RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS OF EFFECTIVE *RHIZOBIUM* SP. ASSOCIATED WITH BEANS CULTIVATED IN BRAZILIAN CERRADO SOILS

Isnia Aparecida de Oliveira[1]; Maria José Vasconcellos[3]; Lucy Seldin[2]; Edilson Paiva[3]; Milton Alexandre Vargas[4]; Nadja Maria Horta de Sá[1*]

[1]Departamento de Botânica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil. [2]Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil. [3]EMBRAPA, Centro Nacional de Pesquisa de Milho e Sorgo, Sete Lagoas, MG, Brasil. [4]EMBRAPA, Centro de Pesquisa Agropecuário do Cerrado, Planaltina, DF, Brasil.

ABSTRACT

Efficient bean nodulating *Rhizobium* strains, isolated from different Brazilian cerrado soils, were characterized by RAPD. This study showed great genetic heterogeneity among *R. tropici* and *R. leguminosarum* bv. *phaseoli* strains and allowed the constitution of genetic clusters, besides indicating the most suitable primers for this characterization. The groups of genetically distinct strains can be used in competitiveness studies to select appropriate *Rhizobium* strains for bean inoculation in cerrado soils.

Key words: *Rhizobium tropici*, *Rhizobium leguminosarum* bv. *phaseoli*, bean plants, cerrado soils

INTRODUCTION

Beans are an important staple crop in the cerrado region of Brazil. In 1990, an area of 801,674 hectares was under cultivation, and the production was 431,628 tons (10). However, the 538 kg/ha average production is considered low. The majority of the bean production in this region takes place on small farms where the use of fertilizers, especially nitrogen, is low. In this context, biological nitrogen fixation assumes an increasing important role. However, inoculating beans with effective strains of *Rhizobium* is not always successful due to several factors, including the competition that occurs between the inoculated strains and those already present in the soil which generally have low effectiveness (6, 9). Competitiveness studies that have been conducted to select bacterial genotypes have been limited due to problems in identifying strains that nodulate this legume. In those organisms, similar antigenic structures (cross-reactivity) are common as seen through serologic tests or classic identification methods (11,14).

However, the development and increased availability of molecular biology techniques have made it possible to obtain information regarding the genomic organization and diversity of rhizobia populations in different soils (1, 3, 7). Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between very closely related bacteria. The RAPD technique is a polymerase chain reaction (PCR) based assay that was developed to detect polymorphisms in genomic DNA (19, 20). Besides being simpler and cheaper, this method is as effective as the more labor intensive RFLP for establishing genetic relationships and identifying *Rhizobium* strains (3,17).

In this study, effective native *Rhizobium* strains isolated from bean plants cultivated in different cerrado regions of Brazil were characterized using genomic patterns obtained through RAPD with the objective of identifying genetic groupings (clusters) of the strains.

* Corresponding author. Mailing address: Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departamento de Botânica, Pampulha, CEP 31270-901, Belo Horizonte, MG, Brasil. FAX: (+5531) 499-2673

MATERIALS AND METHODS

Culture media, growth conditions and maintenance of *Rhizobium* strains: The 89 strains of *Rhizobium* used in this study are listed together with their origin and the chemical characteristics of the soil from which they were obtained (Table 1). These strains were previously phenotypically characterized as *Rhizobium tropici* and *R. leguminosarum* bv. *phaseoli* and selected because of their high level of effectiveness in Leonard jars with their homologue host (9). The strains were grown in liquid manitol medium yeast extract (YM) at 29°C and maintained in the same medium containing agar (YMA) at 4°C (18).

