

Promotion of maize growth using endophytic bacteria under greenhouse and field conditions

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Abstract

The effect of *Bacillus subtilis* isolates before being used as inoculants is very important. They present various impacts on promotion and characteristics of plant at different stages of growth. This experiment was carried out on maize under greenhouse conditions with eleven treatments of endophytic bacteria and three repetitions. The better isolates under greenhouse conditions were used in the field experiment with four best treatments and six replicates. The traits evaluated under both conditions were height, shoot and root dry matter, and the nitrogen and phosphorus content in the plant and soil and the total number of bacteria in the soil. Under greenhouse conditions, the groups that received *Bacillus* spp. showed higher amount of soluble phosphorus and total numbers of bacteria compared to control. Under field condition, isolate BS-290 increased the nitrogen and phosphorus concentrations in shoot dry matter, phosphorus concentration in dry soil and the total number of bacteria in the soil, compared to the control. Isolate BS-320 increased the phosphorus concentration in plants and maize yield, which strongly suggests its use as a biological inoculant for maize crops. BS-248, BS 290 and BS-320 isolates promoted the highest maize growth, compared to the other isolates. The results showed that *Bacillus subtilis* isolates that promote plant growth did not increase similar traits in all plants and the behavior has been highly isolate-dependent. This finding shows that the isolates had a preference and certain specificity on each individual trait.

Keywords: *Bacillus subtilis*, phosphorus solubilization, nitrogen-fixing, inoculation.

Abbreviations: SDM_shoot dry matter; RDM_root dry matter; CFU_colony forming units; IAA_indole acetic acid; PSM_phosphate solubilizing microorganisms.

Introduction

Brazil is the third largest maize producer and the second largest exporter worldwide, making this country a great competitor of the USA and China according to the USDA (2016). Maize is considered as one of the main cereal crops based on its production, especially as a winter crop (autumn and winter). This productivity demonstrates the strategic importance of Brazil offering this product, considering the increase in maize worldwide (CONAB, 2018).

To maintain a high maize crop yield, fertilizers rich in nitrogen and phosphorus have considerable economic importance due to their direct relationship with production costs. Under certain conditions, fertilizers can have environmental impacts such as soil salinization, underground water contamination and river eutrophication (Nguyen et al., 2019). Considering these factors, it becomes crucial to search for strategies to reduce the use and improve the absorption efficiency of fertilizers without reducing yield or promoting environmental damage.

In the search for alternatives to reduce this problem, many advances have used microorganisms that are able to fix nitrogen in the soil and solubilize phosphorus for plants (Knoth et al., 2013; Zafar et al., 2012). In this context, the use of plant growth-promoting rhizobacteria (PGPR) is

promising. The benefits can occur directly on plant growth and indirectly by reducing the incidence of phytopathogens (Abdallah et al., 2017).

Among the plant growth-promoting rhizobacteria, some species are able to enter the plant through the stomata and roots to become established as endophytes (Azevedo, 1998), which can be classified as endophyte plant growth-promoting rhizobacteria. Although free-living rhizobacteria and endophytic bacteria are living in different ecological niches but they use similar mechanisms to promote plant growth (Doty, 2013). PGPR can promote plant growth through several mechanisms, such as nitrogen fixation, phytohormone production, siderophore synthesis, and phosphorus solubilization. Furthermore they have indirect benefits to plant development, such as biocontrol and disturbing quorum sensing (Bhattacharyy and Jha, 2012)

Among PGPR, the genus *Bacillus* is comprises diverse species of rhizospheric and endophytic bacteria with the ability to synthesize phytohormones that can favor root growth by increasing root hair (Angulo et al., 2014). Moreover, their application to maize can contribute to plant growth, development and nutrition (Araujo and Guerreiro, 2010; Lin et al., 2018). *Bacillus* spp. are gram-positive bacteria that

produce endospores resistant to adverse conditions, such as high temperature, solar radiation, and dehydration, which contribute to their use as agricultural biofertilizers (Bergey and Holt, 1994). Thus, the use of *Bacillus* spp. as a biological inoculant is an alternative to increase fertilization efficiency, aiming to reduce the use of mineral fertilization.

