Brazilian Journal of Microbiology (2023) 54:397–406
https://doi.org/10.1007/s42770-022-00874-3

SOIL AND AGRICULTURAL MICROBIOLOGY - RESEARCH PAPER

Bacillus subtilis and *Bacillus licheniformis* promote tomato growth

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Received: 23 July 2022 / Accepted: 10 November 2022 / Published online: 24 November 2022

Abstract

Bacillus spp. are widely marketed and used in agricultural systems as antagonists to various phytopathogens, but it can also benefit the plant as plant growth promoters. Therefore, the longer presence of the bacterium in the rhizosphere would result in a prolonged growth-promoting benefit, but little is yet known about its persistence in the rhizosphere after seed coating. The objectives of this study were to evaluate the tomato growth promotion mediated by *Bacillus licheniformis* FMCH001 and *Bacillus subtilis* FMCH002 and the survival rate of these bacteria both in shoots and in the rhizosphere. The *Bacillus* strains used throughout this study were obtained from Quartzo® produced by Chr. Hansen. The application of a mixture of *B. subtilis* and *B. licheniformis* (Quartzo®) at concentrations 1×10^8, 1×10^9, and 1×10^{10} CFU mL^{-1}, as well as the application of *B. subtilis* and *B. licheniformis* individually at concentration 1×10^8 CFU mL^{-1}, increased fresh and dry masses of shoot and root system, volume of root system, and length of roots of tomato plants when compared to control. Both *Bacillus* strains produced IAA after 48 h of in vitro. *Bacillus* colonies obtained from plant sap were morphologically similar to colonies of *B. subtilis* and *B. licheniformis* strains and were detected in inoculated on plants and not detected in control ones. A similar pattern was obtained through DNA-based detection (qPCR). Therefore, *B. subtilis* and *B. licheniformis* were able to produce auxin, promote tomato growth, and colonize and persist in the rhizosphere.

Keywords Plant growth promotion rhizobacteria · PGPR · *Bacillus* · *Solanum lycopersicum*

Introduction

Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that promote plant growth, protect crops against plant pathogens, and improve soil health [1–5]. The use of PGPR is a sustainable tool to mitigate the dependence on chemical fungicides and fertilizers and/or increase agricultural production [6].

Among the PGPR, *Bacilli* are recognized as the most important species [7–10], being widely marketed and used in agricultural systems as biofertilizers and/or antagonists to various phytopathogens [11, 12]. *Bacillus* spp. produce spores which are resistant to several environmental conditions [13, 14]. Besides, effectively colonizing roots, they are also efficient biocontrol agents being recognized by the ability to promote plant growth [15–22]. *Bacillus*-mediated plant growth promotion can occur through direct and indirect mechanisms [23–25]. Indirectly, *Bacillus* spp. minimize the problems that phytopathogens cause to plant growth, consequently increasing plant health [23, 26]. Directly, *Bacillus* spp. produce siderophore and phytohormones (indole-3-acetic acid, cytokinin, gibberellic acid), solubilize phosphorus and other nutrients that stimulate plant growth, and increase root volume [20, 27–34]. The change in the architecture of the root system also influences the plant's ability to exploit the soil by improving water and nutrient uptake [35].

Several species of *Bacillus* have been reported in the promotion of plant growth, such as *B. velezensis* AP-3 and *Bacillus* spp. in tomato [19]; *B. subtilis* in tomato [36] and additionally in okra and spinach [37]; *B. velezensis* AP-3, S2547, and S2545 in cotton [20]; *B. simplex* in pea [38]; *B. amyloliquefaciens* in *Lemna minor* [27]; *B. licheniformis* in chrysanthemum [39], tomato [40], and maize [11]; and *B.*

Responsible Editor: Luis Nero

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cenocepacia in tomato [40]. In many of these publications, it was observed that the *Bacillus* isolates produced phytohormones and siderophores and/or solubilized phosphorus.

Several *Bacillus* strains are applied in biological control and in the promotion of plant growth [41]. However, the success of these microorganisms in an agricultural application depends on the colonization efficiency and persistence in the rhizosphere of plants, which are considered one of the great challenges for biological control and one of the main reasons for instability in the activity of bacterial inoculants in the soil [42]. Rhizosphere colonization is crucial for PGPR and plant interactions [43].

