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Assessment of the mycorrhizal community in the rhizosphere of maize (*Zea mays* L.) genotypes contrasting for phosphorus efficiency in the acid savannas of Brazil using denaturing gradient gel electrophoresis (DGGE)

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ABSTRACT

Community structure of indigenous arbuscular mycorrhizal fungi (AMF) in the rhizosphere of tropical maize genotypes contrasting for phosphorus efficiency was evaluated using denaturing gradient gel electrophoresis (DGGE). Fragments of AMF ribosomal DNA (rDNA) were amplified using nested PCR with fungal universal primers and ITS (internal transcribed spacer) specific primers for Acaulosporaceae, Glomaceae and Gigasporaceae. ITS-Acaulosporaceae and Glomaceae-specific primers and DGGE were efficient in differentiating the composition of mycorrhizal communities. Maize genotypes had a greater influence on the rhizosphere mycorrhizal community than the level of P in the soil. DGGE profiles of maize roots revealed bands that were present only in P efficient genotypes (L3 and HT3060), suggesting that some mycorrhizal groups were stimulated by P efficient maize genotypes. *Acaulospora longula*, *A. rugosa*, *A. scrobiculata*, *A. morrowiae* and *Glomus caledonium* were found only in the rhizosphere of P efficient maize genotypes cultivated in low P soils. A greater number of mycorrhizal DGGE bands were found in soil samples from no-till maize than conventional tillage. Effective mycorrhizal colonization of crops may influence the maize yield under Brazilian savanna acid soils by modulating the capacity of different cultivars to tolerate P deficiency.

1. Introduction

Low phosphorus (P) availability limits plant growth in many acid soils of the tropics such as the Oxisol soils of the Brazilian acid savannas (Cerrado), which are characterized by low pH, low P, and high P fixation capacity (Novais and Smyth, 1999; Hinsinger, 2001). Arbuscular mycorrhizal fungi (AMF) have an important function in soil nutrient acquisition and mobilization, principally phosphorus. The role of AMF for nutrient acquisition is due to extending the root system by increasing the surface area for nutrient uptake and enhancing the ability of the plants to scavenge for scarce and immobile nutrients, particularly P (van der Heijden et al., 1998; Smith et al., 2003; Koide and Mosse, 2004; Gosling et al., 2006). The external mycelium mass can be as much as 3% of root mass and approximately 10–100 m of mycorrhizal mycelium can be found for each centimeter of root length (Cardoso and Kuyper, 2006). As a symbiotic

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system, the fungi receive carbon sources from the host plant and provide nutrients to the plants (Grigera et al., 2007). AMF also improve the soil aggregate stability, as their extraradical hyphae can bind to soil particles mechanically and chemically through the exudation of glomalin (van der Heijden et al., 1998; Wright et al., 2005).

AMF root colonization in terms of contribution to nutrient acquisition differs markedly among fungal species, isolates, and host plant genotypes (Abbott and Robson, 1991; Marschner, 1995, 1998; Koide, 2000; Siqueira et al., 2002; Wright et al., 2005). Several reports have described positive effects of mycorrhizal association under low P soil conditions in cereals, such as improvement in maize plant development and yield (Clark and Zeto, 1996). However, little information is available regarding the interaction of maize genotypes contrasting for P acquisition efficiency with AMF present in the rhizosphere of tropical maize.

According to Gosling et al. (2006) arbuscular mycorrhiza can be used in agriculture to increase crop yields while minimizing the requirements on chemical fertilizers. In spite of numerous studies claiming substantial yield increases, mycorrhizal technology is still far from being routinely applied in agricultural practices. The main reasons for this are problems in identifying and tracking fungal species in the field, the poor understanding of the basic biology of AMF and the inability to grow these obligatory biotrophic fungi in pure cultures and in trap cultures. Selection of appropriate AMF, production of inocula in quality and quantity, and the ecology of mycorrhiza inoculation are critical issues for the application of AMF technology in agriculture production (Simon et al., 1992; Gianinazzi et al., 2002; Barea et al., 2005a,b).

Cloning, sequencing and real-time PCR of products amplified with AMF-specific primers have contributed to the characterization of culture-independent AMF communities (Simon et al., 1992; Helgason et al., 1998; Redecker et al., 2000; Jansa et al., 2008; Liang et al., 2008). Molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) of ribosomal DNA (rDNA) fragments amplified from total community DNA have been widely used to evaluate the composition of bacterial and fungal communities (Muyzer and Smalla, 1998). The combination of DGGE with sequencing of amplified DNA fragments can be applied to analyze phylogenetic relationships of the community members. Few studies (Kowalchuk et al., 2002; Öpik et al., 2003; de Souza et al., 2004; Ma et al., 2005; Liang et al., 2008) have used this approach to study mycorrhizal fungi communities in the soil and rhizosphere.

The objective of this study was to evaluate the indigenous mycorrhizal community structure in the rhizosphere of maize genotypes contrasting for P acquisition efficiency and the effects of the maize cultivars on the fungal community structure in the acid savanna soils with high and low P. A set of PCR primers which amplify ribosomal DNA from five genera in the Glomeromycota (Redecker, 2000) was used to study a wide portion of AMF colonization. Additional evaluations of AMF colonization in maize roots and spore number in rhizosphere were made by stereomicroscopic visualization.

