

Rhizobium-Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in an Arid Climate

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THE NITROGEN FIXATION PROCESS

The element nitrogen, or “azote,” meaning “without life,” as Antoine Lavoisier called it about 200 years ago, has proved to be anything but lifeless, since it is a component of food, poisons, fertilizers, and explosives (277). The atmosphere contains about 10¹⁵ tonnes of N₂ gas, and the nitrogen cycle involves the transformation of some 3 × 10⁹ tonnes of N₂ per year on a global basis (244). However, transformations (e.g., N₂ fixation) are not exclusively biological. Lightning probably accounts for about 10% of the world’s supply of fixed nitrogen (301). The fertilizer industry also provides very important quantities of chemically fixed nitrogen. World production of fixed nitrogen from dinitrogen for chemical fertilizer accounts for about 25% of the Earth’s newly fixed N₂, and biological processes account for about 60%. Globally the consumption of fertilizer-N increased from 8 to 17 kg ha⁻¹ of agricultural land in the 15-year period from 1973 to 1988 (107). Significant growth in fertilizer-N usage has occurred in both developed and developing countries (238). The requirements for fertilizer-N are predicted to increase further in the future (306); however, with the current technology for fertilizer production and the inefficient methods employed for fertilizer application, both the economic and ecological costs of fertilizer usage will eventually become prohibitive.

For more than 100 years, biological nitrogen fixation (BNF)

has commanded the attention of scientists concerned with plant mineral nutrition, and it has been exploited extensively in agricultural practice (50, 91). However, its importance as a primary source of N for agriculture has diminished in recent decades as increasing amounts of fertilizer-N have been used for the production of food and cash crops (238). However, international emphasis on environmentally sustainable development with the use of renewable resources is likely to focus attention on the potential role of BNF in supplying N for agriculture (91, 238). The expanded interest in ecology has drawn attention to the fact that BNF is ecologically benign and that its greater exploitation can reduce the use of fossil fuels and can be helpful in reforestation and in restoration of misused lands to productivity (50, 301).

Currently, the subject of BNF is of great practical importance because the use of nitrogenous fertilizers has resulted in unacceptable levels of water pollution (increasing concentrations of toxic nitrates in drinking water supplies) and the eutrophication of lakes and rivers (19, 91, 301). Further, while BNF may be tailored to the needs of the organism, fertilizer is usually applied in a few large doses, up to 50% of which may be leached (301). This not only wastes energy and money but also leads to serious pollution problems, particularly in water supplies.

NITROGEN-FIXING ORGANISMS

Organisms that can fix nitrogen, i.e., convert the stable nitrogen gas in the atmosphere into a biologically useful form, all

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belong to a biological group known as prokaryotes. All organisms which reduce dinitrogen to ammonia do so with the aid of an enzyme complex, nitrogenase. The nitrogenase enzymes are irreversibly inactivated by oxygen, and the process of nitrogen fixation uses a large amount of energy (91, 244). Nitrogenase activity is usually measured by the acetylene reduction assay, which is cheap and sensitive (91, 141, 301). 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; 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 (91, 301, 361). This wide variety of diazotrophs ensures that most ecological niches will contain one or two representatives and that lost nitrogen can be replenished.

SIGNIFICANCE OF BIOLOGICAL N_2 FIXATION TO SOIL FERTILITY

BNF is an efficient source of nitrogen (238). The total annual terrestrial inputs of N from BNF as given by Burns and Hardy (49) and Paul (235) range from 139 million to 175 million tonnes of N, with symbiotic associations growing in arable land accounting for 25 to 30% (35 million to 44 million tons of N) and permanent pasture accounting for another 30% (45 million tons of N). While the accuracy of these figures may be open to question (301), they do help illustrate the relative importance of BNF in cropping and pasture systems and the magnitude of the task necessary if BNF is to be improved to replace a proportion of the 80 to 90 million tonnes of fertilizer-N expected to be applied annually to agricultural land by the end of the decade (238, 239). 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 (50). BNF can play a key role in land remediation.

An examination of the history of BNF shows that interest generally has focused on the symbiotic system of leguminous plants and rhizobia, because these associations have the greatest quantitative impact on the nitrogen cycle. A tremendous potential for contribution of fixed nitrogen to soil ecosystems exists among the legumes (46, 238, 313). There are approximately 700 genera and about 13,000 species of legumes, only a portion of which (about 20% [301]) have been examined for nodulation and shown to have the ability to fix N_2 . Estimates are that the rhizobial symbioses with the somewhat greater than 100 agriculturally important legumes contribute nearly half the annual quantity of BNF entering soil ecosystems (313). Legumes are very important both ecologically and agriculturally because they are responsible for a substantial part of the global flux of nitrogen from atmospheric N_2 to fixed forms such as ammonia, nitrate, and organic nitrogen. Whatever the true figure, legume symbioses contribute at least 70 million tonnes of N per year, approximately half deriving from the cool and warm temperature zones and the remainder deriving from the tropics (46). Increased plant protein levels and reduced depletion of soil N reserves are obvious consequences of legume N_2 fixation. Deficiency in mineral nitrogen often limits plant growth, and so symbiotic relationships have evolved between plants and a variety of nitrogen-fixing organisms (116).

Most of the attention in this review is directed toward N_2

fixation inputs by legumes because of their proven ability to fix N_2 and their contribution to integral agricultural production systems in both tropical and temperate climates (238). Successful *Rhizobium*-legume symbioses will definitely increase the incorporation of BNF into soil ecosystems. *Rhizobium*-legume symbioses are the primary source of fixed nitrogen in land-based systems (313) and can provide well over half of the biological source of fixed nitrogen (313).

Atmospheric N_2 fixed symbiotically by the association between *Rhizobium* species and legumes represents a renewable source of N for agriculture (239). Values estimated for various legume crops and pasture species are often impressive, commonly falling in the range of 200 to 300 kg of N ha^{-1} year $^{-1}$ (238). Yield increases of crops planted after harvesting of 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 were estimated to be about 65 to 335 kg of N ha^{-1} year $^{-1}$ (313) or 23 to 300 kg of N ha^{-1} year $^{-1}$ (339). However, the measured amounts of N fixed by symbiotic systems may differ according to the method used to study N_2 fixation (279). Inputs into terrestrial ecosystems of BNF from the symbiotic relationship between legumes and their rhizobia amount to at least 70 million tons of N per year (46); this enormous quantity will have to be augmented as the world's population increases and as the natural resources that supply fertilizer-N diminish. This objective will be achieved through the development of superior legume varieties, improvements in agronomic practice, and increased efficiency of the nitrogen-fixing process itself by better management of the symbiotic relationship between plants and bacteria.

The symbioses between *Rhizobium* or *Bradyrhizobium* and legumes are a cheaper and usually more effective agronomic practice for ensuring an adequate supply of N for legume-based crop and pasture production than the application of fertilizer-N. The introduction of legumes into these pastures is seen as the best strategy to improve nitrogen nutrition of the grasses. Large contributions (between 75 and 97 kg of N ha^{-1} in 97 days of growth) by *Stylosanthes guianensis* were found (333). ^{15}N data suggested that over 30% of the N accumulated by the grass in mixed swards could be derived from nitrogen fixed by the associated legume (333). Other recent studies (199) revealed that the nitrogen contribution of *Arachis hypogaea* to the growth of *Zea mays* in intercropping systems is equivalent to the application of 96 kg of fertilizer-N ha^{-1} at a ratio of plant population densities of one maize plant to four groundnut plants.

Actinorhizal interactions (*Frankia*-nonlegume symbioses) are major contributors to nitrogen inputs in forests, wetlands, fields, and disturbed sites of temperate and tropical regions (313). These associations involve more than 160 species of angiosperms classified among six or seven orders. The contributions of fixed nitrogen to native as well as managed ecosystems by the actinorhizal symbioses are comparable to those of the more extensively studied *Rhizobium*-legume interactions. Typical contributions by *Alnus* associations are 12 to 200 kg of N ha^{-1} year $^{-1}$, and those by *Hippophae* associations are 27 to 179 kg of N ha^{-1} year $^{-1}$ (27).

The above overview clearly indicates the significance of *Rhizobium*-legume symbioses as a major contributors to natural or biological N_2 fixation. Therefore, the following discussion centers on the behavior of these symbioses under severe environmental conditions and also for applications in arid regions.

EFFECTS OF SEVERE CONDITIONS ON NITROGEN FIXATION

Environmental Conditions

Several environmental conditions are limiting factors to the growth and activity of the N₂-fixing plants. A principle of limiting factors states that "the level of crop production can be no higher than that allowed by the maximum limiting factor" (46). In the *Rhizobium*-legume symbiosis, which is a N₂-fixing system, the process of N₂ fixation is strongly related to the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors (e.g., salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases, and grazing) impose limitations on the vigor of the host legume (46, 239, 315).

Typical environmental stresses faced by the legume nodules and their symbiotic partner (*Rhizobium*) may include photosynthate deprivation, water stress, salinity, soil nitrate, temperature, heavy metals, and biocides (337). A given stress may also have more than one effect: e.g., salinity may act as a water stress, which affects the photosynthetic rate, or may affect nodule metabolism directly. The most problematic environments for rhizobia are marginal lands with low rainfall, extremes of temperature, acidic soils of low nutrient status, and poor water-holding capacity (44). Populations of *Rhizobium* and *Bradyrhizobium* species vary in their tolerance to major environmental factors; consequently, screening for tolerant strains has been pursued (176). Biological processes (e.g., N₂ fixation) capable of improving agricultural productivity while minimizing soil loss and ameliorating adverse edaphic conditions are essential.

Salt and Osmotic Stresses

Salinity is a serious threat to agriculture in arid and semiarid regions (252). Nearly 40% of the world's land surface can be categorized as having potential salinity problems (69); most of these areas are confined to the tropics and Mediterranean regions. Increases in the salinity of soils or water supplies used for irrigation result in decreased productivity of most crop plants and lead to marked changes in the growth pattern of plants (69). Increasing salt concentrations may have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress (313). Soil infertility in arid zones is often due to the presence of large quantities of salt, and the introduction of plants capable of surviving under these conditions (salt-tolerant plants) is worth investigating (86). There is currently a need to develop highly salt-tolerant crops to recycle agricultural drainage waters, which are literally rivers of contaminated water that are generated in arid-zone irrigation districts (129). Salt tolerance in plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes. Salinity decreases plant growth and yield, depending upon the plant species, salinity levels, and ionic composition of the salts (86).

As with most cultivated crops, the salinity response of legumes varies greatly and depends on such factors as climatic conditions, soil properties, and the stage of growth (70–72). Variability in salt tolerance among crop legumes has been reported (353, 354). Some legumes, e.g., *Vicia faba*, *Phaseolus vulgaris*, and *Glycine max*, are more salt tolerant than others, e.g., *Pisum sativum*. It has been reported that some *V. faba* tolerant lines sustained nitrogen fixation under saline condi-

tions (6, 72). Other legumes, such as *Prosopis* (105), *Acacia* (367), and *Medicago sativa* (7), are salt tolerant, but these legume hosts are less tolerant to salt than are their rhizobia.

The legume-*Rhizobium* symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia (98, 330, 354, 365). Salt stress inhibits the initial steps of *Rhizobium*-legume symbioses. Soybean root hairs showed little curling or deformation when inoculated with *Bradyrhizobium japonicum* in the presence of 170 mM NaCl, and nodulation was completely suppressed by 210 mM NaCl (323). Bacterial colonization and root hair curling of *V. faba* were reduced in the presence of 50 to 100 mM NaCl or 100 to 200 mM polyethylene glycol as osmoticum (352, 365); the proportion of root hairs containing infection threads was reduced by 30 and 52% in the presence of NaCl and polyethylene glycol, respectively. The effects of salt stress on nodulation and nitrogen fixation of legumes have been examined in several studies (6, 9, 86, 98, 159, 223, 330, 352). The reduction of N₂-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules (86, 159, 337) and a reduction in cytosolic protein production, specifically leghemoglobin, by nodules (85, 86). The depressive effect of salt stress on N₂ fixation by legumes is directly related to the salt-induced decline in dry weight and N content in the shoot (72). The salt-induced distortions in nodule structure could also be reasons for the decline in the N₂ fixation rate by legumes subject to salt stress (302, 352, 360). Reduction in photosynthetic activity might also affect N₂ fixation by legumes under salt stress (122).

