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Review Article

Recent Advances in Biological Nitrogen Fixation in Agricultural Systems

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The environmental concerns due to the increasing amount of the reactive forms of nitrogen in atmosphere, originating from the manufacture and use of chemical fertilizers have resulted in a re-focus on the importance of biological nitrogen fixation (BNF), particularly by legumes. The worldwide contribution of BNF is estimated at 105 Tg yr^{-1}. Nitrogen fixation is catalysed by the enzyme nitrogenase. Advances have included discovery of alternative nitrogenases, elucidation of more details on the mechanism of action, discovery of novel nitrogenases, existence of nitrogenase siblings in eukaryotes etc. There are fresh efforts to engineer cereal plants to nodulate and fix nitrogen. Discovery of common signalling mechanisms between mycorrhization and nodulation, discovery of cereal endophytes and advances in plastid physiology have given a fresh impetus to such approaches. However, the contribution of agronomically significant quantities of N through endophytes still requires more rigorous evidences. Advances in molecular biology have included a greater understanding of the native diversity of rhizobia and together with advances in plant breeding for selection of high nodulating genotypes, presents an attractive opportunity to maximize BNF. Recent advances include the discovery of novel rhizobial species, emphasis on study of climate change and adaptation to various abiotic stresses like high temperature, drought and salinity. This review focuses on major developments in BNF in last two decades with emphasis on rhizobia. The beneficial effects of applying organics and of rhizobial inoculation practices are discussed to bring out the strategies to improve the contribution of BNF in agriculture.

Key Words: Climate Change; Endosymbiosis; Fertilizer; Nitrogenase; Nodulation; *Rhizobium*; Salinity; Soybean

Introduction

Nitrogenous fertilizers have revolutionized crop yields and food availability worldwide. Of all the previous century's technological marvels, the Haber-Bosch process of manufacturing ammonia has made the most difference to our survival and it is considered the most valuable invention of the previous millennium [1]. However, this has come at a substantial economic and environmental cost. Industrial nitrogen fixation is a $100 billion per year global industry, growing and bringing with it the concomitant environmental nitrogen pollution effects. Intensive cropping with the use of high analysis fertilizers coupled with a concomitant reduction in recycling of organics or other wastes has led to a decline in the organic carbon levels in soils [2], impaired soil physical properties, reduced soil biodiversity and aggravation of demand for nutrients that are not applied. All of these are contributing to stagnating yields, reduced factor productivity and impaired soil health [3]. Not surprisingly, there is a renewed emphasis now on biological technologies like composting, legume biological nitrogen fixation (BNF), biofertilizers, integrated nutrient management, biopesticides etc. There is also world-wide consensus that sole dependence on chemical inputs based agriculture may not be sustainable in

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the long run and only integrated plant nutrient systems (IPNS) involving a combination of fertilizers, organics and microbial inoculants is essential to sustain crop production, preserve soil health and soil biodiversity. This mini-review keeps in mind the objectives of the proceedings of the Indian National Science Academy of concentrating on areas of contemporary scientific interest, evaluating advances and newer approaches in the field. An excellent review of the past work in this area is available [4]. The focus of this review is particularly on developments and issues in the last two decades - BNF inventories, nitrogenase biochemistry, endophytic nitrogen fixation, rhizobial taxonomy, GMO's, and effects of climate change and abiotic stress.

Global Estimates of BNF

There are a lot of concerns about the increasing amount of the reactive forms of nitrogen (Nr) in atmosphere [5]. Since 1970, world population has increased by 78% and reactive nitrogen creation has increased by 120%. Efforts for N budgeting for 2050 AD show that human activities are increasingly dominating the N budget at the global and regional scales. In addition, the terrestrial and open ocean N budgets are essentially disconnected, leading to the accumulation of fixed forms of N in most environmental reservoirs [6]. The largest uncertainties in our understanding of the N budget at most scales are the rates of natural biological nitrogen fixation, the amount of Nr storage in most environmental reservoirs and the production rates of N_2 by denitrification [6].

After photosynthesis, nitrogen fixation is considered as the second most important process influencing primary productivity and is the basis of all life on earth. Annually, approximately 2.5×10^{11} kg NH_3 is fixed from the atmosphere through BNF (by legumes and cyanobacteria) and approximately 8×10^{10} kg NH_3 are manufactured by ammonia industry [7]. Further, lightning may also contribute approximately 1×10^{10} kg NH_3/year worldwide. Currently, approximately 2 tonnes of industrially fixed nitrogen is needed as fertilizer for crop production to equal the effects of 1 tonne of nitrogen biologically fixed by legume crops. Therefore, biologically fixed nitrogen influences the global nitrogen cycle substantially less than industrially fixed nitrogen. World population has been increasingly relying on nitrogen fertilizers in order to keep up with the demands of food. World demand for total fertilizer nutrients is estimated to grow at 2% per annum between 2011 and 2015; the demand for nitrogen is forecast to grow annually by 1.7% globally and by 2.6% in south Asia [8]. Globally, BNF in natural terrestrial ecosystems contributes about 107 Tg of nitrogen (1 Tg = 1 million tonnes) while marine N fixation contributes 121 Tg of nitrogen each year [6]. Cultivation-induced BNF in agricultural crops and fields adds 33 Tg per year [9]. The break-up is: a symbiotic BNF by *Rhizobium* associated with seed legumes- 10 Tg (range 8-12 Tg), leguminous cover crops (forages and green manures)-12 Tg/yr, non-*Rhizobium* N fixing species-4 Tg/yr, cyanobacteria in wet rice fields 4-6 Tg and endophytic N fixing organisms in sugarcane- 1-3 Tg. Thus, total terrestrial nitrogen fixation is 140 Tg N/year. It is estimated that cultivation induced BNF may have risen to 40 Tg/yr due to increase in soybean and meat production [5]. Relative to cultivation induced BNF, about 3 times as much N was fixed as ammonia by the Haber-Bosch process, about 100 Tg N per year of ammonia in 1995 of which about 86% was used to make fertilizers [10]. The industrial fixation of nitrogen is increasing each year with the setting up of more plants and it reached to 121 Tg by 2006 [11] with the worldwide manufacture of N fertilizer at 105 Tg. Although the conclusions drawn by Mulvaney [2] about depletion of soil organic nitrogen by synthetic nitrogenous fertilizers are disputed, there can be no disagreement about their conclusion that a transition may be required toward agricultural diversification using legume-based crop rotations, which provide a valuable means to reduce the intensity of ammoniacal fertilization with the input of less reactive organic N.

Herridge *et al*. [12] calculated annual inputs of fixed N to be 2.95 Tg for the pulses and 18.5 Tg for the oilseed legumes. Soybean is the dominant crop legume representing 50% of the global crop legume

area and 68% of global production. They calculated soybean to fix 16.4 Tg N annually, representing 77% of the N fixed by the crop legumes. They estimated annual N_2 fixation input of 12-25 Tg (pasture and fodder legumes), 5 Tg (rice), 0.5 Tg (sugar cane), <4 Tg (nonlegume crop lands) and <14 Tg (extensive savannahs), totalling an overall estimate of 50-70 Tg N fixed biologically in agricultural systems. From the average values of BNF in legumes, cereals, oilseed, fibre, horticultural and fodder crops in India cultivated over 190 million ha a conservative estimate for BNF inputs in Indian agriculture, based on the responses measured in experiments conducted in the All India Coordinated Research Project on Biological Nitrogen Fixation (AICRP-BNF) amounts to 3.68 Tg every year. A conservative estimate for BNF inputs from forests, permanent pastures and grazing lands, miscellaneous tree crops, culturable wastelands, and fallow lands amounting to 121 m ha, works out to 0.52 Tg per year [13].