Thus, the present study evaluated the effect of endophytic *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus velezensis* bacterial isolates as plant growth promoters in maize crops under two conditions of greenhouse and field.

Results and Discussion

Traits measured

The experiment showed that some bacterial isolates significantly affected ($p < 0.05$) plant height and SDM (Figures 1 A-B). The increase in plant height was obtained with BS-320 (97.6 cm), whereas the plant height in the control was 76 cm. Plants inoculated with BS-263 were shorter than the control plants.

For SDM and RDM, significant differences were observed between BS-320 and BS-248 isolates and the control. For SDM, the BS-263 isolate resulted in a lower height than the control (Figures 1A, C).

Usually, plant height, SDM and RDM are promoted by the presence of phytohormones. Although phytohormones promote plant growth, some microorganisms produce these compounds to benefit themselves. Phytohormones increase the plant root contact surface, allowing considerable contact between plants and microorganisms, increasing the efficiency, by which plants obtain nutrients and the carbohydrates from plants. Increased RDM is a direct consequence of the action of phytohormone-producing microorganisms on roots, which promote an increase in root efficiency to obtain water and nutrients from the soil (Wang et al., 2010; White et al., 2014). Interestingly, the BS-263 isolate reduced plant growth. This result was unexpected since BS-263 is also a *B. subtilis* isolate with many characteristics of plant growth promotion, including IAA production.

Phytohormones

An *in vitro* test is necessary to determine the abilities of microorganisms (Rocha et al., 2017). In the *in vitro* test, the BS-320 isolate produced $12.41 \mu\text{g}$ of IAA mL^{-1} , whereas the BS-263 isolate produced $11.32 \mu\text{g}$ of IAA mL^{-1} (data not shown). However, *in vitro* results sometimes do not occur under soil conditions. Interestingly, the BS-263 isolate, even as an IAA producer, limited plant development under soil conditions.

Some results regarding the use of plant-growth microorganisms are inconsistent (Gouda et al., 2018). Certainly, this inconsistency occurs due to natural variation in the parameters analyzed. Many studies are needed to understand how isolates with many plant growth-promoting characteristics can inhibit plant growth. These isolates under specific conditions likely act as plant competitors via nutrient uptake. The plant and BS-263 interaction was competitive.

Root microbiota members appear to serve additional host functions through the acquisition of nutrients from the soil

for plant growth. Rhizodeposition appears to fuel an initial substrate-driven community shift in the rhizosphere, which converges with host genotype-dependent finetuning of the microbiota profiles in the selection of root endophyte assemblages (Bulgarelli et al., 2013). These results strongly suggest that slight genotypic differences among isolates, including their soil establishment and plant interaction abilities are responsible for the different plant growth levels promoted by each isolate. The abilities of these isolates, such as IAA production, nitrogen fixation and phosphorus solubilization are important but do not guarantee plant growth promotion.

The interactions between plants and microorganisms are influenced by a wide variety of factors, such as genotype, growth state, physiological status, plant tissue type, agricultural practices, and environmental conditions, such as temperature, water availability and nutrients (Compant et al., 2010). More studies are needed to understand the different effects of bacterial isolates of the same species on plant growth promotion.

The chlorophyll content varied from 15.34 to $35.17 \mu\text{g mL}^{-1}$, and the nitrogen content in SDM varied from 2.10 to 3.12 g kg^{-1} . However, there was no statistically significant difference ($p < 0.05$) among the treatments for either parameter.

Plants that received BS-320, BS-290 and BS-248 isolates presented higher nitrogen content in the roots, compared to control plants. Interestingly, the same isolates that promoted higher RDM also promoted higher nitrogen content in the roots, except for the BS-290 isolate for RDM (Figure 1 C and Figure 2 A). Moreover, the BS-320 isolate promoted higher SDM (Figure 1 B). Under field conditions, higher nitrogen contents were promoted by BS-248 and BS-290 (Figure 3 A). These results show that some isolates similarly promote high RDM, SDM and nitrogen contents. There is a positive correlation between these parameters and the presence of phytohormones produced by microorganisms. Phytohormones are organic substances that can promote, inhibit, or modify the growth and development of plants at low concentrations (Damam et al., 2016). Phytohormones promote root cell proliferation by overproducing lateral roots and root hairs with a concomitant increase in nutrient and water uptake (Sureshbabu et al., 2016). In this context, the BS-320 isolate was the most competent.

