Chapter 7
Biological Nitrogen Fixation in Cereals Crops: A Bacterial Perspective

S. Garcha and P. K. Maan

Abstract Nitrogen (N_2) is one of the essential requirements of all living forms. The crop requirement is generally met by cheaply available chemical fertilizers. Endophytic bacteria of cereal crops as rice, wheat, etc. have the natural ability to fix atmospheric nitrogen. A few of them have established a mutually beneficial association with the plants. This chapter lists commonly encountered endophytic microbes of rice and wheat and the major enzymes catalyzing N_2 fixation, an energy intensive reaction. Nitrogenase enzyme is sensitive to the presence of oxygen. Microbial cells have devised a method to lower oxygen concentration for optimal performance of the enzyme. Further, genetic control of nitrogen fixation is explained mentioning the genes and their respective functions. Quality assurance and longer shelf life of such biological products can go a long way in consolidating market share of biofertilizers. Engineering plant microbe communication can facilitate manipulation for greater efficacy of nitrogen fixation.

Keywords Biological nitrogen fixation · Cereal crops · Associative nitrogen fixation · Nitrogen-fixing enzymes · Genetics of nitrogen fixation · Bio-inoculants

7.1 Introduction

Nitrogen (N) plays a crucial role in growth of all living forms. It oscillates between organic and inorganic forms. This change is brought about by phenomena of biological nitrogen fixation (BNF) carried out by microorganisms – free-living in the soil or present in associative or symbiotic relationship with the plants. These include *Rhizobium*, *Azorhizobium*, *Azotobacter*, *Azospirillum*, etc. Nonsymbiotic nitrogen-fixing bacteria that live in the rhizosphere (Dobereiner 1997) and/or endophytically (Hecht-Buchholz 1998) often increase yields of cereals and other

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crops. Free-living N-fixing bacteria play an important role in plant development on account of nitrogen fixation and supply of growth activators (Ahmed and Kibret 2014). BNF thus plays an important role in maintaining soil fertility (Vance and Graham 1995).

Wheat and rice along with maize are the cereal crops that are the mainstay of global food security. Unlike legumes, cereals do not have any close association with N_2-fixing bacteria. Hence these crops cannot be grown with reliance on BNF alone and are mostly dependent on chemical N fertilizer for their N needs. *Azotobacter*, a free-living nitrogen-fixing bacterium (Martinez-Toledo 1985) which is used as a biofertilizer in the cultivation of many cereal crops, fixes annually about 60–90 kg N/ha and may be used in crop production as a substitute for a part of mineral N fertilizer. Among the field crops, wheat and sugar beet are most responsive to N nutrition (Bogdanovic et al. 2005). Inoculation with *Azotobacter* replaced up to 50% of urea-N for wheat grown in a greenhouse trial under aseptic conditions, and the effect of inoculation with *Azotobacter* varies depending on the species and strain of N-fixing bacteria, physicochemical soil properties, N fertilizers applied, climatic conditions, and wheat cultivar (Milosevic and Jarak 2005). The increase is construed as a result of BNF, as well as the production of antibacterial and antifungal compounds, growth regulators, and siderophores (Pandey and Kumar 1989).

Azotobacter and *Azospirillum* were found to fix nitrogen in cereals and increase yield up to 27% in wheat (Khandan and Namvar 2013) and in the process they fixed 15–20 Kg N/ha. Cyanobacteria in rice field fixed N_2 in the range of 20–30 Kg N/ha (Singh 2014). Inoculation of rice with *Azospirillum lipoferum* increased yield up to 6.7 g/plant, nearly 1.8 t/ha (Balandreau 2002). *Azospirillum brasilense* and *A. lipoferum* contributed 7–12% of wheat plant N by BNF as was evidenced by N^{15} tracer studies (Malik et al. 2002).

7.2 Associative Nitrogen-Fixing Bacteria in Wheat and Rice: Diversity and Phylogeny

The N_2 requirement of cereals is well established and the N_2 requirement is met by application of chemical fertilizers. Unfortunately, intensive use of chemical fertilizers leads to eutrophication, leaching, pollution of groundwater, alteration of soil organic matter, etc. It also has adverse effect on soil microflora and fauna (Mano and Morisaki 2008). The need can be met at least partially by application of beneficial bacteria. Endophytic bacteria associated with cereals and grasses include *Achromobacter*, *Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Campylobacter*, *Corynebacterium*, *Derxia*, *Desulfovibrio*, *Enterobacter*, *Erwinia*, *Herbaspirillum*, *Klebsiella*, *Lignobacter*, *Mycobacterium*, *Methylosinus*, *Pseudomonas*, *Rhodospirillum*, *Rhodopseudomonas*, and *Xanthobacter* (Wani 1990). The various species isolated and identified from rice and wheat are summarized in Table 7.1.

Table 7.1 Endophytic species identified in rice and wheat

Crop	Endophyte	References
Rice	*Herbaspirillum seropedicae, H. frisingeuse, H. rubrisubalbicans*	Elbeltagy et al. (2000)
	Klebsiella pneumonia	
	Bacillus pumilus, Bacillus subtilis, Bacillus polymyxa	
	Azospirillum brasilense, Azospirillum lipoferum, A. amazonense, A. oryzae	Salamone et al. (2010), Elbeltagy et al. (2000), Vargas et al. (2012), Baldani et al. (1986), and Xie and Yokota (2005)
	Azorhizobium	
	Rhizobium leguminosarum	Yanni et al. (1997) and Mirza et al. (2001)
	Bradyrhizobium japonicum	
	Pseudomonas	Salamone et al. (2012)
	Burkholderia	Singh et al. (2006) and Vargas et al. (2012)
	Clostridium	Choudhury and Kennedy (2004)
	Azotobacter	Choudhury and Kennedy (2004)
	Serratia marcescens	
Wheat	*Azospirillum brasilense,*	Anastasia et al. (2011)
	Azospirillum zeae	Kennedy et al. (1997)
	Azotobacter vinelandii	Aly et al. (2012)
	Pseudomonas stutzeri	Anastasia et al. (2011)
	Klebsiella pneumoniae 342	Iniguez et al. (2004)
	Mycobacterium	Conn and Franco (2004)

Benefit of inoculation with diazotrophic bacteria *Burkholderia kururiensis* M130 and *Azospirillum brasilense* sp245 was observed to be influenced by plant and bacterial genotypes (Vargas et al. 2012; Malarvizhi and Ladha 1999; Shrestha and Ladha 1996). Early plant responses are believed to involve ethylene signaling (Vargas et al. 2012), and ethylene reponse (ER) pathway is demonstrated to participate in the early stages of the establishment of the association between Poaceae and beneficial diazotrophic bacteria. Different bacteria trigger different patterns of ethylene receptor function in various rice genotypes. Inoculation of rice cultivar IR 42 with *Azospirillum brasilense* sp245 resulted in an increase in the level of four ERs and also 143% increase in number of lateral roots. However, its response to inoculation with *B. kururiensis* M130 was different.

