

Published in:

Hungria, M.; Campo, R.J.; Mendes, I.C.; Graham, P.H. Contribution of biological nitrogen fixation to the N nutrition of grain crops in the tropics: the success of soybean (*Glycine max* L. Merr.) in South America. In: Singh, R.P.; Shankar, N.; Jaiwal, P.K., eds. **Nitrogen nutrition and sustainable plant productivity**. Houston, Texas: Studium Press, LLC, 2006. p.43-93.

Contribution of biological nitrogen fixation to the N nutrition of grain crops in the tropics: The success of soybean (*Glycine max* L. Merr.) in South America

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Summary. The high cost of mostly imported N fertilizers in South America has necessitated an approach to crop production that emphasizes biological nitrogen fixation (BNF). In this chapter we discuss the contribution of symbiotic nitrogen fixation to the production of soybean (*Glycine max* (L.) Merrill). Soybean was introduced into South America at the end of the 19th century, but significant commercial crop expansion only began in the 1960s. Today the area under cultivation accounts for almost half of world soybean production. BNF has always been a significant concern in this production system with programs for both strain selection and the identification of plant genotypes with superior symbiotic performance beginning in the late 1960s, and still a major research focus. The expectation is that strain selection matched to genotype improvement should

permit yields of 4,000 kg ha⁻¹ or more, without the need of N fertilization. Nowadays most soils cropped with soybean have high *Bradyrhizobium* populations from previous inoculations, but also exhibit significant diversity. This includes variation in morphological, physiological, genetic and symbiotic traits resulting from adaptation to the soils, as well as horizontal gene transfer. More efficient and competitive strains have been identified and selected from this population. Positive responses to reinoculation are reported in soils with 10³ cells g⁻¹ of soil or higher, such that in 74 field trials performed in Argentina yield was enhanced by a mean of 14% in comparison to the non-inoculated treatment, while in 29 field experiments performed in Brazil reinoculation increased yield by 8%. In Brazil, rates of nitrogen fixation in soybean can exceed 300 kg of N ha⁻¹, providing from 69 to 94% of total plant N. Benefits due to the release of N to the following crop have also been reported. Starter- or post-flowering N fertilization has not resulted in increased yield, emphasizing the efficiency of the biological process. The increased use of micronutrients and especially fungicides in contact with the inoculants is an ongoing problem, with the survival of *Bradyrhizobium* on the seed sometimes drastically affected. High soil temperature and low soil moisture contents may also limit biological nitrogen fixation in the tropics, but no tillage systems can appease these stresses. Most importantly, the strategies reported here may be applied in developing or industrialized countries, by large or small landholders, and for subsistence or cash legume crops.

Key words: *Bradyrhizobium*, inoculants, nitrogen fixation, nodulation, soybean.

Running title: N₂ fixation with soybean in South America.

1. Introduction

Estimates are that in the past half century almost 25% of the 8.7 billion ha of agricultural land, permanent pastures, forests and woodlands on earth have undergone degradation (Pinstrup-Andersen, 2003). Crop sustainability has been affected by this progressive degradation with the situation especially critical in the tropics, where the usually fragile soil structure and the low levels of soil organic matter, and the high cost of chemical fertilizers have resulted in nutrient depletion, soil erosion and decline in

biological activity (Cassman, 1999; Hungria and Vargas, 2000; Giller, 2001; Norse, 2003). Degradation is accentuated where rural poverty and population pressures lead to the use of marginal land and to the adoption of improper agricultural practices. The situation is likely to become worse over the next three decades, as the world population increases to some 8 billion people around 2030, necessitating production increases in a region already under stress (Fresco, 2003). Improved production methods and the appropriate use of technology will be needed to improve productivity, ensure food security, and maintain environmental quality.

Nitrogen (N) and phosphorus (P) are often the most limiting nutrients to plant growth worldwide and their continued supply as chemical fertilizers will certainly be needed if world food needs are to be met. However, more efficient and selective fertilizer use, especially of N, will be essential, both because of the cost of these fertilizers and their contribution to environmental pollution (Graham and Vance, 2003; Norse, 2003). Efforts must be made to improve the contribution of the more environmentally friendly biological nitrogen fixation (BNF) to crop production. Worldwide some 44 to 66 million metric tons of N₂ are fixed by agriculturally important legumes annually, with another 3 to 5 million metric tons fixed by legumes in natural ecosystems, providing nearly half of all the N used in agriculture (Smil, 1999; Graham and Vance, 2003). That contribution could be increased by improving the nutrition of legumes, by attending to edaphic constraints such as soil acidity and drought, and by plant breeding considering the symbioses with rhizobia (Graham and Vance, 2003; Graham *et al.*, 2004).

Although N₂ fixation rates of 1-2 kg N ha⁻¹ day⁻¹ should be possible in all legumes (Giller, 2001), a level that would satisfy most of the legume's need for N, the low cost of fertilizer N in developed countries has led to greater dependence on them as a source of N, with a corresponding reduction in the contribution of N₂ fixation to soybean and bean yield in these regions (van Kessel and Hartley, 2000). In contrast, the relatively high cost of N fertilizers throughout much of South America, has necessitated a reliance on BNF. In this chapter we will discuss the South American experience in the search for improved N₂ fixation in soybean (*Glycine max* L. Merr.).

2. Crop expansion in South America

Soybean is considered one of the oldest crops in the world, emerging as a domesticated plant in the eastern half of northern China around the 11th century BC. The cultivated form of soybean is thought to have been introduced into Korea around the 1st century AD, and to Japan around the 3rd century AD. A secondary gene center occurs in Indonesia, the Phillipines, Vietnam, Thailand, Malaysia and Burma (Morse, 1950; Hymowitz, 1970). Soybean is thought to have been introduced into Europe before 1737.

Glycine spp. were introduced into South America at the end of the nineteenth century. First reports in Argentina date from 1880 (Miró, 1989), with *Soja hispida* (*Glycine soja*) and *Soja ochroleuca* introduced into Brazil in 1882 (D'utra, 1882, 1899), and seeds from the USA, Argentina and Japan brought to Paraguay in the 1920s (Alvarez, 1989). Limited experimentation was undertaken with soybean in both Argentina and Brazil in the earlier 1900s, but the first commercial crops were not planted in southern Brazil until the 1940s. Soybean cultivation in Brazil increased in the 1960s, with further dramatic increases in the 1970s with the opening of the "Cerrado" region, an edaphic type of savanna occupying 207 million ha and representing 25% of Brazil's surface. Soybean cropping in both Argentina and Paraguay also dates from 1970s, with cropped areas also increasing in the last decade in Bolivia, and more recently in Uruguay. Most breeding programs for the crop in South America have been conducted in Brazil and Argentina, with soybean cropping in Brazil continuing to expand. In 2003/2004, grain production estimates for Brazil were of 57.7 Mg of grains, with a mean yield of 2,737 kg ha⁻¹. This compares with a production of 65.8 Mg and a mean yield of 2,630 kg ha⁻¹ in the USA (CONAB, 2004; FAS, 2004). Finally, Brazil, Argentina, Paraguay and Uruguay created in 1991 the Mercosur (the Common Market of the South) for economy, politics, and education, including free trade, and the area is now responsible for nearly 50% of world soybean production, estimated at 204 million tons in 2003/2004; further increases are expected in the following years (CONAB, 2004; FAS, 2004).

3. Selection of *Bradyrhizobium* strains

Studies undertaken during the initial period of soybean crop expansion in Brazil,

noted that fields of soybean that were not inoculated had poor nodulation (Silva, 1948; Stamford, 1972; Lopes *et al.*, 1976a, 1976b; Peres, 1979; Vargas and Suhet, 1980; Lopes and Giardini, 1981; Suhet *et al.*, 1981; Freire, 1982; Vargas *et al.*, 1982a). Commercial inoculants were introduced, mostly from the USA, but often failed because of inappropriate storage, delays in custom clearance, and strain/variety interaction. In the 1960s a national soybean commission determined that BNF was an important trait and needed to be considered in breeding activities. The evaluation of the performance of selected foreign strains with local cultivars started soon thereafter, generally with strains brought from the University of Wisconsin, University of Illinois, USDA and University of Sydney (Silva, 1948; Freire and Vidor, 1981; Lopes and Giardini, 1981). A list of the strains recommended for use in commercial inoculants was first published in 1956 (Hungria *et al.*, 1994, 2005a).

SEMIA 566 (= BR 40) (SEMIA - “Seção de Microbiologia Agrícola” - refers to nomenclature of the National collection of rhizobia, with MIRCEN - Microbial Resources Centre Network - at the Centro de Pesquisa de Fixação Biológica do Nitrogênio, in FEPAGRO - Fundação Estadual de Pesquisa Agropecuária, Porto Alegre, Rio Grande do Sul, Brazil) was one of the first strains “selected” in Brazil. It was isolated from a nodule on the cultivar Hardee, after inoculation with material distributed by Dixie Inc., probably an inoculant from Nitragin (Milwaukee, USA) (J.R.J. Jardim Freire, personal communication). SEMIA 566 belongs to the same serogroup as USDA 123 (=311b123, =TAL 376, =ACCC15036) and effectively nodulated all of the Brazilian cultivars released by that time. It was commercially recommended from 1966 to 1978, and greatly contributed to the successful establishment of the crop in the southern region of Brazil.

A second strain, SEMIA 587 (= BR 96), was isolated in 1967 from soybean nodules collected in Santa Rosa, Rio Grande do Sul (J.R.J. Freire, personal communication). It also proved effective and competitive in a number of field trials (Freire and Vidor, 1981; Freire *et al.*, 1983) and from 1968 to 1975 was recommended together with SEMIA 566 and SEMIA 543, for the inoculation of soybean in Brazil.

