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Maize endophytic bacteria as mineral phosphate solubilizers

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Genet. Mol. Res. 16 (1): gmr16019294
Received September 15, 2016
Accepted December 7, 2016
Published February 16, 2017
DOI http://dx.doi.org/10.4238/gmr16019294

ABSTRACT. In the present study, we demonstrated the *in vitro* activity of endophytic phosphate-solubilizing bacteria (PSB). Fifty-five endophytic PSB that were isolated from sap, leaves, and roots of maize were tested for their ability to solubilize tricalcium phosphate and produce organic acid. Partial sequencing of the 16S rRNA-encoding gene showed that the isolates were from the genus *Bacillus* and different species of Enterobacteriaceae. The phosphate solubilization index on solid medium and phosphate solubilization in liquid medium varied significantly among the isolates. There was a statistically significant difference ($P \leq 0.05$) for both, the values of phosphate-solubilizing activity and pH of the growth medium, among the isolates. Pearson correlation was statistically significant ($P \leq 0.05$) between P-solubilization and pH ($R = -0.38$), and between the gluconic acid

production and the lowering of the pH of the liquid medium at 6 (R = 0.28) and 9 days (R = 0.39). Gluconic acid production was prevalent in all the PSB studied, and *Bacillus* species were most efficient in solubilizing phosphate. This is the first report on the characterization of bacterial endophytes from maize and their use as potential biofertilizers. In addition, this may provide an alternative strategy for improving the phosphorus acquisition efficiency of crop plants in tropical soils.

Key words: *Zea mays*; Endophytic bacteria; Phosphate-solubilizing bacteria; Organic acids

INTRODUCTION

The use of phosphate fertilizers (P-fertilizers) is essential for providing phosphorus as a nutrient for healthy plant growth (Pinto et al., 2013). The majority of the fertilizer-derived phosphorus is retained in the solid phase of the soil as precipitates with aluminum, iron, calcium, and organic matter or is adsorbed on the surface of clay particles, which makes it unavailable for plant uptake (Hinsinger, 2001; Novais et al., 2007; Gurikar et al., 2016). The use of natural sources of phosphate fertilizers is limited by the low ion-exchange capacity of the acidic tropical soils (Edwards et al., 2016). In addition, soluble phosphate chemical fertilizers are costly and may cause eutrophication. Phosphate-solubilizing bacteria (PSB) could play an important role in supplying phosphate to plants in an eco-friendly and sustainable way (Oliveira et al., 2009; Gomes et al., 2014).

PSB, also referred as phosphobacteria, are found ubiquitously in soil and their numbers vary depending on the type of soil (Mohammadi, 2012). PSB can solubilize phosphorus from organic and inorganic sources by the action of organic acids and extracellular enzymes that are secreted into the soil (Chen et al., 2006; Park et al., 2011; Baliah et al., 2016; Gurikar et al., 2016). PSB releases low molecular weight organic acids, which solubilizes mineral phosphates and reduces the pH of soil (Goldstein, 1986; Whitelaw, 2000; Pérez et al., 2007; Gomes et al., 2014). PSB have been widely tested as biofertilizers and inoculants to increase crop yield (Karpagam and Nagalakshmi, 2014). Currently, different species of bacteria such as *Azotobacter chroococcum*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megaterium*, *Arthrobacter ilicis*, *Escherichia coli*, *Pantoea agglomerans*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Microbacterium laevaniformans*, and *Micrococcus luteus* have been identified as P-fertilizers (Kumar et al., 2014).

Endophytic PSB is more competitive than non-endophytic or facultative microorganisms inside the host plant since the endophyte-plant interaction is the result of an evolutionary process that is controlled by genes of both organisms (Rosenblueth and Martínez-Romero, 2006). In fact, almost all endophytes represent a group of soil bacteria, which can colonize plants without inducing the host defense pathway. Thus, the distinction between free-living soil bacteria, the rhizosphere population, and endosymbionts of a host plant may represent a true continuum, with microbes able to move between the soil, the rhizosphere, and inside the root (Farrar et al., 2014). Indeed, several species of *Bacillus* and *Pseudomonas* use the nutrient niche in the rhizosphere and change from a free-living condition to an endophytic state (Rosenblueth and Martínez-Romero, 2006). Bacterial endophytes that were injected into stems moved to the roots and the rhizosphere, thereby confirming the existence of a continuous

shift in microbial community within the root microbiome (Gaiero et al., 2013). Furthermore, bacterial endophytes can be transported from the seeds into the roots and tissues, reducing the need of continuous inoculations (Johnston-Monje and Raizada, 2011). However, the ability of endophytic bacteria to solubilize phosphates in tropical and sub-tropical soils is not well-studied.