P content

Regarding the P content in SDM and in the soil, BS-320 and BA-001 isolates promoted the highest P content for both parameters, whereas the BS-290 isolate promoted the highest P content in soil only. Under field conditions, the highest P content in SDM was promoted by BS-320 (Figure 3 B), whereas for BS-290, there was no statistically significant difference among treatments for the P content in the soil (Figure 3 C).

Phosphorus is the second most important nutrient after nitrogen and is required for plant growth. This nutrient is an essential element in all living systems, and phosphate-solubilizing microorganisms (PSM) play a very important role in phosphorus nutrition by enhancing its availability to plants through its release from inorganic and organic soil

Table 1. Description of the treatments.

Treatments	Isolates	ID code
1	<i>Bacillus amyloliquefaciens</i>	BA-001
2	<i>B. velezenses</i>	BV-188
3	<i>B. subtilis</i>	BS-248
4	<i>B. subtilis</i>	BS-263
5	<i>B. subtilis</i>	BS-274
6	<i>B. subtilis</i>	BS-287
7	<i>B. subtilis</i>	BS-290
8	<i>B. subtilis</i>	BS-291
9	<i>B. subtilis</i>	BS-309
10	<i>B. subtilis</i>	BS-320
11	Control	Control

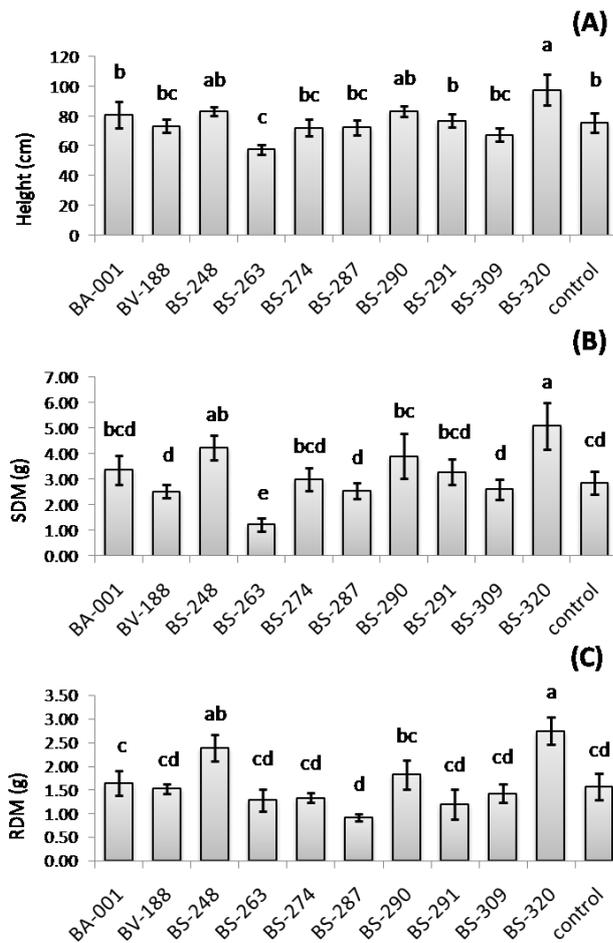


Fig 1. Height (A), shoot dry matter (SDM) (B) and root dry matter (RDM) (C) of maize plants inoculated with 10 endophytic isolates of the genus *Bacillus* and a control without inoculation from 60 DAS. Bars represent average standard errors. Means with different letters are significantly different according to Duncan's test ($p < 0.05$).

Table 2. Results of soil analyses.

