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ASSESSMENT OF COWPEA RHIZOBIUM DIVERSITY IN CERRADO AREAS OF NORTHEASTERN BRAZIL

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ABSTRACT

In order to contribute for the optimization of biological nitrogen fixation (BNF) associated with cowpea in Cerrado areas in the Northeast region of Brazil, this work aimed to analyze the diversity of rhizobial populations in eight areas of Cerrado, during a soybean and rice-cowpea rotation. Morphological traits (mucous production and colony morphology), genotypic analyzes (ARDRA 16S) and intrinsic resistance to antibiotics were determined for a collection of isolates captured using cowpea as a host-plant. The morphological data showed a inverse correlation ($p < 0.05$) between the number of legume (soybean and cowpea) crops, according to the history of each area, and rhizobium diversity, estimated by the Shannon-Weaver index. ARDRA data showed that native Cerrado areas were exclusively colonized by *Bradyrhizobium elkanii,* corroborating previous data. In the areas where legumes were grown, we observed two distinct situations: where soybean only were grown, a high proportion of *B. japonicum* was found, and where soybean and cowpea were grown, we observed more *B. elkanii*. The analysis of antibiotic resistance revealed five different profiles. High percentage of antibiotic resistant *Bradyrhizobium* spp. isolates were found in the areas cultivated for a long time, whereas the native area and areas with a few crops had fewer resistant strain. There was an inverse relationship between intrinsic antibiotic resistance and rhizobial diversity, while the last decreases as more legume crops are introduced into the area, the former increases, suggesting that the presence of legumes may provide ecological conditions to select specific rhizobium groups, which acquire competitiveness traits and become successfully established.

Key words: diversity, *Bradyrhizobium*, Cerrado, cowpea, antibiotic

INTRODUCTION

Cowpea (*Vigna unguiculata* (L) Walp), because of its adaptability to severe climatic conditions, is a typical crop of the Brazilian northeast semi-arid region, where it represents the main protein source for the low-income population. According to Freire Filho *et al.* (6), the crop generates almost 2.5 million jobs, being nutritionally important to 27 million people. Besides its nutritional value, cowpea also presents high genetic variability, high tolerance to edapho-climatic stress, high productivity and a high capacity for benefiting from the symbiosis with rhizobia, receiving most of its nitrogen from biological fixation (BNF). Due to these traits, cowpea is an especially important genetic resource for the semi-arid region and a source of genes for genetic engineering projects (4).

These outstanding traits have also attracted the attention of farmers in the Northern frontier areas of the Brazilian Cerrado. Strategically, cowpea is sown in recently clear-cut areas, after a rice crop, and is also grown in bi-annual rotation, as alternative to soybean. This rotation allows cowpea to benefit from fertilizer

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residues, and optimizes the nutrient cycling in the system. The introduction of the plant at the end of the rainy season does not seem to cause further problems and, according to farmers, the grain yield levels of around 1500 kg.ha^{-1} are usually achieved.

Despite the considerable capacity for obtaining N from BNF (21), the inoculation of cowpea with efficient rhizobium strains does not necessarily result in yield increases. One reason for inoculation failure is the low symbiotic specificity between cowpea and rhizobia. Inoculated strains often fail to compete with indigenous rhizobium for nodulation sites on the roots (12). For this reason, selection and use of specific inoculants has been commonly neglected (13). Nevertheless, recent research on rhizobium ecology in dry-land areas of the Brazilian Semi-arid region indicated a positive response to inoculation of cowpea (11). In this region, the crop is sown at the beginning of the rainy season, when the native rhizobium population is very low, due to the severe stress imposed by the dry season. Under these conditions, the low rhizobium population observed is not sufficient to promote efficient nodulation. Without competition from native rhizobia, the use of efficient strains as inoculants has been showed to promote yield increases (11). This observation shows the need for a better knowledge of ecological parameters, such as the dynamics of indigenous rhizobial populations, survival and competitiveness.

This work aimed to analyze the diversity of rhizobium populations in recently clear-cut areas of the Cerrado, during a rice-cowpea and soybean rotation, using genotypic (ARDRA) and morphologic/phenotypic characterization methods, in order to contribute for further studies on the optimization of BNF associated with cowpea in Cerrado areas in the Northeast region of Brazil.

