Diversity of *Paenibacillus* spp. in the Rhizosphere of Four Sorghum (*Sorghum bicolor*) Cultivars Sown with Two Contrasting Levels of Nitrogen Fertilizer Assessed by *rpoB*-Based PCR-DGGE and Sequencing Analysis

COELHO, MARCIA REED RODRIGUES[1], FABIO FARIA DA MOTA[1],
NEWTON PORTILHO CARNEIRO[2], IVANILDO EVÓDIO MARRIEL[2],
EDILSON PAIVA[2], ALEXANDRE SOARES ROSADO[1], AND LUCY SELDIN[1]*

[1]*Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
[2]*EMBRAPA/CNPMS – Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisas de Milho e Sorgo, Sete Lagoas, MG, Brazil*

Abstract The diversity of *Paenibacillus* species was assessed in the rhizospheres of four cultivars of sorghum sown in Cerrado soil amended with two levels of nitrogen fertilizer (12 and 120 kg/ha). Two cultivars (IS 5322-C and IS 6320) demanded the higher amount of nitrogen to grow, whereas the other two (FBS 8701-9 and IPA 1011) did not. Using the DNA extracted from the rhizospheres, a *Paenibacillus*-specific PCR system based on the RNA polymerase gene (*rpoB*) was chosen for the molecular analyses. The resulting PCR products were separated into community fingerprints by DGGE and the results showed a clear distinction between cultivars. In addition, clone libraries were generated from the *rpoB* fragments of two cultivars (IPA 1011 and IS 5322-C) using both fertilization conditions, and 318 selected clones were sequenced. Analyzed sequences were grouped into 14 *Paenibacillus* species. A greater diversity of *Paenibacillus* species was observed in cultivar IPA 1011 compared with cultivar IS 5322-C. Moreover, statistical analyses of the sequences showed that the bacterial diversity was more influenced by cultivar type than nitrogen fertilization, corroborating the DGGE results. Thus, the sorghum cultivar type was the overriding determinative factor that influenced the community structures of the *Paenibacillus* communities in the habitats investigated.

Keywords: Molecular ecology, nitrogen fixation, *Paenibacillus*, *rpoB* gene, *Sorghum bicolor*

Corresponding author
Phone: 55-21-2562-6741; Fax: 55-21-2560-8344;
E-mail: lseldin@micro.ufrj.br, lucy@seldin.com.br

Microbial communities in soil play a key role in agro-ecosystems through regulating the dynamics of organic matter decomposition and nutrient recycling, including biological nitrogen fixation. The availability of nitrogen often limits productivity of agricultural systems, which is also known to depend greatly upon the functional process of soil microbial communities [16]. However, the structure of soil microbial communities and how it responds to changes in the environment are not very well understood. On the other hand, the structural and functional diversity of microbial populations found in the rhizosphere of different plants have been extensively studied, mainly considering plant growth-promoting rhizobacteria (PGPR), which exert positive interactions with plant roots [7, 17, 18, 24, 42, 45]. Plants might select functional groups of beneficial microorganisms by root exudation [8, 19], and an increased or altered rhizodeposition may selectively stimulate and enrich certain groups of microorganisms, leading to new community structures [20].

Within the 75 species of the genus *Paenibacillus* (NCBI homepage, October 2006), nine species (*P. polymyxa, P. durus, P. graminis, P. macerans, P. borealis, P. odorifer, P. wynnii, P. brasilensis,* and *P. peoriae*) harbor strains that are considered PGPR because of their capability to fix atmospheric nitrogen [10, 38]. They are free-living bacteria that inhabit the soil and the rhizosphere of a variety of plants. Besides fixing nitrogen, some of these *Paenibacillus* species can influence plant growth and health by the production of phytohormones, chitinases [21], proteases [1, 26, 40], antimicrobial compounds [26, 39, 44], and solubilizing phosphate [42]. Studies have shown that the

rhizosphere of many non-leguminous plants, especially of *Poaceae*, is colonized by one or more genera of non-symbiotic diazotrophs, including *Paenibacillus* strains [4]. Although the amount of nitrogen fixed by free-living nitrogen-fixing microorganisms can be considered small compared with the symbiotic diazotrophs, free-living nitrogen fixers are present in the majority of soils and are sometimes considered as the dominant source of nitrogen [22, 23].

