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Isolates of *Bacillus* sp. from garlic: effect on corn development and plant growth-promoting mechanisms

William Gilberto Balbinot[(1)], Sabrina Rodrigues[(1)] and Glória Regina Botelho[(1)*]

[(1)] Universidade Federal de Santa Catarina, *Campus* Curitibanos, Curitibanos, Santa Catarina, Brasil.

ABSTRACT: Corn and garlic are important crops to Curitibanos region (state of Santa Catarina – Brazil), often planted in alternate cropping seasons. Production costs are high, especially due to N fertilizer, since they are highly demanding in N. In addition to reducing economic costs, the search for environmentally sustainable technologies has stimulated the study of interactions between plants and growth-promoting microorganisms. Rhizobacteria, e.g., *Bacillus* sp., have been presenting as growth-promoting microorganisms. Five isolates of garlic rhizosphere from 27 individuals of the Plant Growth Promoting Microorganisms group collection were tested on corn under field conditions, comparing to two levels of nitrogen fertilization: 120 and 60 kg ha^{-1}. The *Bacillus* collection was also evaluated *in vitro* for phosphate solubilization, production of IAA (Indole Acetic Acid), extracellular enzymes, and inhibition of *Sclerotium cepivorum*. For plant height and stalk diameter, the inoculation of the EB16 isolate showed similar results to the fertilization with 120 and 60 kg ha^{-1} of N in corn. Both EB16 and EB02 isolates increased corn ear diameter and the yield was similar to that observed in the treatment with 60 kg ha^{-1} of N, indicating their potential as growth-promoters. All strains of the collection produced IAA, and most of them solubilized calcium phosphate and produced lipases and urease. Forty-eight percent of the isolates inhibited *S. cepivorum*. The EB01, EB15, EB17, and EB27 were positive for three of the four mechanisms analyzed. During these evaluations, it was observed that EB02 and EB16 produced equivalent amounts of IAA, suggesting that more than one growth-promoting mechanism is involved in the efficiency of corn development induction.

Keywords: rhizobacteria, *Zea mays* L, merril. *Allium sativum*, growth-promoters.

*** Corresponding author:**
E-mail: gloria.botelho@ufsc.br

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INTRODUCTION

In the region of Santa Catarina plateau, where is located the Curitibanos county, in Santa Catarina State, Brazil, agriculture is an important activity, with 2,061 farms, of which 71.85 % are focused on small-scale farmers (CEPA, 2018). The main crops are soybeans, corn, beans, garlic, and onions (CEPA, 2018). Corn (*Zea mays* L.) and garlic (*Allium sativum* L.) are alternately cropped in many farms: corn is grown in spring/summer and garlic, in autumn/winter. A common feature of both crops is the high N requirement (Cruz et al., 2008; Lucini, 2010). Nitrogen fertilization is one of the most expensive agricultural inputs to the production costs (Silva et al., 2005; Silva, 2017). Besides the economical aspect, the environmental impact caused by excessive and continued use of these inputs must be considered (Resende, 2002).

There is a demand for sustainable agricultural systems that could add value to farm products, in addition to reducing pressure on the environment. For this reason, ecological methods that maintain productivity are studied. One of the alternatives to reduce these costs is the use of Plant Growth Promoting Microorganisms (PGPM), such as nitrogen-fixing bacteria (Fukami et al., 2018), Plant Growth-Promoting Rhizobacteria (PGPR) (Leoncio and Botelho, 2017; Turatto et al., 2018,), and mycorrhizal fungi (Berbara et al., 2006). These microorganisms can play a relevant and strategic role to ensure high productivity at low cost, in addition to environmental benefits (Valadares-Inglis and Lopes, 2019). Several PGPR have been studied for their ability to stimulate plant development (Souza et al., 2015; Fukami et al., 2017). Among the benefits are BNF (Biological Nitrogen Fixation) (Fukami et al., 2018), ability to solubilize natural phosphates (Souchie et al., 2007; Ayyaz et al., 2016), production of plant hormones, such as indole acetic acid (IAA) (Cattelan, 1999; Ayyaz et al., 2016), production of extracellular enzymes, siderophores production (Compant et al., 2013; Batista et al., 2018), and antibiosis (Khan et al., 2018; Turatto et al., 2018).

Many plant-growth-promoting rhizospheric bacterial genera have been described, and *Azospirillum*, *Pseudomonas*, and *Bacillus* are the most studied (Ayyaz et al., 2016; Fukami et al., 2017; Botelho et al., 2019; Mpanga et al., 2019). The genus *Bacillus* is part of the main rhizobacteria group, which can induce plant development either directly (Lima et al., 2011; Leoncio and Botelho, 2017) or indirectly (Braga Júnior et al., 2017; Fira et al., 2018). These rhizobacteria influence germination, development, and yield of the crop due to several common mechanisms to PGPR (Kupper et al., 2003; Beneduzi et al., 2012; Khan et al., 2018; Turatto et al., 2018).

Twenty-seven *Bacillus* isolates was obtained from garlic rhizosphere samples grown in Nfb medium (Leoncio and Botelho, 2017). Five selected isolates showed potential for corn growth induction like N fertilization at a greenhouse experiment (Leoncio and Botelho, 2017). For this reason, this study aimed to evaluate the performance of these *Bacillus* sp. under field conditions and to analyze the mechanisms for promoting plant growth *in vitro*.

