J. Microbiol. Biotechnol. (2012), **22**(4), 437–447
http://dx.doi.org/10.4014/jmb.1109.09007
First published online February 4, 2012
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Biochemical and Molecular Characterization of High Population Density Bacteria Isolated from Sunflower

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Received: September 7, 2011 / Revised: November 26, 2011 / Accepted: November 27, 2011

Natural and beneficial associations between plants and bacteria have demonstrated potential commercial application for several agricultural crops. The sunflower has acquired increasing importance in Brazilian agribusiness owing to its agronomic characteristics such as the tolerance to edaphoclimatic variations, resistance to pests and diseases, and adaptation to the implements commonly used for maize and soybean, as well as the versatility of the products and by-products obtained from its cultivation. A study of the cultivable bacteria associated with two sunflower cultivars, using classical microbiological methods, successfully obtained isolates from different plant tissues (roots, stems, florets, and rhizosphere). Out of 57 plant-growth-promoting isolates obtained, 45 were identified at the genus level and phylogenetically positioned based on 16S rRNA gene sequencing: 42 *Bacillus* (*B. subtilis, B. cereus, B. thuringiensis, B. pumilus, B. megaterium*, and *Bacillus* sp.) and 3 *Methylobacterium komagatae.* Random amplified polymorphic DNA (RAPD) analysis showed a broad diversity among the *Bacillus* isolates, which clustered into 2 groups with 75% similarity and 13 subgroups with 85% similarity, suggesting that the genetic distance correlated with the source of isolation. The isolates were also analyzed for certain growth-promoting activities. Auxin synthesis was widely distributed among the isolates, with values ranging from 93.34 to 1653.37 μM auxin per μg of protein. The phosphate solubilization index ranged from 1.25 to 3.89, and siderophore index varied from 1.15 to 5.25. From a total of 57 isolates, 3 showed an ability to biologically fix atmospheric nitrogen, and 7 showed antagonism against the pathogen *Sclerotinia sclerotiorum.* The results of biochemical characterization allowed identification of potential candidates for the development of biofertilizers targeted to the sunflower crop.

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Keywords: Endophytic bacteria, plant growth-promoting bacteria, *Bacillus*, *Methylobacterium*, 16S rRNA gene

World agriculture is under a new paradigm: to achieve sustainability without decreasing productivity. The increase of the global population, changes in climate, the needs to reduce greenhouse gas emissions, and the need to increase crop productivity can be considered the main future challenges in agriculture. In this sense, understanding the ecology and dynamics of plant-associated bacteria, as well as their implications for plant physiology and development, is of great importance. Such microbial communities play important roles in plant nutrition, the efficiency of nutrient use, and the protection of crops against biotic and abiotic stresses. Several bacterial groups isolated from different plant species have been previously described, and many of them have demonstrated plant growth-promoting properties. Such bacteria are generally termed “plant growth-promoters” [11, 14, 21].

Plant growth-promoting bacteria (PGPB) are generally found colonizing the intercellular spaces of the plant (in which case they are termed endophytic bacteria) or colonizing the rhizosphere (termed rhizobacteria). Virtually all plant species are associated with one of these groups, making the PGPB of great ecological and biotechnological importance. Several mechanisms of growth promotion have been identified [39]. The main genera of PGPB include *Azotobacter*, *Azospirillum*, *Acetobacter*, *Pseudomonas*, *Bacillus*, *Gluconacetobacter*, *Herbaspirillum*, and *Burkholderia* [22]. From these genera, several commercial formulations are available for use as inoculants for commercial crops, mainly developed for grasses [3].

Oilseed crops have gained increasing importance in agribusiness owing to their versatility of use in human and animal food, as well as biofuel. Nevertheless, studies

regarding the PGPB species in association with oilseed crops are scarce, with few reports performed for the sunflower crop [8, 9]. Sunflower seeds contain a high-quality oil that accounts for 39% to 49% of a seed's total mass, with the yield influenced by the plant genotype and soil nutrients, specifically nitrogen and phosphorus. Both nutrients are directly correlated to the seed and oil yields, as well as oil quality [19].

Improvement of nutrient uptake and/or plant nutritional status can be achieved by the use of selected bacteria, as observed with legumes and grasses [6]. However, large-scale application of inoculant PGPB on commercial crops suffers from inconsistency in field trials, probably due to the numerous and complex interactions that influence the compatible establishment of a PGPB host–plant association. For this reason, bioprospection of PGPB in association with different plant species can identify the isolates most likely to form compatible interactions with specific plant species, and in this way the growth-promoting effect of the association can be fully utilized.

The aim of this work was to contribute to the knowledge about high population density bacterial diversity in association with the sunflower, which was accomplished through isolation, identification, and evaluation of the genetic diversity, characterization of the cultivable PGPB found in association with sunflower, and identification of potential isolates to develop inoculant formulations.

Materials and Methods

Plant Material and Bacterial Isolation

Two different plant genotypes (Aguará 3 and Helio 251) were used in the present study to isolate bacterial strains associated with different tissues of sunflower. Plants of both genotypes were grown in a low fertilizer input system at the Embrapa Soybean experimental field, located in the Paraná State, Brazil. These genotypes were chosen because of their recommended growth conditions in medium to low soil fertility and their wide suitability in Brazilian edaphoclimatic conditions. Five plants of each genotype were sampled at the flower bud phase (E 4) and split into rhizosphere soil, roots, stem, and florets. The plant tissues were surface-sterilized by immersion in 2% hypochlorite solution for 15 min and rinsed 3 times for 15 min with sterile distilled water. Five grams of each plant tissue or rhizosphere soil was ground in 1:10 (w/v) saline solution (0.9% NaCl) and serially diluted up to 10^{-7}. One hundred microliters of the 10^{-5} to 10^{-7} dilutions was used to inoculate semisolid N-free media LGI, NFb, and JMV (in 3 replica vials for each dilution), and Dygs solidified rich medium was also inoculated to estimate the "total" culturable bacteria [7]. The inoculated media were incubated at 28°C for 7 to 8 days, and then a bacterial count was performed using the most probable number (MPN) method or the colony-forming unit (CFU) method. Bacterial colonies and pellicles present in the higher dilutions were streaked on Dygs medium for purification. Purified bacterial isolates were maintained in liquid glycerol:Dygs [1:1 (v/v)] at −20°C until used for molecular and biochemical characterization.