2. Materials and methods

2.1. Field experimental design and sampling

The field experiment was performed in an Oxisol soil (Brazilian savanna biome—Cerrado), during the summer (December through March), at Embrapa Maize and Sorghum located in Sete Lagoas, Minas Gerais, Brazil, at latitude 19°28′S and longitude 44°15′W, at an altitude of 732 m. The local climate is the Aw, according to the Köppen classification, with a mean temperature 22 °C, mean annual rainfall 1300 mm, and a mean relative humidity of 70%. The experiment was a 2 × 8 factorial, soil P levels and genotypes respectively using a randomized complete block design with three replications. The maize genotypes consisted of three P efficient hybrids (HT3060, HS228xL3, HS20x723), two P inefficient hybrids (HS2841x5046, HS26x1113), two efficient inbred lines (L228, L3) and one inefficient inbred line (L22), classified as differing in phosphorus acquisition efficiency according to Parentoni and Souza Júnior (2008). Each experimental plot consisted of 2 rows 5 m long with 0.8 m between rows and 0.2 m between plants, which were planted in two conventionally managed systems: high phosphorus (high P), with 29 mg kg^{-1}, determined using a Mehlich I extraction (Embrapa, 1997) and a low phosphorus (low P), with 3 mg kg^{-1} (Mehlich I). The soil was a clay texture red Oxisol with pH of 5.2 (soil/water ratio, 1:2.5 [w/v]), organic matter (3%); Al (0.25), Ca (2.29), Mg (0.36) $cmol_c\ kg^{-1}$ (dry mass) of soil in a 1N KCl extraction and 62 mg kg^{-1} of K, in a Mehlich I extraction. The original available P status in this soil was 3 mg kg^{-1} (representing approximately only 30% of the critical level of P for maize causing severe P stress in these tropical soils) and the high P managed system was correct to 29 mg kg^{-1} obtained after fertilization with superphosphate (45% P_2O_5) broadcast and incorporated into the soil at 20 cm depth. The available P was determined before the samples were taken.

The samples were composed of the roots and soil adhering to the roots of five random plants within each plot, collected from a depth between 0 and 20 cm for each of three replicates, 60 days after sowing during the flowering stage. The roots were shaken vigorously to separate the rhizosphere soil from the roots. The bulk soil samples were taken between rows from low and high P conditions of the treatments described above. Additional soil samples were taken from a no-till maize field and native savanna vegetation as controls. The samples used for DNA extraction were stored at 4 °C during transport to the laboratory, where the samples were sieved to remove plants debris, transferred to sealed glass flasks, and stored in nature at −20 °C for 4 months until DNA extraction.

2.2. Spore isolation and spore counts

Arbuscular mycorrhizal fungi spores were extracted from rhizosphere soil samples of each genotype in triplicate from 100 g of rhizosphere soil by sucrose centrifugation and flotation and collected on 250, 125, 63 and 32 mm mesh wet sieves according to the methodology described by Clapp et al. (1996).

2.3. AMF staining and root examination

Individual roots of each genotype were picked randomly from the root samples collected, and cut into 1 cm length pieces. One hundred root fragments for each sample were analyzed for the presence of AMF mycelia after root staining. Before the staining, roots were clarified in 5% (w/v) KOH for 1 h, followed by acidification with 1% HCl (v/v) overnight. The roots were stained with 0.05% Trypan Blue (w/v) for 20 min in lactoglycerol acid and were visualized under stereomicroscope. Colonization percentage was assessed using the grid line intersect method (Giovannetti and Mosse, 1980).

2.4. Spore and total community DNA extraction

DNA was extracted from 500 mg of soil of a pooled sample composed of the three replicates using the protocol described in the soil DNA extraction and purification kit (FastDNA SPIN kit for soil, Bio 101 Inc., La Jolla, CA, USA). DNA of AMF isolated spores was used as a control in all denaturing gradient gel electrophoresis (DGGE). Isolates from the Cerrado biome of *Acaulospora morrowiae* (grassland isolate), *A. serobienta* (coffee isolate), *Gigaspora margarita* (maize isolate), *Glomus clarum* (grassland isolate), *G. etunicatum* (grassland isolate) and *Scutellospora* sp. (maize isolate) were provided by Prof. J.O. Siqueira, Federal University of Lavras, Minas Gerais, Brazil. DNA from spores were extracted directly from trap cultures of vermiculite containing pure culture of the spores using the FastDNA SPIN kit for soil according to the modified protocol described by Chellius and Triplett (1999).

2.5. Total AMF-specific PCR conditions

PCR amplification of AMF 18S ribosomal genes fragments was performed using a nested PCR with fungal universal primers, NS1 and NS4 (White et al., 1990) for the first step and AMF-specific primer, VANS1 and NS21 (Simon et al., 1992) for the second step. The VANS1 primer was attached with CG clamp (Muyzer and Smalla, 1998). Amplification reactions were performed in a final volume of 50 μl using 50 μM of each dNTP, 2.5 mM $MgCl_2$, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1% (v/v) formamide, 0.2 μM of each primer, 20 ng of DNA and 1 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR conditions were: 95 °C for 2 min, followed by 40 cycles of first step and 25 cycles of second step (nested) at 94 °C for 20 s, 55 °C for 35 s, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. Amplified fragments were separated on a 1.5% (w/v) agarose gel, stained with ethidium bromide (1 μg ml^{-1}), and visualized under UV Eagle Eye II (Stratagene, La Jolla, CA, USA).

