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Bacterial and Fungal Communities in Bulk Soil and Rhizospheres of Aluminum-Tolerant and Aluminum-Sensitive Maize (*Zea mays* L.) Lines Cultivated in Unlimed and Limed Cerrado Soil

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Liming of acidic soils can prevent aluminum toxicity and improve crop production. Some maize lines show aluminum (Al) tolerance, and exudation of organic acids by roots has been considered to represent an important mechanism involved in the tolerance. However, there is no information about the impact of liming on the structures of bacterial and fungal communities in Cerrado soil, nor if there are differences between the microbial communities from the rhizospheres of Al-tolerant and Al-sensitive maize lines. This study evaluated the effects of liming on the structure of bacterial and fungal communities in bulk soil and rhizospheres of Al-sensitive and Al-tolerant maize (*Zea mays* L.) lines cultivated in Cerrado soil by PCR-DGGE, 30 and 90 days after sowing. Bacterial fingerprints revealed that the bacterial communities from rhizospheres were more affected by aluminum stress in soil than by the maize line (Al-sensitive or Al-tolerant). Differences in bacterial communities were also observed over time (30 and 90 days after sowing), and these occurred mainly in the Actinobacteria. Conversely, fungal communities from the rhizosphere were weakly affected either by liming or by the rhizosphere, as observed from the DGGE profiles. Furthermore, only a few differences were observed in the DGGE profiles of the fungal populations during plant development when compared with bacterial communities. Cloning and sequencing of 16S rRNA gene fragments obtained from dominant DGGE bands detected in the bacterial profiles of the Cerrado bulk soil revealed that Actinomycetales and Rhizobiales were among the dominant ribotypes.

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Aluminum (Al) toxicity has been recognized as a major limiting factor for plant productivity in acidic soils [27]. As the soil pH drops to below 5, toxic forms of aluminum (Al^{3+}) become soluble in the soil solution, interfering with a wide range of physical and cellular processes, which leads to the inhibition of root growth and consequently the reduction of crop yield. Acidic soils constitute approximately 40% of the arable land on Earth [27] and, in Brazil, Cerrado soil represents over 2 million km^2, corresponding to about 22% of the territory of the country [10]. As agriculture is continuously being increased in this area, Al toxicity is a cause for great concern.

One alternative to minimize the problem of Al toxicity is to identify and/or develop plants with improved tolerance to Al. In nature, different plant species and genotypes have developed resistance mechanisms and are able to grow healthily in soils with high concentrations of Al [4, 22]. Moreover, efforts are continuously being made worldwide to produce genetically modified plants with enhanced resistance to Al [28]. For example, the breeding program developed by the Brazilian Agricultural Research Corporation (EMBRAPA) has produced maize lines that show differences in Al tolerance [7, 33]. Understanding the mode of action of these tolerance mechanisms has been the focus of ongoing research in the area, and the exudation of Al-chelating organic acids into the rhizosphere has been proposed as a potential strategy used by plants to avoid Al toxicity [27].

Previous studies have demonstrated that root exudates can selectively influence the growth of bacteria and fungi

in the rhizosphere by altering the chemistry of the soil in the vicinity of the plant roots and by the liberation of selective growth substrates for soil microorganisms [6]. Microbial communities can vary in structure depending on soil type, plant, species, nutritional status, age, stress of the plant and other environmental factors [8, 11, 16, 17, 29, 38].

An alternative to prevent the occurrence of Al toxicity in acid soils is to apply lime to raise the soil pH. However, liming may also affect the activity and composition of microbial populations [39], and the microbial responses to lime can vary with soil type encompassing the kind and amount of organomineral colloids [14, 21]. Although liming is a common agricultural practice, relatively little is known about its impact on the microbial communities in tropical soils.

A range of studies on the microbial community structures of soil and/or rhizospheres have been performed on the basis of DNA extracted directly from these habitats [8, 11, 17, 36, 38]. The diversity of target genes, such as the 16S and/or 18S rRNA genes, can be assessed by means of molecular fingerprinting approaches such as PCR-DGGE. This is a strategy to overcome the limitations imposed by the cultivation-based techniques that allow the analysis of only a minor fraction of the microbial community [20].

Maize (*Zea mays* L.) is widely cultivated throughout the world. Based on the Food and Agriculture Organization of the United Nations, the United States produces almost half of the world's harvest. However, China, France, South Africa, Indonesia, and Brazil are also important producing countries. In 2004, close to 145.2 million hectares of maize were harvested worldwide, yielding a production value of more than US$ 70.5 billion (http://faostat.fao.org). The primary use for maize is as a feed for livestock, forage, silage, or grain. The grain also has many industrial uses, including transformation into plastics and fabrics. Maize grain can be hydrolyzed and enzymatically treated to produce a sweetener, and can also be fermented and distilled to produce ethanol. Increasingly, ethanol is being used as an additive in gasoline for motor fuels to reduce the emission of pollutants (http://www.ncga.com/WorldOfCorn/main; http://en.wikipedia.org/wiki/maize). To overcome the problem of maize yield reduction due to Al toxicity, one or both strategies (*i.e.*, introduction of improved acid soil-tolerant lines and the amelioration of soil acidity using liming) are currently in use. However, the effects on the microbial community present in soil amended with lime are unknown, specifically in the Cerrado soils, and also in the rhizospheres of maize lines showing different tolerance levels to Al.

In this study, we evaluated the influence of soil liming (*i.e.*, reducing the amount of exchangeable Al^{3+} from 30% to 0%) on the structure of bacterial and fungal communities in bulk soil and the rhizospheres of Al-sensitive and Al-tolerant maize lines at two plant growth stages (*i.e.*, 30 and 90 days after sowing) by PCR-DGGE fingerprinting. Furthermore, we attempted to identify dominant members of these bacterial communities.