Nucleic acid-based analyses of bacterial communities, mainly approaches based on cloning and sequencing of 16S rDNA fragments, have been used to overcome biases of cultivation-dependent methods and to provide data from spatial and temporal variations of rhizosphere bacterial communities [14, 43]. However, the use of the 16S rRNA gene to study the diversity of bacterial communities is sometimes limited by the presence of multiple copies of this gene in many bacterial groups, as in *Paenibacillus* [32]. Therefore, the gene encoding the RNA polymerase beta subunit, *rpoB*, has been introduced in taxonomic studies and community analyses of bacteria as an alternative to the 16S rRNA gene, because it exists in a single copy in all bacterial genomes studied so far and contains conserved as well as variable regions. Furthermore, *rpoB* has been shown to be more discriminative than the 16S rRNA gene [27] and has been used to identify nitrogen-fixing *Paenibacillus* [28, 29] and other bacterial genera [13, 27, 36]. In denaturing gradient gel electrophoresis (DGGE) analysis, it is expected that the PCR product of *rpoB* of a species will result in a single band, making it possible to distinguish different species [11]. The *rpoB* gene as a target for PCR-DGGE analysis has been already used in different research fields as, for example, to study bacterial diversity of a tropical soil [34], lactic acid bacteria evolution during winemaking [37], and the ecology of *Yersinia* spp. in food [9].

Sorghum bicolor is an important crop because of its large productivity of grains and utilization as animal ration. In Brazil, it has emerged as an excellent alternative for agricultures in the northeast region of the country by its ability to grow well during the dry season. Different sorghum cultivars have been developed in traditional breeding and genetic programs by EMBRAPA-CNPMS to reduce nitrogen utilization. Among 400 sorghum cultivars sown in Cerrado soil with low levels of nitrogen, 24 cultivars were considered "efficient," which means that they reached their maximum growth without showing the characteristic symptoms of deficiency of nitrogen (reduced growth and yellow leaves). The remaining ones were considered "inefficient", as they demanded high levels of nitrogen to grow well. In this study, four sorghum cultivars were used, two considered as "efficient" (IPA 1011 and FBS 8701-9) and two "inefficient" (IS 5322-C and IS 6320). These four cultivars were sown in Cerrado soil with low and high levels of nitrogen, 12 kg/ha and 120 kg/ha, respectively, and the diversity of *Paenibacillus* populations was evaluated by

molecular methods as *rpoB*-based PCR-DGGE and clone library. The results will demonstrate whether there is an influence of the cultivar and the amount of nitrogen fertilization on the composition of *Paenibacillus* populations.

MATERIALS AND METHODS

Sorghum Cultivars and Experimental Conditions

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. The local climate is the savannah type, with a mean temperature in the coldest month above 18°C, according to the Köppen classification. The soil planted with four different cultivars of sorghum (*Sorghum bicolor*) was classified as a typical Distrophic Red Latosol, Cerrado stage, with a very clay texture (coarse sand 6%, fine sand 4%, silt 12%, and clay 78%). Physical and chemical analyses of the soil showed low organic matter content (3.27 dag/dm³), a pH value of 6.2, and P, K, Ca, and Mg contents of 11, 80, 5.85, and 0.87 cmolc/dm³, respectively. The four types of sorghum cultivars (IPA 1011, FBS 8701-9, IS 5322-C, and IS 6320) were chosen based on their agricultural importance. Briefly, they can be described as follows: IPA 1011, white grain, three-dwarf, 84 days from planting to flowering, and a grain productivity of 5,490 kg/ha; FBS 8701-9, white grain, three-dwarf, 63 days from planting to flowering, and a grain productivity of 5,270 kg/ha; IS 6320, white grain, one or two-dwarf, 93 days from planting to flowering, and a grain productivity of 1,020 kg/ha; IS 5322-C, white grain, short three-dwarf, 56 days from planting to flowering, and a grain productivity of 930 kg/ha. IPA 1011 and FBS 8701-9 are considered as "efficient" (E) cultivars under nitrogen stress, capable of growing well at low concentrations of nitrogen in the soil, and IS 5322-C and IS 6320 are considered as "inefficient" (I) cultivars under nitrogen stress, showing characteristic symptoms of the lack of this nutrient (reduced growth and yellow leaves). The experimental plots consisted of two lines of 5 m of length with spaces of 0.8 m between lines and 0.2 m between plants, made in two replicates. The cultivars were planted randomly in each line. One line received 12 kg of nitrogen per hectare (low concentration of nitrogen) and the other 120 kg of nitrogen per hectare (high concentration of nitrogen). Five plants of each sorghum cultivar were harvested 90 days after sowing and the roots shaken to remove the loosely attached soil. The adhering soil of the five plants was pooled and considered as the rhizosphere soil. Samples were kept at –20°C before DNA extraction.