DNA extraction and RAPD analysis: Bacterial DNA genomic was extracted using the method described in Sá *et al.* (13). DNA amplification reactions of the strains were conducted in a Perkin-Elmer 9600 thermocycler. The amplification reaction was composed of 40 cycles with each cycle having the following steps: denaturation at 94°C for 15 sec., annealing at 35°C for 30 sec. and polymerization at 72°C for one min. An additional cycle for extension was conducted at 72°C for 7 min. Each reaction mixture contained 25µl composed of the following: 2.5 mM of $MgCl_2$ 10 mM of Tris KCl, 0.01 mM of each DNTP (DATP, DCTP, DGTP, DTTP), 1U of Taq DNA polymerase, 0.4 µM of primer, 11µl of H_2O and 25 ng of DNA. The following 27 primers from the Operon Kit (Operon Technologies Inc.) were tested: OPA-05, OPA-08, OPA-09, OPE-02, OPE-03, OPE-04, OPE-05, OPE-06, OPF-04, OPF-17, OPG-07, OPG-14, OPM-07, OPM-04, OPO-09, OPO-11, OPO-14, OPO-16, OPP-06, OPP-08, OPP-17, OPP-18, OPS-17, OPW-04, OPW-10, OPW-15. Amplification products were separated using electrophoresis in 1.2% agarose gels. The gel was visualized by ethidium bromide staining (ETBR) and then photographed. The ETBR stained bands were considered present (1) or absent (0) and only intense, reproducible bands were considered. This data was used to determine the genetic distance between the strains. Average linkage (UPGMA - unweighted pair group method with averages) dendrograms were generated using the Statistica program (Copy Rhigt STATSOFT Inc. 1993 - UNMN 06/74). Principal component analysis (PCA) was also performed with the specific combined data between more effective strains of the two species studied (9), in relation to the tested random primers, using the option factor analysis. In this method, 3 independent consecutive factors are extracted and each consecutive factor maximizes the variability not captured by the preceding factor.