Conditions	pH	OM	P (resin)	K	Ca	Mg	H+Al	BS	CEC	V
	CaCl ₂	g dm ⁻³	mg dm ⁻³mmol _c dm ³						%
Greenhouse	6.5	11	20	0.7	19	5	17	24.4	42.8	64
Field	6.0	23	29	16	10	11	10	21	25.4	58

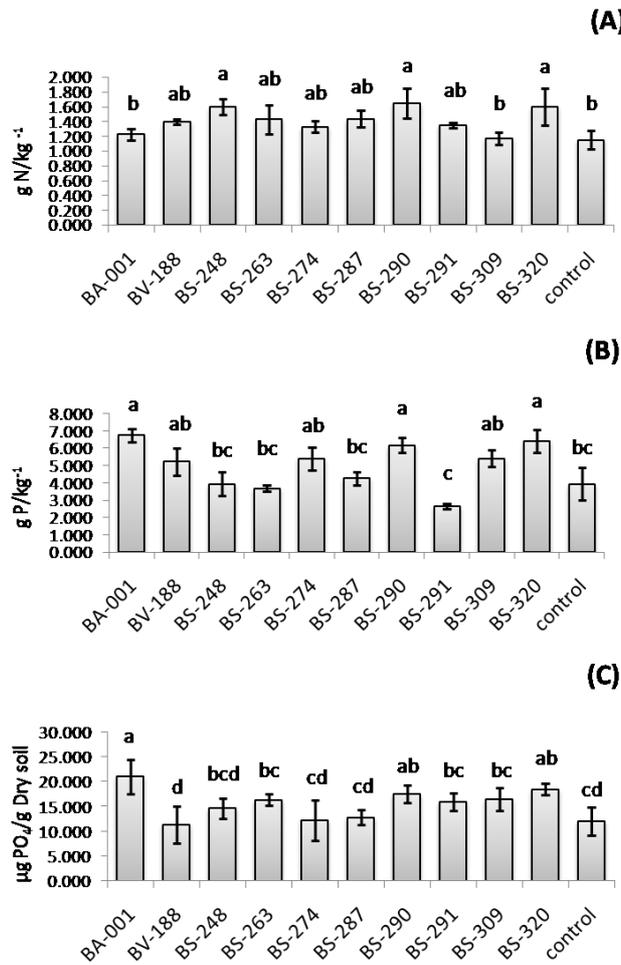


Fig 2. Nitrogen content in roots (A) and phosphorus content in shoots (B) of maize plants and soluble P content in soil (C) of vases inoculated with ten endophytic isolates of the genus *Bacillus* and a control without inoculation from 60 DAS. Bars represent average standard errors. Means with different letters are significantly different according to Duncan's test ($p \leq 0.05$).

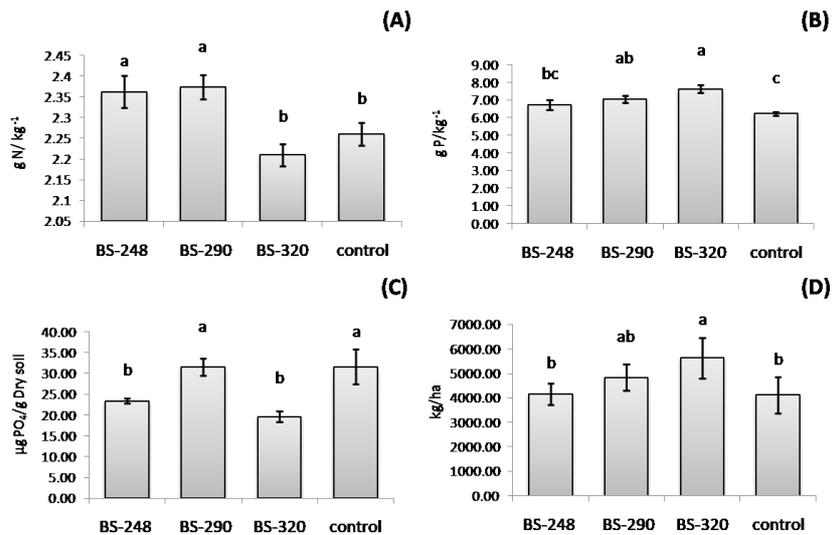


Fig 3. Nitrogen content in shoots (A), phosphorus content in shoots (B), soluble P content in the soil (C) and yield (D) of maize plants inoculated with three *B. subtilis* strains (BS-248, BS-290 and BS-320) and a control without inoculation. Bars represent average standard errors. Means with different letters are significantly different according to Duncan's test ($p \leq 0.05$).