The main goals of the present study were to isolate and characterize endophytic PSB from maize and to test their ability of solubilizing tricalcium phosphate (TCP) *in vitro*.

MATERIAL AND METHODS

Experimental sites and isolation of endophytic bacteria

This study was carried out in 2013, at Embrapa Maize and Sorghum Research Center, Sete Lagoas, Minas Gerais State, Brazil.

Leaves and roots of maize plants were disinfected according to the method described by Araújo et al. (2000), macerated in liquid nitrogen, diluted with 0.85% sodium chloride (w/v) and homogenized for 30 min. The sap samples extracted under positive pressure were diluted with saline solution prior to inoculation (Figure 1). All samples were pre-selected by incubation at 25°-28°C for up to 10 days on the National Botanical Research Institute phosphate (NBRIP) solid medium (Nautiyal, 1999).

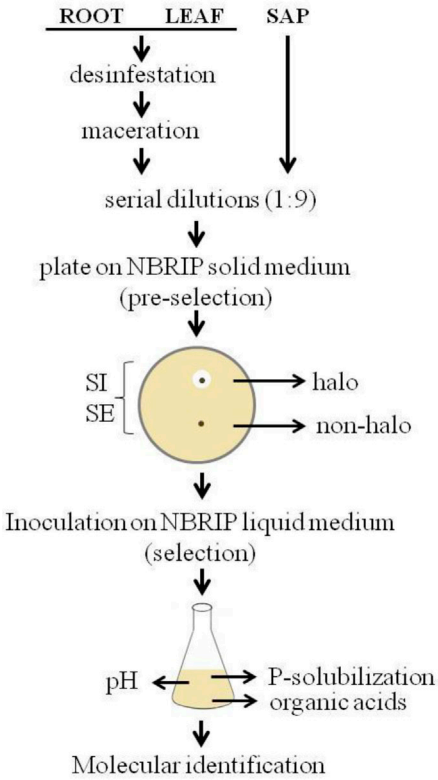

Figure 1. Schematic representation of the protocol used for selecting maize PSB using the NBRIP medium.

Determining the phosphate-solubilizing efficiency of endophytic bacteria on NBRIP solid medium

The selected isolates were tested for their ability to solubilize phosphate on NBRIP agar containing 40 g/L dextrose and TCP as the sole source of phosphate. The phosphate solubilization index (SI) was estimated by the following equation: SI (mm) = (halo zone + colony diameter) / colony diameter. The isolates were classified as low (SI < 2), middle (2 < SI ≤ 4), and high solubilizer (SI > 4) according to Berraquero et al. (1976).

Determining the phosphate-solubilizing efficiency of endophytic bacteria in NBRIP liquid medium

Aliquots of 0.1 mL of each isolate cultured in trypticase soy broth were incubated and subsequently transferred to the NBRIP medium containing 40 g/L glucose and incubated with stirring at a temperature of 30°C. The optimum times of incubation for maximum phosphate solubilization were six and nine days as revealed by previous tests (data not shown). The pH values were measured in water and the phosphorus content released in the culture supernatants was determined by the ammonium molybdate colorimetric method.

Quantification of organic acid production by maize endophytic bacteria

Organic acids were quantitated by high-performance liquid chromatography; a 30 cm x 7.8 mm Supelcogel C-610H column (Sigma-Aldrich, St. Louis, IL, USA) was used in the Shimadzu Prominence Model LC-20A (Shimadzu, Japan) apparatus. The separation of acids occurred at 65°C with 5 mM H_2SO_4 as the mobile phase, at a flow rate of 0.6 mL/min. The Shimadzu RID-10A differential refractive index detector with the cell temperature at 45°C was used to measure the concentration of individual organic acids in the samples. The peak wavelength separation area generated by refractive index was calculated by using the standard curve for each acid.

Molecular identification of maize endophytic bacteria

Identification of the 55 selected PSB isolates by partial sequencing of the 16S rRNA-encoding gene was performed with the bacterial universal primers F968 (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and R1401 (5'-CGG TGT GTA CAA GAC CC-3'). The PCR conditions used were according to that described in Figueiredo et al. (2009). The amplicons were purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer instructions (Applied Biosystems, Foster City, CA, USA), and the reactions were run on an Applied Biosystems automatic sequencer ABI-3100 (Applied Biosystems). Sequence editing and contig sequences were generated with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and similarity among sequences was evaluated by the BLASTn program, which was run against all available DNA sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov). Nucleotide sequences of bacteria were deposited in GenBank.

