Article

Bacillus spp. on the growth and yield of *Capsicum chinense* Jacq.

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Abstract

Plant growth-promoting rhizobacteria are an alternative to improve the production and yield of horticultural crops such as habanero pepper in Yucatan. Eleven strains of the genus *Bacillus*, characterized by their properties related to the promotion of plant growth, were evaluated, finding the production of indole acetic acid from 0.046 to 5.45 µg ml^{-1}, phosphate solubilization indices from 2.1 to 2.76 mm and from 13.01 to 55.82 mg L^{-1} of soluble phosphorus and ACC deaminase activity. Of which four strains with the best characteristics were selected for their properties with the promotion of plant growth, using as a model the habanero pepper, in which it was obtained that the strain of *Bacillus subtilis* CBMT51 promoted the growth of habanero pepper seedlings, improving in the number of leaves, leaf area and biomass of the seedlings by 37.1, 30% and 34.6%, respectively. In greenhouse tests with the same strain, an increase in the number of fruits and crop yield of 79.5% and 58.8%, respectively, was observed, in relation to the control. With *B. subtilis* CBMT2 being the strain that improved some growth variables such as final height (56%), number of shoots (92%) and total dry biomass (86%) with respect to the control. In conclusion, the results of this work show the potential of the strain of *B. subtilis* CBMT51 to be used as a biofertilizer in the production of habanero pepper.

Keywords: *Bacillus subtilis*, ACC deaminase activity, indole acetic acid, phosphate solubilization.

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Introduction

Habanero pepper (*Capsicum chinense* Jacq.) occupies a very important place in the diet of the Yucatecan population, for its organoleptic characteristics, it has become a symbol of pungency for the rest of the cultivars, for its high content of capsaicinoids, property of the fruit that has led to the planting of more areas, looking for more efficient technologies for the development of this crop (Pérez-Gutiérrez *et al*., 2008). Fertilization is one of the practices of great importance for obtaining quality seedlings, thus ensuring good growth, development and yield of habanero pepper, which is why optimizing the amount of fertilizer is a measure used to avoid soil deterioration and reduce the impact of fertilization on the environment (Noh-Medina *et al*., 2010).

Within soil fertility, microorganisms play an important role, not only for nutrient recycling, but also for beneficial associations with plants that improve nutrient availability (Jacoby *et al*., 2017). In agricultural ecosystems, the genus *Bacilllus* is one of the most studied for its ability to produce toxins against insects, antibiotics and antifungals against bacteria and fungi (Villareal-Delgado *et al*., 2018) and for promoting plant growth by optimizing the use of fertilizers, which makes it a viable alternative to make efficient use of these mineral resources (Souchie *et al*., 2006).

Among the biochemical and metabolic activities that *Bacillus* spp. strains employ to promote plant growth is the fixation of atmospheric nitrogen (Yousuf *et al*., 2017), the production of auxins, which induce root development (Garay-Arroyo *et al*., 2014), the solubilization of phosphates (Corrales *et al*., 2014), the production of siderophores that favor the assimilation of iron by plants (Aguado-Santacruz *et al*., 2012) and the enzyme ACC deaminase that degrades the precursor of ethylene, to reduce biotic and abiotic stress (Esquivel-Cote *et al*., 2013).

Plant growth-promoting bacteria, due to their various mechanisms of action, can help in plant nutrition, improving physiological characteristics in the crop and increasing their yield, thereby reducing the impact of excessive use of fertilizers. Therefore, in addition to promoting plant growth, they can reduce the recommended dose of chemical fertilization by up to 50% (Hernández-Leal *et al*., 2011). In this study, the properties of *Bacillus* spp. to solubilize calcium phosphate, produce indole acetic acid, siderophores and ACC deaminase activity, associated in the promotion of growth and yield of *C. chinense* Jacq., were determined.