MATERIALS AND METHODS

Experimental Conditions, Soil, and Maize Lines

The field experiment was carried out at EMBRAPA Maize and Sorghum, Sete Lagoas, Minas Gerais, Brazil, located at latitude 19° 28' S and longitude 44° 15' W, at a height of 732 m above sea level. The local climate is the typical savannah climate type, according to the Köppen classification. The soil was classified as a typical Distrophic Red Latosol, Cerrado stage, with a clayey texture (coarse sand 6%, fine sand 4%, silt 12%, and clay 78%). High amounts of exchangeable acid cations (namely Al^{3+} and H^{+}) in this area reach levels that are toxic to many plants. Before sowing, half of the experimental field was limed with ground dolomitic limestone (2.5 tons per hectare) to alleviate aluminum toxicity and the other half was kept unlimed. Physical and chemical analyses of the unlimed and limed soils were performed before sowing and are presented in Table 1. Three lines of maize (Cateto 237/67, L16, and L3) were obtained after at least eight endogamic generations in the Breeding Program of EMBRAPA (MG, Brazil) and were chosen based on their tolerance of aluminum (Al), determined *in vitro* (data not shown). They are classified as highly Al-tolerant (L3), moderately Al-tolerant (Cateto 237/67), and Al-sensitive (L16). The experimental plots consisted of three rows of 5 m length with spaces of 0.8 m between rows and 0.2 m between plants, made in three replicates. The three maize lines were planted randomly in each row and in limed and unlimed soils. Thirty and 90 days after sowing, five plants of each maize cultivar were harvested and the roots shaken manually to remove the loosely attached soil. The soil adhering to the roots of the five plants was pooled and considered as the rhizosphere soil. Bulk soil (nonroot-associated soil) samples were also collected 30 and 90 days after sowing. The samples were kept at −20°C until DNA extraction.

Table 1. Physical and chemical characteristics of unlimed and limed soils.

Soil sample (0–20 cm)	pH	H+Al	Al	Ca	Mg	O.M.	Sat
Unlimed soil	5.0	9.20	0.95	1.72	0.25	37.8	30
Limed soil	6.3	3.58	0.10	4.92	1.71	35.1	0

Soil samples were characterized as described in the manual of methods of EMBRAPA [12].
H+Al=potential acidity ($cmol_c/dm^3$); Al=Al^{3+}, exchangeable acidity ($cmol_c/dm^3$); Ca and Mg=availability of Ca^{2+} and Mg^{2+} ($cmol_c/dm^3$); O.M.=organic matter expressed in g/kg soil; Sat=Aluminum saturation in %.

Quantification of Plant Growth

Plant growth was quantified based on the grain yield and the dry mass (DM) of three replicates (constituted of five plants) per experimental treatment (limed and unlimed soil) of the Cateto, L3, and L16 maize lines 90 days after sowing, using the methodology described by the AACC [1]. The average DM values obtained were compared by the Tukey test at 1% and 5% probability levels.

DNA Extraction from Bulk and Rhizosphere Soils

DNA was extracted from bulk and rhizosphere soil samples (0.5 g of each) using the Fast DNA Spin Kit for soil (Qbiogene, BIO 101 Systems, CA, U.S.A.) according to the manufacturer protocol. DNA concentrations were determined spectrophotometrically using a GeneQuant apparatus (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The DNA extracts were also visualized on 0.8% (w/v) agarose gels to assess their integrity and purity.

PCR Amplification of Bacterial 16S rRNA Genes

Fragments of 16S rDNA (correspondent to the V6-V8 region of the *E. coli* 16S rRNA gene) were PCR amplified with primers 968F-GC (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G AAC GCG AAG AAC CTT AC-3') and 1401R (5'-CGG TGT GTA CAA GAC CC-3') as described by Nübel *et al.* [31]. The 50-µl reaction mix contained 1 µl of template DNA (corresponding to approximately 15 ng), 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2.5 mM $MgCl_2$, 0.2 µM of each dNTPs, 1.25 U of *Taq* DNA polymerase (Promega, Madison, WI, U.S.A.), and 0.2 µM of each primer. The amplification conditions were 1× (2 min, 94°C), 35× (1 min, 94°C; 1.5 min, 48°C; 1.5 min, 72°C), and a final 10 min extension at 72°C. Negative controls (without DNA) were run in all amplifications. The PCR products were visualized by agarose gel electrophoresis (1.4% gel) followed by staining with ethidium bromide. Amplicons were stored at −20°C until DGGE analysis.

PCR Amplification of Fungal 18S rRNA Gene Fragments

Amplifications of 18S rRNA gene fragments of fungal communities were obtained using the semi-nested protocol described by Oros-Sichler *et al.* [32]. The 25-µl reaction mix contained 1 ml of template DNA (corresponding to approximately 15 ng of DNA), 0.2 µM of both primers NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') and EF3 (5'-TCC TCT AAA TGA CCA AGT TTG-3'), 10 mM Tris-HCl, pH 8.3, 10 mM KCl, 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 2% (w/v) dimethyl sulfoxide, and 2 U of *Taq* DNA polymerase. The amplification conditions were 1× (5 min, 94°C) followed by 25× (30 s, 94°C; 45 s, 47°C; 3 min, 72°C) and a final extension at 72°C for 10 min. Amplicons obtained in this first PCR reaction were diluted in water (500×) and used as template for a second amplification with primers NS1 and FR1-GC (5'-CCC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC G AIC CAT TCA ATC GGT AIT-3'). The reaction mixture was the same as described above. The annealing temperature during the PCR cycling was increased 1°C and the number of cycles was decreased from 25 to 20. Negative controls (without DNA) were run in all amplifications. PCR products were visualized by agarose gel electrophoresis (1.4% gel) followed by staining with ethidium bromide. Amplicons were stored at −20°C until DGGE analysis.

DGGE

DGGEs were carried out using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad Laboratories, Munich, Germany). PCR products (10–15 µl) were applied directly onto 6% (w/v) polyacrylamide gels in 1× TAE buffer (40 mM Tris-acetate [pH 7.4], 20 mM sodium acetate, 1 mM disodium EDTA) containing a denaturing gradient of urea and formamide varying from 45% to 65% and from 25% to 40% for 16S rDNA and 18S rDNA analysis, respectively. The gels were run for 15 h at 60°C and 100 V (16S rRNA gene fragments) and for 16 h at 60°C and 150 V (18S rRNA gene fragments). After electrophoresis, the gels were stained for 30 min with SYBR Green I (Invitrogen - Molecular Probes, SP, Brazil) and photographed under UV light by using a STORM apparatus (Amersham Pharmacia Biotech).