Although the root nodule-colonizing bacteria of the genera *Rhizobium* and *Bradyrhizobium* are more salt tolerant than their legume hosts, they show marked variation in salt tolerance. Growth of a number of rhizobia was inhibited by 100 mM NaCl (350), while some rhizobia, e.g., *Rhizobium meliloti*, were tolerant to 300 to 700 mM NaCl (99, 146, 214, 272). Strains of *Rhizobium leguminosarum* have been reported to be tolerant to NaCl concentrations up to 350 mM NaCl in broth culture (5, 45). Soybean and chickpea rhizobia were tolerant to 340 mM NaCl, with fast-growing strains being more tolerant than slow-growing strains (96). *Rhizobium* strains from *Vigna unguiculata* were tolerant to NaCl up to 5.5%, which is equivalent to about 450 mM NaCl (216). It has been found recently that the slow-growing peanut rhizobia are less tolerant than fast-growing rhizobia (124). Rhizobia from woody legumes also showed substantial salt tolerance: strains from *Acacia*, *Prosopis*, and *Leucaena* are tolerant to 500 to 850 mM NaCl (188, 364, 367). In addition to NaCl, MgCl and chlorides are more toxic than sulfates (96). It has been reported (167) that the growth of *R. meliloti* was severely inhibited by Mg²⁺ ions, whereas Na⁺ and K⁺ ions had little inhibitory effect.

Many species of bacteria adapt to saline conditions by the intracellular accumulation of low-molecular-weight organic solutes called osmolytes (77). The accumulation of osmolytes is thought to counteract the dehydration effect of low water activity in the medium but not to interfere with macromolecular structure or function (292). Rhizobia utilize this mechanism of osmotic adaptation (42, 43, 292, 295, 362). In the presence of high levels of salt (up to 300 to 400 mM NaCl), the levels of intracellular free glutamate and/or K⁺ were greatly increased (sometimes up to sixfold in a few minutes) in cells of *R. meliloti* (43, 167, 189), *R. fredii* (118, 119, 350), *Sinorhizobium fredii* (307), and rhizobia from the woody legume *Leucaena leucocephala* (349), K⁺ strictly controls Mg²⁺ flux during osmotic shock. An osmolyte, *N*-acetylglutaminyl-glutamine amide,

accumulates in cells of *R. meliloti* (292, 294, 295); the accumulation of these osmolytes is dependent on the level of osmotic stress, the growth phase of the culture, the carbon source, and the presence of osmolytes in the growth medium.

The disaccharide trehalose plays a role in osmoregulation when rhizobia are growing under salt or osmotic stress (96, 151). Trehalose accumulates to higher levels in cells of *R. leguminosarum* (45) and peanut rhizobia (124) under the increasing osmotic pressure of hypersalinity. Fast-growing peanut rhizobia accumulate trehalose in the presence of many carbon sources (mannitol, sucrose, or lactose), but the slow growers accumulate trehalose only when cultured with mannitol as the carbon source. In a medium supplemented with 400 mM NaCl, the content of trehalose increased intracellularly throughout the logarithmic and stationary phases of growth of peanut rhizobia (123). The disaccharides sucrose and ectoine were used as osmoprotectants for *Sinorhizobium meliloti* (132). However, these compounds, unlike other bacterial osmoprotectants, do not accumulate as cytosolic osmolytes in salt-stressed *S. meliloti* cells.

One salt or osmotic stress response already identified in rhizobia is the intracellular accumulation of glycine betaine (189, 272, 293). The concentration of glycine betaine increases more in the salt-tolerant strains of *R. meliloti* than in sensitive strains (189, 293). The addition of sodium salts to bacteroids of *Medicago sativa* nodules increased the uptake activity of the exogenously added glycine betaine (113). These osmoprotective substances may play a significant role in the maintenance of nitrogenase activity in bacteroids under salt stress. When externally provided, glycine betaine and choline enhance the growth of *Rhizobium tropici*, *S. meliloti*, *S. fredii*, *R. galegae*, *Mesorhizobium loti*, *M. huakuii*, and *Agrobacterium tumefaciens* (40). However, the main physiological role of glycine betaine in the family *Rhizobiaceae* seems to be as an energy source, while its contribution to osmoprotection is restricted to certain strains. Another osmoprotectant, ectoine, was as effective as glycine betaine in improving the growth of *R. meliloti* under adverse (0.5 M NaCl) osmotic conditions (308). Ectoine does not accumulate intracellularly and therefore would not repress the synthesis of endogenous compatible solutes such as glutamate and trehalose; it may play a key role in triggering the synthesis of endogenous osmolytes (308). Therefore, at least two distinct classes of osmoprotectants exist: those such as glycine betaine or glutamate, which act as genuine osmolytes, and those such as ectoine, which act as chemical mediators.

The content of polyamines, e.g., homospermidine, increases in salt-tolerant cells and acid-tolerant strains of *R. fredii* (118). This polyamine may function to maintain the intracellular pH and repair the ionic imbalance caused by osmotic stress. Osmotic stress (shock) results in the formation of specific proteins in bacteria. Botsford (42) reported that the production of 41 proteins was increased at least 10-fold in salt-stressed cells of *Escherichia coli*. The formation of osmotic shock proteins was only recently found in cells of rhizobia. Zahran et al. (364) reported the appearance of new protein bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of rhizobia from woody legumes grown under salt stress. The synthesis pattern of proteins and amino acids (free or total) changes in cowpea rhizobia after high-salt (10% NaCl = 1.64 M NaCl) stress treatment (362, 365a). The Na⁺, K⁺, and Mg²⁺ concentrations are increased in cells of cowpea *Rhizobium* under salt stress. These organic osmolytes (amino acids) and the inorganic minerals (cations) may play a role in osmoregulation for this *Rhizobium* strain. Zahran et al. (362) extended their work on this halotolerant strain of cowpea *Rhizobium* and examined its cell morphology and ultrastructure

under salt stress (1.64 M NaCl). The rhizobial cells responded to high-salt stress by changing their morphology: the cells appeared as spiral or filament-like structures, and the cell size greatly expanded. The cell ultrastructure was severely affected, the cell envelope was distorted, and the homogeneous cytoplasm was disrupted. It has been reported (51) that cells of a strain of *R. meliloti* appeared with irregular morphology at potentials below -0.5 MPa. Strains of rhizobia from different species modified their morphology under salt stress, and rhizobia with altered morphology have been isolated from salt-affected soils in Egypt (363). High osmotic stress (0.2 to 1.44 MPa) modified the synthesis pattern of extracellular and capsular polysaccharides of *R. leguminosarum* bv. trifolii (45). The colonies of *R. meliloti* EFB1 grown in the presence of 0.3 M NaCl show a decrease in mucoidy, and in salt-supplemented liquid medium this organism produces a 40% lower level of exopolysaccharides (193). The synthesis pattern in SDS-PAGE of lipopolysaccharides (LPS) from various species of rhizobia from cultivated legumes (355) and from woody legumes (364) was modified by salt, in the presence of which the length of side chains increased. Changing the surface antigenic polysaccharide and LPS, by salt stress, might impair the *Rhizobium*-legume interaction. LPS are very important for the development of root nodules (38, 312).

Successful *Rhizobium*-legume symbioses under salt stress require the selection of salt-tolerant rhizobia from those indigenous to saline soils (354). *Rhizobium* strains isolated from salt-affected soils in Egypt failed to nodulate their legume host under saline and nonsaline conditions (359a). These rhizobia showed alterations in their protein and LPS patterns (355). The genetic structure of these bacteria may also be changed (356) since they showed little DNA-DNA hybridization to reference rhizobia. The *Rhizobium* strains that are best able to form effective symbiosis with their host legumes at high salinity levels are not necessarily derived from saline soils (305). Graham (133) reported that salt-tolerant strains of rhizobia represent only a small percentage of all strains isolated and identified; therefore, further research in selecting salt-tolerant and effective strains of rhizobia is strongly recommended. In fact, and as indicated in recent reports, some strains of salt-tolerant rhizobia are able to establish effective symbiosis, while others formed ineffective symbiosis. Isolates of *R. leguminosarum* from the lentil-growing regions of the Southern Nile Valley of Egypt were salt tolerant but were not effective in N₂ fixation (212). Mutant strains of *R. leguminosarum* bv. viciae, which grow at 200 mM NaCl, formed ineffective nodules on roots of *V. faba*. These nodules failed to express nitrogenase activity (63). Some strains of *Rhizobium* tolerated extremely high levels of salt (up to 1.88 M NaCl) but showed significantly decreased symbiotic efficiency under salt stress (223).

Inoculation of legumes by salt-tolerant strains of *R. leguminosarum* bv. trifolii and *R. meliloti* enhanced nodulation and N content under salt stress up to 1% NaCl (95). Salt-tolerant strains isolated from *Acacia redolens*, growing in saline areas of Australia, produced effective nodules on both *A. redolens* and *A. cyclops* grown in sand at salinity levels up to 80 mM NaCl (75). The growth, nodulation, and N₂ fixation (N content) of *Acacia ampliceps*, inoculated with salt-tolerant *Rhizobium* strains in sand culture, were resistant to salt levels up to 200 mM NaCl (370). Under saline conditions, the salt-tolerant strains of *Rhizobium* sp. formed more effective N₂-fixing symbiosis with soybean than did the salt-sensitive strains (97). An important result was obtained from the recent work of Lal and Khanna (188), who showed that the rhizobia isolated from *Acacia nilotica* in different agroclimatic zones, which were tolerant to 850 mM NaCl, formed effective N₂-fixing nodules on

Acacia trees grown at 150 mM NaCl. It was concluded from these results that salt-tolerant strains of *Rhizobium* can nodulate legumes and form effective N₂-fixing symbioses in soils with moderate salinity. Therefore, inoculation of various legumes with salt-tolerant strains of rhizobia will improve N₂ fixation in saline environments (370). However, tolerance of the legume host to salt is the most important factor in determining the success of compatible *Rhizobium* strains to form successful symbiosis under conditions of high soil salinity (75). Evidence presented in the literature suggests a need to select plant genotypes that are tolerant to salt stress and then match them with the salt-tolerant and effective strain of rhizobia (70, 329). In fact, the best results for symbiotic N₂ fixation under salt stress are obtained if both symbiotic partners and all the different steps in their interaction (nodule formation, activity, etc.) resist such stress (34, 122, 364).

The use of actinorhizal associations to improve N₂ fixation in saline environments was also studied but not as extensively as *Rhizobium*-legume associations. One of these actinorhizal associations (*Frankia-Casuarina*) is known to operate in dry climates and saline lands and was reported to be tolerant to salt up to 250 to 500 mM NaCl (67, 94). *Casuarina obesa* plants are highly salt tolerant (254), but growth under saline conditions depends on the effectiveness of symbiotic N₂ fixation. Successful plantings of *Casuarina* in saline environments require the selection of salt-tolerant *Frankia* strains to form effective N₂-fixing association.

Soil Moisture Deficiency

The occurrence of rhizobial populations in desert soils and the effective nodulation of legumes growing therein (164, 165, 336) 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 to increase as the moisture stress is relieved (313). It is well known that some free-living rhizobia (saprophytic) are capable of survival under drought stress or low water potential (117). A strain of *Prosopis* (mesquite) rhizobia isolated from the desert soil survived in desert soil for 1 month, whereas a commercial strain was unable to survive under these conditions (284). The survival of a strain of *Bradyrhizobium* from *Cajanus* in a sandy loam soil was very poor; this strain did not persist to the next cropping season, when the moisture content was about 2.0 to 15.5%. The survival and activity of microorganisms may depend on their distribution among microhabitats and changes in soil moisture (231). The distribution of *R. leguminosarum* in a loamy sand and silt loam soil was influenced by the initial moisture content (245). Moderate moisture tension slowed the movement of *R. trifolii* (139); the migration of bacteria ceased when water-filled pores in soil became discontinuous as a result of water stress. The migration of strains of *B. japonicum* from the initial point of placement was found to be very limited (335); the effective strains migrated into the soil to a greater extent than the ineffective strains did.