Extraction of DNA from Rhizosphere Soil

DNA was extracted from rhizosphere soil of sorghum cultivars (0.5 g of each sample) by using the commercial

kit "FastDNA Spin Kit for soil" (Q.Bio gene, BIO 101 Systems) according to the manufacturer. DNA concentrations were determined spectrophotometrically using a Gene Quant apparatus (Amersham Pharmacia Biotech). The DNA was visualized on 0.8% (w/v) agarose gels [41] to assess its purity and molecular size.

PCR-DGGE Analysis

For amplification of *rpoB* gene sequences, a primer set consisting of a forward primer (*rpoB*1698f) and a reverse primer (*rpoB*2041r) was first used as described by Dahllöf *et al.* [11], yielding a product of 375 bp. The amplification conditions were 1× (5 min, 95°C), 6× (30 s, 94°C; 1.5 min, 40°C; 1.5 min, 72°C), 19× (30 s, 94°C; 1.5 min, 50°C; 1.5 min, 72°C), with a final 10 min extension period at 72°C. The 50 ml reaction mix contained 1 ml of template DNA (50 to 100 ng), 10 mM Tris-HCl, pH 8.3, 10 mM KCl, 25 pmol of each primer, 2.5 mM of each dNTPs, 20 mg of BSA, 2.6 mM $MgCl_2$, and 5 U of *Taq* polymerase. For amplification of *Paenibacillus rpoB* gene sequences, a seminested approach was used. The amplification products of the first PCR for the *rpoB* gene were used as template for a second amplification using the forward primer (*rpoB*1698f) with a GC clamp [11] and the reverse primer (*rpoB*PAEN) as described by Mota *et al.* [29], yielding a product of about 280 bp. The amplification conditions were 1× (5 min, 95°C), and 25× (30 s, 94°C; 1.5 min, 40°C; 30 s, 72°C), with a final 10 min extension period at 72°C. Negative controls (without DNA) were run in all amplifications and PCR products were visualized by 1.4% agarose gel electrophoresis followed by staining with ethidium bromide. DGGE was performed with the Dcode Universal Mutation Detection System (Bio-Rad Laboratories). PCR products (10–15 µl) were applied directly onto 6% (w/v) polyacrylamide gels in 1× TAE buffer (20 mM Tris-acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM disodium EDTA) containing a linear gradient from 40 to 70%. The gradients were formed with 6% (w/v) acrylamide stock solutions [31] that contained either no denaturant or 80% denaturant [the 80% denaturant solution contained 7 M urea and 40% (v/v) formamide deionized with AG501-X8 resin (Bio-Rad)]. The gels were electrophoresed for 16 h at 60°C and 65 V. After electrophoresis, the gels were stained for 30 min with SYBR Green I (Molecular Probes) and photographed under UV light by using the STORM apparatus (Amersham). Some bands were retrieved from the gels (marked in Fig. 1), reamplified, and sequenced using primers *rpoB*1698f and *rpoB*PAEN. The *rpoB* sequences obtained were identified by BLAST-N analyses using the GenBank database.

