

## Review

# Effective factors on biological nitrogen fixation

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Accepted 17 January, 2012

Although relationships among plant, biological N<sub>2</sub> fixation, and response to soil and environmental conditions have received considerable coverage in the scientific literature, a comprehensive summary and interpretation of these interactions with specific emphasis are lacking. Fluctuations in pH, nutrient availability, temperature, and water status, among other factors, greatly influence the growth, survival, and metabolic activity of nitrogen fixation bacteria. The subsequent inhibition of nitrogenase would result in O<sub>2</sub> accumulation in the infected zones, inducing the decrease in nodule permeability. Poor nodulation of legumes in arid soils is likely due to decreases in population levels of rhizobia during the dry season. Fixation, therefore, also tends to decrease with legume age, mainly because of the concomitant increase in soil N. Calcium deficiency, with or without the confounding influence of low pH also affects attachment of rhizobia to root hairs. Rhizobia may have different tolerances to soil acidity factors than the host plant. Relatively, high-root temperature has also been shown to influence infection, N<sub>2</sub>-fixation ability, and legume growth. Also, root nodulation by the bacteria can be dependent on the formation of mycorrhiza.

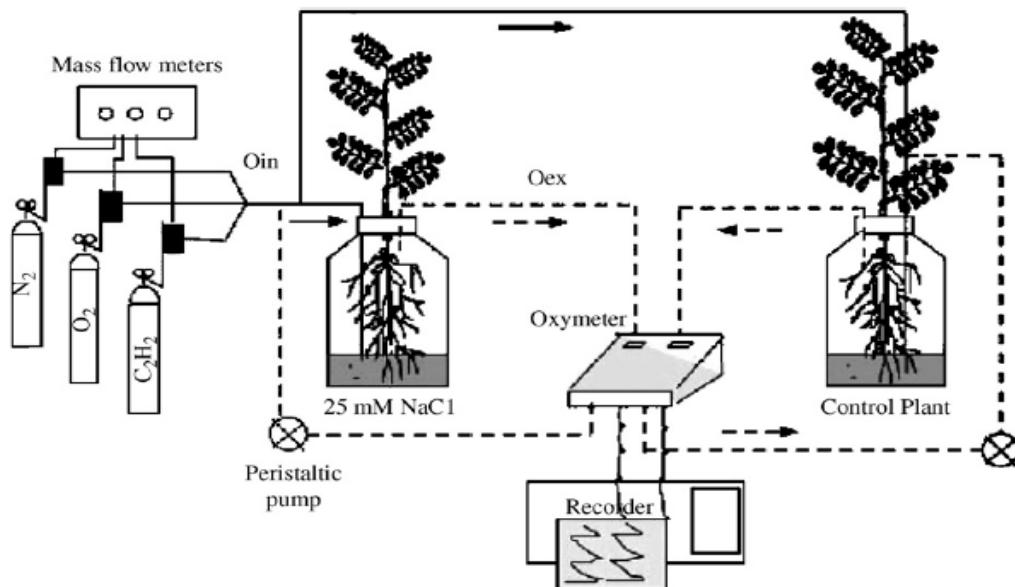
**Key words:** Legume, nitrogen fixation, rhizobia, root, stress.

## INTRODUCTION

Biological Nitrogen Fixation (BNF) is an efficient source of fixed N<sub>2</sub> that plays an important role in land remediation. Interest in BNF has focused on the symbiotic systems of leguminous plants and rhizobia because these associations have the greatest quantitative impact on the nitrogen cycle. Deficiency in mineral N often limits plant growth and as such, symbiotic relationships have evolved between plants and a variety of N<sub>2</sub>-fixing organisms. The symbiotically fixed N<sub>2</sub> by the association between *Rhizobium* species and the legumes represents a renewable source of N for agriculture. Values estimated for various legume crops and pasture species are often impressive, commonly falling in the range of 200 to 300 kg N ha<sup>-1</sup> per year. This underlines