One of the immediate responses of rhizobia to water stress (low water potential) concerns the morphological changes. Mesquite *Rhizobium* (284) and *R. meliloti* (51) showed irregular morphology at low water potential. The modification of rhizobial cells by water stress will eventually lead to a reduction in infection and nodulation of legumes. Low water content in soil was suggested to be involved in the lack of success of soybean inoculation in soils with a high indigenous population of *R. japonicum* (156). Further, a reduction in the soil moisture from 5.5 to 3.5% significantly decreased the number of infection threads formed inside root hairs and completely inhibited

the nodulation of *T. subterraneum* (345). Similarly, water deficit, simulated with polyethylene glycol, significantly reduced infection thread formation and nodulation of *Vicia faba* plants (352, 365). A favorable rhizosphere environment is vital to legume-*Rhizobium* interaction; however, the magnitude of the stress effects and the rate of inhibition of the symbiosis usually depend on the phase of growth and development, as well as the severity of the stress. For example, mild water stress reduces only the number of nodules formed on roots of soybean, while moderate and severe water stress reduces both the number and size of nodules (342).

Symbiotic N₂ fixation of legumes is also highly sensitive to soil water deficiency. A number of temperate and tropical legumes, e.g., *Medicago sativa* (7, 21), *Pisum sativum* (5), *Arachis hypogaea* (286), *Vicia faba* (5, 138, 365), *Glycine max* (89, 179, 251), *Vigna* sp. (233, 331), *Aeschynomene* (15), and the shrub legume *Adenocarpus decorticans* (215) exhibit a reduction in nitrogen fixation when subject to soil moisture deficit. Soil moisture deficiency has a pronounced effect on N₂ fixation because nodule initiation, growth, and activity are all more sensitive to water stress than are general root and shoot metabolism (14, 365). The response of nodulation and N₂ fixation to water stress depends on the growth stage of the plants. It was found that water stress imposed during vegetative growth was more detrimental to nodulation and nitrogen fixation than that imposed during the reproduction stage (236). There was little chance for recovery from water stress in the reproductive stage. Nodule P concentrations and P use efficiency declined linearly with soil and root water content during the harvest period of soybean-*Bradyrhizobium* symbiosis (115). More recently, Sellstedt et al. (279) found that N derived from N₂ fixation was decreased by about 26% as a result of water deficiency when measured by the acetylene reduction assay.

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. Laboratory studies have shown that sensitivity to moisture stress varies for a variety of rhizobial strains, e.g., *R. leguminosarum* bv. *trifolii* (117), *R. meliloti* (51), cowpea rhizobia (232), and *B. japonicum* (196). Thus, we can reasonably assume that rhizobial strains can be selected with moisture stress tolerance within the range of their legume host. Optimization of soil moisture for growth of the host plant, which is generally more sensitive to moisture stress than bacteria, results in maximal development of fixed-nitrogen inputs into the soil system by the *Rhizobium*-legume symbiosis (313).

Drought-tolerant, N₂-fixing legumes can be selected, although the majority of legumes are sensitive to drought stress. Moisture stress had little or no effect on N₂ fixation by some forage crop legumes, e.g., *M. sativa* (175), grain legumes, e.g., groundnut (*Arachis hypogaea*) (331), and some tropical legumes, e.g., *Desmodium intortum* (13). One legume, guar (*Cyamopsis tetragonoloba*), is drought tolerant and is known to be adapted to the conditions prevailing in arid regions (332). Variability in nitrogen fixation under drought stress was found among genotypes of *Vigna radiata* (248) and *Trifolium repens* (262). These results assume a significant role of N₂-fixing *Rhizobium*-legume symbioses in the improvement of soil fertility in arid and semiarid habitats.

Several mechanisms have been suggested to explain the varied physiological responses of several legumes to water stress. The legumes with a high tolerance to water stress usually exhibit osmotic adjustment; this adjustment is partly accounted for by changing cell turgor and by accumulation of some osmotically active solutes (112). The accumulation of specific organic solutes (osmotica) is a characteristic response of plants

subject to prolonged severe water stress. One of these solutes is proline, which accumulates in different legumes, e.g., *Glycine max* (120) and *Phaseolus vulgaris* (172). In these plants, positive correlations were found between proline accumulation and drought tolerance. Other compounds, e.g., the free amino acids and low-molecular-weight solutes such as pinitol (*o*-methylinositol), accumulate in several tropical legumes under drought stress (112, 185). Potassium is known to improve the resistance of plants to environmental stress. A recent report (269) indicates that K can apparently alleviate the effects of water shortage on symbiotic N₂ fixation of *V. faba* and *P. vulgaris*. The presence of 0.8 or 0.3 mM K⁺ allowed nodulation and subsequent nitrogen fixation of *V. faba* and *P. vulgaris* under a high-water regimen (field capacity to 25% depletion). It was also shown that the symbiotic system in these legumes is less tolerant to limiting K supply than are the plants themselves. Species of legumes vary in the type and quantity of the organic solutes which accumulate intracellularly in leguminous plants under water stress. This could be a criterion for selecting drought-tolerant legume-*Rhizobium* symbioses that are able to adapt to arid climates.

High Temperature and Heat Stress

High soil temperatures in tropical and subtropical areas are a major problem for biological nitrogen fixation of legume crops (210). High root temperatures strongly affect bacterial infection and N₂ fixation in several legume species, including soybean (218), guar (22), peanut (180), cowpea (249), and beans (154, 241). Critical temperatures for N₂ fixation are 30°C for clover and pea and range between 35 and 40°C for soybean, guar, peanut, and cowpea (210). Nodule functioning in common beans (*Phaseolus* spp.) is optimal between 25 and 30°C and is hampered by root temperatures between 30 and 33°C (241). Nodulation and symbiotic nitrogen fixation depend on the nodulating strain in addition to the plant cultivar (22, 218). Temperature affects root hair infection, bacteroid differentiation, nodule structure, and the functioning of the legume root nodule (265, 266).

High (not extreme) soil temperatures will delay nodulation or restrict it to the subsurface region (133). Munns et al. (221) found that alfalfa plants grown in desert environments in California maintained few nodules in the top 5 cm of soil but were extensively nodulated below this depth. Nodulation of soybean was markedly inhibited at 42 and 45°C during 12-h and 9-h days, respectively (186), with no correlation between the ability of plant strains to grow at high temperature and to induce nodulation under temperature stress. Piha and Munns (241) reported that bean nodules formed at 35°C were small and had low specific nitrogenase activity, and Hernandez-Armenta et al. (148) found that transferring nodulated bean plants from a daily temperature of 26 to 35°C markedly inhibited nitrogen fixation. Some soybean varieties appear somewhat more heat tolerant, with nitrogen fixation being severely inhibited only by daytime temperatures higher than 41°C (187). The acetylene reduction activity of nodulated roots excised from unstressed bean plants (*Phaseolus*) was strongly diminished at 35 or 40°C when plants were nodulated by heat-sensitive or heat-tolerant strains (210).

For most rhizobia, the optimum temperature range for growth in culture is 28 to 31°C, and many are unable to grow at 37°C (133). However, 90% of cowpea *Rhizobium* strains obtained from the hot, dry environment of the Sahel Savannah grew well at 40°C (93). Strain adaptation to high temperature has also been reported by Hartel and Alexander (144) and Karanja and Wood (173). The latter authors found that a high

percentage of the strains that persisted at 45°C lost their infectiveness. They attributed these losses in infectiveness to plasmid curing. Heat treatment of *R. phaseoli* at 35 and 37°C resulted in mutant strains lacking a plasmid DNA implicated in the synthesis of melanin and is related to the loss of symbiotic properties of these bacteria (36). Screening of *R. leguminosarum* bv. *phaseoli* showed that some strains were able to nodulate *Phaseolus vulgaris* at high temperatures (35 and 38°C) but that the nodules formed at high temperatures were ineffective and plants did not accumulate N in shoots (154).

Rhizobial survival in soil exposed to high temperature is greater in soil aggregates than in nonaggregated soil and is favored by dry rather than moist conditions (133). Ten inoculant strains of *Rhizobium* spp. examined by Somasegaran et al. (297) showed a gradual decline in population during 8 weeks of incubation at 37°C, while exposure to 46°C was lethal to all strains in less than 2 weeks. A decrease in the infectivity of cowpea rhizobia following storage at 35°C has also been documented (343). High soil temperature could contribute to the frequency of noninfective isolates in soil; Segovia et al. (278) noted that such noninfective isolates actually outnumbered those that were infective in the rhizosphere of bean. *R. leguminosarum* isolates from lentil plants in the Southern Nile Valley of Egypt were tolerant to 35 to 40°C; however, these heat-tolerant rhizobia formed less effective symbiosis with their legume hosts (212). Several heat-tolerant N₂-fixing bean-nodulating *Rhizobium* strains (which grow at 40°C) have been described recently (155, 210).

Heat shock proteins have been found in *Rhizobium* (1) but have not been studied in detail (133). The synthesis of heat shock proteins was detected in both heat-tolerant and heat-sensitive bean-nodulating *Rhizobium* strains (210) at different temperatures. An increased synthesis of 14 heat shock proteins in heat-sensitive strains and of 6 heat shock proteins in heat-tolerant strains was observed at 40 and 45°C, respectively (210). Heat-tolerant rhizobia are likely to be found in environments affected by temperature stress. Rhizobia isolated from the root nodules of *Acacia senegal* and *Prosopis chilensis*, growing in hot, dry regions of Sudan, had high maximum growth temperatures (44.2°C) (364, 367). Heat stress (35 and 40°C) changed the pattern of LPS mobility of some strains of tree rhizobia, as shown by SDS-PAGE (364). The same authors found that temperature stress consistently promoted the production of a protein with a relative mobility of 65 kDa in four strains of tree legume rhizobia. The 65-kDa protein that was detected under heat stress was heavily overproduced. This protein was not overproduced during salt or osmotic stress (364), which indicates that it is a specific response to heat stress.

Soil Acidity and Alkalinity

Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity (41, 65, 73, 133). Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic N₂ fixation (41, 47). It has been recently reported (207, 309) that pasture and grain legumes acidify soil to a greater extent and that the legume species differ in their capacity to produce acids. Legumes and their rhizobia exhibit varied responses to acidity. Some species, like lucerne (*M. sativa*), are extremely sensitive to acidity, while others, such as *Lotus tenuis*, tolerate relatively low soil pH (73). Soil acidity constrains symbiotic N₂ fixation in both tropical and temperate soils (220), limiting *Rhizobium* survival and persistence in soils and reducing nodulation (47, 136, 157). Rhizobia with a higher

tolerance to acidity have been identified (136). These strains usually but not always perform better under acidic soil conditions in the field (134). It has been found that *R. loti* multiplied at pH 4.5 but *Bradyrhizobium* strains failed to multiply (68); the acid-tolerant strains of *R. loti* demonstrate a comparative advantage over acid-sensitive strains in the ability to nodulate their host legume at pH 4.5. *R. tropici* and *R. loti* are moderately acid tolerant (344), while *R. meliloti* is very sensitive to acid stress (47, 318). However, *R. meliloti* WSM 419 has recently been shown (65) to perform satisfactorily in the field in acidic soils (pH 5.0 to 5.5). Strains of a given species vary widely in certain cases in their pH tolerance. The fast-growing strains of rhizobia have generally been considered less tolerant to acid pH than have slowly growing strains of *Bradyrhizobium* (134), although some strains of the fast-growing rhizobia, e.g., *R. loti* and *R. tropici*, are highly acid tolerant (68, 78, 134, 344). Recent reports, however, support the existence of acid-tolerant fast-growing strains, since both fast- and slow-growing strains of *Vigna unguiculata* which are tolerant to pH values as low as 4.0 have been isolated (216). The basis for differences in pH tolerance among strains of *Rhizobium* and *Bradyrhizobium* is still not clear (73, 134), although several workers have shown that the cytoplasmic pH of acid-tolerant strains is less strongly affected by external acidity (60, 62, 131, 230). Aarons and Graham (1) reported high cytoplasmic potassium and glutamate levels in acid-stressed cells of *R. leguminosarum* bv. *phaseoli*, a response which is similar to that found in osmotically stressed cells. Differences in LPS composition, proton exclusion and extrusion (60, 62), accumulation of cellular polyamines (118), and synthesis of acid shock proteins (150) have been associated with the growth of cells at acid pH. The composition and structure of the outer membrane could also be a factor in pH tolerance (134). Studies on the genetic basis of tolerance to low pH suggest that at least two loci of either megaplasmid or chromosomal location for pH genes are necessary for the growth of rhizobia at low pH (60–62). Acid tolerance in *R. loti* (73) was related to the composition and structure of the membrane, and acid-tolerant strains showed one membrane protein of 49.5 kDa and three soluble proteins of 66.0, 85.0, and 44.0 kDa. The expression of these proteins increased when the cells were grown at pH 4.0. The same authors (73) suggested that acid tolerance in *R. loti* involves constitutive mechanisms, such as permeability of the outer membrane, together with adaptive responses, including the state of bacterial growth and concomitant changes in protein expression.

