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Heighten Biological Nitrogen Fixation and Improving Efficiency of Bio-inoculants in Pulses

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Biological nitrogen fixation and sustainable agriculture:

Nitrogen is an essential plant nutrient. It is the nutrient that is most commonly deficient in soils, contributing to reduced agricultural yields throughout the world. Nitrogen can be supplied to crops by biological nitrogen fixation (BNF), a process which is becoming more important for not only reducing energy costs, but also in seeking more sustainable agricultural production. Nitrogen fixing micro-organisms could therefore be an important component of sustainable agricultural systems.

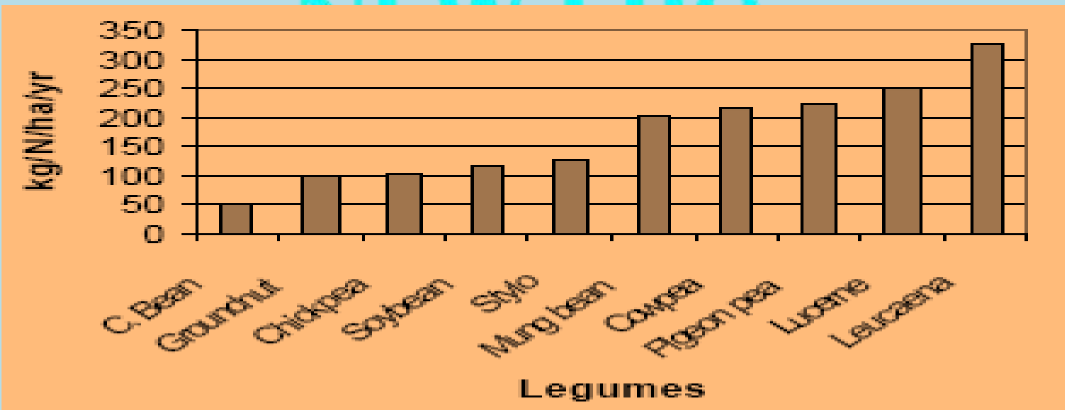

Fig.1 Average amounts of nitrogen fixed by various legumes (kg/N/ha/yr)

A recent consultation by FAO has reviewed the current knowledge on BNF in agriculture, highlighted the importance of many potentially viable but under-used opportunities for the adoption of BNF technologies and concluded that better efforts must be made to promote BNF-based farming systems in developing countries, including farmer-led experimentation and participatory agricultural extension methods to promote the use of appropriate BNF technologies.

The objective of this paper was to explore and discuss the possibilities for enhancing N_2 fixation in pulses and their microbial symbionts (*Rhizobium and* VAM fungi).

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Table 1. History of research on nitrogen, BNF and biofertilizers

Year	Event
1836	Identification of nitrogen as a nutrient for plants
1886	Hellriegel and Wilfarth demonstrated the ability of legumes to convert N_2
1888	First rhizobia were isolated from nodules
1893	Isolation of *Clostridium pasteurianum* (Free-living N fixers)
1895	First commercial inoculant (Nitragen)
1901	Isolation of *Azotobacter*
1913	Carl Bosch performed Haber's ammonia synthesis on an industrial scale
1946	Second commercial inoculant (Azotogen)
1953	Identification of two nitrogen fixing bacteria: *Beijerinckia fluminensis* and *Azotobacter paspali*
1969	Positive results for $^{15}N_2$ uptake by cyanobacteria
1972	Isolation of *Enterobacter cloacae* from corn roots
1975	Isolation of *Spirillum sp.* and demonstration of their nitrogenase activity
1984	Nitrogen fixation in Methanogens (archaea)
2011	The European Nitrogen Assessment provides the first integrated and comprehensive look at Nitrogen use in Europe
2012	Database of all *nifH* sequences available in the Genbank nucleotide database

BNF between past failures and future prospects:

Despite poor success in the past there is a now widespread optimism that modern technologies and holistic approaches can release substantial impact from BNF technologies. BNF is already making a significant contribution in the total N2 fixed globally (90 million tons/year) but the need for its improvement and widespread application in agriculture has never been more urgent than it is today, especially for the improvement of the most vulnerable cropping systems in developing countries. There is also a growing database of experience showing the dramatic knock-on effects for human health.