MATERIALS AND METHODS

Soil samples

Eight composite soil samples were collected from a Cerrado area located in the State of Piauí (Table 1). Seven areas had been recently brought into agricultural production and one covered by typical (native) Cerrado vegetation was used as control. Each soil sample consisted of ten sub-samples, taken from 0 to 20 cm depth, which were fully homogenized. Cropping history and soil analysis of the experimental areas are shown in Table 1. Soil analyses were performed according to Silva *et al*. (18).

Host plants for rhizobium trapping

Soil samples were mixed with sand (2:1) and placed in pots (300 g) kept in a greenhouse. Three cowpea cultivars, BR14, CE315 and BR17, commonly cultivated in the Cerrado, were used as trap host for rhizobia. Each cultivar was sown in duplicate for each soil sample. Seeds were surface sterilized by immersion in ethanol (70%; 30 sec), hydrogen peroxide (5%; 3 min) and washed 10 times with sterilized water. Seeds were sown and 0.25 l of Norris & Döbereiner's nutrient solution (10) were added weekly during the whole growth period of the plants. Sterilized water was added whenever needed. The plants were harvested 40 days after sowing. Roots were washed, nodules were detached and dried in flasks containing silica gel.

Isolation of rhizobia from nodules and morphological characterization

Five per cent of root nodules of each treatment were surface sterilized by ethanol (70%; 30 sec), NaOCl (5%; 4 min) and washed 10 times with sterile water. Crushed nodules were streaked onto yeast manitol agar medium (YMA) containing bromothymol blue (5) and incubated at 28ºC. The morphological traits evaluated comprised mucous production and colony morphology, pH change of the medium during growth of the isolates and growth rate. Mucous morphology analysis was based on type, elasticity and appearance, while colony morphology parameters were diameter, form, elevation, transparency and color.

Confirmation of nodulation

Cowpea cv. BR17 seeds were surface sterilized by ethanol (70%; 30 sec), NaOCl (5%; 3 min) and washed 10 times with sterile water. Seeds were sown in Leonard jars containing a sand: vermiculite (2:1) substrate. The isolates representative of each area and of each morphological group were cultivated in YM (5) media for 3 days at 28ºC and used as inoculants. Plant inoculation (1ml plant^{-1}; 10^8 cells ml^{-1}) was performed in triplicate, 3 days after sowing. Norris & Dobereiner's nutrient solution (0.25 l) was added weekly and sterilized water was added whenever needed. The plants were harvested 40 days after sowing, when the presence of nodules was observed in the roots.

DNA amplification by PCR

Rhizobial isolates representative of the each morphological group and reference strains obtained from Embrapa Agrobiologia culture collection (*Azorhizobium caulinodans*, BR5410; *Bradyrhizobium elkanii*, BR29; *B. japonicum*, BR111; *R. etli*, BR10026; *Rhizobium tropici* IIA BR10016 and BR112, *Sinorhizobium fredii*) were cultivated in YMA for 3 days, submitted to DNA extraction by heating (95ºC; 5min) and used for amplification reaction with the Y1 and Y3 primers amplify approximately 1500bp of the 16S rDNA (7,9,19).

Amplified ribosomal DNA restriction analysis (ARDRA)

Restriction patterns for the amplified 16S rDNA were determined using 9 endonucleases: *Alu* I, *Dde* I, *Hae* III, *Cfo* I, *Hinf* I, *Msp* I, *Nde* II, *Rsa* I and *Taq* I (9), according to the enzyme manufacturer's instructions. The restricted fragments were analyzed by agarose gel (3%) electrophoresis and visualized by UV light after ethidium bromide staining.

Table 1. Cropping history and soil characteristics of the Cerrado areas used in this study.

Areas	Cropping history	pH	Al^{+3}	Ca^{+2}	Mg^{+2}	P	K^{+}
			$cmol_c.dm^{-3}$			$mg.dm^{-3}$	
I	Native cerrado	4.4	1.7	ud	ud	ud	28
II	Rice – 98 and 99	5.2	0.3	2.1	1.5	4	78
III	Soybean – 99	4.9	0.8	1.4	1.3	3	62
IV	Rice – 97; Soybean(1) – 98; Rice – 99	4.9	0.6	2.2	0.7	19	26
V	Rice – 97; Soybean(1) – 98 and 99	4.9	0.8	2.0	1.2	3	23
VI(2)	Rice – 97; Soybean(1) – 98; Cowpea – 99	4.8	0.5	1.8	0.8	13	28
VII(2)	Rice – 95 to 97; Soybean(1) – 98; Cowpea – 99	4.9	0.7	1.8	1.2	20	78
VIII(2)	Rice – 93 to 97; Soybean(1) – 97 and 98; Cowpea – 99	5.0	0.2	3.3	0.9	8	90

*Undetected; (1) soybean was always inoculated; (2) soil samples were collected during the cowpea crop cycle.