Identification and Molecular Diversity of Bacterial Isolates

Total DNA was extracted from the sunflower isolates using the phenol:chloroform method [35] and used for molecular analysis. The ribosomal 16S rRNA gene was partially amplified in PCR reactions using the universal primers 27F and 778R [32]. The amplification products were electrophoresed on 1.0% agarose gel and photographed. The amplicons were purified using the PureLink PCR Purification Kit (Invitrogen Life Technologies, Carlsbad, USA) according to the manufacturer's instructions and were sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Inc.). The sequencing output was analyzed using the Ribosomal Database Project Classifier [41] to identify the strains at the genus level, and a phylogenetic tree was constructed to specifically position each strain in the genera. Nucleotide sequences were aligned using Clustal 2.0.12 [20] and refined by manual correction using Bioedit 7.0.5.2 [13]. Ribosomal gene sequences of type species were downloaded from the Ribosomal Database Project (RDP) databank and used to infer phylogenetic relationships. Bayesian trees were constructed using MrBayes 3.1.2 software [31]. Markov Chain Monte Carlo (MCMC) searches were run until the standard deviation of split frequencies fell below 0.01, with trees sampled every 250 generations. The first quarter of the trees generated (25% of the trees) were discarded to build the phylogram.

The 16S rRNA gene sequences allow only limited diversity analysis. Therefore, genotypic diversity was further evaluated using the random amplified polymorphic DNA (RAPD) technique using the primers M13 [15], 1254 [1], and P2 and P3 [23]. Band patterns from the amplified RAPD products were digitally photographed, and data were analyzed using GelQuest (version 2.1.2) software. Each reaction was performed in triplicate. Consistent polymorphism patterns were used to calculate the Jaccard coefficient and subjected to the unweighted pair group method with arithmetical averages (UPGMA) analysis to construct a dendrogram using the NTSYS-pc (version 2.1) software package.

Determination of Plant Growth-Promoting Ability

The ability to biologically fix atmospheric nitrogen was evaluated by growing the strains in tubes containing NFb semisolid N-free media and incubating for 3 days at 28°C [30]. The isolates that presented positive growth and pellicle formation were selected and transferred to fresh NFb semisolid N-free media. This procedure was repeated 5 times consecutively to assure each isolate's ability to fix nitrogen. Bacterial isolates considered diazotrophs were submitted to molecular detection of the *nif*H gene through PCR amplification using the primers described by Piceno *et al*. [28]. The presence of a single amplicon of the expected size (approximately 469 base pairs) was assumed as an amplification of the *nif*H gene. The diazotrophic bacterium *Azospirillum brasilense* AbV5 was used as a positive control for both assays.

The Salkowski assay for quantification of indolic compounds was applied to estimate the production of indole acetic acid (IAA) by the sunflower isolates. The IAA concentration in cultures was normalized to the total protein content, and the results were expressed as μM IAA per μg of protein according to the procedure of Rodrigues *et al*. [29]. Experiments were performed in triplicate.

Siderophore production was tested using the method of Schwyn and Neilands [37], with modifications. One drop of bacterial cultures grown in Dygs liquid medium was inoculated onto plates containing

Table 1. Culturable bacterial community and number of isolates of each colony morphology colonizing different tissues in two sunflower genotypes.

	Sunflower hybrid Aguará 3		Sunflower hybrid Helio 251	
	Number of isolates	Log CFU/g	Number of isolates	Log CFU/g
Rhizosphere	3	6.95	15	6.73
Roots	3	6.23	10	5.51
Stem	9	5.39	5	5.47
Florets	8	4.36	4	4.74
Total of isolates	23		34	

Counts are the means of five replicates.

Dygs solid medium supplemented with Chrome Azurol S (CAS), 72.9 mg CTAB, and 5 ml of Fe^{+3} solution (1 mM $FeCl_3{\cdot}6H_2O$, 10 mM HCl), and plates were incubated for 3 days at 28°C. Detection of orange color in a halo around the colonies was assumed as the ability to produce siderophores. The ratio between the halo diameter and the colony diameter, named siderophore index (SI), was used to rank the isolates based on siderophore production. Experiments were performed in triplicate.

To address the potential for phosphate solubilization, each bacterial strain was streaked on NBRIP plates and cultivated for 7 days at 28°C [27]. The presence of transparent haloes surrounding the bacterial colonies indicated the phosphate solubilization potential. The ratio between the halo diameter and the colony diameter was used to determine the P-solubilization index (PSI) and to rank the isolates according to the phosphate solubilization potential. The measures were performed in triplicate.

The bacterial strains were tested *in vitro* for antifungal activity against *Sclerotinia sclerotiorum* by dual culture test. Seven-day-old *S. sclerotiorum* mycelia grown on PDA (Acumedia) culture medium were transferred to one side of Petri dishes containing Dygs medium, and then 2-day-old bacterial colonies grown on Dygs solid medium were streaked on the opposite side of the assay plates. Antifungal activity was assessed after 10 days of incubation in the dark at room temperature. Bacterial isolates that maintained the fungal mycelia more than 1 cm away were considered as antagonists of *S. sclerotiorum*. To confirm and quantify the plate assay, a pairing method on standard potato-dextrose was used according to Araújo *et al*. [2]. Pure bacterial and fungal cultures were used as controls, and the experiments were performed in triplicate.