The past research on BNF in most crop research institutes focused on only some aspects such as microbial inoculation, few crop species and cropping systems, with very little attention to the integrated management of soil nutrients in the various agro ecological zones and the socioeconomic impacts of technologies. For example, adaptation studies of symbiotic and non-symbiotic systems to environmental constraints had been scarce and insufficient. The use of BNF technologies has also been often discouraged by national policies, including subsidies of chemical fertilizers, which reduce the economic competitiveness of BNF. Very little work has been done in assisting NARS to develop appropriate strategies and understand socioeconomic issues for BNF technology adoption. As a result, it has been difficult to implement a holistic approach for sustainable crop productivity and soil fertility improvement.

For many poor farmers, BNF is still a viable, cost-effective alternative or complementary solution to industrially manufactured N fertilizers. In addition to their great economic interest and indirect impact on human health, most BNF technologies have the potential to generate global environmental benefits by reducing greenhouse gas emissions and water pollution and promoting more sustainable use of agricultural land. Research on BNF, particularly the *Rhizobium*-legume symbiosis, and on plant molecular genetics have recently made significant progress, opening new possibilities to design strategies aimed at enhancing N2-fixing capacity in both leguminous and non-leguminous crops.

Strategies to enhance BNF in agricultural systems:

There are several methods available to stakeholders working on enhancement of N_2 fixation;

1. Host plant management
2. Selection of effective strains able to fix more nitrogen
3. Use of different agronomic methods that improve soil conditions for plant and microbial symbionts.
4. Inoculum production and Inoculation methods

Host plant management:-

Plant selection:

The amount of nitrogen fixed by legumes varies widely with host genotype, *Rhizobium* efficiency, soil and climatic conditions. The effectiveness of various legume species and their micro-symbionts has been provided in several publications. The nitrogen fixing potential of a number of different legume species and their microsymbionts is showed that when effective rhizobial populations are present either naturally or from inoculation, and there are no other major yield-limiting factor, plant selection is a potential method to enhance BNF. Substantial genotype variability on nodulation and nitrogen fixation were well evidenced. The high values for nitrogen fixation were observed on adapted cultivars and breeding lines when the environmental conditions were favorable. These can be used either directly as cultivars for production or in breeding programme to enhance nitrogen fixation in their cultivars.

Plant improvements:-

***Genomics and new opportunities for BNF technologies*:**

Genomics is revolutionizing research and commerce in the life sciences, and offers the development of a new paradigm in the application of BNF technologies. Despite the whole genome sequencing of rice and *Arabidopsis*, legume species such as *Lotus japonicus* and *Medicago truncatula* have recently been chosen as models systems for genomics and functional genomics, particularly to accelerate the research on symbiotic N2 fixation. The scientific emphasis of this research will generate a global understanding of the expression, regulation, dynamics and evolution of the symbiotic properties, which will certainly open new opportunities for application and result in high socioeconomic impacts. An important issue, however, will be the future share of developing countries in the genomics revolution and breakthroughs in BNF technologies. Several institutes have successfully applied BNF technologies to enhance soil fertility in developing countries, through agro forestry practices (ICRAF), research on asymbiotic BNF in rice (IRRI), and the improvement of legume nodulation (ICRISAT, IITA, CIAT). For example, the development of new varieties of pigeon pea at ICRISAT, as N2-fixing, drought tolerant and P-efficient user legume, and their introduction in cropping systems have made a tremendous impact in the semi-arid tropics.

Although the direct molecular modification of a host plant or microsymbiont has to result in the improvement of N_2 fixation at the field level, several approaches offer promise and some of those are:

- ✓ **Host transformation to modify host range**.

In a recent study transgenic Lotus plants transformed with the soybean lectin gene became susceptible to infection by *Bradyrhizobium japonicum*

- ✓ **Host modification to synthesize opines**.

Because *Rhizobium* strains vary in their ability to use opines, genetic engineering of legumes or other plants for opine synthesis may result in the enhancement growth of rhizosphere organism with the ability to utilize this substrate.

- ✓ **Genetic transformation of plants for enhancement malate dehydrogenase (MDH) synthesis in roots and nodules.**

Malate is the primary plant carbon source used by bacteroid, and is also a factor in plant adaptation to P and Al stress. Alfalfa transformed with a MDH gene having high efficiency in malate synthesis, exuded more organic material into the rhizosphere and fixed more N_2 than the wild type.

