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Diversity of maize (*Zea mays* L.) rhizobacteria with potential to promote plant growth

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Abstract

Plant growth-limiting factors, such as low nutrient availability and weak pathogen resistance, may hinder the production of several crops. Plant growth-promoting bacteria (PGPB) used in agriculture, which stimulate plant growth and development, can serve as a potential tool to mitigate or even circumvent these limitations. The present study evaluated the feasibility of using bacteria isolated from the maize rhizosphere as PGPB for the cultivation of this crop. A total of 282 isolates were collected and clustered into 57 groups based on their genetic similarity using BOX-PCR. A representative isolate from each group was selected and identified at the genus level with 16S rRNA sequencing. The identified genera included *Bacillus* (61.5% of the isolates), *Lysinibacillus* (30.52%), *Pseudomonas* (3.15%), *Stenotrophomonas* (2.91%), *Paenibacillus* (1.22%), *Enterobacter* (0.25%), *Rhizobium* (0.25%), and *Atlantibacter* (0.25%). Eleven isolates with the highest performance were selected for analyzing the possible pathways underlying plant growth promotion using biochemical and molecular techniques. Of the selected isolates, 90.9% were positive for indolepyruvate/phenylpyruvate decarboxylase, 54.4% for pyrroloquinoline quinine synthase, 36.4% for nitrogenase reductase, and 27.3% for nitrite reductase. Based on biochemical characterization, 9.1% isolates could fix nitrogen, 36.6% could solubilize phosphate, 54.5% could produce siderophores, and 90.9% could produce indole acetic acid. Enzymatic profiling revealed that the isolates could degrade starch (90.1%), cellulose (72.7%), pectin (81.8%), protein (90.9%), chitin (18.2%), urea (54.5%), and esters (45.4%). Based on the data obtained, we identified three *Bacillus* spp. (LGMB12, LGMB273, and LGMB426), one *Stenotrophomonas* sp. (LGMB417), and one *Pseudomonas* sp. (LGMB456) with the potential to serve as PGPB for maize. Further research is warranted to evaluate the biotechnological potential of these isolates as biofertilizers under field conditions.

Keywords Plant growth-promoting bacteria · Rhizobacteria bacteria · Inoculants · *Zea mays* L. BOX-PCR

Introduction

Brazil, the USA, and China, are the largest producers of maize (*Zea mays* L.) [1], a major cereal crop worldwide [2]. The intensification of maize production, high degree of nutrient removal from agricultural areas, lack of adequate nutritional management of soil, and adoption of monoculture demand continuous replacement of nutrients for crops [3]. To solve this problem, fertilizers, particularly chemicals, must be used, which incur high production costs and cause environmental pollution. Given the risks associated with the indiscriminate use of these inputs, such as the eutrophication of soil and groundwater or emission of greenhouse gases (GHGs) [4], novel tools to promote plant growth are required. An alternative to overcome this challenge is the use of rhizobacteria that can fix atmospheric nitrogen and

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promote plant growth through other microbiological processes [5].

Many genera of plant growth-promoting bacteria (PGPB), including *Pseudomonas*, *Burkholderia*, *Bacillus*, *Bradyrhizobium*, *Rhizobium*, *Gluconacetobacter*, *Herbaspirillum*, and *Azospirillum*, among others, have been identified [6–11]. However, some PGPB may be pathogenic to plants, humans, and other non-human animals, presenting the risk of environmental spread [12]. PGPB may improve root development and nutrient absorption, thereby lowering production costs, reducing fertilizer use, and mitigating environmental impacts [13].

Nitrogen is the major limiting factor for maize biomass production [14, 15]. The use of synthetic nitrogen fertilizers is limited because of high nitrogen loss to the environment through microbial immobilization, leaching, and volatilization [16]. Therefore, bioproducts derived from PGPB [17] have been used as commercial inoculants worldwide to improve productivity [18].

Currently, several PGPB-based products are available on the market for different crops, such as *Azospirillum brasilense* for maize [6, 19], *Rhizobium tropici* for common bean (Phaseolus vulgaris L.) [20], and *Bradyrhizobium japonicum* for soybean (Glycine max (L.) Merr.) [21]. Of note, there is certain affinity between bacterial strains and cultivars [22].

Some key pathways are required to enable the benefits of using PGPB, and specific pathways can be identified via detection of marker genes, such as nitrogenase reductase (*nif*H), which is involved in nitrogen fixation, or phloroglucinol (*phl*D) and pyrroloquinoline quinine synthesis (*pqq*C), which are involved in phosphate solubilization [23], among others. The detection of such genes is the first indication of a candidate PGPB [24]. Moreover, molecular approaches, such as the detection of genes involved in plant growth-promoting mechanisms, have certain advantages, including the greater ease of execution; higher sensitivity, specificity, and reproducibility; and shorter execution time even for a large number of isolates; however, qualitative data must still be evaluated via biochemical approaches [25–27]. In addition, some microorganisms may promote plant growth through other mechanisms, such as the production of enzymes, particularly hydrolytic enzymes (amylase and cellulase, among others), which may interfere with pathogen control [28, 29].

Rhizobacteria possess several properties [30], justifying the global interest in them as PGPB and biocontrol agents, particularly for maize [31]. However, limited studies have been conducted relative to the global area under maize cultivation, and additional research is paramount considering the wide variability in results with many factors, such as climate, natural microbiota, available nutrients, and crop characteristics. Therefore, the use of PGPB should be optimized to specific agroecosystems [32].

To this end, the present study explored the biotechnological potential of bacterial isolates collected from the maize rhizosphere as PGPB using in vitro and in vivo evaluations. Indole acetic acid (IAA) synthesis and pathways relevant to both plant growth promotion and pathogen resistance (through biocontrol or induction of systemic resistance) were also assessed. BOX-PCR was used to evaluate the genetic variability of the collection, and 16S rRNA sequencing was used to identify isolates at the genus level.

Material and methods

Biological material

The bacteria ($n = 282$) used in the present study were previously isolated from the maize rhizosphere by our research group [33]; however, Ikeda et al. [33] included only 217 selected bacteria from over 500 isolates in their study. Therefore, in the present study, we expanded the number of isolates and performed analyses. The isolates were deposited at the Microorganism Genetics Laboratory (LabGeM), Department of Genetics, UFPR, Curitiba, State of Paraná, Brazil.

Bacteria were isolated from the rhizosphere of different maize genotypes cultivated in field trials [33]. Ikeda et al. (2013) cultivated maize in the southern Brazilian region of Campo Largo, State of Paraná. Maize roots were submerged in distilled water for 1 min, followed by immersion in 70% ethanol (v/v) for 1 min, 3% sodium hypochlorite (v/v) for 3 min, and 70% ethanol (v/v) for 30 s, and then washed three times with sterile distilled water for 1 min. Following surface sterilization, the samples were fragmented into five pieces of 8 mm and aseptically transferred to Petri plates containing a nitrogen-free solid culture medium [33]. Growth was assessed daily.