Members of Poaceae family, to which rice and wheat belong, have beneficial interaction with the genera of *Azospirillum*, *Burkholderia*, *Gluconacetobacter*, and *Herbaspirillum* (Suarez-Moreno et al. 2012). They colonize rhizospheric soils and surfaces of their hosts and are also endophytic in roots, intercellular spaces, and vascular tissue. However, they do not form any specialized symbiotic structures as nodules (James 2000). Rice is widely studied as model system in plant-microbe ecology (Hardoim et al. 2008). Wheat has been found to select specific subpopulation of *Bacillus polymyxa* from rhizosphere soil (Mavingui et al. 1992). Soil type and soil bacteria largely determine the nature of endophytic population in wheat

(Conn and Franco 2004). *Klebsiella* sp strain Kp342 has been found to fix nitrogen in wheat (Iniguez et al. 2004). It was shown to aggregate at lateral root junctions of wheat by Dong et al. (2003).

Use of bacterial bio-inoculant preparations in rice, containing *Azotobacter*, *Clostridium*, *Azospirillum*, *Herbaspirillum*, and *Burkholderia*, is well documented. They are believed to possess the ability to assimilate soil N and also exhibit PGPR traits (Biswas et al. 2000; Yanni et al. 1997). *Azotobacter* is an aerobic free-living heterotrophic nitrogen fixer. It has been reported to increase yields 7–20% and fix 11–15 Kg N_2/ha in cereals by Yanni and El-Fattah (1999). *Clostridium* sp., an anaerobic heterotroph capable of fixing nitrogen in the absence of oxygen in wetland rice (Elbadry et al. 1999), acts like that of *Azotobacter*. Population of *Clostridium* increases upon application of rice straw in fields (Kanungo et al. 1997), and application of rice straw is reported to stimulate N_2 fixation by *Clostridium*, up to 5–10 mg N/g of carbon consumed.

Azospirillum is a heterotrophic N_2-fixing bacterium (Roper and Ladha 1995). It can grow both in the rhizosphere soil of graminaceous crops and also intracellularly in the root (Baldani and Döbereiner 1980). *Azospirillum brasilense* sp245 and *Burkholderia kururiensis* M130 have demonstrated endophytic capability also (Suarez-Moreno et al. 2012; Baldani et al. 2000). *Azospirillum brasilense* and *A. lipoferum* have been isolated from the roots and stems of rice (Ladha et al. 1982). Another species, *A. amazonense*, has also been isolated from rice roots (Pereira et al. 1988). However, 85% of *Azospirillum* isolates from rice rhizosphere belong to *A. lipoferum* (Ladha et al. 1987) which results into 32–81% increase in yield under greenhouse (Malik et al. 2002) and around 22% increase under field conditions (Balandreau 2002). There is increase in the height and tiller number and also increased uptake of PO_4^{-3} and NH^{+4} by rice plants (Murty and Ladha 1988). *Azospirillum* is also reported to have biocontrol attributes as reduction in bacterial leaf blight was reported by Islam and Bora (1998). Amount of N fixed by *A. lipoferum* and *A. brasilense* was quantified by Mirza et al. (2000) using N^{15} isotope, and results demonstrate the ability of *Azospirillum* to meet 19–47% of N requirement of basmati and super basmati rice. Colonization of wheat by *Azospirillum brasilense* was demonstrated by Webster et al. (1998). Naringenin and other flavonoids stimulated colonization by *A. brasilense* (Jain and Gupta 2003).

Herbaspirillum is an endophytic diazotroph reported to colonize many plants including cereals like rice, maize, sugarcane, sorghum, etc. *H. seropedicae* was first isolated from Brazil colonizing rice roots (Baldani et al. 1986). In addition to fixing 31–54% of total rice plant Ndfa (N derived from the atmosphere), it also increased shoot and root length, grain yield and grain weight (Baldani et al. 2000), seed germination (Pereira et al. 1988), and root and shoot dry weight (James et al. 2002). Yield increase of 44–90% under greenhouse condition was reported in super basmati rice inoculated with *Herbaspirillum* spp. by Mirza et al. (2000). Basmati and super basmati recorded % Ndfa of 19.5–38.7 and 38.1–58.2, respectively. However, the amount of N_2 fixed by *Herbaspirillum* is reported to vary with rice variety (Gyaneshwar et al. 2002).

Few species of *Burkholderia* are capable of fixing nitrogen including *B. vietnamiensis*, *B. kururiensis*, *B. tubernum*, and *B. phynatum* (Vandamme et al. 2002; Estrada-de Los Santos et al. 2001). *Burkholderia kururiensis* M130 have demonstrated endophytic capability (Suarez-Moreno et al. 2012; Baldani et al. 2000). *B. vietnamiensis* when used in field trials of rice increased grain yield by 13–22% and reduced N fertilizer application by 25–30 Kg N/ha (Tran Van et al. 2000). Under gnotobiotic conditions, these spp. are reported to fix 19% of rice plant Ndfa. Other endophytic spp. of *Burkholderia* fixed 31% of rice plant Ndfa and increased rice plant biomass by 69% (Baldani et al. 2000). However, *B. cepacia* is reported to be of human health risk (Balandreau 2002), and *B. glumae* is known to cause grain and seed rot of rice (Nakata 2002).

Well-known legume symbiont *Rhizobium* influences growth physiology and root morphology of the rice plant. *R. leguminosarum* bv. *trifolii* has the ability to colonize rice (Yanni et al. 1997). It has been demonstrated to biologically fix nitrogen, increase shoot and root growth and grain yield, and decrease chemical N fertilizer usage (Biswas et al. 2000; Yanni et al. 1997). Inoculation of *Rhizobium* in cereals is accompanied by increased presence of phenolic acids like gallic, tannic, ferulic, and cinnamic acids in leaves (Mirza et al. 2001). *Azorhizobium caulinodans* and *Azorhizobium brasilense* enter rice through lateral root cracks and establish in intercellular space within the cortical cell layer of roots. Nodulation in nonlegumes like cereals has been reported by Al-Mallah et al. (1990). It involves facilitating the entry of rhizobia by enzymatically treating the roots.

Cultivated rice varities have exhibited higher N_2-fixing bacteria. Genotypes of the rice plants effect association between rice plants and N_2-fixing bacteria (Malarvizhi and Ladha 1999; Shrestha and Ladha 1996; Ladha et al. 1986, 1987). Magnitude of variation is attributed to soil N_2 status. A rice genotype IR42 is reported to have high BNF trait (Wu et al. 1995). Rice plants have demonstrated ability to utilize biologically fixed N_2 in soils having low fertility compared to soils having high fertility.