- ✓ **Host mutants with improved characters**

Host mutants with improved characters such as disease and insect pest resistant, earlier and later flowering, higher yield, higher protein content or less toxic compounds

Microsymbiont-Rhizobia:

Several important characteristics to be considered for the selection of the rhizobial symbionts are

Nitrogen fixation ability:

The rhizobia involved in nodulation can influence the percentage and amount of nitrogen fixed by the legume/*Rhizobium* symbiosis. There are several methods available to quantify and estimate N_2 fixation. Plant dry weight is usually well correlated to effectiveness in N_2 fixation, when N is the only limiting growth factor. 15N-based methods provide direct evidence for N_2 fixation.

Competitive ability:

The proportion of the nodules formed on a particular host is influenced by the competitive ability of an inoculated *Rhizobium* strain in comparison to indigenous strains, which may vary in their effectiveness.

Thermo-tolerance and survival ability:

Evidence for the involvement of some plasmids in imparting resistance to the adverse condition in some strains of *Rhizobium* was documented. Survival of these strains in soil can be improved by identification of plasmids carrying genes for resistance to environmental stress and transferring them to the highly effective strains.

Characters desirable for the strains for use in commercial inoculants:-

1. Capacity to form nodules and fix nitrogen on the desirable host
2. Ability to compete in nodule formation with native ineffective native population
3. Ability to fix N across the range of environment and tolerate environmental stress
4. Capability to form nodules and fix nitrogen in the presence of soil nitrates
5. Ability to grow well in artificial media, in inoculants carrier and in soil
6. Capacity to persist in soil particularly for seasonal legumes
7. Capability to migrate from the initial site of inoculation
8. Ability to colonize in the soil in the absence of host legume
9. Ability to fix nitrogen with wide host genotypes and genetic stability
10. Compatibility with agrochemicals and low mortality on inoculated seed

Micro symbiont –Mycorrhiza (VAM Fungi):

VAM fungi are obligate symbiont associated with majority of the agricultural crops growing under broad ecological range. Many graminaceous plants and legumes are highly susceptible to VAM colonization. The transfer of nutrients mainly P from the soil to the cell to the root cortex is mediated by intercellular obligate endo symbionts of the genera ***Glomus, Gigaspora,Acaulospora, Sclerocystis and Endogone*** which possess the **vesicles** for storage of nutrients and **arbuscules** for funneling of these nutrients to the plant root system. The fungi produce phosphatases which allow the utilization of the organic phosphorus. VAM also been reported to improve the uptake of micronutrients *viz.*, Zn, Cu, S, Fe and water. P and micronutrients are the key elements for enhancing the BNF; by the way of enhancing the uptake and availability VAM symbiosis indirectly improves BNF in legumes.

Benefits of VAM fungi highly relate to their amount of infection in host plant .Cultivars play important role on VAM symbiosis in crops. So the selection of efficient VAM fungi for the specific host is the one of the critical objectives in this technology. Inoculants production and huge quantity of inoculums requirement is the major limitation for commercialization and adoption of these technology.

Factors affecting BNF: crop management techniques:-

Environmental factors affecting nitrogen fixation include temperature, moisture, acidity and several chemical components of the soil such as nitrogen, phosphorus, calcium and molybdenum content. It is often difficult to isolate the effect of the above factors on inoculation success from their influence on symbiosis and nitrogen fixation.

For example: acidity as well as, calcium, aluminum and manganese concentrations will interact and affect bacterial proliferation, root-hair infection and plant growth. Numerous climatic variables, soil physical properties and agronomic management factors also play a part in controlling N_2 fixation; however, none of those factors should be considered in isolation as all are interconnected in the control of N_2 fixation (Fig 1).

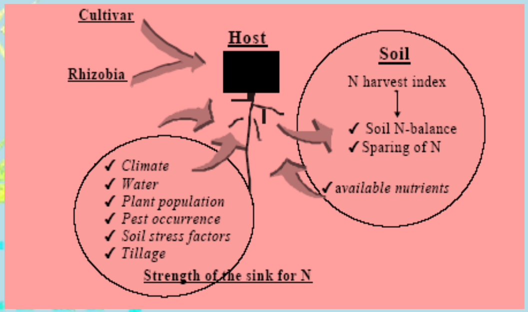

Fig 2. Conceptual model of the major factors that exercise a control on N_2 fixation of grain legumes in a cropping system (adapted from van Kessel & Harley, 2000)

In addition to the competitiveness of the rhizobia in forming nodules and the effectiveness of the *Rhizobium*-host plant to fix N_2, a series of edaphic, chemical and biophysical factors exert a control on N_2 fixation. Management practices like the intensity of tillage or intercropping practices will alter those edaphic, chemical and biophysical factors and therefore influence BNF indirectly (Table 2).

