Investigation note

Improving biometric traits of wheat seedlings with the inoculation of a consortium native of *Bacillus*

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Abstract

Plant growth promoting bacteria (BPCV) represent an alternative for reducing the use of chemical fertilizers and improving their use by agricultural crops. The study determined the ability of a bacterial consortium native to the genus *Bacillus* to promote the growth of wheat seedlings (*Triticum turgidum* subsp. *durum*) under controlled conditions, 30 days after inoculation. The consortium was integrated by strains TRQ8, TE3^T, TRQ65 and TSO9. The molecular identification (based on the 16S RNAr gene) of these strains revealed their taxonomic affiliation to *Bacillus megaterium* TRQ8, *B. cabrialesii* TE3^T, *B. paralicheniformis* TRQ65 and *B. subtilis* TSO9. These strains showed the ability to synthesize siderophores, indoles, and phosphate solubilization, as well as tolerance to thermal stress (43.5 °C), water (10% PEG) and saline (5% NaCl). The inoculation ($4x10^7$ CFU) of said bacterial consortium to wheat seedlings showed a significant increase (compared to non-inoculated seedlings) in the length of the aerial part (28%), root length (25%), total length (28 %), stem diameter (46%), circumference (50%), dry weight of the aerial part (72%) and the biovolume index (57%). The foregoing evidences the capacity to promote plant growth of said bacterial consortium; therefore, the development of future trials under field conditions is crucial to know the feasibility of the design of a biofertilizer containing the study strains for use in current and future agriculture.

Keywords: agriculture, bioinoculant, BPCV, food security, Yaqui Valley.

Reception date: January 2020
Acceptance date: February 2020

Wheat (*Triticum turgidum* subsp. *durum*) is one of the most important crops worldwide (Grijalva-Contreras *et al*., 2016). At present, the production of this cereal requires the use of large amounts of chemical fertilizers (~300 kg N ha^{-1}), whose utilization rate is 20 to 35% (Grageda-Cabrera *et al*., 2018). Which leads to economic and environmental problems, such as: eutrophication, emission of greenhouse gases, and loss of soil fertility (de los Santos-Villalobos *et al*., 2018; Liu and Xie, 2019). Therefore, the generation of sustainable agro-biotechnological alternatives that allow maintaining or increasing agricultural yields decreasing the negative effects on the environment, human health, and economy of the producer is decisive.

In recent years, interest in the generation of integral agricultural systems has emerged; through the application of plant growth promoting bacteria (BPCV), which are a group of bacteria that have the ability to improve the health and quality of plants, restoring soil fertility (Rojas-Solis *et al*., 2016; Valenzuela-Aragon *et al*., 2019). The positive effects shown by BPCV have been strongly associated with various mechanisms of action i) direct, associated with plant growth, such as: the production of phytohormones (auxins, cytokinins, and gibberellins), volatile organic compounds (acetoin, and sulfur derivatives), biological nitrogen fixation, cycling and nutrient solubilization (Moreno-Resendez *et al*., 2018; Valenzuela-Ruiz *et al*., 2018); and ii) indirect, associated with crop resistance to phytopathogens, through the production of lipopeptides, antibiotics, or lithic enzymes (Villarreal-Delgado *et al*., 2018; Villa-Rodríguez *et al*., 2019).

At present, there is a growing interest in the formulation of these biotechnological products from one or more species or bacterial genera with phyto-stimulant capabilities, called bacterial consortia (Jha *et al*., 2013). The objective of the study was to evaluate the wheat growth promoting capacity, under controlled conditions, of a native bacterial consortium composed of strains of the genus *Bacillus*, which were isolated from the Yaqui Valley, Sonora, Mexico (region that contributes 50% to the production of this cereal nationwide).