Characterization of isolates

Isolated fingerprinting with BOX-PCR

For the extraction of genomic DNA, the isolates were grown in 3 mL of Luria Bertani (LB) liquid medium for 18 h at 30 °C with agitation at 200 rpm, and the DNA was extracted as described by Szilagyi-Zecchin et al. [34]. DNA quality was accessed by agarose electrophoresis. DNA quantity was determined using a NanoDrop devise (Thermo Fisher Scientific, USA) and standardized to 50 ng.

The initial characterization of the isolates was performed by BOX-PCR using the A1R primer (5′-CTACGGCAAGGC GACGCTGACG-3′, Invitrogen™) [35]. The reaction was conducted under the conditions described by Kaschuk et al. [36] with required modifications of extension temperatures

and time. The reaction mixture (25 µL) contained 200 ng DNA, 10 × PCR buffer, 0.5 U·µL^{-1} Taq DNA polymerase, 0.2 µM primer, 0.2 mM dNTPs, and 3 mM $MgCl_2$. The amplification conditions were as follows: initial denaturation at 95 °C for 7 min; 35 cycles of 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 3 min; and final extension at 72 °C for 4 min.

The amplified fragments were separated by 0.7% agarose gel (25 cm × 20 cm, pH 8.0) electrophoresis at 140 V for 120 min. The gels were stained with ethidium bromide, visualized under UV light, and photographed. The band pattern was manually defined, comparing the presence and absence (transformed into binary matrices) of bands among the isolates. The dissimilarity of BOX-PCR fingerprints was calculated using the Jaccard (J) coefficient [37] with the vegan package in R [38], and clustering was performed using the bootstrapped (10^4 generations) unweighted pair group method with arithmetic mean (UPGMA) algorithm with the pvclust package in R [39]. A dendrogram was constructed using the ggtree package in R [40, 41].

Genus identification

The isolates were identified by amplifying the partial sequences of the 16S rRNA gene using the fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) primers [42]. The reaction was conducted under the conditions described by Menna et al. [43], with required modifications of amplification time and temperature. The reaction mixture (25 µL) contained 200 ng DNA, 10 × PCR buffer, 0.5 U·µL^{-1} Taq DNA polymerase, 0.2 µM primers, 0.2 mM dNTPs, and 3 mM MgCl2. The amplification conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2 min; and final extension at 72 °C for 10 min. The obtained fragments were sequenced using Big Dye with the ABI3500 DNA Sequencer, as described by Kimoto et al. [44].

The obtained sequences were compared those available in the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (http://www.bacterio.net) [45–47] using the BLAST tool [48], aligned using Muscle software [49], and edited using BioEdit 7.2.5 [50] and MEGA 7 [51]. Bayesian phylogenetic trees were generated using MrBayes 3.2.7a [52], incorporating the evolutionary model indicated after testing with jModel Test [53, 54] and performing simultaneous runs for random trees for 10^7 generations to reach an LnL deviation of 0.01 or below. The trees were visualized using FigTree 1.4.

Evaluation of plant growth-promoting characteristics

The plant growth-promoting potential of 57 representative isolates from each group of the BOX-PCR profile was evaluated. The experiment was performed in a climatic chamber (Walk-In Chamber). The experimental design was completely randomized, and the experiment was performed in triplicate.

Seeds of a commercial hybrid maize cultivar (AG 8780, Agroceres®) were manually treated with an insecticide (250 mL 100 kg^{-1} of seeds), containing carboxin and thiram (belonging to the carboxanilide and dimethyldithiocarbamate chemical groups, respectively, Vitavax-Thiram®) as active ingredients, and a fungicide (1.5 L 100 kg^{-1} of seeds) containing imidacloprid and thiodicarb (belonging to the neonicotinoid and oxime methylcarbamate chemical groups, respectively, Cropstar®) as active ingredients, using a seed treater 36 h before sowing. As the positive controls, seeds were inoculated with a commercial formulation containing Azospirillum brasilense (AzoTotal®, Total Biotecnologia; AbV5 and AbV6 strains; 2×10^8 CFU mL^{-1}; 100 mL 20 kg^{-1} of seeds). As the negative controls, seeds without inoculation were used. The toxicity of the fungicide and insecticide against the bacteria was evaluated by the inoculation of bacteria on solid LB medium containing the same concentrations of the fungicide and insecticide used in the experiment, and no toxicity was observed (data not shown).

For cultivation, the seeds were disinfected with 70% alcohol for 1 min and 3% sodium hypochlorite for 3 min, washed six times with distilled water, inoculated with 1 mL of the bacterial suspension in LB medium (1×10^8 cells mL^{-1}), and sown in autoclaved vermiculite, two per pot (volume, 1.7 L). The plants were maintained at a mean temperature of 28 °C under a 12 h photoperiod daily. Irrigation was applied at 50 mL per pot daily, alternating between sterile distilled water and sterile Hoagland and Arnon nutrient solution [55].

At 13 days after sowing, the plants were thinned to maintain only one plant per pot. After 30 days, the plants were collected, and root growth was measured using a root scanner (WinRhizo® device). For the whole plant analysis, the plants were first stored in paper bags, and the wet mass was measured. The samples were then dried in an oven at 60 °C for 72 h to determine the dry weight.

Homogeneity of variances was determined using the Bartlett test, and the normality of data was assessed using the Kolmogorov–Smirnov test, with Lilliefors correction. Nonparametric data were submitted to the Kruskal–Wallis test [nonparametric single-factor analysis of variance (ANOVA)], and parametric data were subjected to ANOVA. Means were compared using Tukey's test ($p \leq 0.05$). The data were plotted using Sigma®Plot 12.0 (https://systatsoftware.com/), and all statistical analyses were performed using Assistat 7.6 (http://www.assistat.com/indexp.html).