Comparison of DGGE Fingerprints by Hierarchical Cluster and Principal Component Analyses

Matrices indicating the presence or absence (scored as 1 or 0, respectively) of bands in bacterial and fungal fingerprints were constructed for each DGGE gel. Data were further analyzed by multivariate statistical analyses: (1) hierarchical cluster analysis (HCA) and (2) principal component analysis (PCA). Hierarchical cluster analysis was performed using the DICE coefficient of similarity and the unweighted pair group method with arithmetic mean (UPGMA) using the NTSYS software package (version 2.02; Exeter Software, Setauket, U.S.A.). To validate the clusters obtained by HCA, the data of the matrixes were analyzed *via* principal component analysis (PCA) using the software STATISTICA 6.0.

Cloning and Sequencing of DGGE Bands

Selected bands were retrieved from DGGE gels and purified with a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, U.S.A.). After purification, DNA fragments were cloned using the pGEM-T easy vector according to the instructions of the manufacturer (Promega). After transformation of competent *E. coli* JM109 cells, clones were picked and the presence of inserts of the correct size was assessed by PCR using M13f (5'-TAAAACGACGGCCAG-3') and M13r (5'-CAGGAAACAGCTATGAC-3') primers. The clones were sequenced using M13f and M13r primers by an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequences were identified using the Seqmatch facility of the RDPII database (http://rdp.cme.msu.edu/) and BLAST-N facility (http://www.ncbi.nlm.nih.gov/blast) of the National Center for Biotechnology Information with the GenBank nonredundant database.

RESULTS

Physical and Chemical Characteristics of Soils

The physical and chemical characteristics of the soils (untreated and treated with lime) are presented in Table 1. As expected, the limed soil exhibited an increase in the pH value and a decrease in the Al content and in the percentage of aluminum saturation (30% to 0%). An increase of Ca and Mg contents was also observed after the lime treatment.

Plant Growth on Limed and Unlimed Soils

When cultivated in limed soil (0% Al), the three maize cultivars were visually higher and healthier than plants

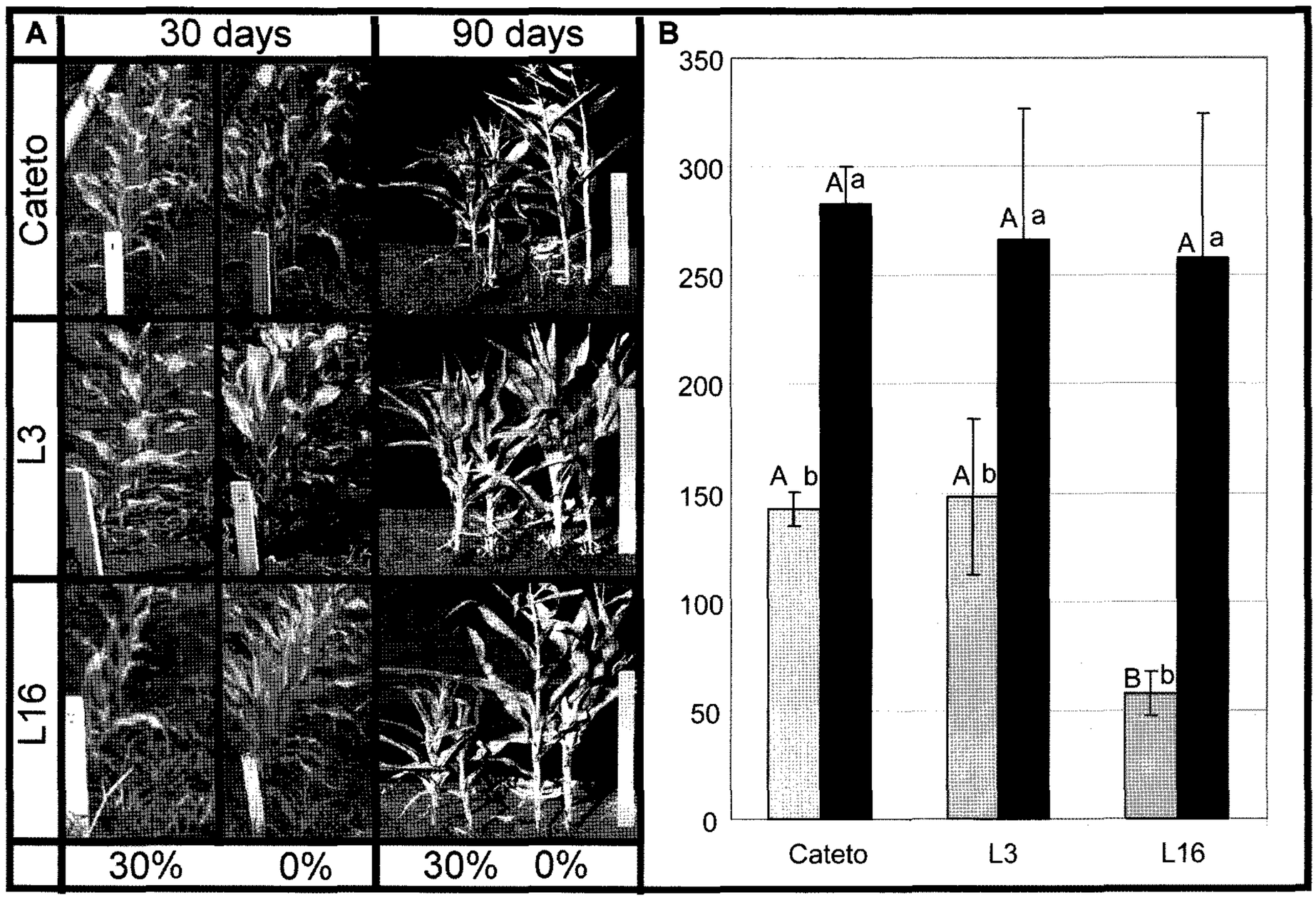

Fig. 1. **A**. Growth of maize lines under Al-stressing (unlimed, 30% Al) and nonstressing (limed, 0% Al) conditions of Cerrado soil, 30 and 90 days after sowing. **B**. Dry mass (g) of Cateto, L3, and L16 maize lines, 90 days after sowing. The averages of the dry mass of five plants of each maize line were compared by the Tukey test at 1% (AB) and 5% (ab).
The black columns correspond to plants sown in limed soil and the gray ones correspond to plants sown in unlimed soil. A–B or a–b represent statistically different samples. Error bars indicate standard error.

