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BACILLUS SUBTILIS ISOLATES WITH DIFFERENT ABILITIES TO PROMOTE PLANT GROWTH IN MAIZE, COTTON AND SOYBEAN CROPS ISOLATION AND CHARACTERIZATION OF BACTERIAL STRAINS

RAFAEL MILANI[1], ROBERTA MENDES DOS SANTOS[1], LAIANA LANA BENTES[1], SAVEETHA KANDASAMY[2], GEORGE LAZAROVITS[1] AND EVERLON CID RIGOBELO[1*]

[1] Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Ciências Agrarias e Veterinárias de Jaboticabal, Via de acesso Prof. Dr. Paulo Donato Catellane, s/n.
[2] A&L Biologicals Agroecology Research Services Center 2136 Jetstream Road London, ON N5V3P5

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Abstract – Plant growth promoting rhizobacteria is a group of bacteria living inside plant tissue or in the rhizosphere, being able to promote plant growth due to their many abilities that directly or indirectly promote plant development. The aim of this study was to isolate and identify bacteria from maize with ability to solubilize phosphorus, fix nitrogen and produce IAA. Then, all isolates were evaluated regarding their abilities to promote plant growth on soybean, maize and cotton crops. In this study, one *Bacillus velezensis*, eight *B. subtilis* and one *Lactococcus lactis* isolates were obtained. Indole acetic acid concentration ranged from 11.32 to 15.23 μg IAA mL^{-1}. For maize, four of *B. subtilis* isolates promoted higher plant height and one isolate promoted higher root dry matter and another promoted higher shoot dry matter. For cotton, one isolate promoted higher plant height, other promoted higher root dry matter another promoted higher shoot dry matter. For soybean, one isolate promoted higher plant height, the other promoted higher root dry matter and the other higher shoot dry matter. In addition, one *B. subtilis* isolate harmed root development for maize crop. These results showed that according to the selection strategy used to select maize bacteria with plant growth promotion characteristics, the largest number of isolates was from *Bacillus subtilis*. Furthermore, *B. subtilis* isolates showed different growth promotion levels in the various cultures tested. This shows the importance of knowing if the correct bacterial isolate is being used for the appropriate plant.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are soil microorganisms able to colonize roots and stimulate plant growth (de Zelicourt *et al.,* 2013). In soil, *Bacilli* represent a large fraction of the microbial community. They are found in the rhizosphere and as epiphytes or endophytes in various crops, including maize, cotton soybean and others (Qiao *et al.,* 2014). Among them, *Bacillus subtilis* is an important species among PGPR, which can be isolated from many environments and adapt to grow in diverse conditions in the rhizosphere (Earl *et al.,* 2008). In addition, *B. subtilis* has been successfully used for several decades with significant effects as a plant growth promoting agent, increasing height, root and shoot dry matter, chlorophyll content and yield (Gao *et al.,* 2013). These effects are due abilities such as auxin production, nitrogen fixation, phosphorus solubilization and antifungal activity (Bhattacharyya and Jha, 2012). In addition, *B. subtilis* has an advantage over other bacteria due to its ability to produce endospore and thus resist to changes in environmental conditions (Qiao *et al.,* 2014). Therefore, isolation and screening of these strains are justified.

However, in many cases, PGPB fail to induce the desired effect when applied to crops (Lugtenberg *et al.,* 2001) or different isolates of the same specie present different results. This could be the result of individual abilities of each isolate to colonize the

rhizosphere. The understanding of the colonization processes is important to better predict how bacteria interact with plants and whether they are likely to establish themselves in the plant environment and the successful PGPB colonization is a requirement to promote plant growth. Furthermore, each isolate presents different abilities to colonize various plant compartments (Compant *et al.*, 2010). Thus, the aim of the present study was to isolate bacteria from maize, to verify their abilities to promote plant growth and to the effects on maize, cotton and soybean under greenhouse conditions.

