Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil

Mariangela Hungria[a,*], Milton A.T. Vargas[b]

[a]*Embrapa Soja, Cx. Postal 231, 86001-970, Londrina, PR, Brazil*
[b]*Embrapa Cerrados, Cx. Postal 08223, 73301-970, Planaltina, DF, Brazil*

Abstract

Biological nitrogen (N_2) fixation is key to sustainable agricultural systems in tropical soils, which are frequently deficient in N. However, high temperature, drought and soil acidity constrain legume root-nodule formation and function in the tropics. In most cases, the microsymbiont is the more affected partner, with plants growing on mineral N usually less sensitive to these stresses. High temperature and moisture deficiency are major causes of nodulation failure, affecting all stages of the symbiosis and limiting rhizobial growth and survival in soil. They may also contribute to undesirable changes in rhizobia, including plasmid deletions, genomic rearrangements and reduced diversity.

Acidity affects several steps in the development of the symbiosis, including the exchange of molecular signals between the legume and the microsymbiont. Liming is effective in overcoming soil acidity and aluminium toxicity, but in Brazil few soils are limed to near neutral pH. Selection of rhizobial inoculant strains that are genetically stable under the often harsh soil conditions of this region is essential, but that task is impaired by a lack of knowledge of tolerance in the microsymbiont. However, good results have been obtained by selecting naturally occurring rhizobia from acid tropical soils affected by water stress and high temperatures. In Brazil, increases in grain yields of common bean and soybean have resulted from inoculation with such stress-tolerant strains. Appropriate soil management practices, e.g. no-till, are other approaches that may decrease soil temperatures and preserve moisture, thereby increasing N_2 fixation. © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Bradyrhizobium; Drought; Nitrogen fixation; Rhizobium; Soil acidity; Temperature stress

1. Introduction

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 (e.g. Giller and Wilson, 1993). Nutrient depletion and soil acidification are just two of the common consequences of inadequate soil management. In this context, biological nitrogen (N_2) fixation is a critical and key process in sustainable land management, where N is the

---

nutrient that most frequently limits crop production. Tropical environmental conditions can constrain N_2 fixation, and need further study.

Six genera of bacteria, collectively called rhizobia, are able to form N_2-fixing nodules on the roots of legumes and the non-legume *Parasponia*. While the great majority of these legume–rhizobial symbioses are found in the tropics, it is only recently that there has been significant progress in understanding constraints to nodulation and N_2 fixation in tropical soils.

This paper discusses the main environmental factors that limit nodulation and N_2 fixation in grain legumes in the tropics, and emphasizes two legumes important for human nutrition and as cash crops in Latin America and Africa: common bean (*Phaseolus vulgaris* L.) and soybean [*Glycine max* (L.) Merr.]. As defined by other authors (e.g. Giller and Wilson, 1993), ''tropics'' as used here includes some subtropical regions. Three main environmental constraints which frequently occur simultaneously are discussed: high temperature, water deficit and soil acidity. Finally, some successful approaches taken in Brazil will be described.

2. High temperature

Maximum soil temperatures in the tropics regularly exceed 40°C at 5 cm and 50°C at 1 cm depth (Eaglesham and Ayanaba, 1984; Hafeez et al., 1991; Lal, 1993) and can limit nodulation (Day et al., 1978; Graham, 1981; Eaglesham and Ayanaba, 1984). Indirect effects of high temperatures on the metabolism of the host plant and direct effects on N_2 fixation have been recognized for a long time (Jones and Tisdale, 1921) and the range of temperature for optimal growth of nodulated legumes are narrower than for the same species receiving abundant mineral N (Joffe et al., 1961; Ferrari et al., 1967; Small and Joffe, 1968; Gibson, 1971; Lie, 1981; Piha and Munns, 1987; Hungria and Franco, 1993). Upper temperature limits for N_2 fixation in tropical legumes vary between 27 and 40°C (Gibson, 1971, 1975; Dart, 1974).

In relation to rhizobial growth, upper limits range between 32 and 47°C, although tolerance varies among species and strains (Pankhurst and Gibson, 1973; Gibson, 1975; Dart et al., 1976; Day et al., 1978; Munévar and Wollum, 1981a; La Favre and

Eaglesham, 1986; Karanja and Wood, 1988b). Death may occur above 37°C, and Fig. 1 shows that a difference of 6°C at 5 cm depth reduced a soybean bradyrhizobial population by more than 10 000 cells g^{-1} soil (Hungria et al., 1997a). Furthermore, physiological and genetic modifications in bacteria, including plasmid deletion (Trevors, 1986) and genomic rearrangement (Soberón-Chavez et al., 1986), probably occur in soils subject to high temperatures.

Because high temperatures decrease rhizobial survival and establishment in tropical soils, repeated inoculation of grain legumes and higher rates of inoculation may frequently be needed. Indeed, in contrast with findings in other regions (e.g. Dunigan et al., 1984; Thies et al., 1991), reinoculation of soybean in Brazil guarantees increases in nodulation, nodule occupancy by the inoculated strains (even when the strain is already present in the soil), N_2 fixation rates and crop yields (e.g. Vargas and Hungria, 1997). This may be due to several factors including reduced survival of rhizobia in the soil, genetic modification or plasmid loss, or to the poor ''physiological state'' of surviving rhizobia. At some sites response to inoculation was obtained even though the naturalized population of rhizobia approached 10^6 cells g^{-1} soil.

The exchange of molecular signals between host plants and rhizobia is also affected by high temperature. At 39°C, the release of *nod*-gene inducers from soybean and common bean roots was decreased (Hungria, 1995; Hungria and Stacey, 1997). The root-infection process is probably the component most affected by high temperatures, with sensitivity located at the nodulation sites (Barrios et al., 1963; Pankhurst and Gibson, 1973; Frings, 1976; Lie, 1974, 1981). High temperature also inhibits root-hair formation, reducing the number of sites for nodulation (Jones and Tisdale, 1921; Frings, 1976); adherence of bacteria to the root hairs (Frings, 1976); root-hair penetration and infection-thread formation (Joffe et al., 1961; Barrios et al., 1963; Pankhurst and Gibson, 1973; Dart, 1974; Frings, 1976). Nodule initiation, rhizobial release from the infection thread, and bacteroid development are also affected (Roughley, 1970; Pankhurst and Gibson, 1973; Vincent, 1980).

If nodules are formed, high temperatures may affect nodule function (Jones and Tisdale, 1921; Dart and Mercer, 1965; Lindemann and Ham, 1979; Munévar and Wollum, 1981b; Hungria et al., 1985, 1989; Piha
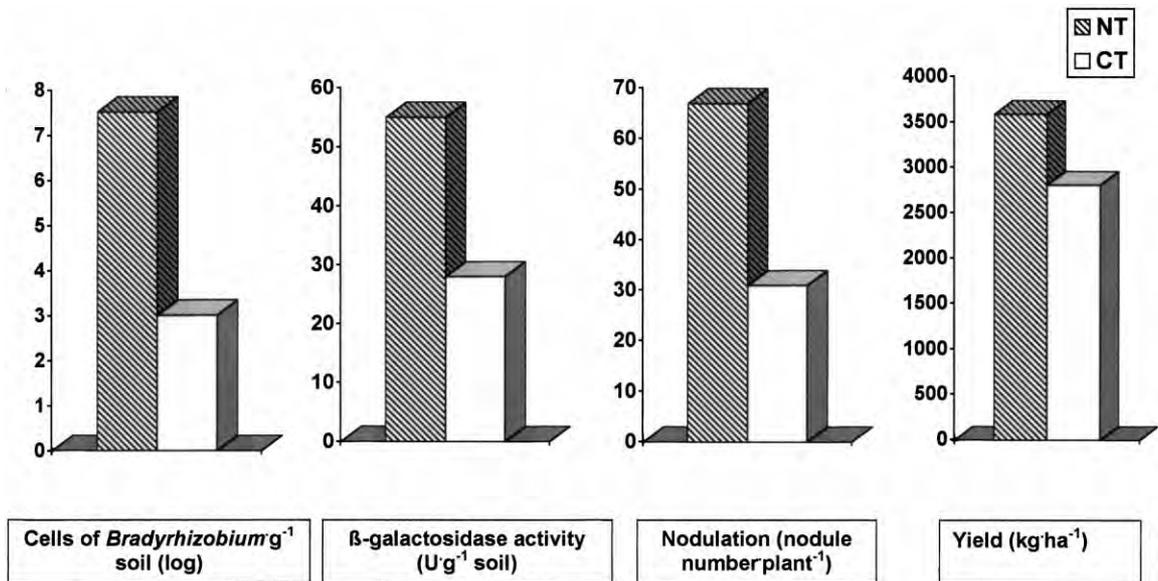
Fig. 1. Number of cells of *Bradyrhizobium*, *nod*-gene inducing activity in the soil solution, nodulation and yield of soybean, cultivar BR-37, inoculated with strain CPAC 15 and grown under the no-till (NT) or conventional till (CT) system. The mean soil temperature at 5 cm depth during the crop season was 6°C below under the NT system. Means of five replicates and for all parameters the NT was statistically significantly higher than the CT (Tukey, $p<0.05$).

and Munns, 1987; Hernandez-Armenta et al., 1989; Hungria and Franco, 1993). Changes that result in suboptimal function include decreased synthesis of leghaemoglobin (Bergersen et al., 1973), a drastic drop in nitrogenase activity (Pankhurst and Sprent, 1976; Hernandez-Armenta et al., 1989; Hungria et al., 1989; Hungria and Franco, 1993), and inefficient allocation of electrons to N_2 reduction, observed as greater H_2 evolution (Rainbird et al., 1983; Hungria and Franco, 1993). Nitrogen assimilation is also affected, with decreased activities of glutamine synthetase and glutamate synthase, and lowered synthesis of ureides (Hungria et al., 1989). Additionally, high temperature accelerates nodule senescence (Pankhurst and Gibson, 1973; Sutton, 1983; Hungria and Franco, 1993).