growing in unlimed soil (30% Al), 30 and 90 days after sowing (Fig. 1A). Moreover, the grain yield and dry mass of each maize line were differentially affected by the aluminum condition of the soil, 90 days after sowing. Maize line L3 exhibited a reduction of its grain yield of about 50% (from 2,474 to 1,243 kg/ha), Cateto of 72% (from 1,182 to 332 kg/ha), and L16 of 95% (from 1,348 to 61 kg/ha) under the aluminum stressing condition of unlimed soil. When the three maize lines were cultivated in limed soil, they revealed similar dry mass values (either in Tukey test 1% or 5% probability levels); however, in unlimed soil, the dry mass values were significantly different for, on the one hand, the Cateto and L3 lines and, on the other hand, L16 (Tukey test 1%, Fig. 1B). For this reason, Cateto and L3 were considered to be Al-tolerant lines and L16 an Al-sensitive one.

Analysis of Bacterial DGGE Fingerprints

DNA was recovered from all rhizosphere and bulk soil samples. Community fingerprints were generated for each sampling event within two growth stages (30 and 90 days). Reproducible DGGE profiles of bacterial 16S rDNA were obtained between duplicate bulk and rhizosphere soil samples (Figs. 2A and 2B). The DGGE profiles were used for the construction of dendrograms using hierarchical cluster analysis (HCA). Thirty days after maize sowing (Fig. 2A), the profiles of lines Cateto, L3, and L16 clustered together, per soil treatment (*i.e.*, limed versus unlimed) (Fig. 2A). The bulk soil samples (limed and unlimed) also clustered together, in a separate cluster. DGGE patterns of bulk soil were more similar to those obtained for the rhizospheres of maize lines grown in aluminum-stressing condition (separated at 66% similarity) than to those from lime-treated soil. At 31% similarity, the different maize lines sown in either the limed or the unlimed soils were separated in the dendrogram. In both cases, the communities associated with lines L3 and L16 were closer to each other than to those of line Cateto. Bulk soil profiles (0 and 30% Al) were separated only at 83% similarity, indicating a low effect in soil of the liming treatment after 30 days.

After 90 days of plant growth (Fig. 2B), the profiles of lines Cateto, L3, and L16 again clustered together in two broad clusters, in accordance with soil treatment (liming versus non-liming). The different maize lines sown in lime and unlimed soils were separated at 61% similarity in the dendrogram. Strikingly, the profiles obtained from lines L3 and L16 in limed soil showed 100% similarity. After the