MATERIALS AND METHODS

Corn development inoculated with *Bacillus* isolates

The *Bacillus* isolates were obtained from the garlic soil rhizosphere brought from Dias farm in Curitibanos (SC) (Leoncio and Botelho, 2017). From a collection of 27 isolates, 14 were identified as belonging to the genus *Bacillus* by 16S RNA gene sequencing. Five isolates from the collection (EB02, EB12, EB14, EB16, and EB23) were evaluated in the greenhouse and showed potential for promoting corn growth and then, they were selected for the field experiment.

The field experiment was carried out at the Experimental Farm of the Universidade Federal de Santa Catarina - *Campus* Curitibanos, in Curitibanos county (SC), Brazil, during the

season 2017/2018 (27° 27' 38.15" S and 50° 50' 32.13" W, with an approximate elevation of 1,000 m). According to Köppen's classification system (2003), the region is under temperate Cfb climate, humid mesothermal, and mild summer. According to Brazilian Soil Classification System (Santos et al., 2018), the soil was classified as *Cambissolo Háplico*, equivalent to Inceptisols (Soil Survey Staff, 2014) and its chemical properties are described in table 1.

To prepare the inoculum, each isolate was inoculated in 250 mL of liquid Luria Bertani (LB) medium and incubated at 30 °C for 24 h. The Colony Forming Units (CFU) reached 10^8 CFU mL^{-1}. Three uninoculated flasks were kept under the same conditions for the control, 50 and 100 % of the N dosage recommended for the crop.

After the incubation, seeds with 30F53 RR (Roundup Ready) gene were grouped in batches of 180 seeds, placed into the inoculated and non-inoculated flasks during 2 h. After that, they were placed on paper towels in trays for drying during 1 h. The entire procedure was performed in laminar flow.

Sowing was performed on October 11, 2017. The experiment was conducted in a randomized block design, with eight treatments (Table 2) and four replicates. The plots were 3 m long by 4 m wide, spaced by 1 m. Plots had nine sowing lines, spaced of 0.50 m. The working area of each plot is the three central lines, with 1.35 m^2. Two seeds were placed in each position to avoid failures. Nine days after sowing (DAS), thinning was carried out to remove surplus plants from double planting.

Nitrogen fertilization was performed with urea (45 % N) at a dose of 120 kg of N (266 kg of urea) per hectare, for full dose (100 %) and 60 kg of N per hectare (133 kg of urea), for half dose (50 %), during the stages V3-V4 of corn development.

The evaluated parameters were plant height (cm), stalk and ear diameters (mm), leaf nitrogen content (g kg^{-1}), and grain yield (kg ha^{-1}). During male flowering, plant height, stalk diameter, and leaf nitrogen content was measured from five plants in the working area of each plot. Height was measured from the base of the plant near

Table 1. Soil chemical properties of the experimental area

Property	Value
pH($CaCl_2$)	5.7
P (mg dm^{-3})	25.39
K ($cmol_c$ dm^{-3})	0.21
Ca^{2+} ($cmol_c$ dm^{-3})	11.68
Mg^{2+} ($cmol_c$ dm^{-3})	5.64
Al^{3+} ($cmol_c$ dm^{-3})	0
H + Al ($cmol_c$ dm^{-3})	3.69
OM (g dm^{-3})	39.9
BS ($cmol_c$ dm^{-3})	17.53
CEC pH 7 ($cmol_c$ dm^{-3})	21.22
Zn (mg dm^{-3})	3.84
Fe (mg dm^{-3})	17.32
V (%)	82.61
Mn (mg dm^{-3})	20.16
Cu (mg dm^{-3})	2.35

pH($CaCl_2$) was measured 1:5 soil:solution ratio (Teixeira, 2017). P, K, Fe, Zn, Cu, and Mn were extracted with Mehlich-1 (H_2SO_4 0.05 mol L^{-1} + HCl 0.125 mol L^{-1}); Ca^{2+}, Mg^{2+}, and Al^{3+} were extracted by KCl 1 mol L^{-1}. H+Al: extracted with calcium acetate at pH 7.0. OM (organic matter) = 1.72 × OC (Organic Carbon) determined by Walkley and Black method modified by Tedesco et al. (1995). CEC pH 7 is the sum of the concentrations of Ca^{2+}, Mg^{2+}, and H+Al.

Table 2. Description of experimental treatments

Treatments	N doses
T1- Control without fertilization or inoculation	-
T2- Without inoculation and top-dressing of 50 % of the required N.	60 kg ha^{-1} of N
T3- Without inoculation and top-dressing of 100 % of the required N.	120 kg ha^{-1} of N
T4- *Bacillus* sp. EB02 isolate inoculation	-
T5- *Bacillus* sp. EB16 isolate inoculation	-
T6- *Bacillus* sp. EB12 isolate inoculation	-
T7- *Bacillus* sp. EB23 isolate inoculation	-
T8- *Bacillus* sp. EB14 isolate inoculation	-

the ground to the last leaf, using a graduated measuring tape. The stalk diameter was determined at the second node above ground using a digital pachymeter. For leaf N, the first leaves opposite to the first ear were removed, in which the middle third was dried in a forced-air convection oven at 45 °C until constant weight and subsequently grinded. The N content was determined according to the procedures of Tedesco et al. (1995) (Nogueira and Souza, 2005).

