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Genetic diversity of *Paenibacillus polymyxa* populations isolated from the rhizosphere of four cultivars of maize (*Zea mays*) planted in Cerrado soil

Fabio Faria da Mota [a], Alberto Nóbrega [a], Ivanildo Evódio Marriel [b], Edilson Paiva [b], Lucy Seldin [a,*]

[a] *Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco I, Ilha do Fundão, CEP 21941-590, Rio de Janeiro, RJ, Brazil*
[b] *EMBRAPA/CNPMS, Sete Lagoas, Minas Gerais, Brazil*

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Abstract

A tropical Brazilian soil (Cerrado) was planted with four cultivars of maize (CMS04, CMS11, CMS22 and CMS36) and the genetic diversity of the *Paenibacillus polymyxa* populations present in their rhizospheres was determined after 90 days of sowing. For that, a total of 67 isolates were identified as *P. polymyxa* by classical biochemical tests and were analyzed for DNA polymorphism with the randomly amplified polymorphic DNA (RAPD) and amplification of repetitive DNA sequences (rep) methods. The amplification patterns obtained using three arbitrary primers and the primer BOXA1R were used separately to construct dendrograms based on the unweighted pair groups method with arithmetic means (UPGMA). Fifty-four genotypic groups were formed when data from different PCR amplifications were combined, showing a high level of genetic polymorphism among *P. polymyxa* strains. A dendrogram based also on combined PCR data, followed by cluster analysis with minimum-variance criteria (Ward) and Euclidean distance, showed that *P. polymyxa* strains could be divided into two main clusters. One cluster was formed predominantly by strains from maize cultivars CMS04 and CMS36, while the other cluster was formed predominantly by strains of maize cultivars CMS11 and CMS22. Multivariate analysis of variance (MANOVA) allowed the correlation between the genetic structure of *P. polymyxa* populations and the different cultivars of maize to be studied. The results showed that the strains isolated from the rhizospheres of the different maize cultivars were significantly different.

Keywords: Genetic diversity; *Paenibacillus polymyxa*; Maize cultivars; Cerrado soil; RAPD-PCR; rep-PCR

1. Introduction

The study of rhizobacteria, which establish positive interactions with plant roots, has increased because of their potential use in sustainable agriculture (Défago et al., 1994). These bacteria considered as plant growth-promoting rhizobacteria (PGPR) can affect plant growth by different ways; for example, by: antagonizing and repressing soil-borne pathogens (by the production of HCN, siderophores, antibiotics and/or competition for nutrients (Défago and Haas, 1990; Glick, 1995; Walker et al., 1998)); fixing atmospheric nitrogen and providing nutrients to the plant (Davison, 1988; Kloepper et al., 1989; Lambert

* Corresponding author. Tel.: +55-21-2562-6741/9989-7222; fax: +55-21-2540-8492/2560-8344.
E-mail address: lseldin@micro.ufrj.br (L. Seldin).

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and Joos, 1989; Lynch, 1990) or inducing plant resistance to diseases after root colonization by PGPR (Timmusk and Wagner, 1999). PGPR when used as seed inoculants can show beneficial effects on plant growth through more than one of these mechanisms.

Among Gram-positive spore-forming bacteria, the species *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*, Ash et al., 1993) has been described as an effective PGPR (Kundu and Gaur, 1980; Chanway et al., 1988; Holl et al., 1988; Holl and Chanway, 1992, Shishido et al., 1995; Petersen et al., 1996). Strains of *P. polymyxa* have been isolated from different soils, rhizospheres and roots from plants cultivated all over the world (Nelson et al., 1976; Jordan et al., 1978; Wullstein et al., 1979; Seldin et al., 1983; Lindberg and Granhall, 1984; Holl et al., 1988; Chanway and Holl, 1991; Mavingui et al., 1992; Heulin et al., 1994; Guemouri-Athmani et al., 2000; von der Weid et al., 2000). Moreover, *P. polymyxa* strains have been shown to be effective in stimulating the growth of different plants by nitrogen fixation and by the production of a wide variety of secondary metabolites, including plant growth-regulating substances (Lebuhn et al., 1997; Nielsen and Sorensen, 1997) and antibiotic compounds (Rosado and Seldin, 1993; Mavingui and Heulin, 1994; Walker et al., 1998). In these studies, different important crop species were used for the isolation and characterization of *P. polymyxa* strains; however, most of these studies were on plants from temperate regions. In tropical countries, where unfavorable climatic conditions are prevalent making their ecosystems unique, the application of *P. polymyxa* strains to seed as a PGPR could be a successful approach. Because strains of this species can form endospores, they can resist a range of environmental stress conditions like high temperatures, dryness or heavy rainfalls, usually present in tropical countries. Therefore, studies on the population structure and intraspecific diversity of *P. polymyxa* are very relevant. In this context, von der Weid et al. (2000) have investigated *P. polymyxa* populations in the rhizosphere of maize (cultivar BR-201) planted in Cerrado soil (a prevalent soil in Brazil) in order to assess their diversity at four stages of plant growth. The resulting data showed that strains isolated during the different stages of maize growth were statistically different. The intraspecific diversity observed in this study may be important to provide the plant with a variety of strains more resistant to stress caused by tropical conditions. However, maize breeding is currently used to obtain cultivars more adapted to tropical conditions. Therefore, additional data are necessary to determine whether the same results obtained by von der Weid et al. (2000) will be found using different maize cultivars. It is unquestionable that the understanding of the relationship between rhizosphere environment and genetic diversity patterns of specific bacterial populations is a requirement for the selection of strains for use as inoculants.

