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Bacillus megaterium: an endophytic bacteria from callus of *Ilex paraguariensis* with growth promotion activities

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ABSTRACT
Endophytic microorganisms live inside healthy plants and their isolation and identification may favor biotechnological applications. We isolated endophytic bacteria from *Ilex paraguariensis* callus cultivated *in vitro* for five months. Sequencing of 16S rRNA region to identify two isolates as *Bacillus megaterium* was used. The presence of bacterial cells in the intercellular spaces of callus cultures was detected by ultra structural analyses. The isolates were also evaluated for indole acetic acid (IAA) production and their potential in plant growth promotion of *Phaseolus vulgaris* L. The synthesis of IAA in the presence and absence of L-tryptophan was detected by colorimetric analyze for both isolates. In the presence of extracts from strains *IPC06* and *IPC*07 the seedlings of *P. vulgaris* grew faster when compared to the control plants without the extract on *in vitro* tests.

Keywords: yerba mate, 16S rRNA, sequencing, tissue culture

Bacillus megaterium: bacteria endofítica de callos de *Ilex paraguariensis* con actividad de promoción del crecimiento

RESUMEN
Los microorganismos endófitos viven dentro de plantas sanas y su aislamiento e identificación pueden favorecer las aplicaciones biotecnológicas. Los microorganismos endofíticos fueron encontrados en callos de *Ilex paraguariensis* cultivados *in vitro* durante cinco meses. Se usó la secuenciación de la región 16S rRNA para identificar dos aislados como *Bacillus megaterium*. La presencia de células bacterianas en los espacios intercelulares de los cultivos de callos se detectó mediante análisis ultraestructurales. Los aislamientos también se evaluaron para la producción de ácido indol acético (AIA) y su potencial en la promoción del crecimiento de las plántulas de *Phaseolus vulgaris* L. La síntesis de ácido AIA en presencia y ausencia de L-triptófano se detectó por análisis colorimétrico para ambos aislamientos. En presencia de extractos de las cepas IPC06 e IPC07, las plántulas de *P. vulgaris* crecieron más rápido en comparación con las plantas control sin el extracto en pruebas *in vitro*.

Palabras clave: yerba mate, 16S rRNA, secuenciación, cultivo de tejidos

INTRODUCTION

Ilex paraguariensis St. Hil (*Aquifoliaceae*), known as yerba mate, is a tree from the subtropical region of South America, present in southern Brazil, Paraguay, Uruguay and northern Argentina (Markowicz *et al*., 2007). It is used for preparing beverages (Bracesco *et al.,* 2011), medicines and cosmetics, due to its stimulant and antioxidants properties, respectively.

Cloning by tissue culture techniques, yerba mate represents an important tool for genetic

breeding of this species. Nevertheless, endophytic bacteria represents a problem, because after some subcultures under *in vitro* culture it kill the explants (Zaniolo and Zanette, 2002; Dutra *et al*., 2008; Luna *et al*., 2013. A recent study based on *in vitro* plants of this specie reveled a rich biota living inside the explants (Luna *et al.,* 2013; Pérez *et al.,* 2016).

Endophytic microorganism, existing in the inner tissues of living plants, have attracted increasing attention among taxonomists, ecologists, chemists and agronomists, mainly for affecting plant growth directly or indirectly. It is common sense, but these microorganisms represent an underexplored reservoir of novel species with potential interest for exploitation in pharmaceutical, agriculture and industry (Hallmann *et al.*, 1997; Qin *et al.*, 2011). In recent years, the plant-bacterial associations have received more attention due to their potential for biotechnological applications (Jin *et al.,* 2014).

For this reason, the aims of this work was to isolate and identify endophytic bacteria from yerba mate *in vitro* callus and to evaluate their potential to produce auxin and promote plant growth.

MATERIALS AND METHODS

Plant material and callus induction

Plants of clone 6-156-6 from *Ilex paraguariensis* grown in the greenhouse, in EMBRAPA Forestry were used. The 2º/ 3º par of leaves were collected in antioxidant solution (0.5% ascorbic acid and 0.5% citric acid). The leaves were washed with neutral detergent in running tap water and disinfested as follow: 10 min in 1% (v/v) Cercobin®, 5 min in 0.05% (v/v) Hg_2Cl_2 and finally the leaves were rinsed three times in sterile distilled water. Afterwards, leaf discs with 2 cm diameter were cut and placed on Petri dishes containing 20 ml autoclaved (1.45 kg $cm^{»2}$ for 20 min) medium.

