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Influence of soil and host plant crop on the genetic diversity of *Azospirillum amazonense* isolates

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Soil type;
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AMOVA

Summary

The genetic structure of *Azospirillum amazonense* populations isolated from the rhizosphere soil and washed and surface-sterilised roots of rice, maize and sorghum plants, cropped simultaneously in two different soils (clay loam and sandy loam) was characterised. Genetic diversity was measured by restriction fragment length polymorphism of the amplified 16S–23S rDNA intergenic spacer region (RISA-RFLP) and cluster analysis. Four genetically distinct clusters of isolates were observed with 78% similarity, suggesting that the *A. amazonense* population was heterogeneous at the strain level regardless of the soil type or host plant. Analysis of molecular variance (AMOVA) demonstrated that the host plant had a highly significant selective effect on the genetic structure of this species, especially on those isolates intimately associated with them, but also to a lesser extent on isolates from the rhizosphere and washed roots. The soil type also had a highly significant selective effect on *A. amazonense* genetic diversity, especially for those isolates from the rhizosphere soil. The selective effect of the soil type combined with that of the host plant suggests that environmental factors, such as soil texture and composition of exudates provided by C_3 or C_4 plants, play major roles in the overall genetic structure of *A. amazonense* populations associated with these cereals.

Introduction

Azospirillum spp. are nitrogen-fixing members of the α-subclass of the Proteobacteria. They have been clustered by phenotypic and molecular techniques, including a detailed 16S rDNA-based

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molecular phylogeny (Stoffels et al., 2001). Besides their broad distribution among soil, grasses, cereals and other plants grown in tropical areas, representatives of this genus have also been found associated with plants from temperate climates (Döbereiner, 1992; Eckert et al., 2001). Currently, seven species have been described in this genus: *Azospirillum brasilense*, *A. lipoferum*, *A. amazonense*, *A. irakense*, *A. halopraeferens*, *A. largimobile*, and *A. doebereinerae* (Hartmann and Baldani, 2003). The species *A. brasilense*, *A. lipoferum*, *A. halopraeferens*, *A. largimobile* and *A. döebereinerae* form one subcluster within the *Azospirillum–Skermanella–Rhodocista* cluster (Eckert et al., 2001; Stoffels et al., 2001), while *A. amazonense* and *A. irakense* form part of another subcluster.

Despite the large number of species within this genus, most of the studies concerning host plant effects and genetic diversity of *Azospirillum* have focused on strains of *A. lipoferum* and *A. brasilense* (reviewed by Kennedy et al., 2004). Early phenotypic characterisations of strains isolated from C_3 and C_4 plants grown in pots or field conditions indicated that C_4 plants were preferentially colonised by *A. lipoferum* while C_3 plants were colonised by *A. brasilense*, suggesting that the host plants could exert a selective effect on the root colonisation of graminaceous plants by these bacteria (Baldani and Döbereiner, 1980). Differences in C_4 and C_3 photosynthetic pathways could be responsible for the preferential bacterial colonisation as observed in the above study. In tropical countries, as in Brazil, most cultivated grasses are C_4 plants, with the exception of the C_3 plants rice and wheat (Heckathorn et al., 1999). Plants with the C_4 pathway are known to produce unique 4-carbon photosynthetic metabolic intermediates (i.e. malate and oxaloacetate). In the tropics, with adequate water, they can maximise the advantages of full sunlight and high temperatures resulting in increased yields of fixed carbon. In contrast, C_3 plants are more adapted to temperate climates. At temperatures above 25 °C, C_3 plants metabolise more carbohydrate than is produced by photosynthesis, resulting in net loss of carbon through photorespiration and a high diversity of carbon-containing exudates into the rhizosphere soil.

The hypothesis that differences in carbon exudates could be responsible for the specificity of *A. brasilense* to C_3 and *A. lipoferum* to C_4 plants was tested by measuring their chemotactic responses to several organic compounds normally present in the rhizosphere, such as amino acids, sugars and organic acids (Reinhold et al., 1985). The authors observed that the chemotactic responses among isolates from two C_4 plants were different from that observed of isolates from C_3 plants. In contrast, Fedi et al. (1992), using exudates released by the roots, suggested that chemotaxis was a general rather than a host plant-specific response. Moreover, studies of the incidence of plant infection suggested that *Azospirillum* species do not have any preference for any particular plant species, but that the plant–*Azospirillum* interaction is a rather active process involving bacterial motility towards root infection sites and the balance between attractive or repelling substances released by the roots (Bashan and Holguin, 1997). In addition, *Azospirillum* strain specificity to host plant or soil type has been inferred from the absence of antigenic similarity among *Azospirillum* strains isolated from the roots of different types of plants (De-Polli et al., 1980; Annapurna and Gaur, 1998; Reis et al., 2004). Clear differences in the ability of *Azospirillum* strains to promote growth, colonisation or contribution to fixed N incorporation in plant and soil systems have been reported (reviewed by Steenhoudt and Vanderleyden, 2000; Bashan et al., 2004).

In spite of a great deal of information about colonisation of plants by strains of *A. lipoferum* and *A. brasilense*, very little is known about the influence of soil or host plant specificity on genetic diversity of *A. amazonense* populations. *A. amazonense* are Gram-negative bacteria, slightly curved in shape, 0.8–1.0 μm in diameter, and motile in liquid media by a single polar flagellum. Optimum growth temperature is 35 °C, and pH range varies from 5.5 to 6.5. They grow well on glucose and sucrose but not on mannitol and glycerol. Salts of organic acids such as malate, succinate, lactate or pyruvate can be used provided that the pH is maintained around 6.0 (Magalhães et al., 1983). This species has been isolated from roots of maize, sorghum, rice and wheat plants as well as forage grasses, roots and stems of sugarcane plants grown in Brazil and other tropical countries (Hartmann and Baldani, 2003). It was also found associated with roots of palm trees from the Amazon region and from pineapple and banana fruits (Weber et al., 1999).