Table 1 Primers used to amplify genes involved in plant growth-promoting pathways in the 11 isolates identified as plant growth-promoting bacteria for maize in the climatic chamber

Gene	Sequence (5′-3′)	Amplification	Reference	
Nitrogenase reductase	*nif*H-F	AAAGGYGGWATCGGYAARTCC ACCAC	1,300 pb	Török and Kondorosi (1981)
	*nif*H-R	TTGTTSGCSGCRTACATSGCCATC AT		
Phloroglucinol synthase	*phl*D-F	ACCCACCGCAGCATCGTTTATGAG C	628 pb	Gardener et al. (2001)
	*phl*D-R	ACCGCCGGTATGGAAGATGAAAAA GTC		
Pyrroloquinoline quinine synthase	*pqq*C-F	CAGGGCTGGGTCGCCAACC	546 pb	Meyer et al. (2011)
	*pqq*C-R	CATGGCATCGAGCATGCTCC		
Indolepyruvate/phenylpyruvate decarboxylase	*ipd*C-F	GAAGGATCCCTGTTATGCGAACC	900 pb	Patten and Glick (2002)
	*ipd*C-R	CTGGGGATCCGACAAGTAATCAGG C		
Nitrite reductase	*nir*K-F	GGMATGGTKCCSTGGCA	514 pb	Braker et al. (1998)
	*nir*K-R	GCCTCGATCAGRTTRTGG		
1-Aminocyclopropane-1-carboxylate deaminase	*acd*S-F	GHGAMGACTGCAAYWSYGGC	792 pb	Blaha et al. (2006)
	*acd*S-R	ATCATVCCVTGCATBGAYTT		

Molecular characterization of genes related to plant growth promotion

To investigate the candidate genes related to plant growth-promoting pathways in the best isolates identified in the root growth assay, DNA was amplified with specific primers (Table 1).

The reaction mixture (25 µL) contained 100 ng DNA, 10 × PCR buffer, 0.5 U·µL^{-1} Taq DNA polymerase, 0.2 µM primers, 0.2 mM dNTPs, and 3 mM MgCl2. The amplification conditions for nitrogenase reductase (*nif*H) [56] were as follows: initial denaturation at 97 °C for 4 min; 1 cycle of denaturation at 96 °C for 20 s, annealing at 65 °C for 30 s, and elongation at 72 °C for 30 s; 2 cycles of denaturation at 96 °C for 20 s, annealing at 62 °C for 30 s, and elongation at 72 °C for 35 s; 3 cycles of denaturation at 96 °C for 20 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 42 s; 4 cycles of denaturation at 96 °C for 20 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 45 s; 5 cycles of denaturation at 96 °C for 20 s, annealing at 53 °C for 30 s, and elongation at 72 °C for 50 s; 25 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 45 s, and elongation at 72 °C for 60 s; and final extension at 72 °C for 10 min. For phloroglucinol synthesis (*phl*D) [57], the amplification followed an initial denaturation at 95 °C for 3 min, 35 cycles of 60 s at 94 °C, 60 s at 60 °C, and 60 s at 72 °C, and final extension at 72 °C for 5 min.

The amplification conditions for phosphate solubilization (*pqq*C) [58] were as follows: initial denaturation at 96 °C for 10 min; 30 cycles of 30 s at 96 °C, 30 s at 63 °C, and 1 min at 72 °C; and a final extension of 10 min at 72 °C. Indole acetic acid synthesis (*ipd*C) [59] was amplified using an initial denaturation step at 94 °C for 5 min; 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C; and final extension at 72 °C for 7 min. The amplification conditions for nitrite reductase (*nir*K) [60] were as follows: initial denaturation at 95 °C for 5 min; 1 cycle of denaturation at 95 °C for 30 s, annealing at 45 °C for 40 s, and elongation at 72 °C for 40 s; 1 cycle of denaturation at 95 °C for 30 s, annealing at 44.5 °C for 40 s, and elongation at 72 °C for 40 s; 1 cycle of denaturation at 95 °C for 30 s, annealing at 44 °C for 40 s, and elongation at 72 °C for 40 s; 1 cycle of denaturation at 95 °C for 30 s, annealing at 43.5 °C for 40 s, and elongation at 72 °C for 40 s; 1 cycle of denaturation at 95 °C for 30 s, annealing at 43 °C for 40 s, and elongation at 72 °C for 40 s; 1 cycle of denaturation at 95 °C for 30 s, annealing at 42.5 °C for 40 s, and elongation at 72 °C for 40 s; 1 cycle of denaturation at 95 °C for 30 s, annealing at 42 °C for 40 s, and elongation at 72 °C for 40 s; 1 cycle of denaturation at 95 °C for 30 s, annealing at 41.5 °C for 40 s, and elongation at 72 °C for 40 s; 1 cycle of denaturation at 95 °C for 30 s, annealing at 41 °C for 40 s, and elongation at 72 °C for 40 s; 1 cycle of denaturation at 95 °C for 30 s, annealing at 40 °C for 40 s, and elongation at 72 °C for 40 s; 20 cycles of 30 s at 95 °C; 40 s at 43 °C; and 40 s at 72 °C; and final extension of 7 min at 72 °C. The amplification conditions for ethylene degradation (*acd*S) [61] were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C; and final extension at 72 °C for 7 min.

Biochemical evaluation of plant growth-promoting parameters

Siderophore production was analyzed as described by Schwyn and Neilands [62] using solid DYGS medium with CAS solution, carefully mixed into 72.9 mg of hexadecyltrimethylammonium (HDTMA) and dissolved in 40 mL of distilled water. The results were considered positive when the color of the medium changed from blue to yellow.

Phosphate solubilization was evaluated as described Sylvester-Bradley et al. [63] using the GL culture medium supplemented with 0.25 g L^{-1} K_2HPO_4 and 1 g L^{-1} $CaCl_2$. The results were considered positive when a halo was formed around the colony.

Biological nitrogen fixation was evaluated as described by Araújo et al. [64] using a nitrogen-free semi-solid JNFb medium. First, the isolates were grown in nitrogen-free solid JNFb medium at 32 °C. After 4 days, the isolates were transferred to ampoules with 5 mL of nitrogen-free semi-solid medium and incubated at the same temperature (120 rpm) for 7 days; the culture was repeated twice. The isolates were successively reinoculated to prevent pellicle formation due to the nitrogen reserves of the accumulated bacteria cells [33]. Bacterial growth was assessed based on the formation of a pellicle on the medium surface.

IAA synthesis was evaluated using the methodology described by Kuss et al. [65], modified with the use of King B medium. Salkowski solution was added to reveal the results. Absorbance was measured at 520 nm using spectrophotometry. The final values were multiplied by the molecular weight of commercial auxin ($C_{10}H_9NO_2$ = 175.19 g mol^{-1}) and expressed in micrograms per milliliter. The data were transformed using $\sqrt{x+½}$.

Amylase production was tested in MM9 medium [66] [200 mL of salt solution (64 g $Na_2HPO_4.2H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5 g NH_4Cl q.s.p. 1,000 mL distilled water), 2 mL of 1 M $MgSO_4$, 10 g glucose, 0.1 mL of 1 M $CaCl_2$, and 15 g agar, q.s.p. 1,000 mL distilled water; pH 7.0] containing 0.5% yeast extract and 1% soluble starch [67]. The result was considered positive when a halo was formed around the bacterial colony following iodine addition.