RESULTS AND DISCUSSION

Random primer genomic DNA amplification of *Rhizobium* strains associated with beans produced patterns with high levels of polymorphism. Fingerprints were strain-specific with certain primers as can be seen in Figs. 1 and 2. Data processing generated dendrograms (Figs. 3 and 4) that suggested 6 or more major divisions based on the genetic relationship between the *Rhizobium* strains of the evaluated species. High degrees of
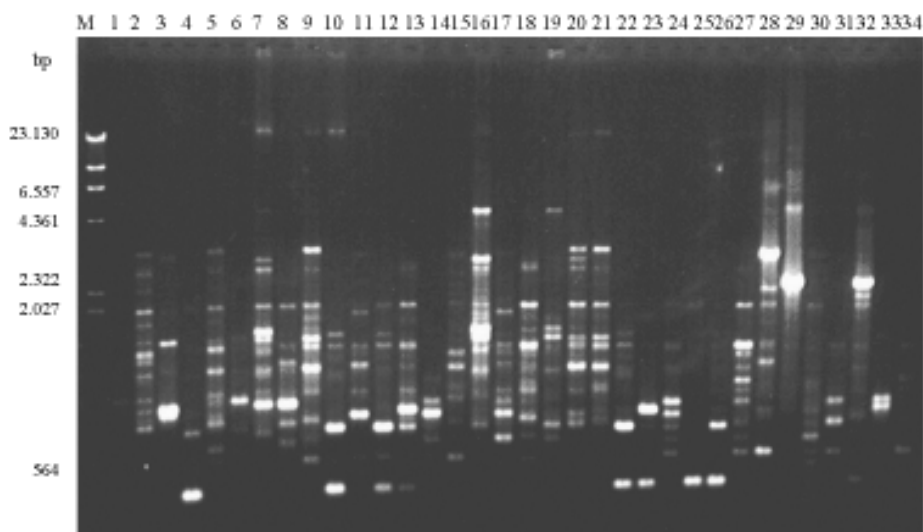
Figure 1. Example of banding patterns obtained from 34 strains of *R. tropici* from Brasilia using primer OPF-04: (M) Kb ladder, (1)CPACH37, (2)CPACH38, (3)CPACH50, (4)CPACH2, (5)CPACH52, (6)CPACH51, (7)CPACH41, (8)CPACH23, (9)CPACH53, (10)CPACH6, (11)CPACH56, (12)CPACH22, (13)CPACH14, (14)CPACH38, (15)CPACH20, (16)CPACH35, (17)CPACH13, (18)CPACH45, (19)CPACH42, (20)CPACH39, (21)CPACH9, (22)CPACH14, (23)CPACH32, (24)CPACH48, (25)CPACH3, (26)FJ1.2, (27)FJ1.22, (28)FJ2.1, (29)FJ2.2, (30)FJ2.4, (31)FJ2.12, (32)FJ2.15, (33)FJ2.21, (34)FJ2.22.
