Isolation of Mexican *Bacillus* Species and Their Effects in Promoting Growth of Chili Pepper (*Capsicum annuum* L. cv Jalapeño)

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Abstract The purpose of this work was to isolate and identify native bacteria from plants collected in the State of Yucatán, México with the ability to promote growth of chili pepper (*Capsicum annuum* L. cv Jalapeño). We identified nine bacterial isolates that belong to five species of *Bacillus* (i.e. *Bacillus subtilis*, *B. flexus*, *B. cereus*, *B. megaterium* and *B. endophyticus*) that produced indoleacetic acid (4.0–24.3 µg/mL) with solubilization index of 1.3–1.6. All the bacterial isolates were evaluated based on their ability to promote growth of chili pepper. Plants inoculated with *B. subtilis* ITC-N67 showed an increase in stem diameter and root volume, whereas inoculation with *B. cereus* ITC-BL18 increased the number of flower buds, fresh biomass of roots and total fresh biomass. Conversely, *B. flexus* ITC-P4 and *B. flexus* ITC-P22 showed deleterious effect on root volume and total biomass. In summary, our data showed that native *B. cereus* TC-BL18 and *B. subtilis* ITC-N67 have potential to be used as growth promoting microorganism for chili pepper, particularly in the state of Yucatán, México.

Keywords *Bacillus* spp. · Growth-promoting · Indoleacetic acid · Phosphate solubilization · *Capsicum annuum* · Yucatán

Plant growth promoting bacteria (e.g. *Bacillus* species) represent a variety of rhizospheric and endophytic microorganisms, which stimulate host growth through the activation of different mechanisms in the plant cell [1]. In particular, *Bacillus* species have the ability to solubilize different chemicals, they are also involved in the synthesis of auxins, productions of siderophores, nitrogen fixation, and in the synthesis of bacteriocins [2–4].

About one-third of total production of pepper in Mexico comes from *Capsicum annuum* cv. Jalapeño. This variety of pepper is cultivated in highly technical production systems in Central and Northern Mexico, and in low-input agroecosystems in southern Mexico and the Yucatan Peninsula [5–7]. Several strains of the genus *Bacillus* have shown growth promoting effects on chili pepper (*C. annuum*) [8, 9]. Inoculation of plants with different strains of *Bacillus* spp. increased not only the fruit weight, but also the number of fruits per plant [10]. Because of the importance of *C. annuum* in México, the purpose of this work was to isolate and identify native bacteria from plants collected in the State of Yucatán México that have the ability to promote growth of chili pepper (*C. annuum* L. cv Jalapeño). Our data shown that *Bacillus cereus* TC-BL18 and *B. subtilis* ITC-N67 have potential to be used as a *C. annuum* L. cv Jalapeño growth promoting probiotic, particularly in the state of Yucatán, México.

To carry out this work, bacteria were isolated from roots of different plants (*Cenchrus echinatus*, *Leucaena leucocephala* and *Sansevieria trifasciata*) collected in the State of Yucatán México using the nitrogen-free semisolid

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malate (NFB) enrichment medium. Bacterial strains that were Gram-positive, catalase positive and endospore forming were used. To detect the Indoleacetic acid (IAA) production, approximately 1×10^8 CFU/mL of each bacterium was inoculated in Luria–Bertani (LB) supplemented with 40 µg/mL tryptophan as precursor of IAA. Samples were incubated (30 °C, 180 rpm, 72 h), centrifuged ($3000 \times g$) for 15 min, and 1 mL of each supernatant was mixed with 1 mL of Salkowski reagent (1.5 mL of 0.5 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 80 mL of 60 % H_2SO_4). Samples were incubated in the dark at room temperature for 30 min, a pink color indicated a positive test. IAA concentrations were determined at 535 nm using a standard curve of IAA (Sigma, St. Louis, MO, USA). To evaluate the phosphate solubilizing activity, 0.8 µL of bacterial culture at 1×10^{-8} CFU/mL was streaked onto Pikovskaya's medium and incubated at 30 °C for 7 days [11]. Isolates that acidified the medium (change in color from blue to yellow) and produced clear zones around colonies were chosen. Phosphate solubilizing capacity of each isolated was calculated using the solubilization index (SI), $\text{SI} = \text{A/B}$, where A is the total diameter (colony diameter + halo diameter), and B is the colony diameter.

