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PLANT MICROBE INTERACTIONS

# Morphological and genetic characterization of endophytic bacteria isolated from roots of different maize genotypes

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**Abstract** Maize is one of the most important crops worldwide, and in Brazil, the state of Paraná stands as its largest producer. The crop demands high inputs of N fertilizers, therefore all strategies aiming to optimize the grain production with lower inputs are very relevant. Endophytic bacteria have a high potential to increment maize grain yield by means of input via biological nitrogen fixation and/or plant growth promotion, in this last case increasing the absorption of water and nutrients by the plants. In this study, we established a collection of 217 endophytic bacteria, isolated from roots of four lineages and three hybrid genotypes of maize, and isolated in four different N-free culture media. Biochemical—comprising growth in different carbon sources, intrinsic tolerance to antibiotics, and biochemical tests for catalase, nitrate reductase, urease, and growth in N-free media in vitro—and genetic characterization by BOX-PCR revealed great variability among the isolates. Both commercial hybrids and homozygous lineages were broadly colonized by endophytes, and sequencing of the 16S rRNA gene revealed the presence of bacteria belonging to the genera *Pantoea*, *Bacillus*, *Burkholderia*, and *Klebsiella*. Qualitative differences in endophytic colonization were detected between lineages and hybrid genotypes.

## Introduction

Beneficial rhizospheric and endophytic associations that occur with the roots of gramineae plants with bacteria include those with capacity of fixing atmospheric nitrogen ($N_2$) and promoting plant growth. Studies of these microorganisms generate important information to understand the biology of the interaction of plants and bacteria and also because they may reveal promising strains useful to the control of plant pathogens, production of metabolites with biotechnological interest, and

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capacity of promoting plant growth and fixing $N_2$, among other advantages concerning agronomic aspects [5, 10, 13, 26].

Maize (*Zea mays* L.) crops need large quantities of nitrogen (N) fertilizer to reach high yields. However, plants can highly benefit from the association with $N_2$ fixing and/or plant growth-promoting bacteria, in this last case contributing to an increase in root surface and growth, improving water and nutrient uptake [e.g., 16, 26]. As maize is broadly used for human and animal consumption, partial reduction of N fertilizer by using rhizospheric and endophytic bacteria would be economically and environmentally relevant. However, for maximizing the benefits, it should be accounted that genotypic variability of maize plants may influence the efficiency of $N_2$ fixation or the capacity of promoting plant growth [e.g., 15, 19, 23].

Although classical phenotyping methods are commonly used to identify microorganisms, they are insufficient to discriminate species and strains, but when associated with molecular techniques, result in reliable microbial identification. Molecular techniques such as BOX-PCR and sequencing of conserved regions of bacterial DNA, with an emphasis on the 16S rRNA have been broadly used in the characterization of $N_2$ fixing and plant growth-promoting bacteria [e.g., 9, 22, 24, 38].

Characterization of bacteria associated with the maize crop may provide valuable information to our still poor knowledge of the plant–bacteria interaction with different genotypes in addition to the possibility of recognizing strategies that may benefit maize management and production. The aim of this study was, thus, to isolate and to characterize, by means of biochemical and genetic methods, a collection of bacteria obtained from roots of different maize genotypes.

## Methods

### Maize genotypes description and plant growth conditions

The following maize genotypes were used in this study: lineages LA, LB, LC, and LD and the hybrids H1, H2, and H3 (derived from the crosses between LA × LC, LA × LB, and LA × LD, respectively). Genotypes were provided by the private company "Semilia Genética e Melhoramento Ltda" (Bateias, Campo Largo, state of Paraná, Brazil).

Forty-eight seeds of each genotype were sown in a complete randomized block design with six replicates, with four plants per replicate. Plants were grown under field conditions, with an average temperature of 21/18 °C (day/night) and manual irrigation every other day. Seventy five days after sowing, one plant of each replicate and each treatment was collected (1×6×7), totalizing 42 root samples.