In this study, we aimed to analyze the genetic diversity of *P. polymyxa* populations associated with the rhizospheres of different maize cultivars, 90 days after plant sowing. The randomly amplified polymorphic DNA (RAPD)- and repetitive sequence-based oligonucleotide primer (rep)-PCR combined with a powerful statistical analysis (multivariate analysis of variance (MANOVA)) were used to examine the genetic diversity of these *P. polymyxa* populations. Those techniques have been extensively used to discriminate strains belonging to the same bacterial species (Paffetti et al., 1996; Di Cello et al., 1997; Rosado et al., 1998; Seldin et al., 1998; Guemouri-Athmani et al., 2000; von der Weid et al., 2000). These approaches will help to elucidate whether the different maize cultivars, commonly used in Brazilian agriculture, select specific bacterial populations to coexist with them or the whether diversity among *P. polymyxa* strains can be considered arbitrary. Indeed, the understanding of the genetic structure of *P. polymyxa* and a possible selection for a specific group of strains by the maize cultivars studied here can be of great agricultural interest.

2. Materials and methods

2.1. Maize cultivars and experimental conditions

Cerrado soil was planted with four different cultivars of maize (*Zea mays*) in Sete Lagoas, city located in the state of Minas Gerais in the southeast region of Brazil. Cerrado is a dark-red distrophic latosol with a clayey texture. It was well described elsewhere as it represents 20% of the territory of Brazil (Dianese et al., 1994; von der Weid et al., 2000). The four types of maize cultivars (CMS04, CMS11, CMS22 and CMS36) were chosen based on their

agricultural importance. Briefly, they can be described as follows: CMS04—a tropical maize, derived from Caribbean, Mexican and Brazilian germplasms, with late cycle and yellow dent grains; CMS11—a tropical maize, derived from Caribbean, Tuxpeño and Mexican germplasms, with yellow semi-flint grains and an intermediate life cycle; CMS22—a subtropical maize, derived from Mexican, Caribbean and American germplasms, with yellow dent grains and intermediate–late cycle; CMS36—a tropical maize germplasm, created from lines of different origins (Catete, Tuxpeño, ETo Amarillo, etc.); it shows good tolerance to acid soil, it has a late cycle and it is much taller than the others. The experimental plots where untreated maize seeds were sown in Cerrado soil comprised two lines of 5 m of length with spaces of 0.90 m between lines and 0.20 m between plants of the same cultivar. The soil was fertilized with 400 kg of a mixture of N, P and K (4:14:8) and 80 kg N/ha were supplied after 45 days of sowing.

2.2. Isolation and maintenance of P. polymyxa strains

Strains of *P. polymyxa* were isolated from the rhizosphere of maize plants from different maize cultivars 90 days after sowing, by the method described by Seldin et al. (1998). Three plants were harvested and the roots shaken to remove the loosely attached soil. One gram of the adhering soil (considered as the rhizosphere) was mixed with 9 ml of distilled water and pasteurized for 10 min at 80 °C. Two-fold serial dilutions were plated onto thiamine–biotin (TBN) agar (Seldin et al., 1984) and incubated for 3 days in Gaspak jars with an atmosphere of 80% N_2, 10% CO_2 and 10% H_2. All bright yellow, convex, shinny and mucous colonies were selected for further purification as described previously (Seldin et al., 1983). The presumptive *P. polymyxa* colonies were stored aerobically on glucose broth (GB) agar slants supplemented with 1% $CaCO_3$ (Seldin et al., 1983).

2.3. Identification of P. polymyxa strains

All isolates from the four maize rhizospheres were identified by using the cultural and biochemical tests proposed by Gordon et al. (1973). Additional morphological characteristics, the fermentation pattern using four carbohydrates (glycerol, D(+)-xylose, L(+)-arabinose and trehalose) and the utilization of citrate and succinate were determined as described in von der Weid et al. (2000). Some strains were also tested using API 50CH kits (Appareils et Procédés d'Identification, bioMérieux sa, Lyon, France), as described in Seldin and Penido (1986), to confirm their identification as *P. polymyxa*.

2.4. Preparation of genomic DNAs

The DNAs were isolated by the method described in Seldin et al. (1998). Cells from 30 ml cultures grown in GB at 30 °C for 16 h were centrifuged (10,000 × *g*, 10 min), resuspended in 2 ml of Tris–EDTA–NaCl buffer (Seldin and Dubnau, 1985) and treated with 1 mg of lysozyme (30 min, 37 °C) and with 1% of sodium dodecyl sulfate (10 min, 65 °C). Further purification steps were those described by Seldin and Dubnau (1985). DNAs were quantified spectrophotometrically using the Gene Quant apparatus (Pharmacia Biotech, Uppsala, Sweden).

2.5. PCR amplifications

2.5.1. RAPD-PCR

Amplification reactions with arbitrary primers were performed in a mix containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 250 μM of each deoxynucleoside triphosphate (dNTP), 0.001% of gelatin, 0.7 μM of the primers OPA-A03 (5′-TTAGCGCCCC-3′), OPD-2 (5′-GGACCCAACC-3′) or OPA-17 (5′-CCTGGAGCTT-3′) from Operon Technologies Inc., 50 ng of target DNA and 1.25 U of *Taq* polymerase (Invitrogen Life Technologies) in a 25 μl final volume. The cycle applied was 45× (1 min, 94 °C; 1 min, 36 °C; 2 min, 72 °C); 4 °C.