Table 2. Summary of some important factors limiting biological nitrogen fixation and their possible recommendations		
Factors affecting BNF	**Effect**	**Recommendations**
Temperature & moisture stress, drought	➢ Survival and poor establishment of rhizobia in soil ➢ Abilities to nodulate and fix nitrogen ➢ Inhibition of nitrogen fixation	➢ Placement of inoculum in deeper soil layers when top soil temperatures are high. ➢ Use of granular soil inoculants was superior under dry conditions. ➢ The surface mulches may conserve moisture and reduce soil temperature. ➢ Use of thermo tolerant strains of Rhizobia to be used.
Nitrogen Fertilization	Generally combined N delays or inhibits nodulation and nitrogen fixation. Because of this adverse effect, N fertilization usually is not recommended for leguminous crops. However there may be situations where N has to be applied, such as to cereals in mixed cropping or rotations and then fertilizer may affect nitrogen fixation of the legume crop.	➢ The development of grain legumes which are less sensitive to mineral N should not be pursued unless there is increase in N-uptake and an improvement in the overall use efficiency of available N. ➢ It is possible to apply small amounts of soil or foliar N fertilizer, which may increase yield without reducing the amount of nitrogen fixed.
Phosphorus deficiency	Decreased nodulation and poor effectiveness of the nodules	External application phosphatic fertilizers(super phosphate/ rack phosphate
Micronutrient deficiency	Poor infective nodules and poor dry matter production	Balanced application of micronutrients like Zinc, Boran, Molybdenum
Pesticides, fungicides and Insecticides and seed exudates	➢ The compatibility of rhizobia with pesticides is poorly understood except for fungicides. ➢ Insecticides have little adverse effect on nodulation when not directly applied on seed. ➢ The effect of herbicides on rhizobial survival is unknown. ➢ Adverse effect of seed exudates	➢ Due to the variability of the effect it is recommended to test the particular *Rhizobium* inoculum and its behavior in respect to the product to be used, before application. ➢ The effect of pesticide on N fixation should be minimized by separate placement of rhizobia and pesticide. ➢ Use of seed amendments improve nodulation under this condition
Intercropping	Increase opportunities for N-use complimentarily, reducing the need for fertilizer-N, either by increasing the availability of soil N or by N transfer.	An increase in the total amount of N_2 fixed could occur when the intercropped legume uses more effectively limited resources
Acid soils or alkalinity/ salinity	Acid / alkaline soils limit agricultural production and nitrogen fixation.	➢ Use of acid-tolerant/ salt tolerant legume cultivars and rhizobium strains. ➢ Soil liming should be limited to achieving a pH at which available aluminum or manganese levels are no longer toxic. ➢ Seed pelleting with gypsum ,rock phosphate, super phosphate etc helps to mitigate immediate effect of soil salinity/ alkalinity.

Factors affecting BNF	Effect	Recommendations
Tillage	When tillage is minimized, lower rates of mineralization and nitrification, coupled with increased N immobilization and a higher potential for denitrification will lead to a decrease in available N.	➢ Limiting tillage can stimulate N demand and N_2 fixation. ➢ Conservation and zero tillage management practices will, therefore, lead to a stimulation of N2 fixation, at least until a new equilibrium between residue input and the rate of decomposition is reached.
Other bioinoculants *viz.*, PGPR, VAM	Synergistic action of beneficial microbial inoculants to Enhancing the abilities to nodulate and fix nitrogen	Use of the consortia of the efficient microbial inoculants
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Inoculation technology and methods

Inoculant strains when applied to the target ecosystem have to compete with all of the negative and neutral microbes presented in the soil. This competition could reduce the efficacy of the final product and therefore methods and strategies to improve *Rhizobium* performance should be studied.

Determining the need for inoculation

In many soils, the nodule bacteria (*Rhizobium* sp) are not adequate in either number or quality. Under these conditions, it is necessary to inoculate the seed or soil with highly effective *Rhizobium* cultures.

- ➢ Inoculation is almost always needed when certain new leguminous crops are introduced to new areas or regions. Host-specific rhizobia are frequently developed for new cultivars or varieties of legumes
- ➢ Many soils are heavily infested with ineffective rhizobia capable of inducing nodulation without host benefit. Under such conditions, a very large inoculum of competitive and highly effective strain of rhizobia is needed to replace the ineffective native rhizobia.