Some authors have suggested that the overall balance between photosynthesis and respiration determines levels of N_2 fixation. While high temperatures increase respiration, with less substrate available to the nodules (Lie, 1971), two studies with common bean have shown that decreases in N_2 fixation rate were not attributable to reduced carbohydrate pools in nodules. Although daytime temperatures of 38/39°C

drastically reduced nitrogenase activity, nodule soluble carbohydrate content was only slightly lower at 38/37°C than at 26/28°C (Hernandez-Armenta et al., 1989; Hungria et al., 1989).

3. Water stress

The tropics encompass both wet (>800 mm rainfall) and dry (<300 mm) regions, but water availability is determined chiefly by the moisture-retaining capacity of the substrate. Usually, at matrix water potentials of −0.5 to −1.5 MPa, growth and nitrogen fixation are affected in tropical areas. Low water availability may be most affected by the removal of deeply rooted vegetation and the conversion of natural forests or savannas to crop production or pasture, with increased runoff and evaporation (Lal, 1993). Shortage of water compromises plant and rhizobial growth, and is a major cause of nodulation failure and low N_2 fixation. Water stress affects rhizobial survival and growth and population structure in soil, the formation and longevity of nodules, synthesis of leghaemoglobin and nodule function. Furthermore, severe stress may lead

to irreversible cessation of N_2 fixation (Sprent, 1971; Vincent, 1980; Walker and Miller, 1986; Venkateswarlu et al., 1989; Stamford et al., 1990; Guerin et al., 1991).

Research has shown that decreases in nitrogenase activity under drought conditions occur in a two-stage process. First, there is a decrease in nodule cortical permeability, enforcing a primary limitation in oxygen supply to the bacteroid zone, restricting respiration and resulting in a simultaneous decrease in nitrogenase activity (Sprent, 1976; Durand et al., 1987; Walsh, 1995). The second step occurs when nodule activity is already drastically reduced, and nitrogenase activity can be constrained by other factors, e.g. leghaemoglobin content (Serraj et al., 1999).

Drought implies that less water is available for the transport of nitrogenous compounds from nodules. Further, since glutamine synthetase activity declines before that of nitrogenase, ammonia and other end products of fixation may accumulate in nodules leading to the complete collapse of N_2 fixation (Hungria, M., unpublished data). A feedback inhibition of N_2 fixation by ureides under water stress conditions has been shown and species transporting high concentrations of these N_2 compounds are likely to be more sensitive to drought stress than those with no or low ureide transport (Sinclair and Serraj, 1995).

In some studies, water stress does not seem to limit N_2 fixation through decreases in the availability of photosynthates. In soybean nodules, nitrogenase activity was almost eliminated with the onset of drought stress, whereas carbohydrate content was not affected (Durand et al., 1987; Fellows et al., 1987). Furthermore, nodule energy-charge recovered rapidly after rewatering, whereas recovery of nitrogenase activity required 4–5 days (Patterson et al., 1979). However, in plants subjected to elevated atmospheric CO_2, N_2 fixation changed from drought sensitive to very drought tolerant (Serraj et al., 1998), therefore indicating that N_2 fixation under water stress was limited by the availability of photosynthates.

Finally, it has been suggested that all three mechanisms, oxygen permeability changes, ureide feedback, and carbon shortage would be involved in the regulation of N_2 fixation under water stress, acting together. At the present moment it is not yet possible to resolve the sequence of mechanisms regulating the stress response (Serraj et al., 1999).

Conditions as a combination of dry and hot conditions, sandy soils or uncovered soils more adversely affect rhizobial survival and growth, and can be crucial for effective nodulation when rains are delayed after planting. Rhizobial strains (Boonkerd and Weaver, 1982) and plant species (Walsh, 1995) vary also in relation to drought tolerance. Finally, agricultural management practices, specially the use of crop residues to cover the soil can temper the effects of drought (Morote et al., 1990), and the positive effects of the no-tillage soil management will be discussed later.

4. Soil acidity

A large proportion of tropical soils have developed from old geological formations. This, combined with climatic conditions, has resulted in highly weathered soils, containing predominantly low-activity clays. These are commonly highly leached, acidic, and infertile, and frequently contain toxic concentrations of available aluminium (Al) and manganese (Mn) (Lal, 1993). Such acid-soil conditions pose problems for the plant, the bacteria and the symbiosis (Giller and Wilson, 1993). The microsymbiont is usually the more pH-sensitive partner. Some rhizobial species can tolerate acidity better than others, and tolerance may vary among strains within a species (Lowendorff, 1981; Vargas and Graham, 1988; Brockwell et al., 1995; Hungria et al., 1997b). The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0 (Jordan, 1984) and relatively few rhizobia grow well at pH less than 5.0 (Graham et al., 1994). Exceptions include strains of *Rhizobium tropici*, *Mesorhizobium loti* and *Bradyrhizobium* sp. *Sinorhizobium meliloti* in particular is highly acid-sensitive (Brockwell et al., 1995), but variation in acid pH tolerance were reported for this species (Graham et al., 1994).

Intrinsic tolerance cannot be predicted from the pH at the site of isolation. When fast-growing rhizobial strains were isolated from nodules of modern and primitive soybean genotypes that had been inoculated with soil from sites in Brazil where the pH ranged from 3.04 to 5.09, only 37% were able to grow in buffered medium at pH 4.0. Sixty percent grew at pH 9.5 (Hungria, M., Chueire, L.M.O., Coca, R., Megías, M., unpublished data).

It appears that acid pH tolerance in rhizobia depends on the ability to maintain an intracellular pH (pH_i) between 7.2 and 7.5, even at acid external pH (Kashket, 1985; O'Hara et al., 1989; Graham et al., 1994). Graham et al. (1994) reported acid-tolerance in *R. tropici* type IIB, CIAT 899 (=UMR 1899), and subsequent studies have identified outer membrane and cytoplasm response (Ballen et al., 1998). The tolerance of *R. tropici* to low pH could explain the high frequency of this species in acid soils of Central America, Brazil and Africa. When the pH_i of *S. meliloti* falls below 7.0 (Perez-Galdona and Kahn, 1994) or 6.5 (O'Hara et al., 1989), growth ceases, suggesting that some essential cytoplasmic process is extremely sensitive to acidity.

Acidity affects early steps in the infection process, including the exchange of molecular signals between symbiotic partners and attachment to the roots. Release of *nod*-gene inducers by soybean and common bean roots was less at pH 4.5 than at pH 5.8 (Hungria and Stacey, 1997), with some nodulation genes, including *nodA*, switched off as the pH falls (Richardson et al., 1988a,b). In addition, McKay and Djordjevic (1993) have reported that low pH can affect the production and excretion of nodulation factors in strains of *R. leguminosarum* bv. trifolii. Other stages of nodule establishment and function are also impacted by acidity, as is the growth of the host plant (Graham, 1981; Munns, 1986).

Low soil pH is often associated with increased Al and Mn toxicity and reduced calcium (Ca) supply. These additional stresses affect the growth of rhizobia (Cooper et al., 1983; Coventry and Evans, 1989; Campo, 1995), of the host legume (Andrews et al., 1973; Kim et al., 1985), and symbiosis (Murphy et al., 1984; Brady et al., 1990; Campo, 1995). Nodulated legumes are more sensitive to Al and Mn toxicity than plants receiving mineral N, e.g. Wouters et al. (1986) observed that an Al-tolerant common bean variety still produced 90% of maximum yield at 73% Al saturation of the exchange complex, while nodulation was completely suppressed at 33% of saturation.