The bacterial strains under study ($TE3^T$, TRQ8, TRQ65 and TSO9) were isolated (by serial dilutions (1:10), using nutritive agar as a culture medium, at 28 °C) from the rhizosphere of the CIRNO2008 variety wheat crop, grown in commercial plots located in the Yaqui Valley, Sonora, Mexico (27° 35’ 53.14” N and 110° 2’ 53.26” W). These strains were preserved in the Collection of Endophytic and Native Edaphic Microorganisms (www.itson.edu.mx/COLMENA) (de los Santos-Villalobos *et al*., 2018).

The $TE3^T$, TRQ8, TRQ65 and TSO9 strains were metabolically characterized for traits associated with the promotion of plant growth, such as: phosphate solubilization, indole production, tolerance to thermal, water, and saline and chlorothalonil stress (Valenzuela-Aragón *et al*., 2019; Díaz-Rodríguez *et al*., 2019). Thus, TRQ8 was the only strain that showed the ability to produce siderophores (8.1 ±0.8%). In addition, it was observed that certain strains under study showed phosphate solubilization capacity ($TE3^T$, 43.2 ±1.7%; TRQ8, 38.0 ±0.9%; TSO9, 54 ±1%), indole production (TRQ8, 12 ±0.4; TRQ65, 28.8 ±0.9 ppm). Finally, all strains showed the ability to tolerate thermal stress (43.5 °C), water [Polyethylene glycol (PEG), 10%, -0.84 mPa)], saline (NaCl, 5%, 6.8 dS m^{-1}) and chemical (Chlorothalonil).

The molecular identification of the strains under study was performed by sequencing the 16S rRNA gene, based on what was reported by Valenzuela-Aragon *et al*. (2019). The sequences obtained were edited with the FINCH TV software (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and were compared with the BLASTn database of the National Center for Biotechnology Information (NCBI), considering the percentage of maximum identity. The 16S rRNA gene sequences of the strains studied were deposited at the NCBI Genbank (https://www.ncbi.nlm.nih.gov/) and published with the accession number: MH057214 (TE3^T), MK493655 (TSO9), MK493638 (TRQ8) and MK493706 (TRQ65).

The DNA sequences obtained were used to construct a phylogenetic tree using the CLC Sequence Viewer software (bio CLC, Aarhus, Denmark), with the Kimura80 evolution model, and the Neighbor-Joining construction model. This construction was done based on 1000 iterations to calculate the percentage of bootstrap. The 16S rRNA gene sequence of *Pseudomonas* sp. (MH569548) was used as 'outgroup'. Thus, strain TRQ65 was taxonomically affiliated with *Bacillus paralicheniformis* (100% coverage and 100% identity) (Valenzuela-Ruiz *et al*., 2019), TSO9 to *B. subtilis* (100% coverage and 100% identity) (Villa-Rodríguez *et al*., 2019), TE3^T to *B. cabrialesii* (100% coverage and 100% identity) (de los Santos-Villalobos *et al*., 2019) and TRQ8 to *Bacillus megaterium* (100% coverage and 99.66% identity) (Robles-Montoya *et al*., 2019). The taxonomic affiliation mentioned above was supported by the value of bootstrap, indicating the percentage at which the configuration of a clade is repeated in 1000 random simulations or iterations (Figure 1).