Pectinase and chitinase production were also evaluated in MM9 medium supplemented with 1% pectin [67] and 0.08% colloidal chitin [68, 69], respectively. For pectin, the result was considered positive when a halo was formed around the bacterial colony following lugol addition. For chitin, the result was considered positive when a halo appeared around the colony following chitin degradation.

Cellulase test were performed as described by Renwick et al. [64]. Cellulase production was revealed by the addition of Congo Red to the mineral culture medium [70] (0.02 g $CaCO_3$, 0.01 g $FeSO_4.7H_2O$, 1.71 g KCl, 0.05 g $MgSO_4.7H_2O$, 4.11 g $Na_2HPO_4.12H_2O$, and 15 g agar; q.s.p. 1,000 mL distilled water) containing 0.5% carboxymethylcellulose.

Esterase production was assayed in a solid esterase/lipase culture medium supplemented with 1% Tween 80 [71]. Protease production was evaluated in skimmed milk and agar medium [72]. For both esterase and protease, the results were considered positive when a halo was formed around the colonies.

Urease production was evaluated in a urease culture medium as described by Dye [73]. The result was considered positive when the culture medium turned blue.

All tests were performed in triplicate. The bacterial culture temperature for all biochemical tests was 32 °C. For IAA analysis, the homogeneity of variances was tested using Bartlett test, and the normality of data was assessed using the Kolmogorov–Smirnov test, with Lilliefors correction. All data were subjected to ANOVA and Tukey's test ($p \leq 0.05$). Statistical analyses were performed using Assistat v. 7.6 (http://www.assistat.com/indexp.html).

Nucleotide sequence accession numbers

The 16S rRNA sequences of the isolates have been deposited in NCBI GenBank (https://www.ncbi.nlm.nih.gov/) under accession numbers MT780814–MT780870.

Results

Characterization of isolates

The band pattern of the 282 isolates was obtained using BOX-PCR. The amplified products were scored in terms of the presence (1) or absence (0) of band at a position in each of the isolates, resulting in 57 different genetic profiles [Fig. 1; values to the left of the nodes represent significant bootstrap values (> 50%)]. The BOX-PCR profiles were considered different when the level of dissimilarity was equal to or higher than 80%.

Dendrogram analysis revealed that the isolates differed in terms of the 57 profiles, separated into three main clusters. The first cluster was formed by LGMB456 (representing one isolate) and LGMB459 (representing three isolates) (*Pseudomonas* spp.), the second by LGMB33 (representing one isolate) (*Bacillus* sp.), and the third by two main subgroups, each subdivided into several branches, representing the remaining 54 profiles (of the remaining 277 isolates).

Based on these results, a single representative isolate was selected at random from each profile, resulting in 57 isolates representing the respective genetic groups.

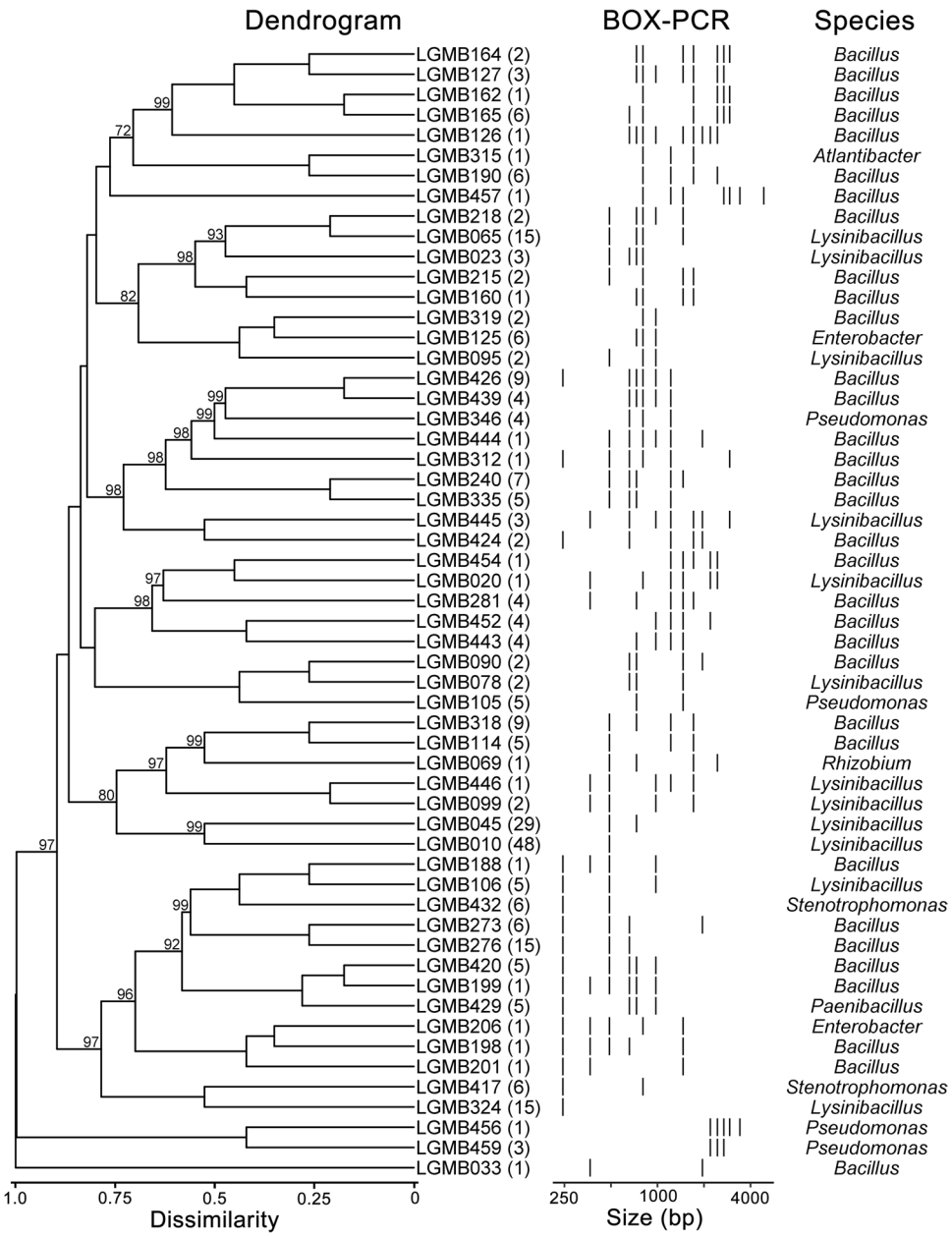

Fig. 1 Dendrogram obtained using BOX-PCR amplification products from maize isolates. Clusters were formed based on 57 distinct genetic profiles. Cluster analysis was performed using the UPGMA algorithm and Jaccard's coefficient. Numbers in parenthesis represent the number of isolates with the same genetic profile and the selected representative. Numbers at nodes represent the bootstrap support of 10^4 generations of clustering (only values exceeding 50% are shown). Genera were identified based on the partial sequence of the 16S rRNA gene using Bayesian analysis

Identification of isolates at the genus level

One representative from each of the 57 BOX-PCR profiles was selected for 16S rRNA sequencing. Based on phylogenetic analysis (Table 2, Fig. S1–S8), the isolates were identified as belonging to the genera *Bacillus* (35 profiles), *Lysinibacillus* (12 profiles), *Pseudomonas* (4 profiles), *Stenotrophomonas* (2 profiles), *Enterobacter* (1 profile), *Paenibacillus* (1 profile), *Rhizobium* (1 profile), and *Atlantibacter* (1 profile).