### Bacteria isolation

Root samples were surface-sterilized [29], and five fragments from each root were aseptically cut and transferred to Petri dish plates containing one of the following solid culture media without nitrogen (N-free media): NFb, JNFb, LGI, or LGI-P [11]. Several controls confirmed that the sterilization procedure was effective, as no bacteria were grown when intact self-sterilized roots were transferred to Petri dishes. Although isolation of $N_2$-fixing bacteria is usually performed in semisolid medium, we have isolated the strains in solid medium to verify the types of colonies obtained from each sample; further tests on semisolid medium were performed, as will be described.

After inoculation, plates were incubated at 30 °C in the dark, and after 7 days, from the plates with positive growth (138 plates), three colonies were randomly taken from each plate for purification by scratching. After reisolation and checking for purity, 217 isolates were maintained for this study.

### Morphophysiological characterization

Basic morphology properties were verified for all 217 isolates [28, 39]. Several strains from the same treatment have shown identical basic morphology properties; therefore, the scope of the study was reduced to 98 strains, based on different treatments and the different morphological properties.

The 98 selected isolates were submitted to the following biochemical tests in vitro: catalase, urease, glucose fermentation, nitrate reduction, growth with different carbon sources (dextrose, fructose, galactose, lactate, malate, mannitol, and sucrose), and intrinsic tolerance to antibiotics (amoxicillin and tetracycline at the concentrations of 50, 150, and 300 $\mu g mL^{-1}$). All tests were conducted in triplicates. A preliminary evaluation of $N_2$ fixation capacity was performed by means of growth of all strains in all four N-free semisolid media (NFb, JNFb, LGI, or LGI-P, with 2 $gL^{-1}$ of agar) [2, 21, 27]. In addition, in order to confirm the property of the strains that confirmed growth in semisolid N-free medium, they were successively replicated ten times to check growth in the absence of N.

### Genetic characterization

Total genomic DNA was extracted from the 98 isolates that were biochemically characterized and amplified by PCR with the primer BOX A1R (5′-CTACGGCAAGGCGACGCT GACG-3′, Invitrogen™) [38], as described before [18]. Gels were stained with ethidium bromide, visualized under UV radiation, and photographed.

**Table 1** Biochemical characterization of 217 isolates obtained from four lineages and three hybrid maize genotypes

| Property | % of isolates |
|---|---|
| Catalase | 100 |
| Nitrate reductase | 81 |
| Urease | 8 |
| Glucose fermentation | 36 |
| Growth in N-free semisolid and solid media (NFb, JNFb, LGI, and LGI-P) | 100 |
| Antibiotic tolerance | |
| Amoxicillin (50 $\mu g mL^{-1}$) | 80 |
| Tetracycline (50 $\mu g mL^{-1}$) | 13 |
| Carbon sources | |
| Dextrose | 96 |
| Fructose | 96 |
| Galactose | 95 |
| Lactose | 92 |
| Malate | 94 |
| Mannitol | 95 |
| Sucrose | 95 |

The results are expressed as the percentage of isolates with positive reaction for each property

When the BOX-PCR profiles of all 98 strains were analyzed together (data not shown), 35 strains were selected that represented different treatments and different clusters in each treatment. Partial sequencing of the 16S rRNA gene of 35 isolates was performed by using the primers Y3 (5′-CTGACCCCACTTCAGCATTGTTC CAT-3′; designed by Dr. J. P. W. Young) and 786f (5′-CGAAAGCGTGGGGAGCAAACAGG-3′, designed by Dr. L. M. Cruz), and the amplification conditions described by Weisburg et al. [40]. Sequencing was performed as described before [25] with the same primers, in a MegaBACE 1000 DNA Analysis System (Amersham Biosciences). Sequence editing and inspection were performed using BioEdit program 7.0 version [14], and then were aligned and compared with sequences from the GenBank by using the BLAST program, BLASTn option [1].