Phenotypic characterisations of *A. amazonense* isolates obtained from C_3 (rice) and C_4 (maize and sorghum) plants were unable to demonstrate a selective effect by plants on *A. amazonense* populations (Baldani J.I., Unpublished data). However, when isolates of *A. amazonense* were clustered by ELISA and Biolog tests, selective effects exerted by different *Brachiaria* genotypes and soil types from two pasture sites were found (Reis et al., 2004). Therefore, molecular techniques

based on DNA analysis have been applied to the analysis of the genetic structure of *A. amazonense* population. The RFLP technique combined with analysis of the 16S–23S ribosomal DNA (rDNA) spacer region (RISA) has been shown to be very useful for examination of the diversity of microbial populations, including differentiation of species at the strain level (Gürtler and Stanisich, 1996).

In this paper, the genetic diversity of *A. amazonense* populations associated with rice, maize and sorghum plants, grown on two soil types, was characterised by RISA-RFLP. In addition, RISA-RLFP matrix data were used to estimate the effect of the soil type and host plant on the *A. amazonense* genetic diversity by the analysis of molecular variance method (AMOVA).

Materials and methods

Bacterial strains, field experimental design, soil and site description

The origin of the reference strains and the 71 isolates with phenotypic characteristics similar to the type strain of *A. amazonense* used in this study are shown in Table 1. The type strain was obtained from the Culture Collection of Embrapa Agrobiologia. Strains were isolated from rhizosphere soil, washed and surface-sterilised roots of rice, maize and sorghum plants grown in two different soil types, located at Embrapa Agrobiologia Experimental Station (22°45′S 43°42′W), in a randomised split plot block design with three replicates. The soil-type characteristics and classification are shown in Table 2. Each isolate used in the study was selected as representative of one plant. Three plants were collected at the same time from each treatment at different growth stages according to the plant development. Maize isolates were obtained at the beginning of flowering and grain filling. Rice and sorghum isolates were collected from plants at the beginning of grain filling, early and late grain maturation. Bacterial isolates were obtained from rhizosphere soil (obtained after manual shaking of the soil adhered to roots), washed roots (roots were homogenised after washing with tap water to remove the adherent soil) and surface-sterilised roots (washed roots were homogenised after immersion of roots in 1% chloramine T solution – 60 min for maize, 30 min for sorghum and 15 min for

Table 1. *Azospirillum amazonense* isolates and reference strains

Reference strains[a]	Origin	Plant	Soil/site
A. amazonense Y2	Sterilised roots	*Hyparrhenia rufa*	Sandy loam/Ecologia
A. amazonense Y3	Roots	*Digitaria decumbens*	
A. amazonense Y6	Sterilised roots	*Pennisetum purpureum*	
Isolates ID[b]			
Ya 64, 76, 80	Rhizosphere soil		Clay loam/Itaguaí
Ya 9, 39, 95	Washed roots	Rice	
Ya 14, 20, 47, 56, 74, 92	Sterilised roots		
Ym 6, 10, 148	Rhizosphere soil		
Ym 4, 11, 54, 69	Washed roots	Maize	
Ym 5, 9, 49, 56, 120, 127	Sterilised roots		
Ys 6, 45, 58	Rhizosphere soil		
Ys 5, 41, 65	Washed roots	Sorghum	
Ys 14, 17, 32, 34, 38, 69, 61	Sterilised roots		
EYa 37	Rhizosphere soil		Sandy loam/Ecologia
EYa 13, 68, 90	Washed roots	Rice	
EYa 22, 44, 75, 18, 73, 88	Sterilised roots		
EYm 67, 92, 97	Rhizosphere soil		
EYm 42, 72, 131	Washed roots	Maize	
EYm 61,133, 145, 15, 68, 95	Sterilised roots		
EYs S-14, 37, 89	Rhizosphere soil		
EYs S-2, 43, 81	Washed roots	Sorghum	
EYs 12, 66, 70, 87,90	Sterilised roots		

[a]Magalhães et al. (1983).
[b]This work.

Table 2. Sampling sites, soils and their edapho-climatic characteristics

Site	Soil classification	pH[a]	0–20 cm depth					Texture	Koeppen classification (climate)
			$cmol_c\,dm^{-3}$			$mg\,kg^{-1}$			
			Al^{3+}	Ca^{2+}	Mg^{2+}	K^+	P		
Itaguaí	Red–yellow podzolic	5.3 (6.5[b])	0.00	2.8		0.12	7.0	Clay loam	Aw[c]
Ecologia	Grey hydromorphic	5.4 (6.4[b])	0.00	1.4		0.18	17.0	Sandy loam	Aw

[a]Soil pH was measured in water (1:2 soil/water ratio).
[b]The Embrapa Experimental Station field was supplemented with 1 $t\,ha^{-1}$ of lime, 160 $kg\,ha^{-1}$ of P_2O_5 and 160 $kg\,ha^{-1}$ of K_2O.
[c]Tropical with drought season in the winter.

rice). The isolates from the rhizosphere soil and washed roots were considered to be from the outer part of the roots, while isolates obtained from surface-sterilised roots were considered to be strictly from the inner part of the roots. The bacterial isolation was performed as described by Döbereiner (1992).

Culture media

Modified potato agar medium contained (per litre): 500 ml of filtered potato juice, 2.5 g sucrose, 1 ml of vitamin stock solution (10 mg of biotin and 20 mg pyridoxal–HCl in 100 ml of distilled water), 2 ml of micronutrients stock solution (200 mg of $Na_2MoO_4 \cdot 2H_2O$, 235 mg of $MnSO_4 \cdot H_2O$, 280 mg of H_3BO_3, 8 mg of $CuSO_4 \cdot 5H_2O$, 24 mg of $ZnSO_4 \cdot 7H_2O$ in 200 ml distilled water), 17 g of Agar from Difco and sufficient distilled water to bring the volume to 1 l. The final pH was adjusted to 6.5. The potato juice was prepared by boiling 200 g of washed potato for 30 min in 500 ml of distilled water (final volume). The juice was then filtered through paper. The composition of LGI broth plus KNO_3 was (per litre): 5 g of sucrose, 0.2 g of K_2HPO_4, 0.6 g of KH_2PO_4, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.1 g of $FeCl_3$, 1 g of KNO_3. The final pH was adjusted to 6.0.