The nature and site of action of Al ions remain undefined. Studies by Johnson and Wood (1987), Matsumoto (1991) and Wood (1995) have indicated that Al ions act by binding to DNA, interfering with cell division. However, since both Al-sensitive and Al-tolerant strains bound similar amounts of Al, a DNA-repair mechanism must exist in tolerant strains. In addition to Al toxicity, acidity may also be related to phosphorus (P) and molybdenum (Mo) deficiencies.

Liming has been considered the most efficient practice in overcoming soil acidity, with some of the benefits to legume crops not only due to increased soil pH, but also to increased availability of Ca to plant, bacteria and the symbiosis. Tn5-induced acid-sensitive mutants of *S. meliloti* were able to grow at low pH only with high concentrations of Ca in the growth medium (Reeve, 1993), and O'Hara et al. (1989) observed that several rhizobial mutants maintained pH_i more effectively when Ca concentration was increased 2-fold. Calcium may affect rhizobial cell wall integrity (Bergersen, 1961; Vincent, 1962; de Maagd et al., 1989; Ballen et al., 1998), some enzymes (Norris et al., 1991; Ballen et al., 1998), or membrane transport systems (O'Hara et al., 1989).

A better understanding of the mechanisms used by H^+-tolerant strains to survive acid stress would be of major importance in defining rational screening procedures. However, genetic studies of acid tolerance, have revealed little. Tiwari et al. (1996b) reported that the *actS* protein in *S. meliloti* may be involved in "sensing" the external environment and in the transmission of a signal that alters gene transcription. Inactivation of the *actS* gene results in the generation of an acid-sensitive phenotype, but tolerance can be restored if the *actS* gene is inserted into the mutant strain. Adjacent to the *actS* gene is *actR* which, when inactivated by Tn5-transposon mutagenesis, results in an acid-sensitive phenotype. *S. meliloti actA* is also required for acid tolerance (Goss et al., 1990; Tiwari et al., 1996a), and *actA* mutants are unable to regulate pH_i (O'Hara et al., 1989). Tiwari et al. (1996a) described a low-pH-regulated gene in *S. meliloti* termed *phrR*, adjacent to *actA*, which is induced 5- to 8-fold by acid conditions (Reeve, W.G., Tiwari, R.P., Wong, C., unpublished, cited by Glenn et al., 1997).

5. Strategies to alleviate high temperature, drought and acid soil limitations

5.1. Strain selection for tolerance to high temperature: a program for common bean rhizobia in Brazil

Physiological differences among plant species and rhizobial species and strains in relation to high tem-

perature, water stress and acidity have been reported and should be considered in selection programs for improving legume yields in areas subject to these stresses (e.g. Ferrari et al., 1967; Gibson, 1975; Munns, 1986; Walker and Miller, 1986; Piha and Munns, 1987; Karanja and Wood, 1988b).

Common bean is an important source of protein in the diets of about 500 million people in Latin America and Africa — especially for those living in poverty — but the plant is very sensitive to high temperatures. The optimum range for the root-nodule symbiosis is considered to be between 25 and 33°C (Barrios et al., 1963; Pankhurst and Sprent, 1976; Graham and Halliday, 1977), but declines in N_2 fixation activity have been observed above 28°C (Hungria et al., 1985; Piha and Munns, 1987; Hungria and Franco, 1993).

In Brazil, nodulation of common bean is usually poor, with failure attributed to high soil temperatures (Hungria et al., 1993, 1997b). The importance of high temperature tolerance in strains selected for use in the tropics has been pointed out by many authors (Dart et al., 1976; Munévar and Wollum, 1981a,b; La Favre and Eaglesham, 1986; Piha and Munns, 1987; Karanja and Wood, 1988b; Hafeez et al., 1991; Hungria et al., 1993). However, rhizobial temperature tolerance is apparently not closely related to the geographic latitude of their origin, or to the host species (Munévar and Wollum, 1981a; Karanja and Wood, 1988b; Hungria et al., 1993). Furthermore, the ability of a strain to grow at high temperatures in vitro does not necessarily correlate with nodulation and N_2 fixation capacity under similar conditions (La Favre and Eaglesham, 1986; Karanja and Wood, 1988b), and varies with host cultivar (Gitonga et al., 1989). Consequently, strain selection for tolerance to high temperature in vitro has not proved to be a promising approach.

Common bean is nodulated by a range of rhizobia species, including *R. leguminosarum* bv. phaseoli, *R. tropici* type IIA and type IIB, *R. etli* and the recently described *R. gallicum* and *R. giardinii*. Several studies have shown that *R. tropici* tolerates significantly higher temperatures than *R. leguminosarum* and *R. etli* (Martínez-Romero et al., 1991; Hungria et al., 1993; Mercante, 1993; Sá et al., 1993), is more acid tolerant (Graham et al., 1994; Anyango et al., 1995), and has a broad host range (Martínez-Romero et al., 1991; Hungria et al., 1993). Most importantly, *R. tropici* shows higher genetic stability, maintaining

symbiotic properties under stress (Soberón-Chavez et al., 1986; Martínez-Romero et al., 1991; Hungria et al., 1993; Segovia et al., 1993; Michiels et al., 1994). Martínez, E., (pers. comm.) estimates that the frequency of genomic rearrangement in *R. tropici* is 100 times less than for *R. etli* and *R. leguminosarum* bv. phaseoli. If plasmid loss is also considered, the genetic stability of *R. tropici* may be at least 1000 times greater than for *R. etli*.

The species of rhizobia that nodulate common beans also vary in N_2 fixation capacity and nodulation competitiveness with this host (Martínez-Romero and Rosenblueth, 1990; Oliveira and Graham, 1990; Hungria et al., 1999a). *R. tropici* is more competitive at low pH (around 5.0) than at pH>6.0 (Vargas and Graham, 1989; Streit et al., 1992; Frey and Blum, 1994). Again, this favors the use of this species in acid tropical soils. Heterogeneity among *R. tropici* strains has been reported for both efficiency in N_2 fixation and competitiveness (Mercante, 1993; Sá et al., 1993; Hungria et al., 1997b, 1999a), indicating that superior strains may be obtained through selection programs.

Genetic stability, especially under conditions of stress is essential for any strain recommended for use in commercial inoculants. This was demonstrated in Brazil in 1994, when one of the two strains used in commercial inoculants, SEMIA 4064 (=UMR 1135, from the University of Minnesota, USA, classified as *R. leguminosarum* bv. phaseoli), lost its ability to fix N_2 in several greenhouse and field experiments. This repeated previous experience with two other strains used in Brazilian inoculants, C-05-0 and V-23, none classified as *R. tropici* (Hungria and Araujo, 1995) and with the Australian inoculant-quality strain CC511 (Graham, P.H., pers. comm.). A selection program was initiated to find efficient, competitive and high temperature-tolerant strains of *R. tropici*, the species considered most likely to be suitable for the soils of Brazil. The first phase of this program examined temperature-tolerant rhizobia isolated from 18 leguminous tree species. Sixty-nine isolates were evaluated for nodulation of common bean. Fourteen isolates from *Leucaena*, *Lonchocarpus* and *Gliricidia* were Nod^+Fix^+ on common bean, and four of these accumulated at least as much N as plants receiving 50 mg N per week when grown at 40°C for 8 h per day (Hungria et al., 1993). Among other strains obtained using *Leucaena* as a trap host, 84% belonged to *R.*

tropici type IIA, and only 16% belonged to *R. tropici* type IIB. However, 67% of the type IIB strains tolerated high temperatures, whereas only 1.7% type IIA strains were temperature tolerant (Mercante, 1993). Unfortunately, no field experiments were conducted with the strains selected in these two experiments. In a parallel study in the State of Paraná, strains isolated using common bean as a trap host, were also tested for tolerance of environmental stresses, ability to nodulate *Leucaena*, for higher N_2 fixation capacity, competitiveness, and yield performance. This screening led to the identification of a strain, PRF 81 (=SEMIA 4080), which is intermediate in properties between *R. tropici* IIA and IIB. Strain PRF 81 is tolerant of high temperatures and acidity in vitro and in vivo, effective in N_2 fixation with common bean and highly competitive against indigenous bean rhizobia. Significant yield increases of up to 900 kg ha^{-1} have been reported at several sites with high populations of indigenous common bean rhizobia (Hungria et al., 2000). Table 1 shows the response obtained by inoculation with PRF 81. The strain has been recommended for use in Brazil since 1998, and superior performance is already increasing farmers' demands for bean inoculants. The responses to inoculation with PRF 81 encourages further efforts to identify genetically stable strains that

are more tolerant of environmental stresses as well as competitive and efficient with common bean in tropical conditions. The broad use of such strains could, at low cost, improve yields and reverse the trend of low bean yields in South and Central America and Africa.