The failure of legumes to nodulate under acid-soil conditions is common, especially in soils of pH less than 5.0. The inability of some rhizobia to persist under such conditions is one cause of nodulation failure (30, 55, 136), but poor nodulation can occur even where a viable *Rhizobium* population can be demonstrated (133, 134). Evans et al. (104) found that nodulation of *P. sativum* was 10 times more susceptible to acidity than was either rhizobial multiplication or plant growth. Some legumes, e.g., *Trifolium subterranean*, *T. balansae*, *Medicago murex*, and *M. 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 (102). Despite this, elevated inoculation levels have enhanced the nodulation response under acidic conditions in some studies (243). The growth, nodulation, and yield of *V. faba* were improved after inoculation with strains of *R. leguminosarum* bv. *viciae* in acid soils (55). 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 af-

ected by acidic conditions in soils (54, 326). Taylor et al. (314) reported that acidity had more severe effects on rhizobial multiplication than did Al stress and low P conditions. They suggested that colonization of soils and soybean roots by *B. japonicum* may be adversely affected by acidity, an effect which will result in reduced nodulation.

The host cultivar-rhizobial strain interaction at acid pH has also been investigated. Munns et al. (221) noted that nodulation and nitrogen fixation by some strains of *Bradyrhizobium* at acidic pH differ with the cultivar of mung bean used. Vargas and Graham (327) examined the cultivar and pH effects on competition for nodule sites between isolates of *Rhizobium* in beans (*P. vulgaris*) under acidic conditions. They found a significant effect of host cultivar, ratio of inoculation, and pH on the percentage of nodule occupancy by each strain. However, it has been suggested (326) that only one of the symbionts needed to be acid tolerant for good nodulation to be achieved at pH 4.5. Inoculation of *Medicago polymorpha* by an acid-tolerant *R. meliloti* strain has extended the area of acidic soils in Western Australia that can be sown with annual legumes to some 350,000 ha (153). The performance of the *R. trifolii-Trifolium pratense* symbiosis under acidic conditions is best when the rhizobial strains were isolated from the most acidic soils, i.e., acid-tolerant strains (191). Rhizobia appear to be vary in their symbiotic efficiency under acidic conditions. Van Rossum et al. (325) compared 12 strains of *Bradyrhizobium* for their symbiotic performance with groundnut in acidic soils and found that some strains were totally ineffective under acidic stress (pH 5.0 to 6.5) while others performed well under these conditions. Acid-tolerant alfalfa-nodulating strains of rhizobia, isolated from acidic soils, were able to grow at pH 5.0 and formed nodules in alfalfa with a low rate of nitrogen fixation (87). The results also demonstrate the complexity of the rhizobial populations present in the acidic soils, represented by a major group of nitrogen-fixing rhizobia and a second group of ineffective and less predominant isolates.

The host legume appears to be the limiting factor for establishing *Rhizobium*-legume symbiosis under acidic conditions. Legume species differ greatly in their response to low pH with regard to growth and nodulation (311). Recently, it has been found that the amount of N₂ fixed by forage legumes on low-fertility acidic soil is dependent on legume growth and persistence (316). However, selection of acid-tolerant rhizobia to inoculate legume hosts under acidic conditions will ensure the establishment of the symbiosis and also successful performance (73, 128, 229).

Recent reports indicated the destructive effects of acidic soils on *Rhizobium*-legume symbiosis and N₂ fixation. Low pH reduced the number of *R. leguminosarum* bv. *trifolii* cells in soils, which resulted in no or ineffective nodulation by clover plants (157). The number of nodules, the nitrogenase activity, the nodule ultrastructure, and the fresh and dry weights of nodules were affected to a greater extent at a low medium pH (<4.5) (328).

In acidic soils with pH of >5.0, where heavy-metal activity is relevant, the presence of available aluminum inhibits nodulation (35, 41). Rhizobia showed varied responses to aluminum toxicity in acidic soils and cultures. Strains of *Rhizobium* (326, 344) and *Bradyrhizobium* (133) that were resistant to aluminum (50 μM) at low pH (>5.0) were identified; however, rhizobia from clover were sensitive to these conditions (344). Johnson and Wood (168) reported that Al was taken up and bound to the DNA of both sensitive and tolerant strains but that DNA synthesis by the tolerant strains of *R. loti* was not affected. However, Richardson et al. (261) found that 7.5 μM Al depressed *nod* gene expression at low pH (4.8).

Legume species vary markedly in their tolerance to Al^{3+} and Mn^{2+} , with some plants being significantly more strongly affected by these ions than are the rhizobia (133). Therefore, for acid soils with high Al content, improvement is achieved by manipulating the plant rather than the rhizobia (314). Nodulation of legumes appears more sensitive to Al than does plant growth (133); at pH 4.5 and with 0.5 mM Ca^{2+} , nodulation in cowpea was delayed by 12.7 μM Al and nodule number and dry weight were severely depressed (20). Availability of Ca^{2+} in acidic soils with a high Al content appears very important for nodulation; a low Ca^{2+} concentration (0.13 mM) at pH 4.5 greatly affected nodule number, nitrogenase activity, and nodule ultrastructure of the common bean, *Phaseolus* (328).

Two strategies have been adopted to solve the problem of soil acidity: (i) selecting tolerant plants, and (ii) liming the acidic soil to ameliorate the effects of acidic conditions. Few cultivated legumes are adapted to low pH levels. The primary protective mechanism of acid tolerance in certain cultivars of lentil (*Lens culinaris*) is excess production of citric, malic, aspartic, glucenic, and succinic acids in root exudate under acidic conditions (247). It has been recently reported that some pasture legumes acidified soils to a greater extent and that the amount of acid produced per gram of shoot dry matter (specific acid production) varied between species and with growth stages, ranging from 44 to 128 cmol/kg of dry shoot matter (309). Similarly, some grain legumes produced large amounts of acids (207), with the production of H^+ ranging between 77 and 136 cmol/kg of dry matter. It has been suggested that Al-tolerant (acid-tolerant) plant species contain and exude more organic acid and other ligands that form stable chelates with Al and thereby reduce its chemical activity and toxicity (114).

In recent studies, trials were performed to study the effects of treating soil acidity by applying lime (at rate of 2,500 kg ha^{-1}) and superphosphate (at rates up to 20 kg ha^{-1}) to acidic soils (239). The amelioration increased the soil pH from 4.5 to 4.9, decreased the concentration of extractable Al and Mn, and improved growth and N_2 fixation of *T. subterranean*. Amelioration of subsoil acidity was also done by applying coal-derived calcium fulvate (324), and this treatment increased the pH more than did amelioration by gypsum, Ca-EDTA, $\text{Ca}(\text{OH})_2$, or CaCO_3 . Previous reports also indicate the importance of liming for improvement of growth and nodulation of legumes in acidic soils, since they indicated that liming raised the pH from 5 to 6.5 and increased the percentage of nodule occupancy of *T. subterranean* (17). However, amelioration by lime and other substances, e.g., carbonate, must be optimized to avoid increasing the pH to a level which would be inhibitory to growth and symbiotic performance of legumes. Applied carbonate was found to react with Na and raise the pH (41). Addition of bicarbonate decreased nodulation, growth, and shoot nitrogen in some grain legumes (311). Nodulation inhibition in *Lupinus angustifolius* grown in a limed sand at a pH of >7.0 has also been reported. Nodulation of groundnut (*Arachis hypogaea*) was also inhibited when plants grew in nutrient solution containing carbonate (309). High pH (>6.0 and up to 10.0) totally inhibited the nodulation of some lupins (310), and the authors suggested that pH values above 6.0 have a specific effect in the impairment of nodulation of lupins. However, rhizobia appear to be more tolerant to alkalinity than do their legume hosts. The number of *R. leguminosarum* bv. trifolii cells was greater in carbonate-treated soil (103); increasing the soil pH increased both the rate at which rhizobia colonized the soil and the frequency of nodule formation. It has also been reported (253) that while germination of pigeon pea was decreased at pH values of >8.8, growth of rhizobia was unaf-

ected up to pH 11.5. These authors also found that uninoculated pigeon pea plants had as good a nodulation as did those grown from plant seeds inoculated with *Rhizobium* in reclaimed alkaline soils in a greenhouse study.

The tolerance of actinorhizal plants to soil acidity and acidic conditions was also reported. Solution culture studies have shown reduced nodulation of black alder (*Alnus glutinosa*) and other actinorhizal plants at low pH. The effect of soil acidity on nodulation of *A. glutinosa* grown in mine soils, limed to various pH values, was also studied (137). The authors found that soil pH was a significant factor affecting nodulation in the mine soil, with the highest level of nodulation occurring between soil pH values of 5.5 and 7.2 and the level being reduced below pH 5.5. There was also evidence of decreased viability of the endophyte (*Frankia*) below pH 4.5 (137). In a recent study, Igual et al. (158) reported a decrease in nodulation of *Casuarina cunninghamiana* at high levels of Al, with the nitrogen-fixing efficiency being decreased from 0.20 to 0.10 mg of N fixed per mg (dry weight) of nodule at 880 μM Al^{3+} . They found that the mean N concentration of nodules was significantly lower at pH 4.0 (1.83%) than at pH 6.0 (2.01%).