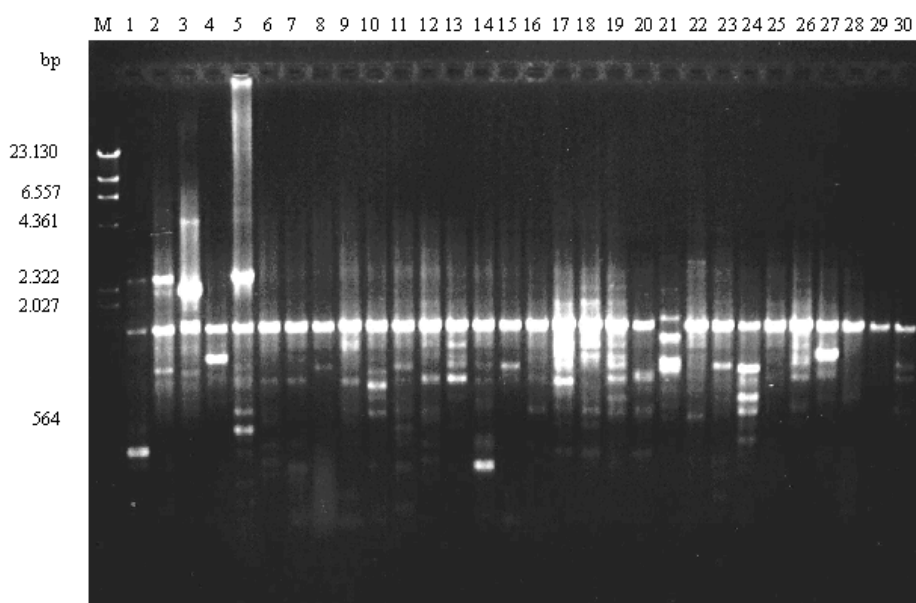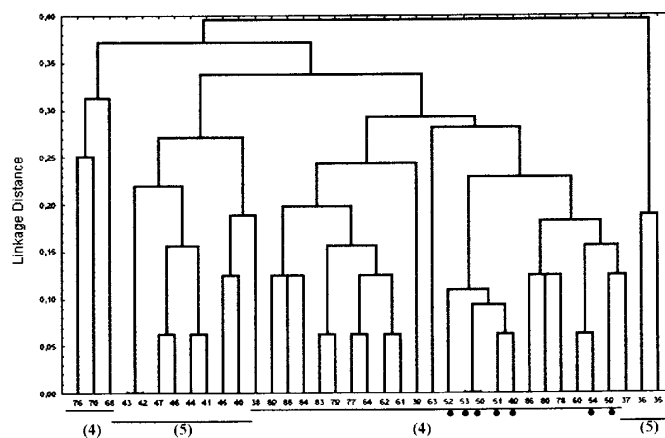
Figure 2. Example of banding patterns obtained from 10 strains of *R. tropici* (lines 1 to 6 and 18 to 21) and 14 *R. leguminosarum* bv. *phaseoli* (lines 7 to 17 and 22 to 30) from S. Lagoas cerrado soil using primer OPF-02: (M) Kb ladder, (1)SLA1.2, (2)SLA1.5, (3)SLA1.6, (4)SLA2.2, (5)SLA2.3, (6)SLA3.2, (7)SLA1.1, (8)SLA1.4, (9)SLA3.13, (10)SLBR2.1, (11)SLBR3.12, (12)SLBR3.13, (13)SLB3.3, (14)SLB3.12, (15)SLB3.13, (16)SLB7.15, (17)SLB10.6, (18)SLP2.13, (19)SLP3.3, (20)SLP4.9, (21)SLP5.9, (22)SLP1.3, (23)SLP1.6, (24)SLP2.2, (25)SLP2.10, (26)SLP4.4, (27)SLP4.7, (28)SLP5.8, (29)SLP19.7, (30)SLP24.1.
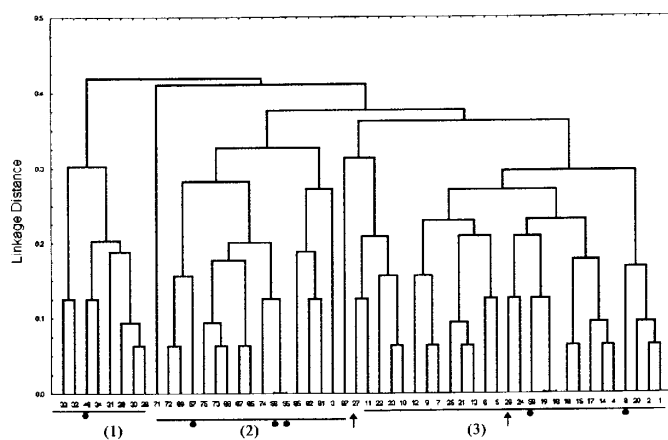
Figure 3. Dendrograms showing genomic relationship between 52 strains of *R. tropici* isolated from cerrado soil as determined by RAPD analysis with 13 different arbitrary primers. (1 and ↑) – Brasilia cerrado soil B, (2) – Sete Lagoas cerrado soil (A, B and C), (3) – Brasilia cerrado soil A, ● commercial strains from different origins.

