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# Intra-Specific Diversity Study of the Nitrogen Fixing Bacterium *Azospirillum amazonense* Isolated from Different *Brachiaria* Species

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**Abstract**

The limitation of nitrogen is considered one of the most important factors for the Brazilian pastures degradation. However, there are evidences that pastures formed by some species of the *Brachiaria* genus could be benefited by the biological nitrogen fixation (BNF), guaranteeing a higher longevity to these pastures. Previous studies showed that the diazotrophic bacteria found in association with these forage grasses were mainly from the *Azospirillum amazonense* species. Since associations between these microorganisms and plants are generally conditioned by the vegetation, it is possible that different *Brachiaria* genotypes can exercise a selective effect on the *Azospirillum* populations. The aim of this work was to study the intra-specific diversity of *A. amazonense* isolates and to establish possible influences of different *Brachiaria* species and edaphoclimatic conditions. The characterisation of the diversity among these isolates was conducted using serological tests (ELISA - Enzyme Linked Immunosorbent Assay) and tests of carbon sources metabolisation (BIOLOG™). These methods were capable to show important diversity among the isolates of *A. amazonense*.

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## 1. Introduction

Among the factors that lead to pastures degradation, limitation of N is considered one of the most important (Oliveira et al., 2001). This nutrient can be removed from the soil-plant system through exportation by animal products (meat mostly), leaching, denitrification and volatilisation of the N deposited in the form of bovine excretions (Russelle, 1996).

Even with observations of nitrogen losses, there are reports indicating that some *Brachiaria* genotypes do not present significant reductions in productivity. The losses of N could be compensated by the biological fixation of the atmospheric nitrogen (BNF), which could be responsible for the introduction from 30 to 40 kg of N $ha^{-1}$ $year^{-1}$ in the soil-plant system (Boddey and Victoria, 1986; Loureiro and Boddey, 1988). In extensive management systems, where the losses are less significant, the quantity of fixed nitrogen can be enough to provide a null or even positive N balance and allow a larger longevity of the pasture with an acceptable productivity.

Most studies on isolation and identification of diazotrophic bacteria in forage grasses occurred between the 60 's and 80 's, with highlight for some species of the *Azospirillum* genus (Neyra and Döbereiner, 1977). Several species of this genus have been related in association with a great number of cereals and forage grasses species cultivated in tropical and tempered climates. *A. amazonense* deserves special attention presenting high incidence and high numbers in association with grasses like *Brachiaria* (Reis et al., 2001) and adaptability to acid pH (Baldani et al., 1997), which is a common characteristic of the Brazilian soils.

Studies on microbial diversity can help in the selection of strains for inoculation (Oliveira et al., 2000) and in the elucidation of the plant-bacteria interaction (Di Cello et al., 1997) as well as indicators of environmental stresses (Kennedy, 1999), such as water deficit, nutrients limitation or land degradation. There are evidences that events like recombinations and mutations contribute significantly for the genetic flexibility considered the main responsible for the adaptation to changes in the environmental conditions (Schloter et al., 2000).

Since the vegetation seems to have an important role in conditioning the diversity of soil microorganisms and of those found inside the plants, it is possible that different genotypes of *Brachiaria* exercise a selective effect on the microbial populations associated to these plants. Thus, in the case of

diazotrophic organisms, this could result in different levels of BNF contribution obtained by each one of these genotypes (Boddey and Victoria, 1986; Loureiro and Boddey, 1988, Reis et al., 2001).

The utilisation of the ELISA methodology in this study is based on the fact that antigenic relations among organisms largely reflect its genotipic relations and according to Hubálek (1982), usually, are linked to their taxonomic similarities. Serology produces general similarity information and it is considered as being a global polyphenic method with great content of information (Sokal and Sneath, 1963). Some serological studies already demonstrated that antigenic markers differentiate *Azospirillum* strains (Sampaio et al., 1978; De-Polli et al., 1980; Annapurna and Gaur, 1998), demonstrating different degrees of hostess specificity among cereals.

Applying the BIOLOG system, according to the differentiated use of carbon sources by microorganisms, the similarity among isolates can be compared by a grouping analysis of the data (Konopka et al., 1998). Yohalem and Lorbeer (1994), studied the intra-specific metabolic diversity among 218 *Burkholderia cepacia* isolates from onion, different soils and clinical samples. Each isolate was characterised by its catabolic ability using the BIOLOG and the authors showed a strong influence of the soil type. Isolates from the same soil frequently showed similarity, nevertheless, isolates from different soils with a history of similar cultivation did not show similarity. Schloter et al. (2000) also used this system to investigate the influence of different management practices on the *Ochrobactrum* spp. microdiversity. These authors founded striking differences in soils with organic cultivation for 40 years when compared with conventional management or with just 2–8 years of organic cultivation.

The aim of the present work was to study the intra-specific diversity of *A. amazonense* isolates derived from associations with Brachiaria roots and to establish possible influences of different *Brachiaria* species and edaphoclimatic conditions, using the ELISA and BIOLOG methodologies.

