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Diversity of a soybean rhizobial population adapted to a Cerrados soil

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Summary

One hundred isolates were trapped by soybean (*Glycine max*) plants inoculated with a soil from the Cerrados, the main producing area in Brazil. The soil was originally void of rhizobia able to nodulate soybean, and 15 years before received inoculant containing *Bradyrhizobium elkanii* strains SEMIA 587 and SEMIA 5019; the area has been annually cropped with soybean since then, but with no further inoculation for the past 7 years. Enormous diversity was observed among the isolates, with thirteen serologically distinct groups, twelve protein and seven lipopolysaccharide profiles; no more than five isolates shared similar characteristics. An unexpected feature was that 48% of the isolates showed multiple reactions with the antisera to the serogroups established in the soils. Also 40% of the isolates reacted with the antiserum to *B. japonicum* strain SEMIA 566, that has never been introduced into the soil, probably due to dispersion from other cropping areas, associated with its high saprophytic competence; 13% of the isolates did not react with any of the antisera. Nodulation and N_2 fixation capacity also varied considerably among the isolates. Although one third of the isolates were fast growers with an acid reaction *in vitro*, and many formed pseudo-nodules on common bean (*Phaseolus vulgaris*), they shared several properties with the *Bradyrhizobium* inoculant strains. A high level of genetic diversity was confirmed when the DNAs were amplified with BOX and RPO1 primers, and several isolates were positioned in far different clusters in the analysis of interspersed repetitive or *nif*-directed sequences. Moreover, serological properties showed higher correlation with BOX than with RPO1 products. The high diversity could be attributed both to lateral transfer of genetic material between inoculant and indigenous strains and to genomic rearrangements during the adaptation to the Cerrados, and may play an important role as a biological buffer, avoiding the dominance of a particular strain.

Introduction

Globally, soybean (*Glycine max* (L.) Merr.) is one of the most important and extensively grown crops. It accounts for 30% of the world's processed vegetable oil, and is a rich source of protein for human consumption, and for the poultry and pork industries; more recently, it has also been employed as a source for biodiesel fuels (Graham & Vance 2003; Hungria *et al.* in press). The current worldwide production of this legume is estimated at 183 Tg, of which Brazil contributes 25% (CONAB 2002). Most of the success of the crop in Brazil is related to its expansion in the 1970s into the 'Cerrados', an edaphic type of savanna occupying 207 million ha and representing 25% of the Brazilian land area, such that in 2001/02 the three main states of this ecosystem, Mato Grosso, Mato Grosso do Sul and Goiás (including the Federal District) cultivated 6.97 million ha and produced 20 Tg of soybean, with a yield average of 2926 kg ha^{-1} (Hungria *et al.* in press).

At the time of crop expansion, the experiments performed in the Cerrados reported zero or close-to-zero nodulation and yellow soybean plants in N-deficient soils, indicating the absence of rhizobial strains able to establish an effective symbiosis. Those experiments showed that inoculation was needed and a selection program was initiated to obtain strains adapted to acidic soils, with low P content and toxic levels of Al; that program continues (Peres 1979; Peres & Vidor 1980; Vargas & Suhet 1980; Vargas *et al.* 1982, 1992; Peres *et al.* 1993; Vargas & Hungria 1997).

The absence of indigenous soybean rhizobia in the Cerrados was recently confirmed using molecular biology tools (Ferreira & Hungria 2002). Studies performed in soybean-growing areas have also identified strains with morphological, physiological, genetic and symbiotic characteristics that differ from the inoculant strains originally introduced into these soils (Hungria *et al.* 1996; Nishi *et al.* 1996; Boddey & Hungria 1997; Hungria *et al.* 1998; Santos *et al.* 1999). Moreover,

those studies were performed with selected strains showing higher capacity for biological N_2 fixation (BNF) and/or competitiveness, thus our understanding of the diversity of naturalized soybean rhizobial populations in the Cerrados is still very poor.

The exploitation of its BNF capability is necessary for the economical competitiveness of Brazilian soybean. Estimates indicate savings of about US $1.95 billion per growing season (Hungria *et al.* in press) that would otherwise be spent on synthetic N fertilizers. In 2001/02, 14 million doses of soybean inoculants were produced commercially; however, to maximize the benefits of BNF, it is necessary to understand the interactions between the naturalized rhizobial population and the inoculant strains. As a first step, the objective of this study was to characterize the diversity within a soybean-nodulating rhizobial population in the Cerrados region. The population studied consisted of 100 isolates trapped by soybean plants inoculated with a soil, originally void of rhizobia able to nodulate soybean, which received inoculant 15 years before. The soil has been cropped annually to soybean since then, with no further inoculation for the last 7 years.