Nitrogenase Biotechnology

The biological fixation of nitrogen is an anaerobic process catalysed by the enzyme nitrogenase and requires a source of reductant, ATP and ammonia assimilating machinery. Enormous progress in almost all aspects of biological nitrogen fixation has been made in the past century, especially in the recent two decades in genetics and biochemistry. Nitrogenase is encoded by a set of operons, which includes regulatory genes such as nif LA, and structural genes such as nif HDK and other supplementary genes. *Klebsiella pneumoniae* has been the *E. coli* of BNF and it is most well studied with respect to the regulation, synthesis and assembly of nitrogenase. A 24kb base pair DNA region contains the entire *K. pneumoniae nif* cluster, which contains 20 genes.

Nitrogenase Assembly

The crystallographic structures of both components of nitrogenase (Mo-Fe protein and Fe protein) have been determined. In *Azotobacter vinelandii*, it was found that the α- and β-subunits of the nitrogenase Fe-Mo protein have similar polypeptide folds [14]. The FeMo-cofactor is completely encompassed by the α-subunit, whereas the P-cluster pair occurs at the interface between α- and β-subunits. The location of the metal clusters and the key steps of nitrogenase functioning have been elucidated but two questions about the nitrogenase mechanism still remain [7]. The role for ATP hydrolysis is to control the electron transfer, "gate" between the two enzyme components. How this is accomplished is not known. Secondly, although it is known that Fe-Mo-cofactor clusters act as the enzyme's substrate binding site and reducing site, but exactly where the substrates bind and are activated remains controversial [7].

Nitrogenase Siblings

Structural similarities have been apparent between nitrogenase and other electron transfer systems, including hydrogenases and the photosynthetic reaction centre [14]. The most important pigment molecule in photosynthesis is chlorophyll *a*, which uses light only at certain wavelengths; more energy can be captured if it uses other accessory pigments to absorb the energy from other wavelengths and pass it to chlorophyll *a*. The reduction of NADPH:: protochlorophyllide (pchlide) is a key step in the synthesis of chlorophyllide (Chl) during the greening of the phototrophic organisms. The process is catalysed by NADPH::protochlorophyllide oxidoreducatse (light operative protochlorophyllide oxidoreducatse or LPOR). With the exception of angiosperms, a dark operative-protochlorophyllide oxidoreducatse (DPOR) operates to convert protochlorophyllide to chlorophyllide. Genes for DPOR were first discovered from the complete chloroplast genome sequence of the liverwort, *Marchantia polymorpha* [15]. A gene designated *frxC* (later renamed *chlL*) was found to have a high degree of similarity with nif H of nitrogenase. Similarly, bchl L, bchl N and bchl B involved in protochlorophyllide reduction in *Rhodobacter capsulatus* [16] and chl L, chl N and chl B from cyanobacteria, algae and gymnosperms exhibit striking homologies to the three sub-units of nitrogenase [17]. This suggested that light independent protocholorophyllide reductase and nitrogenase share a common evolutionary ancestor [18]. The nif H gene codes for the Fe protein of nitrogenase, and is involved in transferring electrons to the MoFe protein (coded by nif D and

nif K) in a reaction that is coupled with the hydrolysis of MgATP. Chl L is similar to Nif H (~ 35%), while chl NB is similar to both nif D and nif K (~ 19%) [19]. From the protein point of view, nitrogenase remains "prokaryotic" while its "sibling" (DPOR) evolves into a higher version (LPOR), ultimately becoming dominant in higher plants [7].

Non-conventional Nitrogenases

Alternative nitrogenases were discovered more than 25 years ago in *Azotobacter vinelandii,* which use vanadium instead of molybdenum in an environment lacking molybdenum. *Streptomyces thermoautotrophicus* was found to be able to fix dinitrogen, but is unusual in having three proteins, a hetero-trimeric molybdenum-containing dinitrogenase (St1), a homodimeric manganese-containing superoxide oxidoreductase (St2) and another hetero-trimeric molybdenum containing carbon monoxide dehydrogenase (St3 or CODH) [20]. These proteins completely differ from the known nitrogenase components and are insensitive to O_2. Compared to conventional or alternative nitrogenases, the St nitrogenase also requires less ATP: $N_2 + 8H^+ + 8e^- +$ (4-12) Mg ATP $\rightarrow 2NH_3 + H_2 +$ (4-12) Mg ADP + (4-12) Pi. St nitrogenase is also not inhibited by carbon monoxide, which is the case for conventional nitrogenase. It is speculated that there may be more such prokaryotic nitrogenases with versatile features waiting to be discovered. Because many prokaryotic enzymes do evolve into the eukaryotic version, Yang and Cheng [21] did not rule out the possibility of the existence of a eukaryotic nitrogenase. If eukaryotic nitrogenase does exist in nature, then it may well be utilizing light as an energy source [22] (LPOR-like nitrogenase: light-utilizing N_2ase (LUN). Thus, efforts are required for searching for a eukaryotic nitrogenase. With hundreds of genome projects having now been completed, leaving on average more than 50% of discovered proteins waiting to be assigned functions, there does seem to be some exciting possibilities [7].

Prospects of Cereal Nitrogen Fixation

There have been intensive research efforts to induce cereals to fix N after the initial high-hopes of the mid-seventies (although the desirability and eventual benefits of such efforts are open to debate). A major effort on rice and other non-legumes in the nineties [23] led to an advancement of the understanding of non-legume BNF but has not produced any "selection" or "bred" variety that can fix a substantial amount of N. Again there are renewed attempts for reinvestment for BNF in cereals (rice, wheat, maize) driven by recent advances in understanding of nitrogen fixation biology [24, 25]. Three approaches are currently considered as promising:

Root Nodulation in Cereals

During bacterial recognition of a suitable plant host, rhizobia and legumes undergo signalling crosstalk, whereby the plant secretes flavonoids that trigger the bacteria to secrete nodulation (Nod) factors that promote nodule formation within the plant. Recently, Myc factors, which are part of the crosstalk between 70 to 90% of terrestrial plants (including cereals) and arbuscular mycorrhiza endosymbiotic fungi, were discovered to be very similar structurally to Nod factors [26]. It was shown that *Glomus intraradices* secretes symbiotic signals that are a mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs), which stimulate formation of arbuscular mycorrhiza in plant species of diverse families (Fabaceae, Asteraceae and Umbelliferae). In the legume *Medicago truncatula,* these signals stimulated root growth and branching by the symbiotic DMI (does not make infections) signalling pathway. It is thought that because Myc-based recognition involves a signalling pathway that is already present within cereals, *engineering cereal nitrogen fixation may be less difficult than originally anticipated* [24] (italics mine). Most rhizobia enter root cells through a complex plant structure called an infection thread. Although further research is needed to understand infection thread development, some legumes and most actinorhizal plants are colonized by symbiotic bacteria through the more primitive root-hair-independent method of crack entry invasion, such as entry at epidermal damage points. It is thought that these routes can be used initially for developing root nodule symbioses strategies in cereals.

Endophytes

It has been known for long that some nitrogen-fixing endophytic bacteria form nodule-independent association with cereal crops [27]. Pursuing this approach will also require screening for new cereal endophytes that fix nitrogen at high rates. Once these endophytes are identified and cultured, they could be used in improved microbial inoculants. However, this has some limitations which are discussed later.