Cloning and Sequencing of PCR Products

rpoB PCR products of 240 bp (without clamp) obtained from the seminested PCR using the forward primer (*rpoB*1698f) and the reverse primer (*rpoB*PAEN) were
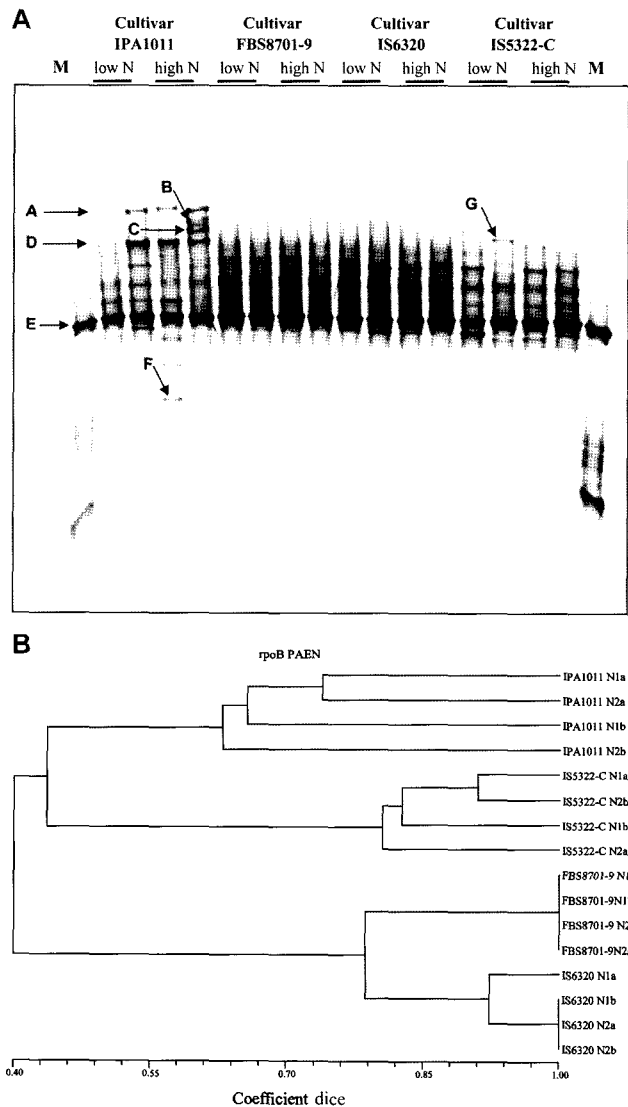
Fig. 1. **A.** DGGE patterns obtained with *Paenibacillus*-specific PCR-DGGE based on the *rpoB* gene of rhizospheres samples from cultivars IPA 1011, FBS 8701-9, IS 6320, and IS 5322-C sown in Cerrado soil amended with low and high levels of nitrogen. Dashes represent each replicate. Arrows indicate the bands that were retrieved from the gel, reamplified, and sequenced. The *rpoB* fragment of *Paenibacillus durus* P3L5[T] was used as a standard (lanes M). **B.** Dendrogram showing the levels of similarity of the *Paenibacillus* communities of rhizospheres samples from cultivars IPA 1011, FBS 8701-9, IS 6320, and IS 5322-C sown in Cerrado soil amended with low and high levels of nitrogen. The dendrogram was constructed with the unweighted pair group method with mathematical averages, and similarity coefficient of DICE. Numbers 1 represent the low level of nitrogen (12 kg/ha) and number 2 represents the high level of nitrogen (120 kg/ha); letters a and b represent the replicates.

purified with a Wizard PCR Preps kit, and DNA fragments were cloned using the pGEM-T easy vector according to the instructions of the manufacturer (Promega). After transformation of *E. coli* JM109 competent cells, clones

were picked and the presence of inserts of the correct size was verified by PCR using M13f (5'-GTAAAACGACGG-CCAG-3') and M13r (5'-CAGGAAACAGCTATGAC-3') primers. Selected clones were sequenced using M13f and M13r primers by an ABI Prism 3100 automatic sequencer (Applied Biosystems). The partial *rpoB* sequences of selected representative clones obtained in this study were compared against the GenBank database by using the algorithm BLAST-N to identify the most similar *rpoB* sequences.