Statistical analysis

The data were analyzed according to a randomized design with three replicates per sample, except for phosphate solubilization in solid medium, where four replicates were used per sample. The analysis of variance for each test was made using the Sisvar 5.3 program (Ferreira, 2010). Significant differences were analyzed by the F test ($P \leq 0.05$), and the means were compared using the Scott-Knott test at 5% probability. To verify the association between P-solubilization and pH, the Pearson correlation analysis was performed using the R software (http://www.R-project.org) at 5% probability.

RESULTS

The maize endophytic PSB used in this study were pre-selected on NBRIP medium containing TCP. To assess the potential of PSB as bioinoculants, we evaluated the SI and phosphate-solubilizing efficiency (SE) on solid medium, and quantified the soluble phosphate and organic acids in liquid medium. The molecular identity of the isolates was determined by partial sequencing of the 16S rRNA-encoding genes.

Selection of maize endophytic PSB on solid NBRIP medium

One hundred and fifteen cultivable endophytic bacteria isolated from roots (64), leaves (23), and sap (28) of maize were tested for their phosphate solubilization efficiency based on either growth or presence of a clear halo zone surrounding the colonies after 10 days of incubation on NBRIP solid medium (Table 1 and Figure 2). Seventy-nine bacterial isolates that grew on solid medium were not able to produce the halo that is typically observed after phosphate solubilization. Nineteen of the 79 non-halo-forming bacteria showed phosphate-solubilizing activity in NBRIP liquid medium (Table 1). Although the majority of the isolates were obtained from the roots (64), the number of PSBs from the three sources were similar (15, 19, and 21, respectively, from roots, leaves, and sap). The re-inoculation of the non-halo forming isolates confirmed their inability to form halo in the solid medium (Table 1). Although the majority of the bacteria were pre-isolated from the roots (64), the number of PSB in liquid medium was similar among the three sources (15, 19, and 19, respectively, from roots, leaves, and sap).

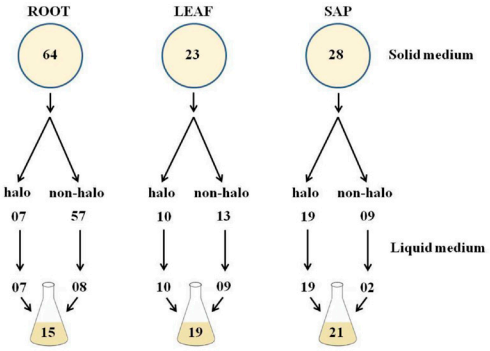

Figure 2. Schematic representation of PSB isolated from different tissues of maize showing the number of halo and non-halo forming isolates and the number of PSB in NBRIP solid and liquid medium, respectively.

The SI varied significantly ($P \leq 0.05$) among the isolates (Table 1). Forty-six isolates had poor SI (0-1.94), whereas only nine had moderate SI (2.0-3.71). Certain isolates that showed relatively low SI on solid medium exhibited higher phosphate-solubilizing activity in the liquid medium, which was similar to the SI values of halo-forming bacteria.

Table 1. Phosphate solubilizing efficiency of maize endophytic bacteria from different sources (sap, leaf, and root) cultivated on solid and liquid media supplemented with tricalcium phosphate (TCP).