Materials and methods

Bradyrhizobium japonicum/B. elkanii *reference strains*

The following strains, which account for practically 100% of the *Bradyrhizobium* inoculant strains established in Brazilian soils, were used as reference: *Bradyrhizobium japonicum* SEMIA 566, SEMIA 586 (=CB 1809), SEMIA 5079 (=CPAC 15, same serogroup as SEMIA 566) and SEMIA 5080 (=CPAC 7, same serogroup as SEMIA 586), and *B. elkanii* SEMIA 587 and SEMIA 5019 (=29w). Information about the strains, their sources and main characteristics is available elsewhere (Boddey & Hungria 1997; Santos *et al.* 1999; Ferreira & Hungria 2002; Chueire *et al.* in press).

Soybean rhizobial isolates

Soil samples, randomly collected at depths of 0–20 cm from a typical Cerrados area in the Federal District, were obtained using soil drills cleaned with alcohol (95%) and flamed between samplings; 20 subsamples randomly collected (0–20 cm) were mixed in a sterile bag. The soils of the area were originally void of rhizobia able to nodulate soybean. They received inoculant 15 years previously and have been cropped annually to soybean since then, but with no further inoculation for the last 7 years. The inoculant used contained the strains SEMIA 587 and SEMIA 5019. The soil samples were passed through a 4-mm sieve and divided for determinations of moisture content and chemical properties, and for rhizobia isolation, with all operations completed as quickly as possible. Moisture content was determined after drying the soil at 105 °C for 4 days, and chemical properties were: pH in $CaCl_2$, 3.87; N(%), 0.16; Al(%), 83; base saturation(%), 2.1; C(%), 2.42; P(mg dm^{-3}), 0.6. The total rhizobial population was estimated by the MPN technique with counting in soybean plants (Vincent 1970) at 10^4 cells g^{-1} soil.

Seeds of soybean cultivar BR 37 were surface-sterilized with alcohol and 10% sodium hypochlorite (Vincent 1970) and germinated for 2 days. Each seedling was inoculated with 500 μl of a 10^{-1} soil dilution (in 0.85% w/v NaCl, agitated with glass beads for 30 min), and 40 replicates were used. Plants were grown as described before (Hungria *et al.* 1998) and 100 nodules were randomly chosen at 35 days after planting from 45 plants. Rhizobial isolates were obtained using standard microbiological methods (Vincent 1970), and culture purity was verified by repeatedly streaking the bacteria on yeast extract-mannitol agar (YMA) medium (Vincent 1970), searching for a single type of colony, and the gram-stain reaction. Bacteria were applied to cultivar BR 37, to confirm effectiveness, and were then stored at −80 °C, with working cultures being maintained on YMA slants at 4 °C. Ninety-seven isolates confirmed the ability to nodulate soybean and were thus used in this study.

Morphological, physiological and serological characterization of the isolates

Colony morphology (colour, mucoidy, transparency, diameter, form, borders, elevation) and acid/alkaline reaction were evaluated after 5 and 7 days of growth on YMA containing bromothymol blue as indicator, in the dark, at 28 °C, after Vincent (1970). Somatic agglutination reactions of each isolate were performed as described by Boddey & Hungria (1997), using antisera to the four dominant serogroups in Brazilian soils: SEMIA 566, SEMIA 586, SEMIA 587 and SEMIA 5019.

Protein and lipopolysaccharide fingerprintings

Protein and lipopolysaccharide (LPS) profiles were determined as described by Ferreira *et al.* (2000) and Hungria *et al.* (2001b), respectively.

BOX-PCR genomic fingerprinting

Bacteria DNAs were amplified by PCR using BOX (Versalovic *et al.* 1994) and RPO1 (Richardson *et al.* 1995) primers, as described by the authors. Banding patterns were photographed and analysed using the Bionumerics program (Applied Mathematics, Kortrijk, Belgium). The Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) clustering method and the coefficient of Jaccard (J) were used.

