

Biological nitrogen fixation with non-legumes: An achievable target or a dogma?

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Nitrogen is most often the limiting nutrient for crop production, since only a fraction of atmospheric nitrogen is made available to the plants through biological nitrogen fixation (BNF). Extending the BNF ability to non-legumes would be a useful technology for increased crop yields among resource-poor farmers. The idea that genetic manipulation techniques might be used to engineer crop plants to fix nitrogen is, of course, not new. However, the more we understand about the biochemistry and physiology of BNF, the less likely it seems that this goal will be achieved by 'simply' transferring the genes for nitrogen fixation to suitable crop species. Induction of nodulation has therefore been the main target of researchers over the past few years. This review briefly describes the progress made towards nodular symbiosis between rhizobia and other free-living atmospheric nitrogen fixers and non-legume crops.

Keywords: Bacteria-plant association, non-legumes, *para*-nodules, nitrogen fixation.

NITROGEN is an essential plant nutrient. It is the nutrient that is most commonly deficient, contributing to reduced agricultural yields throughout the world. Molecular nitrogen or dinitrogen (N₂) makes up four-fifths of the atmosphere, but is metabolically unavailable directly to higher plants or animals. It is available to some microorganisms through biological nitrogen fixation (BNF) in which atmospheric nitrogen is converted to ammonia by the enzyme nitrogenase. According to statistics by FAO (2001), about 42 million tons of fertilizer N is being used annually on a global scale for the production of three major cereal crops, i.e. wheat, rice and maize (17, 9 and 16 million tons respectively). Crop plants are able to use about 50% of the applied fertilizer N, while 25% is lost from the soil-plant system through leaching, volatilization, denitrification and due to many other factors causing not only an annual economic loss of US\$ 3 billion but also cause pollution to the environment. Some of the adverse environmental effects of excessive use of nitrogenous fertilizers are: (i) methemoglobinemia in infants due to NO₃ and NO₂ in waters and food, (ii) cancer due to secondary amines, (iii) respiratory illness due to NO₃, aerosols, NO₂ and HNO₃,

(iv) eutrophication due to N in surface water, (v) material and ecosystem damage due to HNO₃ in rainwater, (vi) plant toxicity due to high levels of NO₂ and NH₄ in soils, and (vii) excessive plant growth due to more available N, depletion of stratospheric ozone due to NO and N₂O. If a BNF system could be assembled in the non-legume plants, it could increase the potential for nitrogen supply because fixed nitrogen would be available to the plants directly, with little or no loss. Such a system could also enhance resource conservation and environmental security, besides freeing farmers from the economic burden of purchasing fertilizer nitrogen for crop production. Thus, a significant reduction in the relative use of fertilizer N can be achieved if atmospheric N is made available to non-legumes directly through an effective associative system with some of the characteristics of legume symbiosis. Recently, several approaches using techniques developed in the area of biotechnology have raised new hopes that success in this secondary objective may yet be realized. It is the authors opinion that there are now sound reasons to anticipate that at least some non-leguminous field crops may also become independent of soil nitrogen. We intend to explain the reasons for this renewed optimism, against the background of knowledge accumulated in the past century that will be relevant to any ultimate success in exploiting these new approaches.

Nitrogen fixation and sustainable agriculture

By definition, biological N₂ fixation is synonymous with sustainability. Advances in agricultural sustainability will require an increase in the utilization of BNF as a major source of nitrogen for plants. Long-term sustainability of the agricultural system must rely on the use and effective management of internal resources. The process of BNF offers an economically attractive and ecologically sound means of reducing external nitrogen input and improving the quality and quantity of internal resources. Clearly, it is not realistic to consider sustainable agriculture on a broad scale in the absence of BNF; research is needed to optimize the contribution of BNF to sustainable agriculture.