2.6. Specific PCR conditions for Acaulosporaceae, Glomaceae, Gigasporaceae families

The first nested PCR step to obtain Acaulosporaceae and Glomaceae-specific amplification products from soil was performed combining the primers NS5 and ITS4 (White et al., 1990). These amplification products were used as a template for a second PCR step using the primers ACAU1660, GLOM1310 (Redecker et al., 2000) with GC clamp specific to Acaulosporaceae and Glomaceae, respectively, in combination with the primer ITS2 (White et al., 1990). Gigasporaceae-specific fragments were obtained after a first step amplification using the primers pair ITS1 and ITS4 and a second step with ITS1 and GIGA5.8R (Redecker et al., 2000) attached with GC clamp. PCR conditions were the same as described above.

2.7. Analysis of PCR products by DGGE

DGGE was performed with the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). A volume of 20 μl of the PCR reaction was applied directly onto polyacrylamide gel in 1× TAE buffer (20 mM Tris–acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM disodium EDTA). Denaturing gradients varied for each primer combination, which were 35–60% for VANS1/NS21 and ACAU1660/ITS2, 25–50% for GLOM1310/ITS2, and 40–70% for ITS1/GIGA5.8R. These gradients were formed with 6% (w/v) acrylamide stock solutions containing 0 and 100% denaturant condition [7 M urea and 40% (v/v) deionized formamide]. Fragments were separated under electrophoresis at 75 V for 16 h in 0.5× TAE buffer at a constant temperature of 60 °C. Gels were silver stained according to Creste et al. (2001) and the images were digitally captured by a Nikon digital camera.

2.8. Sequencing purified fragments from DGGE gels and sequence alignment

Amplified DNA fragments were recovered using a second DGGE gel using the same conditions but stained with ethidium bromide. The most prominent bands were excised and transferred to a microcentrifuge tube containing 50 μl of ultrapure water, incubated at room temperature for two hours and frozen overnight. Some bands were purified and new gels were prepared to test the existence of double bands and contamination. Subsequently, the gel fragment was centrifuged at 11,000 × *g* for 60 s, the supernatant was transferred to a new tube, and 5 μl of eluted DNA was used as a template for an additional PCR reaction with the same conditions. PCR products were separated on a 1.5% (w/v) agarose gel, stained with ethidium bromide (1 μg ml^{-1}), and visualized under UV Eagle Eye II (Stratagene, La Jolla, CA) transillumination. PCR products were purified using QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and sequenced using the kit "Big Dye Terminator v3.1 Cycle Sequencing" (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Nucleotide sequence data were compared with those from the GenBank (http://www.ncbi.nlm.nih.gov/) using the BlastN program (Altschul et al., 1997).

2.9. Data analysis

Percentage values of root colonization and spore counts were transformed using arcsin before factorial analysis of variance (ANOVA) among maize cultivars and phosphorus levels. Tukey's test at 5% significance was applied for comparison of means when appropriate.

DGGE banding patterns were assessed by cluster analysis with a percent disagreement coefficient, and the differences among the profiles of AMF communities were depicted as a

dendrogram constructed by the unweighted pair group with mathematical average method (UPGMA) using the software Statistica version 6.0.

3. Results

3.1. *Mycorrhizal colonization and AMF spores*

The occurrence of indigenous mycorrhizal infection in maize varied among genotypes and P levels in the soil (Fig. 1A and B). Although, the percentage of mycorrhizal colonization was not clearly different between P efficient and inefficient maize hybrids (Fig. 1A), the P efficient inbred lines (Fig. 1B) showed higher mycorrhizal colonization in comparison with the inefficient line for both levels of P. Colonization of the P inefficient line was significantly lower than the efficient lines at both levels of P in the soil (Fig. 1B). L3 under P stress showed an increase in AMF-like infection of about 20% when compared with high P soil.

The recovery of AMF spores from different rhizosphere soils of maize cultivars also varied between genotypes and P levels (Fig. 1C and D). For the maize hybrids, the number of spores was greater in the low P soils, except for the P inefficient hybrid HS26x1113 (Fig. 1C). However, for the P efficient maize inbred line, L3, the total number of spores was superior in the high P soils compared to the low P soils (Fig. 1D).

3.2. *DGGE analysis of AM fungal rhizosphere colonization*

The primers used in this study, VANS1/NS21, ACAU1660/ITS2, GLOM1310/ITS2 and GIGA5.8R/ITS1 amplified PCR products with the expected sizes of 590, 445, 645 and 440 base pairs, respectively. Subsequent DGGE analysis were conducted with pooled samples (no variation was observed between replicates) of PCR products composted from three replicates of the field samples taken from one lane to DGGE. The DGGE patterns for VANS1/NS21 fragments from the maize rhizosphere amplified a wide range of non-AMF taxon, indicating that the VANS1 primer was not able to distinguish the AMF populations between the maize genotypes.

Thus, DGGE analysis was conducted with fungal specific rDNA primers covering three families of Glomeromycota; the Acaulosporaceae, Glomaceae and Gigasporaceae. All different specific AMF primers revealed detectable fragments in the DGGE, from which bands were excised from the gels, reamplified and sequenced (Table 1).

3.3. *Acaulosporaceae and Glomaceae-specific DGGE and sequence analysis of amplified bands*

PCR-DGGE of the Acaulosporaceae/Glomaceae-specific primers for the ITS rDNA gene differentiated all rhizosphere samples (Figs. 2 and 3). Almost all the samples yielded multiple bands, indicating the presence of ribotype variability within the rhizospheres examined.