5.2. How to overcome the acidity problem?

Survival of root-nodule bacteria at acid pH may be affected by prior growth history, particularly medium composition, with an adaptive acid-tolerance response inducible by low pH in some root-nodule bacteria (O'Hara and Glenn, 1994). In *S. meliloti*, growth under mildly acidic conditions improves the performance of cells subsequently exposed to severe acid stress (O'Hara and Glenn, 1994). Inoculants prepared under mildly acid conditions might thus be expected to perform better in tropical acid soils, but that approach has frequently been unsuccessful (Robson and Bottomley, 1991). This is probably because the traits required to survive in soil and effectively nodulate legumes result from a combination of characteristics in addition to the capacity to tolerate H^+ ions.

Variability among common bean varieties (Miranda and Lobato, 1978) and strains (Karanja and Wood, 1988a; Vargas and Graham, 1988) as well as among

Table 1
Response of bean cultivar Carioca to inoculation with six *Rhizobium* strains[a]

Treatment[b]	Greenhouse experiments				
	28/23 (°C)		37/23 (°C)	
	Nodule number (number per plant)	Total N[c] (mg per plant)	Nodule number (number per plant)	Total N[c] (mg per plant)
PRF 81	141.0 a[e]	99.7 a[e]	89.2 a[e]	66 a[e]
CFN 299	81.0 bc	65.1 c	19.0 c	25 c
CIAT 899	128.0 a	85.4 ab	65.3 b	60 ab
CFN 42	95.8 b	75.0 bc	17.2 c	20 c
USDA 2671	68.0 c	40.2 d	9.2 c	10 c
Control[d]−N	0[f]	9.7[f]	0[f]	3[f]
Control[d]+N	0[f]	98.6 a	0[f]	49 b

[a] Greenhouse experiment plants were grown either at optimal temperatures (28/23°C, day/night) or under stress (37/23°C) conditions, in modified Leonard jars containing N-free nutrient solution and were harvested at 45 days after emergence (modified from Hungria et al., 2000).

[b] *Rhizobium* strains: PRF 81 (Brazilian strain with mixed characteristics of *R. tropici* type IIA and type IIB); CFN 299 (*R. tropici* IIA), CIAT 899 (*R. tropici* IIB), CFN 42 (*R. etli*), USDA 2671 (*R. leguminosarum* bv. phaseoli).

[c] N-Kjeldahl (N roots+N shoot+N nodules−N seed).

[d] Non-inoculated controls supplied or not with mineral N (30 mg of N as KNO_3 per plant per week).

[e] Means of five replicates and values followed by the same letter, in the same column, did not show statistical difference (Tukey, $p \leq 0.05$).

[f] Values not considered for the statistical analyses.

soybean varieties (Toledo et al., 1994) and strains (Asanuma and Ayanaba, 1990; Campo, 1995) in relation to tolerance to acidity and Al have been reported. Also significant host/strain interactions at low pH can occur (Vargas and Graham, 1988) and should be considered in tropical acid soils. Chemical mutagenesis can increase the acid tolerance of rhizobia (Rakotoarisoa et al., 1981), but to date no agronomically useful strains have been thus obtained. Another approach is to use acidic soils as the source of root-nodule bacteria. Gemmell and Roughley (1993) observed that rhizobial isolates from acid soils were significantly more tolerant of acidity than were those from neutral soils. Wood and Sheperd (1987) failed to obtain acid-tolerant strains with that method, although successful results were obtained with rhizobia nodulating pasture legumes in Australia and Colombia (Sylvester-Bradley et al., 1983; Howieson et al., 1988).

Acidity is an increasing problem in tropical soils, especially as a result of poor management, and liming is currently the only ameliorative practice extensively used. However, in Brazil, commercial cultivars of soybean and common bean and the rhizobia recommended for commercial inoculants tolerate moderate levels of acidity, and liming to neutral pH is not necessary. Thus, although nodulation is drastically reduced at pH 4.5, good nodulation and N_2 fixation rates can be reached above pH 5.2. Liming must still be used to reach this pH, and to ensure the availability of nutrients such as calcium and molybdenum.

Decreases in soybean and common bean nodulation under acidic conditions may be alleviated in part by supplementation with flavonoid *nod*-gene inducers (Hungria and Stacey, 1997), with studies to examine varietal differences in response or to maximize the expression of rhizobial *nod*-genes under acid conditions still needed. Studies should also consider the impact of acidity and lime application on rhizobial population. In a recent study with common bean grown in a Brazilian soil with a high population of indigenous strains, acidity altered both the rhizobial abundance and genetic diversity, which were lower in the unlimed soil (Andrade et al., 1999). Such changes in population structure may influence responses to inoculation; however, this has not been investigated. Most importantly, as many Brazilian soils show high levels of Al (50–70% saturation),

programs searching for acid-tolerant varieties and strains should continue.

5.3. The soybean bradyrhizobial strains adapted to the Brazilian Cerrados

Genetic engineering is a promising tool for the production of superior rhizobial strains, but the soil will continue to be an important reservoir for efficient, competitive and stress-tolerant strains. Very often the effort required in strain selection proves less than is needed for the development of engineered strains. In Brazil, a successful strain-selection program has been developed for the Brazilian "Cerrados" savanna, an area of 207 million ha (25% of Brazilian land), to which soybean was introduced as a new crop in the 1960s. Today, soybean is Brazil's most important grain export and the main crop in the Cerrados, where growth is strongly dependent on N_2 fixation. This high dependence on N_2 fixation contrasts to practices in northern USA, where plants may derive only 40–50% of their N needs from symbiosis.

The Cerrados were originally free of bradyrhizobia strains able to establish an efficient symbiosis with soybean. The first inoculants carried strain SEMIA 566, commercially recommended from 1966 to 1978. Two other strains, which showed better nodulation with commercial cultivars, then replaced SEMIA 566 (Vargas and Hungria, 1997). More than a decade later, the higher N demands of the newer, more productive soybean genotypes has led to a need for strains with improved capacity for N_2 fixation. However, stressful conditions in the Cerrados — long periods of drought, high temperatures, soil acidity and Al toxicity — limited the choice of strains. To this end, isolation of efficient naturalized strains "adapted" to these inhospitable conditions was attempted from soybean production areas first sown more than 15 years previously. These soils had been inoculated with SEMIA 566 or other strains at that time, but had no further inoculation. Good nodulation was obtained where there were established rhizobial populations, and bacteria isolated from large pink nodules were tested individually for N_2 fixation capacity under greenhouse conditions, and, subsequently, for grain yield in several field trials. An efficient isolate, CPAC 15 (=SEMIA 5079), proved to be superior, increasing soybean yield by up to 750 kg ha^{-1} in comparison

with the commercially recommended strains. This strain has been recommended for soybean inoculation since 1992 (Vargas et al., 1992, 1994).

While CPAC 15 belongs to the SEMIA 566 serogroup, further analyses have shown morphological, physiological and genetic differences between CPAC 15 and SEMIA 566: production of mucus, lower synthesis of indole acetic acid, increased Hai phenotype, and changes in DNA profile after amplification by RAPD or by ERIC/REP-PCR (Hungria and Vargas, 1996; Hungria et al., 1996; Nishi et al., 1996; Boddey and Hungria, 1997). Other selected strains belonging to the SEMIA 566 serogroup are being investigated, with the great majority far more competitive than the parental strain, and some showing higher capacity for N_2 fixation and supporting higher yields (Hungria et al., 1998). These strains also differ from the parental SEMIA 566 in morphological, physiological and genetic characteristics; Fig. 2 shows the ERIC-PCR profiles of selected strains and SEMIA 566 as well as differences in their N_2 fixation capacity. This program has shown that searching for adapted strains allows the selection for tolerance of a variety of environmental stress factors, as well as for higher N_2 fixation capacity and competitiveness, ultimately enabling increases in grain yield.

5.4. Agricultural management practices

Some agricultural practices, like intercropping, multiple cropping, crop rotation and use of cover crops have proven to be microbiologically and economically attractive, improving N_2 fixation and yield under Brazilian conditions and contributing to agricultural sustainability.