At the end of the cycle, all plants of each plot were harvested and the yield was determined. Grain samples were dried at 50 °C to adjust their weight to 13 % humidity and to calculate productivity (kg ha^{-1}).

Data were analyzed by ANOVA (F test) at 5 % significance level and when significant, the Scott-Knott test was performed at 5 % significance level to comparison of means, using the RStudio software (version 1.1.423).

Mechanisms of plant growth promotion of *Bacillus* isolates *in vitro*

For characterization of direct and indirect mechanisms of plant development induction by *Bacillus* sp. collection from garlic, phosphate solubilization, IAA (indole acetic acid) and extracellular enzyme production, and antibiosis were determined.

For *in vitro* phosphate solubilization analysis, isolates were grown into 5 mL of liquid LB medium (pH 7.2) incubated for 72 h at 25 °C. After growth, 100 µL from each strain was transferred into plates containing solid solubilizing medium (Table 3). Each plate was inoculated with four isolates at equidistant points. Each set had five repetitions. Solubilization halos around the bacterial colonies and their diameters were measured at 72-hour intervals. The solubilization index (SI) was given by equation 1 (Chagas Junior et al., 2010).

$$IS = \frac{Halo\ diameter\ (mm)}{Colony\ diameter\ (mm)} \qquad \text{Eq. 1}$$

For *in vitro* indole acetic acid (IAA) production, the isolates were grown into 5 mL of tryptophan-enriched liquid LB medium (0.005g mL^{-1}) during 48 h at 26 °C. Each isolate had three replications. For qualitative analysis, 1 mL of the bacterial suspension was incubated with 1 mL of Salkowski's reagent (H_2SO_4 7.9 mol L^{-1} with 12 g of $FeCl_3$) for 30 min in the dark. After this period, the samples showing pink color were considered positive for IAA production (Marchioro, 2005). Then, it was quantified by spectrophotometry Bel SPECTRO S-2000 in which the optical density (O.D.) was determined at 540 nm. For the standard curve, 3-Indoliacetic Acid (PS) was used at concentrations of 2 µg mL^{-1}, 5 µg mL^{-1}, 8 µg mL^{-1}, 10 µg mL^{-1}, 20 µg mL^{-1}, 50 µg mL^{-1}, 80 µg mL^{-1}, 100 µg mL^{-1}, 200 µg mL^{-1}, 500 µg mL^{-1}, 800 µg mL^{-1}, and 1000 µg mL^{-1}. Each solution was mixed with Salkowski's reagent at a ratio of 1:1 and measured at OD 540 nm.

Table 3. Solubilizing and urease media trials

Trial	Amount	Compound
Solubilizing Medium	7.50 g	Ca_3PO_4
	15.0 g	Glucose
	0.75 g	$(NH_4)_2SO_4$
	0.30 g	NaCl
	0.30 g	KCl
	0.15 g	$MgSO_4.7H_2O$
	0.015 g	$MnSO_4$
	0.015 g	$FeSO_4$
	22.5 g	Agar
	1000 mL	Distilled water
Medium for Urease test	8.0 g	Urea
	0.04 g	Yeast extract
	3.80 g	NaH_2PO_4
	3.64 g	K_2HPO_4
	20 mL	Phenol red 0.18 %
	380 mL	Distilled water
	pH 6.8	

To verify the indirect plant growth promotion mechanisms, the isolates were tested for their ability to produce extracellular enzymes and *in vitro* antibiosis. The pathogen chosen was the fungus *Sclerotium cepivorum* for its virulence to garlic, besides the absence of efficient fungicides for its control.

The extracellular enzymes evaluated were lipase, urease, protease, and chitinase. To lipase analysis, isolates were grown in 5 mL of liquid LB medium for 24 h at a temperature of 26 °C. Then, with a loop, the bacterial suspension was transferred to four equidistant edges in plates containing minimal medium (MM) (Cove, 1966), plus 0.001 % Rhodamine B with 1 % olive oil (Oliveira et al., 2006). Each set of four isolates had five repetitions. The fluorescent spots around or on the bacterial colonies determined enzymatic activity under ultraviolet light (365 nm) (Torquato et al., 2016).

For the urease analysis, each isolate was inoculated in 5 mL of urea medium (Table 2) and kept at 28 °C during a week. Each isolate had five replications. The medium color change, from orange to reddish pink, determined urease production.

For protease analysis, *Bacillus* isolates were grown in 5 mL of liquid LB medium for 24 h at 25 °C. After that, with a loop, each bacterial suspension was transferred into four equidistant edges in plates containing minimal medium (MM) plus 1 % casein (Oliveira et al., 2006). Each set of four isolates had five repetitions. The transparent halo around the colony showed protease production.

For chitinase evaluation, isolates were grown in 5 mL of liquid LB medium for 24 h at 25 °C. After that, with a loop, each bacterial suspension was transferred into four equidistant edges of plates containing MM plus 0.8 % colloidal chitin and 0.078 % NH_4NO_3 (Cattelan et al., 1999). Each set of four isolates had five repeats. Enzyme activity was observed by the transparent halos around the colony.

For the analysis of *S. cepivorum* inhibition *in vitro* (antibiosis), the fungus was transferred to the center of plates containing PDA medium (Potato Dextrose Agar) at 25 °C for three days. Then, an aliquot was placed in the center of another BDA plate and kept at 25 °C for 48 h. Twenty-four hours after fungus inoculation, the isolates were inoculated in 5 mL

of liquid LB medium for a further 24 h. After that, 100 µL aliquots of four isolates were transferred into four equidistant edges in PDA plate. Each set had three repetitions, kept at 25 °C. The evaluations were taken from the first 24 h after isolates inoculation and at 72 h interval for nine days. Initially, only the inhibition halos presence was checked.