## 2. Material and Methods

### *Bacterial isolates*

The *A. amazonense* isolates examined in the present study are listed in Table 1b. For comparative purposes, reference strains of the diazotrophic bacteria *A. amazonense*, *A. brasilense*, *A. lipoferum* and *Herbaspirillum seropedicae* were also included in the study (Table 1a). All the *A. amazonense* isolates were obtained from roots of *Brachiaria humidicola*, *B. decumbens* or *B. brizantha* pastures implanted in the Brazilian Savannah (Santo Antônio de Goiás, Goiás)

Table 1. Reference strains (a) and isolates of *A. amazonense* originated from *Brachiaria* spp. (b) used in this work.

(a)

| Species | Strain | Plant |
|---|---|---|
| *A. lipoferum* | $Sp59^T$ (ATCC 29707) | Wheat |
| *A. brasilense* | $Sp7^T$ (ATCC 29145) | *Digitaria decumbens* |
| *A. brasilense* | $Cd^T$ (ATCC 29729) | *Cynodon dactylon* |
| *A. amazonense* | CBAMC (BR11145) | Sugar cane |
| *A. amazonense* | $Y2^T$ (ATCC 35120) | *Hyparrenia rufa* |
| *H. seropedicae* | $Z67^T$ (ATCC 35892) | Rice |

(b)

| *B. decumbens* | *B. humidicola* | *B. brizantha* |
|---|---|---|
| 37 | 64 | 27 |
| 38 | 77 | 36 |
| 41 | 79 | 47 |
| 53 | 80 | 48 |
| 72 | 81 | 87 |
| 73 | 82 | 94 |
| 76 | 83 | 104 |
| 118 | 84 | 124 |
| 134 | 86 | 125 |
| 137 | 97 | 131 |
| 140 | 107 | 138 |
| 141* | 119 | 139 |
| 142* | 123 | 143* |
| | 127 | |
| | 132 | |
| | 135 | |
| | 136 | |

*With the restriction analysis of the 16S DNAr region these isolates did not group with the other *A. amazonense* isolates (data not show) and should belong to other species.

or Atlantic Forest (Itabela, Bahia) regions. Details of the collection sites are given in Table 2.

Table 2. Edaphoclimatic characteristics of the sampling sites from Goiás and Bahia

| Collection site | Soil classification | $pH/H_2O$ | $Al^{3+}$ | $Ca^{2+}$ | $Mg^{2+}$ | $K^+$ | P | Texture | Koeppen classification (clime) |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0–20 cm $cmol_c.dm^{-3}$ | | | $mg.kg^{-1}$ | | | |
| Goiás 16°28'S 49°17'W | Oxisol (dark red latosol) | 5.3 | 0.1 | 3.0 | 1.7 | 104.0 | 2.0 | Clay loam | Aw (tropical with drought season in the winter) |
| Bahia 16°39'S 39°30'W | Typical Paleudult | 5.8 | 0.2 | 2.6 | 1.5 | 78.0 | 2.0 | Sandy loam | Af (tropical without drought season) |

Ca, Mg and Al extracted in KCl 1N; K and P extracted in Mehlich 1 solution (HCl 0.5 N + $H_2SO_4$ 0.025 N).

*ELISA*

*Production, purification and characterisation of the polyclonal antibodies used in the ELISA tests.* The primary antibodies used in the ELISA tests were produced against *A. amazonense* (reference strain CBAMC and isolates 27, 37, 77), *A. brasilense* (reference strain Sp7), *A. lipoferum* (reference strain Sp59) and *H. seropedicae* (reference strain Z67). The production process and characterisation stages of this material (immunisation and sera collection; determination of the best sera titre; determination of the sera sensitivity; cross-reaction tests) were previously performed according to Reis et al. (2000).

*ELISA procedure.* The procedure adopted for ELISA methodology was the indirect ELISA, proposed by Engvall and Perlmann (1972), with some modifications. This method consisted on growth of the bacterial strains in liquid DYGS medium (Rodrigues Neto et al., 1986) [($g.l^{-1}$): glucose - 2.0; glutamic acid - 1.5; peptone - 1.5; $K_2HPO_4$ - 0.5; $MgSO_4.7H_2O$ - 0.5; yeast extract - 2.0; pH 6.0] at 30°C for 24 h at 150 rpm, centrifugation of 1 ml of these cultured cells at 2000 g for 3 minutes and resuspention in 1 ml of bicarbonate-carbonate buffer (50 mM pH 9.6). The antigen concentration was adjusted to an optical density (O.D.) of 1.0 ± 0.1 read in a wave length of 436 nm. The wells of the microtitre plates were coated with 50 µl of the antigen and incubated in a refrigerator for a period of 16 to 18 hours. After that, the wells were washed once with a wash solution [PBS buffer ($g.l^{-1}$): NaCl - 8.0; KCl - 0.2; $Na_2HPO_4$ $.2H_2O$ - 1.4; $KH_2PO_4$ - 0.2 + 0.05% Tween 20 + 0.5% BSA] and the unspecific reactions were blocked using bovine serum albumin (3% BSA solution in PBS) for a period of 30 minutes at 37°C. An aliquot of 50 µl of the produced serum in the adequate dilution were added to each well and the plate was incubated for 30 minutes at 37°C followed by three washes using 200 µl of washing solution. A 50 µl aliquot of the secondary antibody (goat immunoglobulins IgG conjugated with peroxidase enzyme produced against rabbit immunoglobulins, SIGMA CHEMICAL CO., USA) diluted in the proportion of 1:1000 in PBS were added. After incubation for 45 minutes at 37°C, the plate wells were washed five times with the washing solution. Finally, 100 µl of Signal ABTS (ABTS buffer - stock solution: 1.67g of ABTS buffer in 10 ml of distilled $H_2O$; substratum solution: dilution of the ABTS stock solution 1:10 in distilled $H_2O$ added of 1 mg $ml^{-1}$ of ABTS. Boehringer Mannheim, Germany) were added. Absorbance readings after reaction development were accomplished using a spectrophotometer Labsystem Multskan Plus (Labsystems Oy, Helsinki, Finland) endowed of an interference filter of 405 nm. Control wells with no antigens and/or antisera were included and samples of pre-immune sera were also tested.