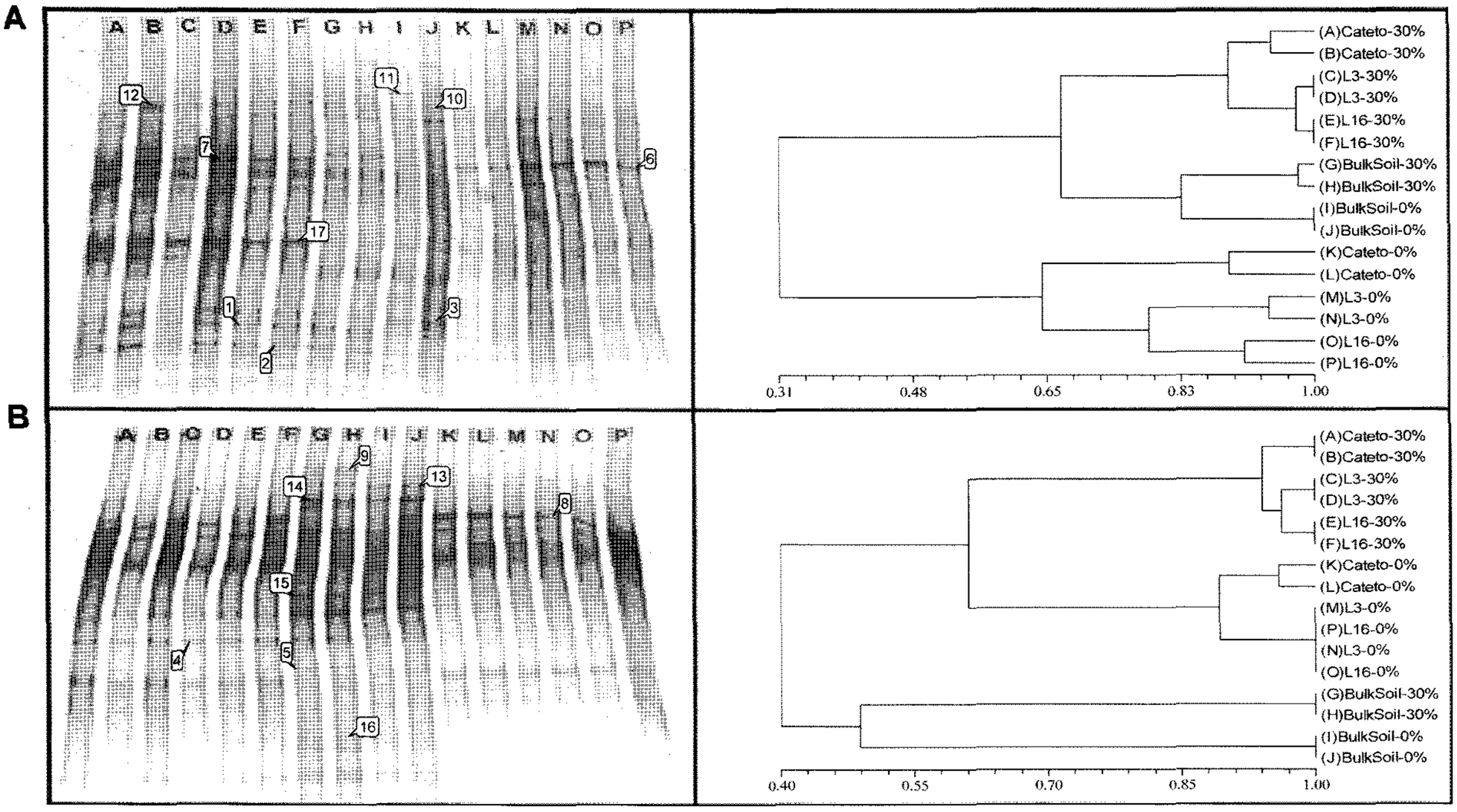

Fig. 2. Denaturing gradient gel electrophoresis (DGGE) fingerprints of bacterial 16S rRNA gene fragments amplified from bulk and rhizospheres (Cateto, L3, and L16 maize lines) soil DNA templates.

Gels obtained for the samplings performed 30 days (**A**) and 90 days (**B**) after sowing with under aluminum-stressing and nonstressing conditions and their respective dendrograms. Lanes A to P in DGGE correspond to the same letters in the dendrograms. Numbers in bullets indicate bands that were extracted from the gels prior to cloning and sequencing analysis.

90-day period, profiles obtained from bulk soil were separated from those from rhizospheres at 40% similarity. Soil profiles (0 and 30% Al) were separated at 49% similarity, indicating the delayed influence of the liming treatment in Cerrado soil.

Analysis of the Sequences of Dominant DGGE Bands

Bands from bacterial profiles (indicated with numbers, Figs. 2A and 2B) were extracted from DGGE gels, their identity (migration behavior) was confirmed, and they were subjected to cloning and sequencing. Their identifications

Table 2. Identification by RDP SeqMatch (Order) and NCBI BlastN (first hit) of bacterial DGGE bands obtained 30 and 90 days after sowing.

Bands	RDP SeqMatch (order)	NCBI BLAST 01/20/2007		
		First hit (Accession number and description)	Max. ident.	E-value
1	Actinomycetales	AY360165.1|*Micromonospora* sp. i19	97%	0.0
2	Actinomycetales	AY917754.1|Uncultured bacterium clone 1969b-35	98%	0.0
3	Actinomycetales	AY326627.1|Uncultured soil bacterium clone 1202-2	97%	0.0
4	Actinomycetales	AY963437.1|Uncultured bacterium clone AS77	100%	2,00E-63
5	Actinomycetales	AY326625.1|Uncultured soil bacterium clone 1309-2	98%	0.0
6	Burkholderiales	DQ129238.1|Uncultured bacterium clone AKIW521	99%	0.0
7	Burkholderiales	AF297697.1|*Telluria mixta*	99%	0.0
8	Burkholderiales	DQ822441.1|Uncultured bacterium clone WHEATSIP	92%	2,00E-61
9	Clostridiales	DQ129281.1|Uncultured bacterium clone AKIW496	94%	0.0
10	Rhizobiales	AJ863369.1|Uncultured bacterium clone 17RHF28	94%	2E-163
11	Rhizobiales	DQ404786.1|Uncultured bacterium clone 661238	100%	2,00E-88
12	Rhizobiales	AY917421.1|Uncultured bacterium clone 1700a-25	98%	0.0
13	Rhizobiales	AY845962.1|Uncultured soil bacterium clone G1-21	98%	0.0
14	Rhizobiales	AJ863369.1|Uncultured bacterium clone 17RHF28	96%	2,00E-163
15	Rubrobacterales	AY395403.1|Uncultured Rubrobacteridae bacterium clone EB1084	92%	4,00E-168
16	Rubrobacterales	AY321277.1|Uncultured bacterium clone SM-OTU59	89%	4,00E-112
17	Xanthomonadales	AY321255.1|Uncultured bacterium clone SM-OTU37	99%	0.0