The medium used for callus induction consisted of ¼ MS (Murashige and Skoog, 1962) salts and vitamins, 3% sucrose, 100 mg l^{-1} myo-inositol, 4.52 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.52 µM zeatin with the pH adjusted to 5.8 prior to the addition of 0.7% agar. The Petri dishes containing the explants were incubated in the dark at 23 ± 2 ºC and subcultured every 30 days.

Isolation and identification of bacteria

Callus from clone 6-156-6, cultivated for 120 days, were placed on solid lysogeny broth (LB) medium (Sambrook *et al.,* 1989) (10 g l^{-1} of NaCl, 10 g l^{-1} tryptone and 5 g l^{-1} yeast extract, pH 7.0) to allow the growth of endophytic bacteria. The Petri dishes were incubated for 3 days in the dark at 23 ± 2 ºC. Afterwards, bacteria growing around the explants were transferred to a fresh solid LB medium and cultivated for another 3 days at the same conditions. Then, single colonies were used for DNA extraction and sequencing (Quambusch *et al.,* 2014).

The phylogenetic assignment of isolates was carried out by sequence analysis of the 16S rRNA gene. Total genomic DNA extraction was done as described by Vicente *et al.* (2008). Silica:celite (2:1) for maceration in CTAB (Cetyltrimethylammonium bromide) and CIA (chloroform:isoamyl alchol) for purification. After that, the DNA was quantified using NanoDrop 1000 (Spectrophotometer Thermo Scientific) and by agarose gel (1%) quantification. For the sequence analysis of the 16S rRNA gene, universal primers Y1f (5'TGGCTCAGAACGAACGCTGGCGGC3') positions 20 to 43 and Y3r (5'TACCTTGTTACGACTTCACCCCAGTC3') positions 1482 to 1507, which are specific for highly conserved regions of 16S rRNA, were used.

The PCR reactions were performed under the following conditions: PCR mixture (25 µl) containing 1.0 U Taq DNA polymerase, 2.0 mM $MgCl_2$, 1x PCR buffer, 2.0 mM of each dNTP, 0.4 mM of each forward and reverse primer (Invitrogen, Brazil) and approximately 50 ng template DNA. It was submitted to 1 cycle of 94 ºC for 2 min, 35 cycles of 94 ºC for 1 min, 58 ºC for 1 min and 72 ºC for 2 min, 1 cycle of 72 ºC for 6 min. After, the amplicons integrity was observed on 1% agarose gel electrophoresis.

Amplicons were cleaned with exonuclease and shrimp alkaline phosphatase (sap) according to the instructions of the manufacturer. Amplicons were sequenced with bigdye terminator cycle sequencing Kit v. 3.1 (Applied Biosystems, Foster city, CA, USA) according

to the instructions of manufacturer, and reaction mixtures were purified with sephadex g-50 fine (Ge health care Bio-sciences, Uppsala, Sweden). Sequences were analyzed on an ABI3130 DNA sequencer (Applied Biosystems).

Alignment and phylogenetic construction

Sequences were edited with the BioEdit software v.7.2.6 and compared to reference sequences at GenBank data sets (Supplement material). The alignment was performed with the MAFFT (Katoh and Standley, 2016), and visual inspection by the MEGA 7 version software (Kumar *et al.,* 2016). The best evolutionary model to each dataset was estimated using the program MEGA v.7. The phylogenetic trees were constructed with 1000 bootstrap replicates using the Maximum Likelihood Implemented in Mega v7 software. New sequences generated in this study were deposited in NCBI GenBank nucleotide database (www.ncbi.nlm.nih.gov).

Ultra- structure analysis

I. paraguariensis callus with 5 months, where the growth of endophytic bacteria was visible under naked eye, were fixed in FAA as mentioned above and dehydrated in ethanol gradient series (70, 80, 90 and 100%, 10 min each step). The 100% ethanol step was repeated twice. After that, the explants were dried using the critical point technique (Baltec CPD 030) and coated with gold. The photomicrograph was taken by a JEOL (JSM 6360-LV) scanning electron microscope (JEOL Ltd, Tokyo, Japan) at the Electronic Microscopy Centre, UFPR.