Isolate	Source of inoculum	NBRIP solid medium		NBRIP liquid medium		
		SI (mm)	SE	P (mg/L)	pH (H_2O)	Gluconic acid (mM)
Control	-	-	-	36.20 i	6.51 a	-
1915	Sap	1.30 f	low	114.19 f	5.25 c	26.23 g
1916	Sap	1.76 e	low	174.52 b	3.97 i	16.39 g
1917	Sap	1.69 e	low	150.47 d	4.41 f	18.49 g
1918	Sap	1.39 f	low	111.35 g	4.68 e	20.58 g
1919	Sap	3.71 a	middle	172.08 b	4.09 h	18.23 g
1921	Sap	1.64 e	low	174.47 b	4.21 g	20.43 g
1922	Sap	2.05 d	middle	176.30 b	3.97 i	16.33 g
1923	Sap	1.33 f	low	111.93 g	5.50 b	202.77 c
1924	Sap	1.94 d	low	166.27 c	3.99 i	20.42 g
1925	Sap	3.61 a	middle	132.88 e	4.73 e	194.59 c
1928	Sap	1.50 f	low	169.23 b	4.03 i	20.17 g
1929	Sap	2.88 b	middle	165.01 c	3.99 i	20.63 g
1930	Sap	2.06 d	middle	129.06 e	4.26 g	18.32 g
1931	Leaf	1.42 f	low	134.21 e	4.23 g	30.65 g
1932	Leaf	1.58 e	low	138.29 e	4.32 g	44.67 g
1934	Leaf	1.55 e	low	80.32 h	4.23 g	188.31 c
1935	Leaf	1.90 d	low	137.54 e	4.28 g	94.68 f
1936	Leaf	1.94 d	low	136.88 e	4.30 g	142.50 d
1937	Leaf	-	-	135.90 e	4.24 g	42.58 g
1939	Leaf	1.90 d	low	135.92 e	4.28 g	72.51 f
1944	Root	-	-	129.71 e	4.77 e	143.96 d
1961	Root	2.00 d	middle	124.33 f	4.54 f	20.43 g
1962	Root	2.42 c	middle	141.18 e	4.38 g	22.78 g
1964	Root	-	-	117.14 f	4.82 e	167.17 d
1974	Root	1.00 g	low	140.55 e	5.10 c	127.40 e
1976	Root	1.62 e	low	169.50 b	4.14 h	27.16 g
1979	Root	-	-	159.09 c	5.14 c	148.18 d
1982	Root	1.00 g	low	167.85 b	4.61 f	178.47 c
1984	Root	-	-	119.77 f	4.71 e	146.53 d
2006	Sap	-	-	158.18 c	5.67 b	190.10 c
2008	Sap	-	-	160.38 c	4.69 e	157.99 d
2009	Sap	1.71 e	low	164.23 c	5.12 c	183.71 c
2010	Sap	1.49 f	low	175.38 b	4.37 g	165.60 d
2011	Sap	2.35 c	middle	181.32 a	4.13 h	0
2012	Sap	1.69 e	low	167.89 b	4.16 h	155.47 d
2013	Sap	1.69 e	low	155.11 d	4.51 f	135.72 e
2014	Sap	1.67 e	low	153.38 d	4.53 f	264.17 b
2079	Leaf	-	-	185.55 a	3.90 i	177.38 c
2080	Leaf	-	-	174.53 b	4.26 g	109.45 e
2081	Leaf	-	-	148.47 d	4.42 f	161.17 d
2082	Leaf	-	-	167.97 b	4.94 d	183.75 c
2083	Leaf	-	-	154.11 d	5.34 c	147.01 d
2084	Leaf	1.13 g	low	120.42 f	4.80 e	324.08 a
2085	Leaf	-	-	149.75 d	4.96 d	97.81 f
2086	Root	-	-	104.07 g	5.17 c	127.40 e
2088	Root	-	-	179.39 a	4.73 e	171.46 c
2096	Leaf	-	-	149.01 d	4.98 d	119.50 e
2099	Root	1.64 e	low	139.57 e	4.25 g	85.76 f
2100	Leaf	1.80 e	low	158.63 c	4.03 i	156.21 d
2103	Leaf	1.21 f	low	134.14 e	4.41 f	30.47 g
2105	Leaf	2.06 d	middle	155.19 d	4.31 g	195.12 c
2106	Root	1.24 f	low	191.46 a	4.19 h	136.70 e
2108	Root	-	-	118.24 f	5.00 d	61.80 f
2110	Leaf	-	-	181.98 a	3.90 i	179.49 c
2111	Root	-	-	176.33 b	4.83 e	212.80 c

Mean values followed by the same alphabet do not differ by the Scott-Knott test at 5% probability. Control = sterile liquid medium, P = phosphate. The phosphate solubilizing index (SI) and the solubilizing efficiency (SE) were measured after 10 days of incubation on solid medium; gluconic acid production was measured after 6 days; pH and phosphate solubilization in liquid medium (milligrams of phosphate per liter) were estimated after 9 days of incubation. Data are reported as means of three replicates.