Nutrient Deficiency Stress

Soil salinity and acidity are usually accompanied by mineral toxicity (specific ion toxicity), nutrient deficiency, and nutrient disorder. Salt damage to nonhalophytic plants grown in nutrient solution is often due to the effect of ion imbalance (disorder) rather than the osmotic potential (347). This disorder might occur by specific toxicity of ions such as Na^+ and Cl^- and might be balanced by increasing the concentration of counterions, like K^+ and Ca^{2+} , against Cl^- (127). It has been suggested that K^+ and NO_3^- inhibited Na^+ and Cl^- translocation from the roots to the shoots of *Arachis hypogaea*, so that leaf growth was protected against salt damage (285). The dominant ions in saline waters and saline soils which are available in arid zones are Na^+ and Cl^- . Excess Na^+ often harms nonhalophytes by displacing Ca^{2+} from root membranes and thus changing their integrity and their normal functioning (76). Also, acidic stress markedly affects ion absorption by and growth of roots (320); the membrane structure and function of the roots suffer fatal changes under these stress conditions. The requirement of some essential elements, e.g., Ca^{2+} and P, is increased under severe stress conditions. The requirement of Ca^{2+} for growth of *R. meliloti* was increased under osmotic stress (51). The Ca-depleted cells of *R. leguminosarum* are swollen, lack rigidity, and express an additional somatic antigen normally blocked by side chains of the LPS O antigen (88). High levels of salinity (up to 10% NaCl) decreased the Ca^{2+} content of *Rhizobium* cells (362), and the outer membrane structure of the *Rhizobium* cells was greatly distorted. In the same way, calcium appears significantly more important in cells exposed to low pH (133). O'Hara et al. (230) found that in acid-sensitive strains of *R. meliloti*, 1.2 mM Ca^{2+} was needed for cytoplasmic pH maintenance, and Beck and Munns (32) noted that phosphorus-limited cells or cells grown at low pH needed Ca^{2+} for phosphorus mobilization in the cell. Lack of Ca^{2+} produced some changes in ion transport, which are caused mostly by changes in membrane properties (66), and Ca^{2+} plays an essential role in cell division, elongation, and membrane structure and function (320). At low pH, addition of Ca^{2+} to the incubation medium improves both growth and ion uptake by roots (320); it was also suggested that Ca^{2+} offset the harmful effects of ions such as K^+ and H^+ and of stress. Ca^{2+} seems to have two effects on K transport, (i) control of K^+ permeability and (ii) activation of K^+ uptake through the

acidification of the cytoplasm. Calcium-dependent cell surface components affect the attachment of *Rhizobium* to root hair cells (54, 293). It has been found (365) that salt stress (100 mM NaCl) reduced the attachment to and colonization of root hairs of *V. faba* plants by *R. leguminosarum*; this was attributed to the effect of salt on calcium availability. The effects of salt stress or acidity on calcium availability and on the initial stages of nodule formation will affect the net nodulating capacity of legumes. Both pH (4.5) and aluminum (100 μ M) caused delays in nodulation of *Vigna unguiculata*, particularly at low Ca^{2+} levels (0.3 mM), while at a high calcium concentration (3.0 mM), nodulation was improved (152). The critical Ca^{2+} level for nodule formation in pigeon pea and guar is more than 50 μ M, whereas peanut and cowpea nodulated very well in solution culture with 2 μ M calcium (35). Nodulation and nodule development in cowpea were strongly depressed at low pH (4.5 to 5.5) and low calcium concentration (0.05 to 2.5 mM) (20). Stress conditions may inhibit nodulation of legumes through the inhibition of genetic activity. It has been reported that Ca^{2+} (10 mM) increased *nod* gene induction and expression activities of clover plants 5- to 10-fold at pH 4.5 to 5.2 (261).

Phosphorus is one of several elements which affects N_2 fixation, and, along with N, it is a principal yield-limiting nutrient in many regions of the world (240). Strains of rhizobia differ markedly in tolerance to phosphorus deficiency (33). Rhizobial P deficiency when there is a P deficiency in the soil and rhizosphere is a real possibility, especially under acidic conditions, where dissolved phosphorus salts may be precipitated in the presence of aluminum (133). Slow-growing strains of rhizobia appear more tolerant to low P levels than do fast-growing *R. meliloti*, in particular (33); this bacterium failed to grow at 0.06 μ M P, regardless of the Ca^{2+} concentration, and some strains needed high Ca^{2+} levels to grow at 0.5 and 5.0 μ M P. Phosphate-limited cultures of both fast- and slow-growing rhizobia do take up phosphate 10- to 180-fold faster than cells grown with adequate P (291), and inducible alkaline phosphatase activity was detected in P-limited cells of fast-growing *R. trifolii* strains (290, 291). Recently, it has been reported (18) that free-living *R. tropici* and bacteroids respond to P stress by increasing their P transport capacity and inducing both acid and alkali phosphatases. This P stress response occurred when the medium P concentration decreased below 1 μ M. Leguminous species differ in their phosphorus requirement for growth from 0.8 to 3 μ M (110).

Phosphorus appears essential for both nodulation and N_2 fixation (240, 303). Nodules are strong sinks for P and range in P content from 0.72 to 1.2% (142, 143); as a consequence, N_2 fixation-dependent plants will require more of this element than those supplied with combined nitrogen (56, 57). Nodulation, N_2 fixation, and specific nodule activity are directly related to the P supply (163, 190, 288). Application of KH_2PO_4 (25 mg of P per kg of soil) to acidic soils significantly increased the percent nodule occupancy of *Trifolium subterraneanum* by *R. leguminosarum* bv. *trifolii* (17). The nodulation and N_2 fixation (nitrogenase activity) of *T. vesiculosum* increased significantly after the addition of P (100 mg per kg of soil) and K (300 mg per kg of soil); nitrogenase activity was doubled when the P concentration increased to 400 mg per kg of soil (194). The interaction of P and Zn and their effects on nodulation of legumes under salt stress were studied. Saxena and Rewari (273) found that application of phosphate (20 and 40 ppm) improved the growth and nodulation of chickpea (*C. arietinum*) in the presence of Zn^{2+} (5 ppm) at two levels (4.34 and 8.3 dS/m) of salinity. They suggested that augmentation with Zn^{2+} provided protection to the plant under saline conditions by reducing the Na^+/K^+ ratio in the shoot; the shoot N content

after augmentation with Zn^{2+} and in the presence of phosphate was equal to that of nonsaline control. Differences between cultivars of some legume species with regard to phosphorus requirements have been reported (48). Variability of N_2 fixation under low P availability existed between lines of *P. vulgaris*; high N_2 -fixing and high-yielding progeny lines were detected (240).

Mycorrhizal infection of roots of legumes has been reported to stimulate both nodulation and N_2 fixation, especially in soils low in available P (121, 257). The role of mycorrhizal fungi in the protection of the *Medicago sativa*-*R. meliloti* symbiosis against salt stress was recently studied (26), and it was found that the interaction between soluble P in the soil mycorrhizal inoculum and the degree of salinity in relation to concentration and nodule formation increased with the amount of plant-available P or mycorrhizal inoculum in the soil and generally declined as the salinity in the solution culture increased. Azcon and Elatrash (26) found that the mycorrhizal inoculation protected plants from salt stress more efficiently than did any amount of plant-available P in the soil, particularly at the highest salinity level applied (43.5 dS/m).

Nitrogen fixation by the *Frankia*-actinorhizal symbiosis may be limited by low available P in soils. Sangina et al. (270) observed increased N_2 fixation by *Casuarina equisetifolia* by adding phosphate to P-deficient soil, and Reddell et al. (255) found a greater increase in the yield (wood volume) of *Frankia*-inoculated *Casuarina cunninghamiana* by adding phosphate to soil. Low P status is a frequent limitation to nodulation of actinorhizal plants. It has been reported that symbiotic N_2 fixation of the *Frankia*-*Casuarina* association requires higher P levels than those required for plant growth, when the P concentration in soil is low (270). Genetic variations among species of *Allocasuarina* in relation to P requirement were identified; species showed different nodulation abilities in soils with low available P (270).

Soil Amendments and Ameliorations

Sewage sludge treatment and organic fertilizers. Sewage sludge application to agricultural soils is an economical way of disposal (109, 202, 228). It improves the physical characteristics of the soil (340) and increases organic matter content and essential plant nutrients, in particular N and P (109). Sewage sludge contains numerous components required for microbial growth and may increase the activity of soil microorganisms (299), including rhizobial growth (177). Contaminants associated with certain fertilizers such as sewage sludge may also negatively affect the survival of various soil microorganisms (202). Concern about the use of sewage sludge contaminated by heavy metals has increased. Heavy metals are known to persist in the soil over long periods and have ecotoxicological effects on plants and soil microorganisms (100). There is increasing evidence of adverse effects on microbial processes related to nutrient cycling in these types of soils (228).

Sewage sludge may contain a variety of materials potentially toxic to rhizobia, such as soluble salts (195) and heavy metals (126, 206). Despite the presence of metal-impacted agricultural soils, there have been few studies of metal resistance in rhizobia. A decline in rhizobial populations (e.g., *R. japonicum*) in higher-sludge soils (5 parts soils to 1 part sludge) may be due to the presence of heavy metals which are available during the mineralization of sludge in soils (256). Kinkel et al. (177) examined two genera of soybean-nodulating rhizobia to determine the level of resistance to eight different metals. Marked variations were found with several heavy metals, even for rhizobial strains belonging to the same species. Relatively

large numbers of *R. leguminosarum* bv. trifolii were found in soils treated with organic (e.g., sewage sludge) and inorganic fertilizers; however, these numbers were related to soil pH, and all isolates were equally effective (202). Tong and Sadowsky (319) reported that *Bradyrhizobium* strains were more resistant to heavy metals than were *Rhizobium* strains. However, this observation is not always correct, since it has been found that resistance to tellurite, selenite, and selenate was observed only in *R. meliloti* and *R. fredii* strains but not *Bradyrhizobium* strains (178). More recently, it has been reported (16) that the growth of some bacteria, e.g., *R. leguminosarum* and *Agrobacter tumefaciens*, was affected by copper treatment. The cells of these bacteria remain viable but nonculturable. However, some heavy metals, e.g., nickel, are essential elements in several biological processes, including H₂ oxidation in some bacteria and urea hydrolysis by plants. Klucas et al. (181) found that the addition of Ni (5 to 8 μM) to both the nitrate-grown and symbiotically grown soybean plants resulted in a 7- to 10-fold increase in urease activity in leaves and significantly increased the hydrogenase activity in isolated nodule bacteroids. They also found that free-living *R. japonicum*, cultured under chemolithotrophic conditions, required Ni for growth and for the expression of hydrogenase activity. Therefore, Ni is an important micronutrient element in the biology of the soybean plant and *R. japonicum*. The survival of *B. japonicum* in sludge-amended soils was also studied (195) and the soluble salts of the sludge (not the heavy metals) were shown to be primarily responsible for a short-term reduction in bradyrhizobial populations following sludge application to soil.

Adverse effects of heavy metals on nodulation and N₂ fixation of legumes have been reported for clover (206, 264) and chickpea (348). Giller et al. (126) suggested two possibilities to explain the mechanism by which the elevated metal concentrations eliminated N₂ fixation: (i) one or more of the metals present might have prevented the formation of N₂-fixing nodules by effective *Rhizobium* strains present in the soil or (ii) the metal contamination might have resulted in elimination of the effective *Rhizobium* strains from the soil. Inoculation of white clover plants grown in metal-contaminated soil with an effective strain of *R. leguminosarum* bv. trifolii promoted N₂ fixation, but this did not occur when inoculation was carried out 2 months before sowing, unless a very large inoculation (10¹⁰ cells per g of dry soil) was used (126). It was also found that the plasmid profiles of these isolates were all very similar, indicating a lack of genetic diversity in the population surviving at high concentrations of heavy metals. These strains were all ineffective in N₂ fixation. These authors concluded that white clover rhizobia are unable to survive (or at least unable to remain infective) in the presence of concentrations of heavy metals close to the current Commission of the European Communities guidelines for environmental protection. The survival and the number of effective strains of *R. leguminosarum* bv. trifolii in soils amended with anaerobically digested and undigested sewage sludge (at rates up to 300 m³ ha⁻¹ year⁻¹) were studied (228). *Rhizobium* was found in most of the contaminated soils, apart from the most contaminated treatment in the soil of lower pH, despite the absence of the host plant from the field sward. Lack of nodulation and N₂ fixation for *Trifolium repens* grown in these soils was indicated. Obbard et al. (228) suggested that important effects on the sizes of effective rhizobial populations were determined by soil pH, sludge type and addition rates, and concentration of heavy metals present. The *Rhizobium* symbiosis with *T. subterranean* was recently studied in soil fertilized with sewage sludge, lime, and standard mineral (PK) fertilizer (109). Nodulation was decreased only with the highest rate (60 tons ha⁻¹) of sludge amendments and was