## Data analysis

To analyze differences in the number of isolates obtained for each maize genotype, a factorial analysis was performed using the data from the Petri dishes of each media which had positive result for colony growth in the four N-free culture media, with the Assistat software v. 7.6 (http://www.assistat.com/indexp.html).

The results obtained in the biochemical and genetic characterization were transformed into binary matrices and analyzed by using the Numerical Taxonomy System program version 2.02 [34], generating the dendrograms and the principal component analysis, with Jaccard (J) coefficient, with the algorithm UPGMA and bootstrap analysis with 10,000 repetitions with BOOD software [8].

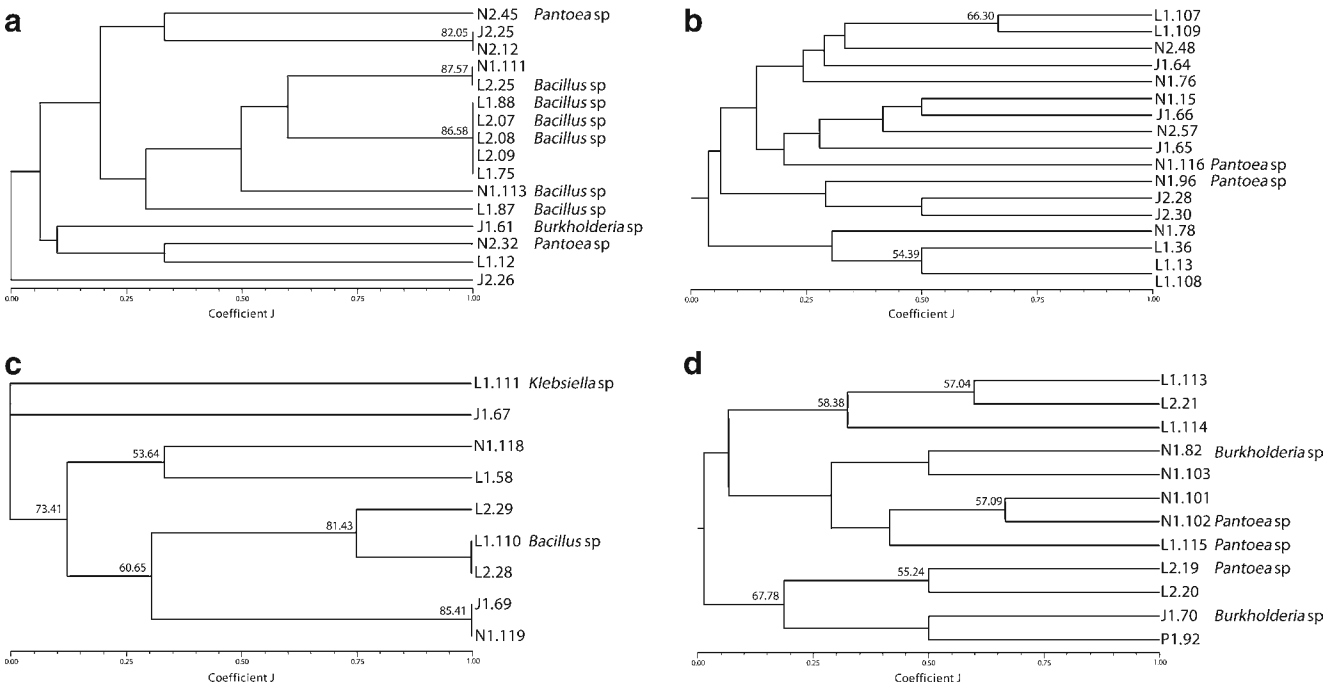


**Figure 1** Dendrogram of genetic characterization by BOX-PCR of isolates from **a** lineage A (LA), **b** lineage B (LB), **c** lineage C (LC), and **d** lineage D. Cluster analysis with the UPGMA algorithm and the coefficient of Jaccard, with the NTSYS software, bootstrap support of 10,000 replicates