DNA extraction

Total cellular DNA was extracted using a boiling method adapted from Cruz et al. (2001). Fresh single colonies, grown on modified potato agar medium, were used to inoculate LGI broth plus KNO_3. Cultures were grown at 30 °C for 48 h, and 50 μl of the cultures were transferred to 0.5 ml microtubes and collected by centrifugation for 15 min at 13,500 rpm (Eppendorf centrifuge model 5415C) at room temperature. The cells were resuspended in 450 μl of sterile Milli-Q filtered water (previously deionised). Each cell suspension was homogenised by vortexing for 30 s, heating in a boiling water bath for 10 min and cooling on ice for 5 min. The crude cell extract was directly used for PCR.

PCR-amplification of the 16S–23S rDNA intergenic spacer region (RISA)

The universal primers used to amplify the ribosomal intergenic region, PHR (5′-TGC GGC TGG ATG ACC TCC TT-3′) and P23 (5′-GGT TCT TTT CAC CTT TCC CTC-3′), were designed based on the consensus sequence described by Arturo et al. (1995). In a final volume of 100 μl, the reaction mixture contained: 4.0 μl of the fresh crude cell extract, 1 × reaction buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM dNTP, 10 pmol of each primer, 2 U of *Taq* DNA polymerase (Invitrogen) and DNAse free water. The PCR process included an initial denaturation step (95 °C, 3 min) followed by 35 cycles (94 °C–1 min; 60 °C–1 min; 72 °C–3 min) and one final extension and cooling step (72 °C–5 min; 15 °C–15 min, respectively). Negative controls were included to check for the presence of false positives due to reagent contamination. Amplified products were separated on 1.2% agarose gels in 1 × TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.5) by electrophoresis at 72 V for 60 min. Amplification products were stained with ethidium bromide, observed with a UV transilluminator and recorded with Polaroid films 667 type.

Restriction of the PCR amplified products

The products of RISA were purified by the Highly Purified PCR Products Purification Kit (Boehringer Mannheim, Germany) to remove PCR reagents and avoid interference on the restriction digestion. The amplification products of each strain were treated

separately with seven restriction enzymes. The *Hae*III, *Alu*I, *Rsa*I, *Cfo*I, *Msp*I and *Eco*RI reactions were incubated for 3 h at 37 °C, and the *Taq*I reactions were incubated at 65 °C for the same time. Each reaction contained, in a final volume 25 μl, 5 U of restriction enzyme, 2.5 μl of 10 × reaction buffer (according to each enzyme requirements) and 10 μl of the purified PCR products. RISA-RFLP products were analysed by horizontal electrophoresis in Multiphor II Unit using 6% polyacrylamide gel and 1 × MOPS as the running buffer according to manufacturer (Pharmacia Biotech). The composition of 50 × MOPS stock solution was 1 M MOPS and 0.25 M Na_2-EDTA. The pH was adjusted to 8.0 by the addition of 32 g of NaOH. DNA fragments were separated by electrophoresis at 200 V for 6 h at 20 °C.

Silver staining of DNA

The DNA was visualised by silver staining using the following procedure: The fixation step was performed by immersing the gel in washing solution (10% ethanol and 0.5% glacial acetic acid) for 3 min twice. Then the gel was transferred to 0.1% $AgNO_3$ staining solution for 10 min, followed by two washing steps in distilled water for 10 s each. The colour development was achieved by washing the silver-stained gel in freshly prepared developer solution (1.5% NaOH and 0.01% $NaBH_4$ in 0.015% formamide), and the process was stopped by washing the gel in 0.75% Na_2CO_3 solution for 5–10 min.

Cluster analyses

A similarity matrix was constructed from the RFLPs for the seven restriction enzymes based upon visual observation of the presence or absence of specific bands with the same migration rate or molecular weight using the Sorensen–Dice coefficient. The NTSYS-pc software package (version 1.8, Exeter Software, Setauket, New York) was used to determine the coefficient of similarity for cluster analyses. The dendrograms were generated using the UPGMA (unweighted pair group method with arithmetic mean) and the reproducibility was assessed by comparison of the overall clustering with others generated by different methods and coefficients (Sneath and Sokal, 1973). Different clustering procedures and similarity coefficients tested were based on single linkage and complete linkage methods, using Sorensen/Dice, Jaccard, Ochiai and Euclidean distance coefficients (Digby and Kempton, 1987).

Analysis of molecular variance (AMOVA)

AMOVA was applied to create a distance matrix between samples in order to measure the genetic structure of the population from which the samples were drawn. The presence (scored as 1) and absence (scored as 0) of restriction markers for each isolate was used to produce a matrix defined by $E = \varepsilon^2_{xy} = n(1-2n_{xy}/2n)$, where $2n_{xy}$ is the number of markers shared by two isolates and n as the total number of polymorphic sites (Excoffier et al., 1992; Huff et al., 1993). In this study, the AMOVA considered variance components, partitioning the variation among soil types, among the host plant species within soil type and among the isolates within host plant species. The isolates origin were also taken into account by analysing all the isolates (analysis 1) or the isolate subpopulations, recovered from the outermost part of roots (analysis 2) or from the innermost part of roots (analysis 3) within each variance component partitioning. Significance levels of variance components were estimated based on permutational analysis and involved few assumptions about the statistical properties of the data. All analyses were performed with the AMOVA program (version 1.55) provided by Laurent Excoffier (Genetics and Biometry Laboratory – University of Geneva).

Results and discussion

The length and number of the 16S–23S rDNA intergenic spacer has been found to be highly variable in many taxonomic groups of bacteria. In different organisms, the number of the amplified products ranged from 2 to 13 but could not always be correlated with the number of the *rrn* coding regions in the genomes (Gürtler, 1999; Fogel et al., 1999). In our study, two amplification products, in the range of 1000 and 1300 bp (data not shown), were observed for the isolates of *A. amazonense*. These products were not separated prior to the restriction digestion. A previous study of five *Azospirillum* species demonstrated that the *A. amazonense* genome is composed of four replicons, two of which contain the 16S rDNA genes, the 2.65 and 1.75 Mbp replicons (Martin-Didonet et al., 2000). Therefore, these observations supported the idea that the presence of two RISA amplified products reflected the number of copies of the 16-23S rRNA intergenic region in the *A. amazonense*.