greater after standard mineral fertilization than after sewage sludge and lime amendments. After 1 year, nodulation of plants grown in soils treated with large quantities of sludge was greater than nodulation of plants grown in soils amended with mineral fertilizer. Improved nodulation after sludge treatment in the second year was attributed to an increase of the rhizobial population and breakdown of soil organic matter. Ferreira and Castro (109) suggested that sewage sludge be used as organic fertilizer, although the nutrient content and the pH of different types of sludge are different. The effects of heavy metals from biosolids on the population and N₂-fixing potential of *R. leguminosarum* bv. trifolii, under two pH regimens, were studied by Ibekwe et al. (157). They found few significant effects of biosolid-borne heavy metals on plants, N₂ fixation, and number of rhizobia at the concentrations of metals studied, as long as the soil pH was maintained near 6.0. Where reduction in rhizobial number and plant parameters was observed, the decrease was attributed primarily to low soil pH and, to a lesser extent, to heavy metal toxicity from biosolids. Smith (296) reported that strains of *R. leguminosarum* bv. trifolii, which are effective in N₂ fixation with *Trifolium repens*, were present in different soils from long-term sewage sludge-treated sites. The rhizobia survived in soil and formed an effective symbiosis with the host plant where the metal concentration in soil increased to 300 mg of Cu kg of soil⁻¹ and 2,000 mg of Zn kg of soil⁻¹. This work demonstrated that nodulation and N₂ fixation by white clover occur in sludge-treated soils containing more than the current United Kingdom maximum permissible concentrations of heavy metals. It appears that some *Rhizobium*-legume associations are able to cope with a high content of heavy metals in soils treated with sludge or organic fertilizers; however, knowledge of the chemical and physical properties of organic fertilizers, e.g., sludge, prior to their application to soil is of the utmost importance (106). This ensures the quality and quantity of the required components and that heavy metals are not above the toxic limit for plants. The characteristics of some local organic fertilizers (e.g., dried sludge and limed sludge) in Egypt were recently studied (106). The tested composts showed high levels of some nutrients (i.e., K, P, B, Fe, Zn, Mn, and Cu) but these fell within the permissible levels. However, the Ni and Cd levels were higher than the permissible levels and should be reduced. Application of organic fertilizers with low concentrations (within permissible levels) of heavy metals will improve soil fertility in reclaimed soils. Furthermore, the legume-root nodule symbiosis can be used as an effective parameter for ecotoxicological evaluation of contaminated soils (341). Substances affecting the macro- and/or microsymbiont vitality, such as certain heavy metals or polycyclic aromatic hydrocarbons (PAHs), reduce nodulation before visible damage of the plant occurs. Wetzel and Werner (341) found a dose-responsive decrease in nodulation of alfalfa after application of CdCl₂, NaAsO₂, fluoranthene, and other PAHs, although PAH-contaminated soil (75 mg/kg) caused only a slight reduction in nodulation of alfalfa.

Fertilizer application. Most combined N available to crop legumes is in the form of NO₃⁻, formed by oxidation of NH₄ from residual fertilizer and mineralization of organic N. Nitrate has been recorded in soils at levels up to 20 mM or 280 ppm (31). Both NO₃⁻ assimilation and N₂ fixation of legumes are strongly dependent on the plant cultivar, bacterial strain, ontogeny, and environmental factors (e.g., soil NO₃⁻ concentration, carbon and water availability, and temperature). Invariably, N₂-fixing activity is confined to areas with low NO₃⁻ availability (31). NO₃⁻ may allow the plant to conserve its energy, since in overall terms more energy is required to fix N₂ than to utilize NO₃⁻. It is therefore necessary that the plant be

able to detect the presence and level of NO_3^- in the rooting medium and to adjust its N_2 fixation accordingly. Symbiotic N_2 fixation in field legumes takes place against a changing background of mineral N availability as a consequence of mineralization, leaching, and, often, fertilizer application (81). Uptake of fertilizer N by plants depends on soil moisture and is higher in normal than in wet soils (with uptakes of 32 and 27%, respectively) as a result of different N-leaching losses (224).

It is widely accepted that the capacity for nitrogen fixation by a nodulating or nodulated legume is influenced, at least in two ways, by mineral nitrogen in the soil in which it is grown. First, the process of nodulation may be promoted by relatively low levels of available nitrate or ammonia, higher concentrations of which almost always depress nodulation (81, 92). Second, the rate of N_2 fixation by an active, growing, and well-nodulated legume is always suppressed by NO_3^- ions (147). It has been established for many years that soil NO_3^- inhibits root infection (8), nodule development (25, 160), and nitrogenase activity (23, 246, 271). Maximum N_2 fixation in a legume requires that the legume be adequately nodulated. Scanty and poorly distributed nodules on the root system do not usually satisfy the nitrogen needs of the plant, resulting in a greater reliance on soil N for growth. Supplemental inoculation to correct initial suboptimal nodulation has been recommended (64, 80). Nodulation and N_2 fixation of soybean have been improved by using this approach. Furthermore, Danso et al. (80) found that the inhibition of soybean N_2 fixation at higher N levels (83 mg of N kg of soil $^{-1}$) was significantly reduced by a second inoculation; this treatment resulted in at least a doubling of both the percentage and total amount of N_2 fixed by soybean plants after the single slurry inoculation.

Variation among strains of *Rhizobium* and *Bradyrhizobium* in their tolerance to the inhibitory effects of combined nitrogen on their population in soil and growth media has been reported (208, 353). Semu et al. (280) showed that nitrogen application of up to 200 kg of N ha $^{-1}$ did not change the population size of *B. japonicum* in a soybean field. In *Rhizobium*-legume symbioses, the formation of nodules is the result of a complex multistep process in which rhizobial attachment to legume root hairs is an early step and may be involved in the control of specificity. Combined N is one of the many environmental factors which limit the development and success of the *Rhizobium*-legume symbiosis in nature and can regulate rhizobial adsorption to host root hairs and root hair infection (82, 90, 219). It has been found that addition of NO_3^- (5 or 16 mM) to the seedling growth medium significantly decreased the number of rhizobial cells adhering to lucerne seedling roots. The attachment of *R. leguminosarum* bv. trifolii and the level of trifoliin A (a lectin) on the root surface of white-clover seedlings grown with high nitrate were also decreased (82, 83). It has been found (283) that excess nitrate did not repress the synthesis of the lectin (trifoliin A) in the root but did affect the distribution and activity of this lectin in a way which reduced its ability to interact with cells of rhizobia. Since it has been suggested (84) that legume lectins confer specificity in the *Rhizobium*-legume symbiosis by interacting with the bacterial symbiont, factors, such as excess nitrate, which affect lectin activity and interaction with rhizobia might inhibit the symbiotic process.

The inhibitory effect of N on nodulation is probably plant mediated; however, differences in tolerance to nitrate and ammonium have also been found between rhizobial isolates when investigated in nodulated systems (225). Gibson and Harper (125) have shown that different strains of *B. japonicum* have varied tolerance to external nitrogen application in their nodulation and nitrogen fixation characteristics. Rhizobia showed

varied responses to combined N with regard to competition for nodule occupancy. Variations in the competitive abilities of three strains of *B. japonicum* for nodulation of soybean (*G. max*) at increasing fertilizer-N concentrations (up to 10 g of N m $^{-2}$) have been reported (201). It has also been reported that combined N altered the nodule occupancy of two strains of rhizobia in soybean (208), while nitrogen treatment had no significant effect on nodule occupancy by three strains each of *B. japonicum* (nodulating soybean) and *R. leguminosarum* bv. phaseoli (2), although nitrogen application reduced the nodule number and mass of both legumes. Recently, it has been found (282) that increased soil fertility (soil N) had no effect on nodule occupancy of chickpea (*Cicer arietinum*).

Nitrogen fertilization is sometimes needed to achieve a substantial yield of legumes (e.g., soybean) when the symbiotic N_2 fixation is unable to provide enough nitrogen (52). However, fertilizer rates exceeding those exerting a "starter nitrogen" effect generally reduce nodulation and N_2 fixation (11). The response of the *Rhizobium*-legume symbiosis to added nitrogen fertilizer is definitely determined by time of application (growth stage), level and form of N, and the legume species (8, 160, 182). Nitrates are more inhibitory to nodulation than is ammonia, especially if added shortly after planting. Application of fertilizer-N (25 mg of N kg of soil $^{-1}$) during sowing was less detrimental to N_2 fixation by *P. vulgaris* than during vegetative growth (217).

Experiments have demonstrated that NO_3^- inhibits nodule formation on legumes primarily as a root-localized effect rather than as a function of whole-plant N nutrition (8, 92). When NO_3^- levels were sufficiently high to completely suppress nodulation on the original root (primary root), there were profuse nodulation and significant nitrogenase activity (C_2H_2 reduction) on the adventitious roots of soybean (92) and nodules were formed on the lateral roots of *V. faba* and *P. sativum* (8). Application of urea (90 kg of N ha $^{-1}$) to soybean plants suppressed nodulation by curtailing the enrichment of *Bradyrhizobium* spp. on the host plant (315). The root system of pigeon pea (*Cajanus cajan*) was poorly developed after application of fertilizer-N (up to 60 kg of N ha $^{-1}$), and this also affected other N_2 -fixing parameters, e.g., nodule number, nitrogenase activity, nodule dry weight, shoot weight, and root and shoot nitrogen (174). However, recent reports suggest a role for the whole process of metabolism of host legumes and their rhizobia in response to applied fertilizer-N. The host legume, but not rhizobia, controls the efficiency of respiration in nodules under normal or stressed conditions (59). Nitrogenase-linked respiration was markedly raised by the addition of nitrate, but the host affected mainly the changes of resistance to O_2 diffusion in nodules, and the presence of combined N depleted the reaction capability of nodules to adjust to changing O_2 levels in the rhizosphere. Salt stress (150 mM NaCl), nitrate stress (10 mM NO_3^-), and drought inhibited N_2 fixation by soybean through the inhibition of nodule enzymatic activity (130). These authors proposed that N_2 fixation in soybean nodules is mediated by both the oxygen-diffusion barrier and the potential to metabolize sucrose via sucrose synthase. The response to the environmental perturbation may involve down-regulation of the nodule sucrose synthase gene.

The inhibitory effect of exogenous nitrate on N_2 fixation has variously been attributed to a direct competition between nitrate reductase and nitrogenase for reducing power (304) or to the fact that nitrite (a by-product of nitrate reductase) inhibits the function of nitrogenase and leghemoglobin (31). The high levels of nitrite and amino acids in nodules of alfalfa plants treated with nitrate (10 mM KNO_3) correlated with reduced leghemoglobin content and nitrogenase activity (74), but this

does not explain directly the mechanism of suppression of nitrogen fixation in alfalfa exposed to nitrate. Low levels of nitrite accumulate in nodules of *V. faba* plants compared to those in roots (53); the root enzymes nitrate reductase (NRA) and nitrite reductase (NiRA) were significantly stimulated by exogenous nitrate, while negative (NRA) or little effect (NiRA) was found for nodules. In a more recent study, treatment of soybean plants with nitrite (10 mM) and salt (150 mM NaCl) did not significantly affect leghemoglobin levels and other enzyme activities (130). In addition, it has been found that the decline in total nitrogenase activity, measured as H_2 production in 20% O_2 -80% Ar, upon exposure to nitrate was independent of the N_2 -fixing efficiency (i.e., the amount of N_2 reduced by nitrogenase) of the symbiosis (39). In fact, the mechanism of inhibition of N_2 fixation (nitrogenase activity) by nitrate and ammonia implies various factors. A comparison of experiments is hampered by the use of different concentrations and durations of fertilizer-N treatment and by the fact that the legumes differ in their sensitivity to NO_3^- and NH_4 (31).