The objectives of this study were to evaluate the tomato growth promotion mediated by *Bacillus licheniformis* FMCH001 and *Bacillus subtilis* FMCH002 and the survival rate of these bacteria both in shoots and in the rhizosphere.

Materials and methods

The assays were conducted at Embrapa Meio Ambiente, Laboratório de Microbiologia Ambiental "Raquel Ghini," located in Jaguariúna, SP, Brazil.

Microorganisms

Bacillus licheniformis FMCH001 and *Bacillus subtilis* FMCH002 strains used throughout this study were obtained from Quartzo® produced by Chr. Hansen (Valinhos, SP, Brazil) and commercialized by FMC Química do Brasil Ltda. (Campinas, SP, Brazil). The isolates and the product formulated with the mixtures of the two *Bacillus* isolates were provided by Chr. Hansen. The isolates were applied separately to demonstrate the potential of each isolate to produce indole-acetic acid and promote tomato growth.

Indole-acetic acid production

To evaluate the production of indole-acetic acid (IAA), the isolates of *B. subtilis* and *B. licheniformis* were multiplied in 250-mL flasks containing 100 mL of Czapek medium (30 g of sucrose, 2 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$, 0.02 g of $FeSO_4$, and 1000 mL of distilled water) supplemented with 0.5 g of L-tryptophan L^{-1} medium. The flasks were incubated on a shaker table (TE-1401, Technal®, Piracicaba, SP, Brazil) at 150 rpm and 25 ± 2 °C for 2 days. Subsequently, the cultures were centrifuged at 7000 rpm for 10 min, the supernatants were collected, and the IAA concentration was determined using the Salkowski reagent (1 mL of 0.5 mol L^{-1} $FeCl_3.6H_2O$ in 50 mL of 35% $HClO_4$), according to Gordon and Weber (1951). The reaction was established by adding 100 µL of culture supernatant or Czapek medium (control), and 100 µL of the Salkowski reagent. Subsequently, the mixture was kept at room temperature for 30 min, and the absorbance was determined in a spectrophotometer at 530 nm. The concentration of IAA in the supernatant was calculated based on a standard curve prepared with IAA (Sigma-Aldrich®, St. Louis, Missouri, USA) (98% purity) at concentrations of 5, 10, 20, 50, and 100 µg mL^{-1}. The mean of the control readings was discounted from the mean of the isolate's readings. The trial was conducted in a completely randomized design with three replications and repeated to confirm the data.

Mixture of *B. subtilis* and *B. licheniformis* on seed germination and emergence of tomato seedlings

Tomato seeds cultivar Santa Clara® were superficially disinfected in alcohol (70%) and in sodium hypochlorite (2.5%) for 2 min each. Then, they were washed three times in sterile distilled water and dried on sterile filter paper in an aseptic chamber. After drying, the seeds were placed in a 250-mL Erlenmeyer flask containing 100 mL of the suspension of Quartzo® (*B. subtilis* and *B. licheniformis*) at concentrations of 0, 1×10^8, 1×10^9, and 1×10^{10} CFU mL^{-1}. After agitation at 100 rpm for 1 h, seeds were dried on filter paper for 1 h. Sowing was carried out in plastic boxes (Gerbox®), on a three-sheet germitest paper (Germiagro, Ribeirão Preto, SP), moistened with distilled water in an amount equivalent to 2.5 times the weight of the dry paper. The boxes were kept in a growth chamber at 25 ± 2 °C for 8 days. Seed germination was evaluated 5 and 8 days after the test was set up, and the results were expressed as percentage of normal seedlings [44]. The radicle protrusion was evaluated 3 days after sowing. The experiment was carried out in a completely randomized design with four replications, each replication represented by a plastic box with 50 seeds. Data were analyzed by regression at 5% probability. The test was repeated twice.