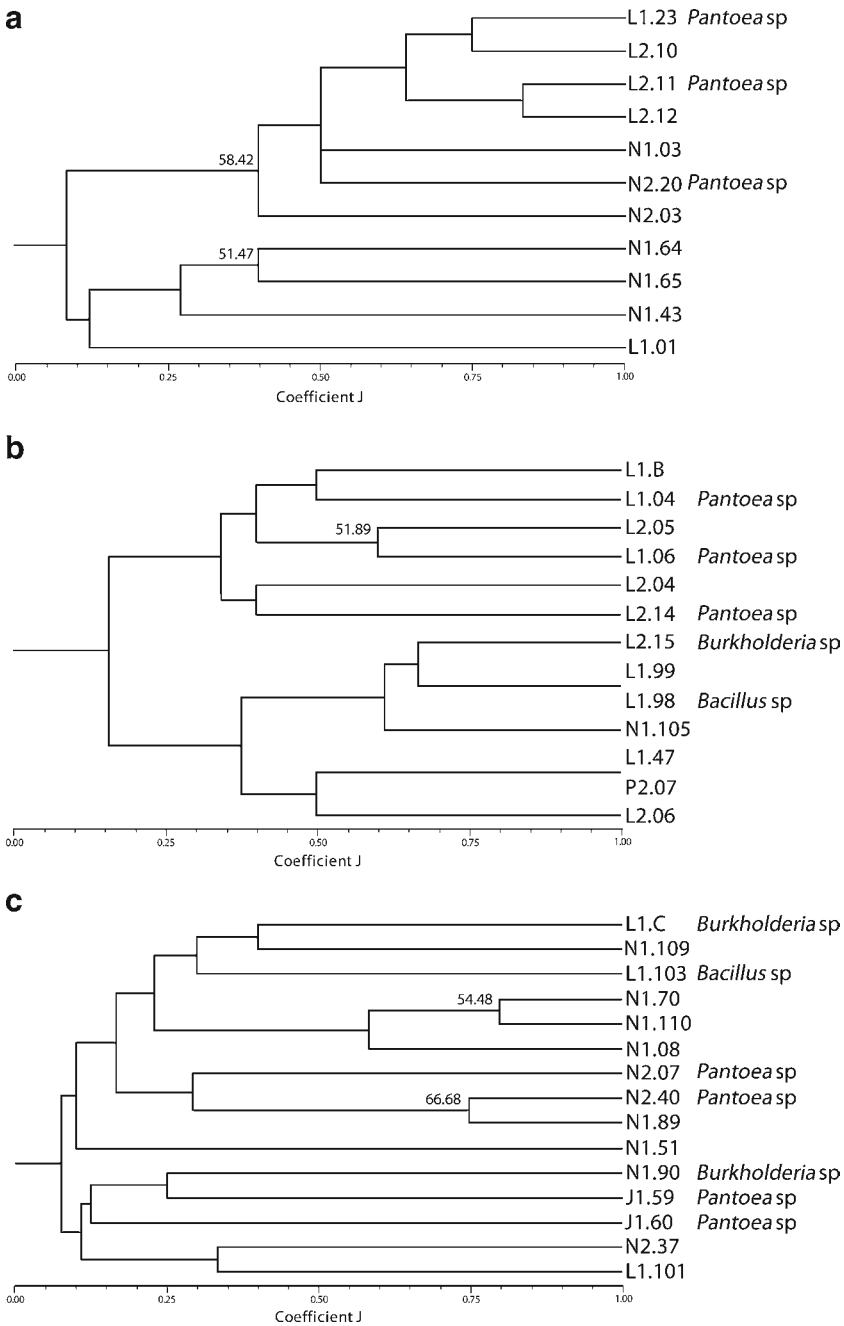


**Figure 2** Dendrogram of genetic characterization by BOX-PCR of isolates from **a** hybrid 1 (LA × LC), **b** hybrid 2 (LA × LB), and **c** hybrid 3 (LA × LD). Cluster analysis with the UPGMA algorithm and the coefficient of Jaccard, with the NTSYS software, bootstrap support of 10,000 replicates

## Results

The number of isolates grown in each of the four N-free culture medium was statistically different ($F$=16.2388, $p$< 0.01, data not shown). On the contrary, no differences were observed in the number of isolates obtained from each of the seven maize genotypes, as well as there were no effects attributed to the interaction of culture media × genotypes (data not shown).