Nitrogen applied in fertilizers usually provides benefit to plants. However, if applied inefficiently, it can also have serious disadvantages in causing pollution. It is difficult to match nitrogen supply to actual requirements of

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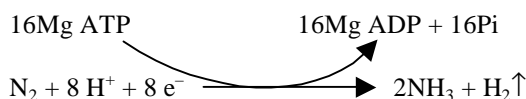
a crop at a given ecosite and any excess may damage this or other ecosites. Excess reduced nitrogen (ammonium) in agricultural or forest ecosystems may lead to their acidification through the process of nitrification, if significant leaching of nitrate occurs. In agricultural ecosystems, however, BNF fixation may usually be expected not to exceed the actual nitrogen requirements of an ecosystem, thus being less likely than fertilizers to cause pollution. Moreover, in the wake of deepening energy crisis and decontrol of fertilizer prices, the crucial role of organics and biofertilizers in sustaining soil productivity and ensuring food security must be realized. Also, our present 'high input' agriculture is not sustainable. The 'plants of the future' for both the developed and developing countries should allow a 'low input' agriculture.

As a society, we must find ways to reduce dependency on fossil fuels for economic, environmental and geopolitical reasons. Development of sources of renewable energy will be particularly important for agriculture. BNF will be a major component in the improvement of agricultural sustainability.

Key features in achieving N₂-fixing activity

The nitrogenase reaction

Chemically, BNF is essentially the conversion of dinitrogen (N₂) to ammonia, catalysed by the enzyme nitrogenase. The reaction catalysed can be represented as follows:



Of course, the process of N₂ fixation is much more complex than this, but the biochemical reaction contains the key ingredients and points to the major requirements. It indicates the dual needs for reducing potential and the substantial energy requirement in the form of ATP. It also suggests the need for a mechanism for ammonia utilization that will simultaneously neutralize the alkalinity generated.

The energy requirement

BNF requires energy. Microbes that fix nitrogen independent of other organisms are called free living. The free-living diazotrophs require a chemical energy source if nonphotosynthetic, whereas the photosynthetic diazotrophs utilize light energy. The free-living diazotrophs contribute little fixed nitrogen to agricultural crops. Associative nitrogen-fixing microorganisms are those diazotrophs that live in close proximity to plant roots (that is, in the rhizosphere or within plants) and can obtain energy materials from the plants. They may make a modest contribution of fixed nitrogen to agriculture and forestry, but quantification of their potential has not been established.

The symbiotic relationship between diazotrophs called rhizobia and legumes can provide large amounts of nitrogen to the plant and can have a significant impact on agriculture.

The symbiosis between legumes and the nitrogen-fixing rhizobia occurs within nodules mainly on the root and in a few cases on the stem. A similar symbiosis occurs between a number of woody plant species and the diazotrophic actinomycete *Frankia*. The plant supplies energy materials to the diazotrophs, which in turn reduce atmospheric nitrogen to ammonia. This ammonia is transferred from the bacteria to the plant to meet the plant's nutritional nitrogen needs for the synthesis of proteins, enzymes, nucleic acids and chlorophyll.

A number of studies have attempted to estimate energy requirements for BNF by comparing the rate of growth of legumes on nitrate with their growth on nitrogen. However, the energy requirement for N₂ fixation is actually almost identical to that required for nitrate assimilation¹, the other main source of nitrogen for most field crops, excepting rice.

Another important experimental approach has been the use of efflux of carbon dioxide from legumes growing with either nitrogen source². In general, greater carbon dioxide efflux has been observed on plants growing with dinitrogen than with nitrate³, suggesting an increased energy demand, as extra carbohydrate required. Unfortunately, none of these studies considered the fact that nitrate-grown plants produce bicarbonate rather than carbon dioxide in assimilating nitrate⁴. When this difference in the metabolism of the two nitrogen sources is taken into account, the apparent difference in energy consumption could disappear.