RESULTS

Bacterial Isolates

A total of 57 bacterial strains were obtained from rhizosphere soil, roots, stem and florets from both sunflower genotypes (Aguará 3 and Helio 251) (Table 1). No significant difference in bacterial density was observed between the sunflower genotypes overall. Nevertheless, the rhizosphere of the cultivar Aguará 3 harbored the highest bacterial population density (up to 8.9×10^6 CFU per gram), whereas the florets of the same cultivar had the smallest population density (up to 2.3×10^4 CFU per gram). A higher number of different colony morphotypes was isolated from the cultivar Helio 251. Out of all the isolates obtained from both sunflower genotypes, 18 came from the rhizosphere soil, 13 from the roots, 14 from the stems, and 12 from the florets (Table 1).

Identification and Molecular Diversity of the Isolates

The RDP Classifier allowed the identification of 42 strains belonging to the *Bacillus* genus plus 3 strains belonging to

Table 2. Characteristics of the bacterial strains isolated from different tissues and the rhizosphere of two sunflower cultivars and characterization of the plant growth-promotion potential.

Isolate[a]	Sunflower genotype	Sampling region[b]	BNF[c]	Antagonist activity[d]	Phosphate solubilization[e]	Indolic compounds[f]	Siderophore production[g]	GenBank ID[h]	Probable species[i]
CA	Helio 251	Stem	No	<1.0	ND[j]	986.93	ND	HM061667	*Bacillus* sp.
CB	Helio 251	Stem	No	<1.0	ND	979.82	2.67	HM061673	*B. amyloliquefaciens*
CC	Aguará 3	Stem	No	1.2	ND	1,148.43	2.48	HM061679	*B. cereus*
CD	Aguará 3	Stem	No	<1.0	ND	874.13	ND	HM061684	*B. cereus*
CE	Aguará 3	Stem	No	<1.0	ND	297.27	ND	HM061689	*Bacillus* sp.
CF	Aguará 3	Stem	No	<1.0	3.28	310.74	1.85	HM061650	*B. cereus*
CG	Helio 251	Stem	No	<1.0	ND	369.83	ND	HM061651	*B. megaterium*
CH	Aguará 3	Stem	Yes	<1.0	ND	265.73	ND	HM061656	*B. cereus*
CI	Aguará 3	Stem	No	1.6	ND	212.29	ND	HM061657	*B. megaterium*
CJ	Aguará 3	Stem	No	<1.0	ND	416.39	2.23	HM061662	*B. subtilis*
CK	Helio 251	Stem	No	<1.0	ND	117.53	ND	HM061674	*B. cereus*
CL	Helio 251	Stem	No	<1.0	1.57	319.68	2.23	HM061668	*B. cereus*

Table 2. Continued.

Isolate[1]	Sunflower genotype	Sampling region[2]	BNF[3]	Antagonist activity[4]	Phosphate solubilization[5]	Indolic compounds[6]	Siderophore production[7]	GenBank ID[8]	Probable species[9]
CM	Aguará 3	Stem	No	1.9	2.97	1,641.16	1.37	NS[k]	Unknown**
CN	Aguará 3	Stem	No	<1.0	ND	821.97	ND	NS	*Bacillus* sp.*
RA	Helio 251	Roots	No	<1.0	ND	953.61	ND	HM061691	*B. megaterium*
RB	Helio 251	Roots	No	<1.0	ND	100.73	ND	HM061652	*B. thuringiensis*
RC	Aguará 3	Roots	No	<1.0	2.72	288.80	1.53	HM061658	*B. megaterium*
RD	Helio 251	Roots	No	<1.0	ND	102.00	1.42	HM061664	*B. megaterium*
RE	Helio 251	Roots	No	<1.0	ND	160.87	2.08	HM061670	*B. megaterium*
RF	Helio 251	Roots	No	<1.0	ND	111.41	5.25	HM061676	*B. thuringiensis*
RG	Aguará 3	Roots	No	<1.0	ND	260.27	1.76	HM061681	*B. megaterium*
RI	Aguará 3	Roots	No	1.6	ND	477.02	ND	HM061686	*B. pumilus*
RJ	Helio 251	Roots	No	<1.0	ND	141.63	ND	HM061692	*Bacillus* sp.
RK	Helio 251	Roots	No	<1.0	1.36	109.26	ND	HM061653	*B. cereus*
RM	Helio 251	Roots	No	<1.0	1.47	122.46	2.78	HM061659	*B. megaterium*
RN	Helio 251	Roots	No	<1.0	1.25	115.41	1.77	HM061665	*B. cereus*
RO	Helio 251	Roots	No	<1.0	ND	141.60	4.67	HM061671	*B. megaterium*
T1AG	Aguará 3	Florets	Yes	<1.0	ND	173.76	2.58	HM061682	*B. pumilus*
T1AH	Aguará 3	Florets	No	<1.0	ND	481.50	3.83	NS	Unknown**
T2AF	Aguará 3	Florets	No	<1.0	ND	190.67	3.70	HM061690	*B. amyloliquefaciens*
T2AJ	Aguará 3	Florets	No	<1.0	ND	368.28	ND	HM061663	*B. cereus*
T2AK	Aguará 3	Florets	No	<1.0	ND	218.20	3.67	NS	*Bacillus* sp.*
T3AL	Aguará 3	Florets	No	1.5	1.57	165.83	2.13	NS	*Bacillus* sp.*
T4AE	Helio 251	Florets	No	1.1	ND	136.46	2.79	HM061675	*B. thuringiensis*
T4AL	Aguará 3	Florets	No	1.5	ND	250.71	3.08	HM061669	*B. thuringiensis*
T5AL	Aguará 3	Florets	No	<1.0	ND	327.59	ND	NS	*Bacillus* sp.*
TSAA	Helio 251	Florets	No	<1.0	ND	750.60	1.90	HM061680	*M. komagatae*
TSAB	Helio 251	Florets	No	1.8	1.50	128.94	ND	HM061685	*B. amyloliquefaciens*
TSAD	Helio 251	Florets	No	<1.0	2.97	447.32	1.19	NS	Unknown**
ZA	Helio 251	Rhizosphere	No	<1.0	3.89	381.62	1.15	HM061648	*B. amyloliquefaciens*
ZB	Aguará 3	Rhizosphere	No	<1.0	ND	347.18	ND	HM061654	*Bacillus* sp.
ZC	Helio 251	Rhizosphere	No	<1.0	ND	641.40	1.49	NS	*Bacillus* sp.*
ZD	Helio 251	Rhizosphere	No	<1.0	ND	610.10	ND	HM061672	*B. amyloliquefaciens*
ZE	Helio 251	Rhizosphere	No	<1.0	ND	ND	ND	HM061678	*B. pumilus*
ZF	Helio 251	Rhizosphere	No	<1.0	ND	ND	ND	HM061683	*B. pumilus*
ZG	Helio 251	Rhizosphere	No	<1.0	ND	110.76	3.50	HM061688	*Bacillus* sp.
ZH	Helio 251	Rhizosphere	No	<1.0	ND	ND	ND	NS	Unknown**
ZI	Helio 251	Rhizosphere	No	<1.0	ND	ND	ND	HM061649	*Bacillus* sp.
ZJ	Helio 251	Rhizosphere	No	<1.0	2.50	93.34	2.35	HM061655	*Bacillus* sp.
ZK	Helio 251	Rhizosphere	No	<1.0	ND	130.47	5.17	NS	*Bacillus* sp.*
ZL	Helio 251	Rhizosphere	No	<1.0	ND	ND	2.88	NS	Unknown**
ZM	Helio 251	Rhizosphere	No	<1.0	ND	1,254.62	ND	HM061687	*M. komagatae*
ZO	Aguará 3	Rhizosphere	No	<1.0	ND	265.00	1.63	HM061660	*B. megaterium*
ZQ	Helio 251	Rhizosphere	No	<1.0	ND	879.23	ND	HM061666	*M. komagatae*
ZR	Helio 251	Rhizosphere	Yes	<1.0	ND	362.24	3.40	HM061661	*B. megaterium*
ZS	Helio 251	Rhizosphere	No	<1.0	ND	279.51	2.26	NS	Unknown**
RP	Aguará 3	Rhizosphere	No	<1.0	ND	1,653.37	ND	HM061677	*B. cereus*

