



## Potential inoculant strains of Brazilian endophytic bacteria for maize (*Zea mays* L.) growth promotion

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### Abstract

The maize (*Zea mays* L.) is one of the most important cereals as a source of energy for human nutrition and animal feed. And the cultivation of maize to be improved with the use of lower-impact resources for the environment and more profitable for farmers. Therefore the use of endophytic bacteria promoting plant growth comes against these ideals. Seven bacteria isolated from maize roots were identified and verified for their ability to promote plant growth under greenhouse conditions. In addition, mechanisms of plant growth promotion were investigated *in vitro*. Sequencing analysis of the 16S rRNA gene revealed five bacterial genera: *Achromobacter*, *Enterobacter*, *Pseudomonas*, *Stenotrophomonas* and *Bacillus*. Among the isolates, *Bacillus* sp. (LGMB227) promoted root length (65.1%), stimulated the aerial part development (39.4%) and *Pseudomonas* sp. (LGMB205) increased the root volume (22.7%). Here we demonstrate that *in vivo* data were corroborated by *in vitro* data, where the largest producer of IAA (63.1 mg/mL) was detected in *Bacillus* sp. LGMB227 that showed as well siderophore and pectinase activity. *Pseudomonas* sp. had the second largest production of IAA (56.1 mg/mL) and was also siderophore positive. Identified that strain LGMB227 of *Bacillus* sp. showed the best performance, with potential use as inoculants for plant growth promotion.

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## Introduction

Maize (*Zea mays* L.) occupies an outstanding position in agriculture, being one of the most important cereals as source of energy for human nutrition and animal feed, besides being one of the main crops for grain production worldwide (FAO, 2009). The world production of maize in 2014/15 was estimated at 988.07 million tons, remaining above the consumption that increased from 944.91 to 968.90 million tons; in this scenario Brazil contributes with 78.4 million tons (CONAB, 2015). Several studies highlight the use of alternative nutrient sources for plants and/or alternative phytopathogens control methods to increase production, and many of them focusing on endophytic microorganisms (Barretti *et al.*, 2008), which are found inside plants without causing diseases (Azevedo, 1998).

To leave inside the tissues gives to these microorganisms the advantage of having higher protection from environmental limitations, increasing the possibilities of a successful response to inoculants (Sharma and Novak, 1998). Endophytic bacteria, presenting potential as growth promoters in maize plants, may contribute to development of technologies aimed to sustainable production. Thus, this study aimed to identify the bacteria isolates and verify their ability to promote corn plant growth *in vivo*, in greenhouse conditions, and to investigate *in vitro* the action mechanisms of bacteria related to growth promotion.

## Material and methods

The study comprised seven endophytic bacterial isolates from maizeroots previously obtained by Ikeda *et al.* (2013), root samples were surface-sterilized according to Glienke-Blanco *et al.* (2002), in which roots were washed with running water and then treated with 70% ethanol (v/v) for 1 min, NaClO 3% (v/v) for 4 min, 70% ethanol (v/v) for 30 s, and rinsed three consecutive times in sterile water. Then aseptically cut, and transferred to Petri dish plates containing solid culture media. Several controls confirmed that the sterilization procedure was effective. The isolates are deposited at the collection

of endophytic bacteria belonging to the Laboratory of Genetic of Microorganisms (LabGeM).

### *Bacterium identification by analyzing the 16S rRNA gene*

The DNA extraction and 16S rRNA sequencing was performed according to Szilagyi-Zecchin *et al.* (2014). Quality verification of bases and assembling of fragments were performed with the Phred Phrap Consed program (Ewing, 1998). Identity of 16SrRNA partial sequences was obtained by comparison with sequences available in the GenBank database, available at NCBI (<https://www.ncbi.nlm.nih.gov/genbank>) using the BLAST<sup>®</sup>N 2.2.29 program (Basic Local Alignment Search Tool, Nucleotide). The 16S rRNA gene sequences were deposited to NCBI GenBank with access numbers from KP276221 to KP276227.

### *Assay in greenhouse conditions*

The experiment was conducted under greenhouse conditions at the Center of Experimental Stands of Canguiri belonging to Federal University of Paraná. Seeds of the commercial maize hybrid SX2530 were supplied by the company Empresa Semília Genética e Melhoramento Ltda. Surface disinfestation of seeds was obtained according to the methodology of Glienke-Blanco (2002). Bacteria were cultivated in King B liquid medium at 30 °C for 24 h (150 rpm), with adjusted concentration of 10<sup>8</sup> cells/mL (Hernández-Rodríguez *et al.*, 2008). One-mL of the resulting suspension was used to inoculate 100 maize seeds. Two plants were maintained per pot with 3 kg of capacity filled with vermiculite. Irrigation was performed using a temporized micro-sprinkler system. Hoagland nutrient solution modified by Gondim *et al.*, (2010) was applied three times a week.