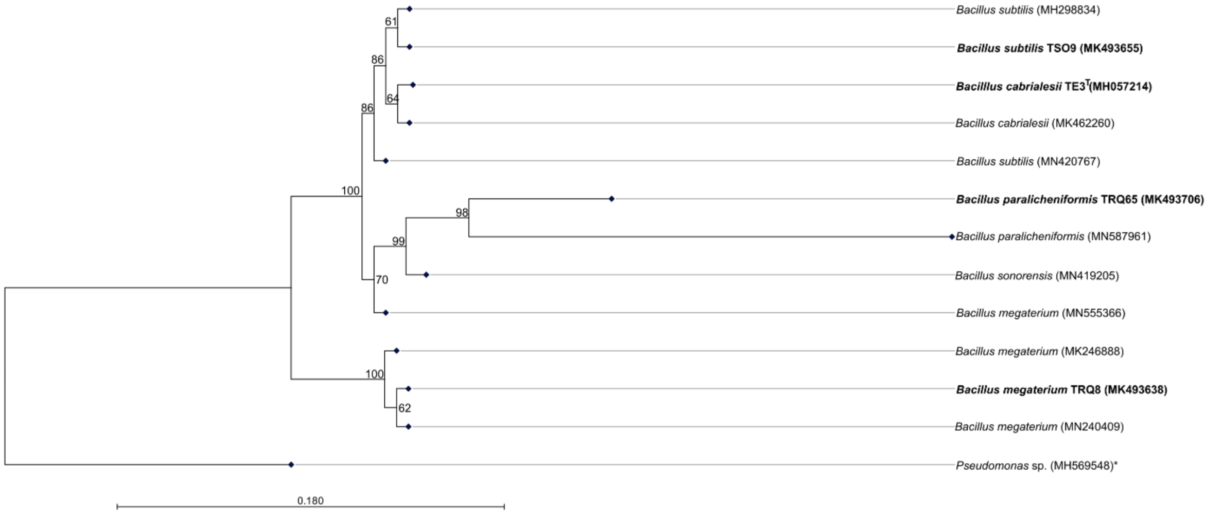

Figure 1. Phylogenetic tree constructed with the 16S rRNA gene sequences of the strains under study, with the Kimura80 and Neighbor-Joining models, clades were determined with 1 000 iterations (bootstrap). *= outgroup sequence (*Pseudomonas* sp. MH569548).

The impact of inoculation of the bacterial consortium composed of *Bacillus megaterium* TRQ8, *B. paralicheniformis* TRQ65, *B. cabrialesii* TE3^T, and *B. subtilis* TSO9 on biometric traits of wheat seedlings (*Triticum turgidum* subsp. *durum*) CIRNO C2008 variety was studied. For this, pre-inoculums of each bacterial strain were made, inoculating ~10^4 colony forming units (CFU) mL^{-1} in 10 mL of sterile nutrient broth (CN, DIBICO$^{®}$) and incubated at 28 °C with continuous agitation

(120 RPM) for 24 h. Subsequently, the inoculums were generated by inoculating 1 mL of the pre-inoculum (optical density at 600 nm: 0.5) in 500 mL of CN and incubating under the conditions described above. Finally, the bacterial cultures were centrifuged at 3 500 RCF, for 15 minutes, performing two washes with sterile distilled water (15 lb, 121 °C, 15 min) and the cell density of each strain under study was adjusted to $1*10^7$ CFU mL^{-1} , with sterile distilled water.

Wheat seeds were superficially disinfected, washing them with commercial soap and soaking them in sterile distilled water (121 °C and 15 lb per 15min) for 24 h, then the seeds were washed with 2% NaCl for 10 min, followed by 3 washes with sterile distilled water. The disinfected seeds were placed in 230 g of a sterile soil mixture (121 °C and 15 lb for 5 days), at a rate of 70:30 (perlite: agricultural soil). The agricultural soil used was collected from commercial plots for wheat production in the Yaqui Valley (hyposodic vertisol, pH 7.74 ±0.05, organic matter <1%, clay texture 40-60%).

A unifactorial design with two treatments was used (two independent repetitions, n= 15 per repetition): i) inoculated seedlings ($4*10^7$ CFU, at 0 and 15 days after planting) by the consortium under study vs; and ii) a negative control (seedlings not inoculated and sprinkled with sterile distilled water). The experiment was carried out in a growth chamber (BJPX-A450, BIOBASE) under 70% relative humidity, temperature of 25 °C (day) and 15 °C (night), and a photoperiod of 14 h light/12 h dark, for 30 days. Once the experiment was completed, the biometric variables evaluated in the seedlings were total length, height, root length, dry weight of the shoot and root, stem thickness, and biovolume index (Thilagar *et al*., 2016).

