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Factors Affecting the Efficiency of Symbiotic
Nitrogen Fixation by Rhizobium

Abdullah M. K. Al-Falih
Riyadh Teachers College, Department of Science (Biology),
P. O. Box 4341, Riyadh 11491, Saudi Arabia

Abstract: Recent reports pointed to a decline in agricultural dependence on symbiotic nitrogen fixation, and in the use of rhizobial inoculants. The aim of the present review was to study the environmental factors that affect the efficiency of symbiotic nitrogen fixation by *Rhizobium* in soil. These factors included pH, salinity, moisture, temperature, microorganisms, organic matter and soil texture. The overall conclusion is that symbiotic nitrogen fixation by *Rhizobium* is a critical biological process. Environmental stresses are generally the limiting factors of the symbiotic nitrogen fixation. With the selection of the appropriate legume and rhizobial inoculant, nitrogen fixation can be increased and concomitantly food production can be improved even under environmentally stressed conditions.

Key words: Nitrogen fixation, *Rhizobium*, environmental, soil, fungi, bacteria pH, salinity, moisture, temperature, microorganisms, fertility

Arable land resources are limited. Meeting food needs in some regions has already led to the adoption of agricultural practices that can degrade the soil, and to the use of land that is marginal for crop production. The situation is most serious in the tropics, due to fragile soil structure, erosion, low soil organic matter and inappropriate farming technologies (Hungria and Vargas, 2000). Biological nitrogen fixation is a critical and key process in sustainable land management, where nitrogen is the nutrient that most frequently limits crop production. Tropical environmental conditions can constrain nitrogen fixation and need further study. To adequately feed world's increasing population it is essential that food production be increased in the third world where it is most needed. This aim is the mandate of the international institutes funded by the Consultative Group on International Agricultural Research and the many national agricultural institutes throughout the tropical world. Moreover, an increasing number of scientists in the developed countries are becoming involved in solving problems which relate directly to tropical agriculture.

The potentially important role of legumes in maintaining soil fertility is well established. Grain legumes provide valuable nutritious seed and when effectively nodulated, can yield in nitrogen-deficient soils where cereals and other non-leguminous crops would barely survive (Eaglesham and Ayanaba, 1984). It is no coincidence that legumes are a component of many of the traditional farming systems throughout the tropics. If food production is to be increased with more productive farming systems, the package of improvement practices is likely to include an increased input of biologically fixed nitrogen to complement the use of fertilizer nitrogen if it is available. It is important to bear in mind, however, that contrary to popular opinion, the growing of a legume crop does not necessarily result in a nitrogen gain for a farming system, except where an effectively nodulated forage legume is ploughed under as a green manure (Eaglesham and Ayanaba, 1984).

If more nitrogen is removed through the harvested grains than was fixed in the nodules, a net depletion results, even if all vegetative residues are ploughed under. The greater the amount of soil nitrogen that is available to inhibit nodulation and fixation and higher the harvest index for nitrogen, the more is the possibility that a nitrogen depletion would occur (Eaglesham *et al.*, 1982). The correct grain legume should be selected for a specific use, it should give high grain yield at the possible expense of some soil nitrogen or less grain yield with the expectation of nitrogen accretion to the soil.

Rhizobial strains inoculation: Inoculation of legumes with superior rhizobial strains in order to improve crop productivity is a common practice in many countries, but often it does not result

in the expected yield increase. A frequent problem is the failure of the inoculum strain to occupy a significant proportion of the nodules (Vlassak and Vanderleyden, 1997). Six genera of bacteria, collectively called *Rhizobia*, are able to form nitrogen-fixing nodules on the roots of legumes and the non-legume Parasponia (Hungria and Vargas, 2000).

Better performing *Rhizobium* strains, at least under laboratory conditions, are being constructed by different methods (Al-Rashidi *et al.*, 1982; Bhardwaj, 1975; Ethiraj *et al.*, 1972; Vlassak and Vanderleyden 1997; Lowendorf and Alexander, 1983). However, until solutions to the nodule occupancy problem are found, these advances would be of limited agronomical benefit. The continued inability to solve this problem is due to its complexity and the inconsistency of the findings. Soils and their microbial inhabitants vary to such a great extent that generalizations about microbial ecology cannot be made. Reviews by Bottomley (1992) and Vlassak and Vanderleyden (1997) give a good picture on factors affecting, Rhizobial survival and competition for nodule occupancy.

Vlassak and Vanderleyden (1997) reported that the inoculant rhizobia must often compete with the indigenous, often ineffective, rhizobial soil population. First, they need to adapt to the prevailing environmental conditions and multiply in the soil and host rhizosphere, then they have to compete on the legume roots for infection sites and nodulation. After harvest, the persistence of the introduced, more efficient strains as soil saprophytes is often desired. However, the latter view is probably not shared by inoculant companies.

Nitrogen fixation: Those involved with rhizobia and legumes should not assume that "improving" nitrogen fixation is necessarily the main priority in increasing legume production (App and Eaglesham, 1982). With judicious use of fertilizers, water management, insect control, disease control, superior cultivars etc. the inputs of biologically-fixed nitrogen may increase concomitantly and spontaneously. On the other hand there is no room for complacency, the natural environment is constantly changing and field crops are subjected to stresses throughout the growth cycle. There exist too many gaps in the understanding, particularly in terms of the responses of biological nitrogen-fixing systems to these stresses.

Nitrogen (N) occurs in the atmosphere as N_2, a form that is not useable by vascular plants. Nitrogen must first be fixed, or reduced, to ammonia (NH_4^+) by prokaryotic organisms such as eubacteria and cyanobacteria. Nitrogen fixation is highly important for the nitrogen nutrition of leguminous plants in natural and agricultural systems. Biological nitrogen fixation is a phenomenon occurring in all known ecosystems and is undoubtedly of greatest agricultural importance.

Symbiotic nitrogen fixation involves different hosts and microsymbionts between legumes and bacteria belonging to the genera Rhizobium, Bradyrhizobium and Azorhizobium. Symbiotic nitrogen fixation is dependent on host cultivar and rhizobia, but as well may be limited by pedoclimatic factors especially those associated with the soil pH (Bordeleau and Prevost, 1994).

Limitation of soil nitrogen: Recent years have witnessed striking discoveries in the genetics and biochemistry of nitrogen fixation, and it is likely that new and exciting finding will be forthcoming in the next decade. Nevertheless, not withstanding the far greater understanding that we now have about certain traits of nitrogen-fixing bacteria and blue-green algae (cyanobacteria), research is moving in a direction that will have only a modest impact on increasing the amount of nitrogen that is fixed in agricultural land (Alexander, 1985). These studies are largely concerned with the genetics and biochemistry of nitrogen fixation and those ancillary physiological processes that have an effect on the fixation, such as energetics and enzyme regulation. However, nitrogen fixation in agricultural ecosystems is rarely limited because of the absence of highly active nitrogen-fixing microorganisms. This point has often been stressed by individuals concerned with the practical aspects of nitrogen fixation, but it seems to have been forgotten under the weight of the voluminous literature on the biochemistry and genetics of nitrogen-fixing organisms (Alexander, 1985).

The limiting factor is not the absence of organisms with the genetic or biochemical potential to bring about appreciable nitrogen gains; rather, it is one or more ecological constraints that hold these organisms in check. The enormous progress made in recent years in understanding the biochemistry and genetics of nitrogen-fixing microorganisms frequently is only leading to improved organisms when no improvement is needed in the near future (Alexander, 1985).

Conversely, little attention is being given to overcome the environmental stresses that prevent the presently available, active nitrogen-fixing microorganisms from doing what the laboratory and greenhouse studies suggest they ought to do. Identifying these stresses should facilitate the development of practical means for overcoming the stresses and help agricultural scientists obtain free-living or symbiotic nitrogen-fixers that not only are active in bringing about the desired reaction in vitro, but can also survive, grow and be beneficial in agricultural environments.

An essential aspect of the strategy to improve the yields of tropical legumes in stressed environments must involve combining stress-tolerant cultivars with stress-tolerant rhizobia. The latter may exist already in the soil or be introduced as seed- or soil-applied inoculants (Brockwell, 1982). Legume screening techniques should be inexpensive and simple in design so that large numbers of plant types can be checked quickly. Of course, preliminary work is required to determine the limits of environmental stress necessary to distinguish types of different tolerances.

Characterization of rhizobia: Possibly the best source of superior rhizobia for use in stressed environments is the soil in that or a similar environment. Having isolated rhizobia from nodules of a plant growing in, or inoculated with, the soil, and after verifying stress resistance by the appropriate screening technique the most effective and competitive isolates may be cultured and added back to the soil in an inoculant to greatly increase their numbers and therefore also their nodulation potential. The rhizobial characteristics of three tropical soils have been examined with the aim of identifying superior strains and of making comparisons of rhizobial diversity in the three soils (Ahmad et al., 1981). The cowpea-nodulating rhizobia were found to be diverse, both within and between locations in terms of colony morphology, salt tolerance, high temperature tolerance, host promiscuity, serological and biochemical characteristics (Ahmad et al., 1981). As far as salinity tolerance is concerned, the available information indicates that legume growth is generally more sensitive to saline conditions than rhizobial growth. In breeding salt tolerance into

legumes, allowance should be made for the possibility that the nodulation process may be particularly saline sensitive. Breeding materials should be screened with low levels of mineral nitrogen available in the presence of appropriate rhizobia.

In general, as an aid to the improvement of agricultural productivity a large range of types of legumes and rhizobia are available. With the selection of the appropriate legume and, if necessary, combining it with the appropriate rhizobial inoculant and by paying due to mineral nutrition and agronomy, legume nitrogen fixation can be increased and concomitantly food production can be improved even under environmentally stressed conditions.

Factors affecting N-fixation: Arid and semiarid soils are generally N-limited and biological soil crusts can often be important sources of N input. For this reason, it is important to understand the factors that influence nitrogen fixation and its release in soil crusts. At given adequate soil nutrients and alkaline pH, the major factors controlling nitrogen fixation rates are temperature, light, and moisture (Belnap, 2001).

Other factors also influence nitrogen fixation rates such as high soil salinity, high soil nitrogen, phosphorus deficiency and soil acidity. are the environmental factors controlling nitrogen fixation in mediterranean rice field Quesada et al. (1997). The overall conclusion is that nitrogen fixation may be an important nitrogen input in the nitrogen cycle, and could lessen pollution problems by lowering the demand for chemical fertilizers. Graham and Vance (2000) wrote a review contrasts the potential contribution of biologically fixed nitrogen to intensive and extensive agricultural systems, and examines opportunities for continued major contributions in the latter. They identified six research and extension areas in which in-depth efforts are still needed and examined some opportunities for improved nitrogen fixation likely to arise through advances in molecular biology.

Arid and semiarid soils are often deficient in nitrogen, therefore biological nitrogen fixation is a key process to agricultural sustainability. However, nitrogen fixation may be limited by different environmental stressful conditions (Hungria and Vargas, 2000). Appropriate soil-management practices for the tropics, e.g. no-tillage, which result in decreases in soil temperature and increase in soil moisture, also benefit nitrogen fixation. Studies are just starting to elucidate mechanisms associated with tolerance to these stress conditions, and effort will be needed to bring such basic information to practical use (Hungria and Vargas, 2000).

Graham (1992) summarized that until 10 years ago, studies of environmental stress and the legume-Rhizobium or Bradyrhizobium symbiosis were restricted to defining the problem, and to using physical or chemical amendments to overcome it, i.e., mulching to reduce soil temperature or liming to ameliorate soil acidity. The identification of bacterial strains and in some cases host cultivars that are tolerant to these stresses opens the way for alternate, lower cost solutions to these problems. Although we will not be able to eliminate many of the stresses currently limiting crop production under low-input conditions, we should be able to identify better host-strain combinations and develop other ways to minimize the impact of stress, giving rise to a more sustainable agriculture. Thus environmental factors can influence the ratio between rhizobia in soil and rhizosphere, and can also influence other steps in the nodulation process, such as attachment, infection and nodule formation, resulting in a different outcome of competition. Various abiotic factors have been reported that significantly influence nodule occupancy (Vlassak and Vanderleyden, 1997).