As pointed out in the "Material and methods," 98 out of the 217 isolates were selected and biochemically characterized based on basic morphological properties. Catalase activity was not discriminatory, as all isolates were positive for this test (Table 1). Growth in culture medium supplied with different carbon sources has also shown that the great majority of the isolates were not selective for specific sources of carbon, but only 36 % were positive for the glucose fermentation test. Intrinsic tolerance to the antibiotics amoxicillin and tetracycline indicated that the last one was the most effective in inhibiting growth, and Table 1 shows the results obtained with the minimum concentration tested. We are showing the minimum concentration tested because it was sufficient to

**Table 2** Identification by partial sequencing of 16S rRNA region from 35 isolates

| Isolate | Accession no | Base pairs | Genus | Identity (%) | *e* value |
|---|---|---|---|---|---|
| L1.103 | JQ811530.1 | 563 | *Bacillus* sp | 100 | 0.0 |
| L1.110 | JQ811531.1 | 574 | *Bacillus* sp | 100 | 0.0 |
| L1.87 | JQ811526.1 | 521 | *Bacillus* sp | 100 | 0.0 |
| L1.88 | JQ811527.1 | 510 | *Bacillus* sp | 100 | 0.0 |
| L1.98 | JQ811528.1 | 583 | *Bacillus* sp | 100 | 0.0 |
| L2.25 | JQ811540.1 | 565 | *Bacillus* sp | 100 | 0.0 |
| L2.07 | JQ811535.1 | 546 | *Bacillus* sp | 100 | 0.0 |
| L2.08 | JQ811536.1 | 573 | *Bacillus* sp | 100 | 0.0 |
| N1.113 | JQ811546.1 | 533 | *Bacillus* sp | 99 | 0.0 |
| J1.61 | JQ811521.1 | 563 | *Burkholderia* sp | 100 | 0.0 |
| J1.70 | JQ811523.1 | 535 | *Burkholderia* sp | 99 | 0.0 |
| L1.C | JQ811534.1 | 560 | *Burkholderia* sp | 100 | 0.0 |
| L2.15 | JQ811539.1 | 561 | *Burkholderia* sp | 99 | 0.0 |
| N1.82 | JQ811542.1 | 629 | *Burkholderia* sp | 99 | 0.0 |
| N1.90 | JQ811543.1 | 556 | *Burkholderia* sp | 100 | 0.0 |
| L1.111 | JQ811532.1 | 597 | *Klebsiella* sp | 100 | 0.0 |
| J1.59 | JQ811519.1 | 588 | *Pantoea* sp | 100 | 0.0 |
| J1.60 | JQ811520.1 | 507 | *Pantoea* sp | 99 | 0.0 |
| J1.68 | JQ811522.1 | 615 | *Pantoea* sp | 99 | 0.0 |
| L1.102 | JQ811529.1 | 536 | *Pantoea* sp | 99 | 0.0 |
| L1.115 | JQ811533.1 | 539 | *Pantoea* sp | 100 | 0.0 |
| L1.23 | JQ811525.1 | 603 | *Pantoea* sp | 99 | 0.0 |
| L1.04 | JQ811524.1 | 481 | *Pantoea* sp | 99 | 0.0 |
| L2.11 | JQ811537.1 | 548 | *Pantoea* sp | 99 | 0.0 |
| L2.14 | JQ811538.1 | 535 | *Pantoea* sp | 100 | 0.0 |
| L2.19 | JX284246 | 585 | *Pantoea* sp | 100 | 0.0 |
| N1.102 | JQ811545.1 | 583 | *Pantoea* sp | 100 | 0.0 |
| N1.116 | JQ811547.1 | 480 | *Pantoea* sp | 100 | 0.0 |
| N1.06 | JQ811541.1 | 554 | *Pantoea* sp | 100 | 0.0 |
| N1.96 | JQ811544.1 | 579 | *Pantoea* sp | 100 | 0.0 |
| N2.20 | JQ811549.1 | 594 | *Pantoea* sp | 100 | 0.0 |
| N2.32 | JQ811550.1 | 509 | *Pantoea* sp | 99 | 0.0 |
| N2.40 | JQ811551.1 | 590 | *Pantoea* sp | 100 | 0.0 |
| N2.45 | JQ811552.1 | 578 | *Pantoea* sp | 100 | 0.0 |
| N2.07 | JQ811548.1 | 537 | *Pantoea* sp | 100 | 0.0 |