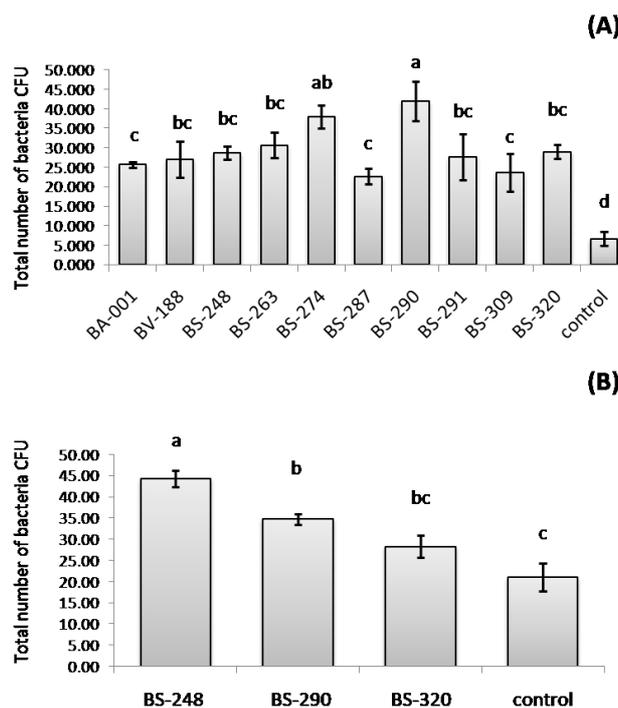


Fig 4. The total number of colony forming units (CFU) of *Bacillus* spp. in the soil of vases inoculated with ten endophytic *Bacillus* strains and a control from 60 DAS in phase I (A) and the number of colony forming units (CFU) of *Bacillus* spp. in the soil under field conditions inoculated with three endophytic *B. subtilis* isolates (BS-248, BS-290 and BS-320) and a control without inoculation in phase II (B). Bars represent average standard errors. Means with different letters are significantly different according to Duncan's test ($p \leq 0.05$).

phosphorus sources by solubilization and mineralization. However, many microorganisms have the ability to solubilize phosphorus, and few are able to increase the P content in plants (Anand et al., 2016). In this context, BS-320, BA-001, and BS-290 isolates were the most competent under greenhouse conditions, and under field conditions. The most competent isolates were BS-320 and BS-290. Interestingly, the BS-320 isolate that promoted the highest P content in SDM also promoted the lowest P content in the soil. These results suggest that this isolate had a close relationship with the plant, making it more efficient in the absorption of phosphorus (Figures 3 B-C).

Most soils have sufficient amounts of plant nutrients, but these compounds are often present in insoluble forms that are unavailable for uptake by plants (Shafi et al., 2017). Rhizobacteria *Bacillus* species secrete a number of metabolites that can strongly increase nutrient availability to plants (Sivasakthi, et al., 2014), including the improvement of the solubilization of sparingly soluble phosphate compounds in soils, resulting in a higher crop yield and increase in the concentration of soluble P (Wang et al., 2014).

The only isolate that promoted a yield increase compared to the control was BS-320. The yield was increased approximately one ton per hectare when maize received the BS-320 inoculant. This result strongly suggests that this yield increase was the result of many characteristics of this isolate, allowing this organism to promote plant growth.

Interestingly, under greenhouse conditions, all treatments that received microbial isolates presented more bacteria than the control, which did not receive any isolates (Figure 4

A). Under field conditions, BS-248 and BS-290 isolates showed more bacteria than control (Figure 4 B). Thus, the isolates had the ability to become established in the soil. This characteristic is important for the isolates to interact with the plant and express their capacity to promote plant growth (Bulgarelli et al., 2013). However, the only isolate that promoted a yield increase was BS-320. This result suggests that bacterial establishment in the rhizosphere does not require a large microbial population but rather efficiency and a close interaction with plants.