Increased N input via fixation is self-limiting, since high soil nitrogen levels inhibit fixation. This limitation could be overcome by using a legume which will continue to fix N_2 in the presence of combined nitrogen. The potential for breeding genotypes of legumes with improved nodulation and N_2 fixation when grown with combined nitrogen was examined. Mytton and Rys (222) and Rys and Mytton (268) demonstrated the existence of heritable genetic factors controlling nodulation and N_2 fixation under sodium nitrate treatment (up to 22.8 mM). Herridge and Betts (149) selected 4 genotypes of soybean from 32 previously tested for tolerance of 2.5 mM NO_3^- . The four genotypes showed the highest levels of symbiotic activity when inoculated with *B. japonicum* and sown into a soil which contained a high level of nitrate (260 kg of N ha^{-1} at depths of 0 to 120 cm) and which was free of soybean rhizobia. Abdel-Wahab and Abd-Alla (4) provided evidence for nodulation and growth variability of soybean cultivars fertilized with different levels of N (up to about 120 kg of N ha^{-1}). Several soybean mutants (101) and a supernodulating genotype of soybean (298) displayed a nitrate-tolerant symbiosis in the presence of fertilizer-N at 40 and 180 kg of N ha^{-1} . Genotypes of *P. vulgaris*, which form a successful symbiosis with *Rhizobium* in the presence of fertilizer-N (at 12.5 mg of N kg of soil $^{-1}$) in saline-sodic soil, were identified (234). Supernodulating genotypes of pea (227) and cowpea (287) with better nodulation and N_2 fixation efficiency under fertilizer-N treatment (20 mM NO_3^- and 120 kg of N ha^{-1} , respectively) were also identified. Different species of legumes showed different responses to the same treatment of fertilizer-N; application of 200 kg of N ha^{-1} decreased N_2 fixation only by 18% in groundnut but by 54% in cowpea (351).

In general, high soil N levels, applied or residual, reduce nodulation and N_2 fixation. To improve BNF by the legumes under such circumstances, the soil N concentration must be managed through inclusion of appropriate nitrate-tolerant high- N_2 -fixing legume crops or the genotype of a given crop (339). Analysis of the different contributions to BNF by legumes discussed in this review suggests that there is a potential to select appropriate legume crops or cultivars of a given legume for specific areas with high soil N contents without decreasing their BNF contribution to the system.

Pesticide application. The use of a vast array of pesticides to overcome the economic losses in agriculture exerts varied environmental stresses on nontarget organisms present in the soil. The maximum benefits of BNF may not be achieved if other constraints are placed on the system. One of the most important and potentially limiting factors to BNF is the use of

herbicides, fungicides, and other pesticides. Herbicides applied to leguminous crops constitute a potential hazard to the establishment and performance of the N_2 -fixing root nodules. It has been reported that foliar application of the herbicides bentazone and MCPA (4-chloro-2-methylphenoxyacetic acid) to red clover (*Trifolium pratense*) at the recommended rates altered the morphology of root hairs and reduced nodule numbers and nitrogenase activity (192). Application of the herbicide dinoseb (2-sec-butyl-4,6-dinitrophenol) to red clover in the field reduced the levels of nitrogenase activity of the plants (191). *Rhizobium* strains isolated from root nodules of red clover plants treated with dinoseb showed resistance to the herbicide when grown in laboratory media containing dinoseb; however, these strains did not improve the symbiotic performance of red clover treated with dinoseb at 200 $\mu g ml^{-1}$ (the recommended rate). Lindström et al. (191) suggested that foliar application of dinoseb did not act directly on the nodules but affected nitrogen fixation by damaging the photosynthetic system of the plant. Reduction in nodulation and N_2 fixation of a legume, e.g., *V. faba*, was found only in cases where herbicide injury to the plant was evident (37). Herbicides have been reported to affect *B. japonicum* growth in vitro and to reduce the nodulation of soybeans under greenhouse conditions (197, 198). Five herbicides tested under Canadian field conditions showed no effect on soybean nodulation and nitrogen fixation (258). The herbicides sethoxydim, alachlor, fluzafop butyl, and metolachlor did not have detrimental effects on N_2 fixation or seed yields when added at the recommended rates for weed control in soybean plantations (183). However, paraquat significantly reduced the amount of N_2 fixed by soybean as measured by the ^{15}N dilution method. Similarly, herbicides were reported to induce reduction in nodulation and N_2 fixation of soybean (162) and bean (276). In most published studies, herbicides varied in the extent of their effect on N_2 fixation. Consequently, the effect of herbicides on N_2 fixation in soybeans must eventually be determined under field conditions, and if the results are to be relevant, the effect must be studied in the area where they are to be used (184). Fungicides are also dangerous to the *Rhizobium*-legume symbiosis. Field rates and higher rates of captan reduced nodulation of and N_2 fixation by *T. repens* (111). Thiram and captan are harmful to nodulation and N_2 fixation of several grain and forage legumes (135, 145, 259).

Rhizobia showed varied in vitro growth under pesticide treatment. Some pesticides are not detrimental to the growth of rhizobia when applied at field rates (200, 213, 322), whereas other pesticides were found to be toxic to rhizobia when applied at low as well as at high rates (28, 267). Certain strains of rhizobia could resist high levels of pesticides by adaptation (171); however, these pesticide-adapted rhizobia may be genetically modified (267). In a recent study on the effects of four pesticides on four species of legumes, Abu-Gharbia (10) showed that the growth of free-living and symbiotic rhizobia is sensitive to pesticide treatment. Variation among legume species with regard to nodulation and N_2 fixation under pesticide treatment may depend on the type and dose of the pesticide, species of *Rhizobium* and legume, and stage of development of the *Rhizobium*-legume symbiosis. High concentrations of Gaucho (an insecticide, imidacloprid) and Vitavax-300 fungicide (carboxin and captan) clearly inhibited the growth of root nodule bacteria under laboratory conditions (211); however, these pesticides did not affect the nodulation or biomass production of *Arachis pintoii*, *A. hypogaea*, *Mucuna pruriens*, or *Desmodium ovalifolium* raised in a greenhouse.

Other chemical contaminants, e.g., PAHs, which might occur as ubiquitous environmental contaminants due to the combustion of fossil fuels, can affect nodulation and N_2 fixation of

legumes (341). One of the PAHs (fluoranthene), found in sewage sludge or beside motorways, was not toxic to the growth of *R. meliloti*. However, when the host legume, *Medicago sativa*, was grown on a solidified fluoranthene-containing medium, the plants exhibited symptoms of toxicity. Plants inoculated with *R. meliloti* showed a dose-responsive decrease in shoot length and inhibition of nodule formation (341). Recently, Ahmed et al. (12) reported the presence of a variety of strains of *R. meliloti* in soils contaminated with PAHs (e.g., aromatic and chloro-aromatic hydrocarbons) and with no history of the presence of indigenous lucerne (*M. sativa*). The population of *R. meliloti* in each of these soils is not homogeneous but is composed of several phenotypically and genetically distinct strains. Interestingly, strains obtained from different sites form an effective symbiosis (N_2 fixation) with *M. sativa*, irrespective of the nature or level of contamination. Recently, it has been reported (140) that a bacterium related to *Rhizobium* can oxidize methyl bromide (MeBr) in a fumigated agricultural soil to CO_2 . These results suggest that soil treatment strategies can be devised whereby bacteria can effectively consume MeBr during field fumigations, which would diminish or eliminate the outward flux of MeBr to the atmosphere. The above findings suggest a possible role of rhizobia in decontamination and recycling of organic compounds and a potential application of these agro-nomically important microbes for environmental cleanup.

NITROGEN FIXATION IN ARID REGIONS

Arid Regions and Arid Climates

About one-third of the land area of the world comprises arid and semiarid climates (169). Arid desert soils were previously considered economically unimportant; however, during the past three decades, the economic and agricultural utilization of arid lands has emerged as a critical element in maintaining and improving the world's food supply (289). Arid lands in Egypt represent about 97% of the total area of the country. Egypt occupies the northeastern part of the African continent, and its total area is a little more than 10^6 km². The whole country forms part of the great desert belt that stretches from the Atlantic across the whole of North Africa through Arabia (366). An extremely arid climate prevails in Egypt; the high temperature, low relative humidity, high evaporation, and scanty rainfall (1.4 to 5.3 mm/year) all contribute to the fact that the greater part of Egypt is barren and desolate desert (3, 366). The desert lands also include saline areas; saline lands represent about 15% of the arid and semiarid lands of the world (281, 358). In saline areas, evaporation greatly exceeds precipitation, and there may be salination to a sufficient degree to eliminate most plants from these habitats (29, 358). Saline lands, like arid lands, have been largely ignored and are usually considered to be abandoned, nonproductive lands. Desert ecosystems are characterized by a lack of moisture and nitrogen, but drought and salt stresses are probably the most important environmental factors that inhibit the growth of organisms in arid and semiarid regions.

Improving the Fertility of Arid Regions

Plant productivity in many arid regions is often limited by low soil fertility; therefore, the nutrient content of the soil should be considered in conjunction with the amount of moisture when selecting plants for deserts (3, 300). One of the suggested treatments for building up the fertility of desert soils in Egypt is by application of a mixture of Nile sediments (clay) and organic manure; however, this suggestion was considered

a nonpractical solution to improve the fertility of desert soils (3), since the huge amount of sediments required for the amelioration of large areas is not available.

Amelioration of low-fertility soils by application of organic fertilizers (e.g., sewage sludge and animal organic manures) is a strategy that has been adopted in many countries in recent years. Recent reports have emphasized the significance of this treatment in improving the physical characteristics of soil and supplying soil with the nutrients required for growth of different organisms. It is also a less expensive means of soil amendment than fertilizer-N (106, 109, 202, 228). Organic fertilizers are rich in several valuable nutrients, but they also contain high levels of toxic heavy metals. Recent reports, however, indicate that heavy metals are probably not the only factors which harm the activity of soil organisms (e.g., plants and microorganisms) but that the presence of soluble salts and the reduction in pH as a result of high levels of ammonia could also suppress the activity of soil organisms (157, 195, 296). In contrast to previous reports, recent reports demonstrate the tolerance of some N_2 -fixing bacteria to conditions created in soils after the application of sewage sludge. Some strains of *R. leguminosarum* bv. trifolii survived and formed an effective symbiosis with clover cultivated in soil treated with sewage sludge containing levels of heavy metals above the maximum permissible concentrations (296). Therefore, we suggest that organic fertilizers be used to ameliorate the newly reclaimed arid lands. Organic fertilizers with low concentrations of heavy metals, containing essential nutrients, and slightly acidic (pH less than 6.0) are recommended. *Rhizobium*-legume symbioses, which are tolerant to moderate levels of acidity, can be grown in these ameliorated soils.

Application of mineral fertilizers, e.g., fertilizer-N, is a common practice to improve soil fertility in developed and developing countries; however, there is increasing concern about serious pollution of drinking water by fertilizers such as nitrates. However, we suggest that fertilizer-N be used to increase fertility in N-poor soils or desert lands in order to achieve substantial yield of legumes when symbiotic N_2 fixation is unable to provide enough N for maximum yield. The application of fertilizer-N, however, requires the selection of N-tolerant legumes. The negative effects of nitrate and other combined nitrogen sources on the symbiosis of *Rhizobium* and legumes are well documented and are discussed above in detail. Variations between strains of rhizobia from the same species in the presence of high concentrations of nitrate were demonstrated, and high concentrations (up to 200 kg of N ha⁻¹) of fertilizer-N had no significant effects on the population size of rhizobia. Recent reports discussed in this review demonstrate the potential of breeding genotypes of legumes, e.g. pea, bean, and soybean, with improved nodulation and N_2 fixation in the presence of combined nitrogen (4, 227, 298, 339). The application of fertilizer-N in arid regions, however, should be optimized to reduce its marginal effects. The inclusion of appropriate nitrate-tolerant N_2 -fixing legumes will maximize the yield of crops and also reduce N losses through leaching.