The objective of this review is to focus on the main environmental factors commonplace in agriculture and how they may affect survival of Rhizobium in soil, the root-nodulation process and the functioning of the effectively nodulated legume. Attention will be given to the environmental stresses that are probably most important in restricting the activity of either indigenous nitrogen-fixers or those that are introduced in nature as a result of inoculation.

The factors that affect the survival, metabolism, and functioning of Rhizobium in soil are as follows:

Soil pH: Much of the arable land of the world is acidic, and widespread agreement exists among agronomists that acidity is a major constraint for legume cultivation in these regions. Moreover, sample evidence exists that the survival and growth of many of the most active strains of Rhizobium that have been tested and the nodulation that these organisms effect are deleteriously influenced at low pH. On the other hand, although many legumes are acid-sensitive, even when growing on fixed nitrogen, many grow well in acid conditions. Even in a single legume species, for example, alfalfa (Jo et al., 1980), tolerances to soil acidity vary among varieties, and plant breeders are currently endeavoring to develop varieties able to cope with significant stress. However, nodulation is more markedly affected at low pH than is root development or plant growth; this has been observed for alfalfa (Jo et al., 1980) and peas (Mulder et al., 1966). Apparently, some phase of the infection process induced by the bacteria is inhibited at low pH, although root development and bacterial growth do not show this high sensitivity (Evans et al., 1980).

In other instances, as with Medicago truncatula, the absence of nodulation in acid soils may result from the inability of R. meliloti to survive or grow (Alexander, 1985). Nodulation failures due to poor survival of rhizobia in acid soils are particularly likely when inoculation is not practiced every year, a common occurrence even in developed countries. Indeed, frequent inoculation of legumes growing in acid soils was recommended some 60 years ago (Bryan, 1923). If nodules are not formed at these low pH values, the plant may develop using the fixed nitrogen, but nitrogen gains will not occur. Serradella and alfalfa, for example, continue to grow at the expense of fixed nitrogen, even when the low pH prevents dinitrogen fixation (Alexander, 1985).

Some species of Rhizobium do not multiply in culture even at moderate acidities, e.g., R. meliloti often does not grow below pH 5.3. Other species have greater tolerances, however and some strains of R. phaseoli are able to multiply at pH 3.8 (Lowendorf and Alexander, 1983). The pH range for survival is expected to be wider than that for replication, but many of the active nitrogen fixers even fail to survive in sterile soil (where suppression is not a result of some harmful microorganisms) at pH 5.2 (Lowendorf et al., 1981). Nevertheless, strains of a single species of Rhizobium vary in their pH sensitivity, whether sensitivity is assessed by growth in culture, survival in sterile soil, or nodulation of host plants (Lowendorf and Alexander, 1983; Lowendorf et al., 1981; Thornton and Davey, 1983). Such strains may be useful as legumes inoculants because they nodulate at pH values at which other strains do not (Mulder et al., 1966; Munns et al., 1979) or because they can survive longer in acid soils.

Acid soils frequently contain levels of Al, Mn, or Fe that may be injurious to nodulation or growth of rhizobia. From the viewpoint of developing resistance in the nitrogen fixing symbiosis, attention has only been given to Al. Rhizobium strains can be selected that vary in their ability to tolerate Al in culture (Alexander, 1985) and differences in sensitivity are also evident in the behaviour of such strains in soil (Hartel et al., 1983). Nevertheless, the Al stress in nature appears to affect the host plants and not the rhizobia.

In an acid soil at Onne in Nigeria (pH 4.6, annual rainfall 2500 mm) the cowpea rhizobial count was 4.5×10^4/g soil, whereas at Maradi in the sahel-savannah zone in Niger Republic (pH 6.1, annual rainfall 600 mm) the count was 4.9×10^2/g soil (Eaglesham and Ayanaba, 1984). Although slow-growing rhizobia are in general more tolerant of low pH than the fast-growers, strains to strain differences exist. Rhizobium meliloti is particularly sensitive to acid conditions. Some slow-growing rhizobia native to acid soils are acid-requiring and grow only at approximately pH 4.5 (Eaglesham and Ayanaba, 1984).

Indeed some species actually grow better at pH 4.0 than in less acidic conditions, e.g. Stylosanthes humilis (Andrew, 1976).

Legumes dependent on the root nodule symbiosis for nitrogen showed a range of responses to low pH, but in general nodulation was reduced or eliminated at pH values below 5 (Munns et al., 1977). In a survey of the effects of liming on eight soils of pH 3.4-4.25 the critical pH for nodule initiation and development in soybean was in the range 4.5-4.8 (Mengel and Kamprath, 1978). For rhizobia optimum pH is neutral or slightly alkaline, but they are differently sensitive to acidity (Yadav and Vyas, 1971a).

Depending on the degree of tolerance to acid conditions, some species respond more strongly than other to lime application. Legumes in acid soils dependent on nodule fixed nitrogen generally derive greater benefit from liming than when there is sufficient mineral nitrogen available (Andrew, 1976). High levels of lime application, where pH is raised to 6-7, can have deleterious effects on plant growth. It appears that acidity is less of a constraint to cowpea rhizobial survival in soil than is desiccation or high temperature (Eaglesham and Ayanaba, 1984).

Adsorption of Rhizobium meliloti in low numbers to alfalfa (Medicago sativa L.) roots was dependent on the presence of divalent cations and required neutral pH (Caetano-Anolles et al., 1989). The adsorption was abolished and viability decreased at pH 6. When pH is lowered, higher Ca concentrations were required to attain similar adsorption levels, including a marked interactive effect between Ca^{+2} and H^+ ions. Caetano-Anolles et al. (1989) showed that low pH appears to affect the stability of binding causing desorption of the previously bound bacteria. Also they suggest that rhizobial binding to the root surface already shows the Ca and pH dependence of alfalfa nodulation, which was generally associated to some event prior to rhizobial penetration of root hairs. Acid soil is a complex problem as it is often correlated with high concentrations of aluminum and manganese, as well as deficiencies of phosphate, molybdenum, and (but less often) calcium. At low pH, nodulation of white clover, subclover, pea, alfalfa and bean is reduced, even in the presence of high rhizobial numbers (Vlassak and Vanderleyden, 1997).

The effect on nodulation of increased available aluminum or manganese, characteristic for acid soils, often very small as compared to the pH effect (Hartel and Alexander, 1983) or these effects are difficult to distinguish. However, nodulation appears more sensitive than plant growth to aluminum (Graham, 1992). Hartel and Alexander (1983) observed superior nodule-inducing capacity by aluminum-tolerant compared to aluminum-sensitive strains after equal survival in an aluminum-rich rhizosphere. Under acidic conditions, the availability of phosphate could be a limiting factor as this element may be immobilized as iron or aluminum phosphates. A delay in infection was observed for nodulation of some legumes when grown under phosphate limitation (Vlassak and Vanderleyden, 1997).

Salinity and alkalinity: Many soils are rich in salt and have high pH values and these areas are often considered to be undesirable for legumes. For example, berseem clover, guar, cowpeas, and lentils nodulate poorly in high saline-alkali soils of India, and peas may be wholly devoid of nodules (Bhardwaj, 1974). Because of these sensitivities, many studies have been performed to establish the effect of salts on the growth of rhizobia in culture. These investigations have demonstrated that inhibition of growth of the bacteria usually requires high salt levels (Alexander, 1985). Comparisons of the sensitivities of microorganisms and plants, moreover, show that the bacteria are able to proliferate at salt levels that do not permit growth of the host (Bhardwaj, 1975). However, it is not correct to assume that as replication bacteria is not seriously affected, no problem related to the symbiosis, exists. First, the rhizobia do not survive in some of these soils, whether it is possible to exploit the differences among Rhizobium strains in salt tolerance for growth or the ability of some rhizobia to "acclimate" or mutate to even higher levels of salt tolerance, as these have been shown several times (Ethiraj et al., 1972; Mendez-Castro and Alexander, 1976), is not now certain. Second, nodulation is more sensitive than root development and may be

affected by salinity in its early phases (Singleton and Bohlool, 1984). So, alkali and salt-induced delays in nodulation of berseem clover and lentils may result in reduced yields (Alexander, 1985), suggest that because strains of Rhizobium exist that are able to survive at high salinities, attention should be given to the influence of salinity on aspects of the symbiosis other than the survival of the bacteria.

In reviewing the literature salinity emerges as a basic problem. The many different notations used to quantify salinity often make it difficult to readily compare data from different studies. Saline soils are common in regions of arid or semi-arid climate where transport of soluble salts to the ocean does not occur because of low rainfall. They are characterized by the presence of high levels of neutral salts in the surface layers resulting from the capillary rise of water when evaporation exceeds precipitation (Eaglesham and Ayanaba, 1984).

The predominant salts are usually sulphates and chlorides of sodium, calcium and sometimes magnesium and small quantities of carbonates and bicarbonates are often present. These soils are only moderately alkaline, with pH about 8, and their agricultural use usually demands irrigation. Rhizobia exhibit a large range of sensitivities to salinity. Three of eleven diverse rhizobial types failed to grow at 0.6% sodium chloride and the other showed a 3.7 fold range in sensitivity in terms of fractional reduction in growth (Singleton et al., 1982).

Rhizobium trifolii appears to be a saline sensitive species, eight strains isolated from Trifolium alexandrinum were completely growth-inhibited at 0.7 percent sodium chloride (Pillai and Sen, 1966) and another six T. alexandrinum isolated had their growth severely reduced or eliminated at 1 percent levels of chlorides and sulphates of sodium, potassium and magnesium (Ethiraj et al., 1972). In contrast five rhizobial strains from Sesbania cannabina, Crotolaria juncea and soybean were uninhibited by 1% levels of these same salts and four of them grew at 3% levels (Yadav and Vyas, 1973).

The growth of six strains isolated from Dolichos lablab was unaffected or actually stimulated at sodium chloride levels up to 1.6 percent (Pillai and Sen, 1973). Four strains of R. meliloti grew well at 3 percent levels of chloride and sulphate salts of sodium, potassium and magnesium. A fifth strains of R. meliloti showed differential tolerance to these salts, it was 17, 49 and 80% inhibited in growth at 3% levels of sodium chloride, magnesium chloride and magnesium sulphate respectively, but failed to grow at only 0.4% potassium chloride and 0.6 percent potassium or sodium sulphate (Singleton et al., 1982).

No correlation was found between the ability of a Rhizobium strains to grow at 0.6% sodium chloride and whether it originated from a saline or non-saline soil (Singleton et al., 1982). Considering this in conjunction with the fact that rhizobia are differentially tolerant to high levels of different salts leads to the conclusion that rhizobial growth studies in saline media should be interpreted with caution. Moreover, rhizobia may survive in soils at salinity levels much higher than those at which growth is restricted or eliminated in media (Singleton et al., 1982).

The addition of salt to three soils of different textures greatly reduced the ability of four strains of R. japonicum to withstand air-drying. Survival was best in the more organic, higher clay soil and there were significant differences in the numbers of the four strains after four weeks (Al-Rashidi et al., 1982). The degree of salinity tolerance of a Rhizobium strain appears to be of only limited use in predicting the effects of salinity on its ability to form root nodules. The nodulation potential of eleven strains of R. meliloti was significantly reduced at 0.4% sodium chloride and eliminated at 0.7% sodium chloride, although none of the strains was significantly growth-inhibited by these salt levels in liquid media (Eaglesham and Ayanaba, 1984). Extract of a saline soil which eliminated infection thread formation in alfalfa did not affect survival of R. meliloti (Lakshmi-Kumari et al., 1974).