To perform the growth-promoting test on *Capsicum annuum* L. cv Jalapeño, seeds were surface disinfected. Seeds were inoculated by immersion in a bacterial suspension (1×10^{-8} CFU/mL) with continuous shaking at 200 rpm for 90 min. For negative control, seeds treated with 0.9 % (w/v) NaCl were used. The seeds were placed in plastic trays with 128 cavities and mixed with a commercial substrate called Cosmopeat (Cosmolcel S.A. San Nicolás de los Garza, Nuevo León, México). After 15 days, each sample was inoculated for a second time with 3 mL of the bacterial suspension as carried out in the first inoculation. The negative control was treated with 3 mL of 0.9 % (w/v) NaCl instead of bacterial suspensions. Once a week, 1 g/L of the commercial fertilizer 17-17-17 (N-P-K) (Royal Garden's®, Guadalajara, México) and 1 g/L of ammonium sulfate, were applied to all samples. Plants were kept for 43 days in a greenhouse at 20–35 °C, 55–75 % relative humidity, and natural light cycles (approximately 11 h light, 13 h dark). After this period, different variables were evaluated (e.g. plant height, stem diameter, etc.). The experiment was set in a completely randomized design, using in each treatments 10 replicates (plants). Data were subject to analysis of variance (ANOVA, StatSoft Inc., Tulsa, OK, USA) and mean comparison by Tukey's Multiple Range Test ($P < 0.05$) using the SAS program for Windows 9.1.

To identify bacterial isolates by 16S rDNA, bacterial DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison MI, USA). 16S rDNA was amplified using the primers Eubact27F (5'-

AGAGTTTGATCMTGGCTCAG-3' and Eubact1492R (5'-TACGGYTACCTTGTTACGACTT-3' (<https://www.macrogenusa.com/support/seq/primer.jsp>). PCR amplifications were carried out as follows: 32 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min 45 s, each, followed by a 7-min termination step at 72 °C. Amplicons were sequencing at Macrogen, USA (Rockville, MD, USA), and the nucleotide sequences were compared using GenBank data from NCBI (www.ncbi.nlm.nih.gov).

Although we did not know the total microbiota associated to the collected roots in this work, as we would need to carry out metagenomic analysis, we were able to isolated different *Bacillus* species that might be involved in promoting growth of chili plants. In this regard, we isolated 46 bacterial strains. From the bacterial isolates, 9 were selected because they grew in NFB medium, were bacilli, Gram-positive, catalase positive, and spore-formers. Bacteria were obtained from 9 different localities from the State of Yucatán, México. One of the key aspects of plant growth promoting rhizobacteria is the production of plant hormones. In this regard, one of the most common and naturally-occurring plant hormone is the indoleacetic acid (IAA). Recently, it has been demonstrated that *Bacillus* strains isolated from rice synthesized IAA in concentrations from 0.1 to 30 µg/mL [3]. Bacterial strains isolated in this work produced IAA in a range from 4.0 to 24.3 µg/mL, which are within the range reported for *Bacillus* spp. isolated from rice roots. *B. megaterium* ITC-BL8, *B. subtilis* ITC-BL11 and *B. subtilis* ITC-BL12 showed the highest IAA production, with values of 16.9 and 24.3 µg/mL, respectively, which were significantly higher than those of the other isolates (Fig. 1).