The accession numbers of 16S rRNA gene sequences deposited in GenBank are presented. The most probable species to which each strain belongs was determined by homology with the partial sequence of the 16S rRNA gene and Bayesian phylogenetic inference.

[a]Isolate codes adopted; [b]Isolates obtained from stems, roots, and florets were considered endophytes; [c]Biological nitrogen fixation (BNF) potential determined by ability to consecutively grow on NFb N-free semisolid medium; [d]Antagonistic activity was determined *in vitro* against *Sclerotinia sclerotiorum*; [e]Phosphate solubilization potential was determined *in vitro* by the ability to solubilize $Ca_3(PO_4)_2$ in NBRIP solid medium; [f]Total indoles in the supernatant of isolates cultured in Dygs liquid medium supplemented with 100 µg/ml DL-tryptophan; [g]Siderophore production determined in Dygs solid medium supplemented with chrome azurol S; [h]Accession numbers of the partial 16S rRNA gene deposited in GenBank; [i]Probable species to which the isolate belongs, as determined by Bayesian phylogenetic analysis; [j]ND, not detected; [k]NS, not sequenced; *Not sequenced isolates assigned based on RAPD profile; **Not sequenced isolates assigned as unknown clustered with lower than 95% similarity based on Jaccard coefficient.

genus *Methylobacterium* (Table 2). The 12 remaining unidentified isolates rendered low sequence quality, and the contigs of these sequences could not be assembled. The phylogenetic analysis of the 16S rRNA gene sequences of 42 strains isolated from sunflower, and some type strains of *Bacillus* species, is presented in Fig. 1. A broad phylogenetic analysis including 182 16S rRNA gene sequences from *Bacillus* type strains deposited in databanks was performed prior to selecting the species presented. Fig. 2 shows the phylogenetic analysis of the 16S rRNA