Legume response to inoculation was largely dependent on:

- Number of rhizobia already established in the soil
- Availability of soil N
- Demand for N by the crop

Enhancing the effectiveness of inoculants

Inoculation technology

The technology should aim at protecting the viability of the microorganism and helping them to occupy the target niches and to express their biological functions. Peat based carrier materials are considered as superior for inoculants production in all respects except for their susceptibility to high temperature, drought and non- availability.

Properties of good inoculant carriers are as follows

- High water holding capacity
- Non-toxic to rhizobia and to retain high viable cell load
- Easy to sterilize by autoclaving or radiation
- Readily and inexpensive available
- Sufficiently adhesive for effectual application to seed
- pH buffering and cation and anion exchange capacity
- Oil based inoculants are superior over peat based inoculants under conditions of drought and high temperature. Dressing of seeds with liquid based inoculants just before sowing also recommended to improving the efficiency of the bioinoculants.
- Polyacrylamide gel was suitable carrier for *Bradyrhizobium* at 35^{o} C for better nodulation.
- Microcapsulation techniques have been successfully used to entrap biofertilizers agents in biodegradable polymers to protect them against storage conditions, oxidation, dryness, UV light and other environmental stresses
- Sterile carriers (Gamma radiated or short wave) with lower water potential proved to help preconditioned biofertiliser inoculant strains to environmental stresses, as well as to support a higher microbial count with a long expiration date.

Inoculation methods

Methods of rhizobial inoculation can have great influence on the amount of N_2 fixed. There are several considerations to be taken into account when optimizing inoculation methods. It has been demonstrated that nodules on the lower part of the root system can fix more nitrogen over the whole growing season than the crown nodules, and they may contribute most of the nitrogen fixed by the legume plant. Farmers applying inoculum on the seed can therefore not expect these bacteria to form nodules on the whole root system. It is likely that applied rhizobia form some or most of the nodules on the crown but other indigenous rhizobia in the soil may form the nodules at greater depth and distance from the crown. It should be possible to enhance N_2 fixation by promoting optimal production of nodules on lateral roots by selecting rhizobia not only for the effectiveness to fix N_2 but also for migration in the soil and along the root under a range of conditions.

Associative nitrogen fixing inoculants has also been developed and commercially produced for crop plants. The output of these inoculants had been inconsistent and more site and crop dependent. Thus, co-inoculants will require extensive *in-vitro* and *in-situ* investigations if the positive attributes associated with each organism are to be effectively exploited.

Role of biotechnology in enhancing the efficacy of inoculants

Biotechnology and gene manipulation techniques were able to provide potential means to improve the commercial inoculants strains. During the last 10 years, extensive studies revealed the genetic determinants and the regulation pathways of most of the microbial functions. Genes that control nodulation (*nod*, *ndv*), nitrogen fixation (*nif*, *fix*), host range (*nod*, *hsp*), surface polysaccharide (*exo*) and energy utilisation (*dct*, *hup*) have been identified. Inoculant strains were able to take advantage of these techniques to produce value-added inoculants.

Road map for successful BNF

Currently, the widespread application of artificial fertilisers is necessary for agricultural output. Numerous issues should be explored by researchers and other stakeholders within an integrated and collaborative paradigm in order to fully utilize BNFs for enhancing soil health and crop quality. Here is a list of the most significant research and development priorities necessary for the widespread use of N-fixing biofertilizers.

- Gain a deeper comprehension of the processes involved in biological nitrogen fixing, relationship selection, and partner fidelity.
- By better understanding the genetic and molecular mechanisms that control symbiotic specificity, it will be possible to increase the range of hosts for symbiotic bacteria.
- Before commercialization, conduct extensive testing on inoculums in a variety of soil types and environmental circumstances.
- Step up your search for highly competitive and productive microbial strains, paying close attention to mixed-strain consortiums as opposed to mono-strain inoculums to capitalise on

functional complementarity in real-world settings.