Phosphate solubilization by maize endophytic PSB in liquid NBRIP medium shows a negative correlation with the pH of the medium

The values of phosphate solubilization for each isolate are expressed as the sum of the absolute values measured on the sixth and ninth days of incubation. There was a statistically significant difference ($P \leq 0.05$) for both, the values of phosphate-solubilizing activity and pH of the growth medium among the isolates (Table 1, Figure 1). The highest and lowest values of phosphorus solubilization were observed, respectively, for isolates 2106 (*B. megaterium*) and 1934 (*Pantoea ananatis*).

Pearson correlation was statistically significant ($P \leq 0.05$) between P-solubilization and pH ($R = -0.38$), and between the gluconic acid production and the lowering of the pH of the liquid medium at 6 ($R = 0.28$) and 9 days ($R = 0.39$). A comparison of the phosphate-solubilizing efficiencies of the isolates in solid and liquid medium, respectively, showed that the majority of bacteria exhibited low phosphate-solubilizing activity in both media (Table 1). The solubility values in the liquid medium, which indicates the relative efficiency of phosphate solubilization of the 55 isolates, are shown in the Figure 3.

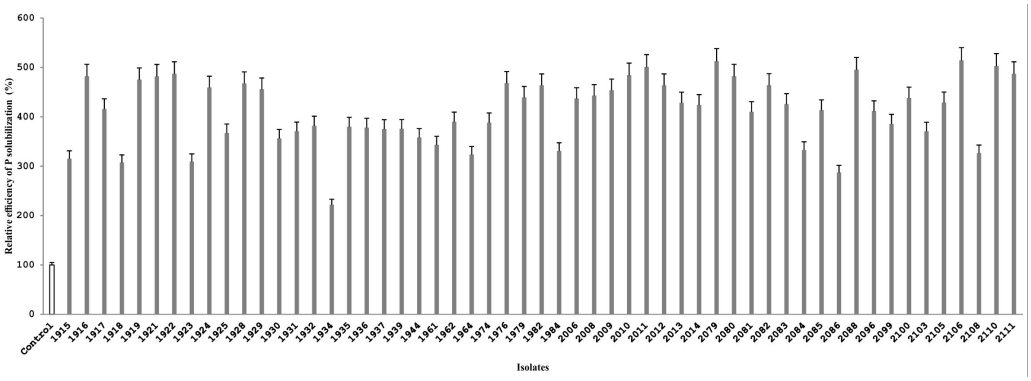

Figure 3. Relative phosphate-solubilizing efficiency of the maize endophytic bacterial isolates cultured in liquid medium with TCP as the sole source of phosphorus. Data (vertical bars) are reported as means ± standard error of three replicates. The empty bar indicates the negative control (culture medium without bacterial inoculum).

Quantification of organic acids revealed that gluconic acid was the predominant acid secreted by PSB

The nature and amount of organic acids secreted by each bacterial isolate varied significantly ($P \leq 0.05$) (Figure 4). The concentration of gluconic acid produced varied between 16.33 and 324.08 mM (Table 1). While 5-ketogluconic acid was not detected in any

of the isolates, gluconic acid was detected in most of the isolates, and acetic acid was detected in only one isolate (Figure 4).

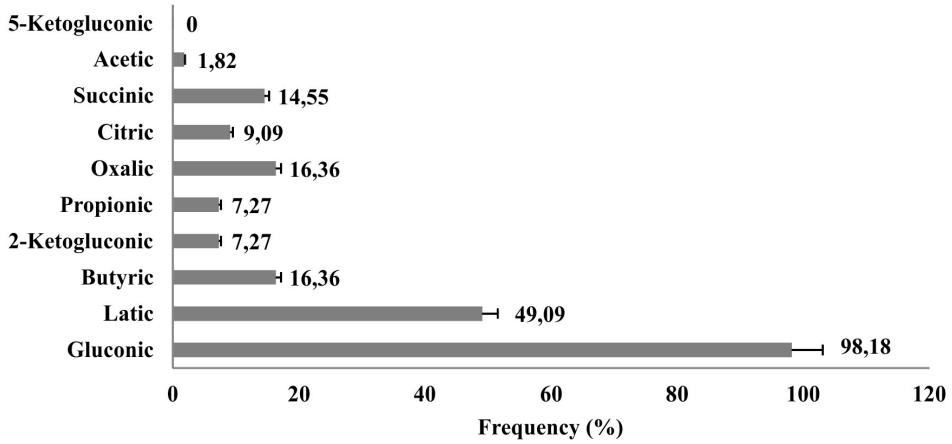

Figure 4. Relative amounts of organic acid produced by the maize endophytic bacterial isolates were determined by high-performance liquid chromatography with refractive index detection. Data (horizontal bars) are reported as means ± standard error of three replicates.