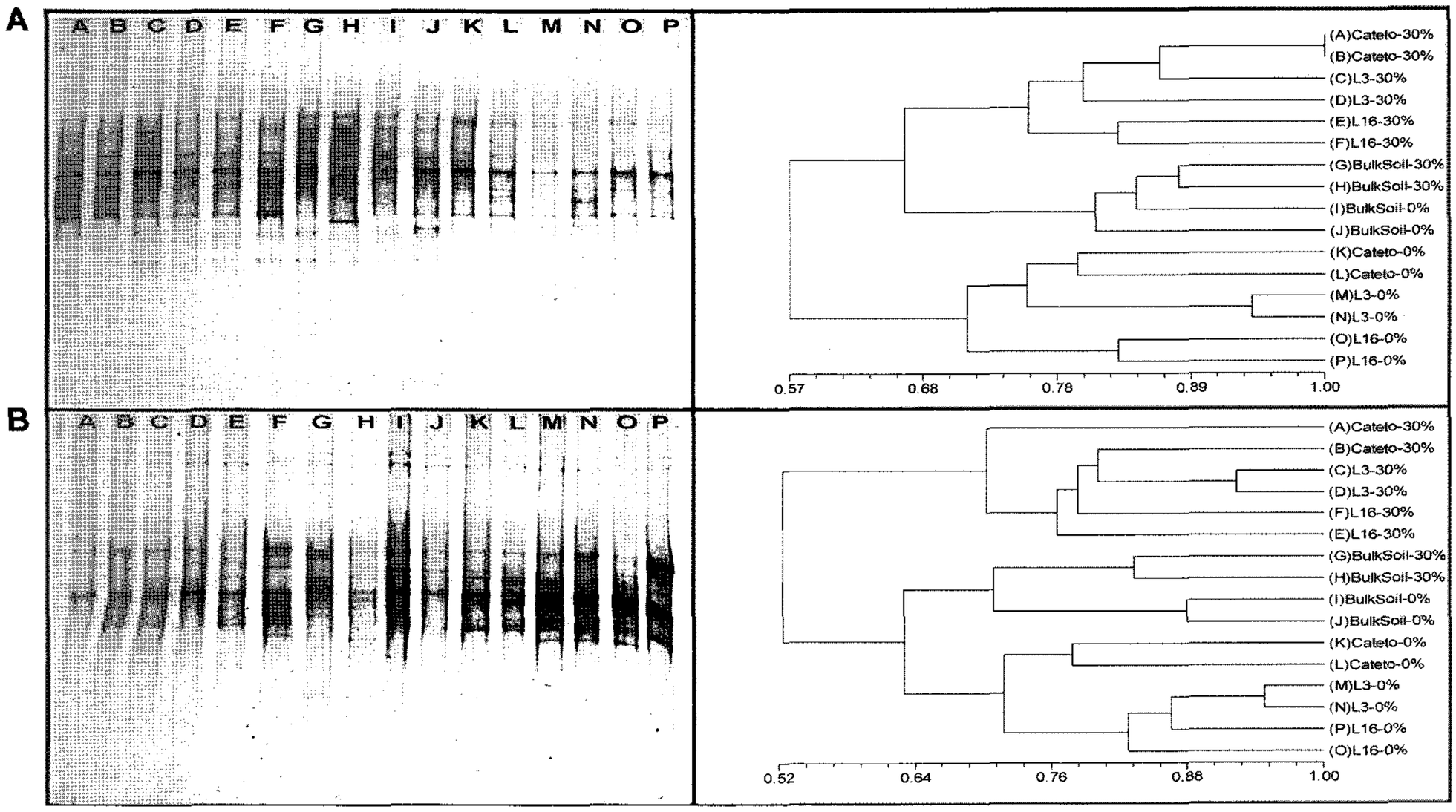

Fig. 3. Denaturing gradient gel electrophoresis (DGGE) fingerprints of fungal 18S rRNA gene fragments amplified from bulk and rhizospheres (Cateto, L3, and L16 maize lines) soil DNA templates.
Gels obtained for the samplings performed 30 days (**A**) and 90 days (**B**) after sowing with under aluminum-stressing and nonstressing conditions and their respective dendrograms. Lanes A to P in DGGE correspond to the same letters in the dendrograms.

are shown in Table 2. After 30 days of plant growth, the profiles obtained from rhizospheres of plant grown under nonstressing aluminum condition (0% Al) showed a dominant band related to Burkholderiales (band 6, Fig. 2A and Table 2). On the other hand, profiles obtained from the different rhizospheres of plants cultivated under stressing conditions (lanes A–F) and from unlimed and limed bulk soils (Fig. 2, lanes G–J) revealed many dominant bands, identified as Actinobacteria (bands 1, 2, and 3) and Rhizobiales (bands 10, 11, and 12) (Fig. 2A and Table 2). A dominant band related to Xanthomonadales (band 17) was observed in unlimed rhizospheres. After 90 days of plant growth, several dominant populations (Fig. 2B, bands 13 and 14) related to Rhizobiales (Table 2) were clearly observed in bulk soil patterns but not in the rhizospheres of fully grown plants. Band 4, identified as Actinomycetales (Table 2), was prevalent in the DGGE profiles from unlimed bulk soil and different corresponding rhizosphere soils (A–H). Band 8, which was observed mainly in the profiles obtained from maize rhizospheres in limed soil, was identified as Burkholderiales. Populations of Clostridiales (Fig. 2B, band 9), Actinomycetales (band 5), and Rubrobacterales (bands 15 and 16) were observed mainly in unlimed bulk soil (Fig. 2B, lanes G–H).

Analysis of Fungal DGGE Fingerprints

As observed for bacterial communities, reproducible DGGE profiles of fungal 18S rDNA were obtained between duplicates of bulk and rhizosphere soil samples (Figs. 3A and 3B). Stable profiles were observed overall. Thirty days after maize sowing (Fig. 3A), the profiles of lines Cateto, L3, and L16 clustered together, per soil treatment (*i.e.*, limed versus unlimed). The bulk soil samples (limed and unlimed) also clustered together, in a separate cluster. DGGE patterns of bulk soil were more similar to those obtained for the rhizospheres of maize lines grown in aluminum-stressing condition than to those from lime-treated soil. Bulk soil profiles (0 and 30% Al) were separated at about 84% similarity, indicating a low effect in soil of the liming treatment after 30 days. At 57% similarity, the profiles from rhizospheres of plants in the limed and unlimed soils were separated in the dendrogram. Hence, the rhizospheres of the three maize lines clearly selected different fungal ribotypes in the limed versus the unlimed soils, resulting in distinct clusters.

After 90 days of plant growth (Fig. 3B), profiles of lines Cateto, L3, and L16 sown in limed soil clustered together. The same situation was observed for the profiles obtained from the rhizospheres of the maize lines sown in nontreated soil. Maize lines cultivated in 0 and 30% of Al were separated at 52% similarity. After the 90-day period, the fungal DGGE profiles obtained from bulk soil were separated from those from rhizospheres from maize grown in limed soil only at 63% similarity. Hence, a great influence of liming in Cerrado soil was apparent. Bulk soil

profiles (0 and 30% Al) were separated at about 71% similarity, indicating a higher effect in soil of the liming treatment after 90 days than 30 days after maize sowing.

Effect of Soil Liming and of Maize Lines on the Microbial Community Structures

Principal components analysis was used to analyze the effect of maize lines and of soil liming on the bacterial and fungal community structures in the rhizosphere and bulk soils (Fig. 4). The microbial communities (both bacterial and fungal) clearly fell into four groups as determined by liming and the presence of a plant rhizosphere; that is, unlimed bulk soil (β), limed bulk soil (γ), rhizospheres under Al-stress (α), and nonstressed (limed) rhizospheres (δ). The only exception was observed in fungal communities from bulk soil analyzed 30 days after plants sowing (Fig. 4C).