the significance of *Rhizobium* and legume symbioses as a major contributor to BNF. Nitrogen fixation, along with photosynthesis as the energy supplier, is the basis of the soil environment under a constant state of change and, as such, can be relatively stressful for both macro- and micro-organisms. Fluctuations in pH, nutrient availability, temperature, and water status, among other factors greatly influence the growth, survival, and metabolic activity of nitrogen fixation bacteria and plants, and their ability to enter into symbiotic interactions (Werner and Newton, 2005). Despite this situation, soils represent one of earth's most productive ecospheres, accounting for a majority of primary and successional productivity. Consequently, microbes, plants, and other soil inhabitants have evolved to adapt to the ever changing and often inhospitable soil environment. Stress factors in soils influence symbiotic nitrogen fixation. However, the reader should be aware that, while some stress factors simultaneously affect both symbiotic partners, water stress, others may differentially influence each partner to

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**Figure 1.** Device for *in situ* measurement of oxygen consumption by nodulated-root applied by L'taief et al. (2007).

a seemingly different degree by different mechanisms. Moreover, both plants and microbes have often adopted different strategies for dealing with these stress factors. Nitrogen fixation provides the basic component of fixed nitrogen as ammonia of two major groups of macromolecules, namely nucleic acids and proteins. Fixed nitrogen is required for the N-containing heterocycles that constitute the essential coding entities of DNA and RNA, which are responsible for the high-fidelity storage and transfer of genetic information, respectively (Werner and Newton, 2005).

#### ENVIRONMENTAL STRESS AND NITROGEN FIXATION

In order to properly discuss how environmental stress factors influence symbiotic nitrogen fixation, it is important to understand how the micro and macro-symbionts interact at the cellular and molecular levels. In some instances, environmental perturbation independently influences the nodulation and nitrogen-fixation processes. Although, here, we only discuss this topic in broad terms, several recent review articles cover this area in much greater detail (Vitousek et al., 2002). As might be expected from the number of genes involved, the induction and repression of bacterial nodulation genes is under tight regulatory control and is a major factor influencing host specificity and response to environmental variables (Spaink et al., 1987). The *nodD* gene can be viewed as a global regulatory gene which, together with plant flavonoid-signal molecules, activates transcription of other inducible nodulation genes (Long,

2001). The flavonoid *nod*-gene inducers are specific for a particular legume-*Rhizobium* interaction and their production is influenced by environmental variables, like plant fertility, pH, and nod factors (Schmidt et al., 1994). Repressor proteins also play a role in *nod*-gene regulation (Stacey et al., 2002). The specific sensitivity of the symbiotic nitrogen fixation (SNF)-dependent legumes to salinity is well documented for initiation, development and function of nodules (Saadallah et al., 2001). An application of salt or drought decreases the nodule permeability. This decrease is associated with a contraction of nodule inner-cortex cells and an increase in acid abscissic content of the nodule (Irekti and Drevon, 2003). The subsequent inhibition of nitrogenase activity and nodule respiration is compensated by raising PO<sub>2</sub> in the nodulated-root rhizosphere (Serraj et al., 1994). Alternatively, the inhibition of the sucrose synthase and various enzymes of the sucrose hydrolysis by salinity have been argued to induce a deficiency of carbon for bacteroids associated with an accumulation of sucrose up to 40 to 70% of the total nodule sugar content. The subsequent inhibition of nitrogenase would result in O<sub>2</sub> accumulation in the infected zones, inducing the decrease in nodule permeability. Additionally, it has been argued that the limitations of O<sub>2</sub> diffusion imposed by structural modifications due to salinity are compensated by the decrease of nodule growth and the formation of a large number of small nodules facilitating the O<sub>2</sub> entry in nodules by increased contact area with external medium. L'taief et al. (2007) applied the device (Figure 1) for the *in-situ* measurement of oxygen consumption by nodulated-root of *Cicer arietinum* in stress condition. Between each confinement, the circuit was opened and