The restriction of the amplified 16S–23S rDNA intergenic spacer regions by seven different endonucleases yielded 52 distinct restriction profiles

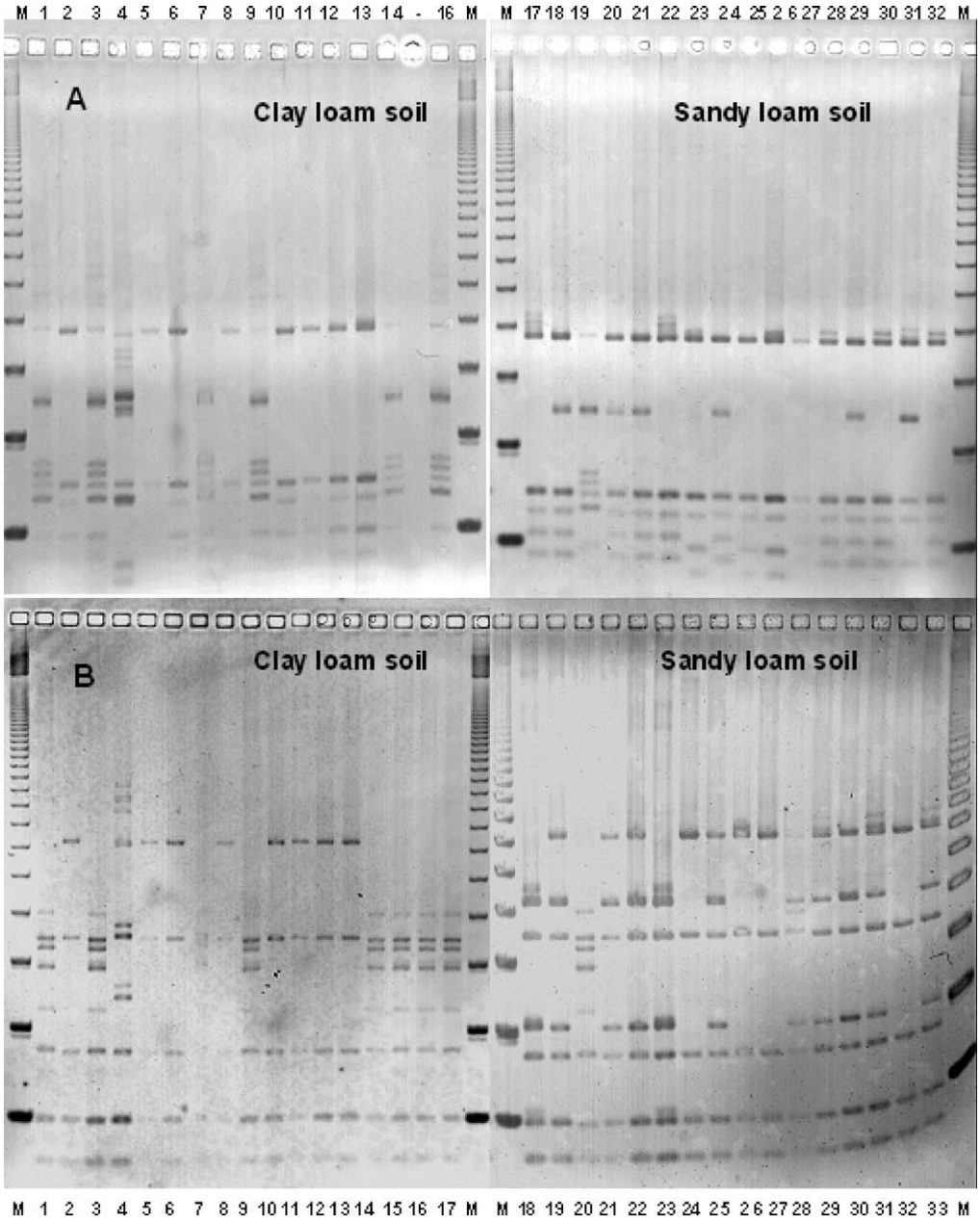

Figure 1. RFLP of 16S–23S rDNA intergenic spacer of *Azospirillum amazonense* isolates. (A) Isolates profiles generated by the enzyme *Cfo*I. Lanes 1–13 – maize/clay loam soil isolates ID: (1) Ym 10, (2) Ym 6, (3) Ym 148, (4) Ym 4, (5) Ym 11, (6) Ym 54, (7) Ym 69, (8) Ym 120, (9) Ym 5, (10) Ym 56, (11) Ym 127, (12) Ym 9, (13) Ym 49. Lanes 14–16 – rice/ clay loam soil isolates ID: (14) Ya 80, (15) – , (16) Ya 76; lanes 17–27 – rice/sandy loam soil isolates ID: (17) EYa 37, (18) EYa 13, (19) EYa 68, (20) EYa 90, (21) EYa 22, (22) EYa 44, (23) EYa 75, (24) EYa 18, (25) EYa 88, (26) EYa 73. Lanes 27–32 – sorghum/sandy loam soil isolates ID: (27) EYs 37, (28) EYs S-14, (29) EYs 89, (30) EYs 82, (31) EYs 43, (32) EYs 81. Lanes 27–32: sorghum/sandy loam soil isolates ID: (27) EYs 37, (28) EYs S-14, (29) EYs 89, (30) EYs 82, (31) EYs 43, (32) EYs 81. B: Isolates profiles generated by enzyme the *Rsa*I. Lanes 1–13 – maize/clay loam soil isolates ID: (1) Ym 10, (2) Ym 6, (3) Ym 148, (4) Ym 4, (5) Ym 11, (6) Ym 54, (7) Ym 69, (8) Ym 120, (9) Ym 5, (10) Ym 56, (11) Ym 127, (12) Ym 9, (13) Ym 49. Lanes 14–17 – rice/clay loam soil isolates ID: (14) Ya 80, (15) Ya 64, (16) Ya 75, (17) Ya 9. Lanes 18–27 – rice/sandy loam soil isolates ID:(18) EYa 37, (19) EYa 13, (20) EYa 68, (21) EYa 80, (22) EYa 22, (23) EYa 44, (24) EYa 75, (25) EYa 18, (26) EYa 88, (27) EYa 73. Lanes 28–33 – sorghum/sandy loam soil isolates ID: (28) EYs 37, (29) EYs S-14, (30) EYs 89, (31) EYs 82, (32) EYs 43, (33) EYs 81. M: 123 bp ladder molecular marker.

comprising a total of 120 markers. The largest number, 23, of polymorphic bands, or bands differing in migration rate or molecular weight, was obtained with the enzyme *Cfo*I (Fig. 1A). The enzymes *Rsa*I and *Msp*I each yielded 18 bands (Fig. 1B and data not shown). The other restriction

enzymes generated fewer polymorphic bands: *Alu*I (16), *Hae*III (16), *Taq*I (10) and *Eco*RI (9). In addition, the enzyme *Cfo*I generated 28 distinct restriction profiles, while the other enzymes produced far fewer distinct profiles: *Msp*I (19), *Rsa*I (18), *Alu*I (18), *Hae*III (13), *Taq*I (12) and *Eco*RI (11).