Symbiotic properties

N_2-fixation capacity: Seeds of cultivar BR 37 were surface-sterilized and individually inoculated with each

isolate, and plants were grown in Leonard jars under greenhouse conditions as described previously (Hungria *et al*. 1998). Plants were harvested 45 days after sowing, nodules were removed and counted and, after drying at 65 °C to constant weight, nodule and shoot dry weights were determined. Total N in shoots was evaluated as described before (Hungria *et al*. 1998). The experiment was performed in a randomized block design with three replicates, and the data were statistically analysed by Tukey's test ($p \leq 0.05$).

Host specificity: The capacity of each isolate to effectively nodulate common bean (*Phaseolus vulgaris* L.) cultivar IAPAR 14 (black seeds) was verified under greenhouse conditions (Hungria *et al*. 1998).

Results and discussion

From a population of a hundred isolates, 97 were confirmed to have nodulation and N_2-fixation capacity after reinoculation of soybean plants and were, therefore, characterized. Isolates showed a wide variation in relation to morphology (data not shown), and 28 were fast growers with acid reaction in YMA medium. The isolation of fast growers from soybean nodules has been reported before in South America, representing from 17 to 24% of populations in Brazil (Hungria *et al*. 2001b) and 25% in Paraguay (Chen *et al*. 2002). In Asian centres of origin of soybean, fast growers have been identified as belonging to the species *Sinorhizobium fredii* and *S. xinjiangensis* (Scholla & Elkan 1984; Chen *et al*. 1988), and in Brazil and Paraguay, the isolates were classified as indigenous *Rhizobium tropici* and rhizobia resembling agrobacteria (Chen *et al*. 2000; Hungria *et al*. 2001a). However, in this study, most of the fast growers shared several characteristics with inoculant strains, as will be discussed.

An unexpected characteristic of this population was its multiple reactions with the antisera of the serogroups established in the Brazilian soils: 48% of the isolates reacted with two or more antisera (Table 1). As expected, the inoculant strains represented the majority of the reactions, with 48 isolates reacting with the antiserum to SEMIA 587 and 37 with that to SEMIA 5019. However, attention should be paid to the serogroup SEMIA 566, since 38 isolates showed reactions against its antiserum. SEMIA 566 was used in commercial inoculants from 1966 to 1978, mainly in the southern region, and since 1992, SEMIA 5079, a variant strain of SEMIA 566 (Vargas *et al*. 1992; Hungria *et al*. 1996; Nishi *et al*. 1996), has been intensively used in the Cerrados. Interesting are the reports that SEMIA 566 has limited saprophytic competence in the first 2 years, but becomes established thereafter (Freire *et al*. 1983; Vargas & Hungria, 1997; Mendes *et al*. 2000). Dispersion of SEMIA 566 from soybean-growing areas, associated with its high saprophytic competence seems to result in the high percentage of reaction with this serogroup, even in areas that had never been inoculated with this strain (Vargas *et al*. 1993; Ferreira & Hungria 2002). Finally, thirteen strains did not react with any of the tested antisera; absence of reaction in 37% of the population was also reported in a study performed in southern Brazil (Ferreira *et al*. 2000).

The most discriminating analysis was the evaluation of protein profile (PP), with 12 different PPs being obtained. PP-III represented 23% of the isolates, and the others included from three to nine strains (Figure 1). Although seven different profiles of lipopolysaccharides (LPS) were observed, most of the isolates (70%) were grouped into LPS-II (Figure 1). The LPS-II profile included isolates from all classes of serogroups, except for that with a mixed reaction with serogroups SEMIA 5019 and SEMIA 566 (Table 1). The high level of diversity observed is readily apparent from Table 1, where the several combinations of serogroups and PP and LPS profiles are shown, with the larger group, with isolates reacting with serogroup SEMIA 587 and

Table 1. Serological reaction, protein (PP) and lipopolysaccharide (LPS) profiles and dominant profiles of PP-LPS within each serogroup class of 97 soybean rhizobial isolates from the Cerrados region.