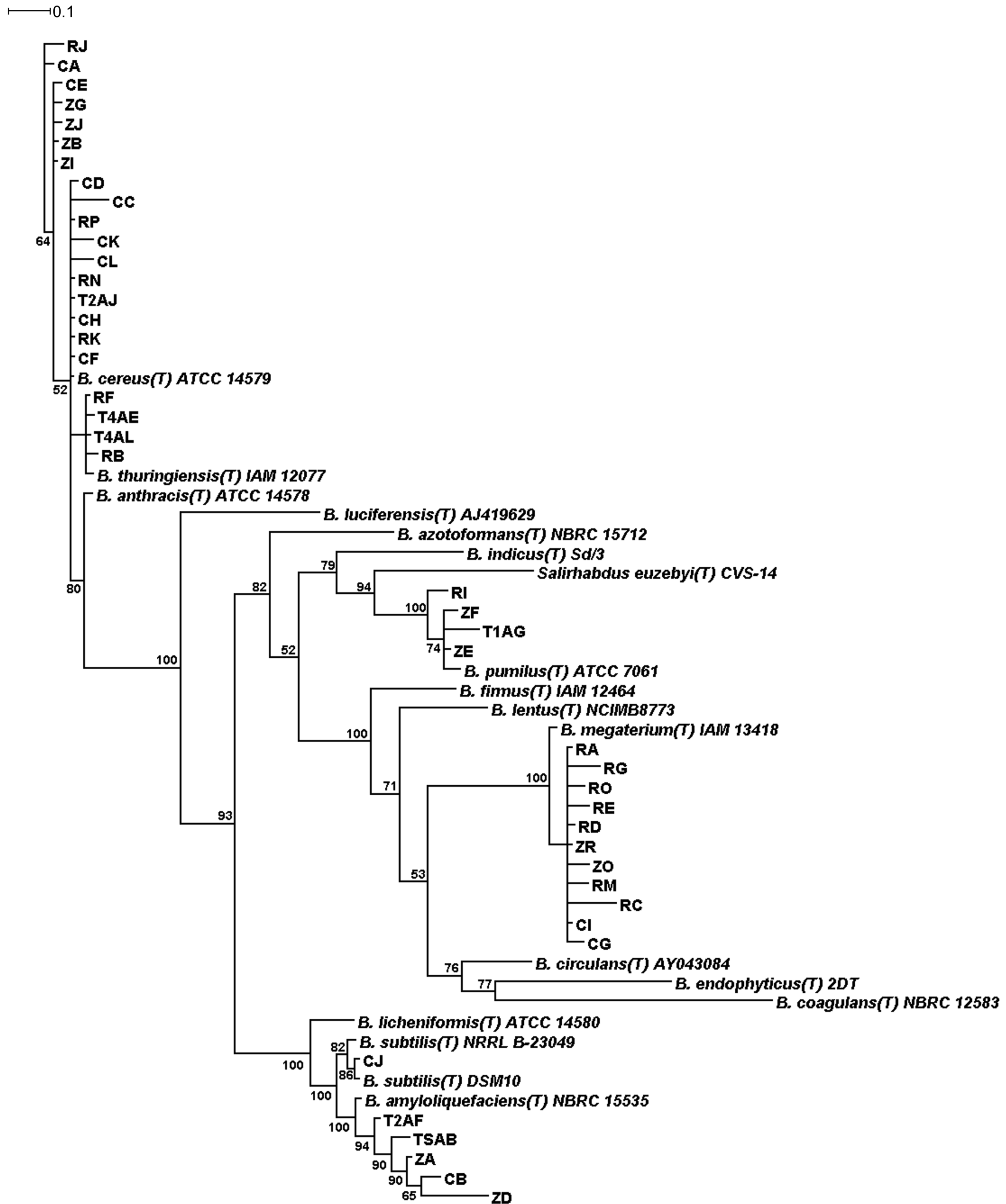

Fig. 1. Bayesian cladistic tree constructed using the 16S rRNA gene sequences of *Bacillus* sp. type strains and sequences of *Bacillus* isolates obtained in this study.
Bootstrap confidence levels greater than 50% are indicated at the internodes.

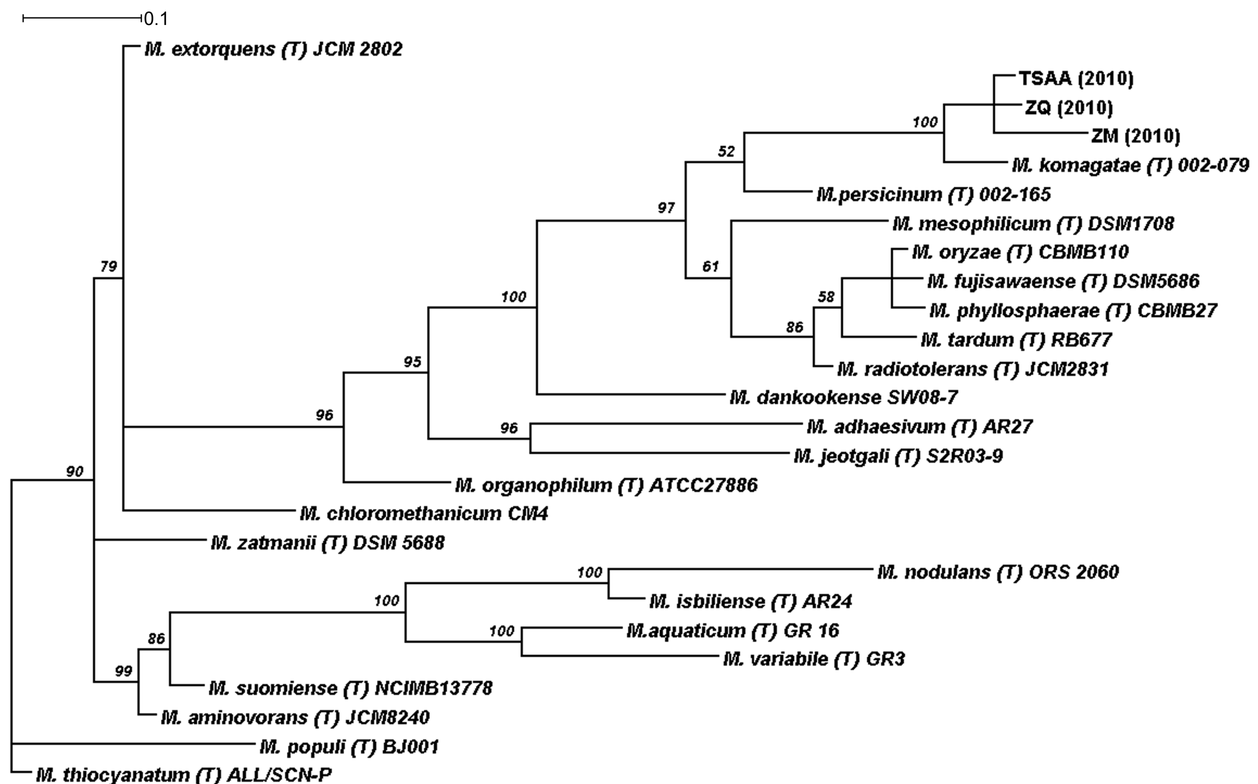

Fig. 2. Bayesian cladistic tree constructed with the 16S rRNA sequences of *Methylobacterium* sp. type strains and sequences of *Methylobacterium* isolates obtained in this study.
Bootstrap confidence levels greater than 50% are indicated at the internodes.

sequences from 3 strains isolated from sunflower and type strains of *Methylobacterium* species. Distance-based phylogeny grouped the new strains into 6 *Bacillus* species and 1 *Methylobacterium* species, in addition to 7 unidentified *Bacillus*.