Transfer of 'Nif' into Organelles

The direct transfer of nitrogen fixation (*nif*) genes to the plant has the advantage of having germline transmission therefore the technology will be in the seed [24]. Achieving this goal will require engineering the complete biosynthetic pathway of the nitrogenase enzyme into cereals. Although many *nif* genes are involved in biosynthesis of nitrogenase and its metal cofactors-iron-molybdenum cofactor (FeMo-co) and P-cluster, *in vitro* biosynthesis of FeMo-co requires only three proteins [28]. Also nitrogenase being highly sensitive, it will also be necessary to find the correct sub-cellular, low-oxygen, environment to allow nitrogenase to function. Chloroplasts and mitochondria are both considered as logical targets since both can provide the high concentration of adenosine 5' -triphosphate and reducing power required for nitrogenase activity. Chloroplast genomes of ferns, mosses, and gymnosperms encode an oxygen-sensitive enzyme related to nitrogenase [29]. Conversely, mitochondria have efficient oxygen-consuming respiratory enzymes, functioning oxygen-sensitive enzymes, and iron sulphur cluster machinery highly similar to the nitrogenase iron-sulphur cluster components [30]. What is lacking is the development of a plant mitochondria transformation method; therefore, the *nif* genes could be transformed into the nuclear genome and the proteins targeted to mitochondria, with expression potentially regulated in a tissue or developmental-specific manner [24]. Moreover, if nitrogen supply and carbon metabolism can be closely coupled, excess nitrogen would not be lost to the environment.

Chloroplast can be a potential site for nitrogen fixation because it carries out photosynthesis and thus provides a major source of ATP. It is also a major site for ammonia assimilation and has both GS and GOGAT pathways. The chloroplast genes are also expressed in a prokaryotic fashion allowing translation of polycistronic messages. The products of the *Chlamydomonas reinhardtii chl* L, N and B genes are structurally similar to the three subunits of nitrogenase, with the strongest sequence identity between *nif* H and *chl* L (nucleotide sequence 43%; putative amino acid sequence 35%) [7]. Therefore the genes required for *chl* L protein activity might activate the *nif* H gene product to obtain Fe protein activity without the requirement of additional genes such as *nif* M, *nif* S or *nif* U. Cheng and his co-workers [31] designed a strategy for introducing *nif* H into chloroplast genome and showed that *nif* H gene product does substitute for the function of *chl* L. However, it is a major challenge to interface plastid physiology with requirements for nitrogenase activity. The technology is available to introduce and express nitrogenase component proteins in plant cells, but there are gaps in knowledge of both plant and microbial physiology.

Advances in Non-Legume BNF

Purely in so as far as research initiatives and methodology for measurement of BNF are concerned, we need to have a critical re-look at the past researches and do some serious brain storming on the way forward. This is particularly true of researches on the contribution of associative symbionts and endophytes. Three well-known organisms associated with non-legume BNF are- *Azotobacte*r, *Azospirillum* and *Gluconacetobacter*. Initially, all of them were thought to promote plant growth by fixation of nitrogen, but it was later realized that it might also be due to the growth-stimulating effects of the bacteria, like production of indole acetic acid and other mechanisms.

Heterotrophic N Fixation

Acetylene-reduction assay has been used widely and indiscriminately for measuring N_2-fixation but this assay has serious errors when applied to rhizosphere-associated or free-living N_2-fixation measurements in soil. The ability of non-symbiotic or associative

or endophytic nitrogen fixation to contribute agronomically significant quantities of nitrogen (~20 kg N ha^{-1}) has been questioned by several workers [32]. The ultimate test of the contribution from N_2-fixation is to measure the net inputs from N_2-fixation in long term experiments (> 10 years); the difficulty of controlling and measuring all of the processes is well known. Positive N balances over long periods in the field in U.K. [33] were attributed to inputs from N_2-fixation by cyanobacteria (blue-green algae). None of the studies, which claim for substantial inputs from root associated or endophytic N_2-fixation, have excluded potential inputs due to N uptake from deep soil horizons or from cyanobacterial N_2-fixation. In controlled experiments, treatment-dependent sources of N have confounded results. For example, substantial amounts of plant-available unlabelled N were released from vermiculite used as potting medium when incubated under warm, moist conditions for several weeks in glasshouse experiments [34]. This leads to highly misleading, treatment-dependent isotope dilution. For example, experiments at ICRISAT, comparing genotypes of sorghum and millet for potential N_2-fixation using ^{15}N-isotope dilution with vermiculite as a medium, indicated differences between genotypes. When soil was collected from fields where ^{15}N-labelling experiments had been conducted three years earlier, so that a reasonably stable isotopic labelling could be guaranteed, these differences disappeared. No significant differences in isotope dilution were detected giving no evidence for associated N_2-fixation. The investigations in Brazil using ^{15}N aided N balances remain some of the best and most closely-controlled studies. But the huge amounts of tap water used for irrigation could contribute up to 20-30 kg N $ha^{-1}y^{-1}$ of unlabelled N [35]. Recent data from a long term experiment in Vertisols for 8 years showed N-accumulation of 5.4 kg/ha/crop season in wheat in 0-15 cm soil that was attributed to non-symbiotic nitrogen fixation [36].

Using 16S-rRNA gene clone libraries of soil [37] it was shown that *Lasiurus sindicus*, a highly nutritive, drought tolerant, perennial grass of Thar Desert of Rajasthan harbours in its rhizosphere Gram-positive bacteria, *Actinobacteria* being the most predominant ones, closely followed by *Firmicutes*. PCR amplification of *nifH* genes revealed a predominance of *Pseudomonas pseudoalcaligenes*. The rhizosphere of *L. sindicus* also harbours a diversity of diazotrophs, some *nif H* sequences showed similarity to *Azospirillum brasilense*, *Rhizobium* sp., and a variety of uncultured nitrogen fixing bacteria. Plant growth promoting associations of cyanabacteria have also been reported to influence wheat growth [38] with a potential for use as inoculants.

Endophytic N Fixation

Rhizosphere of cereals like rice is particularly abundant in species of *Azospirillum* and *Pseudomonas* and in members of the Enterobacteriaceae. In addition, various members of the genera *Alcaligenes, Azotobacter, Burkholderia, Clostridium, Flavobacterium* and *Xanthobacter*-have also been isolated from paddy field soil or wetland rice with regard to endophytic diazotrophs. *Serratia marcescens* inoculation of rice resulted in large numbers of this bacterium within intercellular spaces, senescing root cortical cells, aerenchyma, and xylem vessels, but they were not observed within intact host cells [39]. Although current thinking considers endophytes to make an important contribution of BNF in some grasses, in rice they may be only a relatively small sub-population of a much larger rhizosphere diazotroph population and hence their actual contribution could be minor [40]. For example, the total number of (culturable) diazotrophic endophytes isolated from wetland rice in the Philippines was estimated at only 10^6-10^7 [41]. When it is considered that a soybean (*Glycine max*) plant contains approximately 10^{11} rhizobial bacteroids, on the basis of 10^9 $nodule^{-1}$ [42], the number of diazotrophs living within rice appears to be relatively trivial. James [40] suggested that there are fundamental questions about how efficiently the endophytes can actually function within grasses when no obvious "symbiotic" structures appear to be present, since, in all N_2-fixing symbioses identified so far, specialized organs have evolved to house the diazotrophs, such as nodules on legumes and actinorhizal plants, and leaf cavities in *Azolla*. At present, it is difficult to see how the

apparently random distribution of bacteria within intercellular spaces, aerenchyma, dead cells, and xylem vessels that typifies endophytic associations, can perform functions analogous to those of such highly evolved organs [40].