Very large differences in salt tolerance were found among twelve pasture and forage legumes. Russell (1976) made a survey of the salt tolerance of 31 plant species included temperate legumes, tropical legumes and tropical grasses. The legumes were inoculated by rhizobia, but the excellent growth of the grasses indicated that the soil was abundant in nitrogen. The main salinity values which reduced plant yield by 50% were estimated by a mathematical model to be 7.6, 6.4 and 14.2 (in sodium chloride equivalents: 0.5, 0.4 and 0.9%) for the temperate, the tropical legumes and grasses respectively; mean values of soil salinity which reduced plant yields to zero were 11.9, 12.6 and 21.6 (0.75, 0.8 and 1.4%). The most salt tolerant legume was alfalfa (10.2 mS/cm for half yield and 18.8 mS/cm for zero yield. These levels of salinity which had serious effects on legume growth would not be excessive for many rhizobial strains, as detailed above.

Few studies have been reported on the effects of salt stress on legumes grown with nitrogen supplied in comparison with those dependent on nodule-fixed nitrogen. It is usually difficult, therefore, to appraise to what extent the adverse effects of salinity were on nodulation and fixation rather than on plant growth. The indications were that root-nodule dependent legumes are more saline-labile than when nitrogen-fed. This has been clearly shown in the case of Cicer arietinum, although significant variation in effectiveness among rhizobial strains existed at 0.46% sodium chloride (Lauter et al., 1981). The growth of nodulated Glycine wightii was more adversely affected by a two-week salinity treatment of 8 mS/cm than that of nitrogen fertilized plants (Wilson, 1970). The nodulation process, nodule development and the amount of nitrogen fixed were all inhibited, but were quickly regained after removal of the stress. Tissue injury resulting in leaf abscission occurred only in the nodulated plants, probably the result of nitrogen stress coupled with saline stress, but no nodule loss was noted indicating adaptability of nodules to increases in substrate salinity (Wilson, 1970).

A comparison of salinity effects was made with alfalfa and soybean, using both nodulated and nitrogen-fertilized plants (Bernstein and Ogata, 1966). The two species responded differently to sodium chloride application up to 8 mS/cm. At the highest salinity, alfalfa nodule weight was reduced by only 18% whereas the whole plant weight was reduced by 55% in both the nodulated and non-nodulated plants. At the same salt level soybean nodule weight and nodulated plant weight were both reduced by about 80% whereas nitrogen-fed soybean weight was reduced by only 60%. Clearly the root nodule symbiosis in alfalfa was relatively resistant to saline conditions, while adverse effects on the symbiosis contributed to poor growth in soybean. However alfalfa's salinity tolerance varied with the salt. Potassium chloride had a more strongly adverse effect on alfalfa nodulation than did magnesium chloride. At 0.3%, both salts significantly reduced nodulation but had no effect on seed germination or early seeding growth (Eaglesham and Ayanaba, 1984). Nodulation of the soybean cultivar Amsoy was inhibited at sodium chloride concentrations above 0.6% (9.8 mS/cm) and eliminited at 1.2%. Microscopic examination of the roots showed that at 1% sodium chloride, very few rhizobia were attached to root hairs and very few root hairs were deformed, indicating a salinity-induced lesion in the very early stages of the infection process (Tu, 1981). Detached soybean nodules were sensitive to relatively low levels of salinity, their acetylene reducing activity being reduced by approximately 20% by contact with a sodium chloride solution of 0.25% (Sprent, 1972).

Large variations in sensitivity to salt stress may exist between cultivars of a single species. When salt was applied in irrigation water from three weeks after planting to soybean, two cultivars, Lee and N53-509 were unaffected in their seed yields at a soil salinity of 9.6 mS/cm, whereas four other cultivars including Jackson and Improved Pelican failed to yield (Eaglesham and Ayanaba, 1984). Nitrogen was supplied at 112 kg ha^{-1} so no conclusion may be drawn regarding relative tolerances of the symbiosis. There was no correlation between the effects of salinity on seed germination and its effects on later vegetative and reproductive growth, e.g. at 10.1 mS/cm the germination rates of

Lee, Jackson and Improved Pelican were equally reduced, by 15-20 percent (Abel and Mackenzie, 1964).

A rapid decline in nitrogen fixation by Allocasuarina verticillata occurred when salinity levels increased above 2.3 mS/cm and the loss in nitrogen fixation was strongly correlated with increasing soil salinity (Hopmans et al., 1983). Saline, alkaline and acid conditions are far from optimum for rhizobia. Yadav and Vyas (1971a) found that 2% salinity level was lethal for most Rhizobium sp. and strains of R. trifolii are very sensitive to salinity, but lucerne (Medicago sativa L.) and cowpea (Vigna sinensis L.) are salt resistant. For rhizobia optimum pH is neutral or slightly alkaline, but they are differently sensitive to acidity, while pH of 10 is critical for all Rhizobium sp. (Yadav and Vyas, 1971b).

Alkaline soils can develop from saline soils with low calcium reserves. After a drop in the water table, soluble salts are washed down the profile and exchangeable calcium is replaced by sodium. Soil carbon dioxide forms carbonate and bicarbonate ions and these react with sodium to raise the pH (Webster and Wilson, 1980). Soil conductivity is usually less than 4 mS/cm with the pH in the range 8.5-10. Less intensive leaching of saline soils with higher calcium content can produce saline-alkaline soils with a conductivity of more than 4 mS/cm and a pH usually less than 8.5 (Webster and Wilson, 1980).

Clearly, the constraints to rhizobial survival, nodulation and legume growth pertaining to saline soils also apply to saline-alkaline soils. Very few data have been reported on the effects of high pH on rhizobial growth, nodulation or legume growth. Six legume species, Melilotus parviflora, Sesbania aculeata, Trifolium alexandrinum, Cyamopsis tetragonoloba, cowpea, lentil and pea, were planted in a highly saline-alkaline soil (36.5 mS/cm, pH 10.3) which had been uncultivated for 65 years and were examined for nodules after 60 days (Bhardwaj, 1974). He found nodules on all species except pea, demonstrating that at least some rhizobial types can survive in extreme saline-alkaline conditions for long periods. Soil dilution and plant infection tests indicated that rhizobia capable of nodulating Melilotus parviflora, Sesbania aculeata were in preponderance at 2.8-3.0 x 10^3/g soil. In view of the saline lability of the infection process noted earlier, the observation of nodulation in this soil is surprising. However it may be significant that nodule scoring was delayed until 60 days, rhizosphere effects may have gradually ameliorated conditions sufficiently for the sparse nodulation observed. Beneficial effects of legume roots on both high and low extremes of soil pH have been reported (Eaglesham and Ayanaba, 1984).

None of 17 strains of R. japonicum showed significant growth in liquid media at pH 8.5 (Diatloff, 1970). By contrast, in two surveys of 23 rhizobial isolates from eight diverse legume species all were found to grow well in non-saline conditions at pH values up to 10 (Yadav and Vyas, 1971b). An examination of six Rhizobium species, however, demonstrated their markedly greater liability to carbonates and bicarbonates than to chloride salts (Subba Rao et al., 1974). These findings were supported by a survey of eleven rhizobia; rhizobia which grew well at 3% sodium chloride failed to grow at 0.2% sodium bicarbonate (Yadav and Vyas, 1971a).

Nodulation of alfalfa was reduced to zero in an agar medium containing 0.2% sodium bicarbonate or 0.3% sodium carbonate, but occurred, although delayed, at 0.6% sodium chloride (Subba Rao et al., 1974). Whether the adverse effect of these alkaline salts was mediated by rhizobial death, nodulation failure or poor plant growth is not clear. It appears that the carbonates and bicarbonates in alkaline soils constitute a significant stress factor in addition to high salt content and/or high pH. Some legumes such as alfalfa, fababean and clovers, produce interminate meristematic nodules which are more salt and drought tolerant than the determinate (non-meristematic) nodules formed by soybean and common bean (Bordeleau and Prevost, 1994). Only indeterminate nodules have the potential to regenerate activity in structures affected by stress treatment. Levels of salinity that inhibit the symbiosis between legumes and rhizobia differ from those that inhibit the growth of the individual symbiont. Sodium chloride concentrations that affect the symbiosis between R. meliloti and alfalfa are lower than those that affect the growth and survival of individual alfalfa genotypes or Rhizobium species (Sprent, 1984). Legumes are generally more sensitive to osmotic stress than their microsymbionts are. In contrast to their host legumes, some rhizobia can survive in the presence of extremely high levels of salt both in culture and in soil. Rhizobia isolated from arid lands are better able to nodulate the legume host under saline and drought conditions (Bordeleau and Prevost, 1994). Legumes have long been recognized as either sensitive or only moderately resistant to salinity. Most legumes response to moderate salinity with a decrease in growth. This growth depression can be attributed to the accumulation of toxic ions such as sodium and chloride in plant tissues, where they can disturb enzyme activities. Most legumes respond to the saline stress by exclusion of sodium and chlorine from the leaves.

Bala et al. (1990) isolated fifty seven Rhizobium sp. strains from tree legumes known to grow under saline conditions were screened for their ability to tolerate different levels of salinity ranging from 0-12 dS/m electrical conductivity. Four Rhizobium strains isolated were found to be highly tolerant to salinity (9-12 dS/m). There results showed that the salinity-tolerant rhizobia exhibited better survival, nodulation and nitrogen fixation than salinity-sensitive rhizobia under saline conditions. Thus, biological nitrogen fixation can be significantly increased by inoculating tree legumes with salinity-tolerant rhizobia under saline conditions. Singleton and Bohlool (1983) concluded that reduced nitrogen fixation by nodulated soybeans growing in saline environments was more a result of the effect of salt on leaflets and expansion, than the direct action of salt on the functional processes of the nitrogenase system.

A study was conduced by Surange et al. (1997) to determine the variability among Rhizobium strains isolated from different nitrogen fixing trees in growth response to high temperature, pH and salt concentrations. They reported that the variable response to increases in temperature, pH and salt concentrations was observed. Rhizobium strain isolated from Albizia lebbek survived at 50°C, while Rhizobium strains isolated from Sesbania formosa, Acacia farnesiana, and Dalbergia sissoo were well adapted to grow on pH 12.0. All the Rhizobium strains tolerated salt concentrations up to 5.0%. The tolerance of the legume host was the most important factor determining the success of compatible Rhizobium strains in forming effective symbioses under conditions of high soil salinity (Craig et al., 1991). In the presence of salt at pH 12 a high temperature (45 °C) was tolerated efficiently by Rhizobium sp. NBR1330 isolated from root nodules of Prosopis juliflora grown in alkaline soil (Kulkarni and Nautiyal, 2000).

Vlassak and Vanderleyden (1997) reported that osmotic fluctuation frequently encountered in drying and rewetting soil, and often correlated with conditions of high salinity, are very demanding for microbiological communities, resulting in poor survival and a decline of these populations. Accordingly, poor rhizobial survival or a reduced growth rate due to extreme soil moisture conditions (Pena-Cabriales and Alexander, 1983) or high salt concentrations (Tu, 1981) is common in these soils. Many studies have addressed the response of rhizobial cells to variations in the osmotic potential induced by different osmolytes. On exposure to increased osmolarity, many microorganisms adapt by elevating their intracellular osmotic tension, which is often accomplished by increasing the internal concentration of K^+ and amino acids (Vlassak and Vanderleyden, 1997).