The overall cluster analysis of 71 *A. amazonense* isolates, recovered from the rhizosphere soil, washed and surface-sterilised roots, revealed the existence of five genetic clusters at 78% similarity (Fig. 2). To validate this result all isolates were clustered by other coefficients and methods. The dendrogram derived from the UPGMA method with the Sorensen–Dice coefficient was topologically similar and comprised the same isolates in each group of clusters formed with other clustering algorithms and similarity coefficients tested (data not shown). In addition, the cophenetic correlation coefficients or *r*, which are a test of the ultrametric properties of the data, were also very close to each other when the single linkage method were applied, 0.92 to Sorensen–Dice, Occhiai and Euclidean Distance coefficients and 0.93 to Jaccard coefficient, indicating the validity of this analytical method (data not shown). The relationship between (or among) the predominant clusters and the origin of the soil and plant types was also analysed (Fig. 2). The majority of the isolates obtained from clay loam soil were distributed among clusters I, II and IV, representing 79, 70 and 100% of each cluster, respectively. On the other hand, 91% of *A. amazonense* representatives of cluster III were isolated from host plants cropped in the sandy loam soil (Fig. 2). These results suggested that the soil type exerted a selective and strong influence on the genetic diversity of the *A. amazonense* isolates.

The composition of the clusters was also analysed according to the type of plant, either C_3 or C_4. Here, 11 clusters were observed (clusters a to k). These clusters consisted predominantly or exclusively of isolates from the same soil and plant type (Fig. 2). Isolates from rice, were present in clusters I, II and III, independent of the soil type, suggesting that a high diversity of *A. amazonense* population is associated with this C_3 plant. On the other hand, isolates from sorghum predominated in two clusters (III and IV) and a selective effect of soil type was also observed. The same tendency was observed for other C_4 plants, but the selective effect of soil type in relation to the diversity of maize isolates in clusters I and II was less pronounced. To examine the effect of plant type more closely, cluster analysis was performed considering only the subpopulation of isolates obtained from the surface-sterilised roots or the inner part of the host plant. Evidence of a relationship between a specific cluster and plant type cropped in a specific soil could be observed in clusters *c*, *e* and *j*, specifically for those isolates obtained from rice and sorghum cropped in clay loam soil (Figs. 2 and 3). Only four clusters containing 2, 3 or 4 isolates of the same origin (the same host plant and soil) had over 80% similarity. In this analysis isolates clusters with 100% similarity were considered as representative of one genomic strain. In this case any selective effect exerted by the plant type, as shown by maize isolates obtained from clay loam soil, on their genetic diversity was not taken into account (Fig. 3). On the other hand, three clusters of isolates from the outer part of each host plant cropped in clay soil type were observed (data not shown). These results suggest that the selective effect of the host plant cannot be determined without taking into account the soil type or environmental conditions.

To support these conclusions, AMOVA was applied to *A. amazonense* population and their subpopulations, defined according to their site of isolation – from the outer (rhizosphere and washed roots) or inner part of the plants (surface-sterilised roots). The overall genetic variation among the 71 isolates of *A. amazonense* associated within the soil type and plant species was highly significant ($P < 0.0002$), independent of the partitioning values observed in the variance components (analysis 1, Table 3). These results confirm the cluster analyses presented in Fig. 2. The variation observed among all isolates within the host plant species was highly significant and represented 71% of total observed variation ($P < 0.0002$). Similar results were observed among subpopulations from both the inner and outer part of plant roots (analyses 2 and 3 – Table 3). The soil type also exerted a highly significant ($P < 0.0002$) influence on the genetic variation of the isolates (19%). The effect of soil type was stronger, attributing 24% of the variance, when only the isolates from the outer part of the roots was considered. Among isolates from within the root, soil type accounted of only 16% of the variance, which was significant at $P = 0.0380$. These results suggest that the effects attributed to soil type are more intensely expressed in the subpopulation originating from rhizosphere soil and washed root than in the subpopulation from the inner part of the roots.

As previously shown by the cluster analyses, the effects exerted by the host plant are more easily perceived when isolates from the outer (data not shown) and inner parts of the roots (Fig. 3) are analysed separately. The effects of each host plant on the genetic diversity of *A. amazonense* were