Statistical and Data Analyses

Duplicate samples were analyzed per treatment (sorghum cultivar and level of fertilizer). Analyses of the clone libraries were followed by calculation of the coverage (C), where C is expressed by $1-n_1/N$, in which n_1/N is the ratio of clones that appeared only once (n1) by the total number of clones (N) [6]. An Excel worksheet was used to calculate the Shannon-Wiener index $H'=-\sum_{i=1}^{S}p_i\ln(p_i)$ [25], and Evenness $E=H'/\ln S$ [35], where S is the number of species observed and p_i is the number of clones of a given species divided by the total number of organisms observed. The data from sequence libraries were also submitted to Principal Component Analysis (PCA), using Statistica 6. DGGE data were collected into a matrix indicating the presence or absence (scored as 1 or 0, respectively) of specific bands in DGGE analysis. Cluster analysis of the DGGE profiles was done by using the NTSYS 2.02 software, using the unweighted pair group method with mathematical averages (UPGMA) and correlations were calculated using the Dice coefficient of similarity.

RESULTS AND DISCUSSION

Previous studies have demonstrated that root alterations may interfere in the plant exudation pattern and, thus, affect the structural and functional diversity of rhizosphere microbial communities [12, 42]. The altered root morphology and physiology can be a consequence of the different levels of nitrogen fertilizer amended to the soil. In this study, molecular techniques were applied to study the structural diversity of the *Paenibacillus* community in the rhizosphere of four different sorghum cultivars showing different nitrogen demand to grow. Only one development vegetative stage (90 days after sowing) and the Cerrado soil were used here to avoid the influence of the plant age and the soil type. The growth stage of the plants influencing population shifts in the rhizosphere community has already been demonstrated in maize [12, 18, 42, 43] and in different alfalfa cultivars [5]. In addition, the influence of the soil type in the selection of bacterial communities has been shown in maize [2, 42] and alfalfa [5, 33].

The diversity of *Paenibacillus-rpoB* specific PCR products in rhizosphere samples of four different sorghum cultivars sown in Cerrado soil was first analyzed using the fingerprints of the most dominant populations obtained after separation of *rpoB*-based PCR products in DGGE (Fig. 1A). Banding patterns obtained in DGGE from rhizosphere samples were reproducible among the duplicates, although slight differences were observed. Thirty-two markers (corresponding to different bands in DGGE profiles) were used for the construction of the dendrogram using DICE and UPGMA (Fig. 1B). Profiles of cultivars FBS 8701-9 and IS 6320 clustered together, with only 40% of similarity to IPA 1011 and IS 5322-C. However, only at 79% similarity did all profiles of different cultivars studied cluster separately. Fertilization of the soil with these two contrasting levels of nitrogen did not seem to influence the community of *Paenibacillus* when all cultivars are considered. Mainly in cultivar FBS 8701-9 (considered as "efficient"), the profiles obtained with both levels of nitrogen clustered in 100% similarity. This observation could be explained by the fact that the prevalent *Paenibacillus* communities are being studied and not only the nitrogen-fixing species, which might be more influenced by the nitrogen input in soil. Furthermore, no correlation between the two "efficient" (IPA 1011 and FBS 8701-9) and the two "inefficient" (IS 5322-C and IS 6320) cultivars could be observed (Fig. 1B). This result suggests that the type of cultivar is directly responsible for the differences observed in *Paenibacillus* populations. Similar observations have been described for *P. polymyxa* [30] and *P. durus* [43] when traditional cultivation techniques were used. In those studies, differences between the strains isolated from the rhizospheres of different maize cultivars were clearly demonstrated, indicating an effect of maize cultivar.

DGGE profiles allowed presumptive identification of some of the bands. Thus, seven bands were retrieved from the gels (marked in Fig. 1A), reamplified, and sequenced. BLAST-N analyses were performed and bands were identified as bands produced by organisms related to *P. graminis* (band A), *P. odorifer* (bands B, C, and D), *P. durus* (bands E and F), and *P. campinasensis* (band G). Bands migrating to different positions were assigned as belonging to the same species; these results are in agreement with those presented by Mota et al. [29] and they may represent different strains of the same species. Rhizospheres from all cultivars showed a strong band that could be correlated to *P. durus* because of their comigration with the PCR fragment of the pure culture of *P. durus*. However, this band could not be sequenced because it might represent a mixture of sequences, mainly of *P. durus* and *P. graminis*. *rpoB* and 16S rRNA sequences from both species are considered very similar [3, 28]. Data obtained from sequencing of DGGE bands clearly indicated that the DGGE profiles do reflect the sequences that were also

Table 1. Number of clones identified as members of different species of *Paenibacillus*, obtained from the four libraries (two cultivars of sorghum sown with low and high levels of nitrogen fertilizer).

