model system for analysis of biological process. Horizon Scientific Press, Wymondham, UK, pp 207–224 Berg RH (1990) Cellulose and xylans in the interface capsule in symbiotic cells of actinorhizae. Protoplasma 159:35–43.
44. Normand P, Orso S, Cournoyer B, Jeannin P, Chapelon C,Dawson J, Evtushenko L, Misra AK Molecular phylogeny of the genus Frankia and related genera and emendation of family Frankiaceae. *Int J Syst Bacteriol*,1996;**46**:1–9.
45. Lechevalier MP Taxonomy of the genus Frankia (Actinomycetales). *Int J Syst Bacteriol*, 1994; **44**:1–8.
46. Normand P, Mullin BC Prospects for the study of a ubiquitous actinomycete, Frankia, and its host plants. In: *Pawlowski K, Newton WE (eds) Nitrogen-fixing actinorhizal symbioses*. Springer, Dordrecht,2008; pp 289–303.
47. Wall LG The actinorhizal symbiosis. *J Plant Growth Regul*,2000;**19**:167–182.
48. Wall LG, Berry AM Early interactions, infection and nodulation in actinorhizal symbiosis. In: *Pawlowski K, Newton WE (eds) Nitrogen-fixing actinorhizal symbioses*. Springer, Dordrecht,2008; pp 147–166.
49. Van Ghelue M, Lovaas E, Ringo E, Solheim B Early interaction between Alnus glutinosa and Frankia strain Arl3. Production and specificity of root hair deformation factors. *Physiol Plant*,1997;**9**:579–587.
50. Cérémonie H, Debellé F, Fernandez MP Structural and functional comparison of Frankia root hair deforming factor and rhizobia Nod factor. *Can J Bot*,1999;**77**:1293–1301.
51. Berg, R.H.Cellulose and xylans in the interface capsule in symbiotic cells of actinorhizae. *Protoplasma*,V;**159**:35–43.
52. Berry, M.A. and Sunell A.L.The infection process and nodule development. In: Schwintzer RC, Tjepkema J.D. (eds) The Biological of Frankia and actinorhizal plants. Academic press Inc, San Diego, pp 61–88 Bock JV, Battershell T, Wiggington J, John TR, Johnson J.D. (2001) Frankia sequences exhibiting RNA polymerase promoter activity. *Microbiology*,1990;**147**:499–506.
53. Laplaze, L., Duhoux, E., Franche, C., Frutz, T., Svistoonoff, S. and Bogusz, D. *Casuarinaglauca* prenodule cells display the same differentiation as the corresponding nodule cells. *Mol Plant Microbe Interact*2000; **13**:107–112.
54. Laplaze, L., Gherbi, H., Frutz, T., Pawlowski, K., Franche, C., Macheix, J.J., Auguy, F., Bogusz, D. and Duhoux, E.Flavan-containing cells delimit Frankia-infected compartments in Casuarina glauca nodules. *Plant Physiol*,1999;**121**:113–122.
55. Laplaze, L., Svistoonoff, S., Santi, C., Auguy, F., Franche, C. and Bogusz, D.Molecular biology of actinorhizal symbioses. In: *Pawlowski K, Newton WE (eds) Nitrogen-fixing actinorhizal symbioses*. Springer, Dordrecht,2008; pp 235–259.
56. Svistoonoff, S., Laplaze, L., Auguy, F., Runions, J., Duponnois, R., Haseloff, J., Franche, C. and Bogusz, D.cg12 expression is specifically linked to infection of root hairs and cortical cells during Casuarina glauca and Allocasuarina verticillata actinorhizal nodule development. *Mol Plant Microbe Interact*,2003;**16**:600–607.
57. Gherbi, H., Markmann, K., Svistoonoff, S., Estevan, J., Autran, D., Giczey, G., Auguy, F.,

Péret, B., Laplaze, L., Franche, C., Parniske, M. and Bogusz, D.SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and Frankiabacteria. *Proc Natl Acad Sci USA*,2008; 105:4928–4932.

58. Hocher, V., Auguy, F., Argout, X., Laplaze, L., Franche, C. and Bogusz, D.Expressed sequence-tag analysis in Casuarina glauca actinorhizal nodule and root. *New Phytol*,2006;**169**:681– 688.