The ecological role of endophytes still remains uncertain. The demonstration of the presence of bacteria that actively express nitrogenase genes within a graminaceous plant does not mean that the amounts of N_2-fixtion are of importance. If plants are grown under strong N limitation, conclusions that a high proportion of plant N is derived from N_2-fixation can be highly misleading. For example, in the experiments on N_2-fixation in Kallar grass (*Leptochloa fusca*), the plants were clearly growing under stressed conditions as a weight of only 1 g was reached after 8 months [43]. This was extrapolated to yield BNF of 34 kg N ha^{-1} and from such N starved plants they concluded that 'endophytes' play an important role in N_2-fixation in natural grass ecosystems. This required a huge leap of faith [32]. Contributions of non-symbiotic N_2-fixation for cereals or pastures under agricultural conditions need to be > 20 kg N ha^{-1} yr^{-1} assimilated by the crop to make a useful difference in productivity [32]. Of all the candidate crops, sugar cane remains the most likely candidate, not least because of the abundance of C in a readily utilizable form. Long-term studies conducted for 15 years on commercial sugar cane varieties and a variety of *Saccharum spontaneum* in Brazil showed N accumulation to be persistently high and N balances (60 to 107 kg N ha^{1} yr^{-1}) were significantly positive. The $\delta^{15}N$ of leaf samples were lower than any of the weed reference plants and data obtained from a greenhouse study indicated that this was not due to the cane plants tapping into soil of lower ^{15}N abundance at greater depth. They concluded that Brazilian varieties of sugarcane were able to obtain at least 40 kg N ha^{1} yr^{-1} from BNF [44]. Montanez [45] evaluated the nitrogen-fixing capacity of a range of commercial cultivars of maize by ^{15}N isotope-dilution method. Ndfa ranged from 12 to 33, regardless of N fertilization. BNF was not affected by mineral nitrogen fertilization except in two cultivars. Eleven bacterial isolates from endophytic tissue of maize: seed, root, stem, and leaf, grew on N-free semisolid medium, reduced acetylene and showed the presence of *nif* H gene. 16S rRNA gene sequences and phylogenetic analysis indicated that the bacteria were closely related to *Pantoea*, *Pseudomonas*, *Rhanella*, *Herbaspirillum*, *Azospirillum*, *Rhizobium* (*Agrobacterium*) and *Brevundimonas*. It was concluded that the endophytic diazotrophic bacteria isolated from root, stem, and leaf tissues of maize cultivars may contribute to BNF in these plants.

The role of N_2-fixing endophytes thus remains open to question, though their presence within grasses and cereals is clearly demonstrated by many studies [40], since there is a lack of good quantitative understanding of C allocation to roots and rhizospheres and to endophytic bacteria. Some requirements of future research to determine the amount of N derived from N_2-fixation by endophytes will be to ascertain the relative abundance of non-N_2-fixing bacteria and N_2-fixers within the plants [46]. If large amounts of N_2-fixation were supported in the tissues of sugar cane, then by analogy to the legume-rhizobial symbiosis, a coupling of high rates of respiration would be expected to support intense microbial activity in N_2-fixation, but no such studies have been done. If root-associated or endophytic N_2-fixation is able to provide amounts of N in the range of 40-60 kg N ha^{-1} then benefits in N accumulation should be readily demonstrated in glasshouse experiments under N-free conditions, coupled to strong plant growth, as is the case with N_2-fixing legumes [32]. A detailed and robust quantitative understanding of both the C and N budgets of grasses and their associated rhizosphere and endophytic bacteria is required to properly assess the potential of N_2-fixation in these plants.

Advances in Legume BNF

Legumes have been estimated to contribute 20% of the nitrogen needed for global grain and oilseed production. They can potentially fix about 80% of their own N need and in addition can contribute to the yield of subsequent crops. But all these potential benefits can be harnessed only under certain conditions. Mere inclusion of a legume in a cropping

system does not ensure high BNF. There can be two approaches to harness BNF: first, improved crop, soil and water management to achieve maximum efficiency of BNF and second, *Rhizobium* inoculation or selection of host genotypes to ensure a higher proportion of nitrogen fixation in the plant (P_{fix}). Of these, the first strategy is well known for almost 50 years and continues to play its rightful role. The second approach on host plants selection is more recent. There has also been a considerable revision of rhizobial taxonomy with the discovery of newer nodule inhabitants. The debate on indigenous versus exotic strains of rhizobia is interesting but in most seems settled, since most inoculants are developed from indigenous adapted rhizobia. There is a lot of current interest in the diversity, phylogeny and biogeography of rhizobia.

Selecting for High Nod Legumes

Although there is a large genotypic variability in nodule number and nodule mass in legumes, efforts to use this variability in breeding for improved N_2-fixation has been limited. In addition to the breeding method used for developing a material, absence of any natural selection pressure for nodulation or N_2-fixation during its development may be responsible for the occurrence of the different nodulation types within a material even up to the release stage. This is supported from the fact that during a screening for high nodulating plants at high mineral N in soil, both high and low nodulating plants were observed in 85 out of 90 advanced breeding lines of chickpea [47]. Using appropriate screening procedures several different nodulation types [high nodulating (HN), low nodulating (LN), non-nodulating (NN)] were identified within several chickpea and pigeon pea cultivars [47]. Other studies [48] also indicated plant-to-plant variability within cultivars of groundnut. HN selection generally grew better than the NN and LN selections of a given cultivar. At ICRISAT, the HN-selection of chickpea cultivar G 130 produced 31% more grains than its LN-selection at low soil N (N1) level. The HN-selection of G 130 yielded better even at high soil N (N2) level. At pH 9.0-9.2, a genotype selected for high-nodulation outperformed the four others used in the study of Rao *et al*. [49], (Table 1). Nodulation was reduced in all the five chickpea genotypes as the electrical conductivity increased from 1.1 to 8.1dS m^{-1} but the high nodulating selection CSG 9372 had more tolerance and formed about 3 times more nodules than the salt tolerant line (CSG 8927) even at 6.2 dS $m^{-1.}$

The above studies thus suggest a scope of enhancing N_2-fixation in legumes through host plant selection. For promoting N_2-fixing traits, breeders should grow their legumes at low soil-N (preferably <10 µg mineral N g^{-1} soil) fields, prepared specially for the purpose. Breeders generally handle large numbers of genotypes and materials. In some materials, genes for N_2-fixation may be co-segregating with genes for the other traits. It is likely, therefore, that trait combinations associated with enhanced N_2-fixation will be identified if appropriate assessment methods are applied to the segregating populations. If genetic variation for N_2-fixation existed in breeding populations, the high N_2-fixing lines would be produced as a normal outcome. Developments in the field of genomics (particularly on *Medicago* and *Lotus*) provide a better understanding of the expression and regulation of symbiotic genes [50]. Opportunities have opened up for biotech assisted germplasm enhancement and bio-informatics assisted gene mining and utilization. These may lead to a better-targeted breeding of legumes for high BNF than hitherto possible. Breeding for high N_2-fixation is feasible and should also be on our research agenda [51].

Stem Nodulation

The first event in development of symbiosis is the communication between the partners, rhizobia and the host legume, followed by bacteria gaining entry without triggering defence responses. It is believed that the simplest way of achieving these two aims could have been by bypassing some of the complex signalling required for root-hair infection and gaining entry instead between epidermal cells or via wounds, such as where lateral roots emerge [52]. A few aquatic legumes form nodules on their stem at dormant root primordia. The stem-nodulating legumes described

Table 1: Shoot and root dry mass of five selected genotypes and nodule number and dry mass of nodules of four (the non-nodulating ICC 4918 does not form part of the analysis) genotypes of *Cicer arietinum* grown (60 days) in a saline soil [49]

Genotypes	Shoot dry mass (g per pot)				Root dry mass (g per pot)			
ECe (dSm^{-1})	**1.0**	**3.2**	**6.2**	**8.1**	**1.0**	**3.2**	**6.2**	**8.1**
ICC 4918 (non-nodulating)	0.57	0.61	0.50	0.14	0.26	0.21	0.15	0.03
CSG 8890 (salt-sensitive)	0.76	0.70	0.50	0.32	0.50	0.37	0.19	0.11
BG 256 (check)	1.11	0.90	0.65	0.47	0.46	0.41	0.24	0.13
CSG 9372 (high-nodulation)	1.03	1.22	1.14	0.60	0.38	0.46	0.38	0.22
CSG 8927 (salt-tolerant)	0.93	0.91	0.60	0.54	0.43	0.47	0.31	0.20
LSD P=0.05	0.10 (salinity) 0.12 (genotypes)				0.05 (salinity) 0.06 (genotypes)			