The *B. amyloliquefaciens*-like strains ZA, CB, ZD, TSAB, and T2AF clustered with the type strain, with 16S rRNA gene sequence similarities of 96.5–99.5%. The *B. subtilis*-like strain CJ shared 99.6% sequence similarity to the type strain. The *B. megaterium*-like strains CG, CI, ZR, RA, RD, RE, RG, RM, RO, ZO, and RC shared sequence similarities of 95.3–99.6% to the type strain. The *B. pumilus*-like strains ZF, ZE, T1AG, and RI clustered with the type strain, with sequence similarities of 98.5–99.5%. The *B. cereus*-like strains CL, RN, RK, T2AJ, CC, CK, CD, CH, RP, and CF were positioned closer to the type strain, with sequence similarities of 98.2–100%. The *B. thuringiensis*-like strains RB, T4AL, RF, and T4AE presented with 98.9–99.8% of sequence similarity to the type strain. The strains CA, ZB, ZI, ZJ, RJ, ZG, and CE did not cluster within any *Bacillus* type strain. The *Methylobacterium*-like strains TSAA, ZM, and ZQ grouped with the *M. komagatae* type strain, with 16S rRNA gene sequence similarities of 97.8–98.4% (Fig. 2). Interestingly, these isolates were identified as endophytes on the Helio 251 cultivar only (Table 2).

To compare the genotypic redundancy among the strains, the RAPD-PCR technique was used. A total of 128 RAPD markers were obtained with the primer set used, with fragments varying from 100 to 5,000 bp. The dendogram derived from UPGMA cluster analysis based on the combined similarity matrix presents the genomic diversity among the sunflower isolates (Fig. 3). As a result, 2 main groups were distinguished at 75% similarity, and 13 subgroups were formed at 85% similarity. Group 1 comprised mainly bacterial strains obtained from surface-sterilized plant tissues and hence were considered as endophytes. Group 2 was formed mainly by rhizosphere isolates. At a higher level of similarity, a clear delineation between isolates derived from the same plant tissue could be observed, as well as clonal relationships among some isolates. Subgroups 1 and 2 were formed by endophytic *Bacillus* strains isolated from sunflower stems of both cultivars. Subgroup 3 showed no clear distinction between strains according to the plant tissue of origin. Subgroups 4 and 5 were formed mainly by endophytic *Bacillus* from roots of both cultivars. Strains ZQ and TSAA (both *M. komagatae*-like strains) formed separate clusters at subgroups

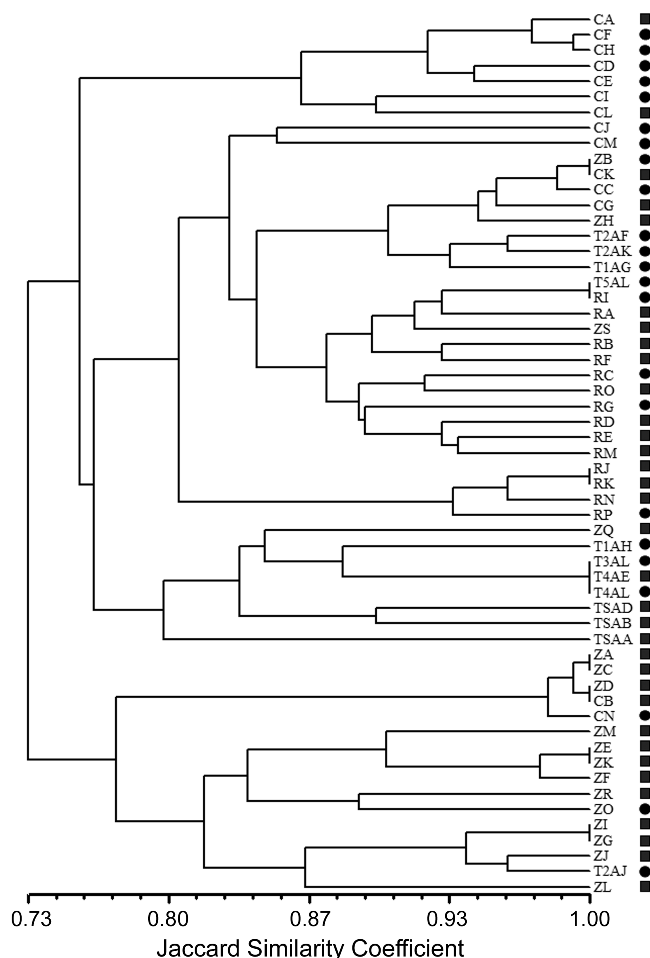

Fig. 3. UPGMA dendrogram based on Jaccard's coefficient from the RAPD profiles of bacterial isolates colonizing different tissues of two sunflower genotypes.

■ Bacterial strains isolated from Helio 251 genotype; ● Bacterial strains isolated from Aguará 3 genotype.

6 and 9, respectively. Subgroups 7 and 8 comprised endophytic *Bacillus* strains obtained from florets. Subgroups 10 and 13 were formed mainly by rhizosphere strains, and subgroups 11 and 12 contained exclusively rhizosphere *Bacillus* (Fig. 3).

Clonal relationships were identified between strains obtained from different sunflower cultivars, as observed with the isolates ZB (rhizosphere, Aguará 3) and CK (stem, Helio 251), which shared 98.7% of 16S rRNA gene sequence similarity, and isolates T3AL (florets, Aguará 3), T4AE (florets, Helio 251), and T4AL (florets, Aguará 3), with these last two sharing sequence similarity of 99.7%. Indeed, clonal relationships among isolates obtained from the same sunflower cultivar but from different plant tissues were also identified, as represented by the strains RI and T5AL (not sequenced), and strains ZD and CB had 97% sequence similarity. Clonally related strains were also identified for the strains RJ and RK (both being obtained from root samples and sharing 99.0% of sequence similarity), rhizosphere strains ZA and ZC (not sequenced), strains ZE and ZK (not sequenced), and strains ZI and ZG (showing 99.4% of 16S rRNA gene sequence similarity).

Plant Growth-Promoting Traits

Bacillus isolates CH, T1AG, and ZR (putative *B. cereus*, *B. pumilus*, and *B. megaterium*, respectively) were considered as having biological nitrogen fixation (BNF) potential (Table 2). Although these isolates grew well in N-free NFb semi-solid medium for 5 consecutive subculturings, the amplification of the *nif*H gene using the primers described previously [28] failed for these isolates. For this reason, the BNF trait was only assumed as BNF potential.