The experiment was conducted with a completely randomized design, where each treatment consisted of two vases, and four replicates. Treatments included seven endophytic isolates, besides the control which was not inoculated. Assessments were performed in two steps. Half of the plants were harvested 15 days after emergence (DAE) and the others at 30 DAE.

Assessed morphometric variables were done according to Szilagyi-Zecchin *et al.* (2015) and the chlorophyll content was measured with a portable chlorophyll detector, as described in Mógoret *al.* (2013). Data were tested for normality using the Kolmogorov-Smirnov test and for homogeneity of their variances by the Bartlett test. Then they were subjected to analysis of variance, and when there was a significant difference, the averages were compared by Duncan test at 5% significance in Assistat® 7.6 Beta program (Silva and Azevedo, 2002).

#### *Enzymatic and physiological characterization of bacteria*

Quantification of bacteria auxin was made with Salkowsky reagent according to Kuss *et al.* (2007). A standard curve was plotted with values from 5 to 80 µg/mL of commercial auxin. To assess the production of siderophores, the universal methodology of Schwyn and Neilands (1987) was applied, using the DYGS culture medium (Rodrigues Neto *et al.*, 1986). To detect lithic enzymes, bacteria were seeded by points in a mineral medium containing carboxymethyl

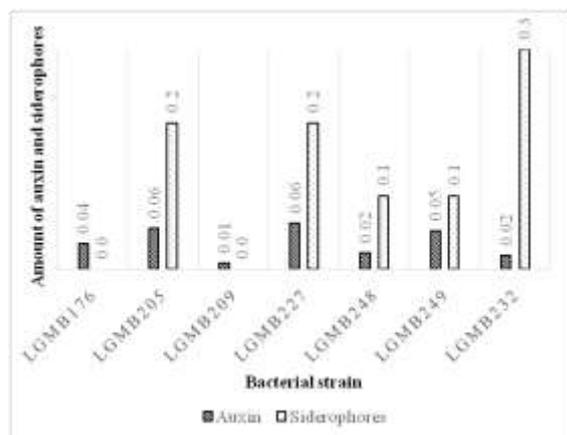
cellulose at 0.5% as only carbon source, to identify cellulases. The methodology described by Hankin and Anagnostakis(1975) was used to identify pectinase, in a M9 Minimal Medium with addition of 0.5% of yeast extract and 0.5% of pectin (v/v).

#### **Results**

After sequence comparison of the gene 16S rRNA we identified five genera of endophytic bacteria: *Achromobacter* (LGMB176); *Enterobacter* (LGMB232 and LGMB248), *Pseudomonas* (LGMB205 and LGMB249), *Stenotrophomonas* (LGMB209), and *Bacillus* (LGMB227) (Table 1). Fifteen days after emergency (DAE), there were no significant differences between the treatments for the assessed variables (data not shown). After 30 DAE, differences could be seen compared to control. *Pseudomonas* sp. (LGMB 205) increased the volume of roots which was 22.74% bigger than in not inoculated plants (Table 2). LGMB 205 was positive to siderophore, and was the second in production of auxin (Fig. 1).

**Table 1.** Molecular identification of the isolates, using partial sequencing of gene 16S rRNA by comparison of sequences of GenBank database.

Isolate	Identity %	Identity (pb)	Access nº	Bacteria species	Identification of the isolated
LGMB176	97	779/799	HF586506.1	<i>Achromobacter insuavis</i>	<i>Achromobacter</i> sp.
	97	779/799	HQ676601.1	<i>Achromobacter xylooxidans</i>	
LGMB205	99	1039/1051	JN210910.1	<i>Pseudomonas fluorescens</i>	<i>Pseudomonas</i> sp.
	99	1038/1051	KC195894.1	<i>Pseudomonas poae</i>	
LGMB209	99	1429/1450	HQ641452.1	<i>Stenotrophomonas pavanii</i>	<i>Stenotrophomonas</i> sp.
	98	1447/1470	JN208898.1	<i>Stenotrophomonas maltophilia</i>	
LGMB227	98	957/978	CP007244.1	<i>Bacillus amyloliquefaciens</i>	<i>Bacillus</i> sp.
	98	955/978	GU826165.1	<i>Bacillus subtilis</i>	
LGMB232	98	1434/1466	JF346886.1	<i>Enterobacter cloacae</i>	<i>Enterobacter</i> sp.
	98	1439/1473	NR042349.1	<i>Enterobacter ludwigii</i>	
LGMB248	99	611/620	KF598982.1	<i>Enterobacter aerogenes</i>	<i>Enterobacter</i> sp.
	98	610/620	KF598981.1	<i>Enterobacter cloacae</i>	
LGMB249	95	710/746	HF545842.1	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> sp.
	95	710/746	HE610888.1	<i>Pseudomonas plecoglossicida</i>	



**Fig. 1.** Biochemical tests on the bacteria strains: production of auxin, and siderophores.

In plants inoculated with *Bacillus* sp. (LGMB227), root length was 65.1% larger than the control, reflecting in a 26.9% increase on root dry biomass. Leaf area in this same treatment was also greater, with increments of 39.4% (Table 2). This strain produced the highest amount of auxin detected and the second one in the production of siderophores (Fig. 1). It is worth pointing out that LGMB227 (*Bacillus* sp.) was the only one showing pectinase activity.

