

Review

Enhancing the biological nitrogen fixation of leguminous crops grown under stressed environments

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Legumes have the ability to establish a symbiotic interaction with soil bacteria, collectively termed as rhizobia. These bacteria can enhance growth and development of associated crops by transferring atmospheric nitrogen into a form that is available for plant growth or by improving nutrient uptake through modulation of hormone-linked phenomena in inoculated plants. Selection of the effective *Rhizobium* strain is the most critical aspect used to achieve maximum benefits from this technology. This review aims to focus on recent findings, thereby highlighting the enhancement of plant growth and nitrogen uptake during the symbiosis between rhizobia and leguminous plants under severe conditions. The potential uses of such microorganisms due to their multifaceted beneficial activities are likely to play an important role in modern high intensive agricultural practices.

Key words: *Rhizobium* spp., legumes, growth promotion, plant protection, sustainable agriculture.

INTRODUCTION

An essential element of agricultural sustainability involves the effective management of nitrogen (N) in the soil environment. This usually implicates at least some use of biologically fixed nitrogen (BNF) because N from this source is absorbed directly by the plants, and so is less susceptible to volatilization, denitrification and leaching. In agricultural settings, about 80% of BNF come from symbioses formed between leguminous plants and species of *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Allorhizobium* (Vance, 1998).

Legumes and rhizobia together fix the atmospheric N and because of this feature, they are often introduced to manage the agricultural ecosystems to improve organic fertility, N economy or farming system flexibility

(Brockwell et al., 1995). Optimal performance of the N-fixing symbiosis depends upon pre-selection of both symbiotic partners for adaptation to the target environment, which may in some form present a challenge to rhizobial survival or nodulation (Sessitsch et al., 2002). Inoculation of stress tolerant strains of rhizobia may enhance the nodulation and nitrogen fixation ability of legumes under stressed conditions. For example, the ability of legumes to grow and survive in saline conditions is improved when they are inoculated with salt tolerant strains of rhizobia (Zou et al., 1995). Rhizobial populations, however, vary in their tolerance ability to major environmental factors (Ulrich and Zaspel, 2000; Wei et al., 2008; Biswas et al., 2008). Thus, the use of rhizobia as biofertilizers under severe conditions is discussed.

BIOLOGICAL NITROGEN FIXATION

Nitrogen is an essential plant nutrient. In agriculture, fertilization with nitrogen products is widely and increasingly practiced to increase the yields of foods (Reinhold-Hurek and Hurek, 2003). The earth's atmosphere contains about 10¹⁵ tonnes of N₂ gas, which cannot be

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Abbreviations: N, Nitrogen; BNF, biologically fixed nitrogen; ROS, reactive oxygen species; CAT, catalase; SOD, superoxide dismutase.

used in this form by most living organisms unless it is reduced to ammonia. The nitrogen cycle involves the transformation of some 3×10^9 tonnes of N_2 per year on a global basis (Postgate, 1982). Biological fixation is the principal process of nitrogen entry into natural ecosystems, while fixation associated with vascular plants usually contributes the greatest quantities of added N (Cleveland et al., 1999). Nitrogen fixation also occurs as a result of non-biological processes (such as, Haber-Bosch and combustion) where lightning alone accounts for about 10% of the world's supply of fixed N (Sprent and Sprent, 1990). Furthermore, the world production of fixed N from dinitrogen used for chemical fertilizer accounts for about 25% of the earth's newly fixed N_2 , while biological processes accounts for about 60%. Significant growth in fertilizer-N usage has occurred in both developed and developing countries (Peoples et al., 1995). The requirements for fertilizer-N are predicted to increase further in the future (Subba-Rao, 1980). However, the use of elevated doses of fertilizers, may have negative and unpredictable effects on the environment, and may cause the contamination of soil, water and natural areas (Sprent and Sprent, 1990). Such impacts pose a serious threat to human and animal health. In addition, developing countries have to face the demand of high costs for such technology and chemical utilization. To circumvent such problems, the use of BNF provides an interesting option for decreasing the reliance on the use of chemical fertilizers.