*Intra-specific diversity studies with ELISA results.* The values of optical density obtained through the ELISA methodology were transformed in percentage of reactivity, considering 100% as the homologous reactions of each

antiserum and 0% the absence of reaction. For grouping analysis a data matrix with 45 lines (tested bacteria) by 7 columns (each antiserum) were built. The values obtained with the reaction of each one of the isolates with the different antisera were compared and the distance among them was estimated by the Bray-Curts coefficient (Rohlf, 1994). The isolates were grouped by the UPGMA (Arithmetic Average Pair Clustering) method (Sneath and Sokal, 1973) and graphically represented by a dendrogram (NTSYS-PC, version 2.1, Exeter Software, USA).

*BIOLOG*

Several isolates were grouped according to the utilisation of 95 different carbon sources. This evaluation was performed using the BIOLOG system protocol.

*Procedure for BIOLOG tests.* The isolates were grown in DYGS solid media for 16–24 h at 30°C. After this period the colonies were carefully taken from the plates with a swab and suspended in an inoculant flowed (BIOLOG, 1999). The concentration of cells was adjusted to 52% of transmittance (±3%) using a spectrophotometer with filter of 590 nm (BIOLOG Inc., USA). Aliquots of 150 µl of this suspension were disposed in each of the 96 wells of the BIOLOG GN2 microplates (BIOLOG Inc., USA). These plates were sealed and incubated for 16–24 h at 30°C. After this incubation period, the pattern of plate wells in purple colour was evaluated. This pattern is characteristic of each microorganism and is a result of the use of different carbon sources. The utilisation of these sources promotes the reduction of a tetrazolium indicator resulting in the formation of the purple color. According to the utilisation of these sources, the similarity among different isolates was compared by grouping analysis of the data.

*Intra-specific diversity studies with BIOLOG results.* For the construction of a binary matrix with the data obtained by this system values of 1 or 0 were attributed, indicating the use or not of a certain carbon source. The utilisation patterns of these sources were compared and their similarities estimated by the Simple Matching coefficient (Rohlf, 1994). The grouping of the isolates and the graphic representation were performed as previously described.

## 3. Results and Discussion

*Evaluation of the A. amazonense diversity based on the ELISA results*

The dendrogram built with grouping of ELISA results showed great diversity among the isolates. The *A. amazonense* bacteria formed 6 groups with at least