Materials and Methods

This study was carried out in two phases (I and II). Phase I corresponded to the experiment conducted in greenhouse conditions with ten *Bacillus* spp. isolates (Table 1) and a control without inoculation for a period of 60 days after emergence (DAE). Phase II corresponded to the experiment conducted under field conditions using three *Bacillus subtilis* isolates, which were selected due to their high performance in terms of most parameters evaluated in the greenhouse conditions, compared to those of the control plants without inoculation.

The seeds were obtained from the commercial 2B587PW hybrid (Dow Agro Sciences).

For phase I, the experiment was carried out in a greenhouse belonging to the Laboratory of Microbiology of the Department of Plant Production - UNESP – campus of Jaboticabal, state of São Paulo (21°14'34"S, 48°17'54"W and 613,41 m a.s.l.).

The bacterial isolates used in this study belonged to the Laboratory of Microbiology collection of FCAV-UNESP, Jaboticabal Campus. Each bacterial isolate was inoculated in 125 mL Erlenmeyer flasks containing 60 mL of nutrient broth (1.0 g/L beef extract; 2.0 g/L yeast extract; 5.0 g/L peptone; 5.0 g/L NaCl, pH of 6.8 ± 0.2), and isolates were incubated at 28°C for 24 hours. Subsequently, samples were standardized by using spectrophotometers at 630 nm (Kloepper et al., 1989). The concentration was adjusted to 1.5×10^8 colony forming units per mL (CFU mL⁻¹).

Three maize seeds were immersed in each bacterial suspension for 30 minutes. Then, the seeds were sown in vases with 5 dm³ of previously sifted Eutrosol soil. Soil fertilization was performed according to chemical analysis (Table 2), and the fertilization recommended for maize crops was applied according to Cantarella et al. (1997). One week after plant emergence, thinning was performed, leaving one plant per pot. The experimental period was 60 days, and bacterial inoculation was performed every 10 days with 10 mL of bacterial suspension per pot, totaling six bacterial inoculations throughout the experiment. The experimental design included randomized blocks with 11 treatments, three replicates and 33 pots. The treatments were 10 *Bacillus* spp. isolates, such as *B. amyloliquefaciens* (BA), *B. velezensis* (BV) and *B. subtilis* (BS), and the control without bacterial inoculation.

Plant height was measured from the base of the plant to the apex with the aid of a tape measure. Then, the plants were collected and separated into roots and shoots. Both parts were incubated in an oven with forced air ventilation at 65°C for 72 hours until they reached a constant weight. Root and shoot dry matter determinations were made on an analytical scale.

The chlorophyll content was measured by spectrophotometer analysis. For this analysis, some leaf squares were cut (1 cm²). Then, each leaf square was cut into smaller pieces and placed in an Eppendorf tube containing 5 mL of dimethylformamide. The tubes were stored in a refrigerator at 8°C for 72 hours in the dark. Then, an aliquot of 3 mL of liquid extract was collected for reading in a spectrophotometer at 470, 647 and 664 nm. The chlorophyll content was determined according to the equation proposed by Wellburn (1994).

For nitrogen determination, the plant material was ground in a Willey-type mill (20 mesh) and submitted to leaf nitrogen analysis according to Bremner (1996) and modified by Bezerra Neto and Barreto (2011), where 0.1 g of the plant sample was weighed and digested using 7 mL of sulfuric acid. Subsequently, 10 mL of distilled water was added. Distillation was performed by using the Kjeldahl method with the aid of 25 mL of NaOH (50%). The distilled material was recollected in 10 mL of boric acid as an indicator solution, resulting in 20 mL of distilled material. Ammonium titration was performed using 0.05 N H₂SO₄ as the standard. The phosphorus concentration (P) was determined by nitro-perchloric digestion followed by the molybdate vanadate colorimetric method according to Haag et al. (1975) with a few modifications (Bezerra Neto and Barreto, 2011). A sample of 0.5 g of plant tissue from the digester tube was added to 5 mL of concentrated nitric acid and 1 mL of concentrated perchloric acid. The mixture was left to rest for one day. Then, complete digestion was performed using a block digester. The material was washed with distilled water

to obtain 50 mL of extract. A reading was performed in a spectrophotometer at 470 nm using 5 mL of extract plus 1 mL of specific reagent composed of a mixture of 5% ammonium molybdate and 0.25% ammonium vanadate.