For more than 100 years, BNF has commanded the attention of scientists concerned with plant mineral nutrition, and has been exploited extensively in agricultural practices (Dixon and Wheeler, 1986). However, its importance as a primary source of N for agriculture has declined in recent times probably due to slow effect of BNF on crops (Peoples et al., 1995). Nonetheless, the use of renewable resources in sustaining crop development, following low input BNF, has renewed the interest of agrarian communities (Dixon and Wheeler, 1986). Furthermore, the expanded interest in its use, which is due to the fact that BNF is ecologically benign, could reduce the use of fossil fuels and may be helpful in reforestation and restoration of misused lands (Sprent and Sprent, 1990).

Nitrogen-fixing organisms

Nitrogen fixation is carried out by numerous prokaryotes, including bacteria, actinobacteria and certain types of anaerobic bacteria. Microorganisms that fix N are called diazotrophs. All organisms reduce dinitrogen to ammonia by an enzyme called nitrogenase. The nitrogenase enzymes are irreversibly damaged by exposure to atmospheric levels of oxygen (Giller and Wilson, 1991). Nitrogenase activity is usually measured by the acetylene reduction assay, which is cheap and sensitive (Sprent

and Sprent, 1990). The ^{15}N isotopic method, which is also used to measure N_2 fixation, is accurate but expensive. A wide range of organisms have the ability to fix nitrogen. However, only a very small proportion of species are able to do so; that is, about 87 species in 2 genera of archaea, 38 genera of bacteria and 20 genera of cyanobacteria have been identified as diazotrophs or organisms that can fix nitrogen (Sprent and Sprent, 1990). This wide variety of diazotrophs ensures that most ecological niches contain one or two representatives and that the lost nitrogen is replenished.

Importance of the biological nitrogen fixation

Most of the N added naturally to soils is from the biological fixation that is symbiotic or non symbiotic in nature. It has been estimated that about 100 Tg N, valued at \$US 40 billion, is required annually for the production of the world's grain and oilseed crops (David and Ian, 2000). The other sources are mainly from lightning discharges, burning of fossil fuels and forest, and from the emission of magmatic gases. Much land has been degraded worldwide, and it is time to stop the destructive uses of land and to institute a serious reversal of land degradation. BNF can play a key role in land remediation. The success of legumes is largely indebted to their symbiotic relationship with specific nitrogen fixing bacteria known as rhizobia. Phylogenetically rhizobia are very diverse, representing several lineages. Rhizobia currently include 12 genera and more than 70 species of α - and β -proteobacteria (Sawada et al., 2003). A tremendous potential for the contribution of fixed nitrogen to soil ecosystems exists among the legumes (Brockwell et al., 1995). There are approximately 700 genera and about 13,000 species of legumes, of which only a portion have been examined for nodulation and is shown to have the ability to fix N_2 (Sprent and Sprent, 1990). Estimates suggest that the rhizobial symbioses, with the somewhat greater than 100 agriculturally important legumes, contribute nearly half the annual quantity of BNF entering the soil ecosystems (Tate, 1995). Legume symbioses contribute at least 70 million tonnes of N per year, and approximately, half is derived from the cool and warm temperature zones, while the remainder is derived from the tropics (Brockwell et al., 1995). Increased plant protein levels and reduced depletion of soil N reserves are obvious consequences of legume N_2 fixation. The deficiency in mineral N often limits plant growth, and so the symbiotic relationships between plants and a variety of nitrogen-fixing organisms (Freiberg et al., 1997) fulfil the N demands of crop plants.

Yield increases of crops planted after harvesting legumes are often equivalent to those expected from application of 30 to 80 kg of fertilizer-N ha^{-1} . Inputs of fixed N for alfalfa, red clover, pea, soybean, cowpea and vetch are estimated to be about 65 to 335 kg of N ha^{-1}

year⁻¹ (Tate 1995) or 23 to 300 kg of N ha⁻¹ year⁻¹ (Wani et al., 1995). The behavior of these symbioses under severe environmental conditions and their applications in arid regions is thus discussed.