New soybean nodulation problems emerged with expansion of the crop to the Cerrado. Initially, it was thought that soybean nodulation failure in the Cerrado was due to increases in the population of antibiotic-producing actinomycetes in soils following

clearing and liming, and to the effect of these organisms on inoculant rhizobia (Coelho and Drozdowicz, 1979; Scotti *et al.*, 1982). From 1976 to 1978 several combinations of strains were suggested including SEMIA 566, SEMIA 527 and SEMIA 532, but proved unsatisfactory. This was probably due to the stressful conditions of the ecosystem – long periods of drought, high soil temperatures (>40°C), low soil moisture, poor soil fertility, soil acidity and Al toxicity.

Another promising strain, 29W (=SEMIA 5019, =BR 29), was isolated in 1979 from a nodule on the soybean line IAC-70-559 (Peres, 1979). Unfortunately there is no information on the inoculant used (J.R.R. Peres, personal communication). In addition to being highly efficient and competitive (Peres, 1979; Freire and Vidor, 1981), strain 29W proved being tolerant to the antibiotics produced by the local population of actinomycetes (Scotti *et al.*, 1982). This was initially considered an important factor in its superior performance. In 1979 the combination of SEMIA 587 and 29W was recommended for use in the Cerrado, a major factor in the establishment of soybeans in this region (Peres, 1979; Peres and Vidor, 1980; Vargas and Suhet, 1980; Vargas *et al.*, 1981), currently the main area of soybean production in Brazil. Today we know that the superior performance of these two strains is probably more related to their compatibility with cultivar IAC-2 than to their antibiotic tolerance (Vargas and Hungria, 1997). Both SEMIA 587 and 29W are still recommended for, and commonly utilized in, commercial inoculants, as they are also effective with more recently released cultivars.

More than a decade later, the higher N demands of the newer and more productive soybean genotypes has necessitated a third wave of strain selection in which the goals have been to identify strains combining greater capacity for N₂ fixation with tolerance to the frequently stressful conditions of the Cerrado region. The emphasis in this phase has been the isolation of efficient and adapted naturalized strains from areas in the Cerrado which had been previously inoculated with SEMIA 566. The first step in selection was to isolate rhizobia from large pink nodules of uninoculated field-grown soybean plants. Each of these rhizobia was then tested for N₂ fixation capacity under laboratory (acetylene reduction method) and greenhouse conditions (plant growth and competitiveness) (Peres *et al.*, 1984) and, subsequently, for nodulation, plant growth and grain yield in field trials. After several crop seasons, the isolate CPAC 15 (=SEMIA 5079, =566a, =DF 24) was

identified, and shown to increase soybean yield beyond that possible with SEMIA 587 and 29W (Vargas *et al.*, 1992, 1993b, 1994a, 1994b; Peres *et al.*, 1993; Hungria and Vargas, 1996).

A second strain selection approach initiated at the same time, tested individual colonies from pure cultures of strain CB 1809 for variants that were of greater efficiency in BNF and also highly competitive. Strain CB 1809 (=SEMIA 586, =USDA 136, =3I1b136, =TAL 379, serogroup 122) was isolated in the USA, but then sent to Australia (Dr. P. van Berkum, personal communication), where it was recommended for use as the inoculant strain for soybean in 1968. It is considered a subculture of USDA 136, which was derived from USDA 122 (Sato *et al.*, 1999). CB1809 was sent from Australia to Dr. J. Döbereiner in Brazil in 1966, classified as exceptional in nitrogen fixation efficiency (Döbereiner *et al.*, 1970; Pedrosa *et al.*, 1972) and recommended for use in commercial inoculants in 1977. Poor nodulation with cultivar IAC-2 (Peres, 1979) led to withdrawal of this recommendation the following year. When dozens of individual colonies of CB 1809 were separately evaluated for N₂ fixation (acetylene reduction activity), competitiveness (nodule occupancy), and plant growth under greenhouse conditions significant variation in these traits was evident. Promising isolates were then evaluated in field experiments in areas of the Cerrado that had not been previously inoculated, and rhizobia were re-isolated from the nodules of plants in treatments showing the best symbiotic performance and grain yield (Santos *et al.*, 1999). Following extensive field testing (Vargas *et al.*, 1992, 1993b, 1994a, 1994b; Peres *et al.*, 1993; Hungria and Vargas, 1996) this approach led to the identification of CPAC 7 (=SEMIA 5080). Both CPAC 7 and CPAC 15 were included in the list of strains recommended for use in soybean inoculation in 1992 (Vargas *et al.*, 1992; Peres *et al.*, 1993), and together with SEMIA 587 and 29W proved to be highly effective in satisfying the N needs of the cultivars released in recent years, as shown in Table 1. The experiments in Table 1 cover the period from 1988 to 2001, and were amongst those with the best soybean yields in each crop season. For the 14 trials reported, mean yield increase due to inoculation ranged from 38 to 1,118 kg ha⁻¹, and averaged 303 kg ha⁻¹, an increase in yield of 11.3% when compared to the non-inoculated treatment. It is noteworthy both that yields of up to 3864 kg ha⁻¹ were obtained in plants dependent on symbiotic N₂ fixation for

growth, and that yield responses to inoculation were obtained in a majority of cases in soils having more than 10³ indigenous rhizobia g⁻¹ soil.

The Brazilian strain selection program continues. The Brazilian national mean yield for soybean has increased from 1,166 kg ha⁻¹ in 1968/69 to 2,737 kg ha⁻¹ in 2003/2004, and further increases are expected. Specht *et al.* (1999) have suggested a genetic potential for soybean of 8,000 kg ha⁻¹, and as crop yields increase so will the demand for N (Specht *et al.*, 1999). Further, more than 90% of the areas cropped to soybean today have been previously inoculated, with many supporting bradyrhizobial populations of 10³ cells g⁻¹.

Table 1. Soybean grain yield (kg ha⁻¹) in non-inoculated or inoculated plots in field experiments conducted from 1988 to 2001¹.

Treatment	year	1988/89 ⁴	1989/90 ⁴	1990/91 ⁴	1993/94 ⁵	1994/95 ⁵	1995/96 ⁵	1995/96 ⁵	1996/97 ⁵	1997/98 ⁵	1997/98 ⁵	1999/00 ⁵	2000/01 ⁵	2000/01 ⁵	2000/01 ⁵
	city	Planaltina	Planaltina	Planaltina	Planaltina	Londrina	P. Grossa	P. Grossa	Cruz Alta	Londrina	Planaltina	Londrina	Jaciara	Londrina	Planaltina
	state	PR	DF	DF	DF	PR	PR	PR	RS	PR	DF	PR	MT	PR	DF
	cultivar	Savana ⁶	Doko ⁶	Doko ⁶	DokoRC ⁷	BR 37 ⁸	BR 37 ⁹	BR 37 ⁸	3cepar4 ¹⁰	BR 37 ¹¹	Celeste ⁷	BR 37 ¹²	UFV-18 ¹³	BRS133 ¹⁴	Celeste ⁷
	no. cells ³	0	0	0	10 ³	1.2.10 ⁴	10 ⁴	10 ³	10 ⁴	10 ⁴	10 ⁴	2.4.10 ⁵	10 ⁵	2.4.10 ⁵	10 ⁴
Non-inoculated		1,269c	2,542b	1,143cd	2,661b	3,318a	2,305b	3,588a	2,347a	3,075b	2,483a	2,810a	2,498b	3,481c	4,102a
SEMIA 587		1,422b	2,747ab	1,150cd									2,668ab	3,681ab	4,200a
29W		1,501bc	2,739ab	1,527a									2,640ab	3,502bc	3,987a
SEMIA 587+29W					2,822a	3,627a		3,647a	2,563a	3,341a	2,875a	2,924a	2,500b	3,704a	4,363a
CPAC 7		1,551ab	3,087a	1,129cd				3,444a					2,888a	3,512bc	4,105a
CPAC 15		1,704a	3,027a	1,351abc				3,402a					2,612ab	3,864a	4,399a
CPAC 7			3,114a	1,403ab	2,888a	3,784a		3,606a	2,525a	3,381a	3,119a	3,089a	2,577ab	3,596abc	4,447a
+CPAC 15															
Mean yield ²		1,489	2,876	1,283	2,790	3,576	3,050	3,614	2,478	3,266	2,826	2,941	2,626	3,620	4,229
Maximum increase ²		435	572	384	227	466	1,139	59	216	306	636	279	390	383	345
Mean increase ²		276	401	169	194	388	1,118	38	197	286	514	196	150	162	148

¹Data followed by the same letter, within the same column, are not statistically different (Duncan, $P \leq 0.05$).

²Mean yield of all treatments, maximum and mean yield increases due to the inoculation in relation to the non-inoculated control.

³Number of cells of *Bradyrhizobium* g⁻¹ of soil.

⁴Mean of four replicates per treatment.

⁵Mean of six replicates per treatment.

⁶Peres *et al.* (1993); ⁷Vargas *et al.* (2002); ⁸Hungria *et al.*, 1997; ⁹Hungria and Vargas (1996); ¹⁰Campos *et al.* (2001); ¹¹Campo and Hungria (1999); ¹²Campo and Hungria (2001); ¹³Loureiro *et al.* (2001); ¹⁴Campo *et al.* (2002).

of soil. This means that future strain selections will need either higher rates or longer periods of N₂ fixation, and marked competitive ability. Both of the selection strategies used in the identification of CPAC 15 and CPAC 7 continue to be used. They are time consuming, but can allow the identification of superior strains without need for genetic manipulation, ensuring that the strains released are accepted by all farmers. Table 2 shows the results obtained in six field experiments where strains derived from SEMIA 566 and CB 1809 were tested. Even though the experiments were performed in soils with high populations of *Bradyrhizobium*, inoculation at Londrina in 2000/2001 and 2001/2002 enhanced yield beyond that possible with SEMIA 566 or CB1809. However, the high yield obtained in the uninoculated plots of the other inoculation trials does suggest that it will be increasingly difficult to identify additional strains with even greater effectiveness and competitiveness.