No. of isolates	Serogroup	Protein profile (PP)	LPS profile	Dominant combination PP-LPS (% of isolates within that serogroup class)
11	SEMIA 587	I, III, VII, IX, XI	I, II, III, V	III–II (45.4%)
09	SEMIA 5019	I, II, III, VIII, IX, XI	I, II, IV	II–I (22.2%)
12	SEMIA 566	I, III, VII, X, XI, XII	II	I–II and X–II (16.6%)
05	SEMIA 586	V, VI, VIII, XII	II	XII–II (40%)
20	SEMIA 587 + 5019	I, III, V, VI, VII, IX, X, XII	II, IV, V, VI, VII	III–II, VI–IV, IX–V, IX–II (10%)
10	SEMIA 587 + 566	I, II, III, IV, V, X, XII	I, II, III, VI	None
01	SEMIA 587 + 586	X	II	Not considered
03	SEMIA 5019 + 566	II, IV, XI	I	None
07	SEMIA 566 + 586	III, IV, V, VII, VIII, IX	II	VII–II (28.5%)
02	SEMIA 587 + 5019 + 566	VI, X	II	None
01	SEMIA 587 + 566 + 586	III	II	Not considered
03	SEMIA 587 + 5019 + 566 + 586	III, V	II, VI	None
13	With no reaction	I, II, III, V	I, II, IV	III–II (30.8%)

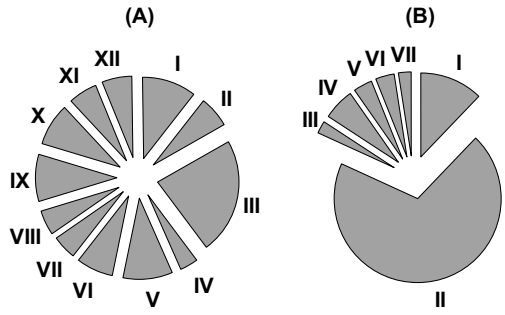

Figure 1. Percent distribution of (A) protein and (B) lipopolysaccharide profiles within a population of soybean rhizobial isolates from a Brazilian Cerrados soil.

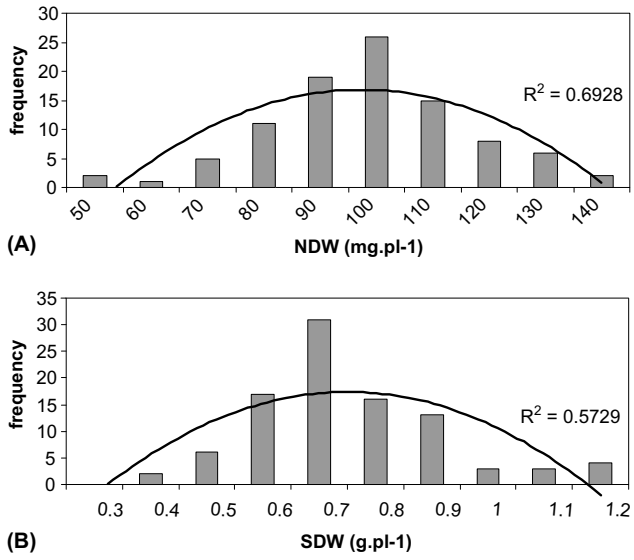

Figure 2. (A) Nodule (NDW, mg pl^{-1}) and (B) shoot (SDW, g pl^{-1}) dry weight of soybean plants of cultivar BR 37 inoculated with 97 soybean rhizobial isolates from a Brazilian Cerrados soil. Plants were grown under greenhouse-controlled conditions and harvested at 45 days after sowing.

showing PP-III and LPS-II profiles, including only five isolates.

In relation to symbiotic performance, when the isolates were used as inoculants for soybean cultivar BR 37, normal distributions for the parameters of nodule number (data not shown), nodule dry weight (Figure 2), shoot dry weight (Figure 2), %N and total N in shoots (data not shown) were observed. Among the 27 isolates that allowed higher accumulation of N in tissues in N-free medium, 10 reacted with serogroup SEMIA 586 (data not shown), confirming the high capacity of N_2 fixation of the strains belonging to this serogroup described before (Neves *et al.* 1985; Vargas *et al.* 1992; Peres *et al.* 1993; Hungria *et al.* 1996; Nishi *et al.* 1996; Hungria *et al.* 1998; Hungria & Vargas 2000). Among the efficient isolates, nine reacted with serogroup of SEMIA 566 (data not shown), usually characterized as less efficient than SEMIA 586 (Boddey & Hungria 1997; Hungria *et al.* 1998; Santos *et al.* 1999; Ferreira &

Figure 3. Pseudo-nodules obtained by the inoculation of common bean plants with some soybean rhizobial isolates from a Brazilian Cerrados soil.