On the other hand, if the diazotrophic organism in an engineered system could have direct access to ample energy supply from the plant, such negative opinions about proposals to engineer non-legumes to fix dinitrogen based on energy requirement could be questioned. Substantial infection of the root cortex by organisms like *Azospirillum* would be a case in point. For such pessimism to be sustained, one would need to dismiss the positive role of leguminous nodules in fixing N₂, which is absurd. To be effective, however, not only penetration but also adequate colonization with the diazotrophs and some kind of symbiosis would be required⁵.

Non-leguminous N₂-fixing systems

A number of non-leguminous plants are recognized to have the ability to fix N₂ either through exogenous or endogenous symbiosis with N₂-fixing microorganisms. This can be a potential source of nitrogen for agriculture and can be of greater economic importance. Some cereal crops of commercial importance like rice, wheat, maize and millets are found to have association with microorganisms that are capable of assimilating atmospheric nitrogen. Fixation of N by bacteria living in symbiosis with leguminous plants is a popular phenomenon. Various attempts have been

made to extend the host range of *Rhizobium* from legumes to non-legumes through plant genetic manipulation. The transfer of *nif* genes along with others for functional nitrogen fixation was considered to be the most suitable strategy to achieve symbiotic N₂ fixation in non-legumes. However, the highly complicated system of gene regulation posed great hindrance in achieving a functional N₂-fixing transgenic system. Genetic engineering through biotechnological means has seen little or no success in achieving the induction of symbiosis between cereals and diazotrophs.

In rice, associative nitrogen fixation supplied 20–25% N of the total need, as reported in one of the studies conducted at IRRI, Philippines⁶. Rice seedlings when inoculated with some strains of *Burkholderia* spp. isolated from rice plants contributed a relatively higher level of N to rice via BNF⁷. *Acetobacter diazotrophicus* could enhance rice growth and growth promotion might be related to the transfer of biologically fixed N although other factors such as auxin production could be involved⁸.

Inoculation of wheat with *Azospirillum brasilense* and *A. lipoferum* significantly increased the yields of foliage, grain and branching of root hairs and gave varying degrees of acetylene reduction⁵ from 48 η mol ethylene h⁻¹ plant⁻¹ to 10 η mol ethylene h⁻¹ plant⁻¹. Wheat *para*-nodules can be readily colonized externally and to some extent internally by the cyanobacterium *Nostoc* spp. strain 2S9B and that such co-existence provides favourable conditions for N₂ fixation⁹.

Acetylene reduction by maize inoculated with *Azospirillum* showed that carbon substrates and oxygen pressure have significant effect on it. Enhanced nitrogenase activity (acetylene reduction assay) was noticed in maize plants treated with *Azospirillum* either alone or with 2,4-D, but higher activity was noticed in the 2,4-D and *Azospirillum*-treated plants¹⁰. The rate of activity varied greatly from 300 to 800 η mol C₂H₄ h⁻¹ g⁻¹ dry wt of roots⁵.

Association of diazotrophs with non-legumes

Azospirillum

Bacteria of the genus *Azospirillum* are associative nitrogen (N₂)-fixing rhizobacteria that are found in close association with plant roots. They are able to exert beneficial effects on plant growth and yield of many agronomic crops under a variety of environmental and soil conditions. In the case of rice, *A. lipoferum* and *A. brasilense* have been isolated from the roots and stems¹¹ and *A. amazonense* has been isolated from the roots¹². Microscopical evidence as to the endophytic nature of *Azospirillum* in rice has been presented¹³ and the colonization of 2,4-D-induced *para*-nodules by an ammonium-excreting mutant of *A. brasilense* has been reported¹⁴. An ammonium-excreting mutant of *A. brasilense* (Wa3) promoted better growth of wheat plants compared to wild type¹⁵ and another strain (C3) was able to transfer the nitrogen fixed directly to the

maize plant¹⁶ and the amount transferred was increased in *para*-nodulating plants obtained by treatment with 2,4-D. In maize plants treated with 2,4-D and *Azospirillum*, cob weight, 100-grain weight and grain cob⁻¹ were found to be maximum and significantly higher¹⁷. Despite these different mechanisms exerted by facultative endophytic diazotrophs in association with graminaceous plants, increases in yield in the range of 5–30% have been observed in several inoculation experiments with *Azospirillum*¹⁸.