In large areas of Brazil, conventional tillage, with the traditional practices of plowing and disking to prepare land, reduces soil organic matter and increases erosion. In contrast, no-till management, with seeds sown directly through the residue of the previous crop, protects the soil against erosion by water, improves soil structure, stability and moisture content, helps in the regulation of soil temperature and, with time, increases soil organic matter content (Derpsch et al., 1991). Since no-till benefits fragile soils particularly, improving physico-chemical and biological properties, its adoption is increasing in Brazil, nowadays occupying some 11 Mha, or 20% of the cropped
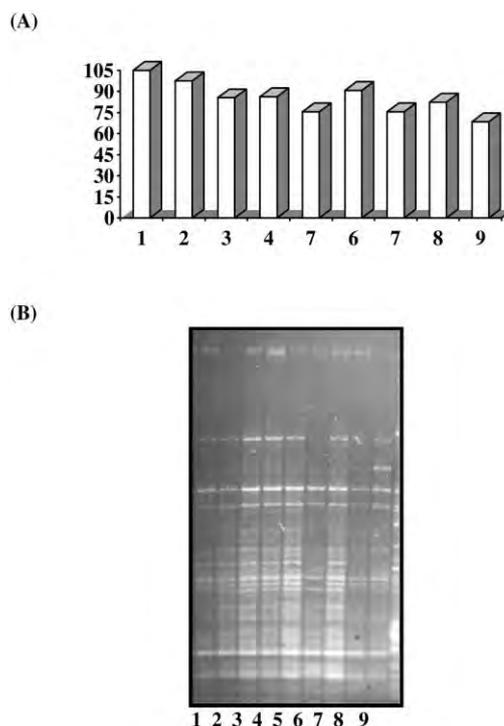

Fig. 2. (A) Total N (mg N per plant) accumulated in soybean, cultivar BR-16, inoculated with seven serogroup SEMIA 566 isolates, with CPAC 15 (also a serogroup SEMIA 566 isolate, column 8) and with SEMIA 566 (column 9). All strains were statistically different from SEMIA 566 (Tukey, $p \leq 0.05$); (B) DNA profile of the strains after amplification by ERIC-PCR. After Hungria et al. (1999b).

area. No-till farming practices result in maximum soil temperatures 5–10°C lower when compared to conventional till, and the soil oscillations are also lower (Lal, 1993). These differences in temperature, added to higher moisture content, may greatly benefit N_2 fixation. Thus, in the absence of inoculation the native population of common bean rhizobia increased from 2.5×10^3 cells g^{-1} soil with conventional till to 3.9×10^4 cells g^{-1} with no-till. Increases were also observed in *nod*-gene inducing activity of the soil solution, nodulation, crop yield, and in the diversity of the rhizobial strains (Hungria and Vargas, 1996). With soybean grown under no-till, not only was nodule number increased as compared with conventional till, but nodulation extended to deeper layers in the soil (Voss and Sidiras, 1985). Furthermore, no-till increased soybean bradyrhizobial population, nodulation and N_2 fixation rates (Fig. 1) with a greater

diversity of strains also found (Ferreira et al., 2000). However, several aspects are yet to be studied. One is that the surface application of lime or gypsum in the no-till system with further leaching over time creates variable pH, Ca and Mg contents in the different soil layers (Caires et al., 1998), and could impact distribution and biodiversity of rhizobia. Consequently, the use of agronomic practices such as no-till may not only improve agricultural sustainability, but also help the establishment of rhizobial populations in soil and enhance genetic diversity. These factors should all contribute to higher yield at little cost.

6. Conclusion

Tropical soils are often deficient in N, therefore biological N_2 fixation is a key process to agricultural sustainability. However, N_2 fixation may be limited by tropical environmental stressful conditions, such as high temperatures, drought and soil acidity. In Brazil, strain selection has been an integral part of both common bean and soybean production focus. Appropriate soil-management practices for the tropics, e.g. no-till, which result in decreases in soil temperature and increase in soil moisture, also benefit N_2 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.

References

Andrade, D.S., Murphy, P.J., Giller, K.E., 1999. Genetic diversity of rhizobial populations nodulating *Phaseolus vulgaris* in acid soils from Brazil. In: Martínez, E., Hernández, G. (Eds.), Highlights of Nitrogen Fixation Research. Kluwer Academic Publishers, New York, pp. 291–294.

Andrews, C.S., Johnson, A.D., Sandland, R.L., 1973. Effect of aluminium on the growth and chemical composition of some tropical and temperate pasture legumes. Aust. J. Agric. Res. 24, 325–339.

Anyango, B., Wilson, K.J., Beynon, J.L., Giller, K.E., 1995. Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. Appl. Environ. Microbiol. 61, 4016–4021.

Asanuma, S., Ayanaba, A., 1990. Variation in acid-Al tolerance of *Bradyrhizobium japonicum* strains from African soils. Soil Sci. Plant Nutr. 36, 309–317.

Ballen, K.G., Graham, P.H., Jones, R.K., Bowers, J.H., 1998. Acidity and calcium interaction affecting cell envelope stability in *Rhizobium*. Can. J. Microbiol. 44, 582–587.

Barrios, S., Raggio, N., Raggio, M., 1963. Effect of temperature on infection of isolated bean roots by rhizobia. Plant Physiol. 38, 171–174.

Bergersen, F.J., 1961. The growth of *Rhizobium* in synthetic media. Aust. J. Biol. Sci. 14, 349–360.

Bergersen, F.J., Turner, G.L., Appleby, C.A., 1973. Studies of the physiological role of leghaemoglobin in soybean root nodules. Biochim. Biophys. Acta 292, 271–282.

Boddey, L.H., Hungria, M., 1997. Phenotypic grouping of Brazilian *Bradyrhizobium* strains which nodulate soybean. Biol. Fert. Soils 25, 407–415.

Boonkerd, N., Weaver, R.W., 1982. Survival of cowpea rhizobia as affected by soil temperature and moisture. In: Graham, P.H., Harris, S.C. (Eds.), Biological Nitrogen Fixation Technology for Tropical Agriculture. CIAT, Cali/Colombia, pp. 207–212.

Brady, D.J., Hecht-Buchholz, C.H., Asher, C.D., Edwards, D.G., 1990. Effects of low activities of aluminium on soybean (*Glycine max*) 1. Early growth and nodulation. In: van Beusichem, M.L. (Ed.), Plant Nutrition — Physiology and Applications. Kluwer Academic Publishers, Dordrecht, pp. 329–334.

Brockwell, J., Bottomley, P.J., Thies, J.E., 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. Plant Soil 174, 143–180.

Caires, E.F., Chueiri, W.A., Madruga, E.F., Figueiredo, A., 1998. Alterações de características químicas do solo e resposta da soja ao calcário e gesso aplicados na superfície em sistema de cultivo sem preparo do solo. R. Bras. Ci. Solo 22, 27–34.

Campo, R.J., 1995. Residual effects of aluminium on *Bradyrhizobium japonicum* in defined medium and soil solution. Ph.D. Thesis. The University of Reading, Reading.

Cooper, J.E., Wood, M., Holding, A.J., 1983. The influence of soil acidity factors on rhizobia. In: Jones, D.G., Davies, D.R. (Eds.), Temperate Legumes: Physiology, Genetics and Nodulation. Pittman, London, pp. 319–335.

Coventry, D.R., Evans, J., 1989. Symbiotic nitrogen fixation and soil acidity. In: Robson, A.D. (Ed.), Soil Acidity and Plant Growth. Academic Press, Sydney, pp. 103–137.

Dart, P.J., 1974. Development of root-nodule: the infection process. In: Quispel, A. (Ed.), The Biology of Nitrogen Fixation. North-Holland, Amsterdam, pp. 382–398.

Dart, P.J., Day, J., Islam, R., Döbereiner, J., 1976. Symbiosis in tropical grain legumes: some effects of temperature and the composition of the rooting medium. In: Nutman, P.S. (Ed.), Symbiotic Nitrogen Fixation in Plants. Cambridge University Press, London, pp. 361–383.

Dart, P.J., Mercer, F.V., 1965. The effect of growth temperature, level of ammonium nitrate, and light intensity on the growth and nodulation of cowpea (*Vigna sinensis* Endl. ex. Hassk.). Aust. J. Agric. Res. 16, 321–345.

Day, J.M., Roughley, R.J., Eaglesham, A.R.J., Dye, M., White, S.P., 1978. Effect of high soil temperatures on nodulation of cowpea, *Vigna unguiculata*. Ann. Appl. Biol. 88, 476–481.

de Maagd, R.A., Wientjes, F.B., Lugtenberg, B.J.J., 1989. Evidence for divalent cation (Ca^{2+}) stabilized oligomeric proteins and covalently bound protein peptidoglycan complexes in the outer membrane for *Rhizobium leguminosarum*. J. Bacteriol. 171, 3899–3995.

Derpsch, R., Roth, C.H., Sidiras, N., Kopke, U., 1991. Controle da erosão no Paraná, Brasil: sistemas de cobertura do solo, plantio direto e preparo conservacionista do solo. GTZ, Eschborn, 268 pp. (Sonderpublikation der GTZ, 245).

Dunigan, E.P., Bollich, P.K., Huchinson, R.L., Hicks, P.M., Zaunbrecher, F.C., Scott, S.G., Mowers, R.P., 1984. Introduction and survival of an inoculant strain of *Rhizobium japonicum* in soil. Agron. J. 76, 463–466.