The correlation between the association of phosphate-solubilization and the type of the acid produced was statistically significant ($P \leq 0.05$) only for oxalic acid at 9 days (Table 2).

Table 2. Pearson correlation coefficient among amounts of soluble phosphate, acidity of medium, and amount of organic acid produced.

	P-solubilization and pH				Organic acid production									
	6 days		9 days		Acetic	Butyric	Citric	Gluconic	2-ketogluconic	Lactic	Oxalic	Propionic	Succinic	Total OA[1]
	P	pH	P	pH										
P (6 days)	1	-0.38*			nc	0.46^{ns}	0.41^{ns}	0.11^{ns}	-0.59^{ns}	-0.06^{ns}	0.44^{ns}	0.57^{ns}	0.21^{ns}	0.19^{ns}
pH (6 days)		1			nc	-0.02^{ns}	0.02^{ns}	0.28*	0.78^{ns}	-0.31^{ns}	-0.37^{ns}	0.49^{ns}	-0.43^{ns}	0.14^{ns}
P (9 days)			1	-0.38*	nc	0.19^{ns}	0.13^{ns}	0.07^{ns}	-0.19^{ns}	0.13^{ns}	0.76*	-0.42^{ns}	0.20^{ns}	0.12^{ns}
pH (9 days)				1	nc	-0.50^{ns}	-0.18^{ns}	0.39*	0.87^{ns}	-0.35^{ns}	-0.43^{ns}	0.13^{ns}	-0.22^{ns}	0.15^{ns}

*Significant at 5% probability; ^{ns}Not significant at 5% probability; Total OA[1]: Total organic acid produced by the maize endophytic PSB; nc: no correlation, P: Phosphate.

Molecular characterization of PSB revealed the presence of *Bacillus* and Enterobacteriaceae

Partial sequencing of the 16S rRNA-encoding gene revealed that all the endophytic PSB isolates belonged to either the genus *Bacillus* (29 = 54.5%) or to different species of Enterobacteriaceae (25 = 45.5%). Twenty-two of the isolates were *B. subtilis*, and eleven isolates that belonged to Enterobacteriaceae were *P. ananatis*. The other bacterial species were *Bacillus amyloliquefaciens*, *Bacillus methylotrophicus*, *B. megaterium*, *B. subtilis*, *Bacillus pumilus*, and *Enterobacter asburiae* (Table 3).

Table 3. Molecular identification using partial sequencing of the 16S rRNA-encoding gene of the PSB isolated from maize.

Isolate	GenBank accession No.	Species identity (99-100%)
1915	KU165802	*Bacillus pumilus*
1916	KU189305	*Klebsiella pneumoniae*
1917	KU165806	*Enterobacter ludwigii*
1918	KU172428	*Bacillus subtilis*
1919	KU172427	*Bacillus subtilis*
1921	KU165807	*Enterobacter ludwigii*
1922	KU189301	*Pantoea ananatis*
1923	KU165803	*Bacillus pumilus*
1924	KU189306	*Klebsiella pneumoniae*
1925	KU189302	*Pantoea ananatis*
1928	KU189308	*Burkholderia gladioli*
1929	KU189309	*Klebsiella* sp
1930	KU189303	*Pantoea ananatis*
1931	KU172436	*Pantoea ananatis*
1932	KU172437	*Pantoea ananatis*
1934	KU172432	*Pantoea ananatis*
1935	KU165821	*Bacillus subtilis*
1936	KU172433	*Pantoea ananatis*
1937	KU172434	*Pantoea ananatis*
1939	KU165805	*Leclercia* sp
1944	KU189307	*Klebsiella pneumoniae*
1961	KU172429	*Pantoea dispersa*
1962	KU165810	*Bacillus subtilis*
1964	KU165811	*Bacillus subtilis*
1974	KU165812	*Bacillus subtilis*
1976	KU165820	*Bacillus subtilis*
1979	KU172438	*Pseudomonas* sp
1982	KU165813	*Bacillus subtilis*
1984	KU172430	*Pantoea dispersa*
2006	KU165818	*Bacillus* sp
2008	KU189304	*Pantoea ananatis*
2009	KU172426	*Bacillus subtilis*
2010	KU165808	*Enterobacter* sp
2011	KU165819	*Bacillus* sp
2012	KU172424	*Bacillus subtilis*
2013	KU172425	*Bacillus subtilis*
2014	KU165804	*Bacillus aerophilus*
2079	KU165822	*Bacillus subtilis*
2080	KU165823	*Bacillus subtilis*
2081	KU189310	*Bacillus thuringiensis*
2082	KU165824	*Bacillus subtilis*
2083	KU165825	*Bacillus subtilis*
2084	KU189311	*Bacillus subtilis*
2085	KU165826	*Bacillus subtilis*
2086	KU189312	*Bacillus amyloliquefaciens*
2088	KU165814	*Bacillus subtilis*
2096	KU189313	*Pseudomonas* sp
2099	KU165815	*Bacillus subtilis*
2100	KU189314	*Serratia* sp
2103	KU172435	*Pantoea ananatis*
2105	KU172431	*Pantoea dispersa*
2106	KU165809	*Bacillus megaterium*
2108	KU165816	*Bacillus subtilis*
2110	KU165827	*Bacillus subtilis*
2111	KU165817	*Bacillus subtilis*