The production of indolic compounds was observed in 52 out of 57 isolates, in a range varying from 93 to 1,653 μM IAA per μg protein (Table 2). The highest value was recorded for the strain RP, phylogenetically related to *B. cereus*. Bacterial strains that presented values of auxin production greater than 850 μM AIA per μg of protein were considered as a high-potential PGPB (Table 3). A total of 9 strains were considered high IAA producers.

The ability to produce siderophores was identified in a total of 32 isolates, based on the observation of a clear halo surrounding the bacterial colonies (Table 2). The siderophore index (SI) varied in a range from 1.15 to 5.25, and strains that presented indices higher than 2.5 were considered as having a high PGPB potential on this trait (Table 3). A total of 14 isolates were considered as high siderophore

Table 3. Bacterial strains with high potential for plant growth promotion (PGP) of the sunflower based on specific traits.

PGP trait	Aguará 3	Helio 251
BNF	CH, T1AG	ZR
Auxin (μM AIA/μg protein^{-} $\geq$ 850)	CC, CD, CM, RP*	CA, CB, RA, ZM, ZQ
Siderophore (SI $\geq$ 2.5)	T1AG, T1AH, T2AF, T2AK, T4AL	CB, RF*, RM, RO, T4AE, ZG, ZK, ZL, ZR
P-solubilization (PSI 2.5)	CF, CM, RC	TSAD, ZA*, ZJ
Antagonism (IH $\geq$ 1.5)	CI, CM*, RI, T3AL, T4AL	TSAB

*Strains with the highest recorded value for each trait studied; Underlined strains are those that presented more than 1 high-potential plant growth-promotion trait.

producers, with the best index being found for the isolate RF, a strain phylogenetically related to *B. thuringiensis*.

Phosphate solubilization was observed in a total of 12 strains. The P-solubilization index (PSI) varied from 1.25 to 3.89, with the higher value observed for the strain ZA, phylogenetically related to *B. amyloliquefaciens* (Table 2). Arbitrary values of PSI were chosen to classify the isolates according to their potential for this trait, with 6 strains presenting a PSI higher than 2.5 and being considered high-potential PGPB (Table 3).

A total of 7 isolates were identified as having antagonistic activity against the pathogenic fungus *Sclerotinia sclerotiorum* (Table 2), 6 of which were considered high-potential PGPB according to the inhibition-halo size (IH). The quantification of the inhibition haloes presented values ranging from 1.2 to 1.9 cm, with the highest antagonistic effect being observed in the *Bacillus* sp. strain CM, not sequenced (Table 3).

Considering the results of plant growth-promotion characterization of the sunflower strains, high-potential candidates for further sunflower inoculation could be selected. From the total of 57 strains obtained from the 2 sunflower genotypes, 5 of them presented at least 2 different plant growth-promotion traits in high potential: endophytic strains T1AG (BNF potential and siderophore production), T4AL (siderophore production and plant protection against *S. sclerotiorum*), CM (auxin synthesis, P-solubilization, and plant protection against *S. sclerotiorum*), CB (auxin synthesis and siderophore production), and the rhizosphere strain ZR (BNF potential and siderophore production).

Discussion

The distribution of the bacterial species associated with plants is controlled by several factors, including plant genotype, plant age, climate conditions, soil characteristics [18], and soil water content. This study presents the diversity of high-density bacterial strains in association with 2 different sunflower cultivars, collected under the same edaphoclimatic conditions. Considering the data, the main factor influencing the diversity of bacterial communities in this study was the plant genotype. No significant differences in bacterial density were observed among the populations associated with both plant genotypes. Nevertheless, the diversity of the bacterial isolates suggested qualitative differences regarding the populations in association with each sunflower genotype. Indeed, the higher level of diversity and higher-density populations seen in the rhizosphere, compared with the endophytic populations, are in agreement with the literature [33].

The cultivar Aguará 3 generated a total of 6 bacterial strains in association with sunflower roots and rhizosphere, corresponding to 10.5% of the total isolated strains. This differed from the below-ground-associated bacterial diversity of the cultivar Helio 251, where a total of 25 different colony morphotypes were isolated (corresponding to 43.8% of the total strains isolated). In contrast, a higher number of different strains were obtained from the aerial tissues of Aguará 3 (17 isolates, 29.8% of the total) than were obtained from Helio 251 (9 isolates, 15.8% of the total). This suggests a lower diversity in a larger population of endophytic bacteria is associated with the Helio 251 genotype (Table 1). The role of plant genotype in shaping the associated bacterial population is unclear, but a similar observation has been previously reported [12] and is in support of our findings. Vogt *et al*. [40] studied 17 sunflower genotypes in respect to morphophysiological characteristics and found a high cophenetic distance between the genotypes Aguará 3 and Helio 251, mainly directed by plant height (172 and 137 cm, respectively) and days to flowering (75 and 67 days, respectively). Genotype-dependent morphophysiological effects, along with signaling pathways involved in plant–microbe communication, are believed to drive the size and composition of nonpathogenic plant-associated bacterial populations [16].

With respect to the bacterial strains defined by the phylogenetic 16S rRNA gene analysis (Fig. 1 and 2), 9 out of 11 *B. megaterium*-like strains were obtained from below-ground tissues (81.8% of *B. megaterium*-like strains) and were mainly from the Helio 251 genotype. On the other hand, *B. cereus*-like strains prevailed in the above-ground tissues (70% of the *B. cereus*-like isolates) and were mainly from the Aguará 3 genotype. Both *B. megaterium* and *B. cereus* are ubiquitous soil bacteria, and have been reported endophytically in field-grown plants [38]. *Bacillus* strains related to *B. pumilus* also showed split distribution of the plant genotype and site of colonization, being found endophytically in Aguará 3 and only on the rhizosphere of Helio 251 (Table 4). Another interesting finding was the isolation of the *M. komagatae*-like strains exclusively from Helio 251 and a single *B. subtilis* from Aguará 3. *M. komagatae* and other *Methylobacterium* strains were recently described as P-solubilizers [17]; however, the isolates described here do not present such a trait. The identification of methylobacteria colonizing field-grown sunflower plants was previously reported [36], but to our knowledge the identification of endophytic *M. komagatae* in sunflower tissues is reported for the first time here. The role of methylotrophs in association with plants is not clearly understood.