Materials and methods

Microorganisms used

Eleven isolates of *Bacillus* spp., from the collection of the Microbiology Laboratory of the National Technological Institute of Mexico-Technological Institute of Conkal were used. The bacteria were activated in nutrient agar (NA) for four to five days at 28 °C until autolysis and were preserved at 4 °C until use. The tests performed were the activity of ACC deaminase, production of indole acetic acid (IAA), production of siderophores and solubilization of calcium phosphate.

Activity of 1-aminocyclopropane 1-carboxylate deaminase (ACC deaminase)

The qualitative activity of the ACC deaminase of the 11 bacteria was performed by using the minimal medium Dworkin and Foster (DF) with ammonium sulfate $(NH_4)2SO_4$ (Penrose and Glick, 2003). First, the strains were inoculated by stria in the DF medium, using $(NH_4)_2SO_4$ as a nitrogen source and after 48 h of growth, they were transferred to plates containing DF medium with 1-aminocyclopropane 1-carboxylate (ACC) (Sigma-Aldrich®) as a nitrogen source, in the absence of ammonium sulfate. The petri dishes were then incubated for 3 days at 30 °C. Bacteria that showed growth were considered to have ACC deaminase activity.

Production of indole acetic acid (IAA)

To determine the microbial production of IAA, flasks containing 50 ml of nutritious broth supplemented with 1 g L^{-1} of L-tryptophan were inoculated with each of the strains at a density of $1x10^8$ CFU ml^{-1}, subsequently, they were taken to an orbital agitator to be incubated for 72 h at 180 rpm and 30 °C. At the end, the supernatant was recovered by centrifugation at 3 000 by gravity (xg) for 15 min. To 1 ml of supernatant, 1 ml of Salkowski reagent was added. The mixture was left to stand at room temperature for 30 min and the concentration of IAA was determined in a spectrophotometer (Genesys 10UV) at 535 nm. To determine the concentration, a standard curve of 5 to 40 µg ml^{-1} of IAA (Sigma) was used (Badía *et al*., 2011; Almoneafy *et al*., 2012).

Siderophore production

To produce siderophores, the strains were previously grown in 7 ml of culture medium containing minimal salts (MS), left for 16 h in stirring at 120 rpm at 30 °C and after this time, 70 µl were taken from the bacterial culture which were transferred back to the MS medium, leaving in stirring for 24-30 h in the same conditions of stirring and temperature. Subsequently, the supernatant was recovered by centrifugation at 8 000 by gravity (xg) for 10 min, 1 ml of it was taken, mixing with 1 ml chrome azurol-S (CAS), the change in the coloration of the CAS reagent from blue to orange was considered positive for the production of siderophores (Alexander and Zuberer, 1991).

Calcium phosphate solubilization

The qualitative activity of phosphate solubilization was performed by inoculating 8 µl of a bacterial suspension of 1x108 CFU ml^{-1} in solid medium Pikovskaya (PKV) (Pradhan and Sukla, 2005). Isolates that formed yellow halos in the culture medium containing bromophenol blue as an indicator were considered positive for phosphate solubilization, whose solubilization index (SI) was determined by the halo diameter (Qureshi *et al*., 2012).

While the quantitative determination was made by inoculating by puncture in 25 ml of NBRIP medium with an initial pH of 7.2, which contained insoluble tricalcium phosphate as the only source of phosphorus (Mehta and Nautiyal, 2001), the cultures were kept in constant stirring at 200 rpm for 5 days at 30 °C. At the end, the cultures were centrifuged at 8 000 by gravity (xg) for 10 min to recover the supernatant, in which soluble phosphorus was determined by the molybdenum blue method (Mussa *et al*., 2009).

Bacillus spp., on the growth of seedlings of habanero pepper (_C. chinense_ Jacq.)

The test was evaluated with the strains CBRF8, CBMT51 and CBMT2 of *B. subtilis* and BL18 of *B. cereus*, which showed better ability to solubilize phosphates, production of IAA, as well as ACC deaminase activity and selection was made based on a cluster analysis. For the test, seeds of habanero pepper *var* naranjo criollo were used, which were disinfected with 2% sodium hypochlorite, followed by three washes with sterile distilled water, then the seeds were sown in polystyrene trays with commercial sterile substrate Cosmopeat®, giving the corresponding agronomic management (Soria *et al*., 2002).