Rhizobia

Soil bacteria of the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* (collectively referred to as rhizobia) interact with leguminous plants to form N₂-fixing nodules. The possibilities of extending the host range of rhizobia to non-legumes were encouraged by the discoveries that *Rhizobium* forms nodules in *Parasponia*¹⁹ and that *Rhizobium parasponium* RP 501 and *Bradyrhizobium* CP 283 induce nodulation in oilseed rape²⁰. In addition, recent evidence shows that rhizobia are capable of colonizing the roots of non-legumes wheat, maize and barley, but the basis of the association between non-legumes and rhizobia is so far not known. Rhizobia were found to have the ability to attach themselves to rice root hairs, elicit deformation of rice root hairs and to form nodule-like structures. Certain strains of *R. leguminosarum* bv. trifolii could colonize intercellularly, multiply and migrate inside growing lateral roots²¹. The stimulation of lateral root development and colonization of lateral root cracks and xylem of rice roots by *A. caulinodans* (ORS 571) has been reported²². In addition to forming nodules after crack entry invasion of emerging lateral roots, it is able to fix nitrogen in the free-living state up to 3% (v/v) oxygen and without differentiation into bacteroids²³. The maize plants inoculated with *A. caulinodans* alone had higher NPK content in grains and stover, and combination of *A. caulinodans* with auxin significantly enhanced the NPK content in the grains and stover²⁴. Nodulated and *Azorhizobium*-treated plants also showed higher chlorophyll content in the leaf and enhanced nitrate reductase activity, leading to higher yield compared to the control plants (non-nodulated)²⁵.

Acetobacter

Acetobacter diazotrophicus was shown to be the major supplier of fixed nitrogen to sugarcane, which accounted for the capacity of certain varieties to be continuously cropped for many years in the same soil in the total absence of added nitrogen fertilizer. Besides fixing nitrogen, *A. diazotrophicus* produces plant hormones such as auxin. Therefore, the bacteria could enhance plant growth by nitrogen fixation and hormone production. The endophyte is passed from one crop to the next through the standard vegetative reproduction by stem pieces (setts)²⁶.

Herbaspirillum

Herbaspirillum is an endophytic nitrogen-fixing organism capable of colonizing intercellular spaces of maize, rice, sorghum and sugarcane. It has been found in the roots, stems and leaves of graminaceous plants. Colonization of rice plants by selected strains of *H. seropedicae* showed that the bacteria first colonize the epidermal cells of the root surface or secondary root emergence. *H. rubrisubalbicans*, another species of *Herbaspirillum*, was isolated from rice plants. Occurrence of *H. rubrisubalbicans* in rice plants may not be a surprise¹³ because inoculation of rice seedlings grown axenically with strain M₄ increased nitrogen accumulation in the plant by about 30%.

Azoarcus

This obligate endophytic diazotroph has been isolated from kallar grass (*Leptochloa fusca*) grown in saline-sodic soils in Pakistan. The strain BH 72 is capable of invading roots of the original hosts as well as rice plants, infecting the cortex region and is capable of expressing nitrogenase genes in the aerenchyma of rice roots²⁷. *Azoarcus* is also able to colonize the interior of sorghum plants by means of its cellulolytic enzymes²⁸.

Role of phytohormones in inducing para-nodules

There are several reports on induction of nodule-like structures termed as *para*-nodules on cereal roots using different plant hormones like 2,4-D, NAA, BAP and zeatin. The term *para*-nodules was introduced by Tchan and Kennedy to describe the chemically induced nodules, since they differ from the naturally occurring legume nodule⁵. These induced nodule-like outgrowths are modified lateral roots with carbon reserves (as starch in amyloplasts) similar to those found in the cortex of roots, and microorganisms are able to modulate or interfere with the development of these outgrowths²⁹.