7.3 Types of Nitrogen-Fixing Enzymes: MO-, V-, Fe-Containing Types

7.3.1 Nitrogenase

Nitrogenase is a complex enzyme system that fixes N_2. It is very sensitive to oxygen. In legume nodules, the protection against oxygen is provided by leghemoglobin, whereas in many cyanobacteria it is provided by a special structure called the heterocyst. Free-living diazotrophs, e.g., *Cyanobacteria* and *Azotobacteraceae*, and symbiotic diazotrophs, e.g., *Rhizobia* and *Frankia*, are

among the organisms that synthesize nitrogenase (Herrero and Flores 2008). Nitrogenase catalyzes the conversion of N_2 to NH^{4+} through this reaction:

$$2N_2 + 8^{e-} + 10H^+ + nMgATP \rightarrow 2NH_4^+ + H_2 + nMgADP + nP_i \quad (n \geq 16)$$

Nitrogenase enzyme consists of two major protein components – dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein). Both of them contain iron (Fe), while dinitrogenase contains molybdenum (Mo) in addition. A cofactor called MoFe-cofactor or MoFe-co is bonded to iron (Fe) and molybdenum (Mo) present in dinitrogenase. MoFe protein is a 230 kDa α_2ß$_2$ tetramer. MoFe protein tetramer contains two pairs of metalloclusters, i.e., two molydenum-iron-sulfur-homocitrate clusters (FeMo-co) and two [Fe_8S_7] clusters (P-cluster) each. The P clusters consist of two iron-sulfur partial cubanes. The P-clusters are located at the interface of α and ß subunits. FeMo cofactor (FeMo-co) contains two components ([Fe_4S_3] and [$MoFe_3S_3$]) bridged by three sulfide bonds. Homocitrate is bonded to the Mo atom. FeMo-co is the actual site of nitrogen reduction. It is enclosed by the three domains of the α subunit. P clusters act as intermediates in the electron transport pathway (Peters et al. 1997; Howard and Rees 1996).

Dinitrogenase (MoFe protein; molecular mass 220,000) is joined with one or two molecules of dinitrogenase reductase (Fe protein; molecular weight 64,000). Fe protein (dinitrogenase reductase) has four iron atoms and is rapidly and irreversibly inactivated by O_2. Fe protein is 64 kDa. A single, regular [Fe_4S_4] cubane is symmetrically coordinated between the subunits by Cys97 and Cys132 from each subunit. This [Fe_4S_4] cluster is the redox-active center involved with electron transfer to MoFe protein. The [Fe_4S_4] cluster of Fe protein oscillates between the reduced and the oxidized state during electron transfer (Merrick 1992). Each Fe protein dimer can bind to two nucleotide molecules (Burgess and Lowe 1996). Binding of Mg-ATP at these sites results in a conformational change in Fe protein. The two subunits rotate toward each other, extruding the [Fe_4S_4] cluster toward the protein surface by 4°A which is an important step in the catalytic cycle of nitrogenase (Schindelin et al. 1997).

The redox potential of the [Fe_4S_4]$^{2+/1+}$ couple is changed from −300 mV to nearly −450 mV by binding of Mg-ATP to reduced Fe protein. An associated Mg-ATP-induced conformational change promotes interaction of Fe protein with MoFe protein. A second conformational change in the Fe protein changes the redox potential of the Fe_4S_4 cluster by another −200 mV. Transfer of single electron from the Fe protein to MoFe protein is now energetically possible. This electron transfer is coupled with the hydrolysis of Mg-ATP (bound to Fe protein) to Mg-ADP and Pi. The nitrogenase complex dissociates in the rate-limiting step of the cycle, after electron transfer and Mg-ATP hydrolysis. Fe protein is then reduced by a low potential electron donor like ferredoxin or flavodoxin in vivo. Mg-ADP is exchanged for Mg-ATP. The catalytic cycle continues till required numbers of electrons have been transferred to completely reduce the FeMo-cobound substrate. Fe protein is the obligate electron donor for MoFe protein in all characterized nitrogenase systems (Burgess and Lowe 1996).

Nitrogenase enzyme functions under anaerobic conditions, i.e., in the absence of oxygen. This character is attributed to the low redox potential required and high reactivity of this enzyme to oxygen. It has a complex oxygen protection system in place. It is evident from the X-ray crystallographic structures for the Fe protein (Schlessman et al. 1998) and MoFe protein (Peters et al. 1997) as well as for two complexes between the two proteins (Rees et al. 1998; Schindelin et al. 1997).

Nitrogenase activity is determined by many factors like temperature, pH, available soil moisture, and presence of oxygen. Nitrogenase activity in wet soils is higher than that observed in soils having moderate to low level of moisture. Also, high levels of available nitrogen are inhibitory to nitrogen fixation. The available nitrogen content of wet, marshy soils is low due to denitrification and leaching of nitrates. As expected, greater rate of acetylene reduction is observed in marshy soils in which crops like rice are grown, compared to soils where other cereal crops are grown. The decreased combined nitrogen in the soil enhances nitrogen fixation (Postgate 1998).

7.3.2 Alternative Nitrogenase

Under certain growth conditions, some nitrogen-fixing bacteria synthesize nitrogenases that contain vanadium (and iron) or only iron in the place of molybdenum. These non-molybdenum nitrogenases are called alternative nitrogenase. Alternative nitrogenases are not synthesized if molybdenum is present in sufficient quantity as the molybdenum nitrogenase is normally the main nitrogenase in the bacterial cell. Alternative nitrogenases serve as a backup mechanism to ensure that fixation of nitrogen can still take place even if molybdenum is not available. Vanadium nitrogenase (VNase) was first described by Bishop et al. (1980) in *Azotobacter vinelandii* and later it was isolated from *A. chroococcum.*

VNase consists of an iron protein, a homodimer with a total molecular mass of $M_r = 64\,000$, and an iron-heterometal (FeV) protein of $M_r = 240\,000$ (Fallik and Robson 1990). A [4Fe–4S] ferredoxin join the two subunits of the iron protein. It has two binding sites for Mg^{2+} ATP. VFe protein has an $\alpha_2ß_2\delta_2$ subunit structure. It has two P clusters located at the interface of the α and ß subunits and two M clusters which are located in the α subunits. The M cluster FeVco is the site for substrate activation. The metal clusters of he VFe protein are analogous to those in the Mo nitrogenase (Chen et al. 1993).