MATERIALS AND METHODS

Isolation of epiphytic and endophytic bacteria

The isolation of strains was performed at "Faculdade de CiênciasAgrárias e Veterinárias de Jaboticabal" (FCAV), Unesp, state of Sao Paulo (21º 14' 05" S, 48º 17' 09" W and 615.01 m a.s.l., using maize plants at V6 stage. The maize plant was collected from the soil using a brush cutter and immediately transferred to the laboratory. Leaves, stem and roots were separated and washed with water to remove soil according to Kuklinsky-Sobral, *et al.* (2004).

Epiphytic and endophytic bacteria were isolated by weighing three grams of plant samples separately, being placed in Erlenmeyer flasks containing glass beads of 0.1 cm in diameter and 50 mL saline solution buffered with phosphate (g $L^{-1)}$ (Na_2HPO_4: 1.44; KH_2PO_4: 0.24; KCl: 0.20; NaCl: 8.00; pH 7.4). The solution was shaken at 150 rpm at 28 ºC for 1 hour. Subsequently, 5 mL of solution were plated on plates with 10% tryptone soybean agar (TSA), being kept at 28 ºC for 15 days. Bacterial colonies were isolated and replicated in inclined tubes with 10% TSA and kept at 28 ºC for 2 days. Then, plates were stored at 4 ºC.

After the removal of epiphytic bacteria, endophytic bacteria were isolated through plant surface disinfection using serial washing. The disinfection process efficiency was verified by collecting of aliquot of water used in the final plant washing process in 10% TSA and kept at 28 ºC for 15 days.

Phosphorus solubilization activity

The fluorapatite solubilization activity was determined by transferring 0.2 mL of suspension at concentration of 1 x 10^7 CFU mL^{-1} to Erlenmeyer flasks containing medium described by Nahas *et al.* (1994), supplemented with 5 g L^{-1} fluorapatite (Araxá apatite). After inoculation, the bacterial solution was incubated with no agitation at 28 ºC for seven days. Four flasks of each bacterial solution were daily removed. Solutions were centrifuged at 9,000 rpm for 15 minutes and the supernatant was collected to determine the phosphate content according to Ames (1966).

Nitrogen fixation

The nitrogen fixation ability was evaluated verifying the bacterial growth in agar medium without NFb nitrogen (Dobereiner *et al.*, 1995). Bacterial growth was observed with the presence of halo, indicating nitrogen fixation.

Indole Acetic Acid Production

Indol acetic acid production was measured according to methodology of (Kuss *et al.*, 2007) with few modifications. Isolates were inoculated in 20 mL of Dextrose Yeast Glucose Sucrose (DYGS) supplemented with 5 mM of L-tryptophan, incubated for 48 hours at 28 ºC under constant agitation at 120 rpm and in the absence of light. Subsequently, 5 mL of each culture were centrifuged at 10,000 rpm for 10 minutes and 2 mL of supernatant were transferred to test tube containing 2 mL of 2% Salkowski reagent (w/v) (0.5 M FeCl3 in 35% of perchloric acid) (Sarwar and Kremer, 1995) and incubated in the absence of light for 30 minutes. IAA production was determined by spectrophotometer at 530 nm and values were obtained through standard curve with known concentrations of commercial IAA. This measurement was performed in triplicate.

PCR reaction – 16S r DNA sequencing

The eight isolates that showed the highest IAA production and presented phosphorus solubilization and nitrogen fixation abilities were selected and had their DNA extracted according to *Quick-DNA Universal Kit (Zymo Research – cat. Nº D4068 e D4069)*. The DNA amplification was done by 16S rDNA at final volume of 25 µL containing all reagent needed to reaction at µL water ultrapure sterile, 11.3; 10 millimolar of primer F, 1.5; 10 millimolar of primer R, 1.5; Taq green Buffer 5x, 5,0; $MgCl_2$, 3,0; 10 millimolar of each dNTP, 1,0; Taq DNA polimerase, 0.2; DNA mold, 1.5).

Genomic DNA was extracted using Quick-DNA Universal Kit (Zymo Research) according to manufacturer's instructions.