**Table 2.** Results obtained from the application of the test of Duncan for the comparison of means of volume (cm<sup>3</sup>) and length of roots (cm), dry mass of roots (g), foliar area (cm<sup>2</sup>), dry mass of the aerial part (g) and chlorophyll content, for the tested treatments. Means followed by the same letter in the column are not statistically different by the test of Duncan at 5% of probability.

Strains	Roots			Aerial part	
	Volume (cm <sup>3</sup> )	Length (cm)	Dry mass (g)	Foliar area (cm <sup>2</sup> )	Chlorophyll content
-	17.23B	1333.20B	1.00AB	271.86BC	51.46B
LGMB176	14.51BC	1105.96B	0.91BC	183.65C	52.96AB
LGMB205	21.15A	1333.42B	1.14AB	315.36AB	50.92B
LGMB209	16.21BC	1021.08B	0.92BC	257.50BC	53.45AB
LGMB227	17.18B	2200.97A	1.27A	379.01A	52.60B
LGMB248	11.15DE	1295.31B	0.71C	219.51BC	44.10C
LGMB249	13.17CD	1354.38B	0.91BC	226.73BC	56.56A
LGMB232	7.826E	1184.33 B	0.94BC	205.94BC	51.53B

**Discussion**

These genera, *Achromobacter*, *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Stenotrophomonas* are common to be found endophytically in maize roots (Pereira *et al.*, 2011; Johnston-Monje *et al.*, 2014; Silva *et al.*, 2014). Some studies have reported the benefits to the maize root system by *Pseudomonas* producing of auxin and siderophores among other substance, like *Pseudomonas* sp. (LGMB205): *P. putida* stimulated the increase of roots (Roca, 2013) and *P. fluorescens* promoted growth of radicles (Montañez *et al.*, 2012).

Roots and aerial part can be stimulated jointly, like done by LGMB227, and Vardharajula *et al.* (2011), determined the same effect in maize plants applying

different bacteria *Bacillus* spp. in hydric stress conditions. But more frequently, is possible to find *Bacillus* stimulating shoots or roots: 39% of increases in roots volume of maize (Szilagyi-Zecchin *et al.* 2014); increments of 47.8% on soybean roots (Araújo *et al.*, 2005); more leaves per plant and biomass production of maize plants (Araújo & Guerreiro, 2010); promotion of shoot growth in two seedlings tomato cultivars with added of 47.7% and 15.5% (Szilagyi-Zecchin *et al.*, 2015).

Those increases in vegetative development of plants may be in relation with cell elongation mechanism promoted by auxins, through which they stimulate synthesis or inhibit the action of the enzymes that act on microfibrils of cell walls, weakening non covalent

bonds between polysaccharides of the wall. This results in increase of plasticity and greater cell elongations, which reflects in a greater extension of roots (Taiz and Zeiger, 2004). Auxins also stimulate growth of secondary roots, thus increasing specific area of water and nutrient absorption by the plant (Radwan *et al.*, 2004).

Bacteria frequently have more than one property to promote growth of plants, besides auxins, the most common are production of siderophores. Siderophores are important compounds for provision of nutrients, because they are often unavailable to the plant due to their low mobility, thus, organic chelates produced by bacteria may increase availability of nutrients in the region near to the roots (Ahmad *et al.*, 2008).

The strain LGMB 277, which produced pectinase beyond auxins and siderophores, could have an advantage over the rest of the bacteria, because pectinase may have contributed to a better observed performance. Efficiency of bacteria in penetrating the host may be advantageous to their establishment and plant growth promotion. Colonization by endophytic bacteria occurs mainly by wounds created in the emergency of lateral secondary roots (Azevedo, 1998). However, penetration can occur even in the absence of wounds. *Enterobacter asburiae* enters in the cotton plants (*Gossypium hirsutum* L.) through active penetration, by enzymatic degradation of the plant cell walls, promoted by cellulases and pectinases (Hallmann *et al.*, 1997). *Klebsiella oxytoca*, capable to producing pectate lyase, when inoculated in wheat (*Triticum aestivum* L.) seeds was able to colonize the internal region of the roots, suggesting that penetration occurs through lysis of pectin layers (Kovtunovych *et al.*, 1999).

Strains with best positive results *in vitro* confirm the effects observed *in vivo*. *Bacillus* sp. (LGMB 227) and *Pseudomonas* sp. (LGMB 205) had good performance in this study increasing length and volume of roots, respectively. But only LGMB 227 produced pectinase and also stimulated the aerial part. So, LGMB227

presented the best performance between the tested strains, showing potential for use in future studies aimed at developing technologies to promote the growth of plants focusing on a more sustainable agriculture.

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