Protein residues required in binding the M and P clusters are conserved as discovered by amino acid sequencing of α and ß subunits of the Mo and V nitrogenases (Fallik and Robson 1990). Close homology of structure as well as function of the clusters has been elucidated by spectroscopic and extrusion studies (Eady 1996; Chen et al. 1993). The P clusters are double cubanes of the composition $[\{Fe_4S_3(Cys)_2\}_2(\mu\text{-}S)_2(\mu\text{-}Cys)_2]$ in the reduced form. V is the component of a complex Fe-S system. It is combined to three iron centers by binding three times to sulfide. It is then bonded to histidine and adjacent alkoxide and carboxylate of homocitrate.

Another type of VNase has been discovered in *A. vinelandii* where α subunit is not present. This nitrogenase has only one FeVco. Also, half of one of the P clusters is missing. The remaining fragment is bonded to a (4Fe–4S) ferredoxin. EPR and redox properties of the $\alpha\, \beta_2$ variant of the VNase are also different (Tittsworth and Hales 1996). Few cyanobacteria and *Anabaena variabilis* have these two alternative nitrogenases (Thiel et al. 1998). One of them is V-dependent nitrogenase. It is encoded by *vnfH*, *vnfDG*, *vnfK*, *vnfE*, and *vnfN* and functions only in the absence of Mo (Lyons and Thiel 1995). The *vnf* genes are expressed only in resistant heterocysts form. Their expression and also that of Mo-dependent *nif1* genes are controlled similarly. The *vnf* genes do not require vanadium for transcription. They are repressed by Mo. The *vnf* genes are constitutively expressed in cells grown in absence of fixed N_2 in Mo lacking mutants. They fix N_2 using V nitrogenase.

7.3.3 Hydrogenase

Many diazotrophs evolve dihydrogen (H_2) during N_2 fixation, which in turn inhibits the N_2 fixation reaction. This inhibitory action of H_2 is overcome by the hydrogenase enzyme which recycles H_2 produced by nitrogenase enzyme in nitrogen-fixing system.

Physiological H_2 uptake has been demonstrated to be similar in *A.vinelandii* and *A. chroococcum*. H_2 is produced when N_2 is reduced to NH^{+4}. It is reoxidized to H^+ by a Ni-dependent hydrogenase. This enzyme is encoded by the 16-gene *hup* cluster in *A. chroococcum* and *hox*/*hyp* cluster in *A. vinelandii* (Enon et al. 1992). Hydrogenase activity is beneficial in *A. chroococcum*. Hup^+ strain MCD-1 has greater ability to survive in controlled, carbon-limited conditions than Hup^- mutant (Yates and Campbell 1989). Nearly equal amounts of protein were produced in chemostat cultures of Hox^+ and Hox^- strains of *A. vinelandii*. Both had similar respiratory activites. Some amount of total respiratory activity was due to H_2 dependent O_2 consumption in the wild type. While fixing N_2 in carbon-limited growth conditions, the hydrogenase enzyme does not prove useful in *A. vinelandii*.

Two types of NiFe hydrogenases are present in cyanobacteria. Genes of these two hydrogenases have been characterized in *Anabaena* sp. PCC 7120, *A. variabilis* and *Nostoc* sp. PCC 73102 (Happe et al. 2000). One type of hydrogenase is an uptake hydrogenase. It has a large subunit encoded by *hupL* and a small subunit encoded by *hupS*. The uptake hydrogenase acts after the start of N_2 fixation (Happe et al. 2000). It is found in the thylakoid membranes of heterocysts. It utilizes the H_2 produced by nitrogenase for energy generation. Uptake hydrogenase lacking mutants, *hupSL* in *A. variabilis* and *hupL* in *Anabaena* sp. PCC 7120, generate greater amount of H_2 after N_2 fixation has begun than the wild-type strains.

The second type of hydrogenase is a bidirectional $NAD(P)^+$-reducing hydrogenase. It can lead to the formation as well as uptake of H_2. Dihydrolipoamide:NAD oxidoreductase component of this enzyme, also known as diaphorase, is encoded by *hox(E)FU*. The hydrogenase component is encoded by *hoxHY*. Nitrogen-fixing

cyanobacteria, e.g., *Anabaena* sp. PCC 7120 and *A. variabilis* possess this enzyme (Axelsson and Lindblad 2002). It is not present in all unicellular cyanobacteria. Whenever present in filamentous cyanobacteria, it can be found in both vegetative cells as well as heterocysts.

7.4 Genetic Basis of Nitrogen Fixation and Its Regulation

Genetical, biochemical, and physiological studies done on *Klebsiella pneumoniae* N_2 fixation system have revealed a number of basic concepts that are common to many diazotrophs (Burris and Roberts 1993; Dean and Jacobson 1992; Merrick 1992). Nitrogen fixation has also been studied extensively in various other diazotrophs such as *Azotobacter* spp. photosynthetic bacteria (Roberts and Ludden 1992), cyanobacteria (Haselkorn and Buikema 1992), *Azospirillum* spp. (Elmerich et al. 1992), Rhizobia (de Philip et al. 1992), and methanogenic bacteria (Lobo and Zinder 1992). Genes involved in nitrogen fixation are summarized in Table 7.2.

nod genes are those genes which direct specific nodulation events in a legume by a strain of *Rhizobium* that along with specificity genes (genes restricting a *Rhizobium* strain to a particular host plant) are borne on large plasmids called "Sym" plasmids. The *nod* gene products are required for the early steps in nodule formation. Ten *nod* genes have been identified in this species, namely, *nod M*, *nod L*, *nod E*, *nod F*, *nod D*, *nod A*, *nod B*, *nod C*, *nod I*, and *nod J*. The *nod ABC* genes are responsible for the synthesis of oligosaccharides, called nod factors, which induce root hair curling and trigger root cell division eventually resulting in the nodule formation. Nod factors contain a backbone of N-acetylglucosamine to which different substituents are linked. Host specificity is determined by the precise structure of the nod factor of a given *Rhizobium* spp. They often show variations in the structural components of their nod factors. However, *nod ABC* genes direct the synthesis of nod factor backbone, whereas variation components are synthesized under the direction of other nod genes. Roots of leguminous plants, unlike those of other plants, secrete large amounts of flavonoids, which act as inducer molecules and presumably trigger *nod* gene expression in nearby rhizobial cells in the soil. Some flavonoids, which are structurally very closely related to *nod* gene inducers (luteolin and eriodictyol), are considered to inhibit *nod* gene expression in certain *Rhizobium* spp.

The *nif* and *fix* genes of *A. caulinodans*, *B. japonicum*, and *R. meliloti* are organized in distinct clusters whose structure and genomic location are specific to the species. Linkage between nitrogen fixation genes in rhizobia is not as firm as in *K. pneumoniae*. In this organism 20 adjacent *nif* genes are organized in eight operons within *ca*. 24 kb of DNA (Arnold et al. 1988).