2.5.2. BOX-PCR

Amplification reactions with BOXA1R primer (Louws et al., 1994) were performed as described by Versalovic et al. (1994) in a mix containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3.75 mM $MgCl_2$, 600 μM of each dNTP, 1 μM of the primer BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′; Louws et al., 1994), 50 ng of target DNA and 1.25 U of *Taq* polymerase. The cycle applied was 1× (7 min, 95 °C); 30× (1 min, 94 °C; 1 min, 53 °C; 8 min, 65 °C); 1×

(16 min, 65 °C); 4 °C. Agarose gel electrophoresis of PCR products was performed with 1.4% agarose in Tris–borate–EDTA buffer (Sambrook et al., 1989) at 90 V for 3 h 30 min at room temperature.

2.6. Statistical analyses

The results of PCR fingerprintings (three arbitrary primers and BOXA1R) were collected into different matrixes indicating the presence or absence (scored as 1 or 0, respectively) of specific bands in each PCR analysis. In each case, a simple matrix was obtained by comparing pair of strains using the simple matching coefficient (SM) and a dendrogram was constructed using the UPGMA. For these analyses, the NTSYS software package (Version 2.02, Exeter Software, Setauket, New York) was used. Using all parameters together, a dendrogram was obtained after cluster analysis with minimum-variance criteria (Ward) and Euclidean distance (Rencher, 1995). The complete matrix (67×77) consisted of 67 isolates analyzed for 77 parameters. When the number of parameters is of the same order as the number of isolates, there is often redundancy in the information obtained and the matrix data can be compressed using new parameters derived by linear combination of the original ones. For that purpose, we used principal component analysis (PCA), a multivariate method which reduces the number of parameters while preserving the maximum of variance of the data (Rencher, 1995). The reduction of parameters is also necessary for the MANOVA test to be discriminative, otherwise the excessive number of parameters compromise the power of the test. The computational calculations of principal components and cluster analysis were done with routines written by A. Nobrega with the software MATHEMATICA (Wolfram Research Inc., Champaign, III). MANOVA of data from RAPD- and BOX-PCR was done with the statistical package NTSYS.

3. Results

3.1. Isolation and identification of P. polymyxa strains

Pasteurized samples from the rhizosphere of four different cultivars of maize (CMS04, CMS11, CMS22 and CMS36) planted in Cerrado soil gave rise to colonies with different morphologies on TBN agar medium, after incubation for 3 days in anaerobiosis. Among them, a total of 70 *P. polymyxa*-like colonies (mucoid, convex and bright yellow) were chosen for purification and identification procedures: 16 strains from CMS04, 20 strains from CMS11, 12 strains from CMS22 and 22 strains from CMS36. The different isolates were designated PM followed by the maize cultivar number (04, 11, 22 or 36) and by progressive numbers of isolation. Sixty-seven strains listed in Table 1 were phenotypically identified as typical *P. polymyxa* strains, based on taxonomic tests proposed by Gordon et al. (1973). Production of acid from glucose, mannitol, arabinose, xylose and glycerol was observed in all 67 isolates and divergent results were obtained with the remaining three isolates (isolated from CMS22). These strains showed negative result for glycerol utilization, indicating that they could belong to *P. peoriae* as described by Montefusco et al. (1993) and Heyndrickx et al. (1996). The observation of positive results for succinate and citrate utilization by these three strains also supported our identification, leading us to exclude them from this study. Some of the remaining 67 strains were tested using the API 50CH kit and the results obtained (data not shown) were those expected for *P. polymyxa* strains, as given by the manufacturer and presented elsewhere (Seldin et al., 1983; Seldin and Penido, 1986; Heyndrickx et al., 1996; von der Weid et al., 2000). These results strongly suggest that all strains used in RAPD and rep analyses belong to *P. polymyxa* species.

3.2. RAPD and rep analyses

The first aim of the PCR analyses was to assess the genetic variability of *P. polymyxa* strains isolated from the rhizospheres of different cultivars of maize. Initially, the DNAs of four *P. polymyxa* isolates (PM04-2, PM11-1, PM22-2 and PM36-3) were amplified by the RAPD technique with 22 different primers (10-mer, OPD series 1–20, OPA-A03 and OPA-C17 from Operon Technologies Inc.). No DNA amplification could be observed with primers OPD-8, -10, -14, -15 and -17 while high molecular weight bands were detected with primers OPD-3, -9, -11, -13 and -19. The more reproducible patterns were

Table 1
Different groups formed by the amplification of total DNA from *P. polymyxa* strains with RADP and rep primers