Durand, J.L., Sheehy, J.E., Minchin, F.R., 1987. Nitrogenase activity, photosynthesis and nodule water potential in soybean plants experiencing water deprivation. J. Exp. Bot. 38, 311–321.

Eaglesham, A.R.J., Ayanaba, A., 1984. Tropical stress ecology of rhizobia, root nodulation and legume nitrogen fixation. In: Subba Rao, N.S. (Ed.), Selected Topics in Biological Nitrogen Fixation. Oxford/IBH Publishing, New Dehli, pp. 1–35.

Fellows, R.J., Patterson, R.P., Raper Jr., C.D., Harris, D., 1987. Nodule activity and allocation of photosynthate of soybean during recovery from water stress. Plant Physiol. 84, 456–460.

Ferreira, M.C., Andrade, D.S., Chueire, L.M.O., Takemura, S.M., Hungria, M., 2000. Effects of tillage method and crop rotation on the population sizes and diversity of bradyrhizobia nodulating soybean. Soil Biol. Biochem., in press.

Ferrari, E., Souto, S.M., Döbereiner, J., 1967. Efeito da temperatura do solo na nodulação e no desenvolvimento da soja perene (*Glycine javanica* L.). Pesq. Agropec. Bras. 2, 461–466.

Frey, S.D., Blum, L.K., 1994. Effect of pH on competition for nodule occupancy by type I and type II strains of *Rhizobium leguminosarum* bv. *phaseoli*. Plant Soil 163, 157–164.

Frings, J.F.J., 1976. The *Rhizobium*-pea symbiosis as affected by high temperatures. Thesis, Medelingen Landbouwogeschool, Wageningen Agricultural University, Wageningen.

Gemmell, L.G., Roughley, R.J., 1993. Field evaluation in acid soils of strains of *Rhizobium leguminosarum* biovar *trifolii* selected for their tolerance or sensitivity to acid soil factors in agar medium. Soil Biol. Biochem. 25, 1447–1452.

Gibson, A.H., 1971. Factors in the physical and biological environment affecting nodulation and nitrogen fixation by legumes. Plant Soil (Spec. Vol.), 139–152.

Gibson, A.H., 1975. Recovery and compensation by nodulated legumes to environmental stress. In: Nutman, P.S. (Ed.), Nitrogen Fixation in Plants. Cambridge University Press, London, pp. 385–443.

Giller, K.E., Wilson, K.J., 1993. Nitrogen Fixation in Tropical Cropping Systems. CAB International, Wallingford, UK, 313 pp.

Gitonga, N.M., Widdowson, D., Keya, S.O., 1989. Interaction of *Phaseolus vulgaris* with thermotolerant isolates of *Rhizobium leguminosarum* biovar *phaseoli* from Kenyan soils. MIRCEN J. 5, 493–504.

Glenn, R.P., Tiwari, W.G., Reeve, W.G., Dilworth, M.J., 1997. The response of root nodule bacteria to acid stress. In: Moniz, A.C., Furlani, A.M.C., Schaffert, R.E., Fageria, N.K., Rosolen, C.A., Cantarella, H. (Eds.), Plant–Soil Interactions at Low pH: Sustainable Agriculture and Foresting Production. Brazilian Soil Science Society, Campinas/Brazil, pp. 123–138.

Goss, T.J., O'Hara, G.W., Dilworth, M.J., Glenn, A.R., 1990. Complementation, characterization and complementation of lesions causing acid sensitivity in Tn5-induced mutants of *Rhizobium meliloti* WSM419. J. Bacteriol. 172, 5173–5179.

Graham, P.H., 1981. Some problems of nodulation and symbiotic nitrogen fixation in *Phaseolus vulgaris*: a review. Field Crops Res. 4, 93–112.

Graham, P.H., Draeger, K.J., Ferrey, M.L., Conroy, M.J., Hammer, B.E., Martínez, E., Aarons, S.R., Quinto, C., 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. Can. J. Microbiol. 40, 198–207.

Graham, P.H., Halliday, J., 1977. Inoculation and nitrogen fixation in the genus *Phaseolus*. In: Vincent, J.M., Whitney, A.S., Bose, J. (Eds.), Exploiting the Legume Symbiosis in Tropical Agriculture. College of Tropical Agricultural Miscel. Publication 145, Hawaii, pp. 313–333.

Guerin, V., Pladys, D., Trinchant, C., Rigaud, J.M., 1991. Proteolysis and nitrogen fixation in faba-bean (*Vicia faba*) nodules under water stress. Physiol. Plant 82, 360–366.

Hafeez, F.Y., Asad, S., Malik, K.A., 1991. The effect of high temperature on *Vigna radiata* nodulation and growth with different bradyrhizobial strains. Environ. Exp. Bot. 31, 285–294.

Hernandez-Armenta, R., Wien, H.C., Eaglesham, A.R.J., 1989. Carbohydrate partitioning and nodule function in common bean after heat stress. Crop Sci. 29, 1292–1297.

Howieson, J.G., Ewing, M.A., D'Antuono, M.F., 1988. Selection for acid tolerance in *Rhizobium meliloti*. Plant Soil 105, 179–188.

Hungria, M., 1995. Efeito das temperaturas elevadas na exsudação de indutores dos genes *nod* pelo feijoeiro e soja. In: Hungria, M., Balota, E.L., Colozzi-Filho, A., Andrade, D.S. (Eds.), Microbiologia do Solo: Desafios para o Século XXI. IAPAR/EMBRAPA-CNPSo, Londrina/Brazil, pp. 368–373.

Hungria, M., Andrade, D.S., Balota, E.L., Colozzi-Filho, A., 1997a. Importância do sistema de semeadura direta na população microbiana do solo. EMBRAPA-CNPSo, Londrina/Brazil, pp. 1–9 (Comunicado Técnico 56).

Hungria, M., Andrade, D.S., Chueire, L.M. de O., Megías, M., 1999a. Characterization of new efficient and competitive strains for the bean (*Phaseolus vulgaris* L.) crop in Brazil. In:

Martínez, E., Hernández, G. (Eds.), Highlights on Nitrogen Fixation Research. Plenum Press, New York, pp. 251–254.

Hungria, M., Andrade, D.S., Chueire, L.M. de O., Probanza, A., Guttierrez-Manero, J., Megías, M., 2000. Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia strains. Soil Biol. Biochem., in press.

Hungria M., Araujo R.S., 1995. Relato da VI reunião de laboratórios para recomendação de estirpes de *Rhizobium* e *Bradyrhizobium*. In: Hungria, M., Balota, E.L., Colozzi-Filho, A., Andrade, D.S. (Eds.), Microbiologia do Solo: Desafios para o Século XXI. IAPAR/EMBRAPA-CNPSo, Londrina/Brazil, pp. 476–489.

Hungria, M., Boddey, L.H., Santos, M.A., Vargas, M.A.T., 1998. Nitrogen fixation capacity and nodule occupancy by *Bradyrhizobium japonicum* and *B. elkanii* strains. Biol. Fert. Soils 27, 393–399.

Hungria, M., Franco, A.A., 1993. Effects of high temperature on nodulation and nitrogen fixation by *Phaseolus vulgaris* L. Plant Soil 149, 95–102.

Hungria, M., Franco, A.A., Sprent, J.I., 1993. New sources of high-temperature tolerant rhizobia for *Phaseolus vulgaris* L. Plant Soil 149, 103–109.

Hungria, M., Hardy, R.W.F., Eaglesham, A.R.J., 1989. Nitrogen fixation and assimilation in common bean (*Phaseolus vulgaris* L.) grown at high temperatures. Book of Abstracts of the 12th North American Symbiotic Nitrogen Fixation Conference, Ames, USA, 30 July–3 August, P-39, p. 106.

Hungria, M., Nishi, C.Y.M., Cohn, J., Stacey, G., 1996. Comparison between parental and variant soybean *Bradyrhizobium* strains with regard to the production of lipo-chitin nodulation signals, early stages of root infection, nodule occupancy, and N$_2$ fixation rates. Plant Soil 186, 331–341.

Hungria, M., Stacey, G., 1997. Molecular signals exchanged between host plants and rhizobia: basic aspects and potential application in agriculture. Soil Biol. Biochem. 29, 519–830.

Hungria, M., Thomas, R.J., Döbereiner, J., 1985. Efeito do sombreamento na fixação biológica do nitrogênio em feijoeiro. Pesq. Agropec. Bras. 20, 1143–1156.

Hungria, M., Vargas, M.A.T., 1996. Exploring the microbial diversity in soil management practices to optimize the contribution of soil microorganisms to plant nutrition. In: Stacey, G., Mullin, B., Gresshoff, P. (Eds.), Biology of Plant–Microbe Interactions. ISMPMI, St. Paul, pp. 493–496.