In *R. leguminosarum* and certain other species of *Rhizobium*, *nif* genes are plasmid-borne. *R. meliloti* carries two extremely large plasmids (megaplasmids) of about 1400 kb (pSym-a or megaplasmid 1) and 1700 kb (pSym-b or megaplasmid 2) (Honeycutt et al. 1993). Both cluster I (consists of 12 genes

Table 7.2 Functions of *NOD*, *NIF*, and *FIX* genes

Gene	Function	References
***nod* gene**		
nod M	Nod factor synthesis	Merrick (1992)
nod L	Determine host range	Gottfert (1993)
nod E	Determine host range	Gottfert (1993)
nod F	Encodes a specific acyl carrier protein used to acylate the nod factor specified by *nod A*	Merrick (1992)
nod D	Encodes a regulatory protein called Nod D that controls transcription of other *nod* genes	Merrick (1992)
nod A	Direct synthesis of nod factor backbone	Gottfert (1993)
nod B	Direct synthesis of nod factor backbone	Gottfert (1993)
nod C	Direct synthesis of nod factor backbone	Gottfert (1993)
nod I	Membrane proteins that help in exporting nod factors	Kondorosi et al. (1991)
nod J	Membrane proteins that help in exporting nod factors	Kondorosi et al. (1991)
***nif* gene**		
nifD	α subunit of dinitrogenase. Forms an α_2 β_2 tetramer with ß subunit interface. FeMo-co, the site substrate reduction, is present buried within the α subunit of dinitrogenase	Dean and Jacobson (1992)
nifK	ß subunits of dinitrogenase. ß clusters are present at ß subunit interface	Dean and Jacobson (1992)
nifH	Fe protein subunit of dinitrogenase reductase. Obligate electron donor to dinitrogenase during dinitrogenase turnover. Also is required for FeMo-co biosynthesis	Dean and Jacobson (1992)
nifN	Required for FeMo cofactor biosynthesis	Allen et al. (1994) and Dean and Jacobson (1992)
nifV	Encodes a homocitrate synthase. Homocitrate is an organic component of FeMo cofactor	Hawkes et al. (1984) and Hoover et al. (1987)
nifB	Required for FeMo cofactor biosynthesis. Metabolic product. NifB-co is the specific Fe and S donor to FeMo-co	Allen et al. (1994) and Shah et al. (1999)
nifQ	Incorporation of Mo into FeMo cofactor. Proposed to function in early MoO_4^2 processing	Imperial et al. (1984)
nifE	Forms $\alpha_2\beta_2$ tetramer with *nifN*. Required for FeMo cofactor biosynthesis	Allen et al. (1994) and Dean and Jacobson (1992)
nifX	Not essential for nitrogen fixation; required for FeMo cofactor biosynthesis	Shah et al. (1999)
nifU	Involved in mobilization of Fe-S cluster synthesis and repair	Yuvaniyama et al. 2000
nifS	Involved in mobilization of Fe-S cluster synthesis and repair	Zheng et al. (1993)
nifY	Associates with MoFe protein and dissociates upon FeMo cofactor insertion	Homer et al. (1993)
nifM	Required for the maturation of *nifH* and Fe protein maturation. Putative peptidyl-prolyl cis/trans isomerase	Dean and Jacobson (1992)

(continued)

Table 7.2 (continued)

Gene	Function	References
nifW	Involved in stability of dinitrogenase. Proposed to protect dinitrogenase from O inactivation	Kim and Burgess (1996)
nifF	Flavodoxin required for electron transfer to the Fe protein, Physiologic electron donor to *nifH*	Thorneley et al. (1992)
nifJ	Pyruvate flavodoxin (ferredoxin) oxidoreductase involved in electron transport to nitrogenase; couples pyruvate oxidation to reduction of the *nifF* product	Shah et al. (1988)
nifA	Positive regulator of *nif* transcription	Dixon (1998)
nifL	Negative regulatory protein	Dixon (1998)
***fix* gene**		
fix LJ	Oxygen-responsive two-component regulatory system involved in positive control of *fixK* and *nifA*	David et al. (1988)
fix K	Positive regulator of *fixNOQP*, *nifA*; negative regulator of *nifA* and *fixK*	Batut et al. (1989)
fix NOQP	Microaerobically induced, membrane-bound cytochrome oxidase	Boistard et al. (1991)
fix GHIS	Redox process-coupled cation pump	Kahn et al. (1989)
fix ABCX	Unknown function; required for nitrogenase activity; FixX shows similarity to ferredoxins	Earl et al. 1987
fix R	Unknown function; not essential for nitrogen fixation	Thony et al. (1987)

nifHDKE, *nifN*, *fixABCX nifA nifB frdX*) and cluster II (consists of 10 genes *fixLJ*, *fixK*, *fixNOQP*, *fixGHIS*) are located on megaplasmid 1 (David et al. 1987). The cluster II genes map at about 220 kb downstream of the *nifHDKE* operon and are transcribed in opposite orientation to it. A functional duplication of the region spanning *fixK* and *fixNOQP* is present at ca. 40 kb upstream of *nifHDKE* (Renalier et al. 1987). A cluster of *nod* genes including the common nod genes (*nodABC*) is located in the 30-kb region between *nifE* and *nifN* (Long 1989). Additional genes required for an effective symbiosis are located on megaplasmid 2 and on the chromosome (Honeycutt et al. 1993).

The *nifD* and *nifK* genes specify α and ß subunits, respectively, of the $\alpha_2ß_2$ FeMo protein (dinitrogenase or component I; Mr 220,000). The homodimeric Fe protein component or dinitrogenase reductase (Mr 60,000) is encoded by *nifH*. In *R. meliloti nifHDK* genes are organized in an operon along with *nifE*. For the synthesis of the FeMo cofactor of component I, the products of the *nifE*, *nifN*, and *nifB* genes are required. The exact biochemical functions of the respective proteins are not known (Dean et al. 1993). The amino acid sequences of the NifE and NifN proteins show a significant similarity to those of NifD and NifK, respectively. It was suggested that the *nifEN* genes originated from duplication of the *nifDK* genes and that the *NifEN* complex may provide a scaffold for FeMo cofactor biosynthesis (Brigle et al. 1987). Downstream of the *nifB* genes of both *R. meliloti* and *B. japonicum* are the genes *fdxN* and *frxA*, respectively, encoding ferredoxin-

like electron transfer proteins. In contrast to *frxA* of *B. japonicum*, the *R. meliloti fdxN* gene is absolutely essential for nitrogen fixation (Ebeling et al. 1988). Another ferredoxin-like protein is encoded by *fixX*; this gene is located downstream of *fixC* in *Rhizobium* spp.