The Acaulosporaceae-specific DGGE banding patterns of the maize hybrids rhizosphere revealed one major cluster, which grouped most of the P efficient hybrids at a genetic distance of 0.26, except for the P efficient HT3060 under low P (Fig. 2A). Additionally, the two P inefficient hybrids were clustered in different groups depending on the level of P in the soil, both of them at a genetic distance of 0.21.

A greater number of bands of the Acaulosporaceae DGGE were observed in the maize lines, but with no clear discriminatory factor of the cluster analysis (Fig. 2B). However,

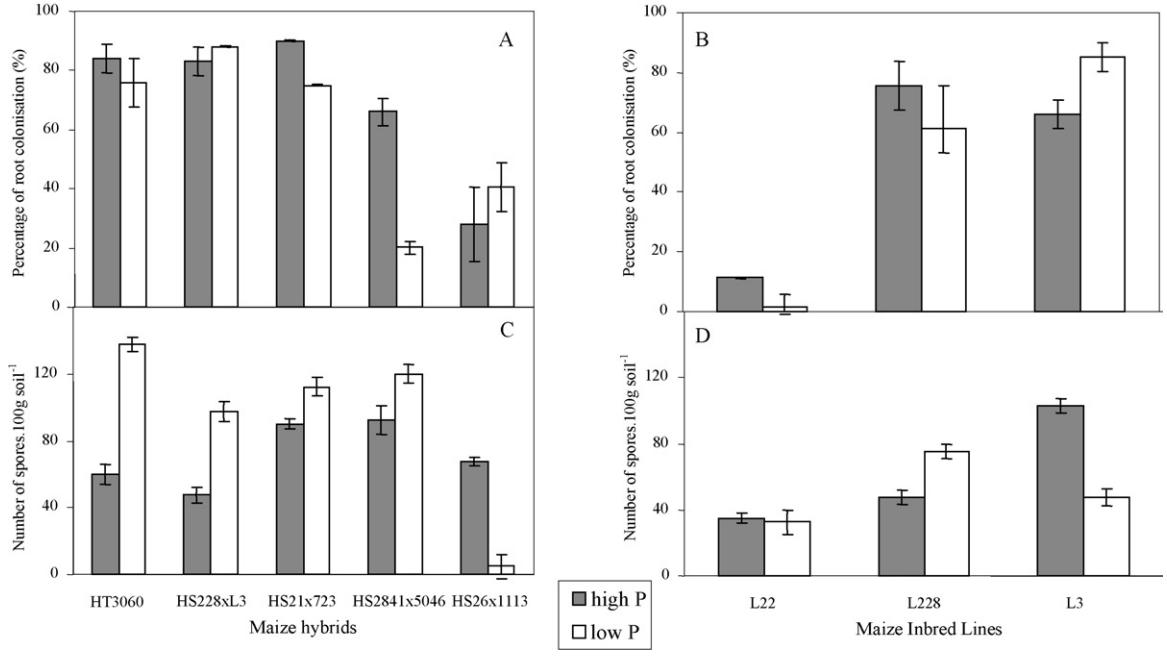

Fig. 1 – Colonization of maize roots growing under low P soil fertilization (3 mg kg^{-1}) and high P (29 mg kg^{-1}) by indigenous mycorrhizal fungi. The hybrids in graphs A and C were: HT3060, HS228xL3, HS20x723 (P efficient), and HS2841x5046, H26x1113 (P inefficient). The inbred lines in graphs B and D were: L228, L3 (P efficient) and L22 (P inefficient). Each data columns represent a mean of three replicates; error bars represent the standard error at the 5% level.

Table 1 – The closest species of the mycorrhiza present in maize rhizosphere that were recovered from DGGE bands.