Maize cultivars	*P. polymyxa* strains	Primers				Groups formed[a]
		OPA-A03	OPA-C17	OPD-2	BOXA1R	
CMS04	PM04-1	23[b]	10	1	13	G.1
	PM04-2	24	3	2	14	G.2
	PM04-3	2	8	3	15	G.3
	PM04-4	22	10	1	15	G.4
	PM04-5	25	3	2	13	G.5
	PM04-6	21	5	3	15	G.6
	PM04-7	22	10	1	15	G.4
	PM04-8	1	1	4	15	G.7
	PM04-9	24	3	2	6	G.8
	PM04-10	22	3	1	15	G.9
	PM04-11	2	2	5	7	G.10
	PM04-12	26	1	6	7	G.11
	PM04-14	2	2	7	15	G.12
	PM04-15	2	4	3	16	G.13
	PM04-16	27	3	2	14	G.14
	PM04-17	28	10	12	13	G.15
CMS11	PM11-1	1	1	13	13	G.16
	PM11-2	2	1	14	4	G.17
	PM11-3	1	1	13	6	G.18
	PM11-4	2	2	8	16	G.19
	PM11-5	11	2	8	16	G.20
	PM11-6	1	1	13	16	G.21
	PM11-7	1	1	13	16	G.21
	PM11-8	2	2	8	16	G.19
	PM11-9	4	3	13	16	G.22
	PM11-10	2	2	8	16	G.19
	PM11-11	3	4	3	16	G.23
	PM11-12	4	5	3	16	G.24
	PM11-13	2	2	8	2	G.25
	PM11-14	2	2	8	2	G.25
	PM11-15	15	1	17	2	G.26
	PM11-16	17	1	13	2	G.27
	PM11-17	17	1	13	2	G.27
	PM11-18	16	1	13	2	G.28
	PM11-21	17	1	13	16	G.29
	PM11-22	11	2	18	2	G.30
CMS22	PM22-2	2	2	8	2	G.25
	PM22-4	4	3	16	3	G.31
	PM22-5	1	1	13	2	G.32
	PM22-6	2	2	8	2	G.25
	PM22-7	2	2	8	13	G.33
	PM22-8	19	1	13	2	G.34
	PM22-9	2	2	8	2	G.25
	PM22-11	20	9	10	5	G.35
	PM22-16	20	9	10	5	G.35
CMS36	PM36-1	5	2	15	7	G.36
	PM36-2	5	2	13	13	G.37
	PM36-3	6	5	3	13	G.38
	PM36-4	5	2	15	7	G.36
	PM36-5	7	1	13	7	G.39

Table 1 (*Continued*)

Maize cultivars	*P. polymyxa* strains	Primers				Groups formed[a]
		OPA-A03	OPA-C17	OPD-2	BOXA1R	
	PM36-6	5	3	13	7	G.40
	PM36-7	5	6	15	8	G.41
	PM36-9	5	6	15	8	G.41
	PM36-10	5	5	3	7	G.42
	PM36-11	8	1	16	7	G.43
	PM36-12	8	1	16	7	G.43
	PM36-13	7	1	9	7	G.44
	PM36-14	18	7	11	11	G.45
	PM36-15	6	5	3	7	G.46
	PM36-16	9	1	15	9	G.47
	PM36-17	10	11	15	12	G.48
	PM36-18	12	1	19	1	G.49
	PM36-19	11	2	5	2	G.50
	PM36-20	10	5	3	10	G.51
	PM36-21	13	5	3	10	G.52
	PM36-22	13	5	3	11	G.53
	PM36-23	14	3	20	12	G.54

[a] Genotypic groups formed by the combination of data from the four primers.

[b] Groups formed based on the comparison of the fingerprints obtained after the amplification of total DNAs with each primer used. Each group is formed by strains presenting the same amplification pattern.

obtained with primers OPD-2, OPA-A03 and -C17 that gave rise to eight, nine and five bands of different sizes (considered as markers), respectively, for a total of 22 RAPD markers. The amplification of these four strains with BOXA1R gave rise to 11 markers (data not shown). Further amplification of genomic DNAs of all 67 *P. polymyxa* isolates with these three arbitrary primers and BOXA1R generated different fingerprints that could be used to identify similarities among strains. Similar fingerprints allowed the grouping of strains when the amplification patterns with each primer were considered and also with the combination of the results obtained with the four primers. Table 1 shows the different groups of strains formed when each primer was used with the 67 strains analyzed and the 54 genotypic groups found when these different PCR groups were combined. Nine genotypic groups were formed by more than one strain. Only one group (G.25) was formed by strains isolated from different maize cultivars (CMS11 and CMS22). The remaining strains were allocated in 45 different genotypic groups.

Amplifications using the primers OPD-2, OPA-C17 and -A03 generated 16, 14 and 21 markers, respectively, while using primer BOXA1R 26 markers were obtained. One band (about 1.2 kb) was common to all 67 strains studied and a band of 570 bp was observed in 66 strains (not being detected only in strain PM36-18) in BOX fingerprints (data not shown). Altogether, 77 markers were used to group the 67 strains based on the comparison of the sizes of the amplification products via gel electrophoresis, using the combination of all the results obtained. None of the RAPD or BOX markers used was specific for strains from a particular maize cultivar. A similarity analysis was then applied based on UPGMA using each of the fingerprints obtained with the four primers to address the diversity among all *P. polymyxa* isolates (Fig. 1A–D). The highest level of polymorphism among strains was achieved when primer OPA-A03 (28 groups at 100% similarity) was used while the opposite situation was observed with primer OPA-C17 (11 groups at 100% similarity). At about 75% of similarity, four groups in OPA-A03, three in BOXA1R, five in OPD-2 and two in OPA-C17 were visible (Fig. 1). At about 50% (in OPA-C17 dendrogram) and 56% (in BOXA1R dendrogram), two strains (PM22-11 and 16) were separated from the others. Using BOXA1R, the strain PM36-18 was also separated from the others at 69% similarity. No phenotypic test used in this study (including API 50CH)