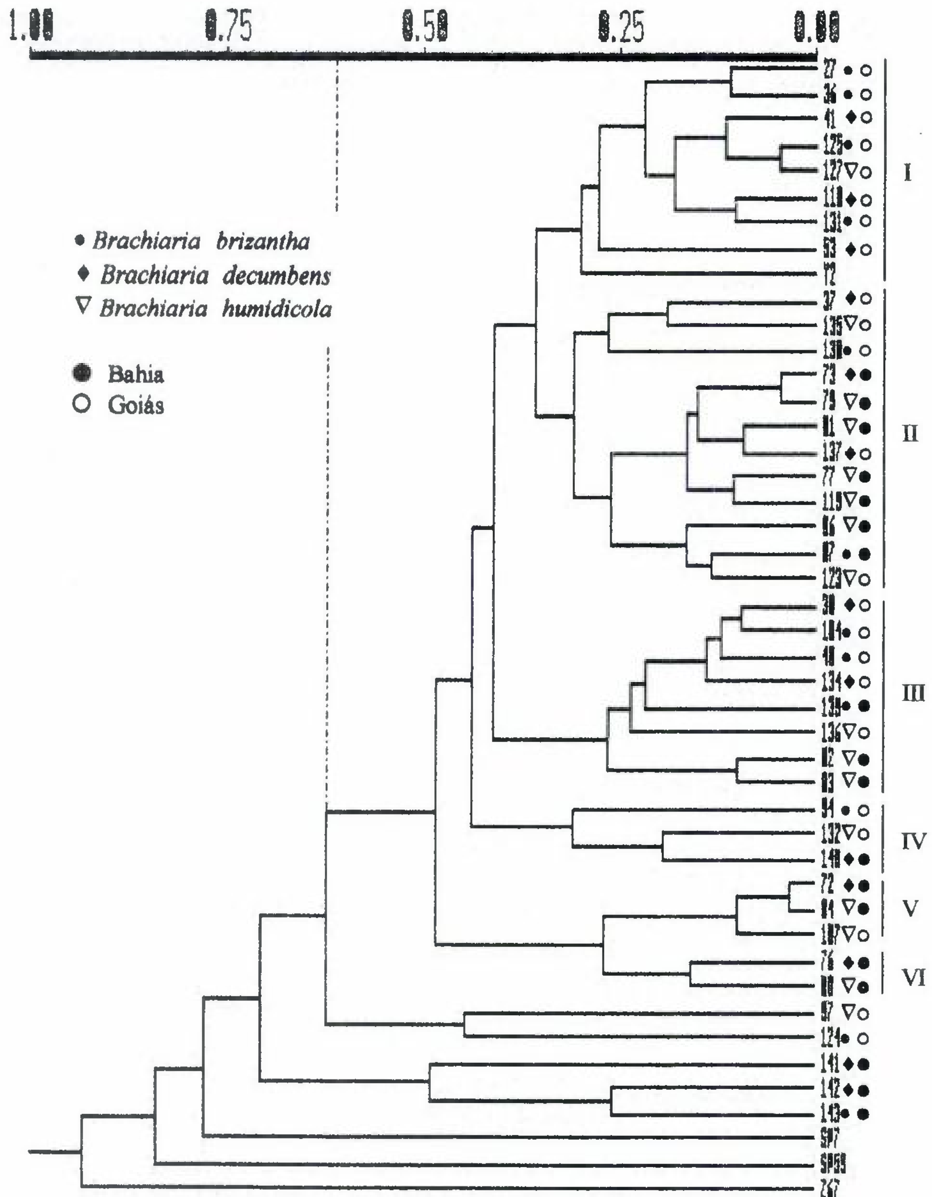



Figure 1. Distance dendrogram of 41 isolates originated from its association with the three studied *Brachiaria* species (evidencing the origin species and collect sites) and of the type strains of *Azospirillum amazonense* (Y2), *A. brasilense* (Sp7), *A. lipoferum* (Sp59) and *Herbaspirillum seropedicae* (Z67). The distance matrix was calculated by the Bray-Curts index from the data obtained with ELISA transformed in percentage of reaction, considering as 100% the homologous reactions of each antiserum. This dendrogram was generated by the UPGMA algorithm.

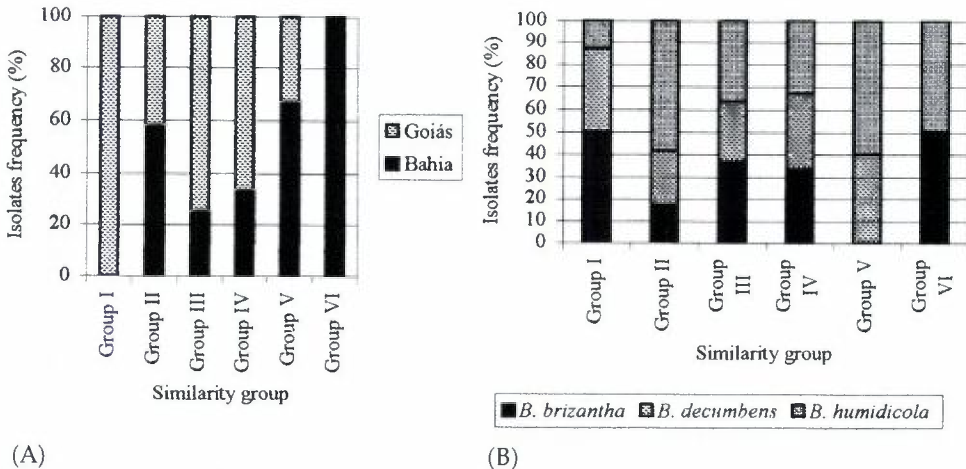



Figure 2. Frequency of the *A. amazonense* isolates in each similarity group, formed by grouping the ELISA results, presented in the Fig. 1, considering the sampling sites (A) and the *Brachiaria* species associated to these isolates (B). The frequencies were obtained dividing the number of isolates from each site (A) or species (B) present in a group by the total number of isolates present in this group.

65% of similarity (Figs. 1 and 2). These results reveal the occurrence of diversity among the isolates in the intra-specific level. The formation of groups with different similarity values may represent a problem for the taxonomic analysis of the isolates at the species level.

Hartmann and Zimmer (1994) reported that components of the *Azospirillum* cellular wall, such as proteins and lipopolysaccharides, allow serological identification in the species or strain levels only with the use of polyclonal or monoclonal antisera. The ELISA technique, together with other immunological methods, were used to determine the antigenic relationship of *Arthrobacter*, *Aureobacterium* and other soil isolates from several areas (Gray and Mansoor, 1996). The result of these evaluations was used to classify these organisms.

The results presented in the Figs. 1 and 2 show, with the use of ELISA, that the experimental sites presented certain influence on the diversity of *A. amazonense* isolates. The influence of the *Brachiaria* species on the diversity of these organisms (Figs. 1 and 2B) does not seem clear. Figs. 1 and 2A show the formation of relatively homogeneous groups in relation to the experimental site from where the isolates were obtained. Observing the Fig. 1, the groups I (8 isolates), III (8 isolates) and IV (3 isolates) are dominated by isolates from Goiás, groups V (3 isolates) and VI (2 isolates) are dominated by isolates from Bahia, while group II (12 isolates) is relatively homogeneous. Unfortunately,

the formation of groups with few isolates (groups IV, V and VI) does not allow a conclusive statement. In addition, in further studies, larger number of isolates should be use.