DGGE band[a]	Closest species		
	Microorganisms[b] (% similarity)	Phylogenetic affiliation	GenBank accession number[b]
Acaulosporaceae-specific DGGE			
1	*Acaulospora scrobicula* (88%)	Acaulosporaceae	AJ306442
2	*A. morrowiae* (88%)	Acaulosporaceae	AJ242500
3	*Glomus intraradices* (94%)	Glomaceae	AF185692
4	*G. intraradices* (97%)	Glomaceae	AF185692
5	*Glomus* sp. 0191 (98%)	Glomaceae	AY174693
6	*A. rugosa* (93%)	Acaulosporaceae	Z14005
7	*Ajellomyces capsulatus*[c] (100%)	Ajellomycetaceae	AF322387
8	Soil clone	–	
9	*Archaeospora* sp. (93%)	Archaeosporaceae	AF452636
10	*A. longula* (89%)	Acaulosporaceae	AJ306439
11	*Archaeospora* sp. (94%)	Archaeosporaceae	AF452636
12	*G. intraradices* (100%)	Glomaceae	AF185692
13	*Archaeospora* sp. (94%)	Archaeosporaceae	AF452636
14	*G. intraradices* (96%)	Glomaceae	AF185692
15	*Glomus* sp. (98%)	Glomaceae	AF452626
16	*A. morrowiae* (88%)	Acaulosporaceae	AJ242500
Glomaceae-specific DGGE			
17	*Glomus caledonium* (98%)	Glomaceae	Y17635
18	*Glomus mosseae* (91%)	Glomaceae	X96828
19	*Glomus* sp. clone 0171 (94%)	Glomaceae	AY174691.1
20	*Glomus* sp. clone W3347 (98%)	Glomaceae	AJ301857
21	*Glomus* sp. clone 0171 (96%)	Glomaceae	AY174691.1
22	*Glomus* sp. clone W3347 (96%)	Glomaceae	AJ301857
23	*Glomus* sp. clone 0171 (95%)	Glomaceae	AY174691.1
24	*G. coremioides* (95%)	Glomaceae	AJ249715
25	*G. coremioides* (96%)	Glomaceae	AJ249715
26	*G. coremioides* (98%)	Glomaceae	AJ249715
27	*Glomus* sp. 5014b25 (90%)	Glomaceae	AF480158.1
28	*G. manihotis* (93%)	Glomaceae	Y17648
29	*Glomus* sp. clone 0171 (96%)	Glomaceae	AY174691.1
30	*Glomus* sp. clone W3347 (92%)	Glomaceae	AJ301857
31	*Glomus* sp. 5014b24 (93%)	Glomaceae	AF480157.1
32	*G. mosseae* (98%)	Glomaceae	X96828
33	*Glomus* sp. 5014b25 (93%)	Glomaceae	AF480157.1
34	*Glomus* sp. clone 0171 (95%)	Glomaceae	AY174691.1
Gigasporaceae-specific DGGE			
35	*Scutellospora cerradensis* (85%)	Gigasporaceae	AB048690.1
36, 37	Soil clone	–	
38	*Gigaspora margarita* (100%)	Gigasporaceae	AY442360.1

[a] DGGE bands designation are as labeled in Figs. 2–4.
[b] Most related isolate from GenBank by BLAST results.
[c] Species belong to Ascomycota order.

the bulk soil (low P) sample and the L22 (low P) rhizosphere sample were clustered at a genetic distance of 0.06 and supported by a 90% bootstrap, indicating that this line, under low P, probably did not influence the AMF community. The most distinct DGGE banding pattern was observed in the Acaulosporaceae community of native Cerrado, suggesting that maize cultivation affected the mycorrhizal Acaulosporaceae community. In addition, the samples from the no-tillage system were clustered at 0.11 genetic distance with a support of 87% bootstrap, distinctly different from the conventional rhizosphere samples.

Sequence analysis did not confirm the family specificity of some bands amplified with Acaulosporaceae-specific primers once species of Ascomycota phylum and the Glomaceae family were identified, as *Ajellomyces capsulatus* and *Glomus* sp., respectively (Table 1). *Acaulospora* species were found in the rhizosphere of the P efficient maize genotypes L3 (low P) and HT3060 (low P), corresponding to *A. scrobiculata* (1), *A. morrowiae* (2), *A. rugosa* (6), and *A. longula* (10) species (Fig. 2, Table 1). Band 8 was found in the rhizosphere of most of the hybrids but its sequence did not align with any sequence deposited in the GenBank, indicating that this sequence belongs to an unknown species. Different bands amplified in some unique DGGE lanes were identified as belonging to the same species, like bands 3 and 4 that corresponded to *Glomus intraradices* (Table 1, Fig. 2).

The dendrogram based on the banding patterns of the Glomaceae-specific DGGE generated a cluster at a genetic distance of 0.18 that included the rhizosphere of the P efficient and inefficient maize hybrids cultivated in high P soils