FACTORS LIMITING THE BIOLOGICAL NITROGEN FIXATION IN ARID CLIMATE

In the *Rhizobium*-legume symbiosis, the process of N₂ fixation strongly depends on the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full N₂-fixation activity, if limiting factors impose limitations on the vigor of the host legume. Several environmental conditions are limiting factors to the growth and activity of the N₂-fixing plants in the Mediterranean region (such as, Tunisia). Tunisia is mostly located in the semi-arid, arid and Saharan climatic zones where the annual rainfall varies from 300 to less than 100 mm (Ben et al., 2009). In Tunisian farming systems, yields of leguminous crops are generally more affected by drought than those of cereal crops. As a consequence, grain legumes cultivation surfaces have decreased in favour of cereal monoculture over the past 20 years.

Water deficiency and drought directly affect persistence and survival of rhizobia in soil, nodule activity and function (Davey and Simpson, 1990), and also limits nodulation through its effects on root-hair colonization and infection by rhizobia (Graham, 1992). Like drought, salinity is a serious threat to agriculture in arid and semi-arid regions, in that plant responses to salt and water stress have much in common. Salinity reduces the ability of plants to take up water, which in turn reduces growth rate along with several metabolic changes identical to those caused by water stress (Munns, 2002). An essential aspect of the strategy to improve the yield of arid legumes in stressed environments must involve a combination of stress-tolerant cultivars and stress-tolerant rhizobia. Breeding and selection of genotypes, tolerant to water stress, were extensively studied for different leguminous plants (Turner et al., 2001). However, BNF capable of improving agricultural productivity while minimizing soil loss and ameliorating adverse edaphic conditions are essential.

Soil salinity

Virtually 40% of the world's land surface can be categorized as having potential salinity problems. Most of these areas are confined to the tropics and Mediterranean regions (World Resources, 1987). High concentrations of sodium chloride lead to marked changes in the growth pattern of plants, while salinity is known to reduce the growth of glycophytes (salt-sensitive species). This effect may result from changes in dry matter

allocation, ion relations, water status, physiological processes, biochemical reactions or a combination of these.

Salt tolerance in plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes (Greenway and Munns, 1980). Survival and growth in saline environments are the result of adaptive processes, such as ion transport and compartmentation, osmotic solute synthesis and accumulation, which lead to osmotic adjustment and protein turnover for cellular repair (Munns and Termaat, 1986; Paul and Cockburn, 1989). Legumes have been suggested as appropriate crops for the enhancement of bioproductivity and the reclamation of marginal lands, because these plants not only yield nutritious fodder, protein-rich seeds and fruits, but also enrich soil nitrogen in symbiotic association with *Rhizobium* (Alexander, 1984). Nodulation and nitrogen fixation in legume-*Rhizobium* associations are adversely affected by salinity, which can preclude legume establishment and growth, or reduce crop yields (Mohammad et al., 1991).

Unlike their host legumes, rhizobia can survive in the presence of extremely high levels of salt and show marked variation in salt tolerance. Some strains are inhibited by 100 mM of NaCl (Singleton et al., 1982; Yelton et al., 1983), whereas strains of *Rhizobium meliloti* and *Rhizobium fredii* grew at salt concentrations above 300 mM (Sauvage et al., 1983; Yelton et al., 1983). Moreover, some *Acacia* and *Prosopis* strains can tolerate up to 500 mM NaCl (Zeghari et al., 2000). Osmotolerant *Rhizobium* strains can support large modifications in the osmolarity without decrease in the number of viable cells (Singleton et al., 1982). Consequently, their multiplication in the rhizosphere of the plant host will not be affected in saline soils, as it is the case in sensitive strains.