Indole acetic acid (IAA) production

The ability of the isolates to produce IAA was determined qualitatively and quantitatively. The isolates were cultured overnight on LB medium at 28 °C, and then 25 µl of this bacterial culture were inoculated on a 50 ml Erlenmeyer flask containing 25 ml of LB medium with or without 0.1% tryptophan. The cultures were incubated for 72 h at 28 ºC at 125 rpm, in the dark. After that, the cultures were centrifuged at 6000 rpm for 30 min, and 2 ml of the supernatant was mixed with two drops of orthophosphoric acid and 4 ml of the reagent of Salkowski (50 ml, 35% perchloric acid, 1 ml 0.5M $FeCl_3$) was added. The mixture was allowed to stand for 25 min. The indole acetic acid (IAA) production was measured qualitatively by the observation of a rose color and quantitatively only in the medium with tryptophan, by the measure of the intensity of the rose color at 530 nm using a spectrophotometer (UV Espectrophotometer 1800, Shimadzu). For determination, a standard curve (from 0-100 µg l^{-1}) was also developed with a standard solution of pure indole-3-acetic acid. The experiment was conducted in triplicate.

Plant growth promotion

The isolates were cultivated separately in LB medium as described above with tryptophan to obtain the bacterial extract. The bacteria optical density (OD_{600}) was determined and controlled to be between 0.8 and 1. The bacterial suspension was centrifuged and the supernatant was then autoclaved. Afterward 2.5% of bacterial extract was added to ¼ MS medium (Murashige and Skoog, 1962) before medium sterilization by autoclave. As control, LB medium without addition of the bacterial extract was used.

Seeds of *Phaseolus vulgaris L.* were disinfected using 1% (v/v) Cercobin for 10 min, and in 0.05% H_2Cl_2 for 5 min. Seeds were then rinsed three times with sterile distilled water and placed in 20 ml glass flasks containing the ¼ MS medium with or without addition of the bacterial extract. Three seeds per flasks with ten replicates were used. All flasks, lidded with plastic film, were incubated in a growth chamber at 23 ± 2 ºC under a 16 h photoperiod. After 20 days root and shoot lengths were measured and the dried weight was estimated (López-Bucio *et al.,* 2007).

Statistical analysis

RESULTS AND DISCUSSION

Isolation and identification of bacteria

Based on the sequence analysis of 16S rRNA region it was possible to identify two endophytic bacteria isolates (IPC06 and IPC07) from yerba mate callus as *Bacillus megaterium* clustered on clade very well supported (100%). The analysis of reference strains deposited in Genebank (http://www.ncbi.nlm.nih.gov/genbank/) revealed that from the total of 1288 sites evaluated for 16S region of RNAr, 923 were conserved,

336 variable, 201 were parsimoniously informative (Pi). The empirical base frequencies were pi (A): 0.25, pi(C):0.22, pi(G): 0.25 pi(T), 0.20, with 1000 bootstrap inferences using *Paenibacillus polymyxa* as outgroup.

The partial 16S rRNA gene sequence of the isolate showed 100% sequence similarity to *B. megaterium* strain CQN-25 (GeneBank accession no. KR347313.1) isolated from *Apostichopus japonicas* (Figure 1).coffee (*Coffea* sp.) (Nunes and De Melo, 2006), ginseng (*Panax ginseng* C.A.Mey.) (Vendan *et al.*, 2010), ginger (*Zingiber officinale* Rosc.) (Chen *et al.*, 2014) and black pepper (*Piper nigrum* L.) (Munjal *et al.*, 2016).