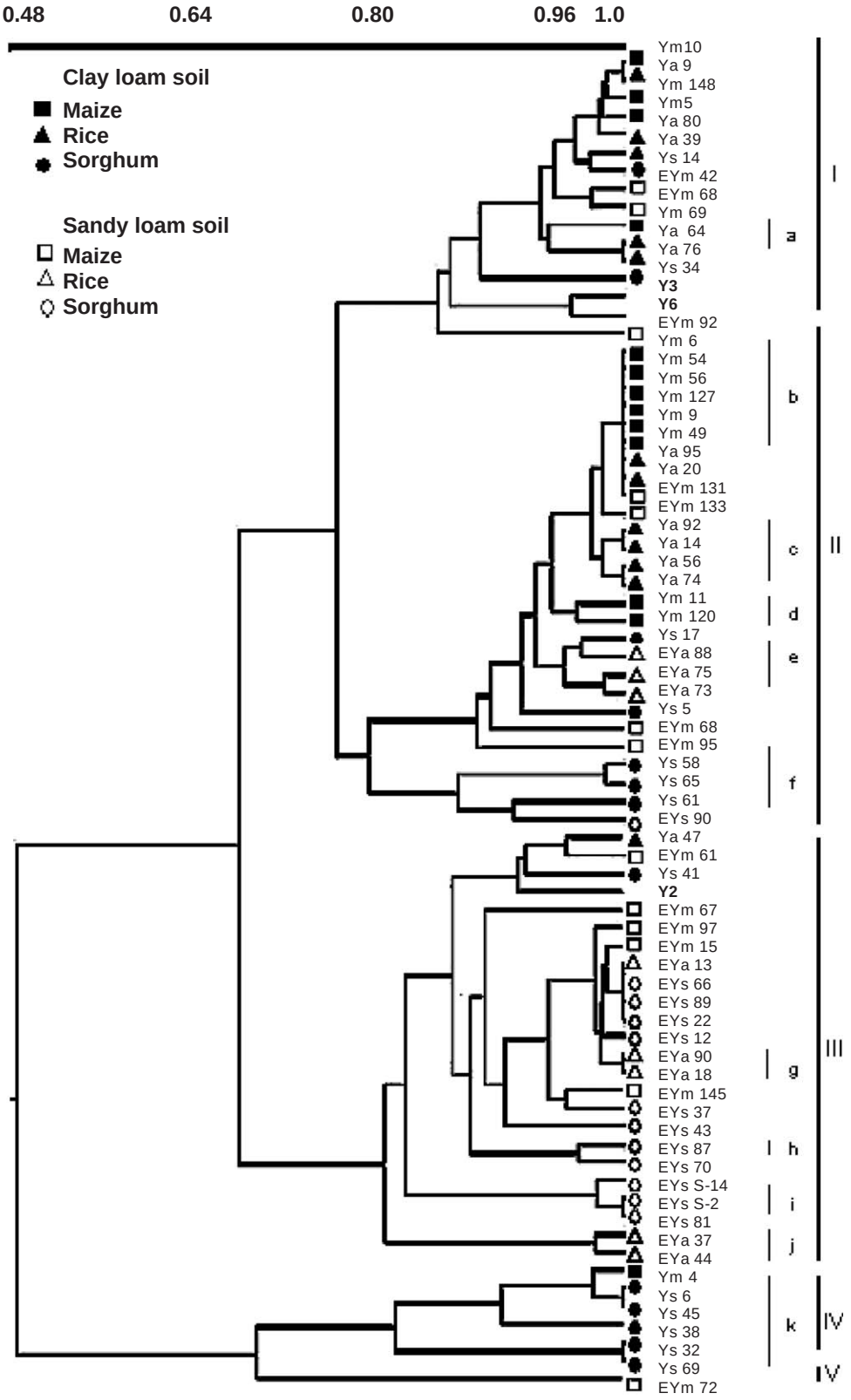

Figure 2. Similarity clustering of the 71 isolates of *Azospirillum amazonense* recovered from rhizosphere soil, washed and surface-sterilised roots. The dendrogram was generated by UPGMA using the Sorensen–Dice coefficient (cophenetic correlation coefficient or $r = 0.93$; $P = 0.0004$). The isolates were identified by the indicated symbols according to soils type or plants origin. Reference strains Y2, Y3 and Y6 are shown in bold.

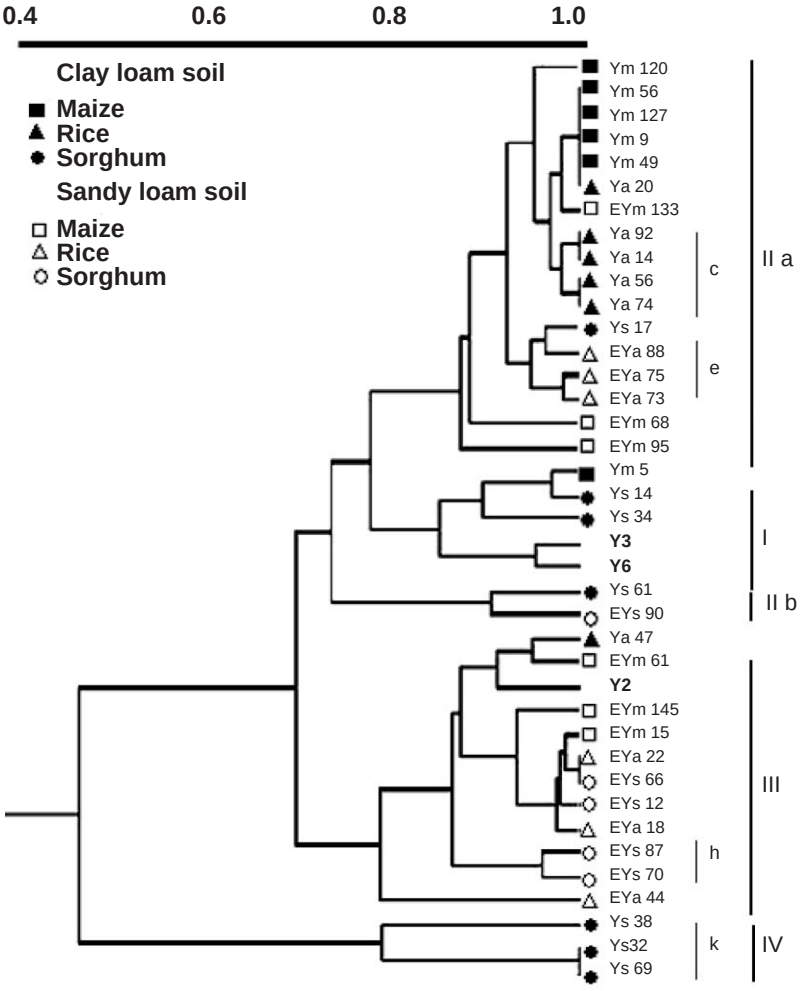

Figure 3. Similarity clustering of the 36 *Azospirillum amazonense* isolates recovered from surface-sterilised roots of different cereals cropped in two soil types. The dendrogram was generated by UPGMA using the Sorensen–Dice coefficient ($r = 0.92$; $P = 0.0004$). Reference strains Y2, Y3 and Y6 are shown in bold. The clusters are identified as in Fig. 2.