FeMo cofactor is synthesized by the expression of several genes in *Klebsiella* including *nifN*, *nifV*, *nifB*, *nifQ*, *nifE*, *nifX*, *nifU*, *nifS*, and *nifY*. Genes *nifS* and *nifU* play a part in the assemblage of Fe-S clusters (Hu and Fay 2007). Maturation of Fe protein is brought about by the products of *nifH*, *nifM*, *nifU*, and *nifS*. *nifE* and *nifN* products function as scaffold for FeMo-co biosynthesis. Various genes have their own specific functions – *nifB* gene product acts as iron and sulfur-containing precursor of FeMo-co, gene *nifQ* is a molybdenum sulfur-containing precursor of FeMo-co, and gene *nifV* encodes homocitrate synthase. It is required for the synthesis of FeMo-co. The gene *nifW* protects the dinitrogenase protein from oxygen inactivation thus stabilizing it (Cheng 2008). *Klebsiella* also contains the genes that mediate electron transport to nitrogenase. The *nifF* gene encodes flavodoxin which transfers electrons to nitrogenase, *nifJ* encodes pyruvate oxidoreductase that transfers electrons to flavodoxin from the pyruvate, *nif A* encodes positive regulatory protein that serves to activate transcription of other genes, and *nifL* acts as repressor of nitrogenase (Beringer and Hirsch 1984).

The *fixABCX* genes were first identified in *R. meliloti* (Earl et al. 1987) in *B. japonicum* (Gubler and Hennecke 1986), *A. caulinodans* (Kaminski et al. 1988), *R. leguminosarum* bv. *viciae* (Gronger et al. 1987), *R. leguminosarum* bv. *trifolii* (Iismaa et al. 1989), and *R leguminosarum* bv. *phaseoli* (Michiels and Vanderleyden 1993). They are organized in a single operon. In *B. japonicum fixA* and *fixBCX* form distinct transcriptional units present in clusters II and I, respectively. The *B. japonicum fixBCX* operon includes a proximal open reading frame (ORF35), which is not essential for nitrogen fixation activity but whose translation significantly stabilizes *fixBCX* mRNA (Gubler et al. 1989). Mutations in any one of the *fixABCX* genes of *R. meliloti*, *B. japonicum*, and *A. caulinodans* totally eliminate nitrogen fixation. It has been proposed that the *fixABCX* gene products may have a role in electron transport to nitrogenase (Earl et al. 1987, Gubler and Hennecke 1986).

The *fixNOQP* genes were first described in *R. meliloti* as a duplicated *fix* region that is linked to the regulatory genes *fixLJ* and *fixK* and whose expression is induced under symbiotic conditions (Renalier et al. 1987). Homologous genes were then identified in *B. japonicum* (Preisig et al. 1993), *R leguminosarum* bv. *viciae* (Hynes et al. 1992), and *A. caulinodans* (Mandon et al. 1993). They are probably organized in an operon in all three species. *R. meliloti* mutant strains deleted for both *fixNOQP* regions and *B. japonicum fixNOQP* mutants have flawed symbiotic nitrogen fixation. A corresponding mutant of *A. caulinodans* retained 50% of wild-type nitrogenase activity under both symbiotic and free-living conditions (Mandon et al. 1993; Renalier et al. 1987). In addition, *B. japonicum* mutants are affected in bacteroid development and exhibit a decreased whole-cell oxidase activity when grown microaerobically or anaerobically. The amino acid sequences of the FixNOQP proteins imply that they constitute a membrane-bound, cytochrome

c-containing heme/copper cytochrome oxidase. It is postulated that this oxidase complex is required to support bacteroid respiration under conditions of low oxygen present in root nodules (Preisig et al. 1993).

Four tightly linked genes, named *fixGHIS*, have been identified by mutational analysis and subsequent DNA sequence determination downstream of the fixNOQP operon in cluster II of *R. meliloti* (Kahn et al. 1989). On the basis of hybridization experiments, homologous genes are found to exist in various members of the genus *Bradyrhizobium*, in *A. caulinodans*, and in *R. leguminosarum* bv. *viciae* and bv. *phaseoli*. FixG is likely to be involved in a redox process, because it contains two cysteine clusters typical of iron-sulfur centers present in bacterial ferredoxins. FixI is homologous to the catalytic subunit of prokaryotic (bacterial) and also eukaryotic ATPases which are involved in cation pumping. It is speculated that FixI is a symbiosis-specific cation pump whose function is coupled to a redox reaction catalyzed by the FixG subunit (Kahn et al. 1989). Further biochemical analysis is required to define the function of the *fixGHIS* gene products in rhizobial nitrogen fixation.

In *B. japonicum* a gene termed *fixR* is located upstream of the regulatory *nifA* gene, and the two genes form an operon (Thony et al. 1987). No *fixR*-like gene has been described so far in other rhizobia, but interspecies hybridization experiments indicate the existence of homologous DNA regions in other slow-growing rhizobia and in the nonsymbiotic bacterium *Rhodopseudomonas palustris* (Thony 1989).

Nitrogen fixation is regulated at the transcriptional level. It is in response to environmental oxygen and ammonium levels. The nitrogenase components are oxygen labile. Bacteria suppress transcription when oxygen levels are high. It is also advantageous to repress the expression of the metabolically expensive nitrogenase system when the cellular level of fixed nitrogen is adequate. The degree to which each stimulus affects transcription is characteristic of the particular diazotroph. Nitrogenase expression in symbiotic diazotrophs is insensitive to ammonium. It is due to the fact that export of ammonium to their symbiont represses ammonium levels.

The expression of *nif* genes in free-living diazotrophs is more sensitive to cellular ammonium levels (Merrick 1992). The paradigm of transcriptional regulation is based on studies on *K. pneumoniae*. In this model, regulation of *nif* gene expression is considered to be based on two elements, an external system involving *ntr* genes and an internal system mediated by *nif* A and *nif* L genes. The *ntr* gene system is responsible for transcription of *nif* genes, while *nif* A and *nif* L genes act as regulatory system through a “switch on” and “switch off” mechanism. Gene *nif* A produces Nif A protein, which activates the *nif I* genes transcription*;* whereas *nif* L genes produces Nif L protein, which inhibits the *nif* gene transcription.

The interrelationship between external (*ntr* genes) and internal (*nif* A and *nif* L genes) systems in *Klebsiella pneumonia* is represented in a simplified way. The protein Ntr A (the product of *ntr* A gene) is a factor of RNA polymerase and allows the latter to bind at *nif* promoters to begin *nif* gene transcription (i.e., N_2 fixation). When ammonia is in an excess in the environment, it inhibits nitrogen fixation through Ntr C protein (the product of *ntr* C gene) and Nif L protein (the product of

nif L gene). In NH_3 excess condition, the Ntr C protein represses the functioning of Ntr L protein, thus "switching off" N_2 fixation. The activity of Ntr C protein is regulated by Ntr B (product of *ntr* B gene). Ntr B is an enzyme that functions both as a protein kinase and as a phosphate; the kinase or phosphatase activity of Ntr B is regulated by the nitrogen status of the cell. When ammonia is limiting, Ntr C protein is activated and promotes the transcription of *nif* A gene to produce *Nif* A protein that "switches on" the N_2 fixation.