discriminate the isolates, but some of the isolates were tolerant to the maximum concentration. Nitrate reductase was positive in 81 % of the isolates, while urease was positive in only 8 %. Finally, the putative capacity of fixing nitrogen in vitro, evaluated by the growth in each one of the four N-free culture media, solid and semisolid (Table 1), was confirmed in 100 % of the isolates (Table 1).

For the genetic characterization, the DNAs of 93 out of the 98 strains studied resulted in profiles with several bands when subjected to the BOX-PCR amplification reaction. Binary matrices were constructed using the band data obtained and dendrograms of similarity were generated. A high level of genetic diversity was observed in both types of maize genotypes, lineages, and hybrids. However, the very low final level of similarity in the clustering analysis of the lineages A, B, and C was noteworthy, with a slight increase in similarity for the hybrids, especially hybrid 2 (Figs. 1 and 2).

Sequencing of about 500 bp (from 480 to 615 bp) concerning the end of the 16S rRNA region was performed for 35 isolates (Table 2). By comparison with the BLASTn deposited sequences, the bacteria could be classified in four genera described as endophytic growth promoters and with potential for nitrogen fixation: *Klebsiella*, *Burkholderia*, *Bacillus*, and *Pantoea*. It is noteworthy that *Pantoea* was present in all maize genotypes used in this study. The identified genera in the sequencing analysis of the 16S rRNA are also shown in Figs. 1 and 2. Finally, Fig. 3 highlights the genera identified in each of the lineages and hybrids used in our study.

## Discussion

Studies reporting the isolation and characterization of endophytic bacteria with potential for plant growth promotion and nitrogen fixation capacity, as well as describing the interaction of these bacteria with the host plant, are of interest for advances in our knowledge about the plant–microbe interactions, as well as due to the potential economic and environmental benefits in agriculture. For example, variety of endophytes have been described in citrus (*Citrus* spp.), cocoa (*Theobroma cacao*), eucalyptus (*Eucalyptus* spp.), soybean (*Glycine max*), and sugarcane (*Saccharum* spp.) [36]. Barreti et al. [4] reported the effects of endophytic bacteria in growth promotion of tomato (*Lycopersicum esculentum* L.), with a significant increase in plant height, leaf area, leaf number, and fresh and dry

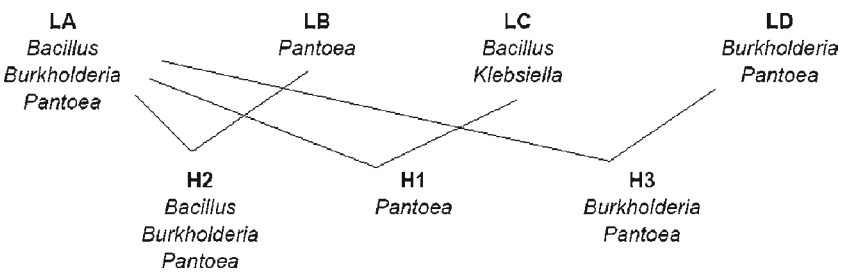


**Figure 3** Genera identified from each maize genotype, lineage (L), or hybrid (H), by partial sequencing of the 16S rDNA