Soils in Argentina were also initially devoid of indigenous *Bradyrhizobium* (Brutti *et al.*, 1998), and as in Brazil, imported inoculants performed poorly (Brutti *et al.*, 1999). The subsequent search for effective strains identified E109 (=SEMIA 5085, =USDA 138, =3I1b138, =TAL 377, =SEMIA 5028, =61A118, serogroup 6) as being effective, and this strain is used in the majority of the commercial inoculants now produced in Argentina. Table 3 shows the results of inoculation experiments in Argentina, in soils with established populations of *Bradyrhizobium*. Inoculation with E109 has increased yield by an average of 749 kg ha⁻¹, or by 24.1% compared to the uninoculated control. The Brazilian strain 29W (=E110) was also effective and competitive with Argentinean cultivars (Brutti *et al.*, 1998, 1999), but CPAC 7 and CPAC 15 were not as effective as E109 (Table 3).

The information from Paraguay and Bolivia is limited, and most of the inoculants used in these countries are still imported from Brazil, Argentina and the USA. However, some Paraguayan isolates have shown outstanding symbiotic performance under greenhouse conditions (Figueredo *et al.*, 2000; Chen *et al.*, 2002), including some evaluations in which non-sterile soils were used (Michajkuk *et al.*, 2001), so there is potential for the selection of efficient and competitive strains for use in local inoculants.

4. Characterization of strains used in commercial inoculants in South America

4.1. Strains SEMIA 587 and 29W

Based on 16S rRNA gene sequence analysis, both SEMIA 587 (GenBank accession number AF234890) and 29W (AF237422), show affinity with *B. elkanii* (Chueire *et al.*, 2003). These strains show 99 to 100% base identity with USDA 121, 101, 94 and 130, as well as with strains indigenous to Korea and Thailand (NCBI, 2004). Both strains have high intrinsic resistance to several antibiotics and accumulate high levels of indole acetic acid (IAA) *in vitro*; absence of hydrogenase and synthesis of rhizobiotoxine (Boddey and Hungria, 1997), traits typical of the species (Kuykendall *et al.*, 1992).

Table 2. Grain yield (kg ha⁻¹) of soybean inoculated with parental (par.) and variant (var.) strains obtained in the Brazilian strain selection program¹.

Treatment	year	1998/99	1998/99	1999/00	1999/00	2000/01 2001/02 (mean)
	city no. cells ⁴	Planaltina ⁵ 10 ³	Londrina ⁶ 1.4.10 ⁴	Londrina ⁷ 2.4.10 ⁵	Planaltina ⁵ 10 ⁴	Londrina ⁸ 10 ³
Non-inoculated (NI) ²		4,207a	3,836a	2,679a	4,647a	1.624c
Non-inoculated + N ²		4,192a	3,434b	2,463a	4,691a	3.525a
SEMIA 566 (parental)						2.610b
CB 1809 (parental)						2.408b
29W+ SEMIA 587		4,102a	4,025a	2,450a	4,583a	3.450a
CPAC 15 + CPAC 7 ³		4,193a	3,995a	2,988a	4,524a	3.425a
CPAC 40 + CPAC 44 ³		4,234a	3,996a	3,031a	4,459a	3.580a
CPAC 42 + CPAC 45 ³		4,236a	3,913a	2,988a	4,666a	3.567a
Mean increases with the new variant strains in relation to NI control		28	118	330	(-84)	1,950
Mean increases with the new variant strains in relation to CPAC 15 and CPAC 7		42	(-40)	22	38	148
Mean increases with 29W, 587, CPAC 7, CPAC 15 and new variant strains, in relation to NI control		(-16)	146	185	(-89)	1,882

¹Data represent the mean of six replicates and when followed by the same letter, within the same column, are not statistically different (Duncan, $P \leq 0.05$).

²Non-inoculated, without or with 200 kg de N ha⁻¹, as urea, split at sowing and at flowering.

³CPAC 15, CPAC 40 and CPAC 42 are variant strains of SEMIA 566; CPAC 7, CPAC 44 and CPAC 45 are variant strains of CB 1809.

⁴Number of cells of *Bradyrhizobium* g⁻¹ of soil.

⁵Vargas *et al.* (2002); ⁶Campo and Hungria (2000); ⁷Campo and Hungria (2001); ⁸M. Hungria and R. J. Campo, unpublished data.

Table 3. Grain yield (kg ha⁻¹) of soybean non-inoculated, inoculated or receiving N-fertilizer in Argentinean soils with established population of *Bradyrhizobium*¹.

Treatments	year city	1996/97 Pergamino	1996/97 La Cruz	1996/97 Las Lajitas	1996/97 Las Lajitas	1997/98 La Cruz	1997/98 Las Lajitas	2000/01 Oncativo	2000/01 Marcos Juaréz	2000/01 ¹ San Agustín	2000/01 ¹ Carabelas	2000/01 ¹ Trenque Lauquen	2000/01 ¹ 9 de Julio
	province cultivar	B. Aires n.a. ⁵	Tucumán n.a. ⁵	Salta n.a. ⁵	Salta n.a. ⁵	Tucumán n.a. ⁵	Salta n.a. ⁵	Córdoba 5436	Córdoba Don Mario 4800 RR	Tucumán A-8000 RR	B. Aires Maravilla 45RR	B. Aires DM 3800RR	B. Aires DM 4700 RR
	no. cells ⁴	10 ³ -10 ⁴	10 ⁴	>10 ³	>10 ⁵	10 ⁵	>10 ³	>10 ⁶					
Non inoculated ²		1,540	944	3,183	3,614	2,316	3,077	3,507	4,103	3,439	4,107	3,060	4,354
Non-inoculated + N ²				3,337	3,786	2,658	3,841	3,903	3,495	3,324	4,792	3,516	4,490
E109		1,780	1,713	3,977	4,473	3,097	4,299	4,362	4,610	3,742	6,077	3,455	4,644
CPAC 7		2,040	1,745	3,853	3,948	2,918	4,237	4,160	4,384	3,368	4,546	3,026	4,496
CPAC 15		2,080	1,791	3,530	4,052	2,780	4,353	3,945	4,534	3,595	4,297	3,039	4,373
Increases due to inoculation with E109 ³		240	769	794	859	781	1,222	855	507	303	1,970	395	290
Increases (%) due to E109 ³		15.6	81.5	24.9	23.8	33.7	39.7	24.4	12.4	8.8	48.0	12.9	6.7

¹Laboratorio Biagro S.A. (2004). Field experiments performed by INTA-BIAGRO. Statistical analyses are not available.

²Inoculated without or with 400 kg of N ha⁻¹, except for the trial in 1996/97 in Las Lajitas, that received 100 kg of N ha⁻¹.

³Increases obtained in relation to the non-inoculated control.

⁴Number of cells of *Bradyrhizobium* g⁻¹ of soil.

⁵Information not available.

4.2. Strains SEMIA 566 and CPAC 15

Based on 16S rRNA gene sequence analysis, strain SEMIA 566 (AF236086) identifies very closely with *B. japonicum*, and differs from its putative natural variant CPAC 15 (AF234888) in just one base pair (Chueire *et al.*, 2003). However, several morphological, physiological and genetic differences between this pair of strains have been reported. CPAC 15 produces more mucus (Nishi *et al.*, 1996; Boddey and Hungria, 1997), increases the number of root hairs (Hai phenotype) (Hungria *et al.*, 1996), and differs from SEMIA 566 in DNA fingerprint when amplified by RAPD (Nishi *et al.*, 1996) or rep-PCR with ERIC and REP primers (Hungria *et al.*, 1998). The superior symbiotic performance of CPAC 15 compared to SEMIA 566 has been confirmed in several studies (Nishi *et al.*, 1996; Boddey and Hungria, 1997; Hungria *et al.*, 1998). Characteristics in which both strains differ from those described for *B. japonicum*, include higher resistance to several antibiotics, higher synthesis of IAA *in vitro*, toxicity symptoms related to rhizobitoxine production, and lack of nodulation with soybean cultivar Hill which carries the *Rj₄* gene (Nishi *et al.*, 1996). Both strains show the Hup⁻ phenotype (Nishi *et al.*, 1996).

4.3. Strains CB 1809 and CPAC 7

Both the parental strain CB1809 (AF236087) and its natural variant CAPC 7 (AF234889) also belong to the species *B. japonicum* (Chueire *et al.*, 2003). CPAC 7 shows high base similarity with strains USDA 110 and USDA 122, as well as with a number of isolates from Northern Thailand. Both CB 1809 and CPAC 7 have a Hup⁺ phenotype, absence of rhizobitoxine symptoms when used as inoculum in sensitive cultivars, and ability to nodulate the soybean cultivar Hill (Nishi *et al.*, 1996). Furthermore, both strains are highly efficient in the process of N₂ fixation (Döbereiner *et al.*, 1970; Neves *et al.*, 1985), though CPAC 7 is more competitive (Hungria *et al.*, 1998; Hungria and Vargas 2000). CPAC 7 also produces more mucus (Nishi *et al.*, 1996; Boddey and Hungria, 1997), increases the Hai phenotype and shows a different profile of nodulation factors (Nod) (Hungria *et al.*, 1996) than the parental strain.

4.4. Strain E109

Strain E109 (=SEMIA 5085) also clusters with *B. japonicum* (AY117677), showing close 16S rRNA gene sequence affinity with strain LMG 6138 and with the type strain USDA 6.

4.5. Genetic diversity of strains in Paraguay

A high level of morphological, physiological, genetic (Chen *et al.*, 2000, 2002); and symbiotic diversity (Figueredo *et al.*, 2000; Michajkuk *et al.*, 2001; Chen *et al.*, 2002) was evident among 78 rhizobia collected from 16 field sites in Alto Parana and Itapua, Paraguay. The 58 *Bradyrhizobium* isolates differed from reference strains in their genetic fingerprints with ERIC-REP-PCR (Chen *et al.*, 2000), but were identical with commercial inoculant strains from Brazil and Argentina on the basis of 16S rRNA sequence analysis.