Resistance to antibiotics

The same isolates analyzed by ARDRA were evaluated for intrinsic resistance to antibiotics. This evaluation was done in Petri plates containing 20 ml of solid BSM medium (22), supplemented with (μg ml^{-1}) chloramphenicol (10, 20 and 40); erythromycin (50, 75 and 100); gentamicin sulphate (10, 20 and 40) or kanamycin (10, 20 and 30); rifampicin (5, 10 and 20); streptomycin sulphate (50, 75 and 100); tetracycline (10, 20 and 40). Antibiotic were prepared according to Sambrok *et al.* (15). Five μl of the each bacterial suspension (0.2 OD; 545 nm), grown in the liquid YMA medium for 5 days and centrifuged (15,000 *g*; 10 min; 3 times) were spot-plated onto the agar surface. The plates were incubated at 28ºC and a control plate without antibiotic was also plated.

Data analyses

The NTSYS-PC V. 2.10 software (Applied Biostatistics) was used to obtain the dendrograms. Similarity matrices of morphological and antibiotic resistance data were calculated using the Simple Matching (SM) index and ARDRA data were analyzed using Jaccard index. Clustering of the three sets of data were performed using UPGMA. Shannon-Weaver's diversity indeces (17) were calculated considering the morphological group, determined by cluster analyses as taxa.

RESULTS AND DISCUSSION

Rhizobium isolation and morphological characterization

Isolation of rhizobium from root nodules rendered at least 30 isolates representative from cowpea cultivated on plants from each area, except for area I, where less than 20 isolates were obtained. The frequent lack of nodules observed in soils from tropical climax forest soil has been associated with a low requirement for N, which is considered not to be the limiting factor under these conditions (3).

Two hundred and twenty rhizobium strains were characterized according to colony morphology and growth time. Colony formation on culture media took at least 4 days for all the isolates. All the isolates but 2, increased the medium pH, which is not common behavior for slow-growing rhizobia (8). These data were used for calculating a similarity matrix by means of Simple Matching index (SM), which was then clustered by UPGMA (dendrogram not shown) revealing 20 morphological groups. From each soil sample, one isolate representative from each morphological group was selected, comprising a total of 50 isolates, which were then tested for nodulation capability. All the isolates tested promoted root nodule formation on cowpea, confirming that they belong to the rhizobium group.

Despite some well-known limitations, morphological characterization may be an important tool for first approaching a rhizobium collection in relation to its diversity (2,22). According to the cluster analysis performed, the majority of the isolates were similar either to *Bradyrhizobium elkanii* or *B. japonicum*. Forty per cent of the isolates displayed mucous production similar to strain BR111 (USDA110), a reference strain for *B. japonicum*, while another group comprising around 30% of the isolates, showed characteristics similar to strain BR29 (SEMIA5019), a reference strain for *B. elkanii*.

Diversity indices

The occurrence of the rhizobium morphological groups in each soil sample, indirectly determined by using cowpea as a host plant for capturing the rhizobia, was used to calculate the Shannon-Weaver's diversity index (17). Each morphological group determined by cluster analyses was considered as a taxon. Fig. 1 shows an inverse correlation ($p < 0.05$) between the number of legume crops, according to the history of each area, and rhizobium diversity. In areas I, and II, where neither soybean, nor cowpea had ever been planted, the highest diversity values were found. On the other hand, the presence

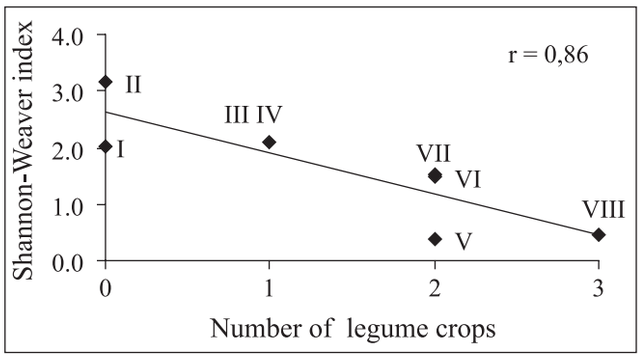

Figure 1. Correlation between number of legume crops (soybean or cowpea) and Shannon-Weaver's diversity index of rhizobia in the Cerrado areas (I to VIII) calculated from morphological rhizobium groups.

of a leguminous crop tended to decrease the diversity of the rhizobium population. These data suggest that the introduction of a leguminous plant is capable of promoting the selection of particular rhizobium taxa. These results are in agreement with data from Coutinho *et al*. (2) that found reduced rhizobial diversity in soil after introduction of soybean. Whether this is under the influence of the plant itself, or due to the use of rhizobial inoculants in all areas where soybean has been cultivated, is anissue for further studies.