	Number of nodules per pot				Dry mass of nodules (mg per pot)			
ECe (dSm^{-1})	**1.0**	**3.2**	**6.2**	**8.1**	**1.0**	**3.2**	**6.2**	**8.1**
CSG 8890 (salt-sensitive)	23.0	10.0	5.6	7.6	8.9	1.7	0.7	0.5
BG 256 (check)	22.2	11.8	13.8	7.6	12.8	5.2	3.3	1.2
CSG 9372 (high-nodulation)	63.2	20.8	21.8	13.0	15.8	5.1	2.8	1.1
CSG 8927 (salt-tolerant)	25.2	15.6	7.2	2.6	5.9	4.4	1.4	0.3
LSD P=0.05	6.5 (salinity) 6.5 (genotypes)				2.7 (salinity) 2.7 (genotypes)			

so far are all members of the genera *Aeschynomene, Sesbania, Neptunia* and *Discolobium* Their rhizobial symbionts belong to *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Azorhizobium***.** *Aeschynomene* are specifically stem-nodulated by photosynthetic bradyrhizobia. The presence of a functional photosynthetic unit in bacteroids and the high expression of the photosynthetic genes observed in stem nodules demonstrate that the bacteria are photosynthetically active during stem symbiosis. Inoculation of a photosynthesis-negative mutant of the *Bradyrhizobium* sp. strain ORS278 symbiont of *Aeschynomene* resulted in 50% decrease in stem-nodule number, which reduced nitrogen fixation activity and plant growth in the same proportion. This result indicated an important role of bacterial photosynthesis in the efficiency of stem nodulation [53].

Rhizobial Taxonomy

Until 1992, there were four genera of root nodulating bacteria: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Azorhizobium.* Later, four more genera added were *Mesorhizobium, Allorhizobium, Methylobacterium* and *Burkholderia*. The nomenclature of some of the old species has been revised [54]. A current list of rhizobia can be found in the supplementary material of a recent review [55]. The study of new geographically dispersed host plants has been a source of many new genera and species and is expected to yield many more. More than 63 species of rhizobia were recognized by 2010 and more are being reported each year. A number of new entrants include *Devosia, Ochrobactrum and Phyllobacterium* in alpha-proteo bacteria and *Burkholderia* and *Cupriavidu*s in beta-proteobacteria. These are not new bacteria but were all along known

to be associated with their specific legumes. However, the nomenclature has improved due to advances made in molecular biology techniques. For soil microbiologists and agronomists who have to source strains for inoculant production to be used in farmers' fields, these fast developing changes definitely pose a challenge [55]. However, for all practical purposes, the symbiotic relationship between the bacteria forming root nodules in a given host legume remains same as ever and does not affect field oriented programs on nitrogen fixation.

Diversity and Biogeography of Rhizobia

There is an enormous amount of research attention on the diversity of rhizobia referred to in the preceding section. There are several recent reports on the molecular diversity and biogeography of soybean rhizobia [56-60] including in Indian soils [61-63]. Diversity is most dependent on previous cropping of an area to a legume and soil factors [60]. For example, large diversities of soybean rhizobia (in countries for which soybean was an exotic crop) could have arisen due to transfer of symbiotic genes from the inoculant strains to indigenous non-symbiotic rhizobia [64, 65]. Chen *et al*. [66] isolated native isolates resembling *Agrobacterium* sp. that were effective in nodulating soybean. The rhizobial population structure in soils is a resultant of the interactions among the bacteria, host plant and environmental factors. Lindstrom *et al*. [55] argued for linking the knowledge of the molecular diversity of rhizobia to the practical requirement of searching for competitive inoculants of rhizobia especially in situations where there is extensive adaptation to the local conditions leading to evolution of native rhizobia.

Knowledge of the biodiversity of rhizobia is thus important for the design of successful inoculation strategies. For example, in north China plain, *Sinorhizobium fredii* was found to be the dominant group (68%) [60]. The phylogeny of symbiotic genes *nodC* and*nifH* defined four lineages among the isolates associated with *Sinorhizobium fredii*, *Bradyrhizobium elkanii*, *B. japonicum* and *B. yuanmingense*, demonstrating the different origins of symbiotic genes and their co-evolution with the chromosome. Clear biogeographic patterns were evident [57] from further analysis of 16 rRNA gene, 16S-23S intergenic spacer, housekeeping genes atpD, recA, and glnII, and symbiotic genes nifH and nodC. All the strains except one were symbiotic bacteria classified into nine genospecies in the genera of *Bradyrhizobium* and *Sinorhizobium*. *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains were found only in neutral to slightly alkaline soils whereas *Bradyrhizobium yuanmingense*, *Bradyrhizobium liaoningense*-related strains and strains of five *Sinorhizobium* genospecies were found in alkaline-saline soils. High soil pH, electrical conductivity, and potassium content favoured the distribution of the *B. yuanmingense* and the five *Sinorhizobium* species but inhibit *B. japonicum* and *B. elkanii*. High contents of available phosphorus and organic matter benefit *Sinorhizobium fredii* and *B. liaoningense*-related strains and inhibit the others groups mentioned above. The symbiotic gene (*nif H* and *nod C*) lineages among *B. elkanii*, *B. japonicum*, *B. yuanmingense* and *Sinorhizobium* spp. were observed in the strains, signifying that vertical gene transfer was the main mechanism to maintain these genes in the soybean rhizobia. However, lateral transfer of symbiotic genes commonly in *Sinorhizobium* spp. and rarely in *Bradyrhizobium* spp. was also detected.

In surveys of soybean rhizobia in Central India during 2002-07 by the All India Network Project on Biofertilizers of the Indian Council of Agricultural Research [67], about 85 % of the isolates showed the presence of the 900 bp RSa fragment indicating the predominance of slow growing *B. japonicum* group and a high level of genetic diversity that revealed 17 main clusters at 20 % level of similarity (Saxena, *et al*. *personal communication*). Concurrent studies [62] revealed eight haplotypes of soybean *Bradyrhizobium* in India based on PCR-RFLP analysis of 16S rRNA and IGS region between 16S and 23S rRNA. They found that genetic diversity was conserved across regions and was wider than expected as per previous studies in various geographic areas. Sequence analysis of IGS and four other functional genes including *nif*H revealed three groups, *Bradyrhizobium liaoningense*, *B. yuanmingense* and

a third group different from other described species but same symbiotic genotype as *B. liaoningense* and *B. japonicum* bv. *Glycinearum* [62]. However, in later studies in our laboratory on native soybean rhizobia in Indian soils, dendrogram of phenotypic diversity based on intrinsic antibiotic resistance and carbon source utilization showed 5 clusters at 52 % level of similarity while that of genetic diversity based on 16S rDNA sequence showed 5 clusters at 63 % similarity. The slow growers belonged to *Bradyrhizobium japonicum* group as well as *Rhizobium radiobacter*, while the fast growers were exclusively *R. radiobacter* [63]. *Bradyrhizobium japonicum* USDA 110, the strain originally introduced on a large scale in Indian soils more than four decades ago, shared 34-81 % phenotypic and 63-83% genotypic similarity with rest of the Indian isolates. Analysis of the micro- and macro-geographical variations in diversity showed conservation of sequences in various soybean-growing areas and also evolution of native strains of rhizobia with varying degree of adaptation among slow as well as fast growing soybean rhizobia.

Studies on biogeography also lead to new discoveries. In different sites of the Northern (Huang-Huai-Hai) plain of China, novel strains belonging to a new species *Bradyrhizobium huanghuai-haiense* were recently reported [68]. Extensive molecular characterization of chickpea rhizobia from Northern India by ERIC-PCR showed them to be most homologous to *M. mediterraneum* [69]. Based on polyphasic taxonomy, other discoveries in India include *Rhizobium pusense*, a novel rhizobial species (NRCPB 10) from the rhizosphere of chickpea in Pusa, Delhi that does not nodulate chickpea and also does not induce tumours in tobacco plants [70]. In addition, *Rhizobium subbaraonis*, an endolithic bacterium from beach sand isolated in an oligotrophic medium, bore 100% similarity to *Rhizobium pusense* [71].