4.6. General comments

Ribosomal sequences, with emphasis on the 16S rRNA gene, have become the method of choice in molecular taxonomy for tracing bacterial phylogenies (*e.g.*, Woese, 1987; Garrity and Holt, 2001). However, there are reports showing that, despite a high level of variability in morphological, physiological and genetic properties, diversity is low in the 16S rRNA sequences of the strains of *Bradyrhizobium* investigated so far (*e.g.*, Molouba *et al.*, 1999; van Berkum and Fuhrmann, 2000). In this context, the high similarity of the 16S rRNA genes of the commercial strains used in Brazil and Argentina does not reflect the differences in several properties already reported in this chapter, including a high level of genetic diversity detected in the analysis by RAPD and rep-PCR with ERIC, REP and BOX primers (Bangel, 2000; Chueire *et al.*, 2000).

5. Ecology of the main serogroups of *Bradyrhizobium* in soils of South America

5.1. Inoculant strain competitiveness and persistence

Among the strains used as inoculant-quality rhizobia for soybeans in Brazil and Argentina, SEMIA 587 has been identified as highly competitive in nodule formation and persistence in soil (Freire, 1977; Freire and Vidor, 1981; Hungria *et al.*, 1994; Vargas and Hungria, 1997), whereas 29W and CB1809 have not (Hungria *et al.*, 1994; Vargas and

Hungria, 1997; Mendes *et al.*, 2004). In Argentina, strain 29W (=E110) required at least three seasons as an inoculant before it was able to dominate in nodule occupancy, but thereafter was able to persist in the soil for up to five years in the absence of the host (Brutti *et al.*, 1998). Results for SEMIA 566 and 29W have been variable. In the 1990s strains belonging to serogroup SEMIA 566 (= serogroup 123) were reported to occupy up to 70% of the soybean nodules in areas that had never been inoculated (Vargas *et al.*, 1993a; Hungria *et al.*, 1994) or even cropped (Ferreira and Hungria, 2002). Other studies have reported that SEMIA 566 survives poorly in soil in the first two years, but become established and highly competitive thereafter (Freire, 1977; Freire and Vidor, 1981; Freire *et al.*, 1983; Vargas and Hungria, 1997; Mendes *et al.*, 2004).

SEMIA 566 is related to the USDA 123 serocluster, considered the most competitive of the indigenous *B. japonicum* strains in the midwestern United States. In this region, serocluster USDA 123 strains usually give rise to 60 to 80% of the nodules formed (Damirgi *et al.* 1967; Ham *et al.*, 1971; Kvien *et al.* 1981; Cregan *et al.*, 1989; Weber *et al.*, 1989), while inoculant rhizobia will usually produce only 10-20% of the nodules formed (Ham *et al.*, 1971; Reyes and Schmidt, 1979; Ham, 1980). This is due less to differences in strain competitiveness than to the limited movement of inoculant rhizobia in the host root system. Nodule dominance by the serogroup 123 does not appear to be due to superior colonization of the host rhizosphere (Moawad *et al.*, 1984). Serogroup 123 has also been reported in Canada (Semu and Hume 1979) and Korea (Kang *et al.*, 1991). In both Brazil and the USA serogroup USDA 123 strains become dominant in soil over time (Streeter, 1994). Replacing persistent strains like USDA 123 and SEMIA 566 with more efficient ones can be difficult, and requires more than one season of inoculation.

To better understand the variability within a serogroup, Boddey and Hungria (1997) studied 17 strains belonging to serogroup SEMIA 566 that had been reisolated from soils in the Cerrado after several years of adaptation. Greater mucus production was shown with 77% of the strains, while 59% had colonies with larger diameter than the parental strain, differing also in intrinsic resistance to antibiotics and to levels of IAA synthesized *in vitro*. The phenogram built with 32 phenotypic traits showed that the majority of the adapted variant strains clustered separately from the parent (Boddey and Hungria, 1997). Changes in polysaccharide production of strains following adaptation to edaphic conditions in the

Cerrado was also observed by Coutinho *et al.* (1999). Adapted strains also varied in relation to N₂ fixation capacity, including nodulation, plant growth and total N accumulated in tissues (Hungria *et al.*, 1998). Remarkable differences were observed in strain competitiveness, evaluated by nodule occupancy with 29W as the co-inoculant, with some adapted strains occupying up to 100% of the nodules. Further, quite distinct rep-PCR profiles (ERIC and REP) were obtained for the parental and adapted strains (Hungria *et al.*, 1998). Streeter (1994) also noted substantial “genetic adjustment” during the period of adaptation, leading to wide genetic diversity in field populations. Differences in competitiveness were also observed between parental and adapted strains, isolated from soils of the Cerrado and belonging to serogroups 532C (=SEMIA 5039, =G3, isolated in Brazil), and SEMIA 5020 (=965, =BR 95, =J5033, received from Japan and tested for N₂ fixation ability by Peres, 1979) (Santos *et al.*, 1999), as shown in Fig. 1.

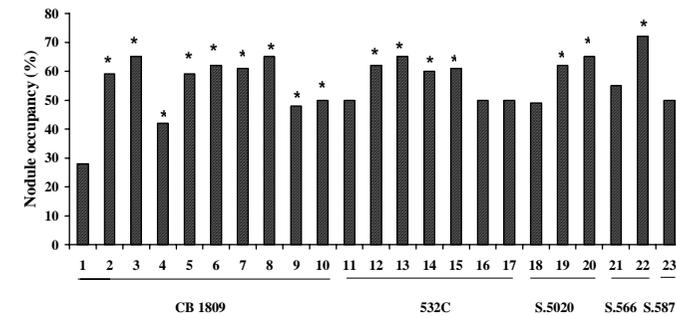


Figure 1. Nodule occupancy (% of nodules occupied by each strain) by variant and parental strains when mixed in a ratio of 1:1 with strain 29W. Occupancy was evaluated serologically using 60 nodules per plant, and five replicates, 45 days after emergence. Strains are represented by the following numbers: Serogroup CB 1809: 1, parental CB 1809; 2, variant CPAC 7; 3 to 10, other variant strains belonging to this serogroup; Serogroup 532C: 11, parental 532C; 12 to 17, variant strains; Serogroup SEMIA 5020: 18, parental SEMIA 5020; 19 and 20, variant strains; Serogroup SEMIA 566: 21, parental SEMIA 566; 22, variant CPAC 15; Serogroup SEMIA 587: 23, SEMIA 587. Within the same serogroup, columns with * indicate that nodule occupancy by the variant strain was statistically different from the parental genotype (Tukey, $P \leq 0.05$). After Santos *et al.* (1999).

5.2. Serogroup of CB 1809

CB 1809 is very effective with Brazilian cultivars (Döbereiner *et al.*, 1970; Neves *et al.*, 1985), but in contrast to the variant strain CPAC 7, is a poor competitor (Hungria *et al.*, 1994, 1998; Hungria and Vargas, 2000).

Additional natural variants of CB1809 have been obtained using the same methodology employed to obtain CPAC 7 (Santos *et al.*, 1999). After a short period of adaptation to the Cerrado these strains showed higher production of mucus, but were similar in lipopolysaccharides (LPS) and protein profiles to the parental strain. Limited polymorphism in the rep-PCR analysis using ERIC and REP primers was also evident (Santos *et al.*, 1999). Differences in symbiotic performance have also been identified, with CPAC 390 yielding 58% more plant N than CB 1809, and increases of up to 132% in nodule occupancy have been obtained with the variant strains CPAC 390 and CPAC 403 (Santos *et al.*, 1999). Figure 1 shows the range in competitiveness of variant strains belonging to serogroup CB 1809.

5.3. Genetic variability due to the adaptation and lateral transfer of genes

Variability in the soybean rhizobia recovered from Brazilian (Hungria and Vargas, 2000) and Paraguayan (Chen *et al.*, 2000, 2002) soils is high. Rhizobial variability has been attributed to several factors, including mutation and recombination in isolated strains and lateral gene transfer. These processes can be affected by interaction with the host plant and by agricultural practices (Sullivan and Ronson, 1995; Martínez-Romero and Caballero-Mellado, 1996; Sullivan *et al.* 1996; Provorov and Vorob'ev, 2000; Silva *et al.* 2003). As pointed out by Schloter *et al.* (2000), adaptation to environmental and cropping conditions could affect the diversity of soybean rhizobia. However, the magnitude of the variability detected in Brazil is greater than has been reported in other areas, *e.g.*, in strains belonging to serocluster USDA 123 in the USA (Judd *et al.*, 1993).

Two aspects of *Bradyrhizobium* population structure in Brazilian soils deserve further investigation: 1) the large differences between putative parental and adapted variant strains, especially in the SEMIA 566 serogroup (Hungria *et al.*, 1998); and 2) recovery of a high percentage of unknown serogroups from fields after some years of soybean cropping

(Freire *et al.*, 1983; Vargas *et al.*, 1993a; Vargas and Hungria, 1997; Ferreira *et al.*, 2000). Contaminant strains, not recorded in the Brazilian germplasm bank, and belonging to the same serogroups as selected inoculant strains could have been part of the first inoculants used in the country and disseminated to the experimental area, or were carried with the seeds, away from older grain producing sites. This seems unlikely as no strain with the same serological reaction and LPS and protein profiles as SEMIA 566 has been found in the Brazilian rhizobia germplasm bank.