16S ribosomal DNA was amplified by polymerase chain reaction (PCR) using P027F (5'-GAGAGTTTGATCCTGGCTAG-3') and 1378R (5'-CGGTGTGTACSSGGCCCGGGAACG-3') primers with the following amplification program: 95 °C for 2 min; followed by 25 denaturation cycles at 95 °C for 30 s, annealing at 63 °C for 1 min and extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. PCR products were purified and sequenced in automated DNA ABI3730 sequencer using P027F and 1378R primers.

Sequences were aligned and edited using the BioEdit 7.0.5.3. software (Hall, 1999) and compared to sequences from the GenBank at NCBI (National Center for Biotechnology Information). Phylogenetic analyses were performed using the MEGA 6.0 software (Tamura *et al.*, 2013).

Plant Experiments

The effect of these bacteria was studied on three crops, 2B587PW corn variety, BRS Esplendor bean variety and Intacta RR PRO soybean variety. Seeds of these varieties were kindly provided by FEPE - FCAV. All varieties were grown in sterilized industrial silica sand with MS nutritional solution (Murashige and Skoog, 1962) throughout the experiment under greenhouse conditions. Experiments with cotton and corn crops were harvested in 25 days and experiments with soybean were harvested in 30 days. The plant traits analyzed were plant height, root and shoot dry matter, shoot nitrogen level and chlorophyll content using chlorophyll meter (CCM200 model). The chlorophyll content was measured in mg cm^{-2}.

RESULTS

The number of epiphytic bacteria isolated from maize was 182 isolates, of which 102 were from leaves, 49 from stem and 31 isolates from roots. The number of endophytic bacteria was 138 isolates, in which 59 were from leaves, 72 from stem and 7 from roots. Bacterial isolates were differentiated according to the colonization tissue and plant growth promoting characteristics (Table1 and Table 2).

All bacterial isolates were qualitatively evaluated and phosphorus solubilization, nitrogen fixation and IAA production of each isolate were measured. Positive control was (*Azospirillumbrasilense*), which produced 22.57ìg IAA mL^{-1}. In this way, 10 isolates that produced at least 50% of the total IAA produced by *A. brasilense* were selected. IAA production ranged from 11.32 to 15.23 µg IAA mL^{-1} (Figure 1a). The phosphorus solubilizing in test tube varied from 0.66 to 1.0 ìg of P $tube^{-1}$ (Figure 1b).

All ten isolates were sequenced and identified. Isolates were identified as *Bacillus subtilis* (eight isolates), *Bacillus velezensis* (one isolate) and *Lactococcus lactis* (one isolate) (Table 3). The construction of phylogenetic trees was based on nucleotide sequences known in databases, which were compared to the nucleotide sequences of all selected isolates (Figure 2).

In growth promotion evaluations in the maize crop, no statistical difference between treatments that received bacterial isolates and control treatment was observed. BS-248 and LL-275 isolates promoted the lowest mean maize plant height, whereas the

Table 1. Number of bacterial epiphytic isolates in corn, as a function of the colonization site and characteristic in the promotion of plant growth.

Characteristic	Colonization			Overall
	Leaves	Stem	Root	
P Solubilization	95 (56%)	47 (28%)	28 (16%)	170
Nitrogen fixation	43 (44%)	35 (36%)	19 (20%)	97
Production of IAA	31 (59%)	14 (26%)	8 (15%)	53

Table 2. Number of endophytic bacterial isolates in maize as a function of colonization site and plant growth promoting characteristics.

Characteristic	Colonization			Overall
	Leaves	Stem	Root	
P Solubilization	50 (41%)	66 (54%)	6 (5%)	122
Nitrogen fixation	30 (54%)	21 (37%)	5 (9%)	56
IAA production	8 (30%)	15 (55%)	4 (15%)	27