The isolates that produced inhibition halos were submitted to the same procedure for subsequent halo measurement. This generated an inhibition degree (GI), determined by equation 2:

$$GI = \left[\frac{\left(\frac{Dtf - Dcf}{Dft} \right)}{Hi} \right] \times 100 \qquad \text{Eq. 2}$$

in which: Dtf is the fungal colony diameter without bacteria (control); Dcf is the fungal colony diameter with each isolate and the inhibition halo (Hi), already discounting the size of the bacterial colony. The final value was multiplied by 100 to obtain the inhibition percentage.

All evaluations were performed in an entirely randomized design (DIC) and the results were analyzed by ANOVA (F test) at 5 % significance level, and when significant, the Scott-Knott test was performed at 5 % significance level to a comparison of means, using the RStudio software (version 1.1.423).

RESULTS

Effect of *Bacillus* sp. isolates on corn development

The isolates EB02, EB12, EB14, EB16, and EB23 tested in the greenhouse showed a positive effect on plant growth, similar to the supplying 50 % of the required N (Leoncio and Botelho, 2017). These isolates were obtained from the medium without N supply, suggesting their BNF capacity. The results stimulated to evaluate their performance under field conditions.

Nitrogen leaf content showed no statistical difference among the assessed parameters. To the others, isolates EB02 and EB16 showed similar performances to 50 and 100 % of the N fertilization recommended for corn (Figures 1 and 2).

Concerning plant height, the EB16 isolate was similar to treatments with 100 and 50 % of required N, and they differed from the other treatments (Figure 1a). The plant growth induction of EB16 isolate reached 12 %, similar to 100 and 50 % of the required N (12.5 and 11.3 %, respectively) to the control. A similar result was observed for stalk diameter, in which the inoculation of EB16 was similar to those obtained at 100 and 50 % of nitrogen doses (Figure 1b).

Regarding ear diameter, isolates EB02 (T4) and EB16 (T5) were similar to top-dressing of 100 and 50 % of the N, and the four treatments differed from the others (Figure 2a). It was observed that the average of inoculated treatments was close to 50 % of N (EB02 - 51.09 mm; EB16 - 52.15 mm; 50 % N - 52.51 mm). These treatments increased the ear diameter by 7 (EB02), 9 (EB16), and 10 % (50 % N) compared to the control.

About the yield, there were differences among treatments. The complete N fertilization (T3) resulted in the highest average yield, recording 11.80 Mg ha^{-1} (Figure 2b). The EB02 (T4) and EB16 (T5) inoculations showed means statistically equivalent to 50 % of the N dose (T2) (EB02 – 9.82 Mg ha^{-1}; EB16 – 9.83 Mg ha^{-1}; 50 % N - 9.13 Mg ha^{-1}). However, the inoculations increased it, on average, by 7.5 % compared to the 60 kg ha^{-1} of N fertilization. These same isolates showed productivity 24.5 % higher than the control, while nitrogen fertilization at half dose increased by 19 %, recording an increase of 5.5 % with the rhizobacteria inoculation.

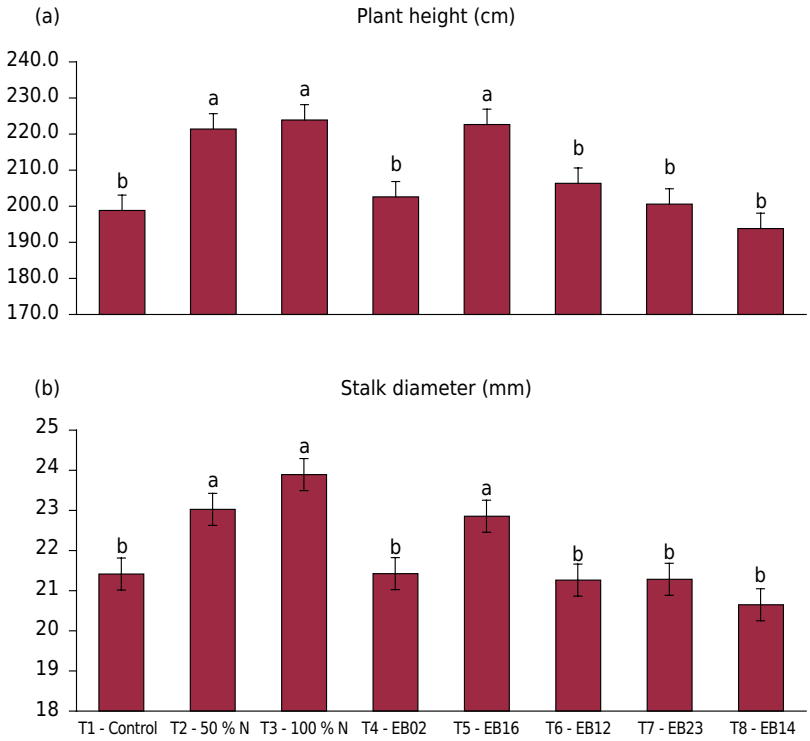

Figure 1. *Bacillus* sp. isolates effect on corn height (a) and stalk diameter (b). Average followed by the same letters does not differ by the Scott Knott test at 5 %.