Differences in the structures of bacterial and fungal communities in unlimed (β) and limed (γ) soils became more pronounced over time. Bacterial populations from both soils split into two groups after 30 days, but a larger distance between the groups could be observed after 90 days (Figs. 4A and 4B, respectively). Thirty days after sowing, the fungal populations in limed and unlimed soils still resembled each other (Fig. 4C). Only after 90 days, these populations clearly clustered separately (Fig. 4D). Furthermore, only small differences were observed among the microbial communities from the rhizospheres of the different maize lines studied (Cateto, L3, and L16), as these communities always clustered together in PCA. However, bacterial communities from rhizospheres in unlimed (α) and limed (δ) soils tended to group more closely together as a result of plant growth (Figs. 4A and 4B). On the other hand, the fungal communities obtained from the rhizospheres in limed versus unlimed soils were stably separated during plant growth (Figs. 4C and 4D). The results obtained from HCA were consistent with those obtained by PCA, revealing an agreement between clustering and ordination.

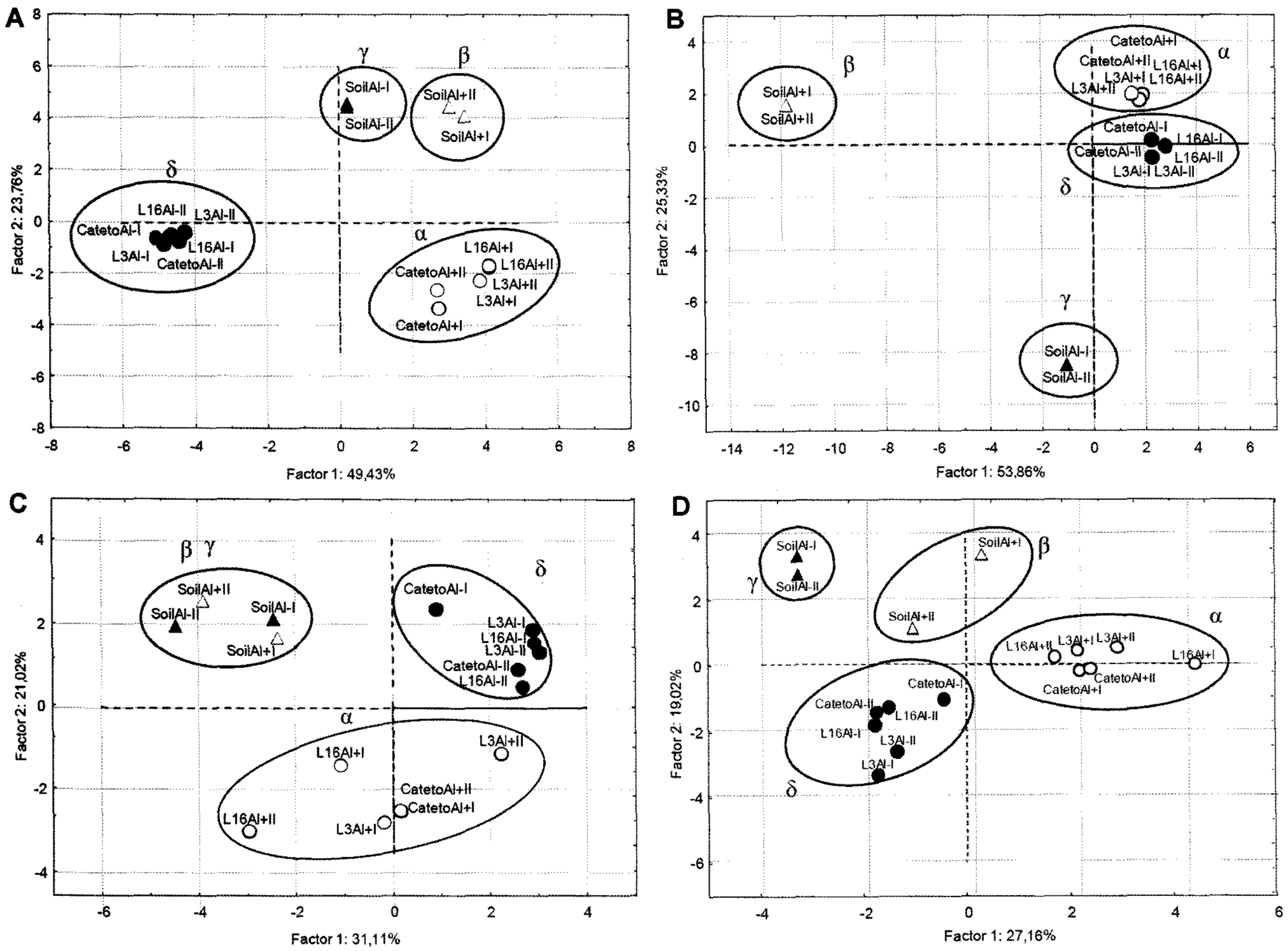

Fig. 4. Principal component analysis of DGGE profiles from microbial communities.
A, **B**. Bacterial communities obtained 30 and 90 days after sowing, respectively; **C**, **D**. Fungal communities obtained 30 and 90 days after sowing, respectively. Groups formed by microbial communities from unlimed soil (β), limed soil (γ), rhizospheres under Al-stress (α), and nonstressed rhizospheres (δ).

DISCUSSION

The microbial communities of the rhizospheres of three maize lines cultivated in limed and unlimed Cerrado soil were investigated using DGGE analysis of PCR-amplified 16S and 18S rRNA genes. This approach provided an overview of the profiles of the microbial communities that emerged in response to the change of soil condition due to liming, which is known to decrease Al toxicity. The importance of soil microbial community structure has already been well documented. It encompasses a range of fundamental processes associated with ecosystem functioning, such as organic matter decomposition, nutrient cycling, plant growth stimulation, pathogenicity, and plant disease suppression [23, 40].