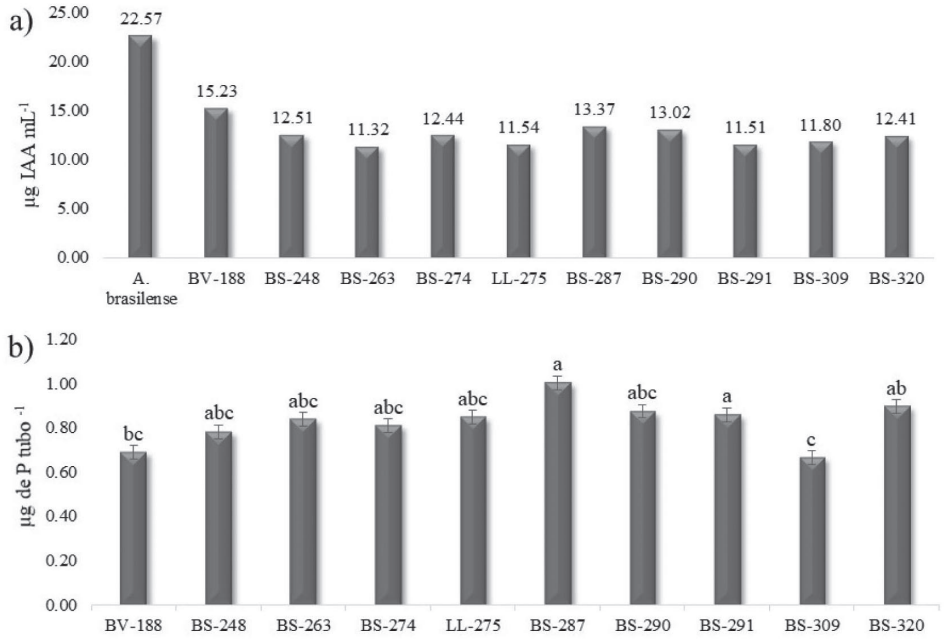

Fig. 1a). Amount of IAA produced by ten isolates with at least 50% of *A. brasilense* production and b) Amount of solubilized P in each test tube µg of $P^{-1.}$ tube.

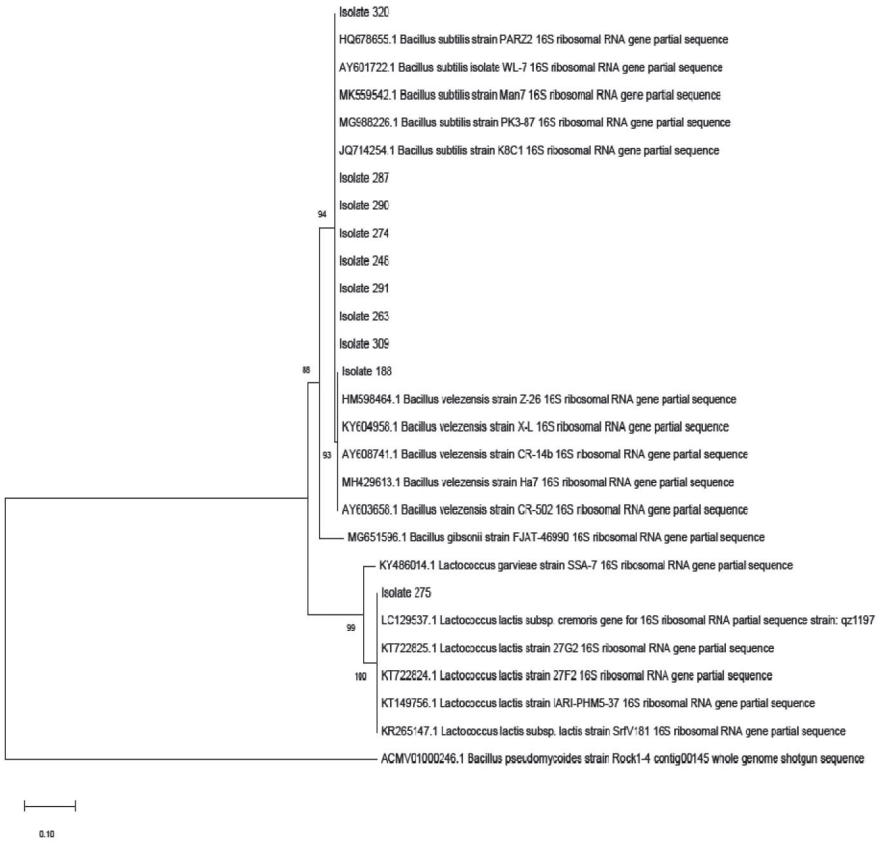

Fig. 2. Phylogenetic tree based on comparison of the sequence of known nucleotides to obtained nucleotide sequences from selected isolates.