Nitrogenase activity is also inhibited, as mentioned earlier, by oxygen. It is to note that Nif L protein contains a molecule of FAD (ferredoxin adenine nucleotide) that is critical for oxygen sensing by the protein. When oxygen exceeds the required level, Nif L protein shuts down the transcription of *nif* genes, and as a result, the synthesis of oxygen-sensitive nitrogenase stops.

Ammonia also regulates nitrogenase activity in certain nitrogen fixers. This phenomenon is called ammonia "switch off" effect. In this case, excess ammonia modifies the structure of dinitrogenase reductase (Fe protein) leading to a loss of nitrogenase activity. When ammonia returns to limiting level, the modified dinitrogenase reductase (Fe protein) converts back to its active form, and the activity of nitrogenase resumes.

The control of *nif* gene expression is focused on NifA (the *nif*A gene product), a σ^{54} (*rpoN* gene product)-dependent transcriptional activator. It is responsible for control of all major *nif* gene cluster transcription. Transcription of *nifA* is under the control of the *ntrBC* gene products. They comprise a global two-component transcriptional activator system which is responsible for cellular nitrogen regulation (Merrick 1992). In the model organism *K. pneumoniae*, the *nifA* gene is co-transcribed with *nifL*. This gene encodes a redox- and nitrogen-responsive regulatory flavoprotein (NifL) which acts as a negative regulator of NifA. This adds another level of regulation in response to oxygen and fixed nitrogen. Oxidized NifL is also sensitive to the nucleotides present in vitro. It exhibits increased inhibition especially in response to ADP (Hill et al. 1996). The means by which NifL inhibits NifA remain unclear.

Deviations from the *K. pneumoniae* model have been found in approximately all nitrogen fixation organisms of interest. In *A. vinelandii* and *Rhodospirillum rubrum*, expression of *nifA* is not regulated by *ntrBC* gene products. It is still not known whether *nifA* expression is under nitrogen control. In *Rhizobium meliloti*, redox-dependent control of *nifA* expression exists in response to *fixL* and *fixJ*, which encode for a two-component regulatory system responsive to oxygen (Merrick 1992). This system is replaced by the *ntrBC* control found in *K. pneumoniae*. *R. meliloti* also lacks NifL. NifA is inhibited by oxygen stimulus (Krey et al. 1992). Interestingly, there is no evidence for NifL in *Rhodobacter capsulatus*. This organism contains *nif*-related genes analogous to *ntrBC*, but the expression of an *rpoN*-like gene is found to be sensitive to oxygen and amount of fixed nitrogen available (Merrick 1992). *R. capsulatus* contains two copies of *nifA*, which respond differently to ammonium (Klipp and Paschen 1998). Nitrogenase transcriptional control mechanisms are separate for different diazotrophs.

An added level of nitrogenase regulation is present in a few free-living diazotrophs due to the metabolically demanding nature of nitrogen fixation. To prevent unproductive nitrogen fixation during energy-limiting or nitrogen-sufficient conditions, the nitrogenase complex is rapidly and reversibly inactivated by ADP-ribosylation of Fe protein. The ADP-ribosylation system has been identified in *R. rubrum* and *R. capsulatus* (purple and non-sulfur photosynthetic bacteria), in *Azospirillum brasilense* and *Azospirillum lipoferum* (microaerophilic, associative bacteria), and also in *Chromatium vinosum* (a purple sulfur bacterium) (Ludden and Roberts 1989). *R. rubrum* remains the model organism under study. The posttranslational nitrogenase regulation was first identified in this organism. The ADP-ribosylation in *R.rubrum* exhibits the roles of the NAD^+-dependent enzyme, dinitrogenase reductase ADP-ribosyltransferase (DRAT), and its partner, dinitrogenase reductase-activating glycohydrolase (DRAG).

ADP-ribosylation of Fe protein occurs at a specific arginine residue (Arg101 in *R. rubrum*). It occurs by the formation of a α-N-glycosidic bond between the guanidino nitrogen atom of arginine and the terminal ribose of ADP-ribose (Ludden and Roberts 1989). Structurally, this ADP-ribose is similar to the modifying groups attached by bacterial ADP-ribosylating toxins. These toxins are the causative agents of cholera and diphtheria. The presence of the ADP-ribose group in nitrogenase inhibits association of Fe protein with MoFe protein instead of blocking electron transfer between complexed Fe protein and MoFe protein (Ludden and Roberts 1989). ADP-ribosylated Fe protein differs from unmodified Fe protein with respect to a few characteristics. The two subunits of the inactive Fe protein dimer are not equivalent because ADP-ribosylation occurs on only one subunit. Modified Fe protein retains the native $[Fe_4S_4]$ cluster. It can be chemically oxidized and reduced and it also retains the oxygen accountability of the active Fe protein. ADP-ribosylated Fe protein cannot hydrolyze Mg-ATP, but it still has the ability to bind Mg-ATP and also to undergo the conformational change that gives access of the $[Fe_4S_4]$ cluster to chelators (Ludden and Roberts 1989). It also plays a role in synthesis and insertion of FeMo-co into MoFe protein (Shah et al. 1988).

The genes encoding DRAT (draT) and DRAG (draG) are co-transcribed from a non-nif operon. This operon includes a third gene (*draB*) of unknown function. The configuration of the draTGB operon is conserved in both *A. brasilense* and *A. lipoferum* (Inoue et al. 1996). *R. capsulatus*, however, lacks draB (Masepohl et al. 1993). DRAT is a 30-kDa monomer with high specificity toward oxidized Mg-ADP-bound Fe protein. It has been found to possess no measurable activity with other arginine residues or even water as the ADP-ribose acceptor (Ludden and Roberts 1989; Halbleib et al. 2000). The amino acid sequence of DRAT is not really comparable to those of the bacterial toxins. Some key amino acid residues are conserved. The Fe proteins from *K. pneumoniae* and *A. vinelandii* are better substrates for *R.rubrum* DRAT than the *R. rubrum* Fe protein itself. These organisms lack the dra operon. There are no measurable reverse or glycohydrolytic reactions catalyzed by DRAT. The removal of the ADP-ribose group is catalyzed by dinitrogenase reductase activating glycohydrolase (DRAG). It restores fully active Fe protein with an intact Arg101 side chain. DRAG is a 32-kDa monomeric

binuclear manganese enzyme that is capable of cleaving the α-N-glycosidic bond of a number of analogs of ADP-ribosylarginine (Ludden and Roberts 1989). Only the reduced Mg-ATP-bound form of ADP-ribosylated Fe protein is a substrate for DRAG (Ludden and Roberts 1989; Halbleib et al. 2000). The exact modes of interaction of DRAT and DRAG with Fe protein are not defined, but it is supposed that each binds the same surface of Fe protein like MoFe protein. Overexpressed DRAT inhibits cellular nitrogenase activity (Grunwald et al. 1995).