At 15 days after germination (DAG), an initial inoculation was performed at the stem level with a bacterial suspension adjusted to $1x10^8$ CFU ml^{-1}. A second application was made 28 days after germination (DAG). Subsequently, the transplant was performed in 32-ounce expanded polystyrene cups with Luvisol-type soil substrate with bovine manure in a 2:1 ratio, previously sterilized. At 60 DAG, height, stem diameter, number of leaves, leaf area, root volume, root length, fresh and dry biomass of the aerial part and root were measured.

A completely randomized experimental design was applied with four treatments (bacterial strains) with 10 repetitions and a control consisting of seedlings without bacterial inoculation. With the results obtained, an analysis of variance (Anova) was performed with a mean comparison test (Tukey, $p\leq 0.05$) with the help of the SAS statistical package version 9.3 for Windows (SAS Institute, 2010).

Effect of _Bacillus_ spp., on the yield of habanero pepper (_C. chinense_ Jacq.)

To evaluate the effect of *Bacillus* strains on the production of habanero pepper, a test was carried out in an asymmetrical greenhouse located in the facilities of the Technological Institute of Conkal. Within which seedlings of 28 DAG were used, with two inoculations as described in the previous section. Subsequently, the transplant was performed in bags of 10 kg, which contained a mixture of Luvisol soil with bovine manure in a 2:1 ratio. A third inoculation was performed eight days after the transplant. Fertilization was carried out based on regional crop recommendations (Soria *et al*., 2002).

The average maximum and minimum temperatures recorded inside the greenhouse were 35.4 and 24 °C, maintaining an average humidity of 73.4%. The plants were kept until 140 days, in which the same growth variables described above were evaluated, additionally, variables related to crop yield were included, such as: number of fruits per plant, polar and equatorial diameter of the fruit and total weight of fruits per plant. A completely randomized experimental design was applied with four treatments (bacterial strains) with eight repetitions and a control consisting of seedlings without bacterial inoculation. With the results obtained, an analysis of variance (Anova) was performed with a mean comparison test (Tukey, $p\leq 0.05$) with the help of the SAS statistical package version 9.3 for Windows (SAS Institute, 2010).

Results and discussion

Activity of 1-aminocyclopropane 1-carboxylate deaminase (ACC deaminase)

In the minimal medium DF, the growth of 10 of the 11 bacterial strains was observed, indicating that they have the ability to produce the enzyme ACC deaminase, with *Bacillus* sp., CBRF4 being the only one that did not show growth (Table 1). It is worth mentioning that differences in the growth of bacteria were observed, the isolates CBLMA4, CBCK44, CBCC58 and CBMT2 showed greater growth in the culture medium. This activity has been reported as part of the characteristics of microorganisms that have been used in the promotion of plant growth in pepper (*C. annuum*) and tomato (*Lycopersicum esculentum*) (Luna Martínez *et al*., 2013; Hernández-Forte *et al*., 2015).

Table 1. Biochemical properties of *Bacillus* spp., related to the promotion of plant growth.