Temperature: Nearly all areas of the world are subjected to rain-free periods for sufficient duration results in extensive drying of soil, and the rhizobia that must survive in these soils in order to bring about nitrogen fixation in the succeeding crop may thereby become too few in number to cause extensive nodulation. With many strains of all species of Rhizobium, a single exposure of the soil to drying reduce the viable population by 99% or more and several cycles of soil wetting followed by drying, a common

phenomenon of nature, reduce the population still further (Alexander, 1985). The conditions under which water is lost from the soil affects the extent of this decline. Without question, prolonged drought or even a short period of drying is a major stress on *Rhizobium*, yet little information exists on the soil properties affecting the reduction in population size (Chao and Alexander, 1981) and on microbial and other factors that are related to differences among strains and soils, although the type and amount of clay are of great importance (Osa-Aftana and Alexander, 1982b). From observations that nearly half of the viable cells of some strains of cowpea rhizobia are not killed in one drying cycle whereas more than 99% of the cells of other strains die under identical circumstances. (Osa-Aftana and Alexander 1982a), it appears that means can be devised to obtain cultures not seriously affected by the drying of soil.

Soil temperatures may increase at seeding time, during the period of plant growth and following harvest; in each instance, the high temperatures may be deleterious. The period after harvest should not be ignored, because survival of the bacteria at that time is important. In tropical and subtropical regions, the temperature near the soil surface is often above 40°C and some times may reach 60°C (Alexander, 1985). Rhizobia are subjected to these temperatures following harvest and before sowing, after planting into hot, dry soil or both. Some strains survive very poorly under such conditions; e.g., *R. trifolii* and strains nodulating *Lotus pedunculatus* and cowpeas (Osa-Aftana and Alexander, 1982b). It is the death of *R. trifolii* in fields of western Australia that is responsible for the poor growth of subterrannean clover in the second year after planting; the rhizobia fail to survive in appreciable number in the hot, dry soil at the end of the first season (Chatel and Parker, 1973). The great decline in growth is not a problem in soils of heavy texture and is prevented to some degree by certain clays. The sensitivity to heat is greater in moist than in dry soil and marked declines are evident in moist soil even at 36°C (Alexander, 1985).

For rhizobia to colonize the rhizosphere and cause nodulation, they must grow. During this time, their sensitivity to temperature is of practical significant. The optimum temperatures for growth in culture vary among strains and species, values between 27-39°C have been noted. The maximum temperatures are generally 35-39°C, but proliferation may take place up to 42°C (Munevar and Wollum, 1981a). Differences in growth and colonizing abilities probably explain why some strains are more active in nodulation soybeans at low temperatures and others are more active at high temperatures (Weber and Miller, 1972).

Studies of *Vicia atropurpurea* and *Medicago tribuloides* confirmed that certain rhizobia form more nodules at low temperatures and others produce more of the nodules at high temperature (Alexander, 1985). Hence, super nitrogen fixers could easily be displaced in nature by strains of lesser effectiveness simply because of their temperature responses. Furthermore, some strains of *R. leguminosarum* fail to induce nodulation at 30°C even though they and their host, peas, grow at that temperature (Alexander, 1985). In addition, the relative activity of the rhizobia is altered by temperature, so that a bacterium that is highly effective at one temperature is less active at different temperatures (Munevar and Wollum, 1981b). For these reasons, greater nitrogen gains probably can be achieved by improvements in the heat resistance of the symbiosis.

Rhizobium strains or species vary greatly in their susceptibility to high temperatures. Thus , *R. meliloti* survives better than *R. trifolii* on seed lying for long periods in hot, dry soil (Brockwell and Phillips, 1970), *R. lupini* and *R. japonicum* are less susceptible than *R. trifolii* when in dry soil at elevated temperatures (Chatel and Parker 1973; Marshall, 1964) and *Rhizobium* strains that nodulate and fix nitrogen similarly on *Cicer arietinum* at low temperatures exhibit differences in nodulation and nitrogen fixing activity at 30°C (Alexander, 1985). Hence, it should be possible to obtain or devise rhizobia able to survive, grow, or fix nitrogen under conditions where elevated temperatures are of importance.

Mean seasonal temperatures are higher in the drier than in the more humid zones of the tropics. In the latter, air temperatures exceeding 38°C are rare, but when the bare surface of a cultivated soil is exposed to direct sunlight its temperature can greatly exceed the air temperature (Eaglesham and Ayanaba, 1984). In seasonal rainfall areas of the tropic, crops are sown at the end of the hot dry period at the start of the rains. After planting there may be dry hot days when germinating seeds and the roots of developing seedlings are exposed to elevated temperatures. In soils low in available nitrogen, nodules of cowpea and soybean are normally initiated within six days of planting and are visible by nine days within a few centimeters of the soil surface (Eaglesham et al., 1982). The upper limits of temperature within which normal patterns of legume nodulation may be expected are still poorly understood for the agriculturally important tropical legumes. The available data indicate that rhizobial survival and/or early nodulation are likely to be adversely affected by tropical soil temperature.

In a screening of 68 rhizobial isolates from tropical legumes for temperature tolerance, only four were able to grow at temperatures in excess of 38°C, and the maximum temperature for growth was 42°C. When incubated in a sandy soil at 40°C, most of these tropical rhizobia died within ten hours (Bowen and Kennedy, 1959). The moist conditions in this experiment possibly contributed to the rapid death of the rhizobia, since it has been shown that rhizobia are likely to be less tolerant of high temperatures in moist soil than in dry, although it is not always the case (Boonkerd and Weaver, 1982).

Strains of *R. trifolii, R. japonicum* and *R. lupini* survived five to six hours at 70-80°C in dry, sandy soils (Chatel and Parker, 1973). The addition of clay to sandy soils improved the high temperature tolerance of fast-growing rhizobia, but not of slow-growing rhizobia. Concomitantly the survival of *R. trifolii* at 70°C in dry soil was better in loamy sands and sandy loams than in red, grey or yellow sands. Rhizobial tolerance of such high temperatures was suggested to be the result of spore formation, but this was later disproved (Eaglesham and Ayanaba, 1984). Twenty strains of the cowpea miscellany were examined for the ability to grow in a mannitol amended silt loam at various temperatures in the range 29-40°C. None grew at 40°C but all grew at 29, 31, 33 and 35°C with the fastest growth rate occurring at 33°C with 16 of them. However all of these strains survived seven days at 42°C in the dry silt loam without significant reduction in numbers (Osa-Afiana and Alexander, 1982b). The growth patterns of 35 strains of *R. japonicum* were examined in liquid media at constant temperatures up to 54°C. The maximum temperature for growth was 38°C shown by four strains, whereas the maximum survival temperature was 48.7°C shown by two other strains. Although significant positive correlations existed between maximum temperature permissive of growth and optimum growth temperature and between maximum permissive temperature and maximum survival temperature, there were however notable exceptions. For example, one strain had the lowest optimal temperature of 27.4°C and the highest survival temperature, of 48.7°C (Munevar and Wollum, 1981a). The growth of soybean and cowpea were more adversely affected by elevated temperatures when dependent on symbiotically fixed nitrogen than when using fertilizer nitrogen (Eaglesham and Ayanaba, 1984).

Legume species show varying tolerance to high temperature in their nodulating abilities. Three cultivars of chickpea (*Cicer arietinum*) inoculated with any one of five rhizobial strains failed to nodulate at a constant temperature of 35°C (Dart et al., 1976). By comparison, cowpea cultivar VITA-3 at a constant 35°C nodulated well with all of ten rhizobial strains, the overall mean nodule number being reduced by only 9.4% in comparison to plants at constant 25°C. Rhizobial strain to strain variation exists in nodulation potential under temperature stress conditions; this has been demonstrated with several host species including chickpea, soybean, cowpea and lotus (Eaglesham and Ayanaba, 1984). A

paper written by Meek et al. (1986) examined the losses of alfalfa (Medicago sativa L.) from irrigation occurred under the combination of high summer temperatures, soil texture and aeration status. Results of this experiment indicate that there is a high probability of damage to grower fields when O_2 stress under high temperature extends over many irrigations.

The most convenient approach to the investigation of temperature effects on nodulation is by the constant temperature technique. The symbiotic performance of different rhizobial strains under temperature stress has been correlated with their ability to grow in pure culture at elevated temperatures. It is interesting to note that the better performance of the rhizobial strains at high temperature was not the result of relatively higher nodule number but of larger nodules.

The link between the ability to grow and ability to retain effectiveness, also shown by R. japonicum (Munevar and Wollum, 1981b), indicates that Rhizobium survival at high temperature may be more critical than the nodulation potential of the host legume. In other words, some legumes may have the potential to nodulate at temperatures above the limit of rhizobial growth and survival. Reduction to almost total nodulation failure in cowpeas resulted from exposure of roots to temperatures of 40-44°C for five hours per day and was directly linked to inoculum death, the first few days after planting being the most critical phase (Eaglesham and Ayanaba, 1984).

In the early weeks of growth and before canopy closure, exposure of nodulated roots to high temperatures is likely to have relatively less effect on the functioning of nodules than on their establishment (Dart et al., 1976). When the soil surface is shaded, after canopy closure, high soil temperatures are unlikely to be a problem. Therefore the time during which high temperature is critical factor in nodulation is short, between planting and the initial rhizobial infection of roots at the crown. At greater depths, rhizobia and secondary nodulation are less likely to be exposed to temperature extremes. Although high temperatures in field conditions are often associated with soil desiccation, reports on the combined effects of these stresses on legume nodulation in controlled conditions were not found. Temperature affects the duration and the magnitude of nitrogen fixation. Graham (1979) reported that the maximum fixation in cultivars of Phaseolus vulgaris L., inoculated with Rhizobium increased from 33.8 (mol C_2H_4 produced/plant/h at 25-35°C to 73.0 8 (mol C_2H_4 produced/plant/h at 15-25°C, but the peak in fixation was increasingly delayed as growth temperature was reduced.

In a study of growth and survival of root-nodule bacteria in legume inoculants stored at high temperatures by Roughley et al. (1995). The workers used different temperature to define experimental storage conditions to study survival of root-nodule bacteria in peat culture. They concluded that the numbers of the clover strain declined significantly at temperatures between 30 and 40°C and those of the medic, lupin and both soybean strains at 40°C. Numbers of all strains exceeded log10^{8.3}/g peat when stored at 35°C for 28 days. Although when stored at 40°C for 2 weeks, the numbers of the five strains declined, they all multiplied rapidly when returned to 25°C. Storage at 40°C for 42 days did not affect the infectivity of the strains. These results indicate that peat-based cultures may be distributed with safety in the tropics from a centralised production centre. Regional reincubation at a favorable temperature (25°C) for growth is recommended when temperatures inside the packaging are likely to have exceeded 35°C for 7 days (Roughley et al., 1995). Hungria and Franco (1993) studied the effects of high temperature on nodulation and nitrogen fixation by Phaseolus vulgaris L. They mentioned that screening of Rhizobium leguminosarum bv. phaseoli strains showed some that were able to nodulate common beans (Phaseolus vulgaris L.) at high temperatures (35 and 38°C/8h/day). The nodulation ability was not related to the capability to grow or produce melanin-like pigment in culture media at high temperatures. However, nodules formed at high temperatures were ineffective and plants did not accumulate nitrogen in shoots.

The survival of Rhizobium leguminosarum, in response to soil desiccation at ambient room temperatures, or at 30°C and to intermittent exposure of the desiccated soil to 50°C, was measured in soils of differing texture, pH and total exchangeable cations (Evans et al., 1993). They concluded that the inter-seasonal persistence of Rhizobium leguminosarum will be strongly affected by the cumulative effects of soil desiccation and high temperature on Rhizobium viability, regulated by soil H^+ concentration and divalent cation exchange capacity, particularly Mg. The probability of high soil temperature eliminating Rhizobium leguminosarum from a soil, or reducing its population to levels inadequate for optimal nodulation, may be greatest when rhizobia numbers are few prior to high temperatures. This is most likely to occur in strongly acid soils and soils of low Mg content.