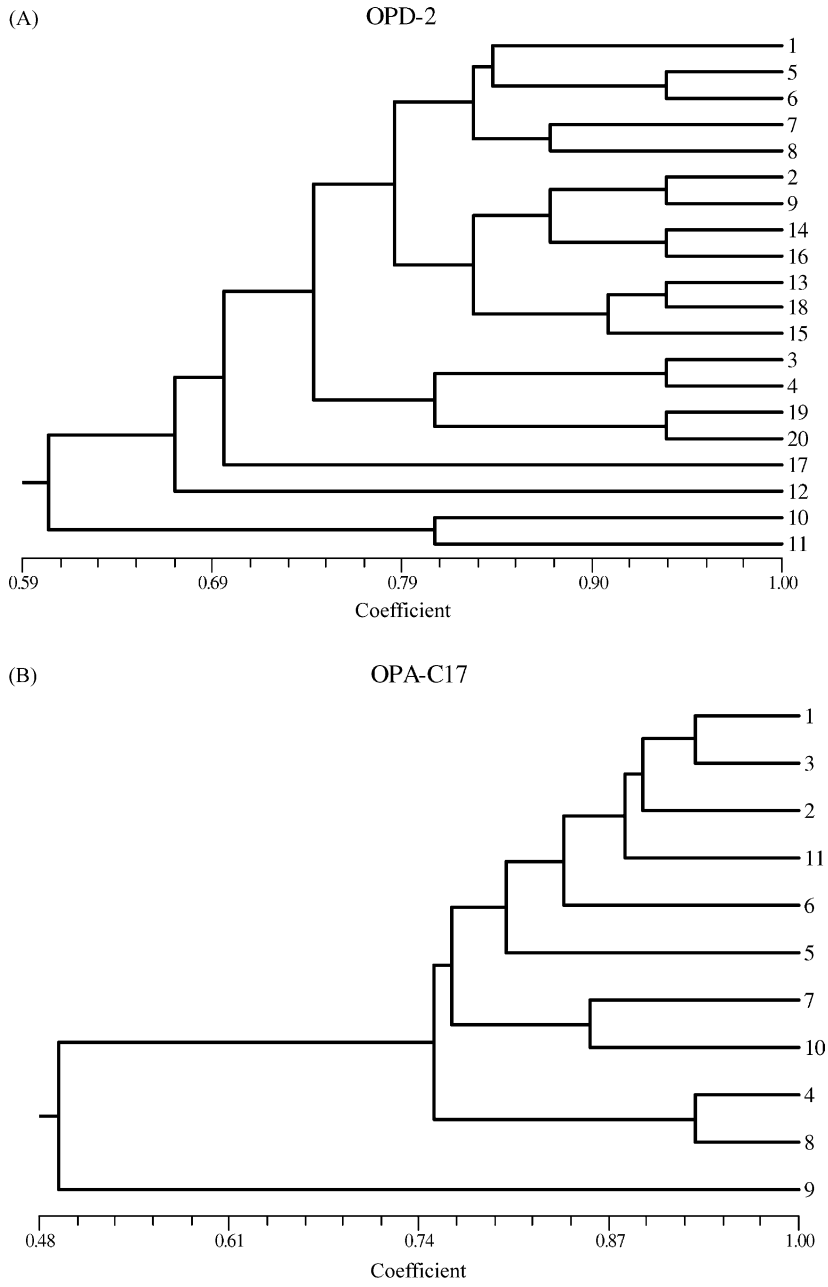

Fig. 1. Dendrograms (UPMGA) based on data from PCR amplifications using primers (A) OPD-2, (B) OPA-C17, (C) OPA-A03 and (D) BOXA1R, showing the similarities between 67 *P. polymyxa* strains isolated from the rhizospheres of four different maize cultivars. Numbers correspond to the group (presented in Table 1), where the isolates were allocated after the amplification with each primer used.