The osmoadaptation of most microorganisms involves the accumulation of K ions and one or more of a restricted range of low molecular mass organic solutes, termed collectively as 'compatible solutes' (Welsh 2000). These solutes are accumulated to high intracellular concentrations, in order to balance the osmotic pressure of the growth medium and maintain cell turgor pressure, which provides the driving force for cell extension. Another investigator demonstrated that during the earlier stages of plant-rhizobia interaction, the host plant reacts to the invasion of the bacteria by the over production of reactive oxygen species (ROS) to initiate the hypersensitive reaction (Santos et al., 2001). The excess of ROS production can drastically damage bacteria and plant tissues. In this process, catalase (CAT) and superoxide dismutase (SOD) were reported to play a key role in the establishment and protection of the symbiosis (Jamet et al., 2003; Santos et al., 2000). Moreover, ROS generation can be enhanced under environmental stresses as salinity, which is considered as a major constraint of symbiotic nitrogen fixation and plant production (Zahran

and Sprent, 1986). The partial reduction of O_2 generates free-radicals whose concentration depends on the levels of O_2 and Fe-protein; whereas O_2^- and its derivative H_2O_2 are eliminated by superoxide dismutase and catalase (Thorneley and Ashby, 1989). The activity of these enzymes doubles when cells fix nitrogen (Dingler and Oelze, 1987). This enzyme converts superoxide radical to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). However, H_2O_2 is itself a cellular toxic product that is scavenged by other antioxidant enzymes, mainly CAT (E.C. 1.11.1.6), which cleaves H_2O_2 to water and O_2 without consuming reductants, and thus, may provide plant cells with an energy-efficient mechanism to remove H_2O_2 (Scandalios et al., 1997). Hydrogen peroxide can be removed also by "non-specific" peroxidases (POX, E.C. 1.11.1.7) which make use of H_2O_2 as an electron donor in metabolising phenolic compounds. These latter enzymes are ubiquitous and are involved in various processes such as, cell growth control and tolerance to environmental stress (Quiroga et al., 2000).

Water deficiency and drought

Water deficiency is a major limiting factor of plant productivity and symbiotic nitrogen fixation in many arid regions of the Mediterranean basin. One of the immediate responses of rhizobia to water deficiency concerns morphological changes (Shoushtari and Pepper, 1985; Busse and Bottomley, 1989). The modification of rhizobial cells by water stress eventually leads to a reduction in infection and nodulation of legumes (Hunt et al., 1981). In addition to its depressive effect on nodule initiation, water deficit also results in the restriction of nodule development and function (Serraj et al., 1999). The occurrence of rhizobial populations in desert soils and the effective nodulation of legumes growing therein (Jenkins et al., 1989), emphasize the fact that rhizobia can exist in soils with limiting moisture levels; however, population densities tend to be lowest under the most desiccated conditions and increase as the moisture stress is relieved. It is well known that some free-living rhizobia are capable of survival under drought stress or low water potential (Fuhrmann et al., 1986). The wide range of moisture levels characteristic of ecosystems where legumes have been shown to fix nitrogen suggests that rhizobial strains with different sensitivity to soil moisture can be selected. *In vitro* studies have shown that sensitivity to moisture stress varies among rhizobial strains (Busse and Bottomley, 1989), while nodulation and N_2 -fixation in alfalfa (*Medicago sativa*) was improved by inoculating plants with competitive and drought tolerant rhizobia (Zahran 1999), suggesting that rhizobial strains can be selected with moisture stress tolerance within the range of their legume host which is generally more sensitive to moisture stress than bacteria.

Several mechanisms have been suggested to explain

the varied physiological responses of several legumes to water stress. Under osmotic stress, a balance between internal and external water potentials can be reached if the cells accumulate compatible solutes or osmoprotectants. These include potassium ions, glutamate, glutamine, proline, quaternary amines (glycine betaine) and sugar trehalose, sucrose and glucosylglycerol. However, compatible solutes help to maintain the stability of proteins during osmotic stress via a 'preferential exclusion mechanism' (Potts, 1994).