Maximum soil temperatures in the tropics regularly exceed 40°C at 5 cm and 50°C at 1 cm depth and can limit nodulation (Hungria and Vargas, 2000). Indirect effects of high temperatures on the metabolism of the host plant and direct effects on nitrogen fixation have been recognized for a long time and the range of temperature for optimal growth of nodulated legumes are narrower than for the same species receiving abundant mineral nitrogen (Hungria and Franco, 1993). Upper temperature limits for nitrogen fixation in tropical legumes vary between 27 and 40°C (Gibson, 1975; Dart 1974).

In relation to rhizobial growth, upper limits range between 32 and 47°C, although tolerance varies among species and strains and death may occur above 37°C (Hungria and Vargas, 2000). As high temperatures decrease rhizobial survival and establishment in tropical soils, repeated inoculation of grain legumes and higher rates of inoculation may frequently be needed. Temperature can affect rhizobial persistence in nodulations during shipment or in storage can influence survival in soil, and can limit both nodulation and nitrogen fixation (Graham, 1992). High temperatures will also delay nodulation or restrict it to the subsurface region, where temperatures are not as extreme. Munns et al. (1977) 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. La Favre and Eaglesham (1986) noted that nodulation of soybean was markedly inhibited at 42°C (12h day) and 45°C (9h day), with no correlation between a strain's ability to grow at high temperature and to induce nodulation under temperature stress. This finding is in contrast to the earlier results of Munevar and Wollum (1981a b) but may reflect differences in the temperatures used. Many laboratories have screened rhizobia for superior tolerance to high temperatures on culture medium as well as under field conditions, but little is known about the specific mechanisms these rhizobia use to cope with high temperatures. The involvement of a cryptic plasmid in the heat tolerance of a strain of R. leguminosarum bv. Trifolii and R. meliloti was suggested by Baldani and Weaver (1992).

Moisture: Climates with alternate wet and dry periods are experienced in vast areas of the tropics. At one extreme is a climate with high total precipitation falling in two peak rainy seasons separated by two short drier spells of reduced rainfall. At the other extreme is a climate of low total rainfall in one short season and a long dry season. In general the seasonal variation in temperature as well as the length and severity of the dry season are greater as one moves from the equator (Eaglesham and Ayanaba, 1984). Distribution of the rains during the wet season can sometimes be uneven resulting in the surface layers of the soil becoming desiccated during crop growth.

Bacterial populations are reduced in size when soils become desiccated (Chen and Alexander, 1973). In a silt loam soil two distinct phases of decline were observed with seven diverse types of slow and fast growing rhizobia. The first phase occurred during the loss of water from the soil, when the bacterial numbers fell rapidly and extensively at an exponential rate. The second phase was a much slower linear decline in numbers after the soil had reached a constant moisture level or the dry state. On wetting and

re-drying the biphasic cycle was repeated. Whether moisture loss was rapid or slow, the same two phases were observed and the final population size was not significantly affected (Eaglesham and Ayanaba, 1984). Slow growing rhizobia survived desiccation in a sandy soil better than did fast-growing types. After overnight drying nine slow-growing rhizobia showed a 0.6-6% survival and nine strains of fast growing showed only a 0.02-2.5% survival (Bushby and Marshall, 1977). After 10 days of drying in a silt loam, survival rates were 0.39-0.92% and 0.39-0.47% for fast and slow growers respectively (Pena-Cabriales and Alexander, 1979). The discrepancy may have resulted from even slightly different clay contents of the two soils, since the addition of montmorillonite to a sandy soil improved the survival of fast-growing rhizobia, but had an adverse effect on slow growers (Bushby and Marshall, 1977). This critical role of clay content in rhizobial survival in soils during hot, dry weather was the key factor in explaining "second year mortality" which occurred with clover and medics but not lupins, in some soils of western Australia (Eaglesham and Ayanaba, 1984).

Moisture level was the dominant factor influencing short and long term survival of R. japonicum strains inoculated into a loamy sand. Different serotypes responded differently but in general, survival over a nine-week period was best at soil water potentials of −0.3 to −1.0 x 10^5 Pa. Cataclysmic declines in population sizes of 2-5 logarithms occurred over the first week at −5 to −15 x 10^5 Pa, water potential levels which were found to be very common in cultivated soils in north Carolina (Mahler and Wollum, 1980). These data clearly illustrated the important role of soil moisture in influencing rhizobial inoculant establishment and performance. Soil type was confirmed as an important interacting factor. Even at low water potentials, rhizobial survive was better in sandy loams, silt loams and sandy clay loams than in sands or clay loams (Mahler and Wollum, 1981). Differences in desiccation sensitive among four strains of R. japonicum were much more pronounced in sand than in soil. Further to rhizobial inoculant performance, as evidenced by subsequent soybean nodulation, survival was better after seven days in the field at moisture levels below wilting point when applied to the soil as a granular peat inoculant rather than to the seed as a powdered peat inoculant or as a liquid inoculant to the seed or soil (Smith and Rio Escurro, 1982).

The greater susceptibility to desiccation of the fast-growing rhizobia was explained not in terms of differences in internal solute concentrations or in water permeability, but rather that the fast growers had a greater affinity for water because of higher surface energy (Bushby and Marshall, 1977). The lower internal water content at low relative vapour pressures of the slow growers was believed to impart greater survival capacity by reducing the activity of those enzymes capable of functioning at low moisture. However it has been suggested that if desiccation is relatively mild, the lower internal water-retaining ability of the slow-growing rhizobia may be disadvantageous because of insufficient moisture for the functioning of vital enzymes within the slow-growing rhizobial grouping, those which produced copious extracellular polysaccharide were found to be more desiccation-sensitive than strains which produced little or none (Eaglesham and Ayanaba, 1984).

There are very few reports on the effects of low moisture on the nodulation process. Reduction of soil moisture in an Australian sandy soil of only 2% from 5.5 to 3.5% represented a large drop in water potential from −0.36 to −3.6 x 10^5 Pa and had very severe effects on root hair infection of Trifolium subterraneum. Nodulation failure at the low moisture level was not a result of the death of rhizobia; in fact rhizosphere population of R. trifolii was not adversely affected. The root hairs assumed a short, stubby form which appeared to be immune to rhizobial penetration, but which became amenable to infection after re-watering (Eaglesham and Ayanaba, 1984). The water potential levels within which nodulation would be expected to occur have not been defined for other legumes. Soybean was relatively unaffected by withholding water at the "nodule initiation stage," but it appears likely that infection had occurred before moisture reached a critically low level. Nodule number and dry weight of cowpea and hyacinth bean were directly proportional to soil moisture level, but caution is required in interpreting these data since nodulation and plant growth were very poor in both species in all moisture treatments (Habish and Mahdi, 1976). Pot grown sitatro and Desmodium intortum subjected to weekly cycles of water stress suffered more in terms of shoot and root growth than in nodule mass or acetylene reduction activity, although the latter was measured under non-stressed conditions. However, nodulation and nitrogen fixing activities were very low even in the non-stressed treatments indicating that plants were utilizing soil nitrogen (Ahmed and Quilt, 1980).

The subject of moisture stress effects on the whole legume has been neglected. No reports of comparison of moisture stress effects on nodulated and nitrogen fixed legumes were found. However it appears that root nodules are particularly sensitive to changes in soil water potential. A drop from −0.55 to −0.7 x 10^5 Pa resulted in significant reduction in nitrogen fixation in soybean (Eaglesham and Ayanaba, 1984). Moisture loss down to 80% of the maximum resulted in proportionate inhibition of acetylene reduction by detached soybean nodules. Below 80% there occurred irreversible damage which correlated with loss of structural integrity in vacuolated non-infected nodule cells (Sprent, 1971). The adverse effects of moderate water stress on nodule activity indicated that considerable day-to-day variation in nitrogen fixation rates occur in field grown legumes simply from changes in whole plant water content with important implications for effects on yields. Decreases observed in acetylene reduction by field-grown cowpeas in Nigeria during daylight hours were believed to have been caused by low relative humidity (Ayanaba and Lawson, 1977).

Nodules on water-stressed clover plants resumed meristematic activity and quickly increased in fresh weight and nitrogen fixing activity after rewatering. Clovers may therefore recover more rapidly from drought than, for example, soybeans whose nodules do not have a localized meristem. Soybean nodules were shed when soil moisture approached the permanent wilting point. The superior drought tolerance of Lotus dorniculatus in comparison with Medicago lupulina and Trifolium repens was correlated with its ability to retain rather than shed its root nodules under dry conditions (Eaglesham and Ayanaba, 1984). Legumes are intolerant to shortage and excess of water and this is primarily due to the ultrasensitivity of the symbiosis to water stress (Sprent, 1984). In dry soils infection is restricted because of the absence of normal root hairs; instead, short, stubby root hairs appear, which are inadequate for infection by rhizobia (Bordeleau and Prevost, 1994). Water stress reduces both nitrogen fixation and respiration of nodules, and within certain limits this reduction is proportional to the degree of water loss of the nodules. During periods of drought, osmotic damage to nitrogen fixation may occur because of the high salt concentrations near or on the nodules. Water stress is also responsible for a decrease of the cytosolic protein content of the nodule and more specifically of leghemoglobin (Bordeleau and Prevost, 1994). Some legumes, such as alfalf, fababean and clovers, produce indeterminate meristematic nodules which are more salt and drought tolerant than the determinate (non-meristematic) nodules formed by soybean and common bean (Sprent and Zahran, 1988). Nitrogen fixation by Allocasuarina verticillata was markedly affected by soil moisture content, optimum activity of acetylene reduction occurring between 25 and 75% of the moisture content at field capacity 33 kPa (Hopmans et al., 1983).

Interaction of rhizobium with other soil microorganisms: Rhizobium strains active in nitrogen fixation appear to be poor competitors with members of the indigenous community of microorganisms in soil and the rhizosphere (Alexander, 1985). Evidence for an effect of indigenous microorganisms on the activity of the root-nodule bacteria in soil comes from several

sources. In an early report, the failure of inoculation of subterranean clover in certain soils of Australia was attributed to the indigenous microbial community preventing colonization of the rhizosphere by the added, active nitrogen-fixing inoculum strain (Hely et al., 1957). Similarly, in areas of New Zealand, it was observed that establishment of white clover was poor, even when lime, fertilizers, or micronutrients were added to the soil. Establishment was successful, however if the size of the indigenous microbial community was reduced by the application of antimicrobial agents. The antimicrobial compounds presumably reduced the activity of components of the indigenous community, thereby permitting the rhizobium to colonize the rhizosphere and bring about nodulation (Beggs, 1961, 1964).

Under more defined conditions in tests of subterranean clover (Harris, 1953) and white clover (Anderson, 1957), it was observed that the nodulation by effective R. trifolii was reduced, delayed, or prevented by fungi or other bacteria. The microorganisms responsible for these deleterious changes usually did not produce antibiotics acting against the rhizobia in culture and hence the harmful effects were attributed to competition. In recent studies, it was observed that rhizobia fail to grow in soil even if a carbon source they can use is added in reasonable amounts (Alexander, 1985). If enormous concentrations of that carbon source are added, proliferation occurs; evidently, the indigenous populations are better able than the rhizobia to make use of low concentrations of the organic compound, but at the high concentrations, some is left for the slow-growing and poorly competitive root-nodule bacteria. Moreover, if antibiotics toxic to many native soil bacteria but not to rhizobia are added to soil, thereby suppressing the potential competitors, rhizobia are able to proliferate and increase markedly in numbers (Pena-Cabriales and Alexander, 1983).