Selection of high-performance isolates using an in vivo *assay*

Dry weight of the whole plants (in g) ($p = 0.0001$) was significantly higher for LGMB324 (*Lysinibacillus* sp.) than that for 38 other isolates belonging to the genera *Stenotrophomonas* sp., *Pseudomonas* spp., *Lysinibacillus* spp., *Rhizobium* sp., *Bacillus* spp., *Enterobacter* sp., and *Atlantibacter* sp. as well as the negative and positive controls but was comparable to that for the 20 other isolates belonging to the genera *Lysinibacillus* spp., *Bacillus* spp., *Pseudomonas* sp., *Stenotrophomonas* sp., and *Paenibacillus* sp. Total root length (in cm) ($p = 0.01063$) was significantly higher for LGMB12 (*Bacillus* sp.) than that for 28 other isolates belonging to genera *Pseudomonas* spp.,

Table 2 Genus-level identification of LGMB isolates used for the inoculation of maize seeds in the climatic chamber. Bayesian phylogenetic analysis of 16S rRNA sequences (Supplementary material Figures S1 to S8) of the LGMB isolates and of species of the corresponding genus available in GenBank was used

Genus/isolate	Close related species
Atlantibacter	
LGMB315(MT780849)	*Atlantibacter hermannii* (JN175345)
Bacillus	
LGMB12(MT780815), LGMB114(MT780828), LGMB125(MT780829), LGMB165(MT780835), LGMB190(MT780837), LGMB198(MT780838), LGMB201(MT780840), LGMB215(MT780842), LGMB318(MT780850), LGMB319(MT780851), LGMB443(MT780862), LGMB452(MT780866), LGMB454(MT780867)	*Bacillus siamensis* (GQ281299),
LGMB218(MT780843), LGMB281(MT780847), LGMB420(MT780856), LGMB424(MT780857), LGMB426(MT780858), LGMB439(MT780861)	*Bacillus velezensis* (AY603658)
LGMB90(MT780823)	Bacillus subtilis (JF749278) *Bacillus tequilensis* (HQ223107)
LGMB33(MT780818), LGMB126(MT780830)	*Bacillus australimaris* (JX680098) *Bacillus safensis* (AF234854) *Bacillus pumilus* (AY876289) *Bacillus zhangzhouensis* (JX680133)
LGMB127(MT780831), LGMB160(MT780832), LGMB162(MT780833), LGMB164(MT780834), LGMB188(MT780836), LGMB199(MT780839), LGMB240(MT780844), LGMB273(MT780845), LGMB276(MT780846), LGMB312(MT780848), LGMB335(MT780853), LGMB444(MT780863), LGMB457(MT780869)	*Bacillus aerophilus* (AJ831844), *Bacillus altitudinis* (AJ831842), *Bacillus stratosphericus* (AJ831841), *Bacillus xiamenensis* (JX680066)
Enterobacter	
LGMB206(MT780841)	*Enterobacter ludwigii* (AJ853891)
Lysinibacillus	
LGMB10(MT780814), LGMB20(MT780816), LGMB23(MT780817), LGMB45(MT780819), LGMB65(MT780820), LGMB78(MT780822), LGMB95(MT780824), LGMB99(MT780825), LGMB106(MT780827), LGMB324(MT780852), LGMB445(MT780864), LGMB446(MT780865)	*Lysinibacillus fusiformis* (AF169537)
Paenibacillus	
LGMB429(MT780859)	*Paenibacillus xylanexedens* (EU558281)
Pseudomonas	
LGMB105(MT780826), LGMB459(MT780870)	*Pseudomonas azotoformans* (D84009), *Pseudomonas lactis* (KP756923)
LGMB346(MT780854), LGMB456(MT780868)	*Pseudomonas koreensis* (AF468452)
Rhizobium	
LGMB69(MT780821)	*Rhizobium massiliense* (AF531767) *Rhizobium oryzihabitans* (MT023790)
Stenotrophomonas	
LGMB417(MT780855), LGMB432(MT780860)	*Stenotrophomonas maltophilia* (FJ971878), *Stenotrophomonas pavanii* (FJ748683)

Bacillus spp., *Lysinibacillus* spp., and *Enterobacter* sp. but was comparable to that for 30 other isolates belonging to the genera *Lysinibacillus* spp., *Rhizobium* sp., *Bacillus* spp., *Atlantibacter* sp., *Stenotrophomonas* spp., *Paenibacillus* sp., and *Pseudomonas* sp. as well as the negative and positive controls.

The length of roots ranging in diameter from 0 to 0.5 mm (in cm) ($p = 0.00736$) was significantly higher for LGMB12 (*Bacillus* sp.) than that for 43 other isolates belonging to the genera *Lysinibacillus* spp., *Stenotrophomonas* sp., *Pseudomonas* spp., *Bacillus* spp., *Enterobacter* sp., *Rhizobium*