To determine whether indigenous *Bradyrhizobium* able to nodulate soybean might exist in soil, but not have been detected before because they did not nodulate the cultivars used at the time of crop expansion, we have carried out additional inoculation studies with a modern cultivar and six non-bred promiscuous soybean genotypes. Each was inoculated with dilutions of soil from fourteen different virgin soils spanning the region from the Amazon to Rio Grande do Sul, all bearing native vegetation. Forty isolates of *Bradyrhizobium* were obtained from seven of the soils, and the analyses of morphological, physiological, phylogenetic and symbiotic properties suggested that 39 of the isolates showed high similarity to seven strains that have been, or are, used in Brazilian commercial inoculants (Ferreira and Hungria, 2002). Only one isolate appeared to be truly indigenous, and it showed poor symbiotic performance (Ferreira and Hungria, 2002). The presence of SEMIA 566 at some distance from areas where it was used as an inoculant might be the result of contamination of seed or agricultural machinery and transport of these organisms from older soybean growing areas to the newer regions of soybean production, or it could be associated with contamination by rain and wind transport.

Non-symbiotic rhizobia can persist in soils in the absence of host legume, acquiring symbiotic genes by transfer of chromosomal symbiotic genes from an inoculant strain upon the introduction of the host legume (Sullivan and Ronson, 1995; Sullivan *et al.*, 1996). To test the possibility of genomic rearrangement and lateral transfer under Cerrado conditions, we trapped and characterized 100 bradyrhizobia from a soil in the Cerrado. The soil was originally void of rhizobia able to nodulate soybean, but 15 years earlier had been inoculated with *B. elkanii* strains SEMIA 587 and SEMIA 5019. It had been annually cropped with soybean, but not further inoculated in the past seven years, and had a population of *Bradyrhizobium* estimated at 10^4 cells g^{-1} soil at sampling. Variation was

significant, with no more than five isolates sharing common characteristics. While 40% of the isolates reacted with antiserum to *B. japonicum* strain SEMIA 566, 13% did not react with any antiserum. Nodulation and N₂ fixation capacity also varied considerably among the isolates, with a high level of genetic diversity also evident from PCR using BOX and RPO1 (related to the *nif* genes) primers. Serological properties showed higher correlation with BOX than with RPO1 products, while isolates clustered differently in repetitive and *nif* cluster analysis. It is suggested that the diversity shown in this study could be attributed to both lateral transfer of genetic material between indigenous and inoculant strains, and to genomic rearrangements during the adaptation process (Galli-Terasawa *et al.*, 2003).

When soybean cultivation began in Brazil, soils were essentially devoid of bradyrhizobia effective for this host. Subsequently, rhizobia could have entered only via inoculation with a very limited number of strains, or as contaminants on introduced seed. That such diverse populations of these organisms are now found in soils that 50 years ago had no soybean bradyrhizobia is striking evidence for the rapid evolution of these organisms under the harsh environmental conditions in the Brazilian Cerrado. This variation justifies the current emphasis for selection among adapted strains reisolated from soils previously inoculated. It also warrants more detailed study of the factors driving change in these organisms, and of the time frame over which changes can occur.

6. Fast growing soybean rhizobia

6.1. Symbiosis of South American soybean genotypes with *Sinorhizobium fredii* and other indigenous fast growing rhizobia

Soybean is also nodulated by fast-growing rhizobia first reported from the People's Republic of China, within the center of origin and diversity of the legume (Keyser *et al.*, 1982). These organisms were initially classified as *Rhizobium fredii* (Scholla and Elkan, 1984) but were later reclassified as *Sinorhizobium fredii* and *S. xinjiangensis* (Chen *et al.*, 1988). Although it was originally thought that *S. fredii* was specific for Asian soybean lines (Keyser *et al.*, 1982; Stowers and Eaglesham, 1984; Devine, 1985), Balatti and Pueppke (1992) later showed that 17% of 194 North American genotypes were effectively nodulated by the *S. fredii* strain USDA 257 (= PCR 257). Some of the claimed advantages of using

fast-growing strains for soybean inoculation include the facility of commercial production, easier establishment in soils, displacement of indigenous *B. japonicum* strains and easier manipulation of genes (Chatterjee *et al.*, 1990; Cregan and Keyser, 1988; Buendía-Clavería *et al.*, 1994).

To determine whether such strains could have potential for inoculation of soybean in Brazil, 80 soybean cultivars from the Brazilian germplasm bank were evaluated for nodulation and N₂ fixation following inoculation with *S. fredii* USDA 205 (=ATCC 35423, =LMG 6217, =PRC 205) and CCBAU 114 (=RT15), or with *S. xinjiangensis* strain CCBAU 105 (=RX22). Sixty-six percent of the Brazilian genotypes formed effective nodules with both *Sinorhizobium* species (Chueire and Hungria, 1997). In the study of Balatti and Pueppke (1992), the frequency of Fix⁺ phenotypes was positively correlated with maturity group, a factor that could have contributed to the very high percentage of Fix⁺ phenotypes observed in this study. Both "Davis" (Dowdle and Bohlool, 1985; Balatti and Pueppke, 1992) and "Paraná" have been reported as cultivars nodulating with fast-growing soybean rhizobia. Since Davis was a progenitor for 20% of the cultivars studied by Chueire and Hungria (1997), and a further 19% included Paraná as a progenitor, it is not surprising that a high percentage of Brazilian cultivars should be capable of nodulation with fast-growing soybean rhizobia. Indeed, both North American (Delannay *et al.*, 1983) and Brazilian (Hiroamoto and Vello, 1986) soybean cultivars come from an extremely narrow range of ancestors.

Fast-growing soybean rhizobia appear to occupy a small, but significant, proportion of nodules recovered from soybean plants in Brazil. Chueire and Hungria (1997) reported 3 to 5% of such nodules occupied by fast-growing strains. Ferreira *et al.* (2000) also found that 5% of the soybean nodules they tested were occupied by fast growing rhizobia. Later reports (Galli-Terasawa *et al.*, 2003) indicated that up to 29% of the rhizobia isolated from soybean nodules in the Brazilian Cerrado and 25% of rhizobia from soybean nodules collected in the two main producing areas in Paraguay (Chen *et al.*, 2002) were fast growers.

To extend these results, a survey was carried out using six Asian and one modern soybean genotypes as trap hosts, with seedlings inoculated with soil from 22 sites that included both undisturbed areas and areas traditionally cropped to soybean. Thirty fast-

growing strains, each effective on both Asian and modern genotypes, were isolated, accounting for 17% of the nodule population in cropped areas and 24% in undisturbed sites (Hungria *et al.*, 2001c). The Brazilian fast-growing isolates differed from *S. fredii* in several characteristics, and each possessed unique protein and LPS profiles (Hungria *et al.*, 2001c). Analysis of 20 fast growing isolates from soybean nodules in Paraguay using rep-PCR (ERIC, REP) (Chen *et al.*, 2000), LPS and protein (Chen *et al.*, 2002) profiles also showed unique strains, none of which showed close affinity to *Sinorhizobium* species on the basis of 16S rRNA gene-sequence analysis (Chen *et al.*, 2000). Some of these isolates showed greater identity with *Rhizobium* genomic species Q, isolated from the native Australian shrub, *Daviesia leptophylla* (Lafay and Burdon, 1998), and with *R. tropici* strain PRF 81 (=SEMIA 4080), isolated from common bean (*Phaseolus vulgaris* L.) nodules in Brazil (Hungria *et al.*, 2000a). Others resembled *Agrobacterium* isolated from root nodules of tropical legume species (Khbaya *et al.*, 1998). In contrast to the findings of Khbaya *et al.* (1998) with *Acacia*, Paraguayan isolates were able to reinfect soybean and produce nitrogen fixing nodules. Brazilian fast-growing soybean rhizobia also group with *Rhizobium* genomic species Q, *R. tropici* PRF 81 and *Agrobacterium* spp. on the basis of 16S rRNA sequence analysis (Hungria *et al.*, 2001b). *R. tropici*, one of a number of species nodulating the common bean is thought to have originated in South America (Martinez-Romero *et al.*, 1991), and to be closely related to *Agrobacterium* spp. (van Berkum and Eardly, 1998). The similarity between *Agrobacterium* and *Rhizobium* has prompted a number of recommendations that they should be combined in the same genus (Young *et al.*, 2001). From these results it appears that most fast-growing soybean-nodulating rhizobia isolated in South America are probably promiscuous symbionts of other native legumes that are also able to nodulate soybean.

6.2. Competitiveness of fast growers

The ability of some fast-growing rhizobia to nodulate South-American soybean genotypes has stimulated the search for strains that could be used in commercial inoculants. However, when 20 of the soybean genotypes that were *fix+* with *S. fredii* and *S. xinjiangensis* (Chueire and Hungria, 1997) were inoculated with equal numbers of *B. elkanii* strain 29W and *S. fredii* USDA 205, most or all nodules were occupied by *B. elkanii*

(Chueire and Hungria, 1997). This finding is consistent with the results of earlier studies (Dowdle and Bohlool, 1985; McLoughlin *et al.*, 1985; Cregan and Keyser, 1988). Hungria *et al.* (2001b) have also reported nitrogen fixation rates with some fast-growing isolates similar to those with the *B. japonicum*/*B. elkanii* strains carried in Brazilian commercial inoculants. Again, however, very few of these strains were able to compete with *B. elkanii* strain 29W. Buendía-Clavería *et al.* (1994) reported that competitiveness of *S. fredii* was related to pH, with *S. fredii* being more competitive than *B. japonicum* at pH 8.1, but the reverse was observed at pH 4.9. Brazilian fast-growing soybean rhizobia, though isolated from soils of pH 3.0 to 5.1 were not competitive at pH 5.1 in the greenhouse, or at pH 5.4 in the field, though their nodule occupancy increased when the pH was raised to 6.8 and 7.9. Competitiveness of *B. elkanii* 29W was not affected by pH. The poor competitiveness of fast-growing soybean rhizobia under acid conditions might explain the difference in frequency of recovery of these organisms in pot trials with pH 6.8 nutrient solution (Ferreira *et al.*, 2000; Hungria *et al.*, 2001c), and in field studies in soils of lower pH (Chueire and Hungria, 1997). As the great majority of Brazilian soils, together with most soils cropped to soybean in South America, have established populations of soybean bradyrhizobia, and are usually of pH 4 to pH 6 even after liming, the limited competitiveness of fast-growing soybean-nodulating rhizobia precludes their recommendation for use in commercial inoculants. However, a search for more competitive and acid tolerant strains is warranted.