DISCUSSION

In the present study, we demonstrated the *in vitro* phosphate-solubilizing activity of endophytic PSB from maize by dissolution of TCP. The selected PSB could potentially be used as bioinoculants to improve phosphorus uptake and maize yield.

The correlation between phosphate-solubilizing activity and low pH of the growth medium was expected since the ability of PSB to convert insoluble phosphorus to a soluble form is directly related to the release of organic acids in the medium (Omar, 1998; Narula et al., 2000; Whitelaw, 2000; Pérez et al., 2007). Similar results were also reported by Chen et al. (2006). The phosphate-solubilizing efficiency is also dependent on the medium used to select the PSB. However, the source of insoluble phosphate in the culture medium is a major issue of controversy regarding the isolation of true PSB.

Although the formation of a clear halo zone around colonies on NBRIP medium is a good preliminary criterion for selecting isolates exhibiting phosphate-solubilizing activity, it should not be considered as the sole test for determining phosphate solubilization. Certain strains that could not solubilize phosphate on the agar plate showed efficient phosphate solubilization in the liquid medium with the same phosphate sources (Nautiyal, 1999; Bashan et al., 2013; Sharma et al., 2013). In our study, among the sixty bacterial isolates that were unable to solubilize phosphate on solid medium, nineteen showed phosphate-solubilizing activity in the liquid medium.

According to Whitelaw (2000), the size of both the halo and the colony correlates with the efficiency of phosphate solubilization. In this study, some isolates that showed relatively low SI on solid medium exhibited phosphate-solubilizing activity in the liquid medium similar to those of the halo-forming bacteria. This result may be explained by the fact that the diffusion of the secreted organic acid depends on the nature of the acid produced and the culture medium (Marra et al., 2012).

According to Bashan et al. (2013), methods using TCP are relatively weak and unreliable for isolating and testing PSB as although it usually identifies many putative strains, further tests may reveal that they are false positives for phosphorus solubilization. Other compounds have also been tested for PSB identification *in vitro*, such as iron phosphate ($FePO_4$), aluminum phosphate ($AlPO_4$), and several calcium phosphates. However, they are even less soluble than TCP in water (Bashan et al., 2013). Therefore, the authors of the study concluded that no metal-phosphate compound should be used for selecting PSB, especially because soils greatly vary in pH and chemical composition. They suggested that the choice of the metal-phosphate substrate for identifying putative PSBs should depend on the type of the soil where the PSB would be used. However, Silva Filho and Vidor (2001) reported a low incidence of $AlPO_4$-solubilizing bacteria among isolates previously identified as efficient solubilizers of TCP. Thus, the phosphate-solubilizing activity is dependent on the medium used to select the PSB, and the choice of the phosphate source should depend on the purpose of the study. We used TCP-containing medium in our study as we intended to select rock phosphate-solubilizing endophytic bacteria that would be used in soil rich in calcium magnesium carbonate [$CaMg(CO_3)_2$] or soil fertilized with rock phosphate from the hydroxyapatite group [$Ca_5(PO_4)_3OH$]. The acidity of the tropical zone soil is corrected with the application of calcium carbonate to the soil surface. Thus, the phosphate group of the fertilizer, which is applied to the soil, also reacts with calcium forming $Ca_3(PO_4)_2$. In addition, since the solubilization of iron or aluminum-complexed phosphate is lower than that of calcium-

complexed phosphate, the use of rock phosphate-solubilizing bacteria is a good choice to increase the availability of phosphorus that has accumulated as $Ca_3(PO_4)_2$ in the soil (Hameeda et al., 2008). In addition, the constant use of synthetic fertilizers has adversely affected the environment, besides increasing the cost of production. The cheaper and direct use of rock phosphate solubilizers is an interesting alternative for poor farmers and it also supports eco-friendly farming and sustainable agriculture (Smalberger et al., 2010).