Table 3. Analysis of molecular variance (AMOVA) of *Azospirillum amazonense* isolates

Source of variation	df[a]	Observed partition % total	*P* value[b]
(1) All isolates			
Among soils	1	19.45	<0.0002
Among plants/soils	4	8.97	<0.0022
Among isolates/plants	65	71.58	<0.0002
(2) Isolates from the rhizosphere and washed roots			
Among soils	1	24.08	<0.0002
Among plants/soils	4	13.14	0.0052
Among isolates/plants	30	62.78	<0.0002
(3) Isolates from sterilised roots			
Among soils	1	16.01	0.0380
Among plants/soils	4	12.20	0.0186
Among isolates/plants	30	71.79	<0.0002

[a]Degrees of freedom.
[b]Probability of having a more extreme variance component determined by non parametrical permutational procedures (number of random permutations = 5000).

significant when the analysis included only the subpopulation from sterilised roots of plants cropped in the clay loam soil (20.94%, $P<0.0242$) but not in the sandy loam soil (5.5%, $P=0.1804$) (data not shown). However, when the subpopulation of isolates from the outer part of the plant was considered, the observed partitioning values among plants (17.10%) and among isolates within plants (82.90%) cropped in the sandy soil reveals that this soil type also exerted a significant effect ($P<0.0272$) on the genetic diversity of this species (data not shown).

The AMOVA results reinforce the hypothesis that the observed genetic diversity of the *A. amazonense* populations structure, based on at least four genetically distinct clusters, is highly heterogeneous and hierarchically influenced by the host plant species, the soil type and to a lesser extent by the interaction of both variance components (analyses 1–3 – Table 3). The differences between the two original environments used to grow the plants could partially explain this behaviour. Furthermore, clay loam and sandy loam soils from the experimental area represent distinct textural properties, water holding capacities and N levels; properties which could be correlated with the survival of *Azospirillum* in soil as reported by Bashan et al. (1995). Other studies with *Azospirillum* spp. from various fractions of fallow soils under the C_4 plant *Pennisetum*, including coarse organic residues, revealed that the diversity of *A. brasilense*/*A. amazonense* genomic species decreased concomitantly with long-term fallow in soil fractions of $F>2000\,\mu M$ and $F=2-50\,\mu M$ (Chotte et al., 2002). Surprisingly, the hot spots for nitrogen fixation could be correlated with soil fractions where *A. brasilense*/*A. amazonense* genomic species are prevalent after 3 years or after 19 years of fallow. Thus, differences in soil properties could in part explain the observed genetic diversity of the *A. amazonense* isolates.

Based on studies of plant infection and reports of occurrence of *Azospirillum* species in several plant species, Bashan and Holguin (1995) suggested that *Azospirillum* strains do not have preferences among particular plant species. However, our data provide information about the existence of selective effects exerted by the soil type and host plant on populations of *A. amazonense*. A few authors suggest that the amount and composition of the root exudates affects the nature and specificity of the soil-bacteria–plant interaction. In addition, the soil properties and textural traits can affect the characteristic impedance and consequently the root exudation process (Reinhold et al., 1985; Latour et al., 1996). The observed partial effects exerted by a specific plant cropped in a specific soil type could be due to a close interaction between the plant and the soil type as suggested by Chotte et al. (2002). Differences in the diversity of fluorescent pseudomonad strains isolated from two soil types were also demonstrated by Latour et al. (1996). The authors suggested that the differences could be ascribed to a higher carbon and iron competition in one soil associated with higher levels of microbial activity and reactivity in the other soil.

The chemotactic responses of 15 strains of *A. lipoferum* and *A. brasilense* strains originating from different C_3 and C_4 plant roots to maize and wheat root exudates suggested a general chemotactic behaviour by azospirilla rather than a specific host-dependent response (Fedi et al., 1992). Reinhold et al. (1985) also investigated the strain-specific chemotactic behaviour of *Azospirillum* and found differences between strains isolated from C_3 and C_4 plants, but commercial chemical compounds were used instead of fractions of native root exudates.

Our study demonstrated that the selective effects exerted by cereals with distinct photosynthetic pathways, C_3 (rice) or C_4 (maize and sorghum) are also dependent on the effect of the soil types on the population of *A. amazonense*. This result is in accordance with previous observations that genetically distinct strains were selectively associated with C_3 or C_4 plants. It is likely that the partial specific association patterns between the isolates and their site of origin result from interactions between all environmental factors determined by the host plant and the physical, chemical and biological properties of soil, especially those related to the composition of root exudates and the surface structure of the root associated with the soil type. In addition, intrinsic factors related to the bacteria could contribute to the observed community pattern, especially the chemotactic behaviour, carbon metabolism and rhizocompetence.

In this study, two statistical approaches, cluster analysis and AMOVA, were used. The potential of AMOVA to estimate the major factor responsible for the genetic diversity within a population has already been shown for populations of *Sinorhizobium meliloti* (Paffetti et al., 1996, 1998). These authors concluded that the genetic diversity within *S. meliloti* was significant among strains and could be attributed to the plant genotype. A similar type of investigation carried out by Dalmastri et al. (1999) showed a dominant effect of soil type on the genetic diversity of maize root-associated *Burkholderia cepacia*. However, no significant effect of the

root compartments (rhizoplane and rhizosphere) on the degree of diversity of this species was observed. In our study, AMOVA was able to estimate the hierarchical level of significance attributable to the host plant and soil type for populations of *A. amazonense*. These two approaches provide new information on the genetic diversity and structure of *A. amazonense* populations. It is also probable that part of the structural differences between the representatives of *A. amazonense* obtained from plants cropped in sandy or clay loam soil was due to distinct modes of interaction with the native microbial communities present in both soils – a subject for future studies.

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References

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

Arturo, A., Odelson, D.A., Hichey, R.F., Tiedje, J.M., 1995. Bacterial community fingerprinting of amplified 16S and 16–23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In: Akkermans, A.D.L., Elsas, J.D.V., Brujin, F.J. (Eds.), Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, pp. 1–8.

Baldani, V.L.D., Döbereiner, J., 1980. Host-plant specifity in the infection of cereals with *Azospirillum* spp. Soil Biol. Biochem. 12, 433–439.

Bashan, Y., Holguin, G., 1995. Inter-root movement of *Azospirillum brasilense* and subsequent root colonization of crop and weed seedlings growing in soil. Microbial. Ecol. 29, 269–281.

Bashan, Y., Holguin, G., 1997. *Azospirillum*–plant relationships: environmental and physiological advances (1990–1996). Can. J. Microbiol. 43, 103–121.