The Oxisol (Goiás) and the Typical Paleudult (Bahia) present significantly different characteristics such as texture and water retention capacity, which could partly explain the results obtained. It is known that phenotypic characters that act as antigenic determinants, such as membrane lipopolysaccharides and proteins suffer great influence of environmental factors, such as aeration degree and iron levels (Davies and Quirie, 1996). It is also known that the clay levels, nitrogen and water retention capacity as well as the levels of thick sand, can be related with the *Azospirillum* viability (Bashan et al., 1995a,b). Latour et al. (1996) demonstrated an intense influence of the soil type on the population structure of *Pseudomonas* isolates, suggesting that the different environmental characteristics could be responsible for the discriminatory and selective effect presented.

*Evaluation of the A. amazonense diversity based on the use of carbon sources (BIOLOG)*

The data obtained from the use of the carbon sources present in the BIOLOG system by thirty two isolates from *Brachiaria* roots, phenotypically characterised as *A. amazonense* (Fig. 3), were used to build a dendrogram. Comparing the results obtained with both methods used in this work, ELISA showed higher distance among the isolates, forming 6 groups ranging from 50% to 65% of similarity. With the BIOLOG, the similarity found among these isolates and the two reference strains of *A. amazonense* (CBAMC and Y2) was at least 85%. Reference strains of other species formed different groups, for *A. brasilense* (Cd, Sp7), *A. lipoferum* (Sp59) and *Herbaspirillum seropedicae* (Z67), all distant at least 60% from *A. amazonense.*

The BIOLOG, which has been used traditionally for the identification of bacteria (Klinger et al., 1992), was effective in the confirmation of the morphological evaluations. Toth et al. (1999), used this system to study the diversity among *Erwinia carotovora* strains, however, it was unable to show a clear difference among the several analysed groups. These authors concluded that *Erwinia carotovora* is nutritionally homogeneous. Ross et al. (2000), comparing the results obtained with BIOLOG, observed a homology higher than 90% between strains of *Pseudomonas brassicareum* and *Pseudomonas thivervalensis*.

The influence of the collection sites on the diversity of *A. amazonense* isolates were not shown by the use of BIOLOG. The major groups presented in the Figs. 3 and 4A, group I (13 isolates), group II (7 isolates) and group IV (5

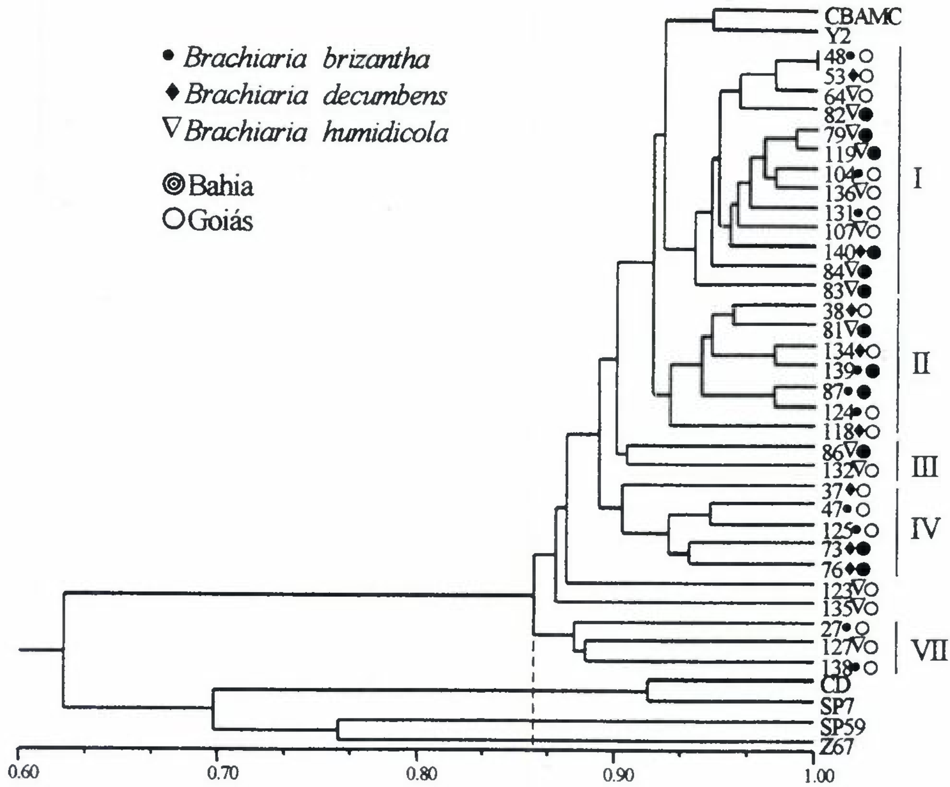
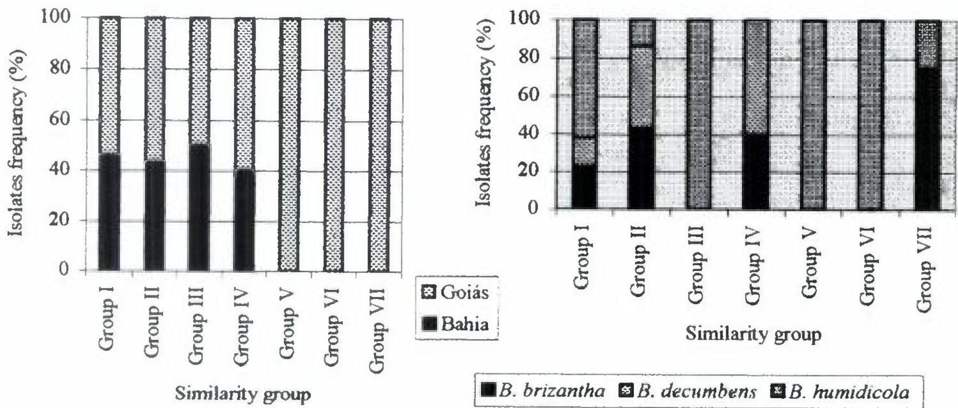