The denaturing gradient gel electrophoresis fingerprints obtained for the bacterial communities analyzed here showed that, regardless of different maize lines, the rhizospheres harbored populations that were determined by the soil conditions where these maize lines were cultivated. This was clearly demonstrated within the first 30 days after sowing and also after 90 days (Fig. 2). The low similarity between the communities associated with maize cultivated in limed versus unlimed soils may be related to the conditions of the plant, given that considerable differences were observed in grain yield and in plant growth after liming of the Cerrado soil, mainly after 90 days of growth (Fig. 1). The data presented here support the contention that the extent to which the plant influences the microbial community structure in the rhizosphere is related to the rhizosphere effect. Depending on the conditions in soil, the plant may respond in different ways. Previous studies have demonstrated that Al-tolerant maize varieties increase the exudation of organic acids, such as citrate and malate, when cultivated with the presence of aluminum [25, 27, 35] or they may show other Al-resistance mechanisms, such as internal detoxification of the aluminum [24, 26].

Differences among the bacterial populations associated with the three maize lines were also observed, but to a lesser extent than that affected by soil liming status. The bacterial populations found in the Cateto line, the Al-tolerant line, were always different from those found with the other lines. Previous studies have also indicated that plant cultivar had the greatest impact on the rhizosphere microflora [11, 17, 18, 36], but soil type was found to be the determining factor in other reports [9, 16, 19]. In our study, a clear influence of soil condition was observed.

Differences were also observed between the sampling times (30 and 90 days after sowing). Thirty days after sowing, rhizospheres of plants grown under Al stress (unlimed soils) showed DGGE profiles more similar to those of bulk soil than to those of plants growing in limed soil (nonstressing conditions). A range of environmental factors, such as soil structure and physicochemical properties, nutrient availability, organic matter content, and pH, has been shown to play a role in soil microbial community dynamics. These factors collectively are responsible in determining the composition of the indigenous soil microflora, which will take part in the process of root colonization [16, 29, 36]. As observed in the present study, the aluminum toxicity due to the low pH of the soil was responsible not only for the reduced growth of maize but also for the selection of the bacterial communities in the soil. Only after 90 days, bacterial populations in limed soil were separated at 49% similarity from those of unlimed soil (Fig. 2B).

Fungi are often dominant in soils when the soil biomass is considered [40]. However, only a few studies on total fungal communities based on independent cultivation methods are available when compared with the number of studies on bacterial communities [2]. The main problem of studying the diversity of fungal communities by cultivation-independent methods used to be the specific amplification of fungal rDNA [32]. Recently, new primer sets and methodologies have been successfully used, allowing reliable DGGE analysis of 18S rDNA of fungal communities [32]. This new semi-nested methodology was applied in our study; the DGGE fingerprints showed that, regardless of the maize line, the rhizospheres harbored different fungal populations in limed versus unlimed soils (Fig. 3). The low similarities between the fungal communities from rhizospheres in limed and unlimed soils may be explained again by the health conditions of the maize lines studied, as clear differences in grain yield and plant growth were observed in relation to liming. Furthermore, the fungal communities shifted only marginally during plant growth (30 and 90 days). No specific bands related to soil condition, maize line, or plant growth stage could be observed in the DGGE profiles. For this reason, bands from the fungal communities were not sequenced.

One advantage of DGGE analysis is the possibility to extract bands from the gels, perform sequence analysis on the purified amplicons, and then to identify members of the community. In this study, after liming of bulk soil, specific high-GC bands affiliated with Actinobacteria became less dominant in the profiles. Actinobacteria populations are usually found in acidic soils [37] and the increase of 1 pH unit observed after liming of soil could have affected their persistence. Bands identified as affiliated with Rhizobiales were detected mainly in bulk soils (unlimed or limed). The rhizobia appear in several different families, and fix nitrogen in association with legume plant roots [15]. On the other hand, in maize (*Poaceae* family), *Azospirillum* and other bacteria, such as *Herbaspirillum* and *Bulkholderia* (Bulkholderiales), are considered as important biological nitrogen fixers [3]. They are also described as plant growth hormone producers (gibberellins and auxins) and nitrogen fixers in other studies [5, 34]. Thirty days after sowing, a predominant population (band 6), which was identified

as affiliated with Burkholderiales (closely related to *Herbaspirillum*), was observed in healthy rhizospheres (limed soil). This population may have a positive effect on the growth of young plants. On the other hand, a band found in rhizospheres in unlimed soil was identified as being affiliated with Xanthomonadales. Stressed rhizospheres are usually more susceptible to opportunistic plant pathogens than those of healthy plants. Moreover, strains of *Xanthomonas* sp. (Xanthomonadales) have been described as maize pathogens [13] and *X. campestris* pv. *holcicola* (Elliott) as the causal agent of bacterial leaf spot in maize. However, there is no indication that this band corresponds to a pathogen, as pathogens and commensals usually have similar 16S rRNA gene sequences.

Principal component analysis is a multivariate statistical technique that summarizes the variability of large data sets using a smaller set of variables, called latent variables or principal components (PCs). The reduction in the number of variables used to describe the data facilitates the interpretation of the relationships between observations [30]. Community patterns represented in PCA resulted in similar groupings of samples as those obtained using HCA, indicating that both data sets are reliable. Multivariate methods thus showed that the effects of liming on the microbial communities of bulk soil increased over time, probably due to long-term dissolution rates of lime materials in soil or slow responses of the microbial communities. Moreover, the data from PCA revealed a quicker effect of liming on the bacterial communities than on the fungal communities of the bulk soils. Therefore, bacterial communities represent more sensitive bioindicators than fungal communities to study the effects of lime treatment on microbial communities of soil.

In conclusion, bacterial communities from the rhizospheres of maize were more affected by soil conditions than by the lines of maize cultivated (Al-sensitive and Al-tolerant). Moreover, differences were observed between sampling times (30 and 90 days after sowing). Thirty days after sowing, rhizospheres of plants in unlimed soils showed DGGE profiles more similar to bulk soil than rhizospheres in limed soils, indicating that plant metabolic stress affects the rhizosphere bacterial community. On the other hand, fungal communities were only weakly affected by liming, and closely related fungal communities were observed among bulk and rhizosphere soils, aluminum condition of soil, maize lines, and time of samplings.

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