The bacterial consortium integrated by *Bacillus cabrialesii* TE3^T, *B. paralicheniformis* TRQ65, *B. megaterium* TRQ8 and *B. subtilis* TSO9 showed a significant increase with respect to the control treatment [Fischer LSD ($p \leq 0.05$), Statgraphics 5.0] on the length of the aerial part (28%), root length (25%), total length (28%), stem diameter (46%), circumference (50%), dry weight of the aerial part (72%) and the biovolume index (57%) (Table 1).

Table 1. Effect of inoculation of a bacterial consortium of the genus *Bacillus* on wheat seedlings, 30 days after inoculation, under controlled conditions. *significant difference ($p < 0.05$) Fisher LSD.

Variable	Treatment	
	Control	Bacterial consortium
Length of the aerial part (cm)	18.59 ±5.41	23.88 ±3.75*
Root length (cm)	7.41 ±2.24	9.33 ±1.16*
Total length (cm)	26.01 ±6.79	33.41 ±5.55*
Stem diameter (cm)	0.15 ±0.04	0.22 ±0.05*
Dry weight of the aerial part (g)	0.05 ±0.02	0.086 ±0.03*
Dry root weight (g)	0.09 ±0.02	0.1 ±0.03
Biovolume index	68.79 ±29.96	177.21 ±52.32*

The bacterial consortium studied increased biomass production and cell elongation in inoculated wheat seedlings (Table 1), which may be associated with the metabolic capacity of the strains, ie. production of indoles (12-28 ppm), siderophores (8%), phosphate solubilization (38-54%), repression of the growth of phytopathogenic microorganisms, and resistance to stress conditions (Villarreal-Delgado *et al*. 2018; Valenzuela-Aragón *et al*., 2019; Díaz-Rodríguez *et al*., 2019; Villa-Rodríguez *et al*., 2019). The inoculation of BPCV producing phytohormones leads to an increase in the length and root biomass of plants, promoting the acquisition of nutrients from the soil and promoting plant growth, even under stress conditions (Valenzuela-Ruiz *et al*., 2018); this beneficial effect is also coupled with a greater bioavailability and mobilization of nutrients towards the plant (Jacoby *et al*., 2017). The ability to solubilize phosphates, as well as siderophores production, has been associated with the increase in the growth rate and yield in wheat, maintaining this attribute under conditions of chemical stress and decreased dose of inorganic fertilization (Munir *et al*., 2019).

The results obtained in this research show the compatibility and ecological/functional role *in vivo* of the metabolic activities detected (and others not described so far) in the strains that make up the bacterial consortium under study (*Bacillus cabrialesii* TE3^T, *B. paralicheniformis* TRQ65, *B. megaterium* TRQ8 and *B. subtilis* TSO9). Thus, the positive effects observed in wheat seedlings inoculated with the developed bacterial consortium can be attributed to the metabolic diversity of the *Bacillus* strains that integrate it, as well as their ability to respond as an organism to the various environmental stressors, which gives an increased capacity for adaptation (synergistic interactions to provide nutrients and stimulate their growth, degradation of inhibitory compounds, microbial competition, among others), compared to individual strains.

Conclusions

Inoculation of a bacterial consortium formed by strains *B. cabrialesii* TE3^T, *B. paralicheniformis* TRQ65, *B. megaterium* TRQ8 and *B. subtilis* TSO9 significantly increased biometric traits in wheat seedlings related to cell elongation, biomass acquisition and efficient use of the water. This suggests that the metabolic attributes shown by the constituent strains of the consortium have a synergistic effect on the acquisition of nutrients, as well as on the regulation of phytohormones in the plant. The results presented suggest evaluating said bacterial consortium under field conditions and developing a biofertilizer for use in current and future agriculture.

Acknowledgments

The authors are grateful for the funding granted by CONACYT to project 257246, 'Wheat interaction x plant growth promoting microorganisms: identifying genes with agro-biotechnological potential'. In addition, the masters and doctorate scholarship granted by the National Council of Science and Technology (CONACYT) to Rosa Icela Robles Montoya and Luis Abraham Chaparro Encinas.

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