For soil evaluations, soil samples were collected from each pot. Samples were divided into two parts. The first part was used for the phosphorus measurement and was sifted and dried at room temperature. The second part was stored in a refrigerator at 8°C to count the total *Bacillus* spp. Soluble phosphorus was measured according to Watanabe and Olsen (1965), where 0.6 g of dry soil was sampled and transferred to Erlenmeyer flasks containing 12 mL of extractor sodium bicarbonate solution and Whatman filter paper. For determination, 2.0 mL of sodium bicarbonate was pipetted, and 0.2 mL of sulfur solution (5 M) and 0.8 mL of B reagent was filtered. Then, the material was incubated at 45°C for 20 minutes. Next, a reading was taken using a spectrophotometer at 820 nm. The counting of *Bacillus* spp. colonies was performed in Petri dishes by serial dilution according to Wollum (1982) associated with the hot shock proposed by Rothfuss et al. (1997) and Barroti and Nahas (2000).

Under greenhouse conditions, the *Bacillus* spp. selected were those that showed significantly higher numbers in the major evaluated parameters. These *Bacillus* were selected for use in field conditions.

Phase II was carried out in field conditions at a farm belonging to UNESP - University. Sowing was performed on 8/03/2017. Soil was classified as a Eutrosol. Before the start of the experiment, soil was collected and chemically analyzed, and its fertility was measured: pH_{CaCl2} = 6; organic matter = 23 g dm⁻³; P_{resin} = 29 mg dm⁻³; K = 16 mmol dm⁻³; Ca = 10 mmol dm⁻³; Mg = 11 mmol dm⁻³; SB = 21 mmol dm⁻³; cation exchange capacity = 25.4 mmol dm⁻³; and V% = 58. The spacing was 0.90 m between rows, and six plants were placed per linear meter. Soil fertilization was based on soil analysis according to Cantarella et al. (1997). Each experimental plot was 6 meters in length per 3.6 m of width. Soil samples were collected before flowering, and the area was 9 m² per plot. Maize was harvested on June 31, 2017, in the same useful area. Treatments that received *B. subtilis* BS-248, BS-290, and BS-320 followed the same methodology described under greenhouse condition. The concentration was 1.5×10^8 CFU mL⁻¹, which was the same as that used under greenhouse conditions. Four applications of bacteria were made every 10 days via soil with the aid of a costal sprayer. Each plot received 240 mL of the bacterial mixture and 1,500 mL of water. The control received water only.

The experimental design was a randomized block design with four isolates and six replicates, resulting in 24 plots with 27 m² each.

The plants were evaluated for shoot and root dry matter and nitrogen and phosphorus concentrations, and the methodology used was the same as that used in the greenhouse conditions. Plant samples were composed of three plants randomly collected in each plot. For nutrient analysis, leaves below the cob were collected.

Yield was evaluated through manual harvesting from each area. Yield data were adjusted to correspond to 13% humidity and transformed into hectares (kg ha⁻¹).

The soil analyses were of the phosphorus content and total number of *Bacillus* spp. according to the methodology described for greenhouse conditions. The soil samples were

collected as compost samples and taken from the used area from each plot. The preparation and storage of these soil samples were the same as described for greenhouse conditions.

The data obtained were submitted to analysis of variance (F test), in which the treatment averages were compared by Duncan's test with a 5% significance level using AgroEstat software version 1.0 (Barbosa and Maldonato, 2010).

Conclusion

Although all isolates presented plant growth promotion abilities, the isolates showed different growth promotion levels. These results were most likely due to slight genotypic differences among isolates. The findings of this study show the importance of understanding the behavior of isolates on individual traits and plant growth. Some isolates, even those that present plant growth promotion characteristics can in some cases unexpectedly inhibit plant development.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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