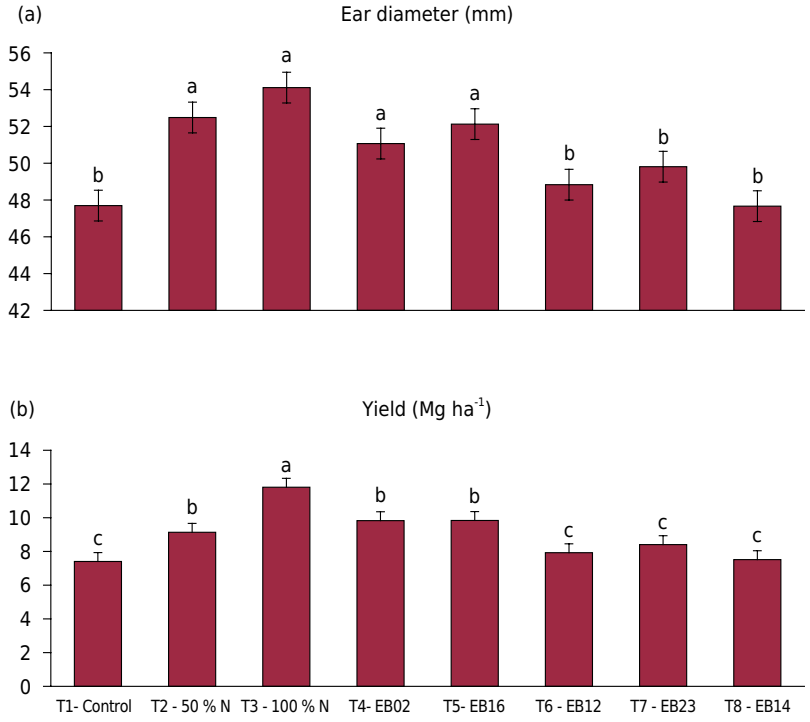

Figure 2. *Bacillus* sp. isolates effect on corn ear diameter (a) and yield (b). Average followed by the same letters does not differ by the Scott Knott test at 5 %.

Plant growth mechanisms of *Bacillus* sp. isolates

From the 27 isolates, 81.5 % (22) could solubilize calcium phosphate (Table 4). Among them, 16 isolates (59 %) showed solubilization halo in all replications, from the first day of observation. Nine isolates had IS greater than 1.0 (EB02, EB03, EB04, EB10, EB17, EB19,

Table 4. Plant-growth promoting mechanisms of *Bacillus* isolates

Isolates	IS	IAA	Lipase	Urease	GI
		µg mL^{-1}			%
*EB01	0.00 g	10.85 d	+	+	12.72 a
*EB02	1.41 b	9.68 d	+	-	0.00 b
EB03	1.65 a	12.23 d	+	-	0.00 b
*EB04	1.44 b	6.13 e	+	+	0.00 b
*EB05	0.48 e	19.03 c	+	+	0.00 b
EB06	0.86 d	13.47 d	+	-	0.00 b
*EB07	0.86 d	11.03 d	+	-	0.00 b
EB08	0.44 f	12.67 d	+	-	0.00 b
*EB09	0.15 f	6.54 e	+	+	0.00 b
*EB10	1.14 c	15.27 d	+	+	0.00 b
*EB11	0.37 f	2.70 f	+	+	0.00 b
*EB12	0.54 f	8.85 e	+	+	0.00 b
EB13	0.00 g	6.36 e	+	-	12.96 a
EB14	0.68 f	24.30 b	+	-	0.00 b
*EB15	0.82 d	34.28 a	+	+	8.98 a
*EB16	0.00 g	14.16 d	-	+	0.00 b
EB17	1.37 b	17.26 c	+	+	21.23 a
EB18	0.87 d	13.01 d	-	+	27.44 a
EB19	1.01 d	4.11 f	-	+	16.54 a
EB20	1.14 c	9.91 d	-	+	13.57 a
EB21	0.75 d	7.56 e	-	-	32.69 a
EB22	0.94 d	3.05 f	-	-	24.04 a
*EB23	1.07 d	19.03 c	+	+	0.00 b
EB24	0.00 g	12.54 d	-	+	19.11 a
*EB25	0.00 g	5.85 e	-	+	32.48 a
*EB26	0.00 g	8.55 e	-	+	17.18 a
EB27	1.13 c	12.91 d	+	+	12.00 a
Control	0.0 0 g	0.00 g	-	-	0.00 b

IS: phosphate solubilization index; IAA: indole acetic acid production; GI: degree of inhibition of *S. cepivorum* development; * *Bacillus* sp. identified by 16S RNA sequencing. Means followed by the same letters in the column do not differ statistically.

EB20, EB23, and EB27). Among them, four isolates (EB02, EB03, EB04, and EB17) stood out with IS above 1.35 and statistically higher. EB03 showed higher IS (1.65), followed by EB04 (1.44), EB02 (1.41), and EB17 (1.37). EB16 isolate had no solubilization halo.

All 27 isolates produced IAA from 2 to 34.28 µg mL^{-1} (Table 4), and mean of 11.90 µg mL^{-1}. The isolates EB15 and EB14 produced the largest amount, 34.28 and 24.30 µg mL^{-1}, respectively. Most isolates (45 %) produced 10 to 19 µg mL^{-1}. Isolates EB02 and EB16 produced 9.68 and 14.16 µg mL^{-1} of IAA, respectively, within this range.