High temperature and heat stress

In arid regions, high soil temperature affects both the free-living and symbiotic life of rhizobia (Zahran, 1999). For most rhizobia, the optimum temperature range for growth is 28 to 31°C, and many are unable to grow at 38°C (Graham, 1992). Some strains of the rhizobia surviving under heat stress may lose their infectivity, due to plasmid curing or alterations in cellular polysaccharides necessary for infection (Zahran, 1999). High soil temperature (35 to 40°C) usually result in the formation of ineffective nodules; however, some strains of rhizobia (for example, *Rhizobium leguminosarum* bv. *phaseoli*) were reported to be heat tolerant, thereby forming effective symbioses with their host legumes (Hungria and Franco, 1993; Michiels et al., 1994). These associations will be more relevant for growing inoculated legumes in arid climates.

Acid soils and soil acidification

Acid soils constrain agricultural production in more than 1.5 Gha worldwide (Edwards et al., 1991), and this damage is related to air pollution and other factors. Despite much controversy, there exists no doubt that air pollution stresses, like 'acid rain' and enhanced N deposition, affect soils by accelerating soil acidification. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they perform symbiotic N_2 fixation (Bordeleau and Prevost, 1994). Acidity is reported to limit both survival and persistence of nodule bacteria in soil, and the process of nodulation itself (Correa and Barneix, 1997). And hence, the failure of legumes to nodulate under acid-soil conditions is common, especially in soils of pH less than 5. The inability of some rhizobia to persist under such conditions is one cause of nodulation failure (Bayoumi et al., 1995), but poor nodulation can occur even where a viable *Rhizobium* population persist (Graham, 1992; Graham et al., 1994). In a study, Evans et al. (1980) found that nodulation of *P. sativum* was 10 times more susceptible to acidity than either rhizobial multiplication or plant growth. Some legumes, for example, *Trifolium subterranean*, *Trifolium balansae*, *Medicago murex* and

Medicago truncatula, showed tolerance to soil acidity as indicated by dry-matter yield; however, the establishment of nodules was more sensitive to soil acidity in most of these plants than was indicated by the relative yields of dry matter (Evans et al., 1990). Despite this, elevated inoculation levels have enhanced the nodulation response under acidic conditions in some studies (Pijnenborg et al., 1991). For example, the growth, nodulation and yield of *Vicia faba* was improved after inoculation with strains of *R. leguminosarum* bv. *viciae* in acid soils (Carter et al., 1994). It appears that the pH-sensitive stage in nodulation occurs early in the infection process and that *Rhizobium* attachment to root hairs is one of the stages affected by acidic conditions in soils (Caetano-Anolles et al., 1989). In another report, Taylor et al. (1991) concluded that acidity had more severe effects on rhizobial multiplication than Al stress and low P conditions. They suggested that colonization of soils and soybean (*Glycine max*) roots by *Bradyrhizobium japonicum* may be adversely affected by acidity which may lead to a decline in nodulation on root systems of tested legumes.

By selection of acid-soil tolerance in both symbiotic partners, annual medics such as *Medicago murex* can be grown symbiotically on soils as acidic as pH 4.3 (Cheng et al., 2002). While the genetic control of acid tolerance in *Sinorhizobium* is becoming increasingly understood (Dilworth et al., 2001), there is little information on the mechanisms contributing to enhanced nodulation at low pH in host species such as *M. murex* in comparison to *M. sativa* (D'Haese and Holsters, 2002). Cheng et al. (2002) reported that the acid-sensitive species of *M. sativa* exhibited delayed nodulation under acid stress relative to the acid-tolerant species of *M. murex*, but that the nodules were eventually formed on both species in the same section of the root.

The establishment of legume symbioses requires the interaction of specific recognition signal molecules produced by both bacterial and plant partners (Denarie et al., 1996). It has been shown that pH affects the exchange or recognition of these signal molecules by both plant and bacterial partners in both the medic symbiosis (Howieson et al., 1992) and clover (*Trifolium pratense*) symbiosis (Howieson et al., 1992). Howieson et al. (1992) noted that root exudates collected at a decreasing pH from acid-tolerant species of *Medicago* resulted in increased *nod* gene induction up to a critical pH which drastically reduced thereafter. Consequently, the root exudates of acid-sensitive species of *Medicago* demonstrated a general reduction in *nod* gene inducing capacity with decreasing pH.