Bacillus subtilis and *B. licheniformis* in promoting the growth of tomato plants grown on rhizotrons

Tomato seedlings (cultivar Santa Clara®, Bragança Paulista, SP) were produced in multicell (200 cells) growing trays containing a commercial substrate (Tropstrato HT Hortaliças®). The seeds were sown without surface disinfection. Twenty-day-old seedlings were transferred to rhizotrons (built in 17.5 diameter PVC tubes, 100 cm long, cut longitudinally in half), containing a mixture of soil and the commercial substrate Terra Nostra® (Tatui, SP) in the proportion of 3:1 (v/v). The soil was collected in the experimental area at Embrapa Meio Ambiente, presenting the following chemical and physical attributes analyzed at 0–20 cm depth: pH in H_2O = 4.3; OM = 32.3 g Kg^{-1}; P = 9.36 mg dm^{-3}; Ca = 3.09 cmolc dm^{-3}; Mg = 1.48 cmolc dm^{-3}; K = 128.55 mg dm^{-3}; SB = 4.95 cmolc dm^{-3}; H + Al = 6.10 cmolc dm^{-3}; *t* = 4.99 cmolc dm^{-3}; V% = 44.54.

The commercial product Quartzo® (*B. subtilis* and *B. licheniformis*) was applied at concentrations of 1×10^8, 1×10^9, and

1×10^{10} CFU mL^{-1}. *B. subtilis* and *B. licheniformis* isolates were applied separately, each at a concentration of 1×10^{8} CFU mL^{-1}. Besides the application of 2.5 mL of *Bacillus* suspension on the seedling substrate, 10 mL of *Bacillus* were applied on container media immediately after transplanting and after 10 and 20 days. The individual isolates of *B. subtilis* and *B. licheniformis* were multiplied in a 125-mL Erlenmeyer flask containing 50 mL of GPL medium (10 g glucose; 10 g peptone; 5 g yeast extract; 3 g NaCl; 1 g of KH_2PO_4; 0.5 g of Mg $SO_4.7H_2O$; 1000 ml of distilled water, pH 6.0), under constant stirring at 150 rpm at 28 ± 2 °C. After 48 h, the content of the Erlenmeyer flask was transferred to a 50-mL Falcon tube and centrifuged for 10 min at 8000 rpm. Subsequently, the supernatant was discarded, and the pellet was resuspended in 0.85% (m/v) NaCl solution. Afterwards, the concentration was adjusted in a spectrophotometer (Shimadzu®; UV-1601 PC) $OD_{540}=2.250$ nm, which corresponds to 1×10^{8} CFU mL^{-1}. This concentration was then confirmed by the serial dilution method.

The assays were conducted in a completely randomized design with five replicates and six treatments. The assay was performed twice, the first between November and December 2019 and the second between May and June 2021. The first and second tests were evaluated 32 and 36 days after transplanting the seedlings, respectively. Plant height, stem base diameter (mm), shoot and root system fresh and dry masses (g), volume of root system (mL) and root length (cm), and chlorophyll contents (using a Chlorophyll Meter SPAD-502 Plus) were evaluated.

Colonization of tomato plants by *B. subtilis* and *B. licheniformis*

A 2.5 mL suspension of the commercial product Quartzo®, at concentration of 1×10^{10} CFU mL^{-1}, is applied according to Table 2 to assess the colonization of *B. subtilis* FMCH002 and *B. licheniformis* FMCH001 strains in the shoots and root system of tomato plants. Fourteen-day-old tomato seedlings Santa Clara® cultivar, previously grown on multicell growing trays (200 cells) containing substrate (Tropstrato HT Hortaliças®) autoclaved three times for 1 h for 3 consecutive days, were transferred to pots containing 500 mL of the same substrate.

The experiment was carried out in a completely randomized design with four replicates. Forty four-day-old plants were collected from two replicates to perform the isolation of endophytic bacteria from the stem and rhizosphere in order to determine colony-forming units of *Bacillus*. From the other two replicates, 45-day-old plants were collected to determine the presence of *B. subtilis* (FMCH002) and *B. licheniformis* (FMCH001) strains in plant roots/rhizosphere via quantitative real-time PCR (qPCR).

The isolation of bacteria was carried out directly from the exuded sap of the plant by cutting the tomato plants transversally and collecting the exuded sap with micropipette. The collected sap was transferred to Petri dishes containing nutrient agar medium (Himidia, 26 Mumbai-India). The presence or absence of *Bacillus* colonies was evaluated after 48 h.