ARDRA Analyses

Sixty-six selected isolates, representing both the different morphological groups and the areas where soil samples have been collected, as well as reference strains, were analyzed by ARDRA. More than 1 isolate was chosen from the larger morphological groups. ARDRA is a fast and simple technique that has been widely used for rhizobium genotypic characterization (9). Restriction analyses of the amplified 16S rDNA were using 9 different endonucleases, yields at least 6 polymorphic patterns (data not shown). Fragments smaller than 100 bp, which could not be properly visualized, were excluded from the analyses. The Jaccard/UPGMA analysis (Fig. 2) showed that the isolates clustered close to the *Bradyrhizobium* reference strains, and far away from other rhizobium genera, such as *Azorhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* and formed two main groups. The largest group G1, consisted of isolates with a restriction pattern 100% similar to strain BR29, which is a typical *B. elkanii* strain. At around 85% similarity level, 4 additional related groups (G2, G3 and G4) were recovered. All together, 37 isolates may be considered to belong to the *B. elkanii* species. The second largest group (G8) had an ARDRA restriction pattern 100% similar to strain BR111, a typical *B. japonicum*, while another 8 small groups (G5, G6, G7, G9, G10, G11, G12, G13 and G14) also clustered with BR111 at around 80% similarity, totaling 24 isolates belonging to the *B. japonicum* species. Five single-membered groups (G15, G16, G17, G18 and G19) were putatively classified as *Bradyrhizobium* sp.

Fig. 3 shows the distribution of the *B. elkanii* and *B. japonicum* species and the *Bradyrhizobium* sp. in each Cerrado area under this study. Rhizobia present in areas I, II, VI and VII belonged predominantly to *B. elkanii*, while in areas III, IV, V and VIII, the majority of isolates belonged to *B. japonicum*. The results from areas I and II agree with data from other authors, where it is considered that in Brazilian soils have well established populations of *B. elkanii*, which seem to be well-adapted and represent a barrier to the introduction of efficient *B. japonicum* strains used as inoculant for soybean (1,13,16). In areas I, and II where leguminous plants have not been cultivated we also found that *B. elkanii* populations are the main rhizobium trapped using cowpea as host plant. On the other hand, in areas where soybean

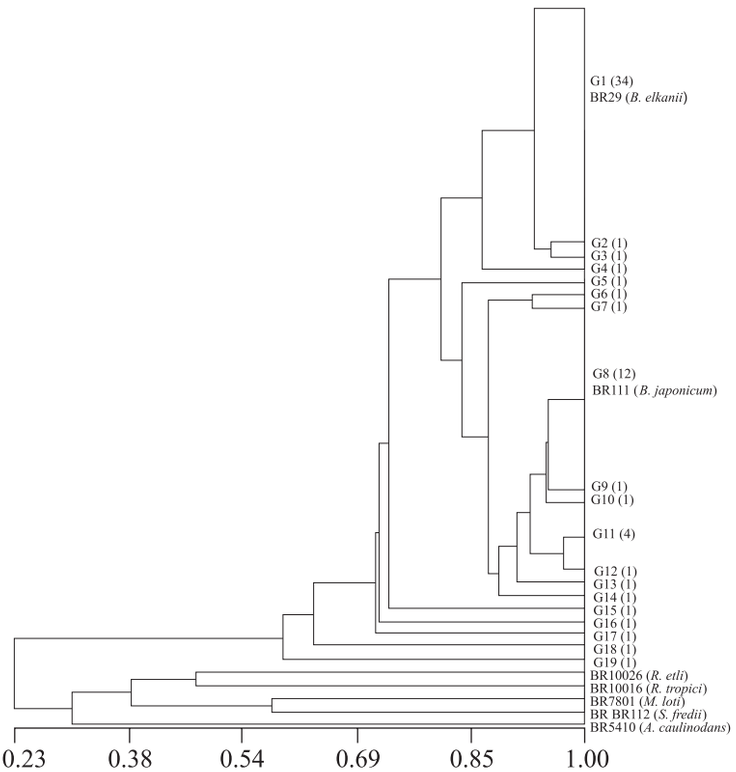

Figure 2. Grouping of selected rhizobia according to restriction fragment patterns of 16S rDNA (ARDRA) derived from Jaccard/UPGMA analysis (cophenetic correlation = 0.98). Number in parentheses indicates the number of isolates.