Biological N_2 Fixation in Arid Regions

BNF is the major way to introduce N into desert ecosystems. BNF in deserts is mediated mainly by some heterotrophic bacteria, associative bacteria, cyanobacteria, actinorhizal plants, and legumes. The N_2 fixed by heterotrophic free-living bacteria is of minor importance as a mechanism for N input in arid soils (3, 361). Associative dinitrogen-fixing bacteria may be potentially important in supplying relatively small amounts of

N to associated grasses in arid lands (346). Bacteria that associate with the roots of C₄ grasses perhaps are the most potentially important in arid climates (300) since such grasses are known to have high levels of water use efficiency. The total amount of N fixed by cyanobacteria may be small; nevertheless, they could contribute to the fertility of arid lands, which are N-limited environments (300). The actinorhizal plants, e.g., the *Frankia-Casuarina* symbiosis, grow very well in hot, dry, and saline environments and fix appreciable amount of N. There are recent reports of desiccation tolerance in *Frankia* spp.; these species also survive and form effective symbiosis at high temperature and low soil moisture (274, 275). A new *Frankia-Atriplex* symbiosis was recently discovered (58) (*Atriplex* is a perennial forage shrub in dry and salt-affected soil in Argentina); this symbiosis is an N₂-fixing mechanism. Further research is needed for an exact quantification of the contribution of actinorhizal plants to BNF in arid regions. *Rhizobium*-legume symbioses represent the major mechanism of BNF in arid lands. This point is discussed in detail in the following sections; however, emphasis is placed on the naturally occurring herb or woody legumes in deserts.

***Rhizobium*-Legume Symbioses and Rehabilitation of Arid Regions**

Drought-tolerant *Rhizobium*-legume symbiosis. Improved cultivars of plants for arid lands must have drought resistance mechanisms to enable them to grow and survive in areas with low moisture availability. In fact, *Rhizobium*-legume symbioses are currently the most important nitrogen-fixing systems, which may have the potential to increase N input in arid lands. The leguminous plants include species or varieties which are extremely well adapted to the drastic conditions of arid lands. Examples are *Medicago sativa*, *Arachis hypogaea*, *Cyamopsis tetragonoloba*, and *Melilotus* spp.; these legumes are known to be adapted to conditions prevailing in arid regions. In addition, a drought-tolerant cultivar of *Phaseolus vulgaris* has recently been identified (250). The dry weight of this legume was not affected by water stress (50 and 30% of field capacity), although the number and weight of nodules as well as N₂ fixation (acetylene reduction) were obviously reduced. However, these legume species require drought-tolerant rhizobia to form effective symbiosis under arid climates. Rhizobia with survival ability, which showed effective symbiotic characteristics with their host legumes (e.g., *Prosopis* rhizobia) in desert soils and arid regions, were identified (117, 164, 165). Effective rhizobia are competitive and able to migrate under conditions of scarce moisture (335). In a recent work, Athar and Johnson (24) reported that two mutant strains of *R. meliloti* were competitive with naturalized alfalfa rhizobia and were symbiotically effective under drought stress. These results suggest that nodulation, growth, and N₂ fixation in alfalfa can be improved by inoculating plants with competitive and drought-tolerant rhizobia. This could be an economically feasible way to increase alfalfa (*M. sativa*) production in water-limited environments.

The N₂-fixing legume-*Rhizobium* symbioses that would be selected are those that grow rapidly when temperature and moisture conditions are favorable (169). These associations could be either annuals or perennials. Peoples et al. (237) reported that perennial pastures containing lucerne (*M. sativa*) provide consistently greater annual herbage production and fix an average of 90 to 150% as much N₂ as do neighboring subterranean clover-based pastures. In the 1994 drought (in Australia), when annual pastures failed, lucerne still managed to fix >70 kg of N ha⁻¹. It is proposed that lucerne-based pastures could represent a more reliable means of improving

soil fertility than annual pastures. A positive correlation was found between proline accumulation and drought tolerance of legumes (112, 120, 172). In a recent study, Straub reported that under mild water stress, soybean plants inoculated with bacteria that were unable to catabolize proline suffered twice the percent decrease in seed yield as did plants inoculated with bacteria that were able to catabolize proline. These results suggest that increasing the oxidative flux of proline in bacteroids might provide an agronomically significant yield advantage when the stress is modest. Various criteria which influence the competition and saprophytic competence of rhizobia under environmental factors such as moisture deficiency should be considered. These criteria were recently reviewed (334) and include rhizobial movement in the soil, inoculum placement, legume root exudates precluding the early stages of nodulation, and competition for nodule induction.

Naturally occurring forage legumes (annuals and perennials) are well nodulated, and their root nodules are active in fixing N₂ (3, 359). These legumes may be found in desert or in cultivated lands as wild plants. Recently, the suitability of rhizobium-inoculated wild herb legumes for providing vegetation cover and improving soil fertility in unreclaimed lands was suggested (166). Isolation of effective rhizobia from wild legumes to inoculate other legume crops is a new strategy to improve the efficiency of the *Rhizobium*-legume symbiosis. The rhizobia of wild legumes may have better traits than the homologous rhizobia. *Rhizobium* strains from *Astragalus cicer* successfully nodulate *M. sativa* and *P. vulgaris* (368). Rhizobia isolated from the wild plants of northern deserts and cultivated lands of Egypt (365a) formed effective nodules on *V. faba* and *P. sativum*.

One of the adaptations of legumes to arid lands (poor in N and P) and those with low moisture availability is their infection by mycorrhizal fungi in addition to *Rhizobium*. Mycorrhizal inoculation has alleviated the effects of drought stress on *Acacia* and *Leucaena* under arid conditions (263). Recent reports support the ability of mycorrhizal infection to improve N₂ fixation by legumes under drought stress (257, 260). *Rhizobium* bacteria, arbuscular mycorrhiza, and plant-growth-promoting bacteria were isolated from a representative area of a desertified semiarid ecosystem in the south-east of Spain (260). Phosphate-solubilizing bacteria could release phosphate ions from soil in association with mycorrhiza (321). The biodegradation of phosphonycin (an antibiotic) by *Rhizobium huakuii* was also reported (205). This bacterium was able to degrade up to 10 mM phosphonycin as a carbon, energy, and phosphorus source and to release inorganic phosphate (P_i). High temperatures may prevail in arid regions; therefore, the drought-tolerant symbiotic systems that are selected should also be heat tolerant. High soil temperature (35 to 40°C) usually results in the formation of ineffective nodules; however, several strains of rhizobia, e.g., *R. leguminosarum* bv. phaseoli, were recently reported to be heat tolerant and to form an effective symbiosis with their host legumes (155, 210). These associations will be relevant for cultivation in arid climates.

Salt-tolerant *Rhizobium*-legume symbiosis. An allied concern with regard to soil moisture in the reclamation of environmentally impacted sites, e.g., arid lands, can be the effect of salinity on the survival of rhizobia in soil systems. Salinity affects the survival and distribution of rhizobia in soil and the rhizospheres of plants (165, 313); however, salt-tolerant rhizobia were isolated from various crop and wild legumes (see above). Some of these rhizobia are tolerant to higher levels of salts, up to 1.8 M NaCl. These salt-tolerant rhizobia underwent morphological and metabolic changes, as well as structural modifications, to cope with and adapt to salt stress. However,

effective salt-tolerant rhizobia were isolated from nonsaline as well as saline environments. Recent reports support the finding that some rhizobia have the potential to form a successful symbiosis with legumes under salt stress. Most of the work done emphasizes the previous understanding about the sensitivity of the host legumes to salt stress. Therefore, salt-tolerant legume genotypes should be selected. In the last few years, rhizobia which form successful N_2 -fixing *Rhizobium*-legume symbioses under salt stress (up to 120 to 150 mM NaCl) have been selected. Examples are strains of *R. meliloti* and *R. leguminosarum* bv. trifolii from forage legumes (95), soybean (97) and woody legumes (75, 188, 370). In a recent study reported by Mashhady et al. (203), *R. meliloti* formed a successful symbiosis with *Medicago sativa* under saline conditions (100 mM NaCl). These rhizobia are local strains isolated from Saudi Arabian soil in arid lands. Also, recent reports point out that rhizobia from naturally growing tree legumes in the deserts are prominent and effective salt-tolerant rhizobia.

Significance of woody (tree)-legume-*Rhizobium* symbioses to the rehabilitation of arid regions. The use of leguminous trees for a variety of food, feed, and fuel wood purposes in semiarid regions has been reviewed (105, 108). Trees of the genera *Acacia* and *Prosopis* are of central importance in the rural economy of many of the world's arid and semiarid areas. Species of both genera provide high-quality animal fodder, timber, fuel wood, charcoal, gums, and other products, as well as contributing to soil stabilization and improvement through N_2 fixation (105). Their particular value in arid zones lies in their extreme resistance to heat, drought, salinity, and alkalinity; they are better able to establish growth in disturbed areas of arid regions than are herb legumes (242). Tree legumes are widely distributed (*Acacia*, in particular, is native to every continent), they are widespread in Africa and the Middle East (209), and many are valuable for fixing atmospheric N_2 . It has been reported that woody legumes, e.g., *Prosopis*, are well nodulated under drought stress; however, the value of many shrubby and woody legumes in arid areas probably lies in their extensive, deep root systems, in addition to their potential to fix N_2 (164, 302, 357). *Prosopis* forms a unique system of deep roots with significant tolerance to water stress (226).

Evaluation of the nodulating ability, N_2 fixation, and agroforestry potential of woody legumes has been the subject of many recent reports (170, 204, 317, 369). These recent studies prove that a high percentage of the trees examined form effective nodules. The N_2 fixed (measured as a percentage of the total nitrogen) in the tree legumes *Leucaena*, *Albizia* and *Cliricidia* ranged from about 20 to 74, 28 to 72, and 44 to 84%, respectively (170). In one of the most recent and interesting studies, Dakora and Keya (79) determined the contribution of some tree legumes to soil fertility. Tree legumes fix about 43 to 581 kg of $N\ ha^{-1}$, compared to about 15 to 210 kg of $N\ ha^{-1}$ for grain legumes (79). This high N_2 -fixing potential makes leaf pruning of these tree legumes an important component of sustainability in agroforestry and soil fertility. It was estimated that in a single year, the pruning of a tree or shrub like *Sesbania sesban* can provide up to 1 ha of cereal crop, up to 448 kg of N, 31.4 kg of P, 125 kg of K, 114 kg of Ca, and 27.3 kg of Mg, thus making the foliage of this legume the "ideal" fertilizer. In arid regions, where soil moisture and low fertility often limit yields, research on neglected symbiotic native tree legumes with nitrate and drought-tolerant traits would constitute a sound basis for increased sustainable production in arid regions.

Tree legumes are nodulated by a diverse group of rhizobia (*Rhizobium* and *Bradyrhizobium*). Some isolates of these rhizobia have been phenotypically and genotypically character-

ized (358, 364, 367); however, the potential of these root nodule bacteria as symbionts with tree legumes has not yet been explored. Woody-legume rhizobia, e.g., *Prosopis* rhizobia, were isolated from the surface layers of desert soils which are usually exposed to fluctuating environmental conditions, e.g., soil moisture, temperature, and salinity (164, 165). Under these conditions, fast-growing rhizobia are more relevant than slow-growing ones. One interesting approach is the usage of rhizobia from woody legumes as an inoculum for crop legumes. Wange (338) succeeded in obtaining effective symbioses between woody rhizobia from *Acacia* and other trees with peanut and cowpea. This symbiosis was more effective than the symbiosis between the trees and their homologous rhizobia. This new approach is the focus of interest of several nitrogen fixation laboratories, including ours.

CONCLUSIONS

This review recognizes the role of BNF as a nonpolluting and more cost-effective way to improve soil fertility compared to other ways, such as fertilizer-N and sewage sludge, with their high levels of toxic metals. The *Rhizobium*-legume symbiosis is superior to other N_2 -fixing systems with respect to N_2 fixing potential and adaptation to severe conditions. Several symbiotic systems of legumes which are tolerant to extreme conditions of salinity, alkalinity, acidity, drought, fertilizer, metal toxicity, etc., were identified. These associations might have sufficient traits necessary to establish successful growth and N_2 fixation under the conditions prevailing in arid regions. In fact, the existence of *Rhizobium*-tree legume symbioses, which are able to fix appreciable amount of N_2 under arid conditions, is fascinating. These symbioses represent the best source of the "ideal" fertilizer in arid regions and therefore command great interest as the subject of future research.

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