Hungria, M., Vargas, M.A.T., Araujo, R.S., 1997b. Fixação biológica do nitrogênio em feijoeiro. In: Vargas, M.A.T., Hungria, M. (Eds.), Biologia dos Solos dos Cerrados. EMBRAPA-CPAC, Planaltina/Brazil, pp. 189–295.

Hungria, M., Vargas, M.A.T., Andrade, D.S., Campo, R.J., Chueire, L.M.O., Ferreira, M.C., Mendes, I.C., 1999b. Fixação biológica do nitrogênio em leguminosas de grãos. In: Siqueira, J.O., Moreira, F.M.S., Lopes, A.S., Guilherme, L.R., Faquin, V., Furtinni, A.E., Carvalho, J.G. (Eds.), Soil Fertility, Soil Biology and Plant Nutrition Interrelationships. SBCS/UFLA/DCS, Lavras, in press.

Joffe, A., Weyer, F., Saubert, S., 1961. The role of root temperature in symbiotic nitrogen fixation. S. African J. Sci. 57, 278–282.

Johnson, A.C., Wood, M., 1987. Deonized distilled water as a medium for aluminium toxicity studies of *Rhizobium*. Lett. Appl. Microbiol. 4, 137–139.

Jones, F.R., Tisdale, W.B., 1921. Effect of soil temperature upon the development of nodules on the roots of certain legumes. J. Agric. Res. 22, 17–27.

Jordan, D.C., 1984. Family III Rhizobiaceae CONN 1938, 321 [AL]. In: Krieg, N.R., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology. William and Wilkins, Baltimore/London, pp. 235–244.

Karanja, N.K., Wood, M., 1988a. Selecting *Rhizobium phaseoli* strains for use with beans (*Phaseolus vulgaris* L.) in Kenya: infectiveness and tolerance of acidity and aluminium. Plant Soil 112, 7–13.

Karanja, N.K., Wood, M., 1988b. Selecting *Rhizobium phaseoli* strains for use with beans (*Phaseolus vulgaris* L.) in Kenya: tolerance of high temperature and antibiotic resistance. Plant Soil 112, 15–22.

Kashket, E., 1985. The proton motive force in bacteria. A critical assessment of methods. Ann. Rev. Microbiol. 39, 219–242.

Kim, M.M., Asher, C.J., Edwards, D.G., Date R.A., 1985. Aluminium toxicity: effects on growth and nodulation of subterranean clover. Proceedings of the 15th International Grasslands Congress, Kentucky, pp. 501–503.

La Favre, A.K., Eaglesham, A.R.J., 1986. The effects of high temperatures on soybean nodulation and growth with different strains of bradyrizobia. Can. J. Microbiol. 32, 22–27.

Lal, R., 1993. The role of no-till farming in sustainable agriculture in tropics. Anais do I Encontro Latinoamericano sobre Plantio Direto na Pequena Propriedade, IAPAR, Ponta Grossa/Brazil, 22–26 Novembro, pp. 29–62.

Lie, T.A., 1971. Symbiotic nitrogen fixation under stress conditions. Plant Soil (Spec. Vol.), 117–128.

Lie, T.A., 1974. Environmental effects on nodulation and symbiotic nitrogen fixation. In: Quispel, A. (Ed.), The Biology of Nitrogen Fixation. North-Holland, Amsterdam, pp. 555–582.

Lie, T.A., 1981. Environmental physiology of the legume–*Rhizobium* symbiosis. In: Broughton, W.J. (Ed.), Nitrogen Fixation, Vol. 1, Ecology. Clarendon Press, Oxford, pp. 104–134.

Lindemann, W.C., Ham, G.E., 1979. Soybean plant growth, nodulation and nitrogen fixation as affected by root temperature. Soil Sci. Soc. Am. J. 43, 1134–1137.

Lowendorff, H.S., 1981. Factors affecting survival of *Rhizobium* in soil. Adv. Microbial Ecol. 4, 87–124.

Martínez-Romero, E., Rosenblueth, M., 1990. Increased bean (*Phaseolus vulgaris* L.) nodulation competitiveness of genetically modified *Rhizobium* strains. Appl. Environ. Microbiol. 56, 2384–2388.

Martínez-Romero, E., Segovia, E., Mercante, F.M., Franco, A.A., Graham, P.H., Pardo, M.A., 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. 41, 417–426.

Matsumoto, H., 1991. Biochemical mechanism of the toxicity of aluminium and the sequestration of aluminium in plant cells. In: Wright, R.J.V., Baligar, C., Murrmann, R.P. (Eds.), Plant–Soil Interactions at Low pH. Kluwer Academic Publishers, Dordrecht, pp. 825–838.

McKay, I.A., Djordjevic, M., 1993. Production and excretion of *nod* metabolites by *Rhizobium leguminosarum* biovar *trifolii* are disrupted by the same environmental factors that reduce nodulation in the field. Appl. Environ. Microbiol. 59, 3385–3392.

Mercante, F.M., 1993. Uso de *Leucaena leucocephala* na obtenção de *Rhizobium* tolerante a temperatura elevada para inoculação do feijoeiro. M.Sc. Thesis. UFRRJ, Itaguai/Brazil.

Michiels, J., Verreth, C., Vanderleyden, J., 1994. Effects of temperature stress on bean-nodulating *Rhizobium* strains. Appl. Environ. Microbiol. 60, 1206–1212.

Miranda, L.N., Lobato, E., 1978. Tolerância de variedades de feijão e de trigo ao alumínio e à baixa disponibilidade de fósforo no solo. R. Bras. Ci. Solo 2, 44–50.

Morote, C.G.B., Vidor, C., Mendes, N.G., Pereira, J.S., 1990. Melhoria da nodulação da soja pela cobertura do solo e inoculação com *Bradyrhizobium japonicum*. R. Bras. Ci. Solo 14, 143–150.

Munévar, F., Wollum II, A.G., 1981a. Growth of *Rhizobium japonicum* strains at temperatures above 27°C. Appl. Environ. Microbiol. 42, 272–276.

Munévar, F., Wollum II, A.G., 1981b. Effect of high root temperature and *Rhizobium* strain on nodulation, nitrogen fixation, and growth of soybeans. Soil Sci. Soc. Am. J. 45, 1113–1120.

Munns, D.N., 1986. Acid soil tolerance in legumes and rhizobia. Adv. Plant Nutr. 2, 63–91.

Murphy, H.E., Edwards, D.G., Asher, C.J., 1984. Effects of aluminium on nodulation and early growth of four tropical pasture legumes. Aust. J. Agric. Res. 35, 663–673.

Nishi, C.Y.M., Boddey, L.H., Vargas, M.A.T., Hungria, M., 1996. Morphological, physiological and genetic characterization of two new *Bradyrhizobium* strains recently recommended as Brazilian commercial inoculants for soybean. Symbiosis 20, 147–162.

Norris, V., Chen, M., Goldberg, M., Voskuil, J., McGurk, G., Holland, I.B., 1991. Calcium in bacteria. A solution to which problem? Mol. Microbiol. 5, 775–778.

O'Hara, G.W., Glenn, A.R., 1994. The adaptive acid-tolerance response in root nodule bacteria and *Escherichia coli*. Arch. Microbiol. 161, 286–292.

O'Hara, G.W., Goss, T.J., Dilworth, M.J., Glenn, A.R., 1989. Maintenance of intracellular pH and acid tolerance in *Rhizobium meliloti*. Appl. Environ. Microbiol. 55, 1870–1876.

Oliveira, L.A., Graham, P.H., 1990. Speed of nodulation and competitive ability among strains of *Rhizobium leguminosarum* bv. *phaseoli*. Arch. Microbiol. 153, 311–315.

Pankhurst, C.E., Gibson, A.H., 1973. *Rhizobium* strain influence on disruption of clover nodule development at high temperature. J. Gen. Microbiol. 74, 219–231.

Pankhurst, C.E., Sprent, J.I., 1976. Effects of temperature and oxygen tension on the nitrogenase and respiratory activities of turgid and water-stressed soybeans and French bean root nodules. J. Exp. Bot. 27, 1–9.

Patterson, R.P., Raper Jr., C.D., Gross, H.D., 1979. Growth and specific nodule activity of soybean during application and recovery of a leaf moisture stress. Plant Physiol. 64, 551–556.

Perez-Galdona, R., Kahn, M.L., 1994. Effects of organic acids and low pH on *Rhizobium meliloti* 104A14. J. Gen. Microbiol. 140, 1231–1235.

Piha, M.I., Munns, D.N., 1987. Sensitivity of the common bean (*Phaseolus vulgaris* L.) symbiosis to high soil temperature. Plant Soil 98, 183–194.