For extracellular enzymes production, the tests for chitinase and protease did not show transparent halos around the bacterial colonies, and also, there were negative results.

From the 27 *Bacillus* isolates, 18 isolates (67 %) were capable of producing lipase (Table 4). The fluorescent points (Figure 3a) indicated their production, as described by Torquato et al. (2016).

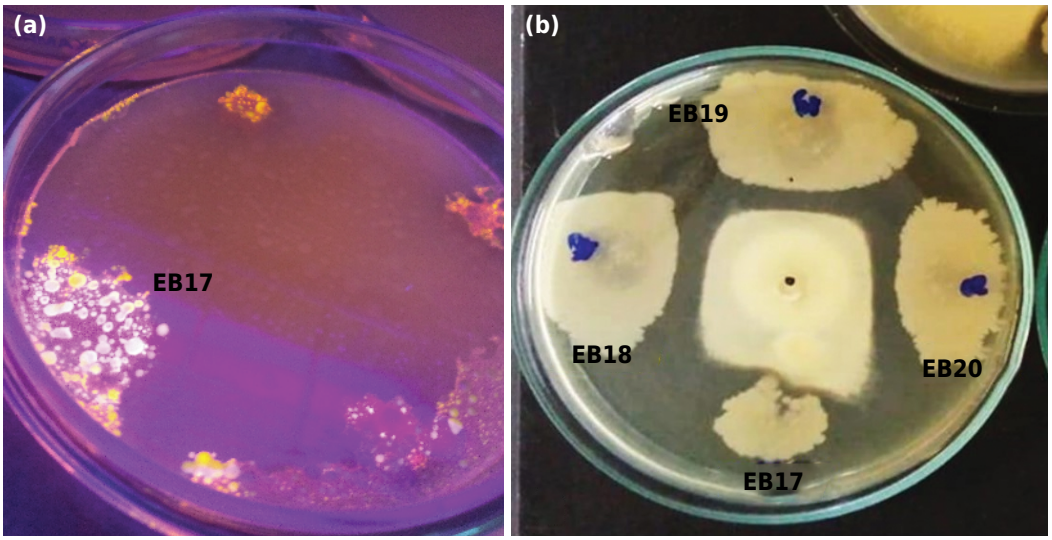

Figure 3. Indirect plant growth promoting mechanisms of *Bacillus* isolates. Lipase production (a). Inhibition of *S. cepivorum* (b).

From the 27 isolates, 18 (67 %) could synthesize extracellular urease (Table 4). The reddish-pink color in the medium indicated enzyme production. Considering the enzymatic activities tested, 11 isolates (40 % of the collection) presented lipases and urease.

The antibiosis assay showed that 13 isolates (48 %) inhibited *Sclerotium cepivorum* growth *in vitro* (Figure 3b). There was a difference in the degree inhibition (GI) among the isolates (Table 4). It was also observed that halo thickness was homogeneous among them. From thirteen isolates that inhibited the fungus, four produced lipases and urease enzymes (EB01, EB15, EB17, and EB27) (Table 4). However, it is not possible to correlate the production of extracellular enzymes to fungus inhibition, since they were also detected at isolates that did not hinder it. Therefore, it is necessary to deepen and expand the analyses. EB02 produced lipase and EB16 produced urease and both did not inhibit *S. cepivorum*.

DISCUSSION

Effect of *Bacillus* sp. isolated on corn development

Plant height and stalk diameter evaluated the efficacy of *Bacillus* isolates inoculation on corn vegetative growth. According to these parameters, the EB16 isolate was as efficient as the N doses tested. Nitrogen is the mineral nutrient most demanded by plants, being directly linked to their development. Plant tissues generally have N levels ranging from 2 to 5 % of dry matter, which is mostly in the organic form. Thus, it is important during the entire plant life cycle (Faquin, 2005).

For this reason, supply and/or stimulating the N uptake is essential, especially in the vegetative growth stages. However, for leaf N content, there was no statistical difference among treatments, including the control. This fact may indicate that other mechanisms beyond the BNF are involved in the inoculation effect.

Regarding height, the inoculation with the EB16 isolate was effective in stimulating plant development as top-dressing 100 and 50 % of required N (Figure 1a), suggesting mechanisms of N supply or absorption. Similar results were reported by Leoncio and Botelho (2017) and Lobo (2018), in which the inoculation with *Bacillus* bacteria demonstrated efficacy in promoting corn growth, both in greenhouse and field conditions. The ability to increase plant height is common for some rhizobacteria. In a study

evaluating the efficiency of corn seed inoculation with *Azospirillum*, with and without nitrogen addition, Braccini et al. (2012) observed that treatments with the bacteria provided higher plant height. This rhizobacterium, in addition to fixing N, is capable of producing IAA (Bashan and De-Bashan, 2010), and this ability positively influences plant development. The EB16 produced IAA more than EB02, but less than EB14 and EB23 (Table 4). Possibly, in addition to BNF and IAA production, the auxin amount produced could be related to its greater efficiency, because it is known that the balance among auxins and other plant hormones is important during several stages of plant development (Taiz and Zeiger, 2004).