Isolates	ACC deaminase	IAA (μg ml^{-1})	SI (mm)	P (mg L^{-1})	pH
CBLMA4	+ + + +	0.05 e	2.22 cd	23.9 ±9.38 cde	5.51 ±0.04 f
CBRF11	+	0.60 bcd	2.68 ab	13.01 ±2.62 e	5.3 ±0.03 ef
CBRF4	-	2.153 b	2.76 a	16.26 ±5.9 de	5.16 ±0.03 de
CBRM9	+ + +	0.518 bcd	2.43 abcd	27.1 ±2.78 cd	5.05 ±0.12 cd
CBCK44	+ + + +	1.697 bcd	2.25 c	23.19 ±1.2 cde	4.99 ±0.02 bcd
CBRF5	+	2.106 b	2.52 abcd	17.06 ±7.43 de	4.96 ±0.07 bcd
CBMT51	+	1.619 bcd	2.51 abcd	55.82 ±3 a	4.9 ±0.1 abc
CBCC58	+ + + +	0.235 cd	2.64 abc	27.2 ±4.25 cd	4.87 ±0.12 abc
CBMT2	+ + + +	5.455 a	2.3 bcd	42.47 ±3.87 ab	4.84 ±0.04 abc
CBRF8	+ +	1.823 bc	2.34 abcd	36.86 ±3.75 bc	4.81 ±0.02 ab
BL18	+ +	4.37 a	2.1 d	50.02 ±0.74 ab	4.71 ±0.03 a
MSD		1.69	0.43	13.94	0.21

Means with different letters in each column are statistically different (Tukey, $p \leq 0.05$). + = activity or growth; ++= growth intensity; +++= lower growth; ++++= higher growth; -= no growth; ACC-deaminase= 1-aminocyclopropane 1-carboxylate deaminase; IAA= indole acetic acid; SI= solubilization index; P= phosphorus; MSD= minimum significant difference.

However, one of the most interesting applications of the strains that have this property is related to the reduction of environmental stress, where the application of *Bacillus* strains reduced the stress caused by soil salinity in corn plants (Misra and Singh, 2020). On the other hand, biotic stress caused by diseases can also be decreased by ACC deaminase activity, where the use of a strain of *Paenibacillus lentimorbus* B-30488 (formerly *Bacillus lentimorbus*) reduces the stress caused by infection by the phytopathogenic fungus *Sclerotium rolfsii* in tomato (Dixit *et al*., 2016).

Production of indole acetic acid (IAA) and siderophores

The 11 bacteria tested produced IAA in a range between 0.046 and 5.45 μg ml^{-1}. The strains CBMT2 and BL18 showed the highest production with 5.45 and 4.37 μg ml^{-1}, respectively (Table 1). Luna-Martínez *et al*. (2013), in their research with strains of *Bacillus* spp., achieved the production of IAA in concentrations similar to those generated in this study, with which they obtained increases in the growth of seedlings of pepper (*C. annuum* L.) and tomato (*S. lycopersicum* L.), such as those generated in *C. chinense* Jacq. in this evaluation. The ability to produce IAA depends on several factors: the bacterial strain, the genes involved that moderate or regulate biosynthetic pathways and the presence of enzymes to convert IAA into its conjugated forms (Patten and Glick, 2002).

Bacillus strains that produce IAA have been reported to promote growth in seedlings of soybean (*G. max*) (Wahyudi *et al*., 2011), wheat (*Triticum aestivum* L.) (Abbasi *et al*., 2011) and tomato (*Solanum lycopersicum* L.) (Almoneafy *et al*., 2012). This hormone modulates cell growth and tissue differentiation, participates in the phenomena phototropism and gravitropism and has an important function during root and xylem formation (Vega-Celedón, 2016). Studies in soil reveal that the microbial production of IAA depends on the presence of its precursor, tryptophan (Sarwar *et al*., 1992). Regarding the production of siderophores, no coloration changes were observed in the CAS reagent, suggesting that under the conditions evaluated the bacteria did not synthesize siderophores (Alexander and Zuberer, 1991).

Calcium phosphate solubilization

In *in vitro* tests, conducted in the laboratory, the 11 bacteria showed different solubilization indices (SI) of calcium phosphate, the greatest activity was observed at seven days of bacterial growth (Wahyudi *et al*., 2011; Almoneafy *et al*., 2012). The SIs found were 2.1 to 2.76 mm, the highest SI was obtained with the strain CBRF4 (Table 1). Qureshi *et al*. (2012) reported SI values between 3.3 and 3.8, higher than those obtained in this study. However, the SIs reported in *Bacillus* spp., in this study agree with those reported by Badía *et al*. (2011).