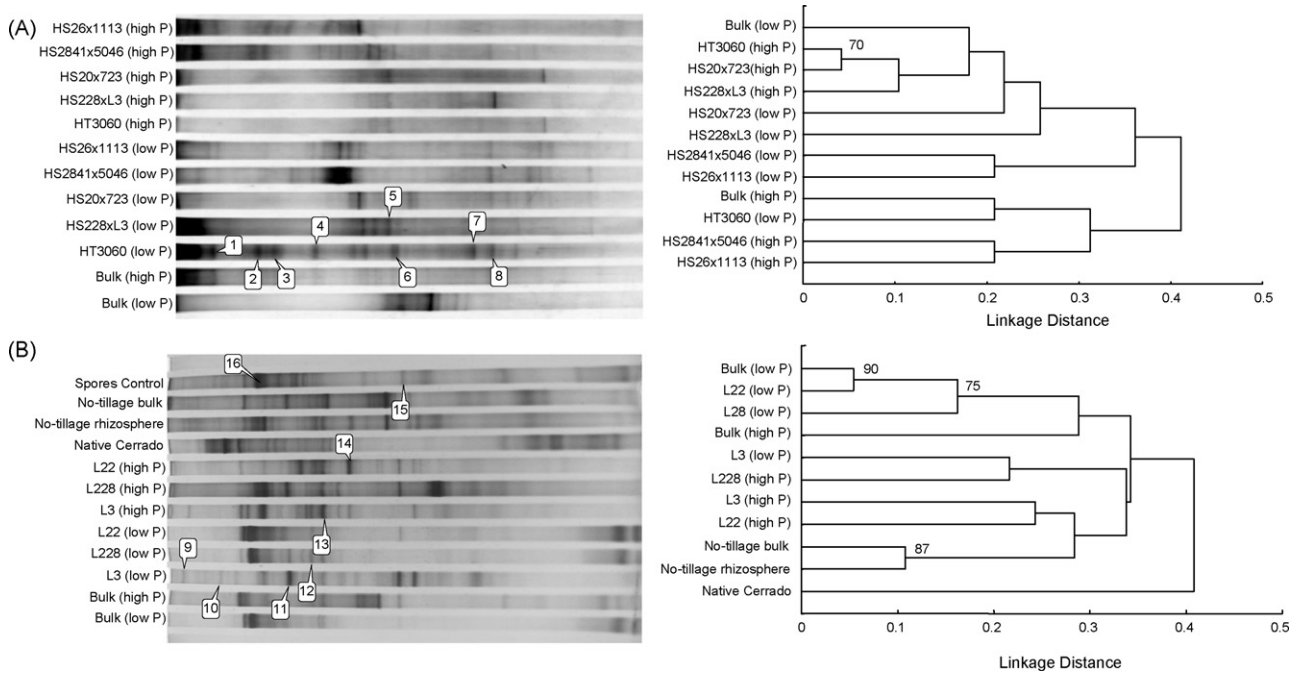

Fig. 2 – Acaulosporaceae-specific PCR-DGGE banding patterns of rhizosphere soil and the dendrogram from maize hybrids (A) and inbred lines (B). The dendrogram was obtained by the unweighted pair group method with mathematical averages (UPGMA). The hybrids in graphs were: HT3060, HS228xL3, HS20x723 (P efficient), and HS2841x5046, H26x1113 (P inefficient). The inbred lines in graphs were: L228, L3 (P efficient) and L22 (P inefficient). Lane spores control, composed by PCR products of *Acaulospora morrowiae* and *A. serobienta* spores. The numbers indicate different bands among the samples identified in Table 1. Bootstrap analyses were performed with 10,000 repetitions.

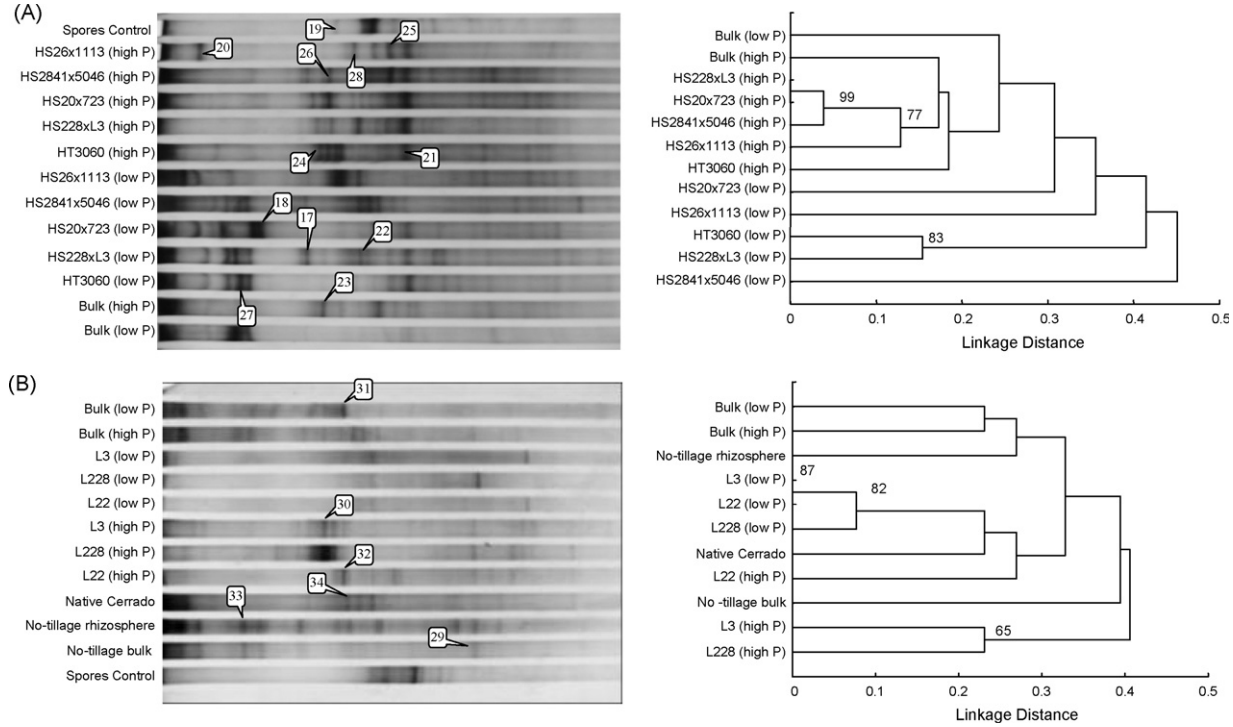

Fig. 3 – Glomaceae-specific PCR-DGGE banding patterns of rhizosphere soil and the dendrogram from maize hybrids (A) and inbred lines (B). The dendrogram was obtained by the unweighted pair group method with mathematical averages (UPGMA). The hybrids in graphs were: HT3060, HS228xL3, HS20x723 (P efficient), and HS2841x5046, H26x1113 (P inefficient). Lane spores control, composed by PCR products of *Glomus clarum* and *G. etunicatum* spores. The inbred lines in graphs were: L228, L3 (P efficient) and L22 (P inefficient). The numbers indicate different bands among the samples identified in Table 1. Bootstrap analyses were performed with 10,000 repetitions.