Table 3. Identification of isolates with phosphorus solubilization, nitrogenase and IAA production abilities.

Isolate code	Identification
188	*Bacillus velezensis*
248	*Bacillus subtilis*
263	*Bacillus subtilis*
274	*Bacillus subtilis*
275	*Lactococcus lactis*
287	*Bacillus subtilis*
290	*Bacillus subtilis*
291	*Bacillus subtilis*
309	*Bacillus subtilis*
320	*Bacillus subtilis*

eight isolates were not different from control (Figure 3a). Regarding shoot dry matter, results were similar to root dry matter, and BS-248 and LL-275 isolates promoted the lowest mean values for root, whereas BS-290 isolate promoted higher shoot dry matter compared to control ($p<0.05$) (Figures 3b and 3c).

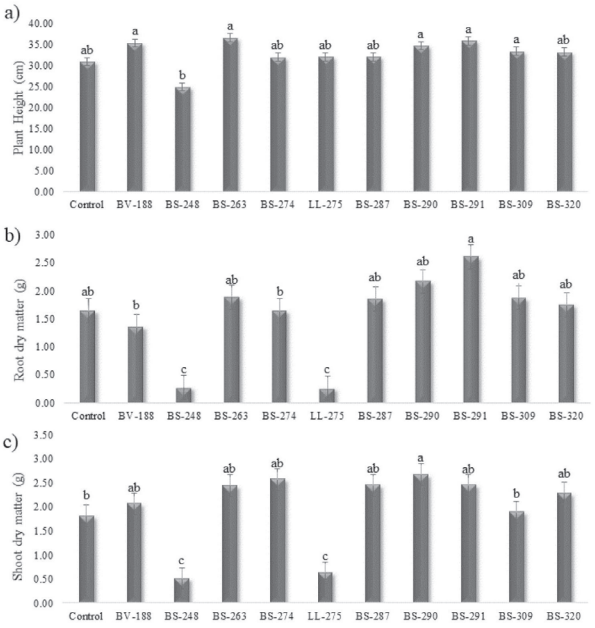

Fig. 3. Mean values for: a) plant height; b) root dry matter and c) shoot dry matter of maize inoculated with 10 isolates. Same letters indicate no statistical difference according to the Duncan test at 5%.

For cotton, BV-188, BS-263, BS-274, BS-309, BS-320 isolates significantly differed from control ($p<0.05$) promoting an increase in plant height (Figure 4a), whereas BS-248 and LL-275 isolates promoted lower root dry matter compared to control. ($p<0.05$). The other isolates did not differ from control for root dry matter (Figure 4b). For shoot dry matter, BS-274 isolate promoted an increase of 48.6% compared to control, whereas the other isolates did not differ from control (Figure 4c).

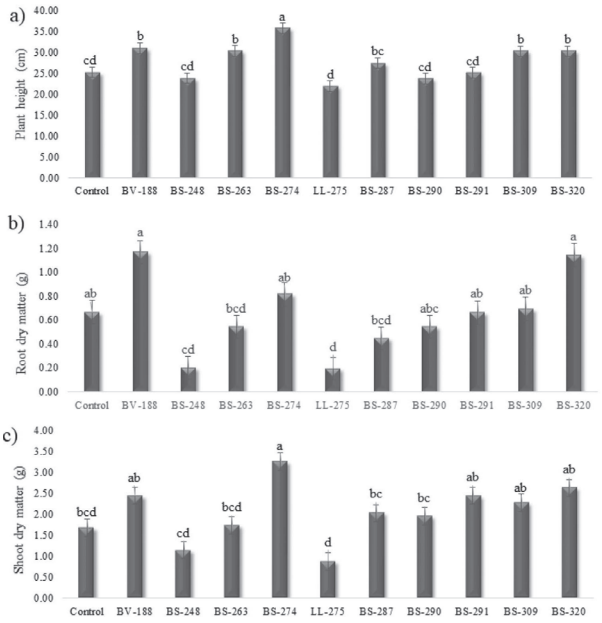

Fig. 4. Mean values for: a) plant height; b) root dry matter and c) shoot dry matter of cotton inoculated with 10 isolates. Same letters indicate no statistical difference according to the Duncan test at 5%.