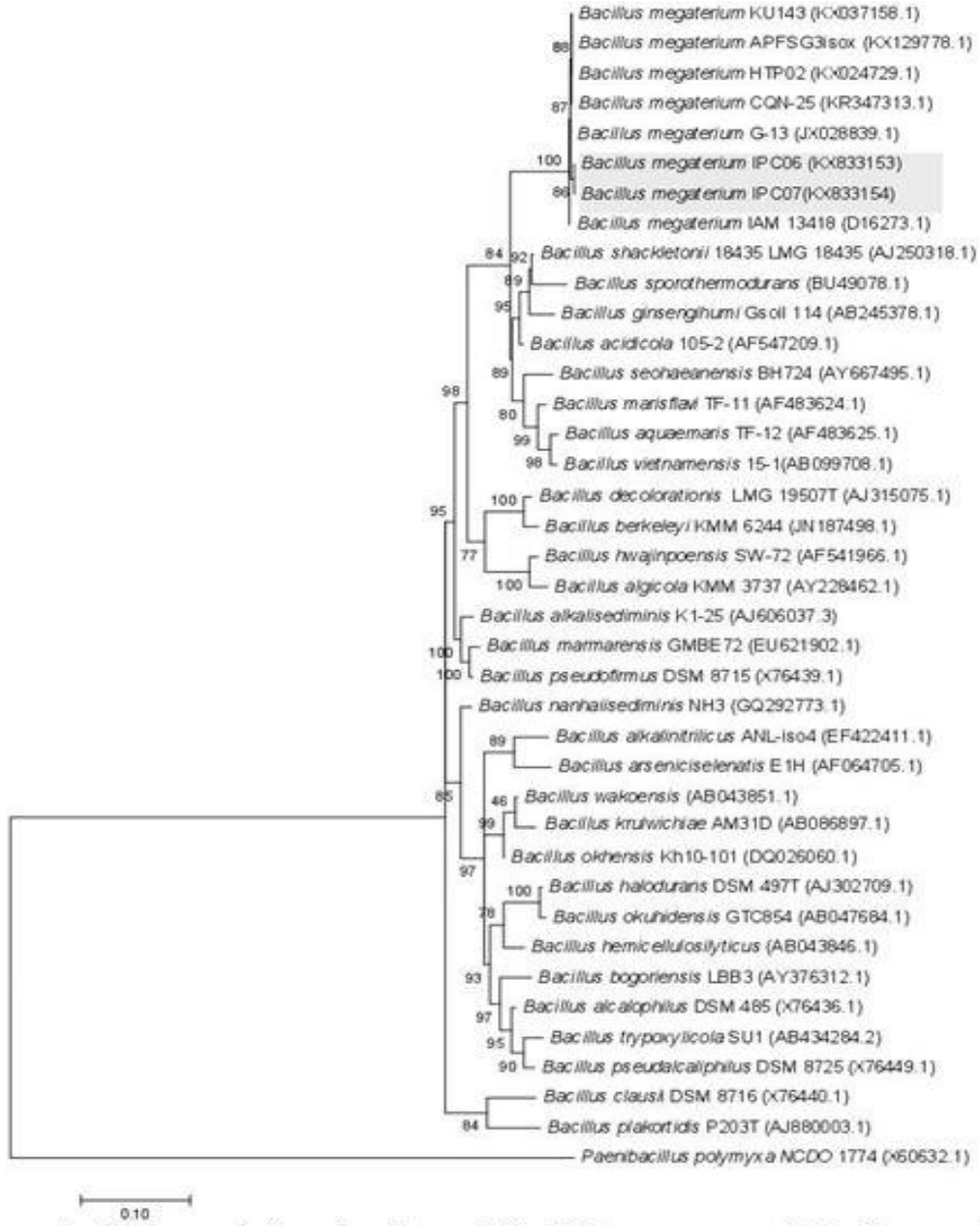

Figure 1. Phylogenetic tree based on 16S rDNA- sequences of *Bacillus* species constructed with maximum likelihood implemented in MEGA 7. ML/MP bootstrap values 80% was displayed above or below each branch. *Paenicillus polymyxa* was used as outgroup.

B. megaterium was first identified by Anton de Bary in 1884 as a soil Gram-positive bacteria (De Bary, 1884) and it is the largest of all bacilli (Korneli *et al.,* 2013). *B. megaterium* is a spore-forming bacterium found in diverse habitats, mostly in soil, but also in seawater, sediment, rice paddies, honey, fish, and dried food (Vary *et al.,* 2007) (Table 1). On the other hand, it has already been identified in several plant species as an endophytic bacteria in cotton (*Gossypium* spp.), sweet corn (*Zea mays* L.) (McInroy and Kloepper, 1995), coffee (*Coffea* sp.) (Nunes and De Melo, 2006), ginseng (*Panax ginseng* C.A.Mey.) (Vendan *et al.,* 2010), ginger (*Zingiber officinale* Rosc.) (Chen *et al.,* 2014) and black pepper (*Piper nigrum* L.) (Munjal *et al.,* 2016).