It was suggested more than 50 years ago that hormones were involved in nodule induction on plant roots and this concept was demonstrated by the use of cytokinin kinetin which could induce pseudonodules on tobacco roots³⁰. It was suggested that cortical cell division, one of the early steps in nodule induction, could be induced by auxins and cytokinins excreted by the infecting rhizobia. In alfalfa roots, localized trans-zeatin produced by *R. meliloti* was found to induce the formation of nodule-like structures³¹. Exogenous application of 2,4-D to either wheat or rice plants induces modified root outgrowths, which results from the induction of meristem³². Furthermore, adding microorganisms can modulate or interfere with the development of these outgrowths²⁹. Auxin application together with *Azospirillum* bioinoculation enhanced polygalacturonase activity of rice roots to a higher level, which resulted

in better root morphogenesis (*para*-nodule) and endorhizospheric colonization of *A. brasilense*³³. Maize seedlings developed nodule-like knots along primary roots and club-shaped tumours at their root tips when treated with 2,4-D. However, well-developed nodule-like tumour knots that can best be described as modified lateral roots (*para*-nodules) emerged only when inoculated with *Azospirillum* along with 2,4-D¹⁰. These observations suggested a close linkage between alterations to the plant cell hormone balance and nodule induction by rhizobia.

Among different plant hormones, 2,4-D a synthetic auxin, was found to be the best in inducing nodular outgrowth in cereals²⁹. The 2,4-D treated plants were shown to carry more associated azospirilla than untreated plants; a dense layer of bacteria was observed covering the surface of young parts of 2,4-D-treated roots and nitrogenase activity was high especially when inoculum was also given. It has been proposed that 2,4-D has an effect akin to bacteria that produce cellulases and polygalacturanases in loosening as well as breaking the intercellular bonds³³. The cells thus released will add to the amount of rhizodeposition with a consequent increase in bacterial colonization of roots.

Establishment of endophytes within the nodules

The term endophyte is used with various meanings in the literature on plant-microbe interactions. Here, we use the term endophyte to describe microbes that have colonized living plant tissue without harm to the host.

The tip regions of healthy roots growing undisturbed in a firm matrix offer no obvious structural features that would allow the casual entry of microbes. In grasses, in which this region has been most studied, the meristematic and elongation zones are completely enclosed in a thick, pellicle-like outer shell, which extends around the distal end of the meristem, separating it from the rootcap. This shell, together with the specialized, closely appressed walls of the meristematic and elongating cells account for the well-documented impermeability of these regions, at least in monocotyledons, to small molecular weight tracers mobile in the apoplast³⁴. This impermeability is seen also in young branch (lateral) root primordia even as they emerge from the parent root³⁵. Thus, the undamaged root surface offers no opportunity for casual entry by rhizosphere microbes, and only those microbes with the ability to hydrolyse their way into these tissues will be able to penetrate. Production of pectolytic and cellulolytic enzymes has been demonstrated in some bacterial species known to be endophytes³⁶. Such bacteria could possibly dissolve their way through non-incrusted parenchyma cell walls. Although undoubtedly openings into root interiors will occur in normal soil conditions because of mechanical breakage by cultivation by browsing fauna or pathogenic microbes, there is no substantial evidence that such cracks are either frequent or normal within undisturbed roots in the soil. The most popular proposed ports for 'crack entry'

have been the postulated openings made by emerging branch roots. Any openings produced by branch root emergence that would allow casual entry of bacterial endophytes, if such openings do exist, are certainly transitory and probably infrequent³⁷. Roots are easily cracked during handling and the possibility of damage during excavation and separation from soil is well known. Much less appreciated is the damage that is caused by simple removal of a root system from a hydroponic solution (or from an agar medium). Root hairs and emerged branch roots are particularly vulnerable, as surface tension and the weight of the medium clinging to these narrow, unsupported structures pull them downward³⁸. The walls of root hairs are thus frequently cracked at their base, and the seals between parent cortex and branch may break at the point of branch exit and also at the union between the endodermis of the parent and the branch root, leading to quite erroneous interpretations of the permeability to nutrients and microbe penetration³⁷.