The method of regulation of DRAT and DRAG is not well understood. However, it is known that the activity of each enzyme is regulated in vivo (Liang et al. 1991). It is widely known that the regulatory signals involve either negative effectors or known assay components. DRAT and DRAG have opposite specificities for Mg-ADP and Mg-ATP-bound Fe protein. Nitrogenase activity regulation is subject to cellular concentration in ATP and ADP levels during cycles of inactivation/activation (Ludden and Roberts 1989). Sensitivity of DRAT and DRAG toward the redox state of Fe protein suggests that DRAT and DRAG may be regulated by sensing the cellular energy and redox status directly from the state of Fe protein (Halbleib et al. 2000). The cellular NAD^+ concentration may also be a positive effector for DRAT (Norén et al. 1997). It appears that the unregulated variants of DRAG have altered divalent cation affinities.

The nitrogenase-inactivating conditions of nitrogen sufficiency (NH^{4+}) and energy limitation give rise to convergent signal transduction pathways. Inhibition of glutamine synthetase unsettles both responses. The cellular concentration of glutamine, however, is relatively unaffected by the modification and demodification of Fe protein (Kanemoto and Ludden 1987). Genetic changes of nitrogen control genes (*glnB, ntrBC*) yield results that do not support the model of narrowly confined signal transduction pathways. It is so because the effects on ammonia response seem to be independent of the darkness response (Zhang et al. 1995).

The response of DRAT and DRAG activities to exogenous inactivation effectors is not species specific. It was evidenced by plasmid-borne *draTG* genes from *A. brasilense* that restored the wild-type phenotype to dra mutants in *R. rubrum* (Zhang et al. 1992). The Fe protein was inactivated in response to darkness and not absence of oxygen. Mutants of *K. pneumonia* having a plasmid with *draTGB* from *R. rubrum* reversibly ADP-ribosylate Fe protein in the presence of exogenous ammonium. Future work in nitrogen fixation will be centered around regulatory strategies in all beneficial agricultural bio-inoculants.

7.5 Impact of Using N_2-Fixing Bio-inoculants: Prospects and Challenges

Bio-inoculants are microbial preparations containing live or latent cells of efficient strains of nitrogen-fixing, phosphate-solubilizing, or cellulolytic microorganisms. They can be applied to seed, soil, or composting areas with the objective of

increasing the population of such microorganisms and accelerating those microbial processes which augment the availability of nutrients to plants. Biofertilizers play a crucial role in long-term sustainability and aid in mitigating the environmental pollution associated with indiscriminate use of agrochemicals. They are seen as one of the best modern tools for sustainable agriculture. An indispensable requirement of sustainable agriculture is the continuous renewal of soil structure and fertility with renewable resources, lessening the need for chemical fertilizers, thus reducing their environmental burden. Intensive agriculture that involves heavy and continuous use of fertilizers has ensured high crop productivity from shrinking agricultural land. The use of fertilizers, including chemical fertilizers and manures, to enhance crop productivity has often negatively affected the complex system of the biogeochemical cycles (Steinshamn et al. 2004). Fertilizer use has caused leaching and runoff of nutrients, especially nitrogen (N) and phosphorus (P), leading to adverse environmental impact (Gyaneshwar et al. 2002).

Microbes are essential in all phases of agricultural practices as in maintenance of soil structure and fertility (Nakas and Hagedorn 1990), in preservation and processing of crops, and in recycling crop residues (Hanson 1996). They contribute a wide range of essential services to the sustainability of all ecosystems, by acting as the primary driving agents of nutrient cycling, regulating the dynamics of soil organic matter, modifying soil physical structure, enhancing the efficiency of nutrient acquisition by the vegetation, and enhancing plant health. These services are not only essential to the functioning of natural ecosystems but constitute an important resource for the sustainable management of agricultural and environmental ecosystems. Microbial intervention in soil fertility, biocontrol, and plant growth promotion involves the introduction of natural or transgenic microbial inoculants. Microbial inoculants or biofertilizers are promising tools in integrated solutions to agro-environmental issues. They possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants (Adesemoye et al. 2008). Thus, the incorporation of biofertilizers plays an important role in improving soil fertility, yield attributing characters of crops, and thereby crop yield. In addition, their application in soil improves soil biota and decreases the use of chemical fertilizers.

7.6 Future Outlook

Elucidation of the regulation of the nitrogen-fixation process in bacteria has advanced considerably due to extensive work done on wide range of model systems as well as expansion in the knowledge of molecular microbiology. This understanding has helped to derive physiological benefits from beneficial rhizospheric microbes. At the molecular level, the understanding of the regulatory processes is increasing especially in those organisms that have a NifA-dependent mode of control. The harvest of pure active NifA proteins (particularly of the O_2 sensitive group) is still a major road block. Newer information on broad outline of the major

signal transduction pathways may be in the public domain very shortly. A lot is yet to be unraveled about Gram-positive diazotrophs. To summarize, the challenge now is to amalgamate the existing knowledge into a whole-cell perspective on the genetic, biochemical, and physiological processes that contribute to successful diazotrophy. While plant biotechnology is making efforts to transfer N-fixing genetic potential in cereals to aid in their N-economy, it may take another 50 years to fructify on a field scale. Till that time, use of N_2-fixing microbes either in close association or as endophyte are good options for a sustainable agriculture.

Many varieties of various plants provide conducive environment to support the growth of endophytes and derive benefits as nitrogen fixation from them (Iniguez et al. 2004; Gutierrez-Zamora and Martinez-Romero 2001; Engerhard et al. 2000; Shrestha and Ladha 1996; Urquiaga et al. 1989). There are still a number of uncertain aspects that need to be focused on as soil microbiomes, soil metagenomics, transcriptomics, and key genes of active microorganisms associated with nodulation, growth regulators, disease suppressing, and nutrient cycling in soil. Development of biofertilizers using microbial consortia containing effective, competitive, and stress-tolerant microbial strains is required. The potential of biofertilizers to supply micronutrients and for biofortification of food crop is yet to be explored. For the development of biofertilizers, public sector should take steps in the future research like monitoring the quality of biofertilizers and their effects on plants and humans. Extension, training program, or short-term diploma programs may be initiated for motivating the farmers to exploit full potential of the biofertilizer technology.

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