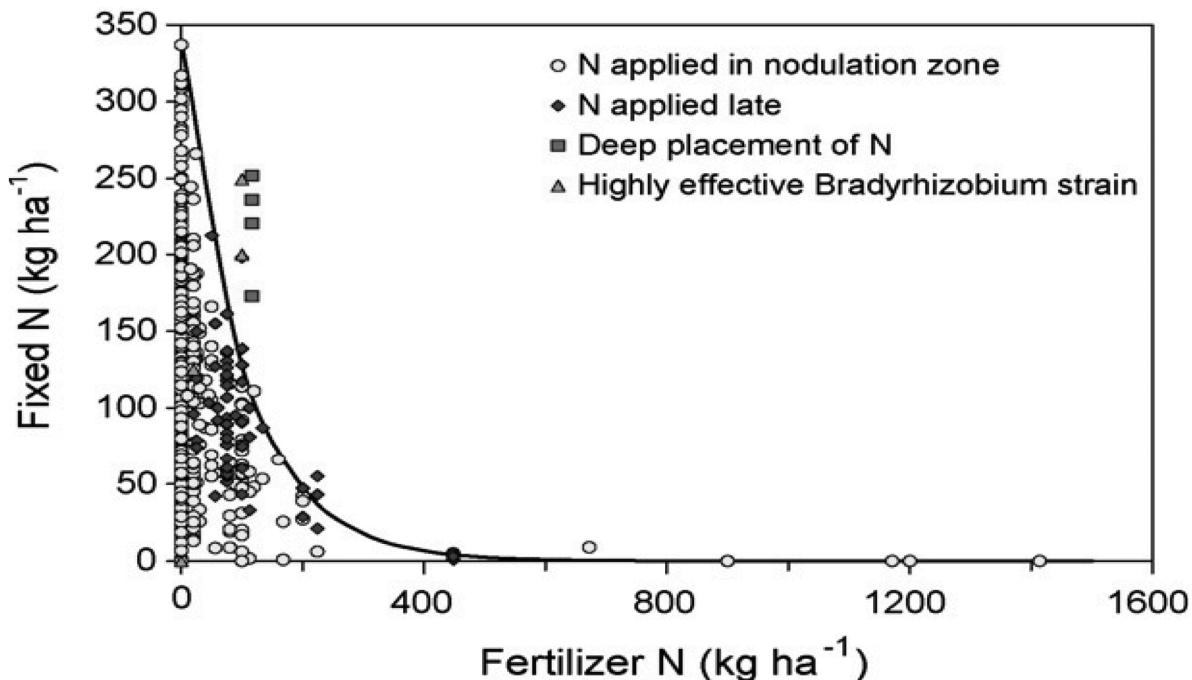


Figure 2. Relationship between nitrogen fixed by soybeans and N applied as fertilizer (Salvagiotti et al., 2008).

washed by a renewed gaseous mixture monitored by mass-flow meters.

Virtually any environmental factor that negatively influences either the growth of rhizobia or the host plant itself has a dramatic impact on symbiotic  $N_2$  fixation. These factors can independently negatively influence the nodulation process itself, and thereby indirectly affect nitrogen fixation, or directly influence plant growth and vigor during post-nodulation events and as such, affect the efficient functioning of the nitrogenase enzyme complex. To facilitate the discussion, we have separated these fundamentally different sets of factors. However, the reader should be aware that, in some instances, some factors simultaneously affect both rhizobia and the host plant.

### Soil water content and stress

Soil water influences the growth of soil micro-organisms through processes of diffusion, mass flow, and nutrient concentration. Soil water is related to soil pore space, and soils containing larger pores and pore spaces retain less water. Thus, soil aggregates having smaller internal pore spaces are more favorable environments for the growth of rhizobia and most soil microbes (Turco and Sadowsky, 1995). Soil-water content also directly influences the growth of rhizosphere micro-organisms, like rhizobia, by decreasing water activity below critical tolerance limits and indirectly by altering plant growth, root architecture, and exudations. Poor nodulation of

legumes in arid soils is likely due to decreases in population levels of rhizobia during the dry season. However, the influence of soil-water activity on plant growth and vigor, and hence, nodulation should not be ignored. Rhizobia have evolved a variety of mechanisms for adapting to osmotic stress, mostly by the intracellular accumulation of inorganic and organic solutes. For example, *R. meliloti* overcomes osmotic stress-induced growth inhibition by accumulating compatible solutes, such as  $K^+$ , glutamate, proline, glycine betaine, proline betaine, trehalose, and the dipeptide, N-acetyl glutamyl glutamine amide (Boscari, 2002). Some compatible solutes can be used as either nitrogen or carbon sources for growth, suggesting that their catabolism may be regulated to prevent degradation during osmotic stress.