Hungria 2002). However, selection within serogroup SEMIA 566 for efficient strains has proven not be a difficult task (Hungria & Vargas 2000). Among the 20 isolates with lower capacity for N_2 fixation, nine reacted with serogroup SEMIA 5019 and seven with SEMIA 587 (data not shown), the two strains originally introduced into the soil of this study. Finally, one interesting symbiotic characteristic observed was the ability of several isolates, mainly those showing a fast growth rate, to infect common bean roots, with the formation of several pseudo-nodules, as shown in Figure 3.

A high level of genetic diversity was shown when the DNAs of the isolates were analyzed by the PCR technique with BOX (interspersed repetitive sequence, Versalovic *et al.* 1994) and RPO1 (*nif*-directed, Richardson *et al.* 1995) primers (Figure 4). With the BOX primer two main clusters were observed at a 20% level of similarity (Figure 4A), while with RPO1 that similarity was of 23% (Figure 4B). In the BOX analysis, the two main groups were not related to slow and fast growers, however, several clusters showed relation to serogroup reaction; that relation was not evident with RPO1 primer (data not shown).

One important feature was that the comparison of genetic variability among isolates within the main clusters showed higher variability with the RPO1 than with BOX primers (Figure 4). As discussed by Provorov & Vorob'ev (2000), one specific feature of nodule bacteria populations is the high degree of panmixia, resulting in recombinant genotypes, and the variability would be primarily manifested in the intensive transfer of symbiotic genes. In this study, the transfer of symbiotic genes is reinforced by the observation that the several isolates occupied quite different clusters in the BOX and RPO1 analyses (Figure 4). One example is the four first isolates of the BOX figure, showing several similarities in the repetitive sequences, by positioned in complete different clusters with the *nif* primer (Figure 4). The PCR products obtained with both primers were also considered in a combined analysis that confirmed the high level the diversity, since the isolates