In the test in liquid medium, the study strains produced concentrations of 13.01 to 55.82 mg L^{-1} (Table 1). The strain *B. subtilis* CBMT51 was the one that showed the highest solubilization with 55.82 mg L^{-1}, this result is similar to that reported for B. *megaterium* (MA06) with a solubilization of phosphorus of 56 mg L^{-1} (Luna-Martínez *et al*., 2013). It has been shown that the production of organic acids by bacteria lowers the pH, which in turn favors the solubilization of phosphates. In this study, a decrease in the final pH of the medium was found, fluctuating between 4.71 and 5.51 (Table 1), which partly explains that the solubilization of phosphates by bacteria is related to the decrease in pH (Mehta *et al*., 2010; Walpola and Yoon, 2013).

Among the organic acids secreted during solubilization activity, lactic, isovaleric, isobutyric and acetic acid have been reported (Metha and Nautiyal, 2001). This activity is of great importance to improve the acquisition of phosphates by the plant, since due to its characteristics, when added in the form of fertilizer to the soil, phosphate is complexed with the calcium, iron or aluminum present, forming insoluble species, not available for the plant (Beltrán, 2014). Prakash and Kumar (2019) report that the inoculation of *Bacillus* sp., STJP (previously characterized as a phosphate solubilizer) in *Mentha arvensis* increased the phosphorus content in roots, stems and leaves, also leading to an increase in biomass, and in the amount of essential oil.

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To perform the scrutiny of the strains with the best plant growth-promoting characteristics, a cluster analysis was carried out, selecting four strains: CBMT51, CBMT2, CBRF8 and BL18. Subsequently, these strains were inoculated in habanero pepper seedlings of 28 DAG. At 60 DAG, and it was found that the plants showed significant differences ($p\leq 0.05$) in height, number of leaves, leaf area, fresh weight and dry weight of the aerial part, root volume and fresh root weight. In the mean comparison test, the strain CBMT51 showed the best effect on plant growth (Table 2), compared to the other treatments and the control.

Table 2. Effect of _Bacillus_ spp., on the promotion of growth of seedlings of _C. chinense_ Jacq. 60 days after transplantation.

Strains	He (cm)	NL	LA(cm^2)	RV(cm^3)	FRW(g)	FWAP(g)	DRW(g)	DWAP(g)
CBRF8	20.2 b	17 b	288.4 b	3.9 bc	3.9 b	7.9 b	0.41 a	1.3 b
CBMT2	21.4 b	21 ab	329.8 ab	4.1 bc	3.9 b	9 b	0.41 a	1.4 ab
CBMT51	25.4 a	25.1 a	421.1 a	5.9 a	5.8 a	11.7 a	0.54 a	1.8 a
BL18	20.9 b	19.4 ab	321.4 b	4.8 b	4.3 b	8.5 b	0.52 a	1.3 b
Control	22.3 ab	18.3 b	323.8 b	3.4 c	4.3 b	8.7 b	0.41 a	1. b
MSD	3.1	4.9	91.6	3.9	1.3	1.9	0.25	0.6

Means with different letters in each column are statistically different (Tukey, $p\leq 0.05$). He= height; NL= number of leaves; LA= leaf area; RV= root volume; FRW= fresh root weight; FWAP= fresh weight of the aerial part; DRW= dry root weight; DWAP= dry weight of the aerial part; MSD= minimum significant difference.

Only in the dry root weight (0.41 to 0.54 g) no differences between the treatments were observed. Several authors reported *Bacillus* spp., as a bacterium capable of promoting growth in *C. annuum* (Luna-Martínez *et al*., 2013; Amaresan *et al*., 2014). Ogugua *et al*. (2018) report that the biomass of chili seedlings, after 35 days, increased 32.3%, similar to that reported in this study (34.6%). Gibberellins are other hormones also produced by strains of *Bacillus*, which have been involved in promoting the growth of chili seedlings, as reported by Gil-Jae *et al*. (2004), which shows the versatility of the genus.