plant weight. In another study, Assumpção et al. [3] described that cultivable endophytic bacterial isolates from soybean (*Glycine max* (L.) Merr.) seeds were capable of synthesizing indol acetic acid, of solubilizing phosphate, and of inhibiting growth and sporulation of pathogenic fungi. Additionally, Lacava et al. [20] characterized endophytic bacterial communities of plants affected by citrus variegated chlorosis (CVC) and observed that the disease development might result from changes in bacterial colonization. In addition, the authors also observed that different endophytic communities might interact to activate plant disease like CVC. There are other reports comprising the isolation and identification of endophytic bacteria from several plant species and, in our study, they represent the first step towards the determination of properties and/or strains with biotechnological potential in agriculture, e.g., as inoculants for grasses, more specifically for lineage and hybrid maize genotypes that represent an important sector of the agribusiness worldwide.

There are reports showing differential colonization of bacteria according to the plant genotype, e.g., in the interaction of *Azospirillum* with wheat (*Triticum aestivum* L.) [7, 17], maize (*Zea mays* L.) [12], and pearl millet [*Pennisetum americanum* (L.) K. Shum.] [6]. In our study, contrary to the results reported by Rodrigues et al. [32] in rice (*Oryza sativa* L.) genotypes, we have not detected quantitative differences in the bacterial endophytic community isolated from different maize genotypes, represented by lineages and hybrids.

Although we have not detected quantitative differences in maize genotype colonization, qualitative differences were revealed, with an emphasis on the results obtained in the analysis of the 16S rRNA. For example, lineage B (LB) as well as hybrid 1 (H1) were only colonized by the *Pantoea* genus, while in lineage C, the genera *Bacillus* and *Klebsiella* were identified, the latter being present only in this lineage. Qualitative differences are highlighted in the scheme of crosses and bacteria genera identified (Fig. 3), with the highest diversity being associated with lineage A and hybrid 2.

In our study, among the 35 sequenced strains, the genera of endophytic bacteria identified—*Bacillus*, *Klebsiella*, *Pantoea*, and *Burkholderia*—have been reported as both growth promoters and nitrogen fixers. In our study, all isolates confirmed in the capacity of growing in four N-free media, even after ten replicates in N-free medium, represent a strong indicative, although not conclusive, of $N_2$ fixation capacity. There are reports that the bacterium *Pantoea agglomerans* can be related to plant growth promotion and seed protection against plant diseases and herbivores [30]. They found that the sequencing of the 16S rRNA discriminated endophytic bacteria from *Eucalyptus* spp: *Pantoea*, *Agrobacterium*, *Brevibacillus*, *Pseudomonas*, *Acinetobacter*, *Burkholderia*, and *Lactococcus*, demonstrating a high level of genetic polymorphism among the endophytic bacteria isolated from stems of *Eucalyptus* spp. The authors also suggested an antagonist action of *Pantoea* and *Burkholderia cepacea* with endophytic fungi. The importance of studies for disease control and plant growth promotion by the selection of endophytic bacteria isolated from plants is noteworthy. The next step of our study will be to try to correlate if the differential colonization of the maize genotypes by genera as *Pantoea* and *Burkholderia* could be associated with lower susceptibility to plant diseases. We should also mention that the diversity observed in our study represents only a small percentage of culturable microorganisms. *Estimates* are that culturable microorganisms might represent only 0.01–1 % of the whole diversity [e.g., 37], what has been confirmed in metagenomics studies [e.g., 31, 33, 35]. However, the importance in our study is that the properties of biotechnological interest from the culturable microorganism could be promptly analyzed.

The qualitative differences in endophytic colonization of roots of maize genotypes reported in our study may, thus, have important effects in plant growth and disease resistance. They also highlight the importance of surveying qualitative differences in microbial community that may vary considerably even in the absence of quantitative differences. Not less important is that the data obtained in comparison of homozygous genotypes are novelty in literature since the analysis of lineages is not common due to lack of access to sources that make up the heterotic pairs of commercial hybrids from plant improvement enterprises.

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