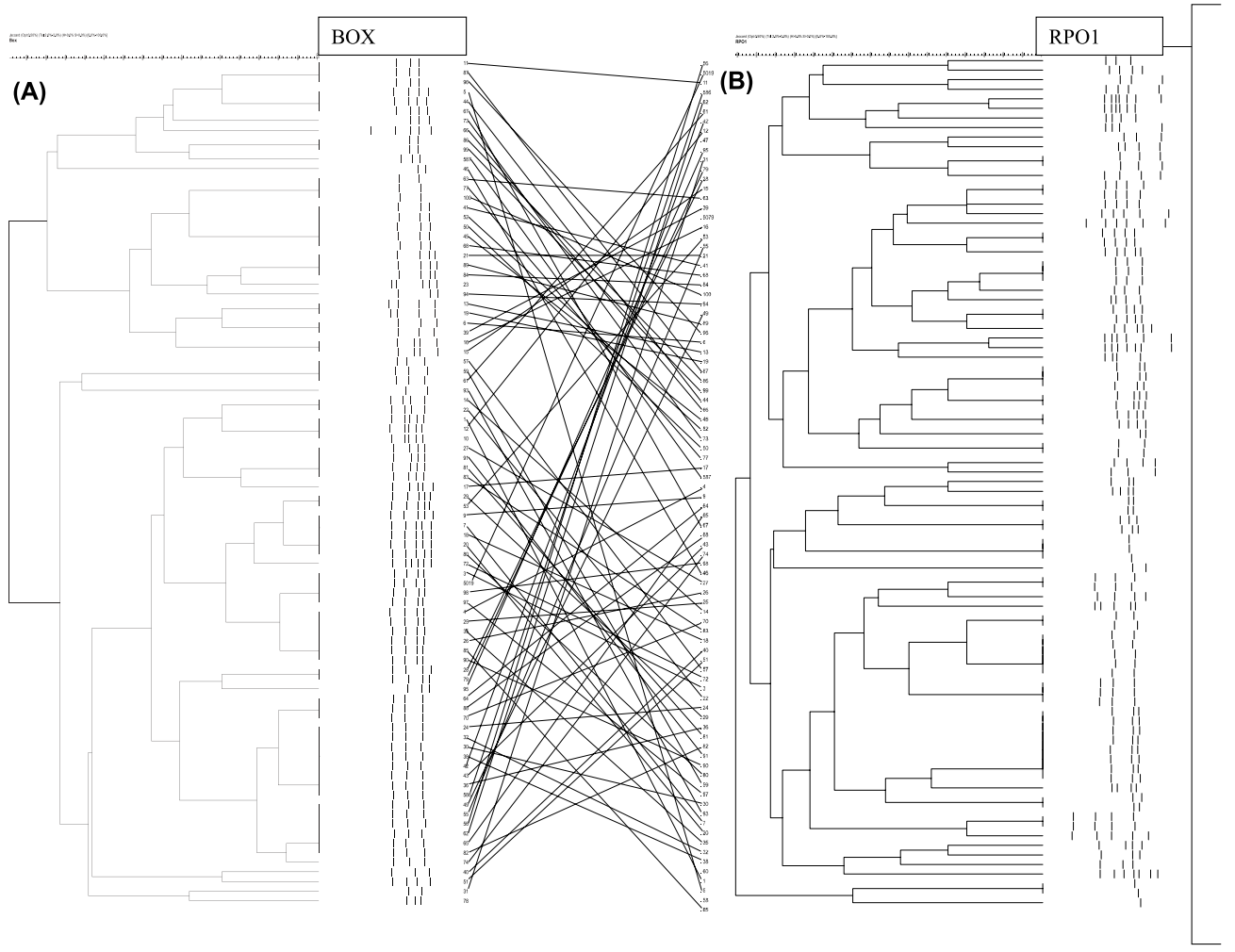

Figure 4. Dendrograms of soybean rhizobial isolates from a Brazilian Cerrados soil, and of representative strains of *Bradyrhizobium japonicum* (586 and 5079) and *B. elkanii* (587 and 5019) species, based on the cluster analysis of (A) BOX and (B) RPO1-PCR products using the UPGMA algorithm and the Jaccard coefficient. The lines indicate the relative position of each isolate in both dendrograms.

were grouped at a very low level of similarity, 23% (data not shown).

Our results provide a remarkable collection of data confirming a high level of diversity within a population of soybean rhizobial isolates from the Cerrados. Variability in rhizobial population has been attributed to several factors, including mutation and recombination in isolated strains and lateral gene transfer to local strains; furthermore, those processes can be affected by the interaction with the host plant and by agricultural practices (Sullivan *et al*. 1995; Martínez-Romero & Caballero-Mellado, 1996; Provorov & Vorob'ev, 2000; Silva *et al*. 2003). One third of the isolates were fast growers and might represent indigenous rhizobia that after 15 years of soybean cropping were able to effectively nodulate this legume. However, as the majority of those fast growers shared serological properties and PP and LPS profiles with *B. japonicum*/*B. elkanii* inoculant strains, transfer of genetic material between indigenous and inoculant strains cannot be discounted. Lateral transfer within the soybean rhizobial-nodulating population was also indicated by both the different clustering position of several isolates in the BOX and RPO1 analyses, and the higher diversity in the symbiotic genes. Finally, as pointed out by Schloter *et al*. (2000), it is also possible that adaptation to the environmental conditions, as well as to the cropping, resulted in the diversity observed. Indeed, variability in morphological, physiological, genetic and symbiotic properties due to adaptation to the Cerrados has been reported before (Boddey & Hungria 1997; Hungria *et al*. 1998; Santos *et al*. 1999). One important consideration is that several reports show that inoculation of soybean in the Cerrados results in increases in nodulation, N_2 fixation rates, and yield, even in soils with high populations of soybean rhizobial strains (10^4–10^6 cells g^{-1} soil) (e.g., Vargas & Hungria 1997; Hungria & Vargas 2000; Mendes *et al*. 2000), which is rarely observed in the United States (Thies *et al*. 1991). It remains to be determined whether a high level of diversity plays a role as a biological buffer, avoiding the dominance of a very competitive but inefficient strain, as occurs with USDA 123 in the United States (Ham *et al*. 1971) and allowing responses to inoculation.

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References

Boddey, L.H. & Hungria, M. 1997 Determination of characteristics of *Bradyrhizobium japonicum* and *B. elkanii* species in Brazilian strains which nodulate soybean. *Biology and Fertility of Soils* **25**, 407–415.