The determination of total bacteria in the rhizosphere was carried out by adding 5 g of roots and rhizosphere substrate in a 125-mL Erlenmeyer flask containing 45 mL of $MgSO_47H_2O$ suspension (0.01 M). After stirring for 5 min on a shaker at 100 rpm and for 5 min in ultrasound, serial dilutions from 10^{-1} to 10^{-5} were performed. Then, 0.1 mL of each dilution was transferred to Petri dishes containing nutrient agar culture medium. The plates were kept in a growth chamber at 28 ± 2 °C, and colony counts were performed after 24 and 48 h. Only counts ranging from 30 to 300 colonies/plate were considered [45, 46]. The assays were carried out in a completely randomized design with three replicates, with each replicate represented by a Petri dish. For the quantification of the total bacteria, a dilution of 10^{-4} was used.

For the quantification of *B. licheniformis* (FMCH001) and *B. subtilis* (FMCH002) by qPCR, the extraction of total DNA from the samples was performed using 180 mg of root. Extraction was performed using the MP Biomedicals™ FastDNA™ SPIN soil kit according to the manufacturer's instructions. The qPCR reaction was performed in a final volume of 12 µL, composed of 5.0 µL of DNA, 6.25 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), and 0.25 µL of each primer. Specific primer pairs were used for each strain. The sequence of the primers and the validation protocols are of FMC proprietary, and the specific quantification of the tested *Bacillus* spp. can be performed in collaboration with the company, if desired. For amplification, an initial denaturation cycle of 98 °C for 3 min was used. Afterwards, 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 25 s were used. All treatments were evaluated in biological and technical triplicate. The values obtained were estimated from standard curves for each strain, obtained from values from 1×10^{3} to 1×10^{8} CFU g^{-1} of root.

Statistical analyses

The data of the assay where the effect of the application of the mixture of *B. subtilis* with *B. licheniformis* on germination and emergence of tomato seedlings were evaluated by regression. Assays of IAA production, of plants grown in rhizotrons, and of bacteria colonization on tomato plants were performed in a completely randomized design and analyzed by comparing means using the Scott-Knott test ($p<0.05$). Statistical analyses were performed using the statistical software RStudio® version 4.0.0.

Results

Indole-acetic acid production

The average production of IAA by *B. subtilis* (FMCH002) and *B. licheniformis* (FMCH001), in the first assay, was 9.60

Table 1 The effect of *Bacillus subtilis* FMCH002 (BS) and *Bacillus licheniformis* FMCH001 (BL) on the growth promotion of tomato plants

	FSM (g/plant)	DSM (g/plant)	FRM (g/plant)	DRM (g/plant)	VR (mL/plant)	LR (cm)	H (cm)	CH	D (mm)
Assay 1									
BL + BS 10^8 UFC mL^{-1}	127.29a	13.33a	33.93a	2.59a	36.0a	74.4a	64.0 ns	35.28 ns	9.21 ns
BL + BS 10^9 UFC mL^{-1}	144.54a	15.24a	33.09a	2.68a	40.2a	79.3a	69.0 ns	33.05 ns	9.06 ns
BL + BS 10^{10} UFC mL^{-1}	131.76a	13.30a	37.66a	3.01a	42.0a	83.8a	69.4 ns	32.00 ns	8.77 ns
BL 10^8 UFC mL^{-1}	145.25a	15.54a	37.10a	2.99a	39.0a	79.0a	65.8 ns	36.31 ns	9.04 ns
BS 10^8 UFC mL^{-1}	118.91a	12.11a	31.15a	2.19a	38.0a	79.8a	66.4 ns	34.37 ns	9,07 ns
Control	93.69b	8.88b	14.76b	0.79b	17.4b	58.2b	56.6 ns	34.70 ns	8.32 ns
C.V. (%)	18.54	20.72	19.68	33.01	24.21	13.77	10.40	8.22	6.55
Assay 2									
BL + BS 10^8 UFC mL^{-1}	115.50b	9.55a	18.04a	1.21b	29.0a	92.6a	58.8 ns	45.46 ns	10.08 ns
BL + BS 10^9 UFC mL^{-1}	141.20a	10.79a	20.86a	1.43b	29.4a	98.0a	58.0 ns	45.10 ns	9.59 ns
BL + BS 10^{10} UFC mL^{-1}	121.00b	9.63a	21.02a	1.43b	36.0a	99.2a	57.0 ns	48.46 ns	9.30 ns
BL 10^8 UFC mL^{-1}	119.40b	10.32a	23.82a	1.83a	37.0a	95.6a	62.6 ns	46.06 ns	9.64 ns
BS 10^8 UFC mL^{-1}	110.60b	10.11a	18.62a	1.40b	32.0a	87.2a	59.2 ns	47.79 ns	8.61 ns
Control	84.60c	6.44b	9.34b	0.66c	11.2b	74.6b	52.4 ns	41.44 ns	10.47 ns
C.V. (%)	11.95	9.32	21.57	23.07	9.84	12.03	9.59	7.44	17.8