Species of *Paenibacillus*	Number of clones			
	IPA 1011 (E-N)[a]	IPA 1011 (E+N)[b]	IS 5322-C (I-N)[c]	IS 5322-C (I+N)[d]
P. graminis	30	41	65	59
P. amylolyticus	21	10	8	10
P. macerans	8	19	2	11
P. thiaminolyticus	5	0	0	0
P. pabuli	4	0	0	0
P. durus	3	6	1	0
P. favisporus	1	0	0	0
P. dentritiformis	2	0	0	0
P. campinasensis	1	4	0	0
P. odorifer	2	0	0	0
P. curdlanolyticus	1	1	0	0
P. sanguinis	0	1	0	0
P. peoriae	0	1	0	0
P. polymyxa	0	0	1	0
Total	78	83	77	80

[a](E-N), efficient cultivar under nitrogen stress, sown in Cerrado soil with low amount of nitrogen (12 kg/ha).

[b](E+N), efficient cultivar under nitrogen stress, sown in Cerrado soil with high amount of nitrogen (120 kg/ha).

[c](I-N), inefficient cultivar under nitrogen stress, sown in Cerrado soil with low amount of nitrogen.

[d](I+N), inefficient cultivar under nitrogen stress, sown in Cerrado soil with high amount of nitrogen. Sequences of all clones were deposited in the GenBank (accession numbers EF176140-EF176457).

found by direct cloning of *rpoB* amplicons generated on the basis of the rhizospheres DNAs.

Two cultivars were further chosen for the construction of *rpoB* libraries: IPA 1011 ("efficient") and IS 5322-C ("inefficient"). Both levels of nitrogen fertilization were also considered and, therefore, PCR products were obtained using the *rpoB*PAEN primer in combination with *rpoB*1698f and four libraries were produced (318 clones). All inserts were sequenced and the resulting sequences (GenBank accession numbers EF176140-EF176457) could be related to one of the sequences from *Paenibacillus* previously obtained [29] by BLAST-N. The results confirmed the great specificity of primers *rpoB*PAEN and *rpoB*1698f for *Paenibacillus*, since 100% of the clones were identified as belonging to this genus (Table 1). In general, considering only the first hit in BLAST-N with *rpoB* sequences, the clones obtained were closely related to *P. graminis* (61%), *P. amylolyticus* (15%), and *P. macerans* (13%). The remaining 11 species were represented with fewer clones in the libraries. The species *P. graminis* was predominant in all libraries, mainly in that obtained from the cultivar IS 5322-C ("inefficient") planted with the low level of nitrogen. The largest diversity of *Paenibacillus* species was observed in the library obtained from cultivar IPA 1011 ("efficient") sown with the low level of nitrogen. Some *Paenibacillus* clones were typical for one cultivar and not found in the other; for instance, clones related to *P. thiaminolyticus*, *P. pabuli*, *P. favisporus*, *P. dentritiformis*, *P. campinasensis*, *P. odorifer*, *P. curdlanolyticus*, *P. sanguinis*, and *P. peoriae* were

observed exclusively in IPA 1011. Moreover, clones closely related to *P. thiaminolyticus*, *P. favisporus*, *P. dentritiformis*, and *P. odorifer* occurred exclusively when the low level of nitrogen was applied to the soil (Table 1), suggesting that the amount of nitrogen added to the soil had influenced the variety of *Paenibacillus* species in the rhizosphere of cultivar IPA 1011. Six species of *Paenibacillus* identified among the clones obtained are considered as nitrogen-fixing species [10]. The species *P. graminis* and *P. amylolyticus* have been observed to be also dominant among clones obtained from the DNA of Cerrado soil [29].