For soybean, no isolates increased plant height compared to control ($p<0.05$), however, there was no significant difference between BS-291 isolate and BS-287 isolate. BS-287 promoted an increase of 41% compared to BS-291 isolate (Figure 5a). Similar results were found for root dry matter, where there was no statistical difference between treatments compared to control ($p<0.05$), however, BS-287 isolate differed ($p<0.05$) from BS-274 and BS-275 isolates, promoting an increase of 68.5 and 81.4%, in root dry matter, respectively (Figure 5b).

For shoot dry matter, there was no statistical difference between treatments; however, BS-287 isolate was higher ($p<0.05$) than BS-248, BS-263, BS-274, BS-275, BS-290 and BS-291 isolates, promoting an increase in shoot dry matter for soybean crop (Figure 5c.)

DISCUSSION

The aims of the present study were to isolate both epiphytic and endophytic bacteria from maize cropto verify their abilities as plant growth promoting agents and to evaluate their effects on

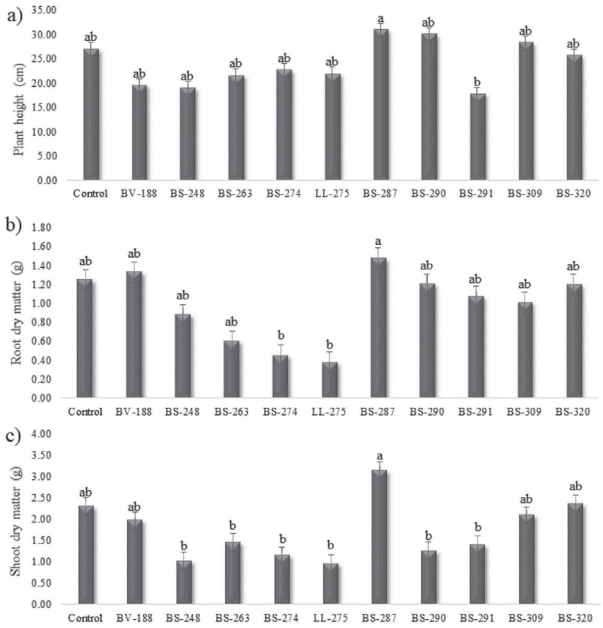

Fig. 5. Means for: a) plant height; b) root dry matter and c) shoot dry matter of soybean inoculated with 10 isolates. Same letters show no statistic difference according Duncan test at 5%.

maize, cotton and soybean under greenhouse conditions using sterile vermiculite.

Most epiphytic and endophytic bacteria isolated from maize were able to solubilize phosphorus, fix nitrogen and produce IAA.

Endophytes are microbes that live within plant tissues without producing negative effects on the host (Bacon and White, 2000). These microbes often benefit plants by imparting biotic and abiotic stress tolerance to hosts (Rosenblueth and Martinez-Romero, 2006). Some endophytes are known to produce anti-pest compounds to protect plants from various pathogens or insects. In many plants, microbe communities may occur in host tissues.

Endophytic bacteria frequently secrete antimicrobial compounds, plant growth hormones, solubilize mineral phosphate and chelate toxic metals in the rhizosphere (Ahemad and Kibret, 2014). *Bacillus* species are also reported as endophytic bacteria in higher plants (Li *et al.*, 2012; White *et al.*, 2014).

Most endophytic bacteria are isolated from leaves (56%) (Table 1), as reported by Orozco-Mosqueda *et al.* (2018). Endophytic bacteria have a more effective action on plant than bacteria from rhizosphere. This is due to the presence of metabolites within the plant tissue produced by endophytic bacteria. These metabolites are in direct contact with plants (Lindow and Brandl, 2003; Penuelas and Terradas, 2014).