Bashan, Y., Puente, M.E., Rodriguez-Mendoza, M.N., Toledo, G., Holguin, G., Ferrera-Cerrato, R., Pedrin, S., 1995. Survival of *Azospirillum brasilense* Cd and Sp-245 in the rhizosphere of 23 soil types. Appl. Environ. Microbiol. 61, 1938–1945.

Bashan, Y., Holguin, G., de-Bashan, L.E., 2004. *Azospirillum*–plant relationships: physiology, molecular, agricultural, and environmental advances (1997–2003). Can. J. Microbiol. 50, 521–577.

Chotte, J.-L., Schwartzmann, A., Bally, R., Monrozier, L.J., 2002. Changes in bacterial communities and *Azospirillum* diversity in soil fractions of a tropical soil under 3 and 19 years of natural fallow. Soil Biol. Biochem. 34, 1083–1092.

Cruz, L.M., Souza, E.M., Weber, O.B., Baldani, J.I., Döbereiner, J., Pedrosa, F.O., 2001. 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa* spp.) and pineapple (*Ananas comosus* (L.) Merril). Appl. Environ. Microbiol. 67, 2375–2379.

Dalmastri, C., Chiarini, L., Cantale, C., Bevivino, A., Tabacchioni, S., 1999. Soil type and maize cultivar affect the genetic diversity of maize root-associated *Burkholderia cepacia* populations. Microbial. Ecol. 38, 273–284.

De-Polli, H., Bohlool, B.B., Döbereiner, J., 1980. Serological differentiation of *Azospirilllum* species belonging to different host-plant specificity groups. Arch. Microbiol. 126, 217–222.

Digby, P.G.N., Kempton, R.A., 1987. Ecological data. In: Digby, P.G.N., Kempton, R.A. (Eds.), Multivariate analysis of ecological communities. Chapman & Hall, London, pp. 1–26.

Döbereiner, J., 1992. The genera *Azospirillum* and *Herbaspirillum*. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.), The Prokaryotes, vol. 3. Springer, New York, pp. 2236–2253.

Eckert, B., Weber, O.B., Kirchhof, G., Halbritter, A., Stoffels, M., Hartmann, A., 2001. *Azospirillum doeberinerae* sp. nov., a nitrogen-fixing bacterium associated with the C_4-grass *Miscanthus*. Int. J. Syst. Evol. Microbiol. 51, 17–26.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.

Fedi, S., Montaini, P., Favilli, F., 1992. Chemotactic reponse of *Azospirillum* toward root exudates of C_3 and C_4 plants. Symbiosis 13, 101–105.

Fogel, G.B., Collins, C.R., Li, J., Brunk, C.F., 1999. Prokaryotic genome size and SSU rDNA copy number: estimation of microbial relative abundance from a mixed population. Microbial. Ecol. 38, 93–113.

Gürtler, V., 1999. The role of recombination and mutation in 16S–23S rDNA spacer rearrangements. Gene 238, 241–252.

Gürtler, V., Stanisich, V.A., 1996. New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. Microbiology 142, 3–16.

Hartmann, A., Baldani, J.I., 2003. The Genus *Azospirillum*. In: Prokaryotes – release 3.12. http://et.springer-ny.com:8080/prokPUB/index.htm.

Heckathorn, S.A., McNaughton, S.J., Coleman, J.S., 1999. C_4 plants and herbivory. In: Sage, R.F., Monson, R.K. (Eds.), C_4 Plants Biology. Academic Press, New York, pp. 285–312.

Huff, D.R., Peakall, R., Smouse, P.E., 1993. RAPD variation within and among natural populations of

outcrossing buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.]. Theor. Appl. Genet. 86, 927–934.

Kennedy, I.R., Choudhury, A.T.M.A., Kecskés, M.L., 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biol. Biochem. 36, 1229–1244.

Latour, X., Corberand, T., Laguerre, G., Allard, F., Lemanceau, P., 1996. The composition of fluorescent pseudomonad populations associated with roots is influenced by plant and soil type. Appl. Environ. Microbiol. 62, 2449–2456.

Magalhães, F.M., Baldani, J.I., Souto, S.M., Kuykendall, J.R., Döbereiner, J., 1983. A new acid-tolerant *Azospirillum* species. An. Acad. Bras. Ciên. 55, 417–430.

Martin-Didonet, C.C.G., Chubastu, L., Souza, E.M., Kleina, M., Rego, F.G.M., Rigo, L.U., Yates, M.G., Pedrosa, F.O., 2000. Genome structure of the genus *Azospirillum*. J. Bacteriol. 182, 4113–4116.

Paffetti, D., Scotti, C., Gnocchi, S., Fancelli, S., Bazzicalupo, M., 1996. Genetic diversity of an italian *Rhizobium meliloti* population from different *Medicago sativa* varieties. Appl. Environ. Microbiol. 62, 2279–2285.

Paffetti, D., Daguin, F., Fancelli, S., Gnocchi, S., Lippi, F., Scotti, C., Bazzicalupo, M., 1998. Influence of plant genotype on the selection of nodulating *Sinorhizobium meliloti* strains by Medicago sativa. Antonie von Leeuwenhoek 73, 3–8.

Reinhold, B., Hurek, T., Fendrik, I., 1985. Strain-specific chemotaxis of *Azospirillum* spp. J. Bacteriol. 162, 190–195.

Reis Jr., F.B., Silva, M.F., Teixeira, K.R.S., Urquiaga, S., Reis, V.M., 2004. Intra-specific diversity study of the nitrogen fixing bacterium *Azospirillum amazonense* isolated from different Brachiaria species. Symbiosis 36, 41–56.

Sneath, P.H.A., Sokal, R.R., 1973. Numerical Taxonomy – The Principles and Practice of Numerical Classification. W.H. Freeman, San Francisco.

Steenhoudt, O., Vanderleyden, J., 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol. Rev. 24, 487–506.

Stoffels, M., Castellanos, T., Hartmann, A., 2001. Design and application of new 16S rRNA-targeted oligonucleotide probes for the *Azospirillum–Skermanella–Rhodocista*-cluster. Syst. Appl. Microbiol. 24, 83–97.

Weber, O.B., Baldani, V.L.D., Teixeira, K.R.S., Kirchhof, G., Baldani, J.I., Döbereiner, J., 1999. Isolation and characterization of diazotrophic bacteria from banana and pineapple plants. Plant Soil 210, 103–113.