Rainbird, R.M., Atkins, C.A., Pate, J.S., 1983. Effect of temperature on nitrogenase functioning in cowpea nodules. Plant Physiol. 73, 392–394.

Rakotoarisoa, R.R., Taillez, R., Guillaume, J.B., 1981. Attention d' un mutant de *Rhizobium meliloti* adapté aux cultures de luzerne en terres acides. Plant Soil 60, 99–110.

Reeve, W.G., 1993. The molecular basis of acid-tolerance in *Rhizobium*. Ph.D. Thesis, Murdoch University, Perth/Western Australia.

Richardson, A.E., Djordjevic, M.A., Rolfe, B.G., Simpson, R.J., 1988a. Effects of pH, Ca and Al on the exudation from clover seedlings of compounds that induce the expression of nodulation genes in *Rhizobium trifolii*. Plant Soil 109, 37–47.

Richardson, A.E., Simpson, R.J., Djordjevic, M.A., Rolfe, B.G., 1988b. Expression of nodulation genes in *Rhizobium leguminosarum* biovar trifolii is affected by low pH and by Ca and Al ions. Appl. Environ. Microbiol. 54, 2541–2598.

Robson, A.D., Bottomley, P.J., 1991. Limitations in the use of legumes in agriculture and forestry. In: Dilworth, M.J., Glenn, A.R. (Eds.), The Biology and Biochemistry of Nitrogen Fixation. Elsevier, Amsterdam, pp. 320–349.

Roughley, R.J., 1970. The influence of root temperatures on the structure and nitrogen-fixing efficiency of the root nodules of *Trifolium subterraneum*. Ann. Bot. 34, 631–646.

Sá, N.M.H., Scotti, M.R.M.L., Paiva, E., Franco, A.A., Döbereiner, J., 1993. Selection and characterization of *Rhizobium* spp. strains stable and capable of fixing nitrogen in bean (*Phaseolus vulgaris* L.). Rev. Microbiol. 24, 38–48.

Segovia, L., Young, J.P.W., Martínez-Romero, E., 1993. Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. 43, 374–377.

Serraj, R., Sinclair, T.R., Allen, L.H., 1998. Soybean nodulation and N_2 fixation response to drought under carbon dioxide enrichment. Plant Cell Environ. 21, 491–500.

Serraj, R., Vadez, V., Purcell, L.C., Sinclair, T.R., 1999. Recent advances in the physiology of drought stress effects on symbiotic N_2 fixation in soybean. In: Martínez, E., Hernández, G. (Eds.), Highlights of Nitrogen Fixation Research. Kluwer Academic Publishers, New York, pp. 49–55.

Sinclair, T.R., Serraj, R., 1995. Dinitrogen fixation sensitivity to drought among grain legume species. Nature 378, 344.

Small, J.G.C., Joffe, A., 1968. Physiological studies on the genus *Trifolium* with special reference to the South African species. II. Influence of root temperature on growth nodulation and symbiotic nitrogen fixation. S. African J. Agric. Sci. 11, 41–56.

Soberón-Chavez, G., Nájera, R., Oliveira, H., Segovia, L., 1986. Genetic rearrangements of a *Rhizobium phaseoli* symbiotic plasmid. J. Bacteriol. 167, 487–491.

Sprent, J.I., 1971. Effects of water stress on nitrogen fixation in root nodules. Plant Soil (Spec. Vol.), 225–228.

Sprent, J.I., 1976. Water deficits and nitrogen-fixing root nodules. In: Kozlowski, T.T. (Ed.), Water Deficits and Plant Growth. Academic Press, New York, pp. 291–315.

Stamford, N., Santos, D.R., Silva, V.M., Santos, C.E.R.S., Monteiro, M.C., 1990. Fixação do N_2 e matéria seca do caupi em dois solos de semi-árido brasileiro submetido à deficiência hídrica. R. Bras. Ci. Solo 14, 283–290.

Streit, W., Kosch, K., Werner, D., 1992. Nodulation competitiveness of *Rhizobium leguminosarum* bv. phaseoli and *Rhizobium tropici* strains measured by glucoronidase (*gus*) gene fusions. Biol. Fert. Soils 14, 140–144.

Sutton, W.D., 1983. Nodule development and senescence. In: Broughton, W.J. (Ed.), Nitrogen Fixation, Vol. 3. Clarendon Press, Oxford, pp. 144–212.

Sylvester-Bradley, R., Ayarza, M.A., Mendez, J.E., Moriones, R., 1983. Use of undisturbed soil cores for evaluation of *Rhizobium* strains for inoculation of tropical forage legumes in a Colombian Oxisol. Plant Soil 74, 237–247.

Thies, J.E., Singleton, P.W., Bohlool, B.B., 1991. Influence of size of indigenous rhizobial population on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl. Environ. Microbiol. 57, 19–28.

Tiwari, R.P., Reeve, W.G., Dilworth, M.J., Glenn, A.R., 1996a. An essential role for *actA* in acid tolerance of *Rhizobium meliloti*. Microbiology 142, 601–610.

Tiwari, R.P., Reeve, W.G., Dilworth, M.J., Glenn, A.R., 1996b. Acid tolerance in *Rhizobium meliloti* strain WSM419 involves a two component sensor-regulator system. Microbiology 142, 1693–1704.

Toledo, J.F.F., Almeida, L.A., Kiihl, R.A.S., Panizzi, M.C.C., Kaster, M., Miranda, L.C., Menosso, O.G., 1994. Genetics and breeding. In: FAO, Tropical Soybean — Improvement and Production. FAO, Rome, pp. 19–36.

Trevors, J.T., 1986. Plasmid curing in bacteria. FEMS Microbiol. Rev. 32, 149–157.

Vargas, A.A.T., Graham, P.H., 1988. *Phaseolus vulgaris* cultivar and *Rhizobium* strain variation in acid-pH tolerance and nodulation under acid conditions. Field Crops Res. 19, 91–101.

Vargas, A.A.T., Graham, P.H., 1989. Cultivar and pH effects on competition for nodule sites between isolates of *Rhizobium* in beans. Plant Soil 117, 197–200.

Vargas, M.A.T., Hungria, M., 1997. Fixação biológica do N_2 na cultura da soja. In: Vargas, M.A.T., Hungria, M. (Eds.), Biologia dos Solos de Cerrados. EMBRAPA-CPAC, Planaltina/Brazil, pp. 297–360.

Vargas, M.A.T., Mendes, I.D.C., Suhet, A.R., Peres, J.R.R., 1992. Duas novas estirpes de rizóbio para a inoculação da soja. EMBRAPA-CPAC, Planaltina/Brazil, pp. 1–3 (Comunicado Técnico 62).

Vargas, M.A.T., Suhet, A.R., Mendes, I.C., Peres, J.R.R., 1994. Fixação biológica de nitrogênio em solos de Cerrados. EMBRAPA-SPI, Brasília/Brazil.

Venkateswarlu, B., Maheshwari, M., Saharan, N., 1989. Effects of water deficit on $N_2(C_2H_2)$ fixation in cowpea and groundnut. Plant Soil 114, 69–74.

Vincent, J.M., 1962. Influence of calcium and magnesium on the growth of *Rhizobium*. J. Gen. Microbiol. 28, 653–663.

Vincent, J.M., 1980. Factors controlling the legume–*Rhizobium* symbiosis. In: Newton, W.E., Orme-Johnson, W.H. (Eds.), Nitrogen Fixation, Vol. II. Symbiotic Associations and Cyanobacteria. University Park Press, Baltimore, MD, pp. 103–129.

Voss, M., Sidiras, N., 1985. Nodulação da soja em plantio direto em comparação com plantio convencional. Pesq. Agropec. Bras. 20, 775–782.

Walker, D.W., Miller Jr., J.C., 1986. Influence of water stress on nitrogen fixation in cowpea. J. Am. Soc. Hort. Sci. 111, 451–458.

Walsh, K.B., 1995. Physiology of the legume nodule and its response to stress. Soil Biol. Biochem. 27, 637–655.

Wood, M., 1995. A mechanism of aluminium toxicity to soil bacteria and possible ecological implications. In: Date, R.A., Grundon, N.J., Rayment, G.E., Probert, M.E. (Eds.), Plant–Soil Interactions at Low pH. Principles and Management. Kluwer Academic Publishers, Dordrecht, pp. 173–179.

Wood, M., Sheperd, G., 1987. Characterization of *Rhizobium trifolii* isolated from soils of different pH. Soil Biol. Biochem. 19, 317–321.

Wouters, J.F.R., Wakana, M., Opdecamp, L., 1986. Sensibilite du haricot *Phaseolus vulgaris* a la concentracion en aluminium des sols de la region des grands lacs. Tropicultura 4, 20–26.