FSM fresh shoot mass, *DSM* dry shoot mass, *FRM* fresh root mass, *DRM* dry root mass, *VR* volume of root, *LR* length of root (cm), *H* height (cm), *CH* chlorophyll (SPAD-502 Plus), *D* diameter (mm), *ns* not significant
Means followed by the same letter do not differ from each other by the Scott-Knott test at 5% probability

and 16.88 µg of IAA mL^{-1} of supernatant, respectively, and in the second assay, the average production was 9.04 and 16.84 µg IAA mL^{-1} of supernatant, respectively. Both *Bacillus* strains produced IAA after 48 h of in vitro growth.

Mixture of *B. subtilis* and *B. licheniformis* on seed germination and emergence of tomato seedlings

Germination and root protrusion of tomato seeds treated with a mixture of *B. subtilis* and *B. licheniformis* increased linearly according to the doses applied (1×10^8 to 1×10^{10} CFU mL^{-1}). The maximum germination of 91.5% was reached for concentrations of 1×10^9 and 1×10^{10} CFU mL^{-1}. Regarding radicle protrusion, these same concentrations reached its plateau at 72.5% protrusion.

Bacillus subtilis and *B. licheniformis* in promoting the growth of tomato plants grown on rhizotrons

The application of a mixture of *B. subtilis* and *B. licheniformis* (Quartzo®) at concentrations 1×10^8, 1 x $\times10^9$, and 1×10^{10} CFU mL^1, as well as the application of *B. subtilis* and *B. licheniformis* individually at concentration 1×10^8 CFU mL^{-1}, increased ($p<0.05$) fresh and dry masses of shoot and root system, volume of root system, and length of roots of tomato plants when compared to control (Table 1, Fig. 1).

In the first assay, *B. licheniformis*, at a concentration of 1×10^8 CFU mL^{-1}, increased the fresh and dry masses of shoots by 55% and 75%, respectively, when compared to control. The mixture of *B. subtilis* and *B. licheniformis*, at a concentration of 1×10^{10} CFU mL^{-1}, increased the fresh and dry masses of the root system by 155% and 280%, respectively, when compared to control. Root system volume and root length were increased by 141% and 44%, respectively, when plants were amended with mixture of *B. subtilis* and *B. licheniformis* (Quartzo®) at 1×10^{10} CFU mL^{-1} concentration (Table 1).

In the second assay, the results were similar to the first assay. However, the application of the mixture of *B. subtilis* and *B. licheniformis* at concentration of 1×10^9 CFU mL^{-1} differed from the other treatments for shoot fresh mass, with an increase of 67% when compared to control. For shoot dry mass, the mixture of *B. subtilis* and *B. licheniformis*, at a concentration of 1×10^9 CFU mL^{-1}, showed an increase of 67% when compared to control. For root system dry mass, *B. licheniformis* at 1×10^8 CFU mL^{-1} differed from the other treatments, showing an increase of 230% when compared to control. *Bacillus* strains also increased root system fresh mass, root volume, and length, differing statistically from control.