Transgenic Rhizobia

The arguments by many that indigenous strains are not effective and that we need genetically modified organisms with superior effectiveness and colonization potential appears to be based on more on belief than on evidence. Genetically engineered rhizobia have not yet been shown in the field to have a remarkable impact on nodule occupancy and yields. Improved nitrogen fixation via the insertion of additional copies of *nif* A was shown in *R. meliloti* by Eric Triplett and workers in USA under field conditions [72]. But the conditions under which these GMO's work is worth noting. They concluded that alfalfa yields can be increased with a genetically engineered strain of *R. meliloti* "*under field conditions of nitrogen limitation, low endogenous rhizobia competitors and sufficient moisture*" [72]. Under these conditions, it can be ascertained with confidence from the Indian work that any native, effective strain would be equally competitive and useful for promoting nodulation and BNF. One cannot but agree with RH Burris who observed in a preface to a book [73] '*rapid progress has been made in understanding the dominance of indigenous strains and in improving the systems for the delivery of rhizobia to legume plants in the field*'. There is a growing acceptance of the need to develop inoculants from among the most competitive and effective of the native strains.

Inter-Strain Competition in Rhizobia

Although it is known that some strains of rhizobia are better than others, our inability to introduce these super strains into nodules limits our ability to fully exploit BNF. This is because of the dominance of the indigenous, adapted strains in soils with long history of cropping to a particular legume. Inoculation of *Bradyrhizobium japonicum* strains in a soil with low background population resulted in 100% nodule occupancy in soybean in the first year but did not persist into second year (< 25% occupancy), since indigenous rhizobia dominated nodulation sites overwhelmingly [74]. In three field sites having indigenous population of < 30,000 or about 17,000 CFU per gram of soil [75], inoculation of *R. leguminosarum* bv. *viciae* showed that with the number of indigenous bacteria > 500, nodule formation on *Vicia faba* by the inoculant strain was severely reduced. However, increases in nodulation and nodule occupancy have also been obtained even in soils with high background rhizobia. Inoculation

of *B. japonicum* strain USDA 110 strain in a Brazilian Oxisol containing 10^5 rhizobia per gram resulted in nodule occupancy of ~50% (up from ~20% in uninoculated), improved nodulation and N uptake [76]. Thies *et al*. [77] reported that significant increases in soybean yield were observed only when no less than a doubling of nodule mass and 66% nodule occupancy by inoculated rhizobia were reached. However, there is a high variability in nodular occupancy by inoculated strains when used in fields for many years. In fields with a long soybean production history, the nodule occupancy by strains from commercial inoculants did not exceed 18% [78] due to the presence of naturalized rhizobia. Gaur *et al*. [79] showed that where soils harboured naturalized populations of ineffective or moderately effective rhizobia, and the problem of inter-strain competition was severe, inoculation of preceding cereal crop (maize) improved the symbiosis in the succeeding legume (green gram or groundnut).

Rhizobium and PGPR Inoculation

Surveys on rhizobial populations in the AICRP on BNF for the major grain legumes have shown the populations to be well below the threshold (~ 1000 cells/g) in all areas and was below 100 cells/g [80] due to the extremely high soil temperature and drying of surface soil layers in summer. In a five-year survey of the entire state of Madhya Pradesh, it was found that wherever rhizobial inoculation was practiced by farmers along with FYM and fertilizer application (IPNS), there was best nodulation and grain yield [67]. In Brazil, although inoculation of cowpea with rhizobia did not significantly alter productivities, N contents or % Ndfa but there was a tendency of lower grain productivities in the non-inoculated plots, which was reflected in lower total and biologically fixed N quantities, indicating that the native strains may be slightly less efficient [81].

Plant growth promoting rhizobacteria are known to improve nitrogen fixation in legumes by promoting nodulation, solubilization of fixed forms of phosphates in soil, production of phytohormones like indole acetic acid and gibberellins, production of siderophores for chelating iron and synthesis of low molecular weight compounds or enzymes that can modulate plant growth and development. PGPR are also reported to produce antibiotics that suppress deleterious rhizobacteria/plant pathogenic fungi or through other unidentified mechanisms, all of which provide a healthy environment for better root growth.

Co-inoculation of *Rhizobium* with phosphate solubilising bacteria (PSB) or arbuscular mycorrhizal fungi (AMF) has been found to be significantly better than rhizobial inoculation alone. Combined use of *Bradyrhizobium*, AMF and PSB increased soybean-yield from 0.95 Mg/ha to 1.21 Mg/ha in control [82]. Non-*Bradyrhizobium* endophytic bacteria (NEB), *Bacillus subtilis* has been isolated from soybean root nodules [83], which increased soybean dry matter upon co-inoculation. Inoculation of soybean with plant growth promoting rhizobacteria *Serratia liquefaciens* or *Serratia proteomaculans* hastened the onset of nodulation in cool season, total fixed N, Ndfa and protein yield [84]. PGPR inoculation was thought to be most effective for those cultivars which have a higher yield potential.

Co-inoculation of AMF (*Glomus fasciculatum*) along with rhizobia significantly improved nodulation, BNF, shoot N and P content and yield of soybean [85-86]. In general, soybean roots that are more thoroughly colonized by AMF are more heavily nodulated by rhizobia. The beneficial influence of mycorrhiza have been attributed to increased IAA and ABA contents in roots, shoots and nodules of the mycorrhizal soybean plants [87], increased size and activity of nodules along with higher photosynthetic nutrient use efficiency in leaves [88] and increased resistance to infection by the pathogen *Pseudomonas syringae* [89].

Similarly, beneficial effect of co-inoculation of *Azospirillum brasilense* and *Bradyrhizobium* in soybean have been known for long [90]. These were attributed to increased number of most active nodules (greater ARA) and increased effectiveness (more leghaemoglobin) [91], greater proportion of N derived from fixation [92] or production of indole acetic acid by azospirilla [93]. The nodule occupancy of *Bradyrhizobium* strain S24 increased from 60%

with single inoculation to 81% upon co-inoculation with an *Enterobacter* isolate EG-ER-2. Another *Enterobacter* isolate KG-ER-1 increased nodule occupancy of bradyrhizobial strain Cog 15 from 77% with single inoculation to 88% upon co-inoculation [94].

In the AICRP on BNF, an on-farm field trial at Aliyarnagar, in Coimbatore district of Tamilnadu, inoculation of *Bradyrhizobium* Tt 9 along with a strain of plant growth promoting rhizobacteria (PGPR) *Pseudomonas* PS 2 at 100% N and P application level gave maximum pod yield [95] (Table 2). Inoculation saved 25% N and P but co-inoculation of *Pseudomonas* was significantly better than inoculation with rhizobia alone, particularly at 75% N and P level.

In an 8-year-long field experiment in a Vertisol, there was additional N uptake of 14.9 kg N ha^{-1} by soybean due to *Rhizobium* inoculation (over control nodulated by native rhizobia) and 20.9 kg N ha^{-1} by wheat crop due to *Azotobacter* inoculation and gain of +38.0 kg N ha^{-1} yr^{-1} to 0-15 cm soil layer after harvest of wheat. The total benefit to crops and soil due to the inoculants was 73.8 kg N ha^{-1} yr^{-1} after one soybean-wheat rotation [36]. This underscored the need to promote awareness for adoption of integrated approach in nutrient management along with use of good quality rhizobial inoculants to promote BNF.