Competition for limiting supplies of nutrients probably is an extremely important interaction among natural microbial communities. Soils are known to be almost invariably deficient in readily available carbon compounds. Members of the genus Rhizobium grow slowly and presumably are not effective competitors in soil. Probably more than is presently realized, competition restricts the activity of nitrogen-fixing organisms, both free-living and those that, like Rhizobium, must compete in the rhizosphere prior to invading the host plant. Means of overcoming or minimizing the impact of soil microorganisms competition, notwithstanding its presumed importance, have not attracted attention. One possible approach is to apply inhibitors to the seed in the hope that these chemicals will suppress the competing organisms but not the nitrogen-fixing species. To obtain such chemicals for seed treatment is not difficult, because many of the commercially important fungicides, including those designed for the treatment of legume seeds, inhibit many bacteria, and the rhizobia can be made resistant to these fungicides. Such an approach has been used with species of rhizobium and with several leguminous species (Alexander, 1985). The chemicals presumably act, at least in part, by inhibiting the indigenous bacteria, thus allowing the introduced root-nodule bacterium to proliferate with a minimum competition (Odeyemi and Alexander, 1977). Like other members of the microbial community in soil, nitrogen-fixing microorganisms are also subjected to the antagonistic and associative effects of other species of bacteria, fungi, actinomycetes, protozoa and bacteriophages. Besides the specific effects of these microorganisms, nematodes and insect predators of nodules also diminish the symbiotic effects in legumes. Nodulation failures or the inability to obtain the desired responses to seed inoculation of legumes with rhizobia have been frequently observed by agronomists and one of the possible reasons for this lack of response in a particular soil environment may be due to a shift in the microbiological equilibrium towards the creation of a rhizosphere microflora predominated by microorganisms antagonistic to rhizobia (Subba Rao, 1984).

A part from the detrimental effects of nitrogen fixation attributable to the inhibitory action of microorganisms towards rhizobia, it would be worthwhile to exploit some of the observed beneficial influence of some microorganisms or their products towards improving plant growth and increasing the number of viable nitrogen-fixing bacteria in the rhizosphere.

Different microorganisms were cultured on agar medium along with rhizobia or their culture filtrates were used to test the inhibitory effects (Subba Rao, 1984). Some of the bacterial species which were inhibitory towards one or more species of Rhizobium included species of Bacillus, Escherichia, Pseudomonas, Xanthomonas, Achromobacter, Flavobacterium, Alcaligenes, Erwinia, Aerobacter, Corynybacterium, Arthrobacter, Brevibacterium, Agrobacterium, Micrococcus, Sarcina and Enterobacter. Some of the actinomycetes which proved inhibitory to rhizobia belonged to the genera, Actinomyces, Streptomyces and Nocardia. Among the inhibitory fungi, Cephalosporium, Alternaria, Aspergillus, Penecillium, Rhizopus, Acrothecium, Fusarium, Rhizoctonia, Curvelaria, Pythium, Trichoderma, Chaetomium, Cladosporium, Mortierella, Scolecobasidium, Sordaria, Thielavia and Mucor may be mentioned.

Understanding the impact of environmental variables on interstrain competition is important to ensure the successful use of rhizobial inoculant. Thies et al. (1992) studied the influence of environmental variables on interstrain competition between applied and indigenous rhizobia and among the three strains comprising the inoculum. Although temperature and soil fertility were correlated with nodule occupancy by inoculant strains in a few cases, the most significant environmental variable controlling their competitive success was the size of the indigenous rhizobial population.

Members of the genera Rhizobium and Bradyrhizobium are not the only inhabitants of the soil and legume rhizosphere, their proportions in the total microbial population depend on various factors, such as soil fertility, field or crop history and the present crop production. Numerous reports on the different behaviours of Rhizobium and Bradyrhizobium sp. in sterile soils, where significant influence of biotic factors on rhizobial survival and nodule occupancy in the field is demonstrated (Vlassak and Vanderleyden, 1997).

The main effect of soil microbial community in the field and legume rhizosphere is the alteration of the size and composition of the rhizobial population, which may result in a numerical advantage for a certain rhizobial strain or subgroup, eventually leading to altered nodule occupancy.

a) Interaction of bacteria with *Rhizobium*. The rhizosphere of legumes contains a large and heterogenous community of bacteria. Interactions of these rhizosphere bacteria between each other and with other rhizobia or bradyrhizobia are complex and poorly studied. In the few studies reported, growth inhibition of some Rhizobium or Bradyrhizobium strains has been observed (Li and Alexander, 1986; Vlassak and Vanderleyden, 1997). Li and Alexander (1986) observed an inhibition of the colonization and nodulation of alfalfa roots by Rhizobium meliloti in the presence of Enterobacter aerogenes, Pseudomonas marginalis, Acinetobacter sp. and Klebsiella pneumoniae, whereas Bradyrhysobium sp. or Micrococcus luteus did not exert such effects on the Rhizobium meliloti-alfalfa interaction. The authors noted a correlation between the growth rate of these rhizosphere bacteria and their effect on Rhizobium meliloti, with the bacterial species that multiplied faster than Rhizobium meliloti being the competitors. It was suggested that these good competitors prevent the rhizobia from obtaining a large part of the excreted carbon needed to support cell growth. The presence of soil bacteria also hindered the colonization of a loamy sand and a silt loam soil by Rhizobium leguminosarum bv. trifolii, making them more susceptible to predation by protozoa (Postma et al., 1990). Sometimes a beneficial or deleterious effect of fluorescent pseudomonalies on rhizobial nodulation is observed (Polonenko et al., 1987). These effects may be caused by the production of antibiotic compounds, siderophores, or plant growth hormones. The enhanced nodule occupancy by

Bradyrhizobium japonicum USDA 110 observed in the presence of fluorescent *Pseudomonas* sp. was attributed to the iron availability and its influence on the siderophore production by the *Bradyrhizobium japonicum* strains (Fuhrmann and Wollum, 1989). Different growth responses and siderophore production by different *Bradyrhizobium japonicum* strains at low iron levels have been reported (Carson *et al.*, 1992).

Some studies have demonstrated the stimulatory effect of *Azospirillum* on rhizobial nodulation (Plazinki and Rolfe, 1985a; Itzigsohn *et al.*, 1993). However, inhibition of nodulation was observed for some strains of *Rhizobium leguminosarum* bv. *trifolii* when coinoculated with an *A. brasilense* strain, resulting in a different nodule occupancy by these strains compared to the nodule occupancy in the absence of *A. brasilense*. An alteration in the auxin concentration brought about by the presence of the *A. brasilense* strain seemed to be responsible for these observed changes (Plazinki and Rolfe, 1985b). *Erwinia herbicola*, often encountered in the seeds of alfalfa, was responsible for a slower nodulation and decreased competitive ability of the HA *Rhizobium meliloti* cells, compared to the LA strains, which were unaffected by this epiphytic bacterium (Handelsman and Brill, 1985). Another mechanism of rhizobia used to compete with rhizosphere bacteria, especially with rhizobia noduling the same hosts, is the production of bacteriocins (Vlassak and Vanderleyden, 1997). These protein-like substances have a highly specific bactericidal or bacteriostatic effect on other bacteria, but do not multiply in the infected cells (Bradley, 1967). The production of bacteriocins has been reported for various rhizobial and bradyrhizobial species, such as *Rhizobium leguminosarum* bv. *trifolii*, *Rhizobium lupini* and *Bradyrhizobium japonicum* (Vlassak and Vanderleyden, 1997).

b) Interaction between *Azotobacter* and other microorganisms: The antifungal properties of *Azotobacter* towards *Fusarium. Alternaria, Ascochyta, Sclerotinia* and other fungi, and the amelioratory influence of *Azotobacter* on several seedling diseases of plants have been reported from time to time (Menkina, 1963; Shende *et al.*, 1975). The antifungal antibiotic principle from *Azotobacter* has been determined as a thermostable, ether-soluble substance which inhibit *Fusarium moniliforme* in culture media (Lakshmi Kumari *et al.*, 1972). Several investigations deal with the associative beneficial effects of *Azotobacter* with other bacteria, especially with phosphate-dissolving bacteria in improving nitrogen fixation (Ostwal and Bhide, 1972; Shende *et al.*, 1973; Ocanpo *et al.*, 1975). In some experiments, the fixation of nitrogen by *Azotobacter* was related to the action of cellulolytic and pectinolytic bacteria (Subba Rao, 1984), growing in association with Azotobacter or to the influence of mixed cultures with other bacteria such as *Rhodopseudomonas* and *Azospirillum* (Tilak *et al.*, 1982).

In field experiments with chickpea (*Cicer arietinum*), the *Azotobacter + Rhizobium* combination increased the mass of nodules and grain yields but similar results were not observed with a *Beijerinckia + Rhizobium* combination (Ravat ana Sanoria, 1976). On the contrary, in potted experiments peat based *Rhizobium japonicum + Beijerinckia* as well as *Azotobacter* increased yield and nodulation of soybean (Apte and Iswaran, 1971). Similarly, French bean plants (*Phaseolus aureus*) established well and produced more nodule when inoculated with *Agrobacterium*, a phosphate solubilizer (Barea *et al.*, 1973). These conflicting reports can be reconciled only on the basis of variations in soil types, inoculum load and environmental factors (Subba Rao, 1984).

c) Bacteriophages in relation to legume symbiosis: There has been no clear-cut evidence to demonstrate the inhibitory effects of bacteriophages on root nodules even though a number of workers, from time to time, have isolated and demonstrated the non-specific lytic action of bacteriophages on rhizobia (Subba Rao, 1984). Some of these reports relate to methods of isolation of bacteriophages from soil and plant roots including nodules and

morphological features, distribution in soils, properties and cross-reaction of bacteriophages between different strains of rhizobia (Takahashi and Quadling, 1961; Schwinghamer and Reinhardt, 1963; Kowalski *et al.*, 1974). The plants investigated for bacteriophages reaction were invariably cultivated grain and fodder legumes such as clovers, *Pisum sativum, Vicia jaba Vicia sativa, Lens esculenta*, soybean and chickpea.

Reports attributing positive detrimental effects of bacteriophage on symbiotic nitrogen fixation have come from the studies on alfalfa and peas (Subba Rao, 1984). On the other hand, doubts have been cast on the role of bacteriophages in minimizing the benefits of legume symbiosis based on the following observations: (1) a strain of bacteriophage could attack only 10-15% of the number of pea and clover rhizobia collected over a large area (2) the absence of specificity between a phage and a strain or species of *Rhizobium* (3) the presence of strains of *Rhizobium* both susceptible as well as resistant to the action of bacteriophages in the same soil sample and (4) the rapid development of strains of rhizobia resistant to bacteriophage action, thereby affording a chance for the natural build up of strains capable of nodulating the host inspite of the presence of lytic bacteriophages (Kleczkowska, 1957).

In another study, it was observed that mutations in *Rhizobium* to bacteriophage resistance may coincide with its morphology and effectiveness in nitrogen fixation. The frequency of occurrence of mutation and stability of the acquired features, however, depends on the variations between strains of *Rhizobium*. It was further observed that ineffective bacteriophage resistant mutants developed rather more readily from effective strains of *Rhizobium* than vice versa, indicating the possibility that the proportion of ineffective strains of *Rhizobium* may increase in soil at the expense of effective strains (Subba Rao, 1984).

Like many bacteria, rhizobia are susceptible to infection by a range of bacteriophages. Different susceptibilities to these rhizobacteriophages exist among rhizobia (Hashem and Angle, 1988) and these differences have resulted in an alteration of the relative rhizobial numbers in broth or peat culture (Schwinghamer and Brockwell, 1978) as well as in soil and on the legume root (Evans *et al.*, 1979; Barnet, 1980), with the resistant strain being favoured while the population of the sensitive strain declines. These proportional changes in rhizobial populations may lead to reduced nodulation of legumes by the homologous rhizobia (Evans *et al.*, 1979; Hashem and Angle, 1988), whereas the number of nodules induced by nonhomologous strains increases.