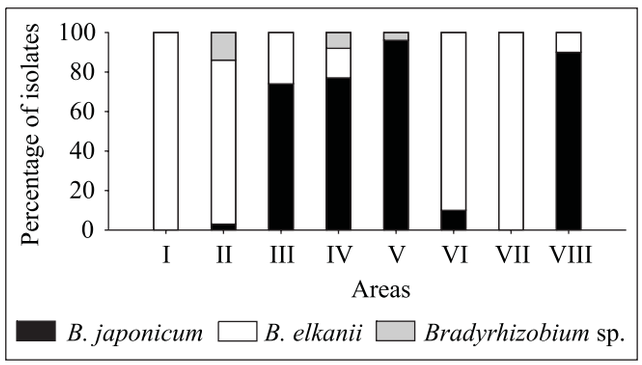

Figure 3. Distribution of the rhizobium species in the Cerrado areas according to ARDRA profiles.

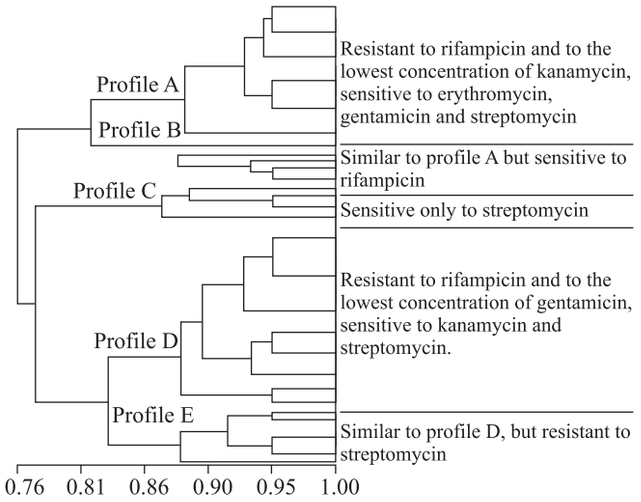

Figure 4. Relationship between selected rhizobial isolates according to their antibiotic resistance profile derived from SM/ UPGMA analysis.

was cultivated and had just been harvested (areas III, IV and V), the main rhizobium population recovered belonged to *B. japonicum*, which might be related to the use of inoculant in these areas. In the three areas where cowpea was still grow (areas VI and VII), *B. elkanii* isolates were found to be dominant. However presence of cowpea was not enough to determine a *B. elkanii* population in area VIII, where it is likely that the two consecutive soybean crops cultivated may have contributed decisively to the establishment of a *B. japonicum* population.

Antibiotic-Resistance Profiling

The same isolates selected for the ARDRA analyses were grown in culture media containing different antibiotics. The resistance data obtained were clustered by UPMGA using the SM index (Fig. 4), showing the formation of 5 main profiles. The main characteristics of each profile were: (A) resistant to rifampicin and to the lowest concentration of kanamycin, sensitive to eritromicin, gentamicin and streptomycin; (B) similar to profile A, but sensitive to rifampicin; (C) sensitive only to streptomycin; (D) rifampicin resistant, and to the lowest concentration of gentamicin, sensitive to kanamycin and streptomycin; (E) similar to profile D, but resistant to streptomycin. All the isolates tested were resistant to chloramphenicol and tetracycline. The last two profiles (D and E) constitute isolates possessing a large range of intrinsic resistance to the different antibiotics tested. In Fig. 5, it is possible to observe that profiles D and E are more abundant in areas where cowpea has already been introduced, suggesting that as more leguminous crops are cultivated, the rhizobium populations may become more competitive. Furthermore, these data may result from the presence of an active rhizosphere, favoring competitive rhizobium populations, as it was the case in areas VI, VII and VIII, where cowpea was under cultivation when soil samples were collected. This observation is especially interesting in area VIII, where isolates recovered were predominantly *B.*