7. Soybean genotypes in Brazil

7.1. Differences among genotypes in N_2 fixation capacity

Most Brazilian soybean cultivars derive from North American genotypes, and there are several earlier studies reporting differences among them in relation to the symbiotic performance (Döbereiner and Arruda, 1967; Vidor *et al.*, 1972; Lopes *et al.*, 1976b; Brose *et al.*, 1979; Freire and Vidor, 1981; Lopes and Giardini, 1981; Vargas *et al.*, 1982b). However, in the 1980s and 1990s, very few studies investigated the variability among genotypes in relation to nodulation and N_2 fixation capacity. Furthermore, although the Brazilian soybean breeding program has always been performed in areas previously

inoculated, and commonly in soils that are limited in N, breeders have often neither inoculated the seeds nor evaluated their symbiotic performance, with more attention being paid to resistance to diseases and yield.

More recently, 152 North American and Brazilian soybean genotypes, belonging to maturity groups VI or higher, and recommended for different regions of Brazil, were evaluated for differences in nodulation and nitrogen fixation. The evaluation was performed first under axenic conditions, with 29w, SEMIA 566 and SEMIA 587 used as single-strain inoculants. Differences among cultivars were shown, with some having up to four times more nodule dry weight and accumulating 2.5-fold total N in tissues. Losses in BNF capacity were also shown, *e.g.*, cultivar Davis, a North American genotype introduced to Brazil in the 1960s, had 30% more nodules and 34% more total N than a recently released cultivar, BR-16, that had Davis as a parent (Bohrer and Hungria, 1998). A decline in nodulation and N₂ fixation was also found when other cultivars frequently used as parental genotype were considered, *e.g.*, Bossier and Paraná (Bohrer and Hungria, 1998; Hungria and Bohrer, 2000).

When the same genotypes were evaluated in pots with an established population of the bradyrhizobia (29W:SEMIA 566:SEMIA 587, 22%:36%:34%), some cultivars accumulated up to twice the nodule dry weight and total N of others. Variation in nodulation, total N and yield was also confirmed for six cultivars grown under field experiment (Hungria and Bohrer, 2000). It is then clear that lack of evaluation of BNF parameters can result in a rapid decline in the symbiotic capacity of recently released cultivars (Bohrer and Hungria, 1998; Hungria and Bohrer, 2000).

7.2. Genetics of nodulation and N₂ fixation

Nicolás *et al.* (2002) crossed four cultivars identified as differing in capacity for N₂ fixation (J-200 and Bossier (high), Embrapa 20 (medium) and Embrapa 133 (low), then evaluated their progeny under soil conditions low in N and with established populations of *Bradyrhizobium*. F₃ plants in each cross showed apparently continuous distribution in N₂ fixation, common for polygenic traits, and this was reflected in the variation in nodulation and plant growth parameters. There was a predominance of genetic additive [*d*] and/or *D* (genetic additive variance) effects for most of the nodulation and plant growth variables

tested, except for nodule dry weight in the cross of J-200 x Bossier. Genetic dominant effects [*h*] and/or *H* (genetic dominance variance) were detected for all variables in the cross Embrapa 20 x Embrapa 133 and for the ratio nodule dry weight/nodule number in Bossier x Embrapa 20. Additive x additive epistatic [*i*] and interaction genotype x microenvironment effects were less important in all experiments. The narrow-sense heritabilities (h_n^2) estimates for shoot dry weight ranged from 0.59 to 0.77, for nodule number from 0.49 to 0.65, and for nodule dry weight from 0.49 to 0.71. Although considered of intermediate magnitude, these were high values when compared to other legumes reported in literature, *e.g.*, h_n^2 for nodule number in mung bean (*Vigna radiata*) ranged from 0.22 to 0.46 (Miller and Fernandez, 1998). The prediction of the genetic potential to generate superior inbred lines for nodulation and BNF capacity indicated that selection could be more effective for the crosses Bossier x Embrapa 20 and Embrapa 20 x Embrapa 133 (Nicolás *et al.*, 2002). F_{7,8} plants with higher BNF capacity obtained from those two crosses are now in national evaluations and new genotypes should be commercially released in 2006.

To identify quantitative trait loci (QTL) influencing nodulation and nitrogen fixation, F₃ plants of the cross Embrapa 20 x BRS 133 were also analyzed using simple sequence repeat (SSR) markers described by Cregan *et al.* (1999). Forty-five polymorphic markers were identified, with 13 of these associated with QTLs. Effects were observed for both nodulation and plant growth parameters studied, ranging from 2 to 9% (Nicolás, 2001). A two-way ANOVA showed six epistatic interactions among non-linked QTLs for nodulation and plant growth, explaining up to 15% of the trait variation and increasing the phenotypic expression from 8 to 28% (Nicolás, 2001). From this data it appears that effective marker-assisted selection using SSR markers may be feasible for the enhancement of BFN traits in soybean breeding programs.

Studies with supernodulating soybean mutants that tolerate nitrate (Carroll *et al.* 1985; Gremaud and Harper, 1989) were not continued, as a preliminary evaluation of these genotypes in Brazil indicated high sensitiveness to water and temperature stresses.

7.3. Exclusion of specific strains by soybean genotypes

CONAB (2004) reported that only 10% of the area planted to soybean in Brazil in the last growing season had not previously been used for this crop. Figures are likely to be similar for the other main soybean-producing countries of South America. A consequence is that most soils will contain a naturalized population in which inoculant strains from the past decade and their variants predominate. As with serocluster USDA 123 in the USA (Caldwell, 1966; Devine, 1976; Cregan *et al.*, 1989; Sadowsky and Cregan, 1992), the established population can make the introduction of more efficient strains very difficult. An approach taken in some studies in the USA has been to identify soybean genotypes which specifically restrict nodulation by *Bradyrhizobium* serogroups that are dominant in the soil, and that instead favor nodule occupancy by the inoculant rhizobia (Caldwell, 1966; Vest *et al.*, 1972; Cregan and Keyser, 1988; Sadowsky and Cregan, 1992). Usually this method has emphasized selection against serocluster 123. However, in a first investigation, nodulation restriction of soybean cultivars to Brazilian naturalized *Bradyrhizobium* strains was not detected (Bohrer and Hungria 1998; Hungria and Bohrer, 2000).

Kvien *et al.* (1981) and Weiser *et al.* (1990) sought cultivars with different levels of preference, or exclusion, for certain serogroups. Using this approach, Hungria and Bohrer (2000) identified 20 Brazilian cultivars showing preference for strain SEMIA 566, or SEMIA 587, or 29W. Under Brazilian conditions, an approach that selects for a limited number of inoculant rhizobia would appear more feasible than one attempting to select against a diverse and evolving population of soil rhizobia.

8. Effects of reinoculation

8.1. Effects on soybean crop

Several of the experiments reported above have involved reinoculation of soils containing high numbers of indigenous rhizobia. Even so we would like to place specific importance on this topic. It is often stated that responses to inoculation of soybean seeds are variable, and in the tropics depend especially on soil fertility, temperature and moisture content. Above all, inconsistent responses to inoculant application are frequently attributed to the rhizobial strains previously applied and naturalized, to the enrichment of indigenous populations due to prior cropping of legumes, or to a combination of both factors (*e.g.*,

Herridge *et al.*, 1987; Sadowsky and Graham, 1998; Thies *et al.*, 1991a, 1991b, 1995). Indeed, there are reports of declines in the response of soybean to inoculation when the numbers of indigenous bradyrhizobia are as low as 10 to 20 cells g⁻¹ of soil (Weaver and Frederick, 1974; Singleton and Tavares, 1986; Thies *et al.*, 1991a, 1991b). In contrast, and as already shown in this chapter, field responses to inoculation in Brazil and Argentina can be obtained even in soils with up to 10⁶ cells of soybean rhizobia g⁻¹ of soil.

In Table 1, eleven out of 14 experiments undertaken in Brazil reported yield response to reinoculation in soils containing 10³ to 2.4 x 10⁴ bradyrhizobia g⁻¹ soil. Reinoculation resulted in yield increases averaging 308 kg ha⁻¹ relative to the not-reinoculated control (2,970 kg ha⁻¹), a gain in grain yield of 10.4%. Similarly, for the five experiments presented in Table 2, mean yield increase following reinoculation was of 422 kg ha⁻¹, or 12.4% in relation to the non-reinoculated control (3,399 kg ha⁻¹). Hungria *et al.* (2000b) report a third set of 13 experiments, performed in soils with established populations of bradyrhizobia where reinoculation allowed statistically significant mean yield increases ranging from 3.8% in southern Brazil to 7.8% in the Cerrado (central west Brazil) (Table 4). Combining these three sets of experiments summing 29 field trials, reinoculation allowed mean yield increases of 8%, a major contribution to the crop.

Table 4. Mean and maximum percentage increases in yield (kg ha⁻¹) and total N in grains (kg N ha⁻¹) due to the inoculation with a combination of the strains *Bradyrhizobium elkanii* SEMIA 587 and *B. japonicum* CPAC 7 (=SEMIA 5080), when compared to the non-inoculated control. The increases were obtained in thirteen experiments performed in two Brazilian Regions, in soils with established population of soybean bradyrhizobia^{1,2}.

Region	Grain yield (% increase)		Total N in grains (% increase)	
	Mean	Maximum	Mean	Maximum
Central West	7.8	23	8.1	25
South	3.8	20	4.3	24

¹After Hungria *et al.* (2000b).

²Each experiment was performed with four to six replicates and after the multivariate analysis the data presented in this table were statistically significant for both grain yield and total N accumulated in the grains (Duncan, $P \leq 0.05$).