Probably, the most effective action of endophytic bacteria on plant is the production of phytohormone, which is responsible for shoot and root development and the production of secondary metabolites, which provide resistance against insects and diseases (Penuelas and Terradas, 2014).

A large number of endophytic bacteria were isolated from leaves. This may be due to variations in the mechanisms and distribution of the microbial population or also due to the ability or preference of each microorganism to inhabit specific niches within the plant tissue (Lugtenberg *et al.*, 2002).

The number of strains with ability to promote plant growth depends on the strategy used to select these strains. There are many studies that have found different number and types of bacteria isolated from maize as a result of different strategies adopted to select endophytic bacteria (Montañes *et al.*, 2012; Gao *et al.*, 2013; Youseif, 2018). The present study selected all isolates with ability to solubilize phosphorus, then isolates with ability to fix nitrogen. Among these, all isolates that produced at least 50% of the IAA produced by *A. brasilense*, in which it was used as the standard isolate, have been selected. Using this strategy, this study isolated eight *B. subtilis* strains, one *L. lactis* strain and one *B. velezensis* strain. Interessante notar o grande número de B. subtilis portando habilidades de promoção de crescimento de plantas vivendo como bactéria endofíticas no milho. It is interesting to note the large number of *B. subtilis* with plant growth promoting abilities living as endophytic bacteria in maize.

B. velezensis is a strain with ability to fix nitrogen, solubilize phosphorus and produce IAA and also plays an important role as biocontrol of *Botrytis cinerea* in chilli (Jiang *et al.*, 2018) and *Glomerellacingulata* in peach (Regassa *et al.*, 2018) *B.velezensis*is genetically and biochemically very close to *B. subtilis.* Pandin *et al.* (2018) suggested to change the strain name, firstly identified as *B. subtilis* to *B. velezensis*, according to phylogenetic analysis. This study showed that these two bacterial strains are very similar (Lahlali *et al.*, 2013).

Although *B. velezensis*and *L. lactis* present many characteristics regarding plant growth promotion, these strains were not able to promote growth on crops used in this study. On the other hand, *B. subtilis* strains isolated in this study presented different plant growth levels on crops evaluated.

L. lactis was classified as lactic acid bacterium, widely used in cheese production and other fermented dairy products (Gutiérrez-Méndez *et al.*, 2008). This bacterium was previously classified as *Streptococcus lactis*(Stackebrandt and Teuber, 1988), with large distribution in the environment and in dairy products (Salama *et al.*, 1995). This bacterium can be isolated from plants (Nomura *et al.*, 2006). Some studies have isolated *L. lactis* from maize (Gutiérrez-Méndez *et al.*, 2008).

Marag and Suman (2018), reported that *L. lactis* promoted an increase in both shoot and root dry matter in maize. These results were different than those found in the present study. This may have occurred due to the use of soil, unlike the present study that used sterile vermiculite.

The plant microbiome is composed of active microorganisms that alter the plant physiology and development and induce the resistance system against pathogens, as well as mechanisms of tolerance to diverse types of stress such as drought, salinity and contaminated soils (Santoyo *et al.*, 2017; Yaish *et al.*, 2017; Yuan *et al.*, 2016).

However, these effects are not performed by any microbiome, but by some microbial species. In addition, some beneficial effects occur due to the synergistic effect of many microorganisms. In this way, some beneficial effects on plants may be lost when only one bacterial strain is used in sterile conditions as in this study (Rojas-Solís *et al.*, 2016; Timm *et al.*, 2016).

Although *L. lactis* did not promote the growth of crops evaluated (maize, cotton and soybean), in cotton and maize, the bacterium harmed their growth, providing plants with lower root dry matter for cotton and lower root and shoot dry matter for maize. These results suggest that there may have been competition between plant and bacterium. It is important to note that this bacterium presented many characteristics regarding the plant growth promotion in test tubes. However, *in vivo* tests revealed that this bacterium harmed plant development. More studies are needed to understand why and how bacteria with characteristics to promote plant growth would impair plant development.