In the case of *Bradyrhizobium japonicum*, these differences in nodule-forming capacity between phage-sensitive and resistant strains resulted in altered nodule occupancy in the presence of rhizobacteriophages (Hashem and Angle, 1988; Vlassak and Vanderleyden, 1997).

d) Interaction of mycorrhizal fungi with *Rhizobium*: Possibilities exist for the beneficial use of vesicular-arbuscular mycorrhizal fungi as inoculants, particularly if *in vitro* culturing becomes possible (Eaglesham and Ayanaba, 1984). Positive results in the field, although not as predictable as in pot experiments, have been demonstrated (Khan 1975; Islam and Ayanaba, 1981). The degree of dependence on mycorrhiza for uptake of phosphate varies with species and with phosphate availability and different mycorrhizas vary in their functional efficiency (Powell, 1976). Whether mycorrhizas of superior efficiency have the potential to improve legume phosphorus nutrition in soils remains to be determined. The introduction of more efficient endophytes for maximum exploitation of soil phosphate will inevitably lead to soil impoverishment unless fertilizer use is possible (Eaglesham and Ayanaba, 1984). Rock phosphates and other inexpensive forms of phosphatic fertilizers can be used in conjunction with mycorrhizas (Mosse, 1973) even in alkaline soils in which the phosphate would be unavailable directly to the plants (Mosse *et al.*, 1976). Several legumes have been found to benefit by the combined inoculation of *Rhizobium* and vesicular-arbuscular mycorrhizal fungi. The

legumes studied extensively in this regard are soybean, lucerne and clovers but other plants such as French bean, groundnut, *Stylosanthes* and cowpea have also been used as test plant to understand this interaction (Subba Rao, 1984). The vesicular-arbuscular mycorrhizal fungi used were species of *Endogone* and *Glomus* and in certain cases vaguely described as vesicular-arbuscular mycorrhizal fungi. *Glomus* sp. appears to be a predominant colonizer of roots and is non-specific with regard to host plants which it infects. The characteristic features taken into account in understanding the vesicular-arbuscular mycorrhizal effects are plant size, yield and phosphorus content in plants grown in fumigated or non-fumigated soil and sterilized or unsterilized soil in pots. In some instances, field-plot experiments have been carried out to verify the results. There appears to be a consensus of opinion that vesicular-arbuscular mycorrhizal inoculation to nodulating legumes generally increases the plant size and yield, although contradictory results have come forth with regard to increased P-uptake by plant tops. The availability of P in soil diminishes with time of incubation of added phosphates in soil and the beneficial effects of vesicular-arbuscular mycorrhizal fungi inoculation seem to be better when phosphates are freshly added to soil. Apart from the observed vesicular-arbuscular mycorrhizal effects on P-uptake, the nitrogen content of plants appears to be enhanced due to better nodulation and symbiotic effects (Schenk and Hinson, 1973; Daft and El-Giahmi, 1976; Abbot and Robson, 1977; Smith and Daft, 1977; Bhagyaraj *et al.*, 1979). Michelsen and Sprent (1994) studied the influence of vesicular-arbuscular mycorrhizal fungi on the nitrogen fixation of legume trees under Ethiopian nursery conditions. In this study they indicated that nitrogen fixation was increased by those vesicular-arbuscular mycorrhizal fungi which also promoted shoot dry weight of legume trees.

Indigenous soil fungi and actinomycetes have frequently been demonstrated to be antagonistic toward rhizobia and bradyrhizobia (Pugashetti *et al.*, 1982; Habte and Barrion, 1984). The production of mycotoxins is primarily responsible for the observed antagonistic effects of soil fungi, although competition, predation and parasitism also may be important as cited by Angle *et al.* (1981). Also the presence of mycotoxins or mycotoxin producing fungi in the soil or on seeds decreases rhizobial populations and reduces nodulation of legume roots, so this way providing a competitive advantage for mycotoxin resistant rhizobia (Angle *et al.*, 1981; Mahmoud and Abd-Alla, 1994).

e) Interaction of protozoa with *Rhizobium*: The decline in rhizobial numbers in soil is accompanied by a fall in the density of protozoa which feed on *Rhizobium*. *Bdellovibrio* is a widespread genus of the protozoa predators in soil (Subba Rao, 1984). Rhizobia however, are not entirely eliminated by protozoa and the numbers of rhizobiz attain normalcy when they survive to the attack of the predators. These observations have been made by studies with *R. meliloti* and *Rhizobium* sp. (cowpea type). In other studies, it was apparent that antibiotic resistant *R. phaseoli* diminished in numbers in natural soil accompanied by a concomitant increase in protozoal numbers although similar effects were not seen in *R. meliloti* inoculated in sterilized soil (Danso *et al.*, 1975; Chao and Alexander, 1981).

Predation seems to be significant in reducing the populations of rhizobia and other bacteria that at times are present in abundance in soil (Alexander, 1985). When artificially high numbers of *Rhizobium* are added to soil, the population size is drastically diminished. This decline is paralleled by the proliferation of indigenous protozoa. The protozoa apparently feed on the large numbers of rhizobia and appreciably reduce their density with out eliminating the bacteria (Danso *et al.*, 1975). Although the high rhizobial densities needed to trigger protozoan predation are not common in natural soils, the root-nodule bacteria proliferate as inoculated seeds imbibe water and the roots begin to develop. *Rhizobium* then begins to grow and may attain large population sizes; as the potential prey for protozoa become more numerous,

the predators begin to feed and thereby prevent the *Rhizobium* population from becoming as large as it would be in the absence of predation. In support of the hypothesis that predation is a significant factor in the rhizosphere is the finding that suppressing protozoa with thiram resulted in enhanced colonization of beans by *R. phaseoli* (Ramirez and Alexander, 1980). In subsequent studies, it was noted that the abrupt fall in population of *R. phaseoli* around germinating seeds and the developing root system of beans was delayed if the seed was treated with chemicals that inhibited the protozoa. The inhibition of protozoa by thiram was accompanied by a high initial frequency of nodules formed by the test *Rhizobium*, a yield increase, and a greater amount of nitrogen fixed by beans (Lennox and Alexander, 1981). In this instance, therefore, nitrogen gains by effective rhizobia are not realized because of a stress on the bacteria, the stress being predation. It is also possible that protozoa may be significant in nonrhizosphere soil, as suggested by data showing that additions of inhibitors of eukaryotes allowed for an increase in size of *Rhizobium* population (Pena-Cabriales and Alexander, 1983). Vlassak and Vanderleyden (1997) reported that the predation is known to be an important factor in soil, and may affect bacterial survival drastically. Among the soil organisms that feed on rhizobia, protozoa appear to be the most important and best-studied group, although a role of other parasites, such as *Bdellovibrio*, in predation of rhizobia has been reported as well (Alexander, 1985).

The number of protozoa in the soil depends on environmental factors such as soil moisture and organic matter content (Pena-Cabriales and Alexander, 1983). When a sufficient number of rhizobia is added to a liquid culture that contains protozoa (Danso and Alexander, 1975), or to a protozoa-rich soil (Danso *et al.*, 1975; Ramirez and Alexander, 1980; Chao and Alexander, 1981; Heynen *et al.*, 1988), the rhizobial population declines drastically while protozoan numbers increase.

f) Virus in relation to legume symbiosis: Nodulation and nitrogen fixation in clover (*Trifolium repens*) were reduced on infection by clover phyllody virus (Joshi and Carr, 1967). Similarly, soybean mosaic virus and bean pod mottle virus influenced nodulation in soybean (Tu *et al.*, 1970). In field bean (*Dolichos lablab*) infected with *Dolichos enation* mosaic virus (DEMV), a reduction in nodule numbers has been reported in natural soil whereas increase in nodule numbers could be observed in infected plants grown in nitrogen-free sand cultures (Rajagopalan and Raju, 1972). On the other hand, in a detailed study on *Dolichos enation* mosaic virus and symbiotic nitrogen fixation in field bean (*Dolichos labalab*), it was observed that infection decreased the levels of total carbohydrates and reducing sugars in roots and nodules of these plants after 24 days of growth. However, the total nitrogen content in these nodulated virus infected plants was more than that of the healthy plants. There was a higher rate of transfer of fixed nitrogen from nodules to the tops of plants as well as roots consequent upon virus infection of the host. Soluble proteins, total soluble nitrogen constituents and the total insoluble nitrogen increased in nodules following virus infection of the legume. The infected plants showed increased nitrogen fixation during 24 to 31 days which was higher than healthy plants and this high level was maintained throughout the growth period (Raju, 1974). These results point out that virus infection promotes nitrogen fixation.

g) Possible benefits of interaction: The associative effects of other microorganisms in soil and in roots with nitrogen-fixing microorganisms could be harnessed in the preparation of carrier-based inoculants. A mixture of beneficial microorganisms and rhizobia could be used in the carrier to obtain better and longer survival of rhizobia both in the carrier and on the inoculated seeds. Bacteria, which produce high amounts of extracellular polysaccharide (slime) and increase the growth of rhizobia can prove extremely beneficial to augment the moisture-retaining capacity of the carrier material during harsh storage conditions.

Phosphorus is essential for successful nodulation and nitrogen fixation in legumes. The dual effect of phosphorus mobilizing vesicular-arbuscular mycorrhizal fungi (VAM) and specific nitrogen-fixing rhizobia is yet another instance of a naturally occurring interrelationship among two beneficial microorganisms on root which could be exploited for better nitrogen as well as phosphorus nutrition of legumes (Subba Rao, 1984). From the standpoint of the microbial contribution to the symbiosis involving legumes and to nitrogen gains in agricultural fields, the factors limiting nitrogen fixation are rarely the absence of highly effective strains of *Rhizobium* or the lack of availability of cyanobacteria potentially active in fixation. The limiting factors are nearly always the absence of strains adapted to ecologically significant stresses in soil (Alexander, 1985).

Therefore, basic and applied research that is designed to increase food production, whether in the long or the short run, should seek to define these stresses and help to find the means to overcome them.

Organic matter and nutrients: The organic matter percentage differs from soil to soil according to the fertility status of each soil. Some of which having about 5 percent, while the other does not exceed 0.5 percent such as arid and semi-arid soils. In fact, the organic matter is a sink of soil nutrients. Most of the nitrogen-fixing microorganisms, other than the Cyanophyta and anaerobic phototrophic bacteria, require a supply of organic carbon as an energy source. The effect of organic matter on soil microbial activity depends on the type of material, its nutrient contents and on the initial fertility of the soil (Jurgensen, 1973). Clark (1967) maintains that it is a lack of suitable organic matter rather than mineral nutrients which limits the growth of heterotrophic bacteria in soil. He pointed out that most of the studies showing a strong microbial response to supplemented minerals were carried out with specific organic amendments and soil low in the nutrient being examined. Additional evidence showing the effects of limitation of organic matter on the growth of microorganisms in soil has been discussed by Gray and Williams (1971).

Information on the relationship of soil nutrients to the activity of heterotrophic soil microorganisms has been obtained by adding various amounts of mineral fertilizers to soil. Such studies have shown that the application of fertilizer generally increases the activity and population size of the soil microflora. In contrast, the response of nitrogen-fixing bacteria to fertilizer treatments has been variable. In some studies no changes were found in azotobacter population when phosphorus and potassium were added to soil, while in others a stimulation was reported (Jurgensen, 1973). Application of mineral fertilizers or liming has increased heterotrophic fixation in some instances but these gains are probably due to changes in the availability of soil organic matter or changes in soil acidity. In contrast soil nutrient levels may be significant for nitrogen-fixing microorganisms in the rhizosphere since this region around plant roots contains higher amounts of organic materials than soil away from the root (Jurgensen, 1973).

Nitrogen fixation by *Allocasuarina verticillata* was strongly influenced by the addition of nitrogen fertilizer and was inversely proportional to the mineral nitrogen status of the soil (Hopmans *et al.*, 1983). In a study of the influence of calcium, nitrogen and pH on alfalfa root growth and nitrogen fixation using the implanted soil mass technique by Rechcigl *et al.* (1987). They concluded that the surface liming increased the root mass of alfalfa in only the implanted soil mass receiving a Ca(OH)$_2$ or KOH amendment, but the other treatments had no influence on nitrogen fixation in the implanted soil mass. Adsorption of *Rhizobium meliloti* in low numbers to alfalfa (*Medicago sativa* L.) roots was dependent on the presence of divalent cations, and required neutral pH (Caetano-Anolles *et al.*, 1989). Adsorption was proportional to Ca and/or Mg concentrations up to 1.5 mM.