(Fig. 3A). However, the low P soils were not consistently grouped, except for the two P efficient hybrids (HT3060 and HS228xL3) that formed clusters based on the bootstrap value of 83%. The greatest *Glomus* spp. DGGE bands in the rhizosphere of maize hybrids was found in low P soils. The AMF populations of two different P efficient maize hybrids under low P (HT3060 and HS228xL3) were grouped at a genetic distance of 0.16 (bootstrap 83%) (Fig. 3B) in contrast with the P inefficient hybrids.

The P availability in the soil seems to be a putative discriminating factor of clustering the Glomaceae populations of the maize lines rhizosphere, which were grouped under low P at a genetic distance of 0.08 and supported by a bootstrap value of 82% (Fig. 3B). The P efficient maize lines rhizosphere, L228 and L3 in the high P soil were also grouped at a genetic distance of 0.23 (65% bootstrap). Additionally, the rhizosphere profiles obtained under high P soil showed exclusive bands that could not be visualized in low P soils (see bands 30, 32, Fig. 3B).

Sequence analysis (Table 1) confirmed the identity of all fragments from Glomaceae-DGGE bands as belonging to the genus *Glomus*, with some bands being exclusively present in the rhizosphere of the P efficient maize genotypes (Fig. 3), such as *Glomus caledonium* in HS228xL3 (low P). However, similarity of some sequences is not sufficiently high (91%) to confirm the species, as shown in Table 1 to *Glomus mosseae*.

3.4. *Gigasporaceae-specific DGGE and sequence analysis of excised DGGE bands*

The specific Gigasporaceae PCR-DGGE yielded bands that provided a low level of discrimination within the different maize samples. The fingerprints generated showed a lower number of bands than the Acaulosporaceae and Glomaceae families. A prevalent monomorphic band was identified as *G. margarita* (band 38, Table 1) in the rhizosphere of all the maize hybrids (data not shown) and lines (Fig. 4). However, an exclusive band (band 35, Fig. 4) presented in the L228 under high P was identified as *Scutellospora cerradensis*. The Gigasporaceae community was not influenced by the P availability in the soil or by the maize genotypes. However, the no-tillage system generated bands that were absent in the conventional crop management and in the native Cerrado (see arrows and band 36, Fig. 4).

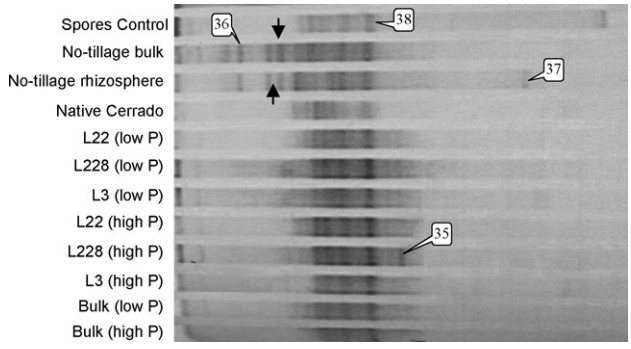

Fig. 4 – Gigasporaceae-specific PCR-DGGE banding patterns of rhizosphere soil of maize inbred lines. The inbred lines were: L228, L3 (P efficient) and L22 (P inefficient). Lane spores control, composed by PCR products of *Gigaspora margarita* and *Scutellospora* sp. spores. The numbers indicate different bands among samples identified in Table 1. Arrows indicate bands present in the no-tillage system and absent in the conventional system and in the native Cerrado.

4. Discussion

The knowledge of the structure and composition of the mycorrhizal community in the rhizosphere is important to evaluate the environmental effects, such as phosphorus stress, on plant species. Preliminary fingerprints of the rhizosphere mycorrhizal community were generated by DGGE using universal primer pairs designed by Simon et al. (1992) to assess the diversity of maize rhizosphere indigenous AMF populations cultivated under low and high P soil. The 18S rDNA region amplified by the VANS1/NS21 primer pair provided a low level of discrimination among the samples and the majority of the sequenced bands were not related to the Glomeromycota phylum. Studies have shown that the VANS1 annealing site is not well conserved within the Glomeromycota (Clapp et al., 1999) and that several newly characterized ancestral lineages of the Glomeromycota do not have this site at all (Redecker et al., 2000). Similar results were observed by Ma et al. (2005) and Öpik et al. (2003) using the AM1 and NS31 primers described by Helgason et al. (1998) and Simon et al. (1992).

Thus, the PCR-DGGE method using specific primers to ITS rDNA fragments designed by Redecker et al. (2000) was better suited to amplify species of Acaulosporaceae, and Glomaceae in the rhizosphere of maize. These data, together with the visual analyses of AMF colonization showed that both maize genotypes and the level of P in the soil influenced the total population density (spores number and AMF root colonization) as well as the AMF species (DGGE bands).

Few differences were found in spore density among P efficient and inefficient genotypes evaluated. In contrast, the Acaulosporaceae and Glomaceae PCR-DGGE profiles showed exclusive bands, found only in the P efficient genotypes under low P. In general, these results showed that AMF root colonization and DGGE profiles in a majority of the efficient maize genotypes available in this study were different from the inefficient maize genotypes AMF community, suggesting that the level of P efficiency of maize genotypes may influence the community of AMF in the rhizosphere. The difference in the occurrence of mycorrhiza species may depend upon root exudations and/or signaling of the maize genotype under P stress. According to Lynch and Whipps (1990), Marschner (1998), Barea et al. (2005b), root exudates are crucial determinants of rhizosphere microorganism diversity. Plants grown in soils with P deficiency can exudate functional substances, such as organic acids, jasmonic acid, phosphatases and phenolic compounds which can stimulate colonization and growth of the mycorrhiza (Gianinazzi-Pearson et al., 1989; Siqueira et al., 1991; Marschner, 1998; Lambais, 2000; Hinsinger, 2001; Koide and Mosse, 2004) and influence host-AMF specificity.