Table 5. Effects of reinoculation of soybean on soybean grain yield. Experiments performed in Argentinean soils with established population of *Bradyrhizobium*. Only field trials yielding more than 2,000 kg ha⁻¹ were considered.

Year	Institution	City	Non-inoculated	Inoculated	Increase (kg ha ⁻¹) ⁵	Increase (%) ⁵
1994/95 ¹	INTA	Marcos Juarez	2,309	2,525	216	9.35
1996/97 ¹	Crea-Darwich	Rincón del Valle	2,111	2,276	165	7.82
1996/97 ¹	Crea-Darwich	Metan (3 trials)	2,726	2,979	253	9.28
1996/97 ¹	Crea-Darwich	La Cocha (2 trials)	3,395	3,918	523	15.40
1996/97 ¹	Crea-Darwich	Burruyaco	2,929	3,500	571	19.49
1996/97 ¹	Maisur S.A.	Arequito	2,442	2,606	164	6.72
1996/97 ¹	Maisur S.A.	Rancagua	2,722	2,879	157	5.77
1997/98 ¹	INTA	Marcos Juarez	3,210	3,450	240	7.48
1997/98 ¹	INTA	Oliveros	3,730	3,810	80	2.14
1997/98 ¹	Maisur S.A.	Rancagua	3,990	4,275	285	7.14
1997/98 ¹	AFA	S.M. de las Escobas	3,940	4,059	119	3.02
1999/00 ¹	INTA	Marcos Juarez	2,250	2,376	126	5.60
1999/00 ¹	Maisur S.A.	Arequito	2,528	2,688	160	6.33
2000/01 ¹	INTA	Bragado	4,393	4,853	460	10.47
2000/01 ¹	INTA	Firmat	3,498	3,855	357	10.20
2000/01 ¹	Don Mario	Chacabuco (2 trials)	3,570	3,958	388	10.87
2000/01 ¹	Don Mario	Silveira	4,802	4,903	101	2.10
2001/02 ¹	Several ²	29 trials ²	2,621	3,022	401	15.30
2001/02 ³	Universidad Nacional de Rio Cuarto and INTA ⁴	12 trials ⁴	2,540	2,822	282	11.10

¹Nitragin Argentina (2004).

²Institutions: Crea, INTA, Maisur, Esc. Inchausti, El Tejar, Univ. Del Rio Cuarto, Basf, UNER, EEA; Sites: America, Gral. Villegas, Rancagua, Inchausti, Saladillo, Rio Cuarto, Isla Verde, Conc. Del Uruguay, Las Lajitas and Monte Redondo.

³Rizobacter Argentina S.A. (2004).

⁴Sites: General Cabrera, Chucul and Olaeta with cultivars A 3901, DM 4800, A 4910, A 5520, A 6040, A 7636. In all sites yield of inoculated treatment was higher than that of the non-inoculated, and yield increases ranged from 59 to 487 kg ha⁻¹.

⁵Statistical analysis not available.

Similarly, in Argentina, Peticari *et al.* (1998) and Devani *et al.* (2003) reported yield responses following reinoculation ranging from 235 to 823 kg ha⁻¹, and 150 to 527 kg ha⁻¹, respectively. Further, for the 12 trials reported in Table 3, all carried out in soils with established population of *Bradyrhizobium*, reinoculation with strain E109 allowed yield increases ranging from 6.6 to 81.5%, and on average of 24%. Mean yield of the treatment inoculated with E109 was 3,852 kg ha⁻¹, or 749 kg ha⁻¹ greater than that of the non-inoculated control. Table 4 reports data from another set of 62 trials performed in Argentina, with grain yield increases of 2.1 to 19.5%. In these trials, weighted mean yield of the reinoculated treatment was 3,133 kg ha⁻¹, and the increase due to reinoculation, 10.7%. This could reflect both the improved competitiveness of some of the newer inoculant strains, and the better inoculant quality, as sterile-peat inoculants represent today the majority of the commercialized inoculant, and often have 10 to 100-fold the number of cells g⁻¹ product of older non-sterile peat products.

Possible explanations for the general lack of response to inoculation in soils containing indigenous bradyrhizobia in the USA could include a higher competitiveness of the bradyrhizobial strains established in North American soils; the practice of reinoculation for “insurance against inoculation failure” rather than for yield increase, with inoculants often haphazardly applied; and lack of emphasis in the USA on the need for inoculation, including the need for care in mixing inoculant cultures and seed protectants. In Brazil, the possibility exists that soil rhizobia could be limited by the environmentally stressful conditions in their response to the presence of the host.

8.2. Introduction of new and more efficient strains in areas with established population

Another important feature of reinoculation is the replacement of persistent strains with more efficient ones, as recently evaluated by Mendes *et al.* (2004). In the first year a soil void of resident bradyrhizobia was separately inoculated with four serologically distinct *Bradyrhizobium* strains, CPAC 15 (serogroup of SEMIA 566 and USDA 123), CPAC 7 (serogroup of CB 1809), 29W and SEMIA 587. In the second year, each of the initial plots was subdivided and either not inoculated, or inoculated with CPAC 7 or CPAC 15. In the third year, the entire area was inoculated with CPAC 7, and after that none of the treatments was inoculated, although soybean cropping was continued. In the treatments

where strain CPAC 7 was used as inoculum in each of the first three years, its representation in nodules increased to 70% in the third year. However, without further reinoculation, nodule occupancy by this strain decreased to only 8% by the sixth year. In treatments first inoculated with strains 29W, 587 and CPAC 15, then reinoculated in the second and third years with CPAC 7, nodule occupancy by CPAC 7 in the third year was 44%, 43% and 25%, respectively. Frequency of CPAC 7 in nodules then declined dramatically in nodules in the years without reinoculation. By the sixth year strains belonging to serogroup SEMIA 566 dominated nodulation, occurring in more than 50% of the nodules of all treatments, including those where it had never been introduced. These results emphasize that reinoculation with a less competitive but more efficient strain (CPAC 7) was necessary to avoid the dominance of CPAC 15.

The Brazilian experience with the common bean (*Phaseolus vulgaris* L.) has been similar. Lack of response to inoculation has often been attributed to significant, but inefficient populations of indigenous common bean rhizobia in soil. However, strain selection programs searching for efficient and competitive strains within the indigenous population (Hungria *et al.*, 2000a; Mostasso *et al.*, 2002) have led to further studies with improved strains. In trials performed in soils with at least 1.1×10^3 resident rhizobia g^{-1} soil, nodule occupancy by inoculated strains was increased by both initial inoculation and reinoculation (Hungria *et al.*, 2000a, 2003). Reinoculation with one of the selected strains, *Rhizobium tropici* H 12, increased nodule occupancy from 25% in 1999 to 55% in 2001 and, in average of six experiments, resulted in yield gains of 437 kg ha^{-1} (Hungria *et al.*, 2003).

9. Main constraints to the biological process

Soybean growers in Brazil and in most sites in South America are usually large-scale producers, and so more likely to have the capital needed to correct soil acidity, aluminum toxicity and low fertility problems. Instead the main limiting factors for those farmers are often environmental stresses: high soil temperatures, with maximum temperatures exceeding 40°C at 5 cm and 50°C at 1 cm depth, and low soil moisture (Hungria and Vargas, 2000). No tillage (NT) management systems have been widely adopted in the

region, and improve soil moisture retention, enhance organic matter content of soil, and help to regulate soil temperature. They also help in erosion control, and improve soil structure and aggregate stability, usually resulting in higher yields (Derpsch *et al.*, 1991; Derpsch, 1998; Hungria *et al.*, 2005a). Because of these benefits the area devoted to NT management systems in Brazil has increased from 2.02 million ha in 1992/1993 to more than 19 million ha today (FEBRAPDP, 2004). NT systems for soybean in Brazil have enhanced rhizobial survival, growth and diversity in soils, showing higher induction activity of nodulation genes, better nodule distribution in the soil profile, better nodulation, N_2 fixation rates and yield (Voss and Sidiras, 1985; Ferreira *et al.*, 2000; Hungria and Vargas, 2000; Hungria *et al.*, 2001c, 2005a).

Probably the main concern to the BNF today is the widespread use of fungicides in contact with the inoculant at sowing. In Brazil, more than 90% of soybean seeds are treated with fungicides, and cell death rates of up to 70% after only two hours of contact with fungicides have been reported, as well as reduction of nodulation under field conditions (Hungria *et al.*, 2005b). Decreases in rhizobial survival on seeds are also found when seeds are treated with the micronutrients molybdenum (Mo) and cobalt (Co), a need in many tropical soils where low soil pH may result in these nutrients not being available to the growing plant (Hungria *et al.*, 2005b). Strategies to improve the compatibility of seed treatment with inoculation were recently discussed and include the recommendation of higher number of cells per seed, inoculation in the furrow and, for the micronutrients, spray application after germination, and production of seeds enriched in Mo (Hungria *et al.*, 2005b). A number of “*Rhizobium*-compatible” fungicides are also available on the USA market, but still need to be tested in more detail.