In *I. paraguariensis, B. megaterium* was recently identified by Pérez *et al.* (2016), in leaves from plants grown under field conditions in Argentina. Besides the authors identified 20 taxa of endophytic bacteria from yerba mate leaves and stems. Another bacterium was also identified from *I. paraguariensis* during *in vitro* culture, *Stenotrophomonas malthophilia* (Luna *et al.,* 2013). This bacterium was isolated from nodal segments of greenhouse-grown plants and hampered the *I. paraguariensis in vitro* propagation by bioreactor.

It was observed in preliminary tests, that the contaminations by endophytic microorganisms are lower in leaves when compared to stems on *in vitro* culture. In addition, it was observed the presence of endophytic bacteria only in a very few callus. The occurrence of bacteria in callus is also lower when compared with other works that used stems on *in vitro* culture from yerba mate (Zaniolo and Zanette, 2002; Dutra *et al*., 2008) (data not shown).

Pérez *et al*. (2016) observed the bacterial diversity in stems and leaves from *I. paraguariensis,* and identified 126 species in stems and 66 in leaves, confirming that the bacterial diversity is higher in stems than leaves from yerba mate. For this reason, our group is currently working to develop regeneration protocols for yerba mate based on regeneration of callus from *in vitro* leaves. Similar report was observed by Jin *et al*. (2014) in a medicinal plant, *Stellera chamaejasme* L. where the diversity of the bacterial community associated with stems was higher than that with leaves.

Ultra- structure analysis

In the ultrastructural analysis it was possible to observe the presence of bacteria on the surface of callus (Figure 2 a). In addition, were also observed a high concentration of bacteria inside the cells (Figure 2 b) and in the intercellular spaces (Figure 2 c) that confirm the endophytic condition of the bacteria.

Endophytes were also observed colonizing other callus cultures. In *Pinus sylvestris* L. biofilms on the surface of bud-derived callus were detected using scanning electron microscopy (Pirttilä *et al.,* 2002). Besides, callus from *Piper* spp., *Taxus baccata* subsp. *wallichiana* and *Withania somnifera* (L.) Dunal also showed the presence of endophytic bacteria (Kulkarni *et al.,* 2007).

In the case of yerba mate, Luna *et al.* (2013) informed the presence of endophytic bacteria in nodal segments. By scanning electron microscopy the authors detected the presence of bacteria in the intercellular spaces of stem cortical parenchyma but no identified the species present. In another specie of the same genera, *Ilex dumosa,* previous report refer also in nodal segments cultivated *in vitro* the presence of endophytic bacteria (Luna *et al.,* 2008). These reports confirm the presence of endophytic bacteria in tissue of *Ilex* plants and the possibility to find isolates with potential for different uses.

Indole acetic acid (IAA) production

Colorimetric analysis by the reagent of Salkowski of stationary phase cultures indicated that both isolates produce auxin in the presence and absence of L-tryptophan (Figure 3).

The reagent of Salkowski used in the colorimetric method allows the oxidation of indole-3-acetic acid (IAA) by peroxidase enzymes (Mayer, 1958). By using this reagent when the answer is positive for IAA production a pink color that varies from light pink to intense pink is obtained, depending on the concentration of the IAA (Meudt and Gaines, 1967). This method is an important option for qualitative and semi-qualitative determination of IAA that assure the presence of the hormone in the supernatant of bacterial cultures (Ambawade and Pathade, 2013).