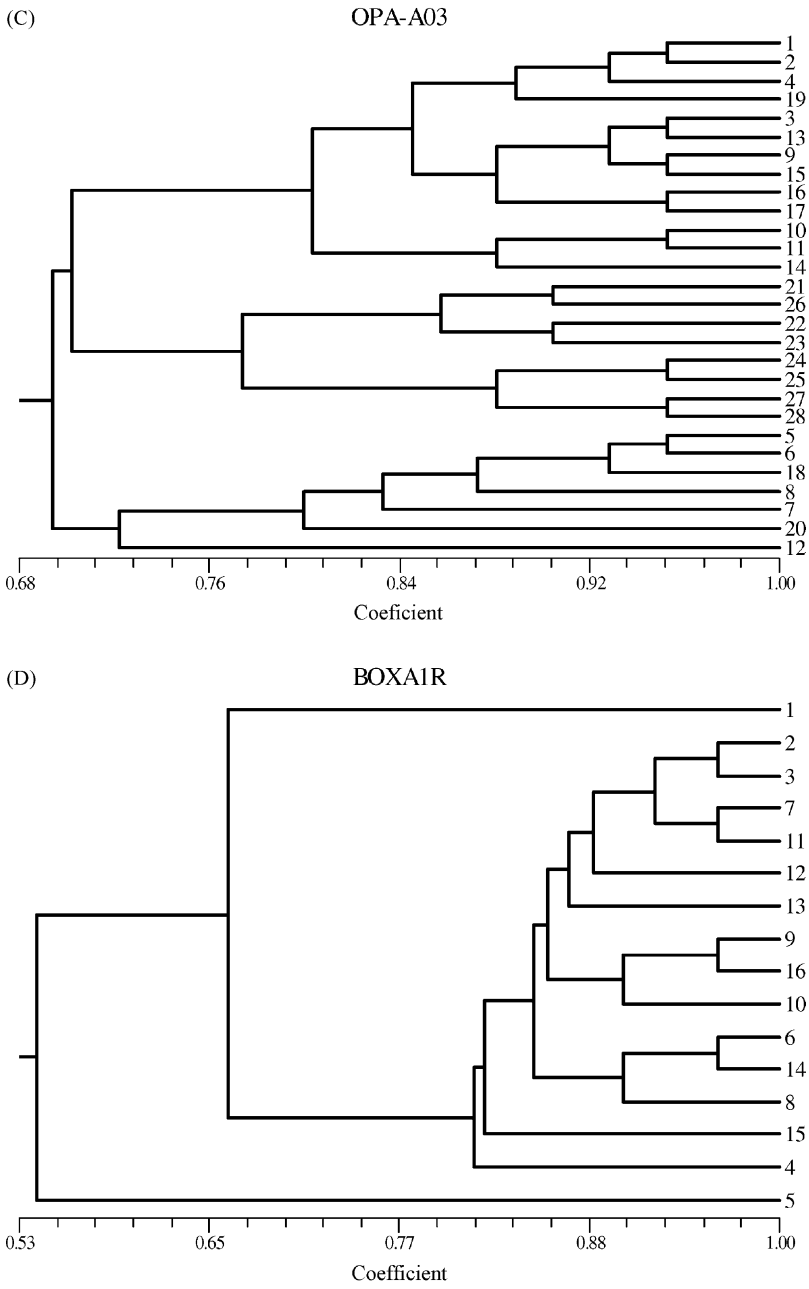

Fig. 1. (*Continued*).

could differentiate those three strains from typical *P. polymyxa* ones.

An overall analysis was also performed to address the heterogeneity among *P. polymyxa* populations present in the rhizosphere of different cultivars of maize in order to understand whether the genetic variability is influenced by the plant cultivar. At this point, the data were clustered according to Ward

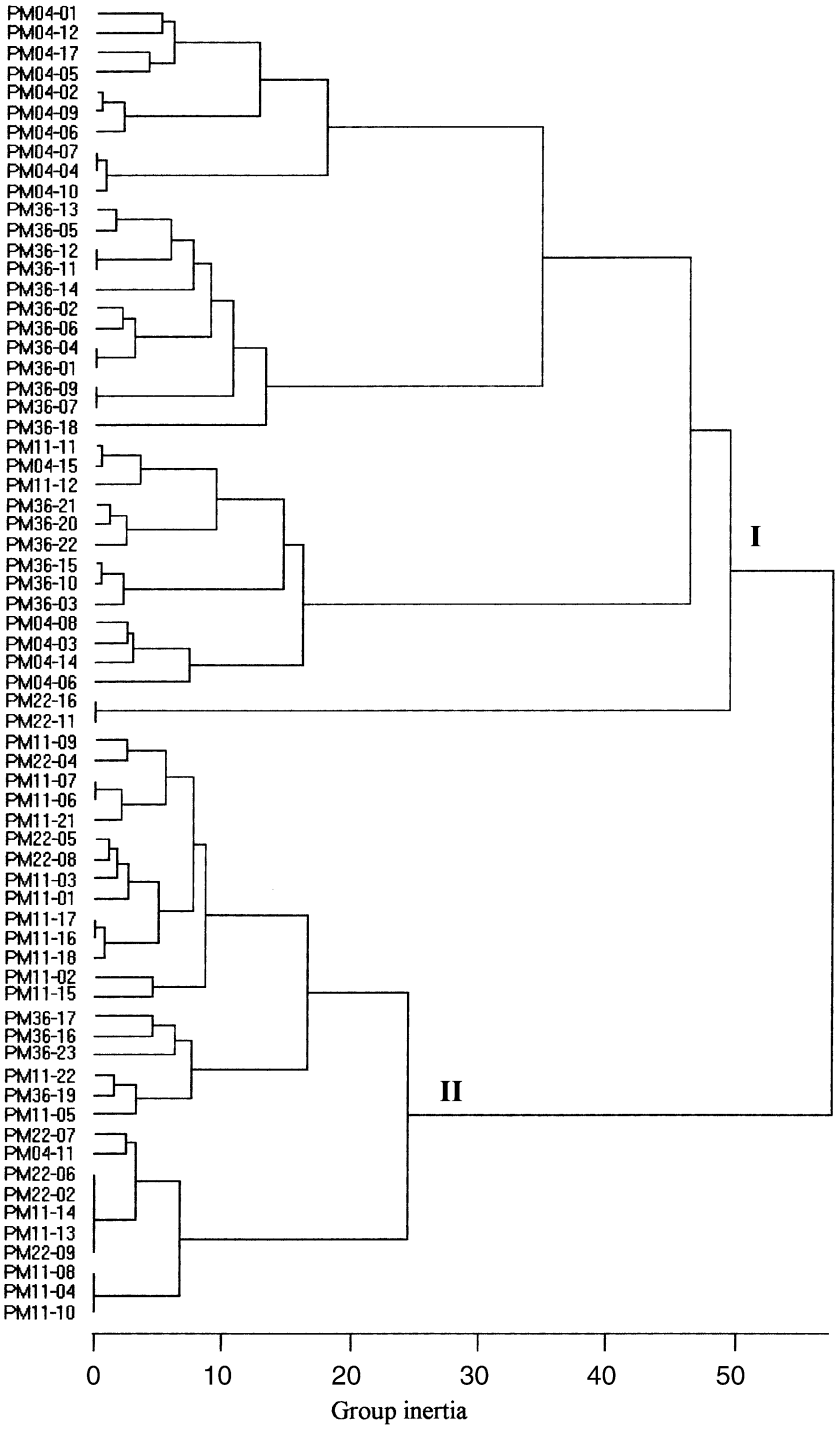

Fig. 2. Dendrogram based on data from RAPD and rep fingerprints, followed by cluster analysis with Ward and Euclidean distance. Strains isolated from the different rhizospheres of maize are those listed in Table 1.