- Enhance the purity of the microbial strains used in commercial inoculums,

Table 3. Some famous marketed microbe-based biofertilizers and target crops

Name of Manufactured Products and Producer (*in Italic*)	Strain	Formulation	Crop suited	Benefits
BioGro *Nguyen Thanh Hien in Hanoi University (Vietnam)*	*Pseudomonas fluorescens* *Bacillus subtilis* *Bacillus amyloliquefaciens* *Candida tropicalis*	Solid in peat	Rice (*Oryza sativa*)	Improve rice yield
Biofix *MEA company limited (Kenya)*	R*hizobium*	Solid	-Soya bean (*Glycine max*) -Common bean (*Phaseolus vulgaris L*) -Alfalfa (*Medicago sativa)*	Cheaper than chemical nitrogen Lighter to transport, requires less labor effective for many crops
Bio N *Nutri-Tech Solutions* (*Australia*)	*Azotobacter* spp.	liquid	Horticulture	Access free atmospheric nitrogen. Increase yield and quality. Reduce soil erosion. Improve water retention. Enhance germination. Promote root growth. Phosphate release
Microbin and Azottein *Egyptian Ministry of Agriculture*	*Klebsiella*, *Bacillus*, *Azotobacter* *Azospirillum*	Carrier material	Barley cultivar Giza	Increased the different plant characteristic increases in grain yield reached approximately 24.8 and 27.2%
Legumefix *Legume Technology (UK)*	*Bradyrhizobium japonicum*	Sterile peat inoculant	Soybean and cowpea	grain yield (12–19%) relative to the control
Leguspirflo *SoyGro* *(South Africa)*	*Azospirillum brasilense*	Liquid	soybean	Inefficient
TerraMax's Micro AZ product *TerraMax (Minnesota, USA)*	*Azospirillum brasilense* *A. lipoferum.*	Liquid	Wheat, Corn and Grain Sorghum	Improve root structure and stimulate root growth Provide biological nitrogen fixation Increases yields Stimulates rooting Increases yields

Nitrofix P *Agro-Input Suppliers Limited (AISL) (Malawi)*	*Bradyrhizobium japonicum* and *B radyrhizobium elkanii*	Dry-inoculum based on gamma-sterilized peat	Soybeans	Promotes an increase in the yield by an average of 14.3–20.3% Reduced the nitrogen requirement
Vault LVL *BASF (Badische Anilin- & Soda-Fabrik) Germany*	*B. japonicum* + *Bacillus subtilis*	Liquid	Soybeans	Biomass yield improved

as well as their density and the formulations used to keep them alive during storage. As a component of inoculant quality, microbial strain performance should also be taken into account.

- The third-party controls and formal registration requirements for commercial inoculums which will improve quality control.
- Boost farmer capabilities through education and the creation of networks for the exchange of reference protocols and BNF knowledge.
- Create innovative inoculation techniques and make it simple for end users to get timely access to high-quality inoculants.
- It is known that the presence of arbuscular mycorrhizae increases the transfer of symbiotically fixed nitrogen through network connections between similar or dissimilar plants, so combining mycorrhizae with BNF will make it easier for nitrogen to move from plants with high fixing potential to low or non-fixing plants.
- Identify the soils that are best suited for the inoculation of N-fixing organisms, paying close attention to soils with low pH and high N concentrations that are known to prevent the formation of nodules. We found many regions with high potentials for inoculation success due to their soil pH (ranges from slightly acidic to neutral, 5.5 to 7.2) and relatively low amounts of nitrogen in the soil by superimposing a nitrogen fertility map onto a pH map.

Conclusions

Biological N_2 fixation is an important aspect of sustainable and environmentally friendly food production and long-term crop productivity. However, if BNF is to be utilized, it must be optimized. In the near future, particularly in developing countries, tremendous opportunities exist for enhancing the BNF capacity of legumes.

There is no simple and easy approach to increase BNF in grain legumes grown as part of a cropping system, under realistic farm field conditions. Numerous climatic variables, soil-physical properties, agronomic management, host-rhiozobia combination and socioeconomic aspects play an important role in controlling BNF.

The use of improved host-rhizobia combination has great potential to increase N_2 fixation. Interaction between a range of traits and N_2 fixing symbiosis will require particular care in breeding and selection programs aimed at alleviating environmental and management practices that reduce BNF.

- Programmes for host plant selection
- Programmes for *Rhizobium* /VAM selection
- Management practices that increase N demand by the host plant is a promising avenue to increase N_2 fixation in grain legumes in a cropping system.

- The most likely management practices to have an impact on BNF are:
  - Improving the overall fertility status of the soil, while maintaining low levels of available soil N.
  - Improving soil structure
  - Conversion from conventional tillage to zero or minimal tillage.

There are several methods available to enhance BNF, as shown in the present paper. None of the approach is better than others in individually; rather work on synergistically combining experience from interdisciplinary research programmes.