59. Gherbi, H., Nambiar-Veetil, M., Zhong, C., Félix, J., Autran, D., Girardin, R., Auguy, F., Bogusz, D. and Franche, C.Posttranscriptional gene silencing in the root system of the actinorhizal tree Allocasuarina verticillata. *Mol Plant MicrobeInteract*,2008;**21**:518–524.
60. Catoira, R., Galera, C., de Billy, F., Penmetsa, R.V., Journet, E.P., Maillet, F., Rosenberg, C., Cook, D. and Dénarié, J.Four genes of Medicago truncatula controlling components of a Nod factor transduction pathway. *Plant Cell*,2000;**12**:1647–1666.
61. Rippka, R., Deruelles, J., Waterbury,J.B., Herdman, M. and Stanier, R.Y.Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol*,1979; 111:1–61.
62. Rai, A.N., Söderbäck, E. and Bergman, B.Cyanobacteriumplant symbiosis. *New Phytol*,2000;**147**:449–481.
63. Buikema, W.J. and Haselkorn, R.Molecular genetics of cyanobacterial development. *Annu Rev Plant Physiol*,1993;**44**:33–52.
64. Ehira, S., Ohmori, M. and Sato, N.Genome-wide expression analysis of the responses to nitrogen deprivation in the heterocyst-forming cyanobacterium Anabaena sp strain PCC 7120. *DNA Res*,2003:**10**:97–113.
65. Shi Y, Zhao W, Zhang W, Ye Z, Zhao J., Regulation of intracellular free calcium concentration during heterocystdifferentiation by HetR and NtcA in Anabaena sp Pcc7120. *Proc Natl Acad Sci USA* 2006; **103**:11334–11339.
66. Yoon HS, Golden JW., Heterocyst pattern formation controlled by a diffusible peptide. *Science* 1998; **282**:935–938.
67. Huang X, Dong Y, Zhao J., HetR homodimer is a DNA binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. *Proc Natl Acad Sci USA* 2004; **101**: 4848–4853.
68. Xu X, Elhai J, Wolk CP., Transcriptional and developmental responses by Anabaena to deprivation of fixed nitrogen. In: Herrero A, Flores E (eds) Cyanobacteria: Molecular biology, genomics and evolution. Horizon Scientific, Norwich, 2008; 383–422.
69. Wang CM, Ekman M, Bergman B., Expression of cyanobacterial genes involved in heterocyst differentiation and dinitrogen fixation along a plant symbiosis development profile. *Mol Plant Microbe Interact* 2004; **17**:436–443.
70. Wong FCY, Meeks JC., The hetF gene product is essential to heterocyst differentiation and affects HetR function in the cyanobacterium Nostoc punctiforme. *J Bacteriol* 2001; **183**:2654–2661.
71. Lumpkin TA, Plucknett DL., Azolla: botany, physiology, and use as a green manure. *Econ Bot* 1980; **34**:111–153.
72. Cohen MF, Sakihama Y, Takagi YC, Ichiba T, Yamasaki H., Synergistic effect of deoxyanthocyanins from symbiotic fern Azolla spp on hrmA gene induction in the cyanobacterium Nostoc punctiforme. Mol Plant Microbe Interact 2002; **9**: 875–882.
73. Dobbelaere, S., Vanderleyden, J., and Okon, Y., Plant growth-promoting effects of diazotrophs in the rhizosphere, *Crit. Rev. Plant Sci*. 2003; **22:** 107-149.
74. Tien, T. M., Gaskins, M. H., and Hubbell, D. H., Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.), *Appl. Environ. Microbiol*.1979; **37:**1016-1024.
75. Bottini, R., Fulchieri, M., Pearce, D., Pharis, R. P., Identification of gibberellins A1, A3 and iso-A3 in culture of *Azospirillum lipoferum*, *Plant Physiology* 1989; **89:**1-3.
76. Omay, S. H., Schmidt, W. A., Martin, P., and Bangerth, F., Indoleacetic acid production by the rhizosphere bacterium *Azospirillum brasilense* Cd under in vitro conditions, *Can. J. Microbiol.* 1993; **39:**187-192.
77. Brandl, M. T., and Lindow, S. E., Environmental signals modulate the expression of an indole-3-acetic acid biosynthetic gene in *Erwinia herbicola*, *Mol. Plant-Microbe Interact*. 1997; **10:** 499-505.