with Euclidean distance. The dendrogram obtained is shown in Fig. 2. Two main clusters, denoted I and II, were observed. Cluster I was made up predominantly of strains from maize cultivars CMS04 and CMS36, while cluster II was made up predominantly of strains from maize cultivars CMS11 and CMS22. Only one strain from CMS04 (PM04-11) could be found in cluster II, while only two strains from CMS11 (PM11-11 and 12) were found in cluster I. Clusters I and II comprised 37 and 30 strains, respectively. Data from PCR analyses were also subjected to PCA, such that only the more important factors were retained for MANOVA. In our case, the first five factors represented 56% of the variance and the first eight factors represented 73% of the variance. For this approach, we split the total *P. polymyxa* population (67 strains) into four sub-populations, corresponding to isolates from each maize cultivar and patterns obtained by RAPD- and rep-PCR of these different sub-populations were tested for identity by MANOVA. Using these eight factors, the scores of comparison between CMS04, CMS11, CMS22 and CMS36 are presented in Table 2. The data clearly showed that the strains isolated from different maize cultivars differ significantly ($P = 1.7 \times 10^{-7}$). MANOVA was also done for all possible combinations of 3×3 and 2×2 with strains from the four cultivars. All 3×3 groupings were significantly different. The majority of 2×2 groupings were not significantly different by MANOVA. Only in two cases when the cultivar CMS36 was compared to CMS04 and CMS11 did the results prove to be significantly different, suggesting that strains isolated from cultivar CMS36 deviate from the others. The diagram presented in Fig. 3 corresponding to the two-dimensional plot of MANOVA scores corroborates this observation.

Table 2
MANOVA of 67 *P. polymyxa* strains from the rhizospheres of the four maize cultivars studied with RAPD- and rep-PCR patterns

Canonical variate analysis	Statistic[a]	*F*-statistic	*P*[b]
Maize cultivars			
CSM04 × 11 × 22 × 36	1.80	3.81	1.7×10^{-7}
CSM04 × 11 × 22	2.13	2.35	0.0075
CSM04 × 22 × 36	2.13	3.36	2.0×10^{-4}
CSM11 × 22 × 36	2.09	2.93	7.6×10^{-4}
CSM04 × 11 × 36	2.09	3.97	1.0×10^{-5}
CSM04 × 11	3.12	2.22	0.058
CSM04 × 22	3.20	1.99	0.114
CSM04 × 36	3.12	3.63	0.0048
CSM11 × 22	3.15	1.68	0.165
CSM11 × 36	3.08	3.61	0.0041
CSM22 × 36	3.15	2.23	0.065

[a] Multivariate test of significance (Wilk's λ-value).
[b] Probability of occurrence of extreme variance.

4. Discussion

It has been extensively demonstrated that bacterial adaptation to a heterogeneous and fluctuating environment, such as the plant rhizosphere is dependent on the genetic diversity of rhizobacterial population (Roszak and Colwell, 1987; Mavingui et al., 1992). This diversity is affected by the amount and composition of organic materials excreted by plant roots which may vary during plant development and in different plant cultivars (Neal Jr. et al., 1970; Neal Jr. and Larson, 1976; Chanway et al., 1988). Different methods are available for the sound estimation of bacterial diversity (Torsvik et al., 1990; Berg et al., 1994; Wong et al., 1996) and they provide essential knowledge for the selection of potential inoculant strains for use in agriculture.

In this study, we analyzed the genetic diversity of 67 *P. polymyxa* strains that were isolated from the rhizosphere of four different cultivars of maize planted in Cerrado soil. Strains of *P. polymyxa* in the maize rhizosphere have already been described (von der Weid et al., 2000), however, no data were provided on the selection of specific sub-populations of *P. polymyxa* in different cultivars. Our isolates were first analyzed on their phenotypic features and this showed that their identification was unambiguous and that only three strains (among the 70 isolates) could belong to *P. peoriae*, the closest species of *P. polymyxa*, as they were not capable of metabolizing glycerol (Montefusco et al., 1993). The remaining 67 isolates showed the same phenotypic characteristics described for *P. polymyxa* species (Gordon et al., 1973).