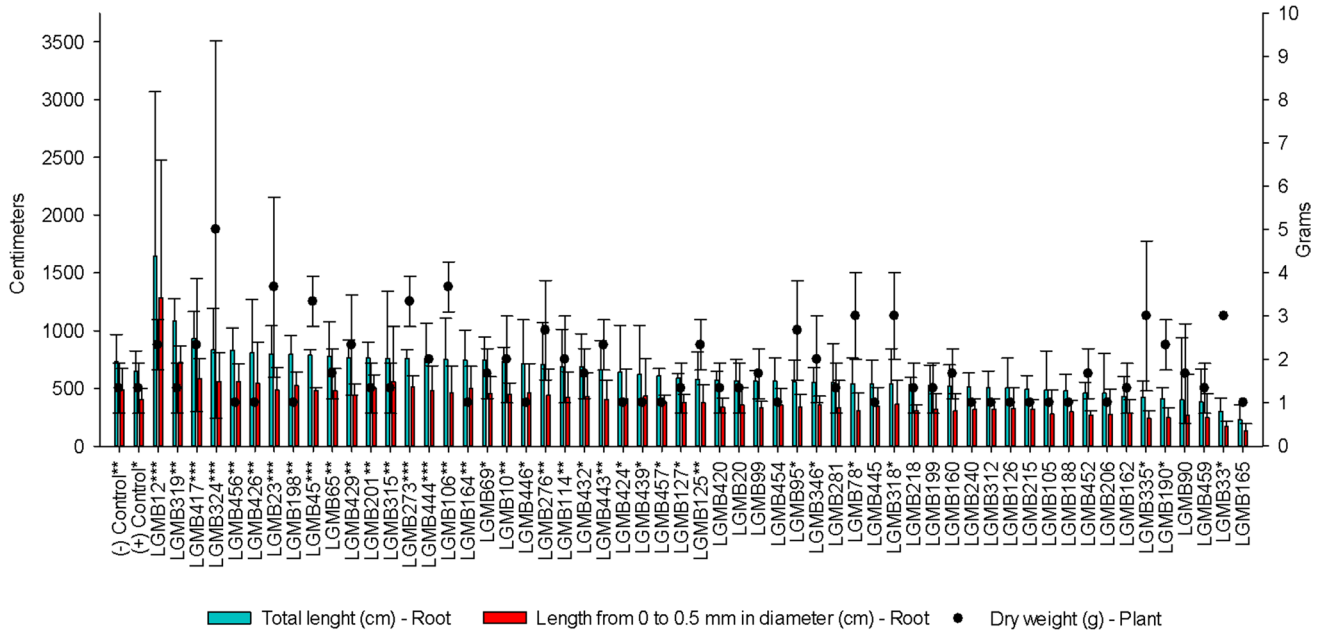

Fig. 2 Evaluation of plant growth-promoting bacteria for maize development after 30 days of inoculation of seeds and growth in a climatic chamber. Dry weight of the whole plants (in g, black dots), total root length (in cm, blue bars), and total length of roots ranging in diameter from 0 to 0.5 mm (in cm, red bars) were evaluated. The data were analyzed with Tukey's test, and a $p < 0.05$ was considered significant. The *Y* axis on the left indicates the total root length and total length of roots ranging in diameter from 0 to 0.5 mm, expressed in centimeters, and the *Y* axis on the right indicates the dry weight of the whole plants, expressed in grams. The controls are represented by (−) negative (no bacterial inoculated) and (+) positive seeds (inoculated with *Azospirillum brasilense* AbV5 and AbV6). The error bars with SD are represented by vertical lines. Each column and dot on the graph represent triplicate analyses of plants whose seeds were inoculated with the LGMB marked on the *X* axis, comprising 177 samples. The asterisks (*) next to the isolate name indicate the number of parameters that were significantly higher: *Significant for one parameter; **significant for two parameters; and ***significant for three parameters

sp., and *Paenibacillus* sp. as well as the positive controls but was comparable to that for 15 other isolates belonging to the genera *Lysinibacillus* spp., *Bacillus* spp., *Atlantibacter* sp., *Stenotrophomonas* sp., and *Pseudomonas* sp. as well as the negative controls. The wet mass of the whole plant (in g) ($p = 0.1662$), total root volume (in cm^3) ($p = 0.1412$), and mean root diameter (mm) ($p = 0.2523$) were comparable across all treatments (Tukey's test, at 5% significance) (Fig. 2).

Based on the analysis of plant growth, 11 isolates stood out, presenting results statistically equal or superior to the other isolates tested for dry weight, total root length, and total length of roots ranging in diameter from 0 to 0.5 mm. LGMB12, LGMB273, LGMB444 (*Bacillus* spp.), LGMB23, LGMB45, LGMB324 (*Lysinibacillus* spp.), and LGMB417 (*Stenotrophomonas* sp.) were significant in terms of all characteristics, whereas LGMB319 and LGMB426 (*Bacillus* spp.), LGMB429 (*Paenibacillus* sp.), and LGMB456 (*Pseudomonas* sp.) were significant in terms of two of the three characteristics (dry weight and total length of roots ranging in diameter from 0 to 0.5 mm; although not significant, total root length exceeded 800 cm).

Genetic and biochemical characteristics involved in plant growth promotion

The 11 isolates selected in the above evaluations, based on the higher root growth of maize plants, were further analyzed to determine the possible pathways involved in plant growth promotion.

Molecular characterization of genes revealed that the isolates likely possess specific plant growth-promoting mechanisms. Therefore, the results of gene expression analyses were confirmed using biochemical tests for phosphate solubilization, IAA production, and biological nitrogen fixation.

Molecular assays detected (Table 3) the marker gene for phosphate solubilization (*pqq*C) in 54.5% of the isolates, and all these isolates could solubilize phosphate in the biochemical test [LGMB12, LGMB273, LGMB426 (*Bacillus* spp.), LGMB23 (*Lysinibacillus* sp.), LGMB417 (*Stenotrophomonas* sp.), and LGMB456 (*Pseudomonas* sp.)]. Moreover, the marker gene for biological nitrogen fixation (*nif*H) was detected in 36.4% of the isolates [LGMB12 and LGMB426 (*Bacillus* spp.), LGMB417 (*Stenotrophomonas* sp.), and LGMB429 (*Paenibacillus* sp.)]. However, in the biochemical test, amplification

Table 3 Qualitative results referring to the presence (+) or absence (−) of genes related to plant growth promotion (gene column) and the related biochemical tests (Bioch. test column) in the 11 isolates identified as plant growth-promoting bacteria for maize in the climatic chamber

Isolate	Genus	Phosphate solubilization		Indol acetic acid			Biological nitrogen fixation		Denitrification	Sidero-phore production
		pqqC gene	Bioch. test	*ipdC* gene	Bioch. test	Quantification* µg mL^{-1}	*nifH gene*	Bioch. test	*nirK* gene	Bioch. test
LGMB12	*Bacillus*	+	+	+	+	3.635^{bcd}	+	-	-	+
LGMB23	*Lysinibacillus*	+	+	+	+	5.310^{abcd}	-	-	-	-
LGMB45	*Lysinibacillus*	-	-	+	+	6.926^{a}	-	-	+	-
LGMB273	*Bacillus*	+	+	+	+	2.525^{de}	-	-	-	+
LGMB319	*Bacillus*	-	-	+	+	4.185^{bcd}	-	-	-	+
LGMB324	*Lysinibacillus*	-	-	+	+	6.006^{ab}	-	-	-	+
LGMB417	*Stenotrophomonas*	+	+	+	+	3.027^{cde}	+	+	-	-
LGMB426	*Bacillus*	+	+	+	+	4.199^{bcd}	+	-	+	+
LGMB429	*Paenibacillus*	-	-	+	+	3.611^{bcd}	+	-	+	-
LGMB444	*Bacillus*	-	-	-	+	0.734^{e}	-	-	-	+
LGMB456	*Pseudomonas*	+	+	+	+	5.452^{abc}	-	-	-	-

*IAA concentration data were analyzed with Tukey's test ($p < 0.05$)

with the *nif*H primers was successful in only one isolate [LGMB417 (*Stenotrophomonas* sp.)] (Table 3). The *ipd*C gene, which encodes a precursor of IAA, was detected in 90.9% isolates, except in LGMB444 (*Bacillus* sp.). In biochemical test, LGMB45 (*Lysinibacillus* sp.) showed higher IAA production, albeit not significantly different from LGMB23 and LGMB324 (*Lysinibacillus* spp.) or LGMB456 (*Pseudomonas* sp.) (Table 3).