The EB16 isolate also increased the stalk diameter as much as the N doses (Figure 1b). Thicker stalks are important for promoting lodging resistance and contribute to increasing productivity by raising the photo-assimilated compounds storage (Kappes et al., 2011). The rhizobacteria effect on that has already been observed by Dartora et al. (2013), who tested the combination of strains Ab-V5 (*A. brasilense*) and SmR1 (*H. seropedicae*). They observed larger stalk basal diameter at the vegetative stage than the control, attributing it to the diazotrophic bacteria association. Santos et al. (2013) observed that NFB (Nitrogen Fixing Bacteria) provided a significant increase in corn stalk diameter, supporting the water and nutrients transport.

Regarding yield parameters, such as ear diameter and grain yield, it was observed that isolates EB16 and EB02 inoculation showed similar efficiency to the application of 120 or 60 kg ha^{-1} of N. The isolates increased the ear diameter, as much as top-dressing of 100 and 50 % of required N, indicating their positive effects that could represent a significant increase in crop yield. Similar results were observed in off-season corn inoculated with *Azospirillum brasilense* associated with different N fertilizer levels (Mumbach et al., 2017). These authors observed that the increase in ear diameter with inoculation was as significant as the N tested levels.

These same isolates showed grain yield similar to that achieved with half of the N fertilization (50 %). Inoculations reached 24.5 % more grain yield than the control, while yield was increased by 19 % with a half-dose N fertilization, indicating an increase of 5.5 % with rhizobacterial inoculations. The results suggested that the bacteria were efficient in promoting productivity gains. Possibly, this effect was related to the two isolates IAA production and its amount (Table 4), since they produced this phytohormone at similar amounts, while the other isolates produced this compound at higher or lower concentrations, differing from these. The yield increase was also observed in other reports with rhizobacteria inoculation. Thus, Lima et al. (2011) found that corn seeds inoculating with *Bacillus subtilis* both improved the development and increased the grain yield. Quadros et al. (2014) found interaction among *Azospirillum* inoculation and corn hybrids or cultivars and concluded that inoculation was more efficient in some hybrids. Because of the promising results, the isolates EB02 and EB16 will be evaluated in production fields in the next harvests, as well as EB15 and EB14 that produced higher IAA amounts (Table 4).

Bacillus sp. isolates plant growth-promoting mechanisms *in vitro*

It was observed that a significant number of isolates had mechanisms of direct action on plant growth tested, phosphate solubilization, and IAA production. Most of the bacteria collection was able to solubilize Ca phosphate and all produced AIA. These abilities are described for several rhizobacteria (Ayyaz et al., 2016; Batista et al., 2018; Fukami et al., 2018). The highest IS four isolates (EB02, EB03, EB04, and EB17) were close to 1.5. The results were similar to those of Karpagam and Nagalaksh (2014). The authors identified 37 microorganisms capable of solubilizing phosphate in tomato rhizosphere samples and six of them had the highest IS, ranging from 1.13 to 2.23. The isolates IS observed at this report were relevant because the solubilization potential is proportional to the inhibition halo and the bacterial colony sizes (Silva, 2017). This

author found *Bacillus subtilis* isolates with indexes greater than 1.5, being similar to those found in this report. In addition to N, phosphorus is the most limiting mineral for plant growth. In the soil, there is a large reserve of this element, but insoluble, which makes it unavailable to plants. However, rhizobacteria, e.g., *Bacillus* sp., has the ability to solubilize this inorganic phosphate, converting it into soluble and absorbable forms for the plant (Arruda, 2012).

Stroschein et al. (2016) found that the major part of PGPR isolates from a collection produced up to 10 µg mL^{-1}, close to those found in this report. However, they also found bacteria from legume nodules and rhizospheric soil capable of producing up to 57.75 µg mL^{-1}. Moreira and Araujo (2013) observed that the 127 rhizospheric isolates from eucalyptus produced IAA and 85 % of them provided amounts from 4 to 8 mg mL^{-1}, much higher than those found by many reports. Florentino et al. (2017) observed the diazotrophic bacterial isolates efficiency to the IAA production that ranged from 3.99 to 46.97 µ mL^{-1}. The highest amount was reached by one of the isolates that was statistically equivalent to the control, *Azospirillum brasilense*, a bacterium known as IAA producer. According to the authors, this variation is reported in other studies, representing the high variability of this phytohormone production by bacterial isolates. It is important to note that several BNF are efficient IAA producers, suggesting their double use to induce plant growth.

Auxins synthesized by rhizobacteria promote an increase in the size and number of secondary root branches, which allows enlarging the root exploring area, suggesting an increase in nutrient absorption by the plant (Ribeiro, 2010). Other physiological processes, such as phototropism, apical dominance, vascular differentiation, and flower and fruit development, are also controlled by auxins (Ori, 2006), which emphasize the importance of microorganisms capable of supplying this plant hormone or its precursors to plants. The positive effect observed at EB02 and EB16 inoculation on corn may be related to one or more physiological processes caused by their IAA production.

It is important to note that EB16 produced 46 % more IAA than EB02 and this isolate improved all the tested parameters. However, EB16 was not able to solubilize phosphate, while EB02 showed significant IS (Table 4). These results suggested an interaction mechanism for the absence and/or intensity of the mechanisms. The study of these interactions will be further examined in future evaluations.

Regarding the indirect mechanisms tested, production of extracellular enzymes, and antibiosis, it was observed that most of the collection produced lipases and urease and almost half inhibited *S. cepivorum* growth, suggesting its potential as bioprotectants. No transparent halos were observed around the bacterial colonies for chitinase and protease tests, indicating absence of enzymes or activity by the isolates. However, many reports described the genus *Bacillus* efficiency in producing such enzymes (Costa et al., 2010; Saber et al., 2015; Rais et al., 2017).