In this study, eight *B. subtilis* were isolated and all of them were evaluated as plant growth promoting agents in maize, cotton and soybean. For maize, the isolate that promoted the best plant growth was isolate 290 (Figure 3c). For cotton, the best isolate was isolate 274 (Figure 4c) and for soybean, there was no statistic difference between isolate and control. Interestingly, although the eight isolates had been identified as *B. subtilis*, their effect as plant growth promoting agents were different for each crop. These results suggest the importance of the affinity between crop and microbial isolate.

Bacillus subtilis has 4,101 genes in its genome (Kunst *et al.*, 1997). Of these, only 192 were indispensable and 4% of the essential genes performed unknown functions (Sonenshein *et al.*, 2002). Many of these genes are involved in the synthesis of secondary metabolites, including antibiotics, which are more typically associated with *Streptomyces* species. The genome contains at least ten prophages or prophage remnants, indicating that bacteriophage infection has played an important evolutionary role in horizontal gene transfer (Kunst *et al.*, 1997). Certainly, the eight *B. subtilis* isolated in this study presented genomic diversity responsible for different abilities for plant growth promotion. It could be explained why isolate 290 was better for maize and not for cotton or soybean. Isolate 274 was better for cotton and not for soybean and maize.

The first challenge when an isolate is inoculated into soil is its establishment in the rhizosphere. In many cases, PGPB fail to induce the desired effects when applied in the field. This might be due to insufficient rhizosphere and/or plant colonization, which is as an important step required for exhibiting beneficial effects (Lugtenberg *et al.*, 2001). On the other hand, *B. subtilis* has many abilities to establish in the rhizosphere like antibiotic production, tolerance to extreme pH and osmotic conditions, colonize the root surface and cause lysis of fungal mycelia (Chauhan *et al.*, 2016). In addition, some plants secrete a wide variety of low molecular weight compounds and macromolecules from their root system, creating a nutritious and physicochemical environment for microbes to develop near the rhizosphere. A few of these excreted molecules act as chemical signals for recruiting bacteria such as *B. subtilis* to the root surface (Otto, 2006). However, different isolates have different abilities to colonize the rhizosphere and express different plant growth promotion levels as shown by *B. subtilis* isolated in this study.

Many factors may be involved in rhizosphere and rhizoplane and are performed by PGPR. These factors may be quimotaxis, bacterial growth rate, quorum sensing, amino acid synthesis, outer membrane protein, agglutinin, type IV pili,

antibiotic secretion, siderophore production, site specific recombinase (Compant *et al*., 2010). On the other hand, the plant may select specific rhizosphere colonizers via root exudation according to its necessity (Compant *et al*., 2010).

Although the eight isolates had been identified as *B. subtilis* and had been isolated from the same place, they presented different abilities to promote plant growth in different crops. The lack of several characteristics of each isolate could explain the low colonization of this isolate in the rhizosphere and endosphere. We need to better understand how these bacteria colonize different plant niches

B. subtilis is a ubiquitous bacterium adapted to grow in several environments within the biosphere. An important characteristic of *B. subtilis* is the ability to form endospore in response to nutrient or environmental deprivation (Sonenshein *et al*., 2002). The endospore can be easily dispersed by wind and germinate in adequate conditions, and can also be isolated in greater amounts than most other spore-forming bacteria from the rhizosphere of a variety of plants (Earl *et al*., 2008). In soil, *Bacilli* represent a large fraction of the microbial community. They are found in the rhizosphere and as epiphytes or endophytes in various crops and due to their characteristics, *Bacilli* have several mechanisms providing beneficial effects on plants (Cherif-Silini *et al*., 2016). It could explain the high number of *B. subtilis* isolates obtained in this study.

The results showed that according to the strategy used in this study to select bacteria with plant growth promoting characteristics, the majority was *B. subtilis*. It shows the importance of this specie for maize. In addition, although most isolates have been from *B. subtilis*, these isolates showed different growth promotion levels in each culture tested, showing the importance of knowing the right microbial isolate for the appropriate plant crop.

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The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

https://repositorio.unesp.br/handle/11449/151225

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