Table 2: Effect of combined inoculation of *Rhizobium/ Bradyrhizobium* and *Pseudomonas* on groundnut [95]

Treatment	Nodule (no. pl^{-1})	Nodule DW (mg/pl)	Pod yield (kg ha^{-1})	% increase over control
100% N and P				
Uninoculated control	20	120	1333	-
Rhizobium (TNAU 14)	43	160	1399	5.0
Bradyrhizobium (Tt9)	42	170	1433	7.5
Pseudomonas (PS2)	33	160	1415	6.2
TNAU 14+ PS2	47	220	1492	11.9
Tt9 + PS2	44	180	1517	13.8
75% N and P				
Uninoculated control	21	100	1001	-
Rhizobium (TNAU 14)	33	160	1042	4.1
Bradyrhizobium (Tt9)	36	210	1083	8.2
Pseudomonas (PS2)	29	160	1024	2.3
TNAU 14 + PS2	39	270	1278	27.6
Tt 9 + PS2	38	190	1351	34.9
L.S.D. (p=0.05)	3	35	69	-

Green Manuring and Residual N Benefits

Green manuring with legumes is an age-old practice to supply biologically fixed nitrogen to subsequent crops grown in rotation. Nitrogen accumulation of 17 annual accessions of *Sesbania* averaged 154 kg N ha^{-1} with nitrogen fixation ranging from 105-150 kg ha^{-1} [96]. *Sesbania cannabina* (‘*dhiancha*’) grown for 45 days and decomposed for 5 days prior to rice transplantation could supply a N fertilizer equivalent of 122 kg N ha^{-1} to rice [97]. Modelling of nitrogen fixation in *Sesbania* showed that nitrogenase activity peaked at 33 days resulting into highest nitrogen accumulation in shoots at 40 days. Seasonal integration of nitrogen fixation over 15-45 days growth period showed that fixation could meet most of the plant demand for nitrogen. Therefore, growing green manure crop beyond 45 days is not useful as they start taking up nitrogen from soil reserves [98], (Table 3). Stem nodulating *Sesbania rostarta* could substitute for 35-90 kg N /ha for rice grown in rotation in Phillipines depending on establishment method and season [99]. Experiments in an alkaline soil showed that *Sesbania rostrata* nodulated profusely (265 nodules per plant) with N uptake at 60 days of 4.6, 23.0 and 59.6 kg in stem nodules, green stem and leaf, totalling 87.3 kg N/ha (D L N Rao, 1988, unpublished). The rotational benefit of legumes and N credit for succeeding cereal crops are widely known. Recent reports on effects of *Rhizobium* inoculated soybean and *Azotobacter* inoculated wheat in a Vertisol showed a fertilizer-nitrogen saving of 30 kg ha^{-1} in wheat [36].

Table 3: Nodulation, nitrogenase activity and N uptake by *Sesbania cannabina* at various growth stages (average daily values plant^{-1}) Modified from [98]

Growth stage (days)	Nodule no.	Nodule mass (mg fr.wt.)	Nitrogenase activity#	Shoot biomass (g DW)	N (%)	N uptake (mg)
15-21	13.7	148	24.0	0.27	2.59	7.1
22-27	19.1	272	28.0	0.60	2.97	17.6
29-34	30.4	520	34.7	1.80	3.00	53.2
36-41	55.9	1287	36.3	3.84	3.09	120.7
43-47	81.6	1459	23.9	9.73	3.32	322.6
51-54	84.0	1378	11.2	13.21	2.80	366.7
57-60	83.3	1609	8.0	22.97	2.83	655.0

#u moles of acetylene reduced g^{-1} fr.wt.nodule

Other Developments in Rhizobia

Some of the other developments include the ability of rhizobia to influence plant growth through the production of growth promoting compounds. Extensive field trials on rice in Egypt indicated an average of 20% yield increase [100]. The ability of rhizobia to influence growth and yield of wheat and rice was reported almost three decades ago [101].

Climate Change and BNF

The goal of any rhizobial inoculant programme is to select an inoculant strain that is not only highly effective in N_2 fixation but also has other desirable attributes for promoting plant growth as well as tolerance to adverse moisture and temperature conditions [58]. With expected warmer and drier future climate scenarios, this will be a major focus of the coping strategies. A review by Thomas *et al*. [102] indicates that symbiotic root associations with N_2-fixing bacteria or mycorrhizal fungi may be stimulated by CO_2 enrichment and result in increased nutrient supply to the plant. The growth response of symbiotic N_2 fixing plants appears to be larger than that of other functional groups. The ability of N_2-fixing plants to respond to elevated CO_2 under conditions of low soil N availability increases their competitive capability with non-fixing plants [103].

An analysis of 165 studies that examined the responses of pastures and rangeland to global change indicates that content of legume increases by about 10% in grass-legume swards with a doubling of atmospheric CO_2 concentration [104]. Overall, elevated atmospheric CO_2 may be expected to stimulate the growth and N_2-fixation of most symbiotic N_2-fixing plants when grown in the absence of any environmental constraints like nutrient deficiency, low temperature or drought. And rising CO_2 levels may offer some protection from drought-induced decreases in N_2 fixation, which will become more prevalent with projected changes in precipitation intensity and frequency that are projected to accompany the rise in CO_2 [105]. There have been very few long-term studies of the response of field-grown legumes to elevated CO_2. This greatly limits characterization of the environmental conditions under which N_2 fixation can or cannot be stimulated at elevated CO_2. The feedback effects of nutrient limitation on N_2 fixation and photosynthesis have not been quantified [105].

The effects of various abiotic stresses are discussed in detail. As a general rule for all stresses, the microsymbiont is most tolerant, followed by the host plant and the most sensitive steps are the various symbiotic interactions.

Temperature Stress

N_2-fixing plants and their associated strains of N_2-fixing bacteria display a remarkable resilience to temperature regimes in both cold and warm climates. High temperature and moisture deficiency are major causes of nodulation failure and the upper limits to rhizobial growth were 32ºC-47ºC and upper limits to N_2 fixation in tropical legumes were lower at 27ºC-40ºC [106]. Our studies show that all the slow and fast growing rhizobia that nodulate soybean could grow equally well at 35°C; a few tolerated 40-45°C among which the fast growers formed a higher proportion. Temperature can also alter the outcome of competition between strains. *Vigna* rhizobia were more competitive at higher temperatures (36ºC) than the *B. japonicum* strains which competed better at low temperature (24-30ºC) [107]. Qi *et al*. [58] found that out of 100 rhizobia of wild soybean in yellow river delta soils in China, four strains exhibited strong tolerance to acidity, alkalinity and salinity, as well as high and low temperatures and also had strong nodulation capacity. A few studies have addressed the interaction of elevated CO_2 and higher temperatures on N_2-fixation and these generally support the hypothesis that the effects of elevated CO_2 will be greatest at high temperatures.

Drought Stress

Optimal symbiosis depends upon the successful survival ability of rhizobia even under adverse environmental conditions. Water stress is a major environmental factor limiting growth and symbiotic N_2 fixation and has been well studied. The strains from arid regions that survive and adapt to such adverse environmental conditions may be effective inoculant strains for crops growing under drought stress [108]. Fewer commercial strains were tolerant to heat and desiccation stresses than field isolates [109]. Desiccation decreases water activity and an increase in osmotic or salt stress elevates the ability of some microorganisms to survive desiccation and some have argued [110] for a need for identification of desiccation resistant microorganisms for use as dry seed inocula. The ability of microorganisms including rhizobia to survive desiccation is related to their ability to cope with a variety of stresses including solute and temperature extremes [110].