Figure 3. Similarity dedrogram of 32 isolates obtained from the association with the three studied *Bracharia* species (evidencing the origin species and collect sites) and reference strains of *Azospirillum amazonense* (Y2 and CBAMC), *A. brasilense* (Sp7 and CD), *A. lipoferum* (Sp59) and *Herbaspirillum seropedicae* (Z67). The similarity matrix was calculated by the Simple Matching Coefficient based on BIOLOG results and this dendrogram was generated by the UPGMA algorithm.

isolates), show certain equilibrium between the isolates originated from Bahia and Goiás.

Besides BIOLOG has been widely used for species characterisation, in some cases this system has also been used to evaluate the intra-specific diversity. Hildebrand et al. (1993) reported a relationship between strains of *Xanthomonas campestris* and the host plant. Therefore, in comparison with *X. campestris*, as well as showed for *E. carotovora* (Toth et al., 1999), *A. amazonense* strains can be considered nutritionally similar. However, when analysing the formed groups, even with this high similarity, an influence of the plant species on the formation of these groups can be noticed, as shown in




(A) (B)

Figure 4. Frequency of the *A. amazonense* isolates in each similarity group, formed by grouping the BIOLOG results, presented in the Fig. 3, considering the sampling sites (A) and the *Brachiaria* species associated to these isolates (B). The frequencies were obtained dividing the number of isolates from each site (A) or species (B) present in a group by the total number of isolates present in this group.

Figs. 3 and 4B, where it is observed that the majority of the *B. decumbens* and *B. brizantha* isolates are distributed among the groups II and IV and the isolates of *B. humidicola* concentrate on the group I. An interesting observation is that *B. decumbens* cv Basilisk (the cultivar from where the isolates were obtained) is actually an intermediate ecotype between the *B. brizantha* and *B. decumbens* species (Maass, 1996). While *B. decumbens* cv Basilisk and *B. brizantha* cv Marandu are tetraploides, *B. humidicola* (commercial genotype) is hexaploide, which according to Valle et al. (2000), allows the inference of the genetic distance among the *Brachiaria* accesses.

The plants in a certain area can influence the diversity of microbial communities due to the variability of the chemical compounds from their exudates (Christensen, 1989). These compounds influence the microbial community altering the chemical composition of the soil in the neighbourhood of the roots and serving as selective substrate for the growth of soil microorganisms. Actually, the variety of organic compounds liberated by the plants has been emphasised as the key factor which influence the microorganisms diversity associated with the rhizosphere of different vegetable species (Bowen and Rovira, 1991, cited by Grayston et al., 1998).

Among the 95 carbon sources included in the BIOLOG system, four of them (D-fructose, D-psychosis, Acetic acid, Formic acid) were emphasised for being more

used by isolates from *B. humidicola* than by isolates from the other two species (Table 3). As discussed previously, it is known that different root exudates originated from different plants exercise a selective pressure over the rizosphere organisms, favouring isolates with specific routes of carbon source utilisation and that are capable to explore different types of these sources. Ross et al. (2000), also discuss as a probable evolutionary factor, that strains from the same species isolated from different plants have acquired the capacity to metabolise different carbon sources.

Table 3. Percentage of *A. amazonense* isolates in the use of four different carbon sources.

| Origin of the isolates | Carbon sources | | | |
|---|---|---|---|---|
| | D-fructose | D-psychosis | Acetic acid | Formic acid |
| *B. brizantha* (10 isolates) | 30% | 50% | 20% | 40% |
| *B. decumbens* (8 isolates) | 37% | 25% | 12% | 25% |
| *B. humidicola* (14 isolates) | 71% | 64% | 64% | 80% |

In the description work of the *Azospirillum amazonense* species (Magalhães et al., 1983), it was reported that D-fructose was not used by these bacteria. However, our results indicate that this source can be used, with prominence for isolates originated from *B. humidicola*. Bagwell et al. (1998), affirmed that in their studies some tested diazotrophic bacteria (*Azotobacter chroococcum*, *Vibrio diazotrophicus* and *Rhizobium meliloti*) produced results with BIOLOG, quite different from its recognised physiological behaviour. These authors believe that the media used for the growth of these bacteria (Bacto Marine Agar) might have been a stress factor generating atypical results.