Two other factors, besides mechanical damage, may be operating to allow entry of bacteria into axenically grown roots challenged by high titres of potential endophytic bacteria. (i) The surface of axenically grown crop roots is always different in appearance from that of field-grown material. Generally, the former are white for much of their length, whereas the latter quickly became beige or brownish behind their growing tips. This colouration in the field-grown roots must derive, at least in part, from the early suberization and lignification of the walls of the surface and subsurface tissues (epidermis and hypodermis) that occur in these roots, presumably in response to both physical and biological stress imposed by the soil environment. The coddled roots in the laboratory environment may be especially vulnerable to the sudden challenge of a heavy inoculum of the applied bacterium, which may thus gain entry by wall hydrolysis and/or cell death. (ii) If the roots are growing in agar-solidified media, marked changes in root tissue architecture and increase in cell death may result, thus facilitating bacterial entry³⁷.

The basic message here is that the entry of bacteria into roots of plants cultured in semi-solid media may not be representative of the situation in undisturbed field-grown plants. Therefore, it is essential that bacterial strains identified as efficient endophytes under laboratory conditions be rigorously evaluated for efficient colonization under field conditions. Further studies are required to determine how endophytes that are present within living tissues in some field-grown crops gain entry.

Scientific challenges

To make BNF more useful, progress must be made to meet numerous scientific challenges.

- No BNF microbial associations or symbioses are known that produce significant amounts of fixed nitrogen for

the major cereals – corn, wheat, barley and sorghum. The symbiotic genes in legumes are being identified. Might these be transferred to cereals to enable them to have nitrogen-fixing symbiosis? Another possibility is the transfer of nitrogen-fixation (*nif*) genes to bacteria that are endophytic in cereal species or to induce nitrogen-fixing bacteria to become endophytic.

- In general, the more efficient nitrogen-fixing strains of rhizobia compete poorly with the rhizobia already in the soil. Can ways be derived to improve the competitive ability of inoculant rhizobia?
- Fertilizer nitrogen generally inhibits BNF in both symbiotic and asymbiotic systems. Can this inhibition be reduced to obtain large contributions of nitrogen from BNF?
- There are many environmental stresses that negatively affect nodulation and nitrogen fixation, such as acid and alkaline soils, nutritional deficiencies, salinity, high temperature, and presence of toxic elements. Can cultivar–strain combinations resistant to these stresses be developed for stressed field conditions?
- In addition to legumes, a number of non-legume woody plants such as casuarinas (*Casuarina* spp.) and alders (*Alnus* spp.) fix nitrogen symbiotically with *Frankia* spp. Research on this system is in its infancy. Advances could be beneficial to forestry and agroforestry.

Conclusion

If root nodules, in essence, are said to be callus formed on plant roots as a result of external stimulus (e.g. mechanical, physical, chemical and biological), then any plant, including non-leguminous plants, can form root nodules, because they all have such a gene. No matter whether on leguminous plants or non-leguminous plants, root nodules once formed, may be either effective or ineffective, this depends on: (i) whether or not the microbes living inside the nodules are capable of fixing N; (ii) whether or not the microbes and the host plant can form a highly harmonious symbiotic N-fixing system, and (iii) the environmental condition in which the host plant is growing. The biological nitrogen fixation phenomenon occurs and exists first in nature and is then understood by man. It is, therefore, advisable to observe and learn the natural phenomenon and interpret the findings in the laboratory. The study of BNF cannot be confined only to such a narrow space as the relationship between a certain host plant and a certain single strain of microbe. Relationship of a plant with other plants and microbes, of the microbe with microbes and organisms, and sunlight, temperature, air, moisture, etc. are factors to be considered in the studies. Sometimes they can even become crucial deciding factors under certain circumstances³⁹.

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