Previous studies of bacterial isolation and diversity in association with sunflower are scarce. Hence, the plant genotype-based comparative analysis provides useful information about the composition of the high density bacterial community associated with this crop. The results presented suggest an active role of the sunflower plants in selectively recruiting such *Bacillus* species from soil and

Table 4. Distribution of the bacterial strains sequenced on different plant tissues and rhizospheres of two sunflower genotypes.

Bacterial strain[a]	Aguará 3				Helio 251			
	Rhizos[b]	Roots	Stems	Florets	Rhizos[b]	Roots	Stems	Florets
Bacillus sp.	1		1		3	1	1	
B. amyloliquefaciens				1	2		1	1
B. cereus	1		4	1		2	2	
B. megaterium	1	2	1		1	5	1	
B. pumilus		1		1	2			
B. subtilis			1					
B. thuringiensis				1		2		1
M. komagatae					2			1
Total	3	3	7	4	10	10	5	3

[a]Bacterial strains identified according to the Bayesian tree constructed using *Bacillus* type strains.
[b]Rhizosphere strains.

supporting its population increase. This effect was previously reported for *B. polymyxa* in wheat [26]. These results cannot be widely interpreted and may not reflect the true picture of the bacterial community associated with the sunflower cultivars, so they must be interpreted according to strict criteria. The use of phenotypic characteristics such as colony morphology to select the bacterial isolates studied here could potentially bias the results. Thus, relative and non-absolute populations were described in this work.

Fürnkranz *et al*. [10] evaluated the bulk bacterial population associated with sunflower and other crops in Bolivian fields, finding rhizosphere populations up to 100 times larger than that reported here but endophytic populations up to 10 times smaller. Such discrepancies could be due to differences in soil and plant genotypes, as well as the methodology used to obtain the isolates. Forchetti *et al*. [9] reported the isolation of 29 bacterial strains from sunflower root tissues, from which 8 were considered diazotrophic and identified by 16S rRNA gene sequences. Although the bacterial population density was not mentioned, the majority of the sequenced isolates was identified as *B. pumilus* homologous, and one isolate showed homology with *Achromobacter/Alcaligenes*. In our results, almost all sequenced bacteria were also identified as belonging to the *Bacillus* genus, divided into 6 different species. The ability of *Bacillus* to associate with and express plant growth-promoting traits in different plant species, including in commercial inoculant strains [25], has already been reported in the literature [4, 34].

The plant growth-promoting activity of *Bacillus* strains includes direct and indirect effects. Direct effects such as nitrogen fixation, phosphate solubilization, and auxin synthesis are considered consistent indicators of potential biofertilizers [3, 39]. Indirect effects, such as antagonism against pathogens and siderophore production, are bacterial traits useful in the development of plant-protection inoculants.

Diazotrophic *Bacillus* was identified based on its ability to grow in semisolid N-free NFb medium in consecutive cycles of subculturing, a procedure well established to selectively isolate diazotrophic bacteria. The discrepancy between the results of the culturing and molecular methods in detecting diazotrophic bacteria could result from variability of this gene, and similar reports are available in the literature [18]. Phylogenetic analysis of the isolates CH, T1AG, and ZR suggests that they belong to *B. cereus*, *B. pumilus*, and *B. megaterium*. Although the detection of the *nif*H gene had been unsuccessful for the tested strains, nitrogen-fixing *Bacillus* strains phylogenetically related to *B. pumilus* were previously isolated in the sunflower [9] and other plants [42]. Nevertheless, the transference of fixed nitrogen by *Bacillus* to the plant has not been determined at this point.

Most of the isolates obtained in this study showed the potential to produce indoles on *in vitro* assay, including the *Methylobacterium* isolates. Among 57 isolates, only the strains ZE, ZF, ZH, and ZI did not present any of the plant growth-promotion traits that were tested. Six isolates with high potential for P-solubilization were identified, and strains related to *B. subtilis* (strain ZA), *B. cereus* (strain CF), *B. megaterium* (strain RC), and *Bacillus* sp. (strains CM, TSAD, and ZJ) equally distributed among the plant genotypes. On the other hand, *Bacillus* strains antagonistic against *S. sclerotiorum* were isolated mainly from Aguará 3, from which 5 out of 6 strains with high antagonistic activity were isolated (Table 3). Siderophore production is considered to play a role in the biological control of pathogens by limiting iron availability. Interestingly, only the *B. thuringiensis*-like strain T4AL was classified as highly antagonistic and a high siderophore producer, suggesting that the antagonistic activity presented by the other strains could be due to the presence of another active metabolite instead of siderophores.

This work reported the isolation, characterization, and identification of PGPB in association with sunflowers grown in the southern region of Brazil. Because effective PGPB must be present in high levels to exert growth-

promoting effects [5, 21], we focused on isolating such bacterial groups. *Bacillus* species predominated in our isolates, suggesting its ecological relevance for the healthy development of sunflowers. Among the PGPB characteristics identified in the *Bacillus* isolates, biological nitrogen fixation, phosphorus solubilization, and antagonism against the fungal pathogen *S. scerotiorum* are of the most interest for developing an inoculant-based formulation. Tropical soils have characteristically low nitrogen levels and high P-immobilization potential, and in some regions, pathogenic soil fungi are the major limitations in sunflower cropping [24]. The *Bacillus* strains described in this work are suitable candidates to develop biotechnological tools, aiming to overcome such limitations and contribute to the sunflower nutrition and the protection against soil pathogens when grown in tropical countries.

Acknowledgments

The authors are grateful to Universidade Estadual de Londrina and Embrapa Soja for logistic support. This work received financial support from CAPES, CNPq, and Fundação Araucária.

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