Regarding the production of organic acids, the type of acid that is produced by each bacterial species depends on complex parameters such as the nutritional and physiological status of the culture and the concentration and composition of nutrients in the medium (Chen et al., 2006; Pérez et al., 2007). Gluconic acid and keto-gluconic acid are the two most common organic acids produced by PSB (Goldstein, 1986). In other studies, gluconic, 2-ketogluconic, oxalic, succinic, citric, and lactic acids were among the most frequent metabolites produced by PSB (Rodríguez and Fraga 1999; Khan et al., 2010; Behera et al., 2016). Various authors found that the acids most frequently produced by PSB are the gluconic acid, 2-ketogluconic, oxalic, succinic, citric, and lactic (Behera et al., 2016). We found significant correlation between organic acid production and reduction of the pH of the medium only for gluconic acid.

In this study, all endophytic PSB isolates were from the genus *Bacillus* and different species of Enterobacteriaceae. These isolates belong to bacterial genera previously demonstrated to have the ability of solubilizing different sources of mineral phosphates (Murugan and Ramesh, 2013). Comparison of data presented in Tables 1 and 3 shows that there is no relationship between the source of the isolates (leaves, sap or roots) or the taxonomic group, and the efficiency of phosphate solubilization. We have previously shown that PSB species from these bacterial groups were also isolated from the soil of maize rhizosphere (Oliveira et al., 2009; Gomes et al., 2014). This indicates that these PSB may be capable of developing a connection between the plant tissue and the rhizosphere soil as mentioned by Farrar et al. (2014). All isolates of the present study belong to bacterial groups that have been previously demonstrated to solubilize different sources of mineral phosphates (Murugan and Ramesh, 2013). Kim et al. (1998) showed that *Enterobacter* solubilizes hydroxyapatite and organic phosphate, and Mohammadi (2012) found that mixed cultures of *Bacillus*, *Streptomyces*, and *Pseudomonas* are more effective in mineralizing organic phosphate. Different species of *Bacillus* and *Pantoea* have been extensively reported as efficient agents for solubilizing insoluble inorganic phosphate from different sources, including $Ca_3(PO_4)_2$, $[Ca_5(PO_4)_3OH]$, and rock phosphate (Pérez et. al., 2007; Oliveira et al., 2009; Kumar et al., 2014; da Silva et al., 2015). In fact, numerous *Bacillus*-based and *Pantoea* sp inoculants that produce phytase or organic acids have already been developed and patented (Kim et al., 1998; Mohammadi, 2012; Kumar et al., 2014).

Two isolates from the sap (1919 = *B. subtilis* and 1925 = *P. ananatis*) showed the highest SI (3.71 and 3.61 mm, respectively) in NBRIP solid medium (Tables 1 and 3). However, none of the isolates was efficient in solubilizing phosphate in NBRIP liquid medium. Four *Bacillus* isolates were very efficient in solubilizing phosphate in the liquid medium (>180.00 mg/L). Interestingly, three of these isolates were previously identified to be non-phosphate-solubilizing candidates on the solid medium (Table 1).

In conclusion, endophytic PSB isolated from maize grown in the Brazilian Cerrado soil exhibited different levels of phosphate-solubilizing activity and gluconic acid production. The gluconic acid production was prevalent in all the PSB studied, and species of the genus *Bacillus* were the most efficient phosphate solubilizers. *Bacillus* species are easily isolated

from maize, suggesting an evolutionary relationship between these organisms (Figueiredo et al., 2009). This indicates that *Bacillus* species may be used for selecting the most adapted and efficient phosphate solubilizers from maize. This is the first report on phosphate solubilizer endophytic bacteria from tropical maize as potential biofertilizers.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by grants from Universidade Federal de São João Del Rei (UFSJ) to Crísia Santos de Abreu, Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Embrapa Milho e Sorgo (CNPMS), and Universidade Federal de Minas Gerais (UFMG).

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