Chen, L.S., Figueredo, A., Pedrosa, F.O. & Hungria, M. 2000 Genetic characterization of soybean rhizobia in Paraguay. *Applied and Environmental Microbiology* **66**, 5099–5103.

Chen, L.S., Figueredo, A., Villani, H., Michajluk, J. & Hungria, M. 2002 Diversity and symbiotic effectiveness of rhizobia isolated from field-grown soybean nodules in Paraguay. *Biology and Fertility of Soils* **35**, 448–457.

Chen, W.X., Yan, G.H. & Li, J.L. 1988 Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *International Journal of Systematic Bacteriology* **38**, 393–397.

Chueire, L.M.O., Bangel, E., Mostasso, F.L., Campo, R.J., Pedrosa, F.O. & Hungria, M. (in press) Classificação taxonômica das estirpes de rizóbio recomendadas para as culturas da soja e do feijoeiro baseada no seqüenciamento do gene 16s RNA. *Revista Brasileira de Ciência do Solo*.

CONAB (Companhia Nacional de Abstecimento) 2002 Retrieved December 10, 2002, from http://www.conab.gov.br/politica_agricola/Safra/Quadro9.xls.

Ferreira, M.C., Andrade, D.S., Chueire, L.M.O., Takemura, S.M. & Hungria, M. 2000 Tillage method and crop rotation effects on the population sizes and diversity of bradyrhizobia nodulating soybean. *Soil Biology and Biochemistry* **32**, 627–637.

Ferreira, M.C. & Hungria, M. 2002 Recovery of soybean inoculant strains from uncropped soils in Brazil. *Field Crops Research* **79**, 139–152.

Freire, J.R.J., Kolling, J., Vidor, C., Pereira, J.S., Kolling, I.G. & Mendes, N.G. 1983 Sobrevivência e competição por sítios de nodulação de estirpes de *Rhizobium japonicum* na cultura da soja. *Revista Brasileira de Ciência do Solo* **7**, 47–53.

Graham, P.H. & Vance, C.P. 2003 Legumes: importance and constraints to greater utilization. *Plant Physiology* **131**, 872–877.

Ham, G.E., Frederick, L.R. & Anderson, I.C. 1971 Serogroups of *Rhizobium japonicum* in soybean nodules samples in Iowa. *Agronomy Journal* **63**, 69–72.

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. *Biology* and *Fertility of Soils* **27**, 393–399.

Hungria, M., Campo, R.J., Chueire, L.M., Grange, L. & Megias, M. 2001a Symbiotic effectiveness of fast-growing rhizobial strains isolated from soybean nodules in Brazil. *Biology and Fertility of Soils* **33**, 387–394.

Hungria, M., Chueire, L.M.O., Coca, R.G. & Megías, M. 2001b Preliminary characterization of fast growing rhizobial strains isolated from soyabean nodules in Brazil. *Soil Biology and Biochemistry* **33**, 1349–1361.

Hungria, M., Franchini, J.C., Campo, R.J. & Graham, P.H. in press The importance of nitrogen fixation to soybean cropping in South America. In *Nitrogen Fixation Research*: *Origins and Perspectives*, ed. Newton, W.E. vol. 7, *Agriculture*, *Forestry*, *Ecology and Environment*, co-ed Werner, D. Dordrecht: Kluwer Academic Publishers.

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. *Plant and Soil* **186**, 331–341.

Hungria, M. & Vargas, M.A.T. 2000 Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Research* **65**, 151–154.

Martínez-Romero, E. & Caballero-Mellado, J. 1996 *Rhizobium* phylogenies and bacterial diversity. *Critical Reviews in Plant Sciences* **15**, 113–140.

Mendes, I.C., Vargas, M.A.T. & Hungria, M. 2000 *Estabelecimento de estirpes de Bradyrhizobium japonicum*/*B. elkanii e seus efeitos na reinoculação da soja em solos de Cerrado*. Planaltina, DF, Brazil: Embrapa Cerrados. (Documentos 20).

Neves, M.C.P., Didonet, A.D., Duque, F.F. & Döbereiner, J. 1985 *Rhizobium* strain effects on nitrogen transport and distribution in soybeans. *Journal of Experimental Botany* **36**, 1179–1192.

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 in Brazilian commercial inoculants for soybean. *Symbiosis* **20**, 147–162.