ROLE OF BIOTECHNOLOGIES IN THE IMPROVEMENT OF NITROGEN FIXATION IN SEVERE CONDITIONS

Symbiotic nitrogen-fixing organisms possess the ability to reduce dependence on man-made forms of N fertilizer.

There is a considerable economic incentive to explore ways to increase the efficiency of BNF as a biofertilizer resource. Although, the microsymbionts are usually modified by the recombinant or classic genetic manipulation, the host plant can also be engineered for increased N₂-fixation efficiency, and so, identification and analysis of host plant genes involved in nodule morphogenesis and functioning has lagged behind that of *Rhizobium* (Morris et al., 1999). A wide array of new and explosive molecular tools, like genomics and proteomics, are currently available and they provide plant breeders several new opportunities to create and manipulate genetic variation to produce improved plants more easily (Morris et al., 1999).

In this context, McCardell et al. (1999) reviewed several strategies to improve symbiotic N₂ fixation suggesting that either structure or regulatory genes could be altered to enhance N₂ fixation. In a study, transgenic alfalfa plants transformed with the soybean or pea lectin genes became susceptible to infection by *B. japonicum* (Van et al., 2001). Since *Rhizobium* strains differ in their ability to use opines (Murphy et al., 1995), genetic engineering of legumes or other plants for opine synthesis may result in the enhanced growth of rhizosphere organisms with the ability to utilize this substrate (Oger et al., 1997; Savka and Farrand, 1997). Malate is the primary plant carbon source used by bacteroids, and is also a factor in plant adaptation to P and Al stress (Johnson et al., 1996).

The modification of bacterial strains for synthesis of the peptide antibiotic trifolitoxin is another area of interest. Trifolitoxin produced by some strains of *R. leguminosarum* bv. *trifolii* is toxic to a wide range of gram-negative bacteria including most rhizobia. While a strain of *Rhizobium etli* expressing trifolitoxin (*tfx*) genes was more competitive for nodulation in unsterilized soil than a close isogenic *tfx*⁻ strain (Robledo et al., 1997), the concern could be the transfer of such genes to other less-effective rhizobia in the soil population. Transfer of a chromosomal DNA from the salt-tolerant *Bacillus* species (which grow at 30% NaCl) into a strain of *R. leguminosarum*, was successful (El-Saidi and Ali, 1993). This rhizobia strain became salt tolerant and grew at about 10 to 15% NaCl. Further, inoculation of lentil, grown in the reclaimed desert soil by this salt tolerant strain of *Rhizobium* improved plant yield and the nitrogen content of soil and plant.

One of the most important and interesting strategy of dealing with salt tolerance in leguminous plants could be the identification and cloning of genes regulating salt stress. However, some of these genes have been cloned in the model of legume *Arabidopsis thaliana* (Shi et al., 2000; Venema et al., 2002), because they encode putative Na⁺/H⁺ antiporters. In saline environments, plants accumulate Na⁺ in vacuoles throughout the activity of tonoplast Na⁺/H⁺ antiporters. The first gene for a putative plant vacuolar Na⁺/H⁺ antiporter (AtNHX1) was isolated from *Arabidopsis* and shown to increase plant

tolerance to NaCl (Venema et al., 2002). Other species are highly specific in their requirements. If nodules do not occur, an appropriate strain of *Rhizobium* inoculum is frequently added to the seed, sometimes as a fine cap or pellet. It has been reported that the introduction of target indigenous species of plants, associated with a managed community of microbial symbionts, is a successful biotechnological tool to aid the recovery of desertified ecosystems (Requena et al., 2001).

CONCLUSION

Biological nitrogen fixation is an important process for providing nitrogen inexpensively to legumes in farming system for increasing the productivity of crops. The BNF system such as rhizobia and legume adapts well under different ecological conditions and fixes considerable amounts of nitrogen. However, under certain adverse environmental situation, the actual impact of rhizobia on legumes is not realized. Therefore, there is an increasing demand for identifying rhizobial species that could also work under stressed soil environment so that the productivity of the inoculated legumes does not suffer under derelict soils.

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