Furthermore, biochemical tests revealed that six isolates produced siderophores [LGMB12, LGMB273, LGMB319, LGMB426, LGMB444 (*Bacillus* spp.), and LGMB324 (*Lysinibacillus* sp.)] (Table 3). In addition, the *nir*K gene, which is involved in denitrification, was detected in 27.3% isolates [LGMB45 (*Lysinibacillus* sp.), LGMB426 (*Bacillus* sp.), and LGMB429 (*Paenibacillus* sp.)]. The evaluated isolates did not possess *phl*D and *acd*S genes, which are responsible for the synthesis of phloroglucinol and degradation of plant ethylene precursor, respectively.

In addition, LGMB426 (*Bacillus* sp.) presented the highest number of evaluated characteristics, being able to solubilize phosphate, synthesize IAA, and produce siderophores.

Production of enzymes with biotechnological applications in agriculture

The enzymatic profiles (Table 4) showed that 90.1% isolates produced amylase, 54.5% produced urease, 72.7% produced cellulase, 18.2% produced chitinase, 81.8% produced pectinase, 90.9% produced protease, and 45.4% produced esterase. LGMB444 (*Bacillus* sp.) exhibited the highest diversity of enzymes (Table 4), being able to produce all enzymes tested, including amylase, cellulase, pectinase, chitinase, urease, esterase, and protease.

Discussion

Microbial inoculants of elite strains may help improve crop productivity through diverse mechanisms, including nitrogen fixation, phosphate solubilization, siderophore synthesis, phytohormone production, and hydrolytic enzyme synthesis (chitinase, cellulase, and protease, among others), which enhance soil fertility and agricultural yield as well as favor plant growth [74–76].

In the present study, high intraspecific diversity was detected among 282 isolates collected from maize rhizosphere based on the BOX-PCR profiles. BOX-PCR fingerprinting is a precise discriminatory technique to determine genetic relatedness and diversity, particularly for the genus *Bacillus*, which shows multiple distinct band patterns [77]. This modality is of great importance in studies comprising a large number of samples [78–80] to identify at the strain level, which is not possible based solely on their morphology [81].

Diversity of maize root bacterial isolates is well-known, and bacteria of the genera *Bacillus*, *Pseudomonas*, *Paenibacillus*, and *Enterobacter* are known to possess the potential to promote plant growth [9, 10, 34, 82, 83]. In the present study, we identified several isolates belonging to these genera as potential PGPB (*Bacillus*, Fig. S1; *Pseudomonas*, Fig. S2; *Paenibacillus*, Fig. S3; and *Enterobacter*, Fig. S4), in addition to bacteria belonging to other genera, including *Rhizobium* (Fig. S5), *Lysinibacillus* (Fig. S6), *Stenotrophomonas* (Fig. S7), and *Atlantibacter* (Fig. S8). These genera are agriculturally important and predominant colonizers of the rhizospheres of various crops, and they possess a broad spectrum of antagonistic activities [84], which is an advantage for bacteria intended for use as inoculants. However, the isolates must be identified to the species level for use as inoculants to exclude the ones pathogenic to plants, humans, and other non-human animals [12].

Table 4 Qualitative results referring to the presence (+) or absence (−) of degradation catalyzed by amylase, cellulase, pectinase, chitinase, urease, esterase, and protease produced by the 11 isolates identified as plant growth-promoting bacteria for maize in the climatic chamber

Isolate	Genus	Amylase	Cellulase	Pectinase	Chitinase	Urease	Esterase	Protease
LGMB12	*Bacillus*	+	+	+	-	-	-	+
LGMB23	*Lysinibacillus*	-	-	-	-	-	+	-
LGMB45	*Lysinibacillus*	+	-	-	-	+	-	+
LGMB273	*Bacillus*	+	+	+	+	+	-	+
LGMB319	*Bacillus*	+	+	+	-	-	-	+
LGMB324	*Lysinibacillus*	+	-	-	-	+	-	+
LGMB417	*Stenotrophomonas*	+	+	+	-	+	+	+
LGMB426	*Bacillus*	+	+	+	-	-	-	+
LGMB429	*Paenibacillus*	+	+	+	-	-	-	-
LGMB444	*Bacillus*	+	+	+	+	+	+	+
LGMB456	*Pseudomonas*	+	+	+	-	+	+	+

For use as a candidate biofertilizer, a bacterium must possess the potential to promote crop growth and increase yield, being active mainly in the rhizosphere [85]. As such, enhanced root growth promotes nutrient absorption, and root length is an indicator of the nutrient uptake and acquisition efficiency of plants [86].

In the present study, the 11 isolates that significantly increased root length were selected to assess the possible mechanisms underlying plant growth promotion. Specifically, inoculation of bacteria that solubilize phosphate can serve as an alternative for the application of fertilizers, since majority of these phosphate solubilizers are present in the soil [87, 88]. A considerable proportion of bacteria tested in the present study could promote plant nutrient uptake by making phosphorous available through inorganic phosphate solubilization (Table 3). Bacteria of the genus *Bacillus*, including *B. amyloliquefaciens*, *B. megaterium*, and *B. subtilis*, isolated from maize [9, 10, 89], rice (*Oryza sativa* L.) [90, 91], *Medicago polymorpha* [92], and soil [93] are phosphate solubilizers, and these bacteria can solubilize phosphate even under salinity stress.

Biological nitrogen fixation is relevant to plant growth. Several bacterial genera can fix nitrogen [11–18]. In the present study, only LGMB417 (*Stenotrophomonas* sp.) showed this possible ability, as evidenced by the detection of the *nif*H gene and the results of biochemical tests. Bacteria of the genus *Stenotrophomonas* are present in different types of soil and rhizospheres of different crops, and they can promote plant growth through biological nitrogen fixation [94].