Effect of _Bacillus_ spp., on the yield of habanero pepper (_C. chinense_ Jacq.)

In the greenhouse test, it was observed that the strain *B. subtilis* CBMT51 presented the best effect by inducing a greater number of fruits and greater fresh weight of them, increasing by 79.5 and 58.8%, respectively, in relation to the control (Table 3). The strain *B. subtilis* CBMT2 induced increased height, stem diameter, number of shoots, total fresh and dry biomass (Table 3). Previous studies reported bacteria capable of promoting plant growth in *C. annuum* (Luna Martínez *et al*., 2013; Amaresan *et al*., 2014). In habanero pepper inoculated with species of *Azospirillum* sp., increases in the aerial and root biomass of the crop were obtained (Canto-Martín *et al*., 2004).

Table 3. Effect of *Bacillus* spp., on the development and yield of *C. chinense* Jacq. 140 days after transplantation.

Strains	He (cm)	SD (mm)	NS	NF	FFW (g)	PFD (mm)	EFD (mm)	TFB (g)	TDB (g)
CBRF8	82.5 bc	12.1 ab	12 b	19.5 b	89.2 b	37.2 a	23.3 a	165.2 b	43.7 ab
CBMT2	129.7 a	13.1 a	20 a	15.4 c	69.6 c	39 a	22.6 a	187.1 a	50.8 a
CBMT51	100.6 b	11.9 ab	15.4 ab	24.6 a	104.5 a	39.7 a	24 a	152.3 b	36.1 ab
BL18	77.6 c	12.2 ab	13.2 ab	18.7 b	93.8 b	40.4 a	24.3 a	159.2 b	46.6 a
Control	83 bc	11.3 b	10.4 b	13.7 c	65.8 c	38.9 a	22.9 a	97.6 c	27.3 b
MSD	21.6	1.8	7.1	2	8.6	5.9	2.5	42	16.5

Means with different letters in each column are statistically different (Tukey, $p \leq 0.05$). He= height; SD= stem diameter; NS= number of shoots; NF= number of fruits; FFW= fresh fruit weight; PFD= polar fruit diameter; EFD= equatorial fruit diameter; TFB= total fresh biomass; TDB= total dry biomass; MSD= minimum significant difference.

On the other hand, inoculations with species of *B. amyloliquefaciens*, *B. licheniformes* and *B. subtilis* in *C. annuum* increased height, number of fruits and yield (Datta *et al*., 2011; Yu *et al*., 2011), changes that could be observed in this study with the application of *B. subtilis* CBMT51. Peña-Yam *et al*. (2016) report that the inoculation of seedlings of chili cv Jalapeño with the strain of *Bacillus cereus* ITC-BL18 increased the number of flower buds, which may also have occurred in the present work with *B. subtilis* CBMT51, which finally led to a greater number of fruits.

The improvement in the growth and development of plants when inoculated with this type of bacteria is mainly associated with the production of indole acetic acid and the solubilization of phosphates (López-Bucio *et al*., 2009), properties that were demonstrated with the strains under study, with the strains *B. subtilis* CBMT51, *B. cereus* BL18 and *B. subtilis* CBMT2 being the ones that showed the best effect on habanero pepper plants for the variables evaluated. The use of *Bacillus* as a biofertilizer not only improves the development and production of plants, but recent studies also show that in chili fruits, it improves their organoleptic quality and increases the content of ascorbic acid and antioxidant activity (Cisternas-Jamet *et al*., 2020).

Conclusions

The bacterial strains under study presented biochemical properties related to the promotion of plant growth. In the evaluation of growth and yield promotion in *C. chinense* Jacq., it was obtained that the strain of *Bacillus subtilis* CBMT51 influenced the growth promotion of *C. chinense* Jacq. seedlings. And it increased the number of fruits and yield of the crop; while the strain of *B. subtilis* CBMT2 increased plant growth and development.

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