To reach the goal of our study, the genetic structure of *P. polymyxa* was investigated by analyzing RAPD and rep markers. The primers and the amplification conditions were chosen to generate reproducible patterns. All experiments were performed at least twice and the results obtained allowed the grouping of the strains when each of the RAPD primers and the primer

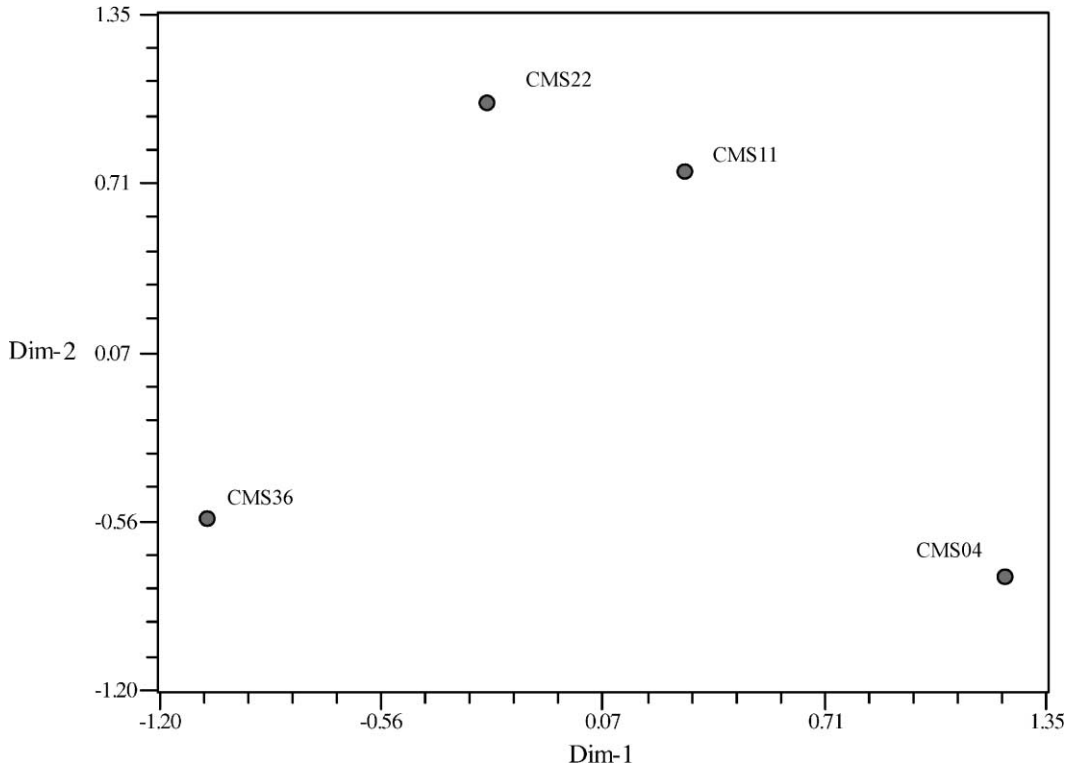

Fig. 3. Two-dimensional plot scores for the four sub-populations of *P. polymyxa* (corresponding to isolates of each maize cultivar) tested by MANOVA.

BOXA1R were considered separately or combined. A high level of genetic diversity was found within the *P. polymyxa* population, yielding a total of 54 distinct groups (Table 1). Data originated from the RAPD and rep results were used to construct dendrograms (Fig. 1A–D) representing the genetic relatedness of the strains. Although the dendrograms do not show phylogenetic relationships, they allow us to define the population considered to be genetically closely related, having originated from a common ancestor or being a co-isolate. The differences between the strains isolated from the four cultivars were clearly shown, independently on the markers used for the construction of the dendrograms. In a previous report, von der Weid et al. (2000) demonstrated that *P. polymyxa* strains isolated from maize (cultivar BR-201) were also heterogeneous. They were able to link plant development to the diversity of *P. polymyxa* populations associated with maize planted in Cerrado soil. Diversity associated with maize growth has been also demonstrated by Seldin et al. (1998) working with *P. azotofixans* and by McArthur et al. (1988) and Di Cello et al. (1997) working with *Burkholderia cepacia*. In the present report, we fixed 90 days after the maize sowing to conduct the isolation procedures, based on the observations of Hamlen et al. (1972). Plant exudates which represent nutritional sources for rhizosphere microorganisms may influence the microbial population; therefore, middle-end stages of maize growth should represent more stable ecosystem when compared to the unstable ecosystem of very young or declining plants.

In order to understand whether the genetic variability was influenced by the maize cultivars, RAPD and rep markers were clustered according to Ward with Euclidean distance (Fig. 2). Two main clusters were observed, one formed by strains from maize cultivars CMS04 and CMS36 and the other by strains from maize cultivars CMS11 and CMS22. Fig. 3

also shows the higher proximity between strains isolated from these two last cultivars. As cultivars CMS11 and CMS22 originated from Mexican and Caribbean germplasms, this could be an explanation for their genetic proximity and, consequently, for the selection of more similar *P. polymyxa* populations to co-exist in their rhizospheres. When data from PCR analyses were further used for MANOVA, the results also showed that the maize cultivar significantly affected the diversity of *P. polymyxa* populations (Table 2; Fig. 3). It was found that strains isolated from the rhizosphere of maize cultivar CMS36 may play an important role in the difference observed in *P. polymyxa* populations isolated from the four cultivars. This suggests that the plant may have selected a sub-population of *P. polymyxa*. It is important to point out that this cultivar is much better adapted to tropical conditions and to the acidic Cerrado soil than the other cultivars. Similar results have been reported by Paffetti et al. (1996) and Carelli et al. (2000) for alfafa cultivars and *Sinorhizobium meliloti* populations and by Garcia de Salomone et al. (1996) for maize-genotype association with *Azospirillum*. In all cases, the cultivar significantly influenced the distribution of genetic variability in specific populations.

The present findings emphasize the importance of the selection of *P. polymyxa* strains for use as inoculants of maize in Cerrado soil; however, further studies are necessary to evaluate their potential as PGPR in the different maize cultivars.

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