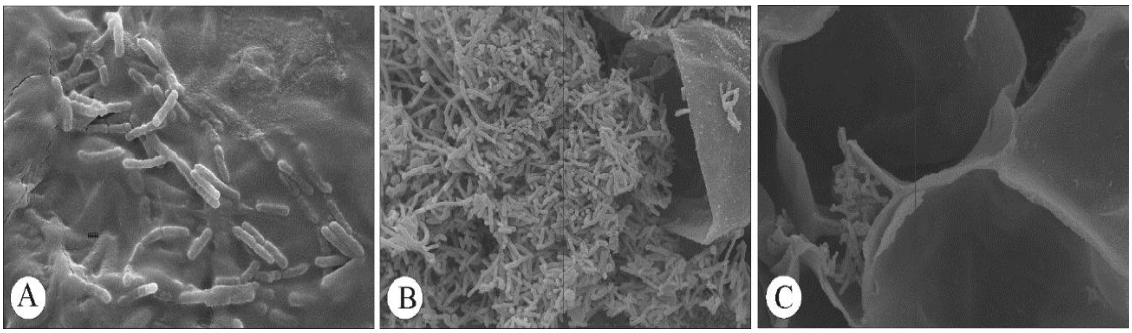

Figure 2. Ultrastructural analysis of yerba mate endophytic bacteria. a) Bacteria on the surface of callus. b) It located in the intercellular spaces of callus. c) Bacterial cells in stem parenchyma cells. a, b) 5000X increase, c) 4.500X increase.

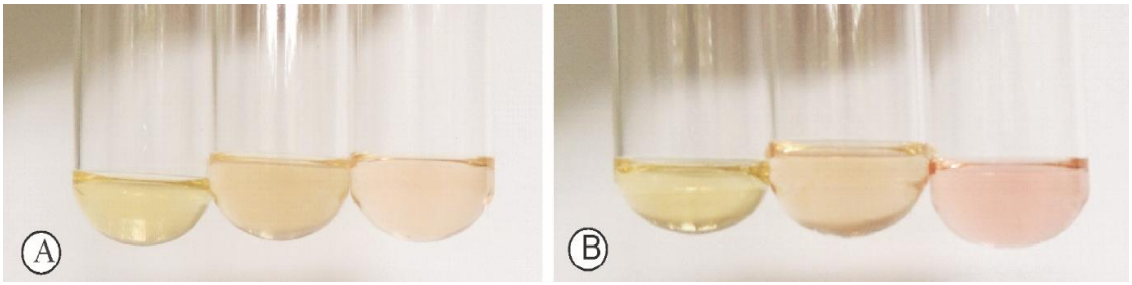

Figure 3. Qualitative result confirmation of IAA production from both isolates. a) Isolate IPC06: control, bacteria growth without, and with tryptophan. b) Isolate IPC07: control, bacteria growth without, and with tryptophan.

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The IAA synthesis occurs by different biochemical pathways: tryptophan-dependent pathways and tryptophan-independent pathway, given that, tryptophan has been identified as a main precursor for IAA biosynthesis pathways in bacteria (Spaepen *et al.,* 2007). The production of IAA in the presence of L-tryptophan was 28.63 µg l^{-1} and 18.22 µg l^{-1} respectively for isolate *IPC06* and isolate *IPC07*. The production of IAA in the absence of L-tryptophan was not evaluated quantitatively.

There are five proposed Tryptophan-dependent auxin biosynthesis pathways in bacteria: the indole-3-acetamide (IAM) pathway which is the best characterized pathway in bacteria, the tryptamine (TAM) pathway, the indole-3-acetonitrile (IAN) pathway, Trp side-chain oxidase (TSCO) pathway and the indole-3-pyruvic acid (IPyA) pathway, the major pathway for IAA biosynthesis in plants (Spaepen *et al*., 2007; Di *et al*., 2016). Besides these fives pathways in bacteria, there is one tryptophan-independent pathway branches from indole-3-glycerol phosphate or indole (Spaepen *et al.,* 2007). However, the intermediate stages, and genes involved in tryptophan-independent pathways remain undefined (Fu *et al.,* 2015). In this study, it was observed that the IAA production in both isolates from *B. megaterium,* occurred in the presence or absence of L-tryptophan, suggesting that this species can produce IAA by both pathways (Spaepen *et al*., 2007).

A bacterial tryptophan-independent pathway was demonstrated in *other strains from B.*

megaterium: B. megaterium (AUX36) (*Aziz et al., 2015), B. megaterium* (MiR-4) *(Ali et al., 2009)* and other species from *Bacillus: B. methylotrophicus* (EB-26), *B. subtilis* (EB-04 and EB-55) and *B. tequilensis* (EB- 87) (Andrade *et al.,* 2014). The activity of bacteria of the genus *Bacillus* is well documented, and several mechanisms have been suggested for the phytostimulatory activity of this group of plant growth promoting bacteria (PGPB). For this reason, the genus is considered very significant in agricultural applications (Ali *et al.,* 2010).