The variability in mycorrhizal responsiveness among different maize cultivars has also been observed by Kaeppler et al. (2000) and Wright et al. (2005). The ability of plants to grow under P stress and the efficiency of mycorrhiza colonization are both strongly influenced by the host genotype (Marschner, 1995; Smith et al., 2003; Wright et al., 2005). Several authors have demonstrated that the maize genotypes used in this study have significant differences in P efficiency (Alves et al., 1999; Parentoni and Souza Júnior, 2008). Some authors have documented increases in maize productivity with AMF colonization (Clark and Zeto, 1996; Kaeppler et al., 2000; Wright et al., 2005) and others have shown the effect of the crop genotype and soil type on the bacteria and fungi community profiles (Gomes et al., 2001, 2003; Mota et al., 2002). This is the first report with these maize genotypes contrasting for P efficiency demonstrating AMF diversity and community structure in the acid savannas of Brazil. The understanding of the mechanisms that increase P acquisition can be very useful in delineating strategies in maize breeding programs to select genotypes which favor early support of large populations of AMF (Alves et al., 1999; Picard et al., 2008), and continued research to characterize AMF community composition including genes controlling host signaling is warranted.

The specific Acaulosporaceae PCR-DGGE was efficient in differentiating AMF populations in the maize rhizosphere samples evaluated in this study. However, sequence analysis did not confirm the identity of some bands amplified with AMF-specific primer (ACAU1660) being identified as species of other groups of fungi and others families of Glomeromycota. Further primer refinement (Redecker et al., 2000) should increase the reliability of PCR-based strategies targeting specific AMF families or genera such as the primer (ACAU1660) was not efficient for the DGGE method used in this study.

Some AMF-like sequences did not match with any sequence deposited in the GenBank, indicating that these sequences may belong to new AMF, still not identified. A double-band pattern of *Glomus* was observed in this study, similarly to that reported by Kowalchuk et al. (2002) and de Souza et al. (2004) for *Glomus* and *Gigaspora* genera, respectively. Some authors have suggested that operon heterogeneity may be responsible for these double bands (Sanders et al., 1995; Clapp et al., 1999; Kowalchuk et al., 2002; Ma et al., 2005). The variability of some rRNA loci in an AMF community may lead to complex DGGE banding patterns from a single spore (Ma et al., 2005). This heterogeneity in rRNA markers within a species or a single individual has been described for a wide range of organisms (Kuhn et al., 2001; Dahllof et al., 2000; Araujo da Silva et al., 2003), including AMF fungi (Clapp et al., 1999; Kowalchuk et al., 2002; Sanders, 2002; de Souza et al., 2004; Liang et al., 2008). The origin of this diversity is still undetermined but the heterokaryotic nuclei of AMF could explain the presence of unusual polymorphisms within ribosomal DNA revealed by DGGE profiles (Kuhn et al., 2001). However, this issue was not addressed in our study because the DNA was extracted from soil samples containing multiple spores.

Mycorrhiza populations of the three AMF families described above was also affected by the cropping system. Clear differences were found between conventional tillage and no-tillage management systems, forming separated groups by the DGGE profiles of Acaulosporaceae and Glomaceae. Additionally, specific bands (for example 33, 36) were obtained from the no-till system for all AMF families evaluated. Greater mycorrhizal populations in a maize no-till system were also found by Jansa et al. (2003). Kabir (2005) evaluated AMF hyphal density and nutrient content in maize cultivated under no-till and conventional tillage for 11 years and found that AMF hyphae length and densities in the maize rhizosphere was highest in the no-tillage treatment. These changes in communities of AMF colonizing maize roots might be due to (1) differences in hyphae maintenance disruption caused by tillage, (2) changes in organic matter and nutrient content of the soil, (3) changes in microbial activity, and/or (4) changes in weed populations in response to the soil tillage system. Different AMF species differ in their symbiotic efficiency (Smith et al., 2003), and thus any induced changes in AMF community structure could lead to changes in crop nutrient uptake and/or productivity.

DGGE fingerprints based upon rDNA fragments amplified by nested PCR from community DNA were effective in studying mycorrhiza community structures in the maize rhizosphere. This analysis was supported by DNA sequencing, except for the Acaulosporaceae primers used and by visualization of mycorrhizal colonization and spore counts. These results clearly show that the maize genotypes and the P level in the soil can influence the mycorrhiza populations, indicating that some mycorrhizal groups are enhanced by efficient maize genotypes cultivated under contrasting P availability in the soil.

Understanding the community structure of the native AM fungi in crop root systems is a prerequisite for effective management in sustainable agricultural systems, especially in acidic soils with low P, typically encountered in the Brazilian Cerrado. The productivity of maize may depend upon the symbiotic effectiveness of the colonizing mycorrhiza and the maize cultivar. The use and understanding of the genetic control of the exudation of mycorrhiza signaling products by the host plant and the use of these metabolites should be helpful in managing useful native AMF populations.

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