Figure 4. Dendrograms showing genomic relationship between 37 strains of *R. leguminosarum* bv. *phaseoli* isolated from cerrado soil as determined by RAPD analysis with 13 different arbitrary primers. (4) – Sete Lagoas cerrado soil (A, B and C), (5) – Janauba cerrado soil, ● - commercial strains from different origins.

variation were observed in the *R. tropici* (6-42%) (Fig. 3) as well as in the *R. leguminosarum* bv. *phaseoli* strains (5-40%) (Fig. 4). The constituted groups formed preferentially with strains from the same location (Table 1), indicating the selection of genetically related sub-populations in relation to the soil characteristics of each sample site. Also within each site,

specially Brasilia and Janaúba, high levels of genetic variation were observed. Using the random primer OPF-04, as shown in Fig. 1, DNA amplification of *R. tropici* strains from Brasilia had intensely polymorphic patterns even though the strains had common bands. A dendrogram generated (data not shown) from the results showed genetic variation levels of 6 to 44% between

Table 1. Identification, origin of 52 effective strains of *R. tropici* and 37 *R. leguminosarum* bv. *phaseoli* and the chemical soil analysis of the isolation sites.

Rhizobium strains	Specie	Site/sub-site	Chemical soil analysis				
			pH H_2O	Al mE/100	Ca mE/100	K ppm	P ppm
(1)CPAC37, (2)CPAC38, (3)CPAC50, (4)CPAC2, (5)CPAC52, (6)CPAC51, (7)CPAC41, (8)CPAC23, (9)CPAC53, (10)CPAC6, (11)CPAC56, (12)CPAC22, (13)CPAC15, (14)CPAC58, (15)CPAC20, (16)CPAC35, (17)CPAC13, (18)CPAC45, (19)CPAC42, (20)CPAC39, (21)CPAC49, (22)CPAC14, (23)CPAC32, (24)CPAC48, (25)CPAC3	*R. tropici*	Brasilia (A)	5.2	0.18	3.32	84	7.5
(26)FJ1.2, (27)FJ1.22, (28)FJ2.1, (29)FJ2.2, (30)FJ2.4, (31)FJ2.12, (32)FJ2.15, (33)FJ2.21, (34)FJ2.22	*R. tropici*	Brasilia (B)	4.7	1.02	0.69	26	1.1
(69)SLA1.2, (71)SLA1.5, (72)SLA1.6, (73)SLA2.2, (74)SLA2.3, (75)SLA3.2,	*R. tropici*	Sete Lagoas (A)	5.6	0.05	3.10	32	7.0
(68)SLA1.1, (70)SLA1.4, (76)SLA3.13	*R. leguminosarum* bv. *phaseoli*						
(65)SLBR2.1, (66)SLBR3.12, (67)SLBR3.13	*R. tropici*	Sete Lagoas (B)	5.8	0.00	3.74	112	12
(60)SLB3.3, (61)SLB3.12, (62)SLB3.13, (63)SLB7.15, (64)SLB10.6	*R. leguminosarum* bv. *phaseoli*						
(81)SLP2.13, (82)SLP3.3, (85)SLP4.9, (87)SLP5.9	*R. tropici*	Sete Lagoas (C)	6.0	0.05	5.84	42	11
(77)SLP1.3, (78)SLP1.6, (79)SLP2.2, (80)SLP2.10, (83)SLP4.4, (84)SLP4.7, (86)SLP5.8, (88)SLP19.7, (89)SLP24.1	*R. leguminosarum* bv. *phaseoli*						
(35)JA1.4, (36)JA1.7, (37)JA1.8, (38)JA2.2, (39)JA2.3, (40)JA2.4, (41)JA2.5, (42)JA2.8,(43)JA3.2, (44)JA3.5, (45)JA4.3, (46)JA6.4, (47)JA6.6	*R. leguminosarum* bv. *phaseoli*	Janauba	6.2	0.00	4.82	1.83	13
(8)SEMIAV23, (48)CIAT899, (55)BR814, (56)BR818, (57)BR855, (58)BR860	*R. tropici* (commercial strains)	different origin					
(49)BR365, (50)BR376 (52)BR10.026, (53)BR10.028, (54)BR576, (59)SEMIA4077	*R. leguminosarum* bv. *phaseoli* (commercial strains)						

the strains. It was possible to detect at least 4 clusters among the analyzed strains from Brasilia and in sub-sites within the region the genetic variation levels were from 9-40% (subsite A) and 7-52% (subsite B). DNA amplification profiles of *R. leguminosarum* bv. *phaseoli* strains from Janaúba showed that in addition to common bands, these strains had bands that allowed for the differentiation of individual strains. The dendrogram resulting from the use of different primers indicated at least 4 distinct clusters and genetic variation of 6-46%. Using OPA-02 primer, *R. tropici* (Fig. 2; lines 1-6 and 18-21) and *R. leguminosarum* bv. *phaseoli* strains (Fig. 2; lines 7-17 and 22-30) from Sete Lagoas produced patterns with high polymorphism, reflected in a dendrogram which showed genetic variation of 6-37%. In samples from the Sete Lagoas region, clusters formed initially by subsite and within them, by *Rhizobium* species. Subsites of this region had similar levels of genetic variation: subsite A 9.5-46.5%; subsite B 16-48%; subsite C 4.5-40%.