Since the BNF is a result of complex physiological and biochemical reactions, it is dependent on the potential genetic expression of the diazotrophic microorganism and the host plant. It is known that the BNF potential can be limited by factors of biotic and abiotic nature. In *Brachiaria*, Loureiro and Boddey (1988) have shown that different plant genotypes obtain variable contributions from the BNF. By using the 15-N isotopic dilution technique for the BNF quantification, these authors showed a superior contribution for *B. humidicola* comparing to *B. decumbens*, *B. ruziziensis* and *B. radicans*. Possibly the results of the intra-specific diversity of diazotrophic bacteria isolated from *Brachiaria* can be considered as one important factor on the biological nitrogen fixation levels associated to these plants.

The use of ELISA and BIOLOG to evaluate the *A. amazonense* isolates showed important diversity among them. The results indicate possible effects exercised by the edaphoclimatic conditions (ELISA) and *Brachiaria* species (BIOLOG) on the *A. amazonense* diversity.

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## REFERENCES

Annapurna, K. and Gaur, Y.D. 1998. Antigenic diversity amongst strains of *Azospirillum* from an Indian soil and their host specificity. *Soil Biology & Biochemistry* **30**: 1217–1219.

Bagwell, C.E., Piceno, Y.M., Ashburne-Lucas, A., and Lovell, C.R. 1998. Physiological diversity of the rhizosphere diazotroph assemblages of selected salt marsh grasses. *Applied and Environmental Microbiology* **64**: 4276–4282.

Baldani, J.I., Caruso, L., Baldani, V.L.D., Goi, S.R., and Döbereiner, J. 1997. Recent advances in BNF with non-legume plants. *Soil Biology & Biochemistry* **29**: 911–922.

Bashan, Y., Puent, M.E., Rodriguez-Mendoza, M.N., Holguin, G., Toledo, G., Ferrera-Cerrato, R., and Pedrin, S. 1995a. Soil parameters which affect survival of *Azospirillum brasilense*. In: *Azospirillum VI and Related Microorganisms: Genetics, Physiology, Ecology*. Fendrik, I., Del Gallo, M., Vanderleyden, J., and Zamaroczy, M., eds., Springer-Verlag, Berlin. pp. 441–449.

Bashan, Y., Puent, M.E., Rodriguez-Mendoza, M.N., Toledo, G., Holguin, G., Ferrera-Cerrato, R., and Pedrin, S. 1995b. Survival of *Azospirillum brasilense* Cd and Sp-245 in the rhizosphere of 23 soil types. *Applied and Environmental Microbiology* **61**: 1938–1045.

Boddey, R.M. and Victoria, R.L. 1986. Estimation of biological nitrogen fixation associated with *Brachiaria* and *Paspalum* grasses using $^{15}N$ labelled organic matter and fertilizer. *Plant and Soil* **90**: 256–292.

Christensen, M. 1989. A view of fungal ecology. *Mycologia* **81**: 1–19.

Davies, R.L. and Quirie, M. 1996. Intra-specific diversity within *Pasteurella trehalosi* based on variation of capsular polysaccharide, lipopolysaccharide and outer-membrane proteins. *Microbiology* **142**: 551–560.

De-Polli, H., Bohlool, B.B., and Döbereiner, J. 1980. Serological differentiation of *Azospirillum* species belonging to different host-plant specificity groups. *Archives of Microbiology* **126**: 217–222.

Di Cello, F., Bevinino, A., Chiarini, L., Fani, R., Paffetti, D., Tabacchioni, S., and Dalmastri, C. 1997. Biodiversity of a *Burkholderia cepacia* population isolated from maize

rhizosphere at different plant growth stages. *Applied and Environmental Microbiology* **63**: 4485–4493.

Engvall, E. and Perlmann, P. 1972. Enzyme-linked immunosorbent assay (ELISA). III. Quantification of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. *Journal of Immunology* **109**: 129–135.

Gray, T.R.G. and Mansoor, E.Y. 1996. The application of serological techniques to the taxonomy of *Arthrobacter* and related organisms. *Microbiology* **142**: 561–563.

Grayston, S.J., Wang, S., Campbell, C.D., and Edwards, A.C. 1998. Selective influence of plant species on microbial diversity in the rizosphere. *Soil Biology & Biochemistry* **30**: 369–378.

Hartmann, A. and Zimmer, W. 1994. Physiology of *Azospirillum*. In: *Azospirillum/Plant Associations*. Okon, Y., ed., CRC Press, Boca Raton. pp. 15–39.

Hildebrand, D.C., Hendson, M., and Schroth, M.N. 19??. Usefulness of nutritional screening for the identification of *Xanthomonas campestris* DNA homology groups and pathovars. *Journal of Applied Bacteriology* **75**: 447–455.

Hubálek, Z. 1982. Numerical comparative serology – the methods. *Journal of Applied Bacteriology* **52**: 307–318.

Kennedy, A.C. 1999. Bacterial diversity in agroecosystems. *Agriculture, Ecosystems and Environment* **74**: 65–76.