The results of Caetano-Anolles *et al.* (1989) suggest that rhizobial binding to the root surface already shows the Ca and pH

dependence of alfalfa nodulation, which was generally associated to some event prior to rhizobial penetration of root hairs. Many investigations have been conducted on the nutrient requirements of various nitrogen-fixing microorganisms. These studies have conclusively shown that N, P, K, Mg, S, Mo, Fe and Co are essential for the growth of nitrogen-fixing microorganisms. Since nitrogen fixation is directly related to growth of the organisms, the nutrients essential for growth will also be indirectly required for nitrogen fixation process.

The only nutrients that seem to be associated directly with the fixation process are Mo, Co and Fe (Follett *et al.*, 1981). Higher amounts of these elements are required when N$_2$ is the nitrogen source as compared with nitrate or ammonia (Jurgensen, 1973). An adequate supply of molybdenum would seem especially important since nitrogen-fixing microorganisms require larger amounts of this micronutrient for nitrogen fixation than for growth on mineral nitrogen (Jurgensen, 1973). Deficiencies of molybdenum in soil have been found to reduce nitrogen fixation by legumes (Follett *et al.*, 1981) but the rhizobia are not as severely limited by a lack of an energy supply as would be the nonsymbiotic nitrogen-fixers. Quastel (1965) maintains that molybdenum may be a limiting factor since the amount available in many soils is below that required for optimum fixation. The availability of molybdenum decreases rapidly with increasing soil acidity and so it would become especially critical in acid soils (Chapman, 1965). However, molybdenum availability is generally high in alkaline soils, soils high in organic matter and young soils derived from volcanic materials (Follett *et al.*, 1981).

Highly acid leached soils such as those derived under forested conditions and acid sandstone-derived soils generally are low in available molybdenum. Follett *et al.* (1981) discussed in detail the role of molybdenum in plant metabolism in nitrate reductase activity. In addition to the important function of molybdenum in nitrate reductase, nitrogenase is the enzyme responsible for the reduction of nitrogen from nitrogen gas (N$_2$) to an oxidation state equivalent to ammonium or amino nitrogen, has also been determined to require molybdenum. The fact of special significance is that legume Rhizobial bacteria require molybdenum for N$_2$ fixation. Molybdenum deficiency in most legumes such as alfalfa and clovers is due to the nitrogen deficiency. Poor supplies of molybdenum lead to poor nitrogen (N$_2$) fixation and plants show a general chlorosis, stunting and lowere leaves may become necrotic and drop off (Follett *et al.*, 1981). Eaglesham and Ayanaba (1984) found that low phosphate levels limited the growth of some rhizobial strains but with less severity than did acid. The growth of microorganisms in soil may be affected not only by a lack of essential nutrients but also by the presence of certain minerals in excess amounts. Aluminium (50 μM) was the most severe factor, stopping growth of 40% rhizobial strains. A complementary study showed that while high manganese (200 μM) and low calcium (50 μM) had adverse effects on slow-growing rhizobia which varied from strain to strain in severity, neither was as severe as aluminium, and strains which were tolerant of aluminium were also tolerant of manganese and low calcium (Mengel and Kamprath, 1978).

Aluminium has most often been implicated as causing soil toxicity problems. reduced microbial activity and organic matter decomposition in soils have been attributed to aluminium toxicity (Jurgensen, 1973). However, aluminium may affect microbial growth at higher pH levels. Although species and cultivars differ in their nutritional needs, legumes have a relatively high phosphorus requirement for optimum growth. Some require significantly more phosphate to reach optimum yields when relying on symbiotically fixed nitrogen in comparison to when supplied with fertilizer nitrogen (Cassman *et al.*, 1981). Little work has been done on root nodulation at low phosphorus levels. Indications are that phosphorus deficiency limits nodulation indirectly by limiting legume growth rather than the infection process (Zaroug and Munns, 1979), although there is evidence to imply that some rhizobia are more able to nodulate at lower

phosphate levels than others (Munns and Mosse, 1980). Nodule development requires adequate phosphorus and nodules accumulate a higher phosphorus content than roots (Eaglesham and Ayanaba, 1984). Providing adequate phosphate fertilizer to a common bean increased the nodule weight nine fold, so the symbiotic nitrogen fixation is highly correlated with supply of phosphorus (Graham and Rosas, 1979).

In an early study, Munns (1977) reported that the scant evidence available indicates that high manganese levels are unlikely to inhibit the growth or survival of rhizobia in acid soils. Manganese toxicity mainly affects legume growth rather than nodulation in particular, and tolerance of manganese varies considerably between and within legume species. However, variations among strains of *Rhizobium phaseoli* were found in their ability to nodulate and fix nitrogen in conditions where manganese was marginally phototoxic (Eaglesham and Ayanaba, 1984), liming alleviated the adverse effects of manganese.

Soil texture: Soil texture classified according to the soil construction that depends on the percentage of sand, clay and silt. However, each soil has definite characteristics that are affect the crops growth. Clay soil has a high water holding capacityis, rich of plant nutrients and a high population of microflora that decomposing organic matter in the soil. On the other hand, sandy soil has low water holding capacity, small amount or nil of organic matter and less population of soil microflora.

Some papers have been published, from an agricultural point of view, correlating nitrogen symbiotic fixation exclusively with soil characteristics (Wilson and Alexander, 1979). However, Quesada et al. (1997) indicated that physical and chemical characteristics of water influenced nitrogen fixation more than the soil properties. The larger number of protective micro-habitats available to soil bacteria in a clay rich soil may influence rhizobial survival, especially in soils exposed to high temperatures or desiccation (Osa-Afiana and Alexander, 1982; Heynen et al., 1988; England et al., 1993). *Cowpea bradyrhizobia* exposed to aluminum toxicity and low pH also showed a different response in different soils (Hartel and Alexander, 1983). Moreover, disturbing an acid soil also results in a decline of the rhizobial population.

It is estimated that in adverse soils, rhizobia are protected in microsites (Nazih et al., 1993). Marshall (1964) showed the clay mineral form was important to survival of *Rhizobium trifolii* in heat-stressed, dry soil. Also, Bushby and Marshall (1977) and Osa-Afiana and Alexander (1982) found that the clay mineral and its concentration affected survival of *Bradyrhizobium japonicum* and *cowpea rhizobia* in desiccating soils. Soil type affected the response of *Rhizobium leguminosarum* to both desiccation and storage at elevated temperatures (Evans et al., 1993). Under these conditions, soils with higher pH and exchangeable divalent cation capacity, but particularly with higher Mg were more favourable to the survival of *Rhizobium leguminosarum*.

Clay amendment of a soil is known to exert a protective effect against predation by protozoa as well; in the clay microniches, bacteria cannot be reached by grazing protozoa (Heynen et al., 1991; Vlassak and Vanderleyden, 1997). As a consequence, the clay content of a soil could influence the size of the rhizobial soil population (Mahler and Wollum, 1981; Ozawa, 1988). The reason for different nodule occupancy in different soils, observed with strains of *Rhizobium leguminosarum* (May and Bohlool, 1983), Leucaena rhizobia (Moawad and Bohlool, 1984) and chickpea rhizobia (Somasegaran et al., 1988). It is possible that a difference in surface charges among strains resulted in different rhizobial behavior in the different soils. A correlation between the surface charge characteristics of a Bradyrhizobium population and the soil in which this population persisted has been observed (Bushby, 1990).

Vlassak and Vanderleyden (1997) studied the factors influencing nodule occupancy by inoculant rhizobia and the environmental factors, such as soil texture, that influence competition and saprophytic competence of rhizobia. They reported that the

inoculation of legumes under field conditions with superior nitrogen-fixing rhizobia does not always result in the desired yield increase. They observed that the inoculum strain fails to occupy a significant proportion of the nodules. The introduced inoculant strains have to compete with the indigenous, often ineffective, nitrogen-fixing rhizobial population at different levels. The success of inoculant depends to a large extent on the ratio of the inoculant cells to indigenous rhizobia. However, intrinsic characteristics of the inoculant and indigenous rhizobia and their responses to abiotic and biotic environmental variables, also influence the outcome of inoculation (Vlassak and Vanderleyden, 1997).

Riffkin et al. (1999) reported that the amount of nitrogen fixed was influenced by different factors depending on soil texture. Soil chemical properties accounted for 31% variation on the medium-textured soils and soil chemical properties accounting for 77% of variation on light-textured soils. Amounts of nitrogen fixed per tone herbage dry matter produced averaged 8.2 kg on the light-textured soils and 7.3 kg on the medium-textured soils. Effect of soil texture on nitrogen fixation were found to be attributed to the different cation exchange and water-holding capacities of the soils and highlight the importance of considering soil type in nitrogen fixation in the study of Riffkin et al. (1999). Soil texture was found to play important role in the survival of four rhizobia isolated in a study by Mahler and Wollum (1981). They stated that the Rhizobia populations were lowest in the sands and clay loam soils. Conversely, populations were usually highest in the sandy loams, silt loams and sandy clay loam soils. Issa and Wood (1995) studied the influence of soil texture on multiplication and survival of chickpea and bean rhizobia in dry soils. Their results showed that there were very significant differences in multiplication and survival in response to the effect of matric potential, rhizobia strain, soil type and the interactions between these factors. However, the effects of soil type and strain were much greater than that of matric potential over the range of potential used. The survival and phenotypic expression of a transposon Tn5 mutant of bean rhizobia (*Rhizobium leguminosarum*) and the corresponding wildtype under increasing moisture stress conditions was studied in two Sonoran Desert soil types (Pillai and Pepper, 1990). Survival studies showed that both biotic and abiotic factors influenced the survival of the mutant and the wild type. This study suggested that Tn5 is a stable useful ecological marker even under moisture stress.In addition the soil texture showed a significant effect on nitrogen mineralization (Egelkraut et al., 2000). So the soils with greater clay concentrations had longer time periods of initial nitrogen immobilization and mineralized less nitrogen from the added materials.

Environmental factors that may influence rhizobial growth and survival in soil were described by Eaglesham and Ayanaba (1984). During this reviw they found that the soil texture and clay content seemed to have some effect on soil population or colonization of the rhizosphere by *Rhizobium* spp. especially strains of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *phaseoli*.

Conclusion: The efficiency of symbiotic nitrogen fixation by *Rhizobium* in soil is a biological process depends on the environmental factors. Various abiotic factors have been reported that significantly influence nodule occupancy. In the past, studies of environmental stress and the legume *Rhizobium* symbiosis were restricted to defining the problem and to using physical or chemical amendments to overcome it.

The limiting factor is not the absence of organisms with the genetic or biochemical potential to bring about appreciable nitrogen gains; rather, it is one or more ecological constraints that hold these organisms in check. The enormous progress made in recent years in understanding the biochemistry and genetics of nitrogen-fixing microorganisms frequently is only leading to improved organisms when no improvement is needed in the near future. Recent years have witnessed striking discoveries in the genetics and biochemistry of nitrogen fixation, and it is likely that

new and exciting finding will be forthcoming in the next decade. However, nitrogen fixation in agricultural ecosystems is rarely limited because of the absence of highly active nitrogen-fixing microorganisms.

In the last 10 years, molecular genetic analysis have increased our understanding of symbiotic nitrogen fixation and strategies to increase nitrogen fixation in the field have been explored. So, studies on gene expression, DNA transfer, and genomic rearrangements will increase our knowledge of this complex ecological system.

Acknowledgments

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