### Soil nutrient

As might be expected, soil nutrient status has a tremendous influence on the symbiosis as well as independent growth and survival of both partners. Fixation, therefore, also tends to decrease with legume age, mainly because of the concomitant increase in soil N. A negative exponential relationship was observed between N fertilizer rate and  $N_2$  fixation when N was applied to 0 to 20 cm of the top soil or to the soil surface (Figure 2). The difference in this relationship arises from a variation in N supply derived from indigenous sources (net soil N mineralization, irrigation, atmospheric deposition), and possibly other factors affecting growth

**Table 1.** Linear relationship among different parameters of peanut and root nodules studied by Basu et al. (2008).

Parameter	Symbiotic N fixation bacteria	Nodule plant <sup>-1</sup>	N content of nodules	LAI	Dry matter	100 kernel weight	Kernel yield
Symbiotic N fixation bacteria		0.45*	0.84**		0.73**		0.87*
Nodule plant <sup>-1</sup>					0.47**		0.62**
N content of nodules					0.65**		0.81**
LAI					0.89**		0.98**
Dry matter							0.88**
100 kernel weight							0.69**
Kernel yield							

and N<sub>2</sub> fixation (for example, soil pH and drought). Thus, in this respect, an unfertilized grass component, in a short-term mixed sward can perform the useful task of competing for available soil N, so that the legume is forced to obtain a greater proportion of N from N<sub>2</sub> fixation (Ledgard and Steele, 1992). It should be noted, that in some cases, nutrient stresses are indirectly caused by changes in soil matric potential or acidity, which limits the nutrient bioavailability, rather than the lack of presence of nutrients *per se*. When considering nutrient limitations to symbiotic nitrogen fixation, one must clearly separate factors affecting growth of the host from those influencing the microbe or the symbiotic interaction. For example, acid and water stress causes alterations in root growth, which can indirectly affect both nodulation and nitrogen fixation. This effect is thought to be mediated by abscisic acid. Mohammadi et al. (2011) evaluated the effect of different soil fertility strategies on absorption metabolism and molecular nitrogen fixation in chickpea. They showed highest nodule weight, nodule number and nitrogen fixation was obtained in co-application of organic manure and biofertilizers treatment. Combined application of compost, farmyard manure and biofertilizers increased dry weight of roots. Compost and farmyard manure (FYM) are organic sources of nutrients, which increase soil organic matter and enhance grain quality (Mohammadi and Rokhzadi, 2012).

Unfortunately, initial soil nitrate content was not measured in many of the published studies. To account for differences in the total supply of mineral N from soil and fertilizer sources, a boundary line for the 0.99 quartile was fitted to represent the maximum attainable level of N<sub>2</sub> fixation as a function of fertilizer-N rate. When no N fertilizer was added, the maximum amount of N<sub>2</sub> fixation reached 337 kg ha<sup>-1</sup>, and as a consequence of the exponential decline, a maximum N<sub>2</sub> fixation of 129 and 17 kg N ha<sup>-1</sup> would be expected if 100 and 300 kg ha<sup>-1</sup> of fertilizer-N were to be applied to the upper soil layer, respectively (Salvagiotti et al., 2008).

Isaac et al. (2011) concluded that, both the number of nodules and the N derived from atmosphere determined by the <sup>15</sup>N natural abundance method did not increase along the P gradient. Phosphorus stimulated growth and increased mineral N uptake from solution without

affecting the amount of N derived from the atmosphere under non-limiting N conditions, *Acacia senegal* N acquisition strategies changed with P supply, with less reliance on N<sub>2</sub>-fixation when the rhizosphere achieves a sufficient N uptake zone. Stress conditions apparently increase requirements for essential elements, such as Ca<sup>2+</sup>, P, and N, in both plants and microsymbionts. Ca<sup>2+</sup> might, in some instances, offset the deleterious influence of low pH on root growth and ion uptake and increase *nod*-gene induction and expression (Richardson et al., 1988). Calcium deficiency, with or without the confounding influence of low pH, also affects attachment of rhizobia to root hairs, and nodulation and nodule development (Alva et al., 1990). Lastly, a calcium-spiking phenomenon is initiated in root-hair cells of legumes by nodulation factors and rhizobia, suggesting that Ca<sup>2+</sup> plays a pivotal role in symbiotic interactions at the molecular level. Poor nodulation of soybeans in acid soil has been attributed to an Al induced Ca deficiency (Biswas et al., 2003). Many studies have shown soil amelioration to be of benefit in peanut production.