The fluorescent spots (Figure 3a) on or around bacterial colonies indicated a positive result as described by Torquato et al. (2016). Analyses on the ability of bacterial strains to produce lipases on solid medium detected 11 of them (Lima, 2004). All belonged to the genus *Bacillus*, especially LTEB11 strain (*Bacillus megaterium*), which were isolated as culture medium contaminant.

In fungi, the cell wall can be composed of polysaccharides, proteins, chitin, cellulose, and lipids. In the cell membrane there is a protein arrangement surrounded by a lipid bilayer. The predominant lipid component is ergosterol (non-polar sterol), responsible for important characteristics such as structure, permeability, and flow modulation. Its absence may cause changes in plasma membrane permeability and growth inhibition. In addition, lipids are involved in the regulation of chitin synthesis (Loguercio-Leite et al., 2006). There are few reports that show the lipase's effectiveness in biological control (Mora et al., 2016).

However, the rhizobacteria's ability to produce lipases may hinder the development of phytogenic fungi, useful to biological control and, consequently, to plant development. In this report, some isolates of *Bacillus* sp. synthetizing lipases also inhibited *Sclerotium cepivorum* development *in vitro* (Table 4; Figure 3), suggesting further analysis.

Urease is an extracellular enzyme produced by soil microorganisms and is responsible for the hydrolysis of urea to ammonium, bicarbonate, and hydroxyls. This ammonium can be absorbed by plants, which generally has a preference for this form at the beginning of their development (Lanna et al., 2010). Most of the *Bacilus* collection of this study was able to produce urease. Similar results were found by Madureira et al. (2014) from isolates from Prata-Anã banana tree rhizosphere, of which 50 % that belong to the genus *Bacillus* were urease positive.

Plant urease can inhibit filamentous fungi and yeast growth. Its performance has been reported against phytopathogenic fungi, such as *Rhizoctonia solani*, *Fusarium* spp., *Penicillium herguei*, *Colletotrichum* spp., and others (Postal et al., 2012). A plant urease isoform showed biological properties independent of its ureolytic activity, e.g., insecticide activity, suggesting that this enzyme may be involved in plant defense processes (Folmer et al., 2004). Thus, it is evident that this enzyme has dual application and can be a mechanism against pathogens, in addition to providing nitrogen to plants, favoring their development twice. The urease produced by microorganisms can have the same action, emphasizing the importance of investigating this enzyme in rhizobacteria.

Among the indirect mechanisms tested, the isolates with higher effectiveness on corn growth in the field, EB02 and EB16, did not inhibit the growth of the fungus. However, once again, interactions of mechanisms were observed, as EB02 produced lipases and no urease was detected, while in EB16 no lipases were found, but it produced urease. This capacity may reinforce the higher EB16 efficiency, especially on vegetative growth parameters (Figure 1).

Correlating the direct and indirect mechanisms tested *in vitro*, it was observed that EB01, EB15, EB17, and EB27 isolates had significant results to at least three of the four trials (Table 4). This ability may be important for the selection of plant promoters and/or protective microorganisms. Barretti et al. (2008) noted that two tomato endophytic bacteria inhibited *Ralstonia solanacearum in vitro* in addition to promote plant growth. This multifunctionality of rhizospheric and endophytic microorganisms is relevant to biofertilizers production (Gomes et al., 2016). However, even those that do not have multiple mechanisms may provide favorable effects, such as those obtained by the EB02 and EB16 inoculation on maize that just had one or two mechanisms detected.

It is important to note that the growth induction mechanism common to the EB02 and EB16 isolates, which effected the corn development, was the production of IAA (Table 4). This remark suggested that this mechanism, associated with their BFN capacity (Leoncio and Botelho, 2017), may be related to the positive results found. It demonstrated the need to deepen further studies on the *Bacillus* sp. plant growth-promoting mechanisms, to be performed *in vitro* and *in vivo*.

CONCLUSION

In the evaluation of plant growth-promoting mechanisms, 40 % of the *Bacillus* collection isolates produced the extracellular enzymes lipases and urease and 48 % inhibited the growth of *S. cepivorum in vitro*. The EB03 strain stood out in phosphate solubilization, with the highest IS. Isolates EB01, EB15, EB17, and EB27 were effective for three of the four mechanisms tested. Among the mechanisms, the production of IAA was the most relevant, mainly for isolate EB15, which stood out and was detected

in isolates EB02 and EB16. These isolates increased the development of corn, as well as fertilizer doses of 120 and 60 kg ha^{-1} of N. The results indicated the potential to increase corn growth and production by inoculation of rhizobacteria, possibly related to the capacity of BNF and production of IAA. These observations can help to establish the management of rhizobacteria, as a method to achieve more efficient and sustainable agriculture.

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AUTHOR CONTRIBUTIONS

Conceptualization: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Methodology: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Software: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Validation: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Investigation: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Resources: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Data curation: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Writing – original draft: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Writing – review and editing: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Visualization: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Supervision: Glória Regina Botelho (lead).

Project administration: William Gilberto Balbinot (equal), Sabrina Rodrigues (equal), and Glória Regina Botelho (equal).

Funding acquisition: Glória Regina Botelho (lead).

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