a) *R. tropici*
● Brasília
❖ S. Lagoas

b) *R. leguminosarum* bv. *phaseoli*
● Janaúba
❖ S. Lagoas
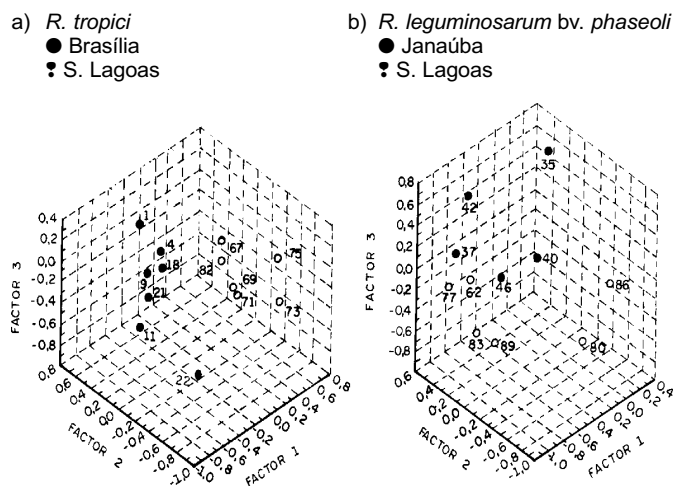
Figure 5. Groups determined using Principal Component Analysis on the specific combing data between the most effective *R. tropici* strains (a) and *R. leguminosarum* bv. *phaseoli* strains (b) in relation to 13 random primers.

Based on the PCA diagrams (Figs. 5a and b), 2 groups can be distinguished among the most effective strains according to origin of the two species studied (*R. tropici* and *R. leguminosarum* bv. *phaseoli*), which confirms the trend observed in the dendrogram analysis. PCA also verified that the *R. leguminosarum* bv. *phaseoli* strains had more variation among themselves than the *R. tropici* did, probably due to the former's higher genetic plasticity (12, 13). In addition, this analysis identified adequate primers for strain identification.

Result of the present study confirm the high degree of genetic diversity found using a variety of methods in bean nodulating *Rhizobium* strains from Mexican (8) and Brazilian soils (6, 13). In general, strains within the *Rhizobium* genus are fast growing, such as there studied here that nodulate beans. They appear to be more taxonomically diverse and ecologically adaptable than the slower growing strains from other genera like *Bradyrhizobium* and *Azorhizobium* These differences probable occur because in the slow growing strains the genetic information, in general, is contained in the chromossomal DNA, while in fast growing strains many genes, including those related to biological nitrogen fixation are located on plasmids which confers dynamic qualities to the genome (5, 12). These characteristics favor recombination between genotypes of the *Rhizobium* population present in soils (14). Even though new bean nodulating *Rhizobium* species have been defined recently (4, 15), additional species should be defined to reflect on the taxonomic level, the large diversity of these bacteria. For now, this study established groups of genetically distinct strains that can be used in competitiveness studies to select appropriate strains for inoculation in cerrado region bean plants.

RESUMO

Caracterização, via RAPD, de estirpes efetivas de *Rhizobium* sp associadas ao feijoeiro cultivado em solos de cerrado no Brasil

Estirpes de *Rhizobium* eficientes na nodulação do feijoeiro, isoladas de diferentes solos da região do cultivo dessa leguminosa nos cerrados brasileiros, foram caracterizadas via RAPD. Esse estudo mostrou grande heterogeneidade genética entre as estirpes de *R. tropici* e *R. leguminosarum* bv. *phaseoli* testadas e permitiu a definição de grupos genéticos, além de indicar os "primers" mais adequados para essa caracterização. Os grupos de estirpes geneticamente distintas podem ser usados em estudos de competitividade, importantes para obtenção de resultados positivos na inoculação dessa leguminosa em solos de cerrado.

Palavras-chave: *Rhizobium tropici, Rhizobium leguminosarum* bv. *phaseoli,* feijoeiro, solos de cerrado

REFERENCES

1. Amarger, N.; Bours, M.; Revoy, F.; Allard, M. R.; Laguerre, G. *Rhizobium tropici* nodulates field - grown *Phaseolis vulgaris* in France. *Plant Soil* 161:147-156, 1994.

2. Kattah, L. S.; Pinto, P. P.; Sá, N. M. H. Caracterização de estirpes de *Rhizobium* nativas isoladas de áreas de cultivo do feijoeiro em solos de cerrado. In: HUNGRIA ET AL, eds. Microbiologia do solo: desafios para o século XXI. Londrina: IAPAR/EMBRAPA – CNPSO, 1995.