78. Abeles, F. B., Morgan, P. W., and Saltveit, M. E., *Ethylene in Plant Biology*. Academic Press, 2nd ed., New York, 1992.
79. Holguin, G. and Glick, B.R., Expression of the ACC deaminase gene from Enterobacter cloacae UW4 in Azospirillum brasilense. *Microb Ecol* 2001; **41**:281–288
80. Glick, B. R., Penrose, D. M., and Li, J., A model for the lowering of plant ethylene concentrations by plant growth promoting rhizobacteria, *J. Theor. Biol*. 1998; **190:**63-68.
81. Honma, M., and Shimomura,T., Metabolism of 1-aminocyclopropane-1-carboxylic acid, *Agric.*

Biol. Chem. 1978; **42:**1825-1831.

82. Belimov, A. A., Safronova, V. I., Sergeyeva, T. A., Egorova, T. N., Matveyeva, V. A., Tsyganov, V. E., Borisov, A. Y., Tikhonovich, I. A., Kluge, C., Preisfeld, A., Dietz, K. J., and Stepanok, V. V., Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase, *Can. J. Microbiol.* 2001; **47:**642–652.
83. Bacilio, M., Rodríguez, H., Moreno, M. Hernández, J. P., and Bashan, Y., Mitigation of salt stress in wheat seedlings by a gfp-tagged *Azospirillum lipoferum*, *Biol. Fertil. Soils* 2004; **40:**188-193.
84. Alami, Y., Achouak, W., Marol., C., and Heulin, T., Rhizosphere soil aggregation and plant growth promotion of sunflowers by an exopolysaccharide-producing *Rhizobium* sp. strain isolated from sunflower roots, *Appl. Environ. Microbiol.*2000; **66:**3393-3398.
85. Amelal, N., Burtan, G., Bartoli, F., and Heulin, T., Colonization of wheat roots by an exopolysaccharide-producing *Pantoea agglomerans* strain and its effect on rhizosphere soil aggregation, *Appl. Environ. Microbiol.* 1998; **64:** 3740-3747.
86. Bezzate, S., Aymerich, S., Chambert, R., Czarnes, S., Berge, O., and Heulin, T., Disruption of the *Bacillus polymyxa* levansucrase gene impairs its ability to aggregate soil in the wheat rhizosphere, *Environ. Microbiol.*2000; **2:**333-342.
87. Heulin, T., Gucker, A., and Balandeau, J., Stimulation of root exudation of rice seedlings by *Azospirillum* strains: carbon budget under gnotobiotic conditions, *Biol. Fertil. Soils* 1987; **4:** 9-14
88. Timmusk, S., Nicander, B., Granhall, U., and Tillberg, E., Cytokinin production by *Paenibacillus polymyxa*, *Soil. Biol. Biochem.* 1999; **31:**1847-1852.
89. Richardson, A.E., Barea, J.M., McNeill, A.M., Prigent-Combaret, C., Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms.*Plant Soil* 2009; **321**: 305-339.
90. Mullen, M.D., Phosphorus in soils: biological interactions. In: D. Hillel, C. Rosenzweig, D. Powlson, K. Scow, M. Singer, D. Sparks (eds).Encyclopedia of Soils in the Environment, Vol.3. Academic Press, Elsevier, Ltd, Oxford, 2005; 210–215.
91. Goldstein, A.H., Krishnaraj, P.U., Phosphate solubilizing microorganisms vs. phosphate mobilizing microorganisms: What separates a phenotype from a trait? In: E. Velázquez, C. Rodríguez-Barrueco (eds). First International Meeting on Microbial Phosphate Solubilization. Springer, 2007; 203–213.
92. Oliveira, C.A., Alves, V.M.C., Marriel, I.E., Gomes, E.A., Scotti, M.R., Carneiro, N.P., Guimarães, C.T., Schaffert, R.E., Sá, N.M.H., Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. *Soil Biol. Biochem*. 2009; **41**: 1782–1787.
93. Chen, Y.P., Rekha, P.D., Arun, A.B., Shen, F.T., Lai, W-A., Young, C.C., Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities.*Appl. Soil Ecol.* 2006; **34**: 33-41.
94. Loper JE, Henkels MD, Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. *Applied and Environmental Microbiology*, 1997; **63** (Suppl 1): 99-105.