Despite the height being higher in treatments with *Bacillus*, no significant differences were observed. When, in the first assay, a mixture of *B. subtilis* and *B. licheniformis* was applied at concentrations of 1×10^9 and 1×10^{10} CFU mL^{-1}, an increment of approximately 22.6%, for both concentrations was observed when

Fig. 1 The effect of *Bacillus subtilis* FMCH002 (BS) and *Bacillus licheniformis* FMCH001 (BL) on the growth of tomato plants 36 days after planting on rhizotrons. BS = *B. subtilis* 1×10^8 CFU mL^{-1}. BL = *B. licheniformis* 1×10^8 CFU mL^{-1}. BL + BS 10^{10}, 10^9, and 10^8 = *B. licheniformis* + *B. subtilis* at concentrations of 1×10^{10}, 1×10^9, 1×10^8 UFC mL^{-1}, respectively

compared to control (Table 1). In the second assay, *B. licheniformis*, at a concentration of 1×10^8 CFU mL^{-1}, increased height by 20% in relation to control. *B. subtilis* and *B. licheniformis* did not increase the stem diameter of the plants nor the chlorophyll content of the leaves.

Colonization of tomato plants by *B. subtilis* and *B. licheniformis*

Bacillus colonies obtained from plant sap, using the technique of isolating bacteria directly, were morphologically similar to colonies of *B. subtilis* and *B. licheniformis* strains inoculated on plants as described by Bettiol et al. [47]. There was no detected bacterial growth from control plants (Fig. 2).

By counting colonies in Petri dishes, we observed that the highest concentration of total bacteria in the rhizosphere was 6×10^7 CFU g^{-1}. In general, the same concentration was observed for all treatments, except the ones with seed treatment and in-furrow application, with concentrations of 2.8×10^6 and 4×10^5 CFU g^{-1} of rhizosphere, respectively. In this case, total bacterial concentration was similar to control (2.0×10^5 CFU g^{-1} of rhizosphere) (Table 2).

Using the qPCR technique, it was possible to quantify the number of bacterial cells g^{-1} of root. Although the *Bacilli* were detected from the biocontrol treated plants, it was not possible to correlate the different forms and times of application with the number of cells of bacterial strains in tomato roots. When the application of the *Bacillus* mixture was performed 7 days after transplanting, bacterial concentration of 7.46×10^9 g^{-1} in root was observed. With two applications of the *Bacillus* mixture, one on the seeds and the other on the container media substrate, 7 days after transplanting, the observed number of bacterial cells was 8.35×10^9 g^{-1} of root. The presence of *Bacillus* was not observed in the water-treated control. For the other treatments, the concentration ranged from 9.2×10^3 to 5.6×10^6 cells g^{-1} of root (Table 3).

Discussion

We describe the potential of *B. subtilis* (FMCH002) and *B. licheniformis* (FMCH001) to promote tomato growth when applied either in a formulated mixture (Quartzo®) and separately. The application of *Bacillus* strains not only promoted root growth and the increase in fresh and dry masses of the tomato root system and shoot, but also stimulated seed germination (Figs. 1 and 2, Table 2). The ability of *Bacillus* spp. to promote plant growth is reported by Franco-Sierra et al. [48], Liu et al. [49], Raji and Thangavelu [40], and Shahid et al. [50] among others.

The success of PGPR depends on an efficient colonization of the root system [51]. *B. subtilis* FMCH002 and *B. licheniformis* FMCH001 isolates efficiently colonized the root system of tomato plants (Tables 1, 2, and 3). The presence of *Bacillus*, both in the stem and in the rhizosphere of such plants, indicates the survival of the isolates (Tables 2 and 3). Kalam et al. [52] demonstrated that active colonization of tomato roots by *B. subtilis* is important for promoting plant growth and nutrition. However, the level of colonization of strains FMCH001 and FMCH002 was influenced by the number and form of application (Tables 2 and 3). The populations of the introduced bacteria in the rhizosphere was similar to that described by Abdallah et al. [41] who reported that a closely related bacterial species (*Bacillus amyloliquefaciens*) colonizing the rhizoplane of tomato plants and was detected at a rate of 1×10^5 to 1×10^7 CFU g^{-1} of root. Actually, it seems that the concentration of the bacterium in the