3. Laguerre, G.; Mavingui, P. Allard, M.; Charnay, M.; Louvrier, P.; Mazurier, S.; Rigottier, L.; Amarger, N. Typing of Rhizobia by PCR DNA Finger printing and PCR - Restriction Fragment Length Polymorphism Analysis of Chromosomal and Symbiotic Gene Regions: Application to *Rhizobium leguminosarum* and its different biovars. *Appl. Environ. Microbiol.* 62: 2029-2036, 1996.

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

5. Martinez – Romero, E. Recent developments in *Rhizobium* taxonomy. *Plant soil.* 161:11-20, 1994.

6. Mercante, F. M. Uso de *Leucaena leucocephala* na obtenção de *Rhizobium* tolerante a temperatura elevada para inoculação do feijoeiro. UFRRJ Itaguaí 149p. (tese de mestrado), 1993.

7. Nour, S. M.; Fernadez, M. P.; Normand, P.; Cleyetmarel, J. C. *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (cicer arietinum L.). *International J. Syst. Bacteriol.* 44:511-522, 1994.

8. Pinero, D.; Martinez, E.; Selander, R. K. Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* bv. *phaseoli*. *Appl. Environ. Microbiol.* 54:2825-2832, 1988.

9. Pinto, P. P.; Kattah, L. S.; Sá, N. M. H. Termotolerancia e eficiência em fixar N$_2$, de estirpes de *Rhizobium* nativas, isoladas de áreas de cultivo do feijoeiro nos cerrados. *XXV Congresso Brasileiro de Ciência do Solo* vol. I, 509 - 510. Viçosa, Brasil, 1995.

10. Pereira, G.; Moreira, L.; Messias, M. O comportamento agropecuário na região nuclear dos cerrados. Planaltina: EMBRAPA - CPAC, 3p. (EMBRAPA - CPAC/PNP). *Avaliação dos recursos naturais e sócio-econômicos dos cerrados*. Projeto 026.9/801/1. Form. 1.3/1992, 1992.

11. Pereira, J. C.; Vidor, C.; Lovato, P. E.; Penteado, A. Symbiosis among field beans and *Rhizobium leguminosarum* bv. *phaseoli* strains sensitive and resistant to antibiotics. *Pesqui. Agropecu. Bras.* 26:1097-1105, 1991.

12. Romero, P.; Brom, S.; martinez-Salazar, J.; Girard, M. L.; Palacios, R.; Davila, G. Amplification and deletion of a nod - nif region in the symbiotic plasmid of *Rhizobium phaseoli*. *J. Bacteriol.* 173:2435-2441, 1991.

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

14. Schmidt, E L.; Bankole, R. O. & Bohlool, B. B. Fluorescent - antibody approach to study of Rhizobia in soil. *J. Bacteriol.* 95:1987-1992, 1968.

15. Schofield, P.R.; Gibson, A.H.; Duman, W.F.; Watson, J.M. Evidence for genetic exchange and recombination of *Rhizobium* symbiotic plasmids in a soil population *App. Environ. Microbiol.* 53:2842-2947, 1991.

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

17. Selenska-Pobell, S.; Euguenieva-Hackenberg, E.; Radeva, G.; Squartini, A. Characterization of *Rhizobium* "hedysari" by RFLP analysis of PCR amplified rDNA and by genomic PCR fingerprinting. *J. Appl. Bacteriol.* 80:517-528, 1996.

18. Vincent, J. M. A manual for the practical study of root - nodule bacteria. 164p. Oxford: Blackwell Science, 1970.

19. Welsh, J.; McClelland, M. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18:7213-7218, 1990.

20. Williams, J. G. K.; Kubelik, A. R.; Livak, K. J.; Raifalski, A.; Tingey, S. V. DNA polymorphisms amplified by arbitrary primers are usefull as genetic markers. *Nucleic Acids Res.* 18:6531-6535, 1990.