Nodulation is highly sensitive to drought stress and sudden drought drastically reduces the functioning of the already formed nodules. Drought stress also reduces the number of rhizobia drastically. The ability of rhizobia to survive in moisture-limited conditions varies among species and among strains within each species depending on the degree of tolerance to temperature. At a matric water potential of –0.5 to –1.5 M Pa, growth and nitrogen fixation are affected in tropical areas [106]. Our studies show that slow growing rhizobial strains of soybean were comparatively more osmosensitive than fast growers. About 30% of the slow growers could not tolerate 25% PEG (~Matric water potential, ψ_w 1.0 M Pa) in culture broth compared to 7% fast growers.

In the few studies that have been conducted with N_2-fixing species, elevated CO_2 at least partially compensated for drought-induced reductions in N_2 fixation by stimulating nodule mass and specific nodule activity. In soybean, elevated CO_2 stimulated the accumulation of nonstructural carbohydrates while reducing concentrations of soluble nitrogen compounds that might reduce N_2-fixation through negative feedback [111-112] suggesting that both N_2-fixing plants and the process of N_2-fixation could become more drought-tolerant as atmospheric CO_2 continues to increase. However, relatively few studies have attempted to assess interactions between elevated CO_2 and other environmental factors on roots, nodules, and nodule activity of N_2-fixing plants.

Athar and Johnson [113] showed that nodulation, growth and N_2-fixation in alfalfa could be improved by inoculation with competitive and drought-tolerant rhizobia. Ben-Romdhane *et al*. [114] showed that water deficiency affects the diversity of nodulating rhizobia; nodulation of chickpea by *Mesorhizobium mediterraneum* was reduced while inefficient nodulation by *Ensifer meliloti* was favoured. Co-inoculation of legumes with mycorrhiza and rhizobia is known to promote nodulation and nodule occupancy [115] and also help withstand drought stress better. In practical terms, BNF can be

improved by modifying the soil hydro-thermal regimes through simple practices like straw mulching, reduced tillage, deep sowing etc [116] and other soil moisture conservation like opening deep furrows (20 cm) after every 3 m distance to capture rain water [117].

Nutrient and Metal Stress

Since nitrogen fixation is strongly limited by available soil phosphorus, soil P will probably severely limit the responses of symbiotic N_2-fixing plants to CO_2 enrichment, and experimental evidence indicates that adequate soil P is required for elevated CO_2 to positively affect symbiotic N_2-fixation. Soybean plants adequately fertilized with P responded to elevated CO_2 with greater nodule mass, specific nodule activity, and total plant N, but soil P deficiency eliminated this response [118]. Similar results were obtained in clover [119]. Correcting soil constraints and alleviating nutrient deficiencies improves BNF inputs significantly as shown in a number of studies, particularly in acid soils. For example, liming increased the grain yield of soybean by 60% in acid soils of Manipur, while Lime + *Rhizobium* improved rhizobial population and grain yield [120]. In acid soils of Orissa [121], application of micronutrients (molybdenum and cobalt) boosted nodulation and BNF in green gram. *Rhizobium* inoculation increased the grain yield (25.7%) resulting in additional N fixation of 7.1 kg N/ha and P uptake of 0.6 kg/ha. Application of micronutrients along with inoculation further enhanced the grain yield dramatically (+78.4%) over uninoculated control resulting in additional N fixation of 24 kg N ha^{-1} and additional P uptake of 3.4 kg ha^{-1} respectively [121]. The adverse effect of heavy metals on rhizobia resulting from the influence of sewage water irrigation in soils and the adverse effects on nodulation parameters has been reported [122].

Salinity Stress

In a future water-limited world, soil salinity problems would increase. Also, due to competition for limited quantities of water with the needs of industry and infrastructure development, agriculture will be increasingly dependent on recycled waters. Legumes are in general, more sensitive to salinity and alkalinity stress than cereals. Rhizobia show marked variation in salt tolerance. A majority of soybean rhizobia are inhibited by 100 mM salt, especially bradyrhizobia. The fast growing strains have been reported to grow at salt concentrations of more than 300 mM. On the other hand, Raza *et al*. [123] showed that all the soybean rhizobial isolates tolerated a NaCl concentration up to 5%. Soybean and chickpea strains were tolerant to 340 mM NaCl with the fast growing strain being more tolerant than slow growing strain [124]. Hungria *et al*. [125] found that most of the fast growing soybean strains produced abundant extracellular polysaccharides, and were tolerant to 0.5 mM NaCl and a temperature of 40°C. Slow growing peanut rhizobia are less tolerant than fast growing rhizobia [126]. *Rhizobium* strains from cowpea (*Vigna unguiculata*) were tolerant to 5.5% NaCl (450 mM) [127].

The inhibitory effect of salinity on root hair infection and symbiotic process leading to decrease in nodulation and nitrogen fixation in legumes is well known. Although some authors argued that it could be overcome by inoculating with salinity tolerant rhizobial strains and even proposed molecular manipulation for transferring 'osm' genes from other bacteria into rhizobia, it is clear that the success of a symbiotic function under stress is more dependent on the tolerance of the host plant rather the microsymbiont. It was shown that the rhizobial strains most effective in normal soils are also the most effective ones under salinity stress [128]. Rao *et al*. [49] further showed using ^{15}N measurements, the crucial importance of selecting tolerant plant varieties to increase BNF under saline stress (discussed above). However, it is pertinent to mention more recent work where it was found that most of the rhizobia nodulating chickpea under water deficiency were NaCl tolerant [114] and inoculation with selected salt tolerant strain of *M. mediterraneum* significantly increased the nodule number and grain yield in field. It was also shown by some workers [129-130] that the ability of legume hosts to grow and survive in saline conditions is improved when they are inoculated with salt tolerant strains of rhizobia. Saxena and Rewari [131] had identified four chickpea

genotypes and *Rhizobium* strains combinations that gave higher grain yields in saline soil.

Strains tolerant to multiple-stresses are useful for inclusion in biofertilizers to both improve their viability during the storage of the inoculants as well as improve survival on seed post-inoculation. Our results on soybean rhizobia show the sensitivity of a major proportion of rhizobia to environmental stresses like temperature, drought and salinity. Very low proportion of strains (5%) that are tolerant to all the three individual stresses (40°C, 40% PEG, 3% NaCl), which thus requires careful selection of rhizobial strains for preparation of superior biofertilizers that are not only efficient but compatible and stress tolerant.

Organic Amendments

Climate change induced drought and increasing temperatures are bound to lead to a reduction in soil organic matter content. Soil organic carbon was the single most important component of soil that explained the variation in rhizobial population in dry land soils [132]. Hence, there is a need to build up populations by addition of organic materials as well as repeated inoculation of the desired strains. Organics have been found to boost the proliferation of *Rhizobium* and enhance nodulation and nitrogen fixation in a number of legumes and oilseeds. Beneficial effect of the combined application of FYM and *Rhizobium* gives synergistic effect on legumes and led to the recommendation 'Apply *Rhizobium* inoculants along with FYM @ 5 t/ha' from the ICAR AICRP on Biological Nitrogen Fixation [95]. Addition of farm-yard manure has been shown to improve BNF in legumes. Nitrogen derived from air (Ndfa) in soybean improved from 46.1% in control to 62.5% at 4 Mg FYM ha^{-1} [133]. Other studies also showed beneficial influence of organics on legumes. FYM @4t/ha +VAM+ *Rhizobium* had best effect on clusterbean yield and soil microbial properties in an arid soil [134].

Conclusions

There is an urgent need to improve the inputs of organics and BNF in Indian agriculture. Development of effective and competitive rhizobial strains tolerant to high temperature, drought, nitrate, acidity and other abiotic stresses is of high priority. Soil management practices such as soil reclamation, correcting nutrient deficiencies, application of organics and screening of segregating material in legumes in low N soils are some of the quickest means to increase the contribution of BNF in Indian agriculture. Where inoculation is not feasible, the selection/breeding for high nodulating cultivars could be an option. Developing transgenic inoculants, nitrogen-independent cereals, and endophytic N fixation requires a very careful re-look and stronger justification for funding.

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