Peres, J.R.R. 1979 *Seleção de estirpes de Rhizobium japonicum e competitividade por sítios de infecção nodular em cultivares de soja* (*Glycine max* (*L.*) *Merrill*). M.Sc. thesis, UFRGS-FA, Porto Alegre, RS, Brazil.

Peres, J.R.R. & Vidor, C. 1980 Seleção de estirpes de *Rhizobium japonicum* e competitividade por sítios de infecção nodular em cultivares de soja. *Agronomia Sulriograndense* **16**, 205–219.

Peres, J.R.R., Mendes, I.C., Suhet, A.R. & Vargas, M.A.T. 1993 Eficiência e competitividade de estirpes de rizóbio para a soja em solos de Cerrados. *Revista Brasileira de Ciência do Solo* **17**, 357–363.

Provorov, N.A. & Vorob'ev, N.I. 2000 Evolutionary genetics of nodule bacteria: molecular and populational aspects. *Russian Journal of Genetics* **36**, 1323–1335.

Richardson, A.E., Viccars, L.A., Watson, J.M. & Gibson, A.H. 1995 Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. *Soil Biology and Biochemistry* **27**, 515–524.

Santos, M.A., Vargas, M.A.T. & Hungria, M. 1999 Characterization of soybean bradyrhizobia strains adapted to the Brazilian Cerrados Region. *FEMS Microbiology Ecology* **30**, 261–272.

Schloter, M., Lebuhn, M., Heulin, T. & Hartmann, A. 2000 Ecology and evolution of bacterial microdiversity. *FEMS Microbiology Reviews* **24**, 647–660.

Scholla, M.H. & Elkan, G.H. 1984 *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybeans. *International Journal of Systematic Bacteriology* **34**, 484–486.

Silva, C., Vinuesa, P., Eguiarte, L.E., Martínez-Romero, E. & Souza, V. 2003 *Rhizobium etli* and *Rhizobium gallicum* nodulate common bean (*Phaseolus vulgaris*) in a traditionally managed milpa plot in Mexico: population genetics and biogeographic implications. *Applied and Environmental Microbiology* **69**, 884–893.

Sullivan, J.T., Patrick, H.N., Lowther, W.L., Scott, D.B. & Ronson, C.W. 1995 Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proceedings of the National Academy of Sciences of the USA* **92**, 8985–8989.

Thies, J.E, Singleton, P.W. & Bohlool, B.B. 1991 Influence of the size of indigenous rhizobial population on establishment and symbiotic

performance of introduced rhizobia on field-grown legumes. *Applied and Environmental Microbiology* **57**, 19–28.

Vargas, M.A.T. & Hungria, M. 1997 Fixação biológica do N_2 na cultura da soja. In *Biologia dos Solos de Cerrados*, ed. Vargas, M.A.T. & Hungria, M. pp. 297–360. Planaltina, DF, Brazil: EMBRAPA-CPAC. ISBN 85-7075-006-4.

Vargas, M.A.T., Mendes, I.C., Suhet, A.R. & Peres, J.R.R. 1992 *Duas novas estirpes de rizóbio para a inoculação da soja*. Planaltina, DF, Brazil: EMBRAPA-CPAC. (Comunicado Técnico 62).

Vargas, M.A.T., Mendes, I.C., Suhet, A.R. & Peres, J.R.R. 1993 Serological distribution of *Bradyrhizobium japonicum* from Brazilian 'Cerrados' areas under soybean cultivation. *Revista de Microbiologia* **24**, 239–243.

Vargas, M.A.T., Peres, J.R.R. & Suhet, A.R. 1982 Adubação nitrogenada, inoculação e épocas de calagem para a soja em um solo sob Cerrado. *Pesquisa Agropecuária Brasileira* **17**, 1127–1132.

Vargas, M.A.T. & Suhet, A.R. 1980 Efeitos da inoculação e deficiência hídrica no desenvolvimento da soja em um solo de cerrado. *Revista Brasileira de Ciência do Solo* **4**, 17–21.

Versalovic, J., Schneider, M., de Bruijn, F.J. & Lupski, J.R. 1994 Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology* **5**, 25–40.

Vincent, J.M., 1970 *Manual for the Practical Study of Root Nodule Bacteria*. Oxford, UK: Blackwell Scientific Publications. (IBP Handbook 15). ISBN 0-63206410-2.