In addition to macro nutrients, the growth and persistence of rhizobia in soils is also influenced by several other nutritional factors (Brockwell et al., 1995). The rhizobia are metabolically diverse and have been shown to use a variety of both plant- and soil-derived compounds for growth. Interestingly, some of the same compounds that support growth have also been shown to be chemotactic and induce *nod* genes. In addition, supplementation of soil and inoculants with glutamate, glycerol, and organic matter has been shown to enhance the survival and numbers of rhizobia in soils and increase both early nodulation and N<sub>2</sub> fixation (Rynne et al., 1994). This result indicates that, although, rhizobia can surely persist in soils, their efficacy can be enhanced by carbon addition, which suggests that they are C limited in the natural state. Results of Basu et al. (2008) indicated a linear relationship between nitrogen fixation and fertilization (Table 1).

### Soil pH stress

The influence of soil pH on the nodulation process has

**Table 2.** The effect of elevated temperature and drought on total dry mass (DM), leaf DM, stem DM, root DM, nodule DM, of nodulated alfalfa plants studied by Aranjuelo et al (2007).

Variable	C25	C28	D25	D28
Total DM (g plant <sup>-1</sup> )	2.2 <sup>a</sup>	1.3 <sup>b</sup>	1.3 <sup>b</sup>	0.5 <sup>c</sup>
Leaf DM (g plant <sup>-1</sup> )	0.5 <sup>a</sup>	0.3 <sup>b</sup>	0.3 <sup>b</sup>	0.1 <sup>c</sup>
Steam DM (g plant <sup>-1</sup> )	0.7 <sup>a</sup>	0.4 <sup>b</sup>	0.3 <sup>b</sup>	0.1 <sup>c</sup>
Root DM (g plant <sup>-1</sup> )	0.9 <sup>a</sup>	0.5 <sup>b</sup>	0.7 <sup>a</sup>	0.2 <sup>c</sup>
Nodule DM (mg plant <sup>-1</sup> )	39 <sup>a</sup>	43 <sup>a</sup>	23 <sup>b</sup>	16 <sup>b</sup>

C25 and C28: fully watered plants grown under control (25 °C) and elevated (28 °C) temperature; D25 and D28: partially watered plants grown under control (25 °C) and elevated (28 °C) temperature. Each row represents the mean of 40 plants. Means with identical letters (a-d) are not significantly different ( $P > 0.05$ ).

been extensively examined in part due to the World's large number of acid soils. Low soil pH is generally accepted as an indicator of conditions under which some other soil properties may limit crop growth rather than as a primary cause of poor growth. In addition to the direct effects of soil acidity, factors on plants, growth of legumes may be reduced indirectly through depression of nodulation and nitrogen fixation. In this regard, rhizobia may have different tolerances to soil acidity factors than the host plant. Nodules formed by the rhizobia may not fix nitrogen or their fixation rate may often be inadequate. Worldwide, more than 1.5 g ha<sup>-1</sup> of acid soils limit agriculture production (Graham et al., 2000) and as much as 25% of the earth's croplands are impacted by problems associated with soil acidity. Brockwell et al. (1991) reported a nearly 10<sup>-3</sup> decrease in the number of *S. meliloti* in soils with a pH<6 compared to those with a pH>7.0. As found for soil moisture, there is a range of effects of soil pH on rhizobia, but relatively few grow and survive well below pH values of 4.5 to 5.0. Although the micro-symbiont appears more pH sensitive than the host partner (Hungria and Vargas, 2000), acidity also influences both the growth of the legume plant and the infection process. This effect is, in part, most likely due to both a disruption of signal exchange between macro- and micro-symbionts and repression of nodulation genes and excretion of Nod factor in the rhizobia. Interestingly, nodulated legumes appear more sensitive to metal toxicity by Mn and Al than to their N-fed control counterparts (Hungria and Vargas, 2000).