Plant growth promotion assay

Statistical analysis of data revealed that the *B. megaterium* strains had significant effect on growth of *P. vulgaris* under *in vitro* conditions when compared with control (Table 1). Although similar results were observed for the two isolates tested (increase in shoot length and root length) here only present the results of strain IPC06. Similarity, increase in shoot and root fresh weight was observed. These results demonstrated that application of bacterial extract in culture medium favored the growth of bean seedlings (Figure 4).

Regarding the root development, it was observed an increase in number and length of secondary roots and in the number of thin roots. It is well known that the division, expansion and differentiation of root cells and root elongation is influenced by IAA (Martinez-Viveros *et al.,* 2010).

Table 1. Dry weight (g) and length (cm) of bean plants growth in medium with extract from endophytic bacteria (*Bacillus megaterium* IPC06) from *Ilex paraguariensis*.

Variable	Seedlings without extract (control)	Seedlings inoculated with IPC06 extract
Dried weight root (g)	0.112 ± 0.035	0.164 ± 0.059 *
Dried weight stem (g)	0.158 ± 0.040	0.219 ± 0.072 *
Root length (cm)	5.716 ± 3.840	7.866 ± 3.810 *
Shoot length (cm)	7.753 ± 5.000	10.900 ± 4.460 *

Values shown represent the average of ten replicates ± standard deviation. Asterisks are used to indicate significant differences by t test (p<0.05)

Figure 4. Effect of *Bacillus megaterium* on growth of bean seedlings. a) bean plant growth in medium ¼ MS without *B. megaterium*. b) bean plant grown on medium ¼ MS supplemented with 2.5% of *B. megaterium*.

Other studies demonstrated the activity of bacteria in plant root formation. Similar response was reported by López-Bucio *et al.* (2007), where the inoculation of *B. megaterium* (UMCV1) in beans showed significant increase in the fresh and dry weights of plants. The plant-growth promotion was also related to modifications in root architecture. For *Triticum aestivum* L.*,* the use of *B. megaterium* was reported to increase the root length, root number and root mass of the germinated seeds (Aziz *et al.,* 2015).

Besides promoting the growth and development of the roots, the extract of *B. megaterium* accelerate the plants development when compared with the control. In addition to the IAA production, the endophytic bacteria may promote plant growth by a wide variety of mechanisms (Yu *et al.,* 2016), including phosphate solubilization, nitrogen fixation, ammonia production and through the production of other phytohormones, such as cytokinins and gibberellic acids (Pereira *et al.,* 2016).

The economic use of *B. megaterium* could be highly versatile. *B. megaterium* is known for plant growth promotion, its ability against plant pathogens and biocontrol (Munjal *et al.,* 2016).

The success of *B. megaterium* use depends of plant and the isolate used. For example, in *Arabidopsis* it increased plant biomass, improved root system architecture, and augmented photosynthetic capacity (Zhou *et al.,* 2016). For *Vigna radiate (L.) Wilczek it* caused significant increase in number of roots *(Ali et al., 2009). In* peppermint (*Mentha piperita* L.), plants inoculated with *B. megaterium* isolates manifested increase in plant height, number of leaves and branches, biomass and nutrient content, compared to non-inoculated plants (Sandeep *et al.,* 2011). Nevertheless, in other plants such us *Eucalyptus*, the use of strain EUCB 26, with high production of IAA, had a negative effect on rooting, weight and length (Paz *et al.,* 2012).

On the other hand, application of *B. megaterium* in tea (*Camellia sinensis* (L.) Kuntze) plants, promoted its growth, introduced the antagonistic activity to fungal pathogens and reduced sclerotial blight of tea caused by *Sclerotium rolfsii*. In addition, significant increases in phenolic compounds of tea plants were observed (Chakraborty *et al.,* 2015).

According to the results, the endophytic bacterial strains from *I. paraguariensis* are promising microorganisms for promoting root and plant development.

CONCLUSION

Endophytic bacterial strains of *B. megaterium* isolated from callus of *I. paraguariensis* produce IAA and promote the growth of bean seedlings under *in vitro* conditions. This strains has potential as plant growth promoters.

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