Iron is another essential nutrient for plant development. Therefore, siderophore production is critical. Iron acts as an enzyme cofactor in biochemical pathways involved in several plant physiological processes, such as respiration, photosynthesis, and biological nitrogen fixation [23]. In the present study, over half of the isolates evaluated [LGMB12, LGMB273, LGMB319, LGMB426, LGMB444 (*Bacillus* spp.), and LGMB324 (*Lysinibacillus* sp.)] could produce siderophores, indicating their significance in the context of plant growth. Tropical soils are characterized by intense geochemical weathering of primary minerals in the substratum rock, leading to the formation of stable secondary minerals, such as hematite and goethite (iron oxides) [95]; thus, Brazilian soils are rarely iron deficient. PGPB that can produce siderophores are capable of biocontrol, since the siderophores produced by these isolates have a greater affinity toward iron present in the soil [96]. This gives rise to competition among microorganisms for establishment in the rhizosphere, which can further prevent the proliferation of harmful microbes due to iron deficiency and loss of their pathogenicity [97]. *Bacillus* spp. and *Enterobacter* spp. isolated from iron-enriched soils were proven promising candidates for siderophore production [98]. Moreover, bacteria of the genus *Stenotrophomonas* play pivotal roles in biogeochemical processes, such as sulfur and nitrogen cycle [99–101]. In addition, *Stenotrophomonas* spp. can produce different forms of siderophore [102, 103].

IAA production is crucial for plant development [102]. Bacteria of the genera *Enterobacter*, *Bacillus*, and *Pseudomonas* can produce IAA [84]. This phytohormone promotes cell stretching, division, and differentiation [104]. However, optimum levels of IAA are critical, as the roots are extremely sensitive to auxin. As such, when present in trace amounts, IAA can activate plant responses, but at high concentrations, it can produce inhibitory effects [105]. In the present study, variations in IAA production were observed among the tested isolates. However, in some cases, we noted discordance between the results of biochemical and molecular analyses. For instance, LGMB444 could produce IAA according to the results of biochemical test, but the gene encoding the precursor of this phytohormone was not detected in molecular assays, perhaps because of the lack of primer specificity or the presence of an IAA residue in the bacteria itself [106, 107]. The opposite trends were observed for LGMB12, LGMB426, and LGMB429, which showed positive results of gene amplification but negative results in the biochemical tests (no pellicle formation in the semi-solid medium); this may be attributed to the incompatibility between the species analyzed and the conditions of biochemical assays [108].

Genes involved in the biological control of phytopathogens, such as *phl*D and *acd*S [109, 110], were also investigated. However, these genes were not detected in any isolate. According to Bruto et al. [111], not all PGPB possess the *phl*D gene, and the *acd*S gene is rather rare in genera of the order Enterobacterales (e.g., *Enterobacter*, among others). Few genera that have evolved close relationships with plants (e.g., *Dickeya*) express these genes [112].

Another mechanism underlying plant growth promotion includes the production of enzymes, such as amylases, which represent one of the most important groups of enzymes [113]. In the present study, LGMB12, LGMB273, LGMB319, LGMB426, LGMB444 (*Bacillus* spp.), LGMB45, LGMB324 (*Lysinibacillus* spp.), LGMB417 (*Stenotrophomonas* sp.), LGMB429 (*Paenibacillus* sp.), and LGMB456 (*Pseudomonas* sp.) produced amylase, indicating their ability to degrade and process raw materials or synthesize related products [114].

Hydrolytic enzymes directly affect the activity of soil microbiota (microbial biomass and basal respiration, among others). These enzymes are involved in the biocontrol of pathogens as well as the decomposition of organic compounds and release of nutrients in the soil, which can enhance soil quality and ultimately improve crop fields [115, 116]. In addition, other enzymes, such as chitinase, cellulase, and protease, among others, play vital roles in agriculture. For instance, these enzymes can enable biocontrol against

many fungi through cell wall lysis [25, 26, 117]. The second most frequently detected enzyme in the present study was protease, produced by LGMB12, LGMB273, LGMB319, LGMB426, LGMB444 (*Bacillus* spp.), LGMB45, LGMB324 (*Lysinibacillus* spp.), LGMB417 (*Stenotrophomonas* sp.), and LGMB456 (*Pseudomonas* sp.). Proteases break down disrupted proteins to recycle their amino acids for use in other functions related to the regulation of plant growth, development, and defense [118].

Furthermore, LGMB12, LGMB273, LGMB319, LGMB426, LGMB444 (*Bacillus* spp.), LGMB417 (*Stenotrophomonas* sp.), LGMB429 (*Paenibacillus* sp.), and LGMB456 (*Pseudomonas* sp.) produced both cellulase and pectinase, which are involved in the lysis of pathogen cell wall, inducing plant systemic resistance during pathogen colonization [119]. LGMB23 (*Lysinibacillus* sp.), LGMB417 (*Stenotrophomonas* sp.), LGMB444 (*Bacillus* sp.), and LGMB456 (*Pseudomonas* sp.) produced esterase, which can inhibit the growth of pathogens, thus minimizing their detrimental effects on plants [120]. Only LGMB273 and LGMB444 (*Bacillus* spp.) produced chitinase. Isolates that can produce chitinase are potential biocontrol agents [121, 122]. Urease is another enzyme of extreme importance, and half of the isolates tested in the present study [LGMB45, LGMB324 (*Lysinibacillus* spp.), LGMB273, LGMB444 (*Bacillus* spp.), LGMB417 (*Stenotrophomonas* sp.), and LGMB456 (*Pseudomonas* sp.)] produced this enzyme. Through the inoculation of PGPB in the rhizosphere, urease is involved in the conversion of nitrogen for assimilation by plants, promoting growth and biocontrol activity [123].

Conclusion

In the present study, we explored the diversity of bacteria isolated from maize rhizosphere to evaluate their plant growth-promoting potential. Gene sequencing revealed the presence of the genera *Atlantibacter*, *Bacillus*, *Enterobacter*, *Lysinibacillus*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, and *Stenotrophomonas*. Eleven bacteria stood out in in vivo assays. Among these, five isolates [LGMB12, LGMB273, LGMB426 (*Bacillus* spp.), LGMB417 (*Stenotrophomonas* sp.), and LGMB456 (*Pseudomonas* sp.)] showed the most promising results and the greatest potential for plant growth promotion in maize owing to their biochemical and enzymatic characteristics. Thus, further research is warranted to test the biotechnological potential of these isolates under field conditions, exploring their utility as alternative biofertilizers for these crops. Moreover, it would be interesting to identify the bacteria to the species level, to exclude the ones pathogenic to plants, humans, and other non-human animals and elucidate the growth-promoting mechanisms within the plants.

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