### Soil temperature

Temperature has a marked influence on survival and persistence of rhizobial strains in soils. For example, cowpea rhizobia from the hot dry Sahel-savannah of West Africa grow at 37°C, and more than 90% of the strains isolated from this region grew well to 40°C (Werner and Newton, 2005). The influence of temperature on rhizobia appears to be both strain and soil dependent. For example, *Bradyrhizobium* sp. was

less susceptible than *R. leguminosarum* bv. *trifolii* to high soil temperatures, but addition of montmorillonite and illite remediated this problem in sandy soils. Soil temperature also greatly influences competition for nodulation (Triplett and Sadowsky, 1992). This effect may, in part, be due to a temperature-induced delay in nodulation or the restriction of nodules to the sub-surface region. Aranjuelo et al. (2007) evaluated effect of elevated temperature and water availability on CO<sub>2</sub> exchange and nitrogen fixation of nodulated alfalfa plants. They showed that plant and nodule dry weight affected by temperature (Table 2).

Relatively high-root temperature has also been shown to influence infection, N<sub>2</sub>-fixation ability, and legume growth (Hungria and Franco, 1993) and has a strong influence on specific strain and cultivar interactions (Arayankoon et al., 1990). It appears that every legume and *Rhizobium* combination has an optimum temperature relationship, which is around 30°C for clover and pea, between 35 to 40°C for soybean, peanut and cowpea, and between 25 to 30°C for common bean (Long, 2001).

### MYCORRHIZAL INTERACTION

Although, the interaction between *Rhizobium* and *mycorrhizal* fungi was previously reported, less attention was paid to the joint symbiosis of *Frankia*, *Mycorrhizae* and *Hippophae tibetana*, especially in identification of the joint symbiosis. A century ago, Janse was the first to report the coexistence of certain bacteria and fungi colonizing the root system of legume plants. The bacteria produce nodular structures on the root and the fungi established a type of "root infection" that was later recognized as mycorrhizal development. Further, Asai (1994) stated that root nodulation by the bacteria can be dependent on the formation of mycorrhiza. Nowadays, both the widespread presence of the AM symbiosis in nodulated legumes and the role of AM in improving both nodulation and *Rhizobium* activity within the nodules are universally recognized and a great deal of work has been carried out on the tripartite symbiosis among legume-mycorrhiza-rhizobia. Relevant current information that

has arisen since our previous reviews will be summarized here by discussing: (1) the fundamental studies of the formation and functioning of the tripartite symbiosis; (2) strategic studies related to agricultural systems; (3) strategic studies related to the revegetation of degraded ecosystems; (4) biotechnological developments for integrated management; and (5) future trends for this research area. In natural conditions, AM fungi and *Rhizobium* colonize the root almost simultaneously but the two endophytes do not seem to compete for infection sites. In certain cases, previous inoculation with one of the endophytes can depress the development of the other. This has been mainly attributed to competition for carbohydrates when host photosynthesis is limited. When this occurs, AM fungi usually show a competitive advantage for carbohydrates over *Rhizobium* (Brown and Bethlenfalvay, 1988; Xiao et al., 2010).

## CONCLUSION

Environment condition such as temperature, pH, nutrient availability and soil condition had a significant difference in nitrogen fixation. Despite many decades of progress and the acquisition of a large amount of useful information, the physiological and molecular bases for the tolerance of legume-microbe symbiotic systems to environmental stress remains largely unknown and empirical in nature. Although, understanding these processes was originally thought to be straightforward and tractable, we have learned that we now have more questions than answers. This situation is perhaps due to the fact that abiotic stresses independently and differentially influence the host legume, the rhizobia, and the symbiotic couple. So where do we go from here? Clearly, more work needs to be done on the underlying molecular bases for tolerance to stress factors in both legume and microbes. Further research in the genomics and proteomics of macro- and micro-symbionts will accelerate development of this area by providing a wealth of information on how both host and microbes respond to environmental perturbations. However, these further studies need to be combined with traditional plant breeding and microbial selection efforts in order to rapidly define and utilize microbial and host genetic loci that are involved in tolerance to a large number of environmental stresses.

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