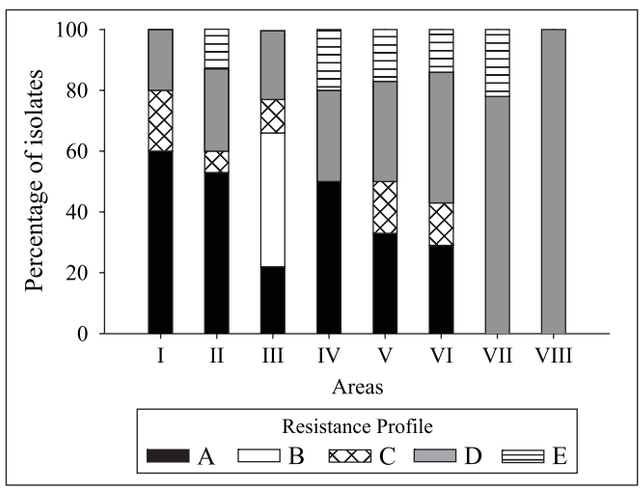

Figure 5. Distribution of antibiotic resistance profile in rhizobium obtained from Cerrado areas.

japonicum, having high intrinsic resistance to antibiotics, a species that is generally recognized for not being competitive and sensitive to most of the antibiotic tested (14). As has been pointed out before, the cropping history may have somehow promoted the selection of competitive *B. japonicum* isolates capable of persisting in soil during the cowpea cultivation, contrary to what was observed in areas VI and VII. Vargas *et al*. (20) also found competitive *B. japonicum* isolates persisting in the Cerrado years, after inoculant application. On the other hand,

in the climax Cerrado area (area I), despite *B. elkanii* being the predominant species, isolates were mainly sensitive to most of the antibiotics tested, suggesting that under these conditions, there may be competition for specific niches. *B. elkanii* has already been reported to be the predominant *Bradyrhizobium* species in tropical soils (13). There is an inverse relationship between intrinsic antibiotic resistance and rhizobium diversity, while the last decreases as more leguminous crops are introduced in the area, the former increases, suggesting that the presence of legumes provides ecological conditions to specific rhizobium groups which, acquire competitiveness determinant traits and become successfully established. A specific intrinsic resistance antibiotic profile (profile B), representing bacteria sensitive to rifampicin, appeared only in area III, which may to be a local effect.

In this work, we studied rhizobium populations present in recently clear-cut Cerrado areas. The predominant *Bradyrhizobium* species were found related to the history of legume cultivation, suggesting that the introduction of a new crop or inoculant may represent a modification of the native rhizobium population. Furthermore, a general shift to rhizobium population possessing high antibiotic resistance level was associated to areas where cowpea was being cultivated. The understanding of the native rhizobial population diversity will ultimately contribute to the selection of inoculants for soybean and cowpea in the Brazilian Cerrado.

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RESUMO

Avaliação da diversidade de rizóbios nodulantes de caupi em áreas de Cerrado do nordeste do Brasil

Com o objetivo de contribuir com a otimização do processo de fixação biológica de nitrogênio (FBN) na cultura do caupi (*Vigna unguiculata* (L) Walp) no Cerrado do nordeste brasileiro, a diversidade de isolados de rizóbio obtidos em oito áreas de Cerrado com rotação de cultura bianual com soja, arroz e caupi. Foram realizadas caracterizações morfológicas (produção de muco e morfologia das colônias), genotípicas baseadas em ARDRA do 16S rDNA e resistência a antibióticos. Os resultados da caracterização morfológica mostraram uma correlação inversamente proporcional ($p < 0,05$) do índice de diversidade de Shannon-Waver com o número de cultivos de leguminosas (caupi e soja). Os dados de ARDRA mostraram que no Cerrado nativo somente foram observados isolados de *Bradyrhizobium elkanii*, corroborando com dados da literatura. Nas áreas onde haviam sido cultivadas leguminosas ocorreram dois fatos distintos; onde somente cultivou-se soja houve maior proporção de *B. japonicum* e onde cultivou-se soja e caupi, ocorreu maior proporção de *B. elkanii*. A análise de resistência a antibióticos mostrou cinco diferentes perfis de resistência. Maior resistência de *Bradyrhizobium* spp. foi encontrada em áreas cultivadas há mais tempo, e menor na área nativa e/ou áreas com poucos cultivos. De forma geral, pode-se observar uma relação inversa entre a diversidade de rizóbios e a resistência a antibióticos. Como a menor diversidade foi observada em áreas com maior número de cultivos de leguminosas, sugere-se que a presença da leguminosa pode favorecer condições ecológicas específicas, nas quais determinados grupos de rizóbios adquirem características competitivas importantes para seu estabelecimento.

Palavras-chave: diversidade, *Bradyrhizobium*, Cerrado, caupi, resistência a antibiótico

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