Other factor that contributes to the plant growth promoting effect is phosphate solubilization, where the production of organic acids may be linked to this effect [12]. The nine *Bacillus* (100 %) isolates described in this study did not show significant difference in the phosphate solubilization index, from 1.3 to 1.6 (Fig. 1). This index is higher compared with that reported in *Bacillus* isolates from rice [2]. Nevertheless, the solubilization index of *Bacillus* strains in this work were lower than those obtained with species of *Bacillus* that promote growth of cotton, i.e. 1.3–1.6 versus 3.3–3.8 [13]. Interestingly, some strains (e.g. ITC-LB8, ITC-BL11 and ITC-BL12) that produce IAA and solubilize phosphate were not able to promote growth of chili pepper. This result suggests that other factors, and not only those determined here, might be responsible for the growth-promoting effect observed [14].

The effects of the 11 bacterial isolates (9 strains isolated in this work and 2 strains from our stock collection) on the growth of *Capsicum annuum* plants were evaluated. Significant differences in some of the variables were observed. Plants inoculated with isolate ITC-N67 showed

Fig. 1 **a** Production of indole acetic acid by *Bacillus* spp. after 72 h of incubation in nutrient broth supplemented with L-tryptophan. **b** Phosphate solubilization of different strains of *Bacillus* spp. after 7 days of incubation in Pikovskaya medium. Standard errors were

calculated based on ten replicates. In each figure, bars with different letter (a–e) are significantly different as determined by Tukey's multiple range test ($P < 0.05$)

Fig. 2 Comparative growth of *C. annuum* L. cv Jalapeño seedling, untreated (C) or treated with *B. endophyticus* (ITC-BL14), *B. subtilis* (ITC-N67) and *B. cereus* (ITC-BL18) after 43 days under greenhouse conditions

significantly higher ($P < 0.05$) stem diameter and root volume, whereas plants inoculated with isolate ITC-BL18 showed higher number of flower buds, fresh biomass of roots and total fresh biomass, compared with the control (Table S1; Fig. 2). In contrast, we observed a negative effect of bacterial isolates ITC-P4 and ITC-P22 on root volume and total biomass (Table S1). Previous studies have shown the effect of *Bacillus* spp. to promote the growth and yield of *C. annuum*. For example, inoculation of *B. subtilis* MA12 and MA17 of *C. annuum* seedling increased biomass by 37 and 16 %, respectively [12]. In other studies, inoculation of *Bacillus* sp. BECS7 and *B. licheniformis*

BECL5 increased the number of flower buds, the root length and leaf area in *C. annuum*. We have found similar results with the inoculation of *B. cereus* ITC-BL18 and *B. subtilis* ITC-N67, and the effect might be attributed to the synthesis of IAA [8, 9, 14].

B. subtilis and *B. pumilus* are the most frequently isolated rhizobacteria followed by *B. licheniformis*, *B. cereus* and *B. amiloliquefascens* [15]. Here, in order to identify the rhizospheric bacterial strains, we amplified the 16S rDNA obtaining amplicons of ~1.5 kbp, which were sequenced and compared with GenBank data in NCBI. As such, we identified *B. subtilis* (one isolate), *B. flexus* (two isolates), *B. cereus* (three isolates), *B. megaterium* (two isolates), and *B. endophyticus* (one isolates). The bacterial isolates CBRF12 and ITC-N67, which belong to our stock collection, were identified as *B. subtilis* (Table S2). Those reports and our results confirm the high diversity of *Bacillus* spp. in the rhizosphere, in particular among grass species. In conclusion, we isolated and identified nine native *Bacillus* spp. strains, which synthesize IAA and solubilize phosphate, both parameters that appear to be linked to the growth promoting effect on *C. annuum*. Our results showed that *B. cereus* ITC-BL18 and *B. subtilis* ITC-N67 have a high potential to be used as a probiotic promoting biomass of *C. annuum* L. cv Jalapeño, particularly in the state of Yucatán, México.

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