Klinger, J.M., Stowe, R.P., Obenhuber, D.C., Groves, T.O., Mishra, S.K., and Pierson, D.L. 1992. Evaluation of the Biolog automated microbial identification system. *Applied and Environmental Microbiology* **58**: 2089–2092.

Konopka, A., Olivier, L., and Turco Jr., R.F. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microbial Ecology* **35**: 103–115.

Latour, X., Corberand, T., Laguerre, G., Aallard, F., and Lemanceau, P. 1996. The composition of fluorescent pseudomonad populations associated with roots is influenced by plant and soil type. *Applied and Environmental Microbiology* **62**: 2449–2456.

Loureiro, M.F. and Boddey, R.M. 1988. Balanço de nitrogênio em quatro gramineas do gênero *Brachiaria*. *Pesquisa Agropecuária Brasileira* **23**: 1343–1353.

Maass, B.L. 1996. Identifying and naming *Brachiaria* species. In: *Brachiaria: Biology, Agronomy and Improvement*. Miles, J.W., Maass, B.L., and Valle, C.B., eds. CIAT, Cali, Embrapa-CNPGC, Brasilia.p. ix–xiii.

Neyra, C.A. and Döbereiner, J. 1977. Nitrogen fixation in grasses. *Advances in Agronomy* **29**: 1–38.

Oliveira, I.A., Vasconcellos, M.J., Seldin, L., Paiva, E., Vargas, M.A., and de Sá, N.M.H. 2000. Random amplified polymorphic DNA analysis of effective *Rhizobium* sp. associated with beans cultivated in Brazilian cerrado soils. *Brazilian Journal of Microbiology* **31**: 39–44.

Oliveira, O.C. de, Oliveira, I.P. de, Ferreira, E., Alves, B.J.R., Miranda, C.H.B., Vilela, L., Urquiaga, S., and Boddey, R.M. 2001. Response of degraded pastures in the Brazilian Cerrado to chemical fertilisation. *Pasturas Tropicales* **23**: 14–18.

Reis, V. M., Reis Jr., F.B., Salles, J.F., and Schloter, M. 2000. Caracterisation of different polyclonal antisera to quantify *Herbaspirillum* spp. in Elephant Grass (*Pennisetum purpureum* Schun.). *Symbiosis* **29**: 139–150.

Reis, V.M., Reis Jr., F.B., Quesada, D.M., Oliveira, O.C.A., Alves, B.J.R., Urquiaga, S., and Boddey, R.M. 2001. Biological nitrogen fixation associated with tropical pasture grasses. *Australian Journal of Plant Physiology* **28**: 837–844.

Rodrigues Neto, J., Malavolta Jr., V.A., and Victor, O. 1986. Meio simples para o isolamento e cultivo de *Xanthomonas campestris* pv. Citri Tipo B. *Summa Phytopatologica* **12**: 16.

Rohlf, F.J. 1994. NTSYS-pc - Numerical taxonomy and multivariate analysis system. State University of New York.

Ross, I.L., Younes, A., Harvey, P.R., Achouak, W., and Ryder, M.H. 2000. Genetic diversity and biological control activity of novel species of closely related *Pseudomonads* isolated from wheat field soils in South Australia. *Applied and Environmental Microbiology* **66**: 1609–1616.

Russelle, M.P. 1996. Nitrogen cycling in pasture systems. In: *Nutrient Cycling in Forage Systems*. Joost, R.E. and Roberts, C.A., eds. Potash and Phosphate Institute, Manhattan, Kansas, USA, pp. 125–175.

Sampaio, M.J.A., de Vasconcelos, L., and Döbereiner, J. 1978. Characterization of three groups within *Spirillum lipoferum* Beijerinck. *Ecological Bulletin* **26**: 364–365.

Schloter, M., Lebuhn, M., Heulin, T., and Hartmann, A. 2000. Ecology and evolution of bacterial microdiversity. *FEMS Microbiological Review* **24**: 647–660.

Sneath, P.H.A. and Sokal, R.R. 1973. *Numerical Taxonomy – The Principles and Practice of Numerical Classification*. Freeman, W.H. (ed.), San Francisco, 573 pp.

Sokal, R.R. and Sneath, P.H.A. 1963. *Principles of Numerical Taxonomy*. Freeman, W.H. (ed.), San Francisco, 359 pp.

Toth, I.K., Bertheau, Y., Hyman, L.J., Laplaze, L., López, M..M., McNicol, J., Niepold, J., Persson, P., Salmond, G.P.C., Sletten, A., Van der Wolf, J.M., and Pérombelon, M.C.M. 1999. Evaluation of phenotypic and molecular typing techniques for determining diversity in *Erwinia carotovora* subspp. Atroseptica. *Journal of Applied Microbiology* **87**: 770–781.

Valle, C.B., Euclides, V.P.B., and Macedo, M.C.M. 2000. Características das plantas forrageiras do gênero *Brachiaria*. In: Anais do 17° Simpósio sobre manejo da pastagem: a planta forrageira no sistema de produçåo. Piracicaba - FEALQ, pp. 65–108.

Yohalem, D.S. and Lorbeer, J.W. 1994. Intraspecific metabolic diversity among strains of *Burkholderia cepacia* isolated from decayed onions, soils, and the clinical environment. *Antonie Van Leeuwenhoek* **65**: 111–131.