10. Quantification of the N_2 fixation contribution to the soybean and release of N to the following crop

Unkovich and Pate (2000) and Giller (2001) suggested a potential for N_2 fixation in soybean of $360\text{--}450 \text{ kg of N ha}^{-1}$, levels that would allow yields at current levels of seed protein of up to $5,600 \text{ kg ha}^{-1}$. Yields higher than $4,000 \text{ kg ha}^{-1}$ have already been obtained in both Brazil and Argentina using BNF as the main source of N (Tables 1 to 4). In a trial

at Carabelas, Argentina, in which E109 was used as inoculant, yield achieved 6,000 kg ha⁻¹ (Table 3), and in Londrina, Brazil, soybean under the NT system yielded 5,890 kg ha⁻¹, with a contribution from BNF estimated at ≈300 kg of N ha⁻¹ (Zotarelli, 2000).

van Kessel and Hartley (2000) analyzed 362 soybean trials performed mainly in USA and Australia over a 25 year period, in which ¹⁵N isotopic dilution or N difference with non-nodulating isolines methods were used to estimate the contribution of BNF to soybean. In this study, soybean derived an average of 59% of its N from BNF, a value similar to the 53% reported for 33 commercial soybean fields in Australia (Unkovich and Pate, 2000). Although there are fewer quantification studies under field conditions in South America, a recent survey of data obtained in Brazil using ¹⁵N isotopic dilution, δ¹⁵N, N balance and N-ureides techniques, indicated higher contributions of BNF to soybean growth and yield than in the USA and Australia, with the percentage of plant N derived from fixation (%NDF) ranging from 69 to 94%. Estimated rates of BNF ranged from 109 to ≈300 kg of N ha⁻¹ crop cycle⁻¹ (Hungria *et al.*, 2005a). Mean yield in field trials performed for three crop seasons with recent soybean cultivars under the NT system in Londrina, Brazil, was 4,927 kg ha⁻¹, with %NDF estimated by the N-ureides methodology, contributing to 83% of plant N, or 290 kg of N ha⁻¹ (Zotarelli, 2000; Hungria *et al.*, 2004).

Increasing N harvest index has also been a trait sought by plant breeders, but results in higher exportation of N to the grains with less N left in the soil (Alves *et al.*, 2003). Root N has rarely been considered in N quantification studies carried out so far, but may represent 30 to 35% of total plant N (Peoples and Herridge, 2000; Khan *et al.*, 2002). Indeed, recent field evaluations performed in Londrina, Brazil, demonstrated that the soybean roots left 30 kg of N ha⁻¹ to the following crop (B.R.J. Alves, unpublished data).

The N released by the soybean can benefit the following crop. In Londrina, yields of wheat (*Triticum aestivum*) cropped after soybean inoculated with strains CPAC 7, 29W, or SEMIA 587 produced 262, 396 and 108 kg ha⁻¹, respectively, more than in the parcels where soybean had not been inoculated (Nishi and Hungria, 1996). Similar results were obtained in other crop seasons in both Londrina and Ponta Grossa (Hungria *et al.*, 2001a). Likewise, in Passo Fundo, southern Brazil, measurements over six crop seasons indicated

that mean wheat yield was 403 kg ha⁻¹ greater after soybean than after maize (Wiethölter, 2000).

11. N fertilizers and soybean yield

The compilation by van Kessel and Hartley (2000), referred in the previous item, concluded that the %NDF in soybean has declined from 65% (analysis of 196 trials) to 54% (166 trials) since 1985. The authors attributed the lower symbiotic performance to an increased use of N fertilizers. Such data emphasize the importance of the management that has been taken place in South America, maximizing the contribution of the biological process.

Soybean represents a high profit crop in South America with increasing pressure for farmers to purchase N fertilizer. In Argentina, N fertilization at planting has not shown grain yield responses. and at reproductive stages (R1, beginning bloom, to R3, beginning pod) it has shown variable results, according to soil N availability, crop growth and development, and yield potential (Garcia, 2004). However, starter doses of N at sowing (less than 30 kg N ha⁻¹) have resulted in yield increases where soybean is sown following the harvest of high-yielding wheat crops (>5,000 kg ha⁻¹) in southeastern Buenos Aires (Garcia, 2004).

In Brazil, starter N doses as low as 20-40 kg of N ha⁻¹ may decrease nodulation and N₂ fixation, with no benefits to yield (Vargas and Hungria, 1997; Crispino *et al.*, 2001; Hungria *et al.*, 2001a; Mendes *et al.*, 2003). Indeed, in more than 50 experiments where inoculation and fertilization with 200 kg of N ha⁻¹ have been compared (split application of N at sowing and flowering), no increases in yield due to N-fertilizer use have been observed (Hungria *et al.*, 2000b, 2001a). Similarly, there were no benefits when N-fertilizer was applied at a rate of 400 kg N ha⁻¹, split across ten applications (Nishi *et al.*, 1996; Nishi and Hungria, 1996).

In six field experiments performed in Londrina and Ponta Grossa, Brazil, soybean produced an average of 3,200 kg ha⁻¹, with no yield increase when 50 kg of N ha⁻¹ was applied at the R1 or R3 growth stage (Crispino *et al.*, 2001). These results were confirmed in the next two crop seasons (unpublished data), as well as in Jaciara, in the Brazilian

Cerrados (Loureiro *et al.*, 2001). It is a striking contrast that in northern and central China, the center of genetic origin of soybean, N₂ fixation alone cannot meet the N requirement for maximum yield (Gan *et al.*, 2002, 2003). In those areas, best results were obtained with top dressing of N fertilizer (50 kg ha⁻¹) at V2 (the unifoliolate node and the first two trifoliolate leaf nodes with unfolded leaflets), and especially at R1 stage (Gan *et al.*, 2002, 2003). These authors mention that the inefficient use of N fertilizer can often result in high costs, while the reported yields are lower than generally obtained in South America.

12. Economic return from soybean inoculation

Economic returns on inoculation and biological N₂ fixation with soybean in South America are outstanding, with the Brazilian case used here as an example. For each 1,000 kg of soybean produced the plant requires approximately 80 kg of N (65 kg allocated to seeds having ~40% protein and 15 kg N left in roots, stems and leaves). Thus, to achieve the national mean yield of 2,737 kg ha⁻¹, N requirement of the crop would be of ~220 kg N ha⁻¹. Brazilian soils are poor in N and usually supply only 15 to 30 kg of N crop⁻¹, thus the plants would need an additional ~200 kg N ha⁻¹. To supply this amount as N fertilizer in an environment where N utilization efficiency rarely reaches 60%, a soybean crop would require ~330 kg N or ~710 kg of urea (46.64% of N) to achieve equivalent yields. At a price of US\$ 0.20 kg⁻¹ of urea, the cost of applying N fertilizer would be ~US\$ 142 ha⁻¹, or in excess of US\$ 3 billion nationally for the 21 million ha cropped with soybean in 2003/2004. BNF clearly plays a key role in the commercial success of this crop.

It is also important to emphasize that the application of only 30 kg of N ha⁻¹ would imply in a cost of US\$ 13.3 ha⁻¹ to the farmer, representing ~US\$ 280 million to the country per growing season, certainly decreasing competitiveness of the national product (Hungria *et al.*, 2005a).

13. Inoculant production and use in South America

The first production and distribution of inoculants for legumes in Brazil dates from 1930 in São Paulo (Lopes and Giardini, 1981). Rio Grande do Sul began to produce agar

inoculants in 1949, and peat based inoculants in 1955, but industrial scale production did not begin until the 1960s (Freire and Vidor, 1981). Control of inoculant quality in the country was a main concern of researchers from the very beginning (Freire and Vidor, 1981; Lopes and Giardini, 1981), with official control of inoculant quality established by the Federal Government in 1975. The initial standard was 10⁷ cells g⁻¹ (Lopes and Giardini, 1981), and Freire and Vidor (1981) noted that at recommended rates of application, this would supply 7,000 cells seed⁻¹. Legislation today requires that inoculants must contain 10⁹ cells g⁻¹ or ml⁻¹, allowing 600,000 cells seed⁻¹, and be void of contaminants at the 10⁻⁵ dilution; researchers are already recommending the application of 1 million cells seed⁻¹. Nowadays, 50% of the inoculants commercialized in Brazil are peat-based and the remainder are liquid or gel formulations. Often, however, inoculants not based on peat can perform poorly under tropical conditions, and cell protectants against stress can become a main factor defining symbiotic performance (Hungria *et al.*, 2005b). Furthermore, other constraints must be investigated, *e.g.*, if the type of inoculant formulation can alter the relative competitiveness of the strains (Streeter, 1994).

As a consequence of the positive results obtained with soybean inoculation and reinoculation, and of the expansion of cropped area, the number of doses sold in both Brazil and Argentina increases every year (Fig. 2), and estimates are that in both countries inoculation is practiced by about 60% of the farmers. In Paraguay, roughly 15 to 20% of the farmers inoculate soybean (Chen *et al.*, 2002), but that percentage is increasing. In Canada, due to the reported benefits, inoculation is also practiced by nearly 60% of the farmers, while in the USA the estimates reach only 15% of the cropped area (Hungria *et al.*, 2005a). As the costs of transports and inputs are much higher in South America, the soybean growers need to maintain their focus on N₂ fixation as the only source of N for the crop.

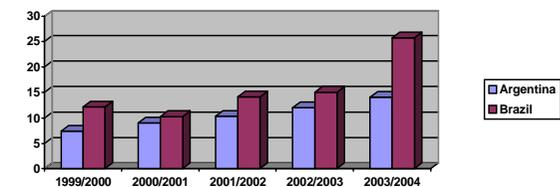


Figure 2. Doses of inoculants (million) commercialized in Argentina and Brazil for the soybean crop in the last five crop seasons.

14. Conclusions

Nitrogen fixation plays a key role in the agricultural sustainability of soybean production in the tropics, increasing productivity, ensuring food security, and reducing contamination of the environment with chemical sources of N. In this chapter, the successful adoption of soybean as a major crop in the region has been shown to depend in large part on the success of approaches that stress BNF as a major component of soybean crop production in South America. These approaches resulted in higher yields based exclusively on the biological process. Most important, the strategies reported here may be applied in developing or industrialized countries, by large or small landholders, and for subsistence or cash legume crops.

15. Acknowledgements

The authors thank to Dr. Décio L. Gazzoni and Dr. George G. Brown for suggestions on the manuscript. M. Hungria acknowledges a research fellowship from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). The authors dedicate this chapter to Dr. Milton A.T. Vargas, for his outstanding research in the Brazilian Cerrado, to Dr. Fabio de Oliveira Pedrosa, for the remarkable scientific support to our group in the last decade, and to the memory of Dr. Johanna Döbereiner.

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