When statistic analyses were performed using the data of *rpoB* libraries, the Shannon-Wiener index values showed the highest (1.764) and the lowest (0.586) diversity of *Paenibacillus* populations in the cultivars sown in soil amended with the low level of nitrogen (E-N and I-N, respectively). Both values observed for the "efficient" (E=IPA 1011) cultivar were higher than those observed for the "inefficient" (I=IS 5322-C) cultivar. However, the lowest evenness value was observed in IS 5322-C amended with the low level of nitrogen, which is more affected by nitrogen limitation (Table 2). To check whether the size of the soil clones libraries was reflecting the real diversity, the coverage index of the clones was checked according to Chelius and Triplett [6]. The four clone libraries obtained in this study covered at least 96.2% of the total diversity, considering the BLAST-N identification (Table 2). Finally, data from clone libraries were also subjected to PCA. Three factors represented 100% of the variance (factor

Table 2. Statistical analyses of the clone libraries.

Cultivars/level of nitrogen fertilization[a]	Shannon-Wiener[b]	Evenness[c]	Coverage[d]
E+N	1.437	0.691	0.964
E-N	1.764	0.735	0.962
I+N	0.757	0.689	1.000
I-N	0.586	0.364	0.974

[a]E (cultivar IPA 1011)+N=efficient cultivar under nitrogen stress sown in Cerrado soil with a high amount of nitrogen (120 kg/ha), E-N=efficient cultivar under nitrogen stress sown in Cerrado soil with a low amount of nitrogen (12 kg/ha), I (cultivar IS 5322-C)+N=inefficient cultivar under nitrogen stress sown in Cerrado soil with a high amount of nitrogen, I-N=inefficient cultivar under nitrogen stress sown in Cerrado soil with a low amount of nitrogen.

[b]Shannon-Wiener index calculated by $H'=-\sum_{i=1}^{s} p_i \ln(p_i)$ [25].

[c]Evenness calculated by $E=H'/\ln S$ [35].
[d]Coverage calculated by $1-n_1/N$ [6], as described in Materials and Methods.

1=42.1%; factor 2=32%, and factor 3=25.9%). For this approach, *Paenibacillus* populations were considered in each clone library obtained. Fig. 2 corresponds to the three-dimensional plot of PCA, which demonstrates that the cultivar considered as "efficient" (IPA 1011) deviated from the "inefficient" (IS 5322-C), but the level of nitrogen fertilizer contributed less to the variance.

The present findings emphasize the importance of the cultivar in the selection of specific *Paenibacillus* populations to coexist in the rhizosphere and, therefore, they have to be taken into account in the selection of *Paenibacillus*
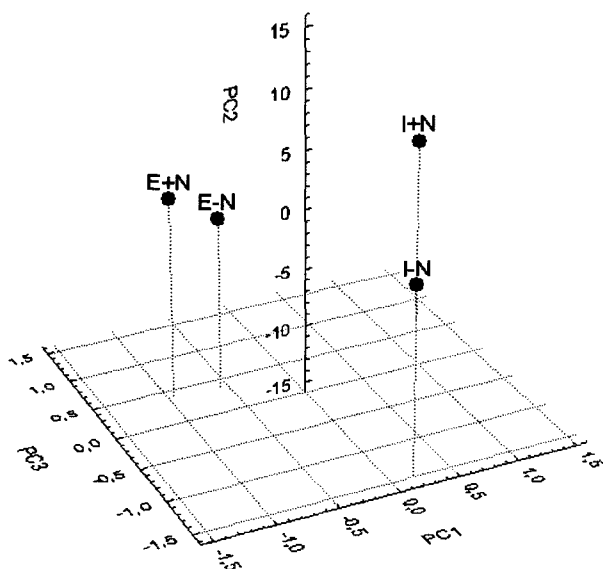
Fig. 2. Three-dimensional plot scores for the four populations of *Paenibacillus* (corresponding to clone libraries of cultivars IPA 1011 and IS 5322-C) sown in the two levels of nitrogen, tested by Principal Component Analysis (PCA).

strains for using as sorghum inoculants. Similar results have been reported by Garcia de Salomone *et al.* [15] for maize-genotype association with *Azospirillum* and by Carelli *et al.* [5] for alfalfa cultivars and *Sinorhizobium* populations. On the other hand, the level of nitrogen fertilization used in Cerrado soil does not seem to influence significantly the distribution of *Paenibacillus* populations. Further studies are necessary to evaluate not only the structural diversity of the genus *Paenibacillus* but also the functional diversity.

Acknowledgments

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