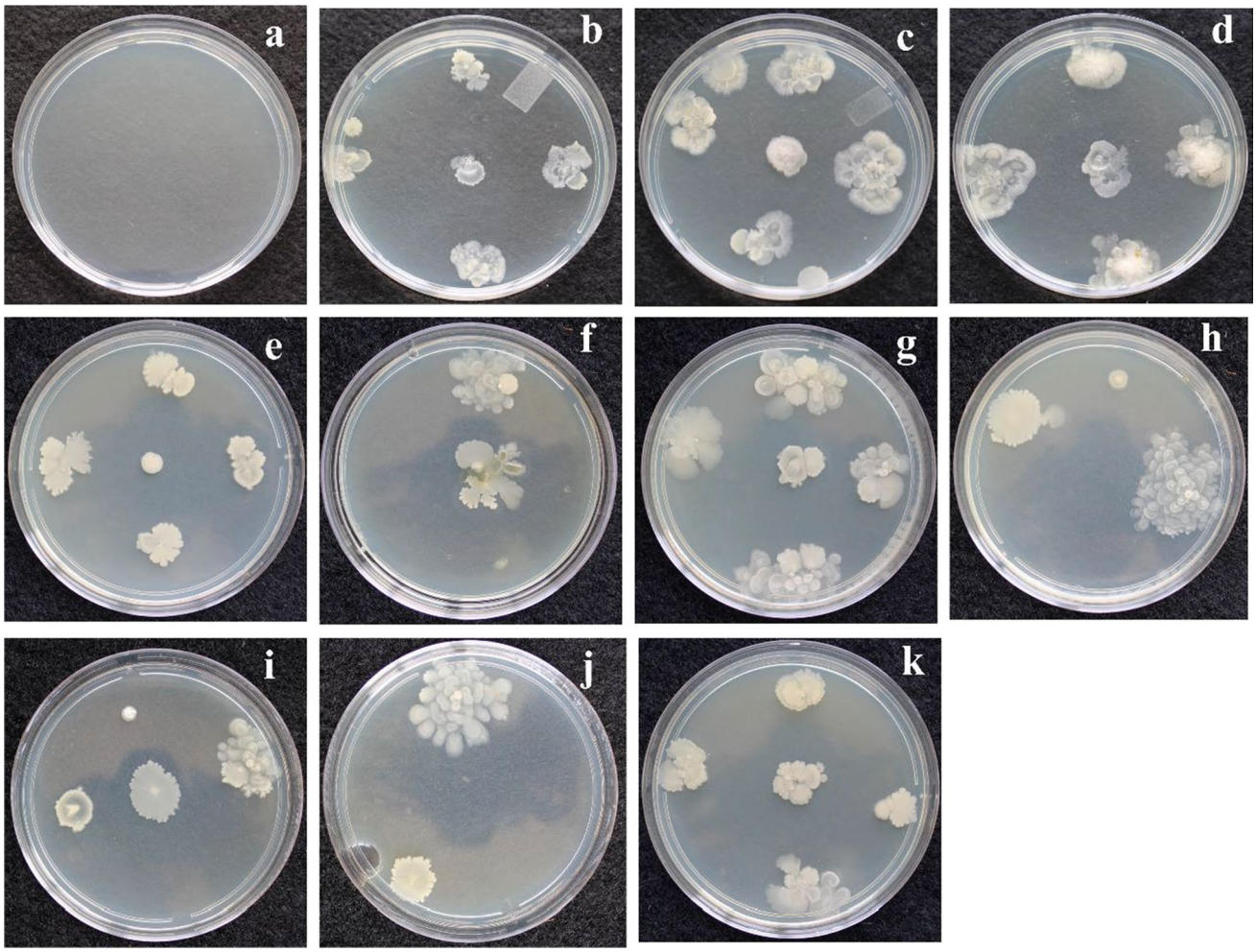

Fig. 2 Colonies of bacteria isolated from the sap exuded after the cross section of the tomato stem obtained from different forms and application times of the mixture containing *Bacillus subtilis* FMCH002 and *Bacillus licheniformis* FMCH001 (Quartzo®) at a concentration of 1 × 1010 CFU mL-1. **a** Control; **b** application on the seeding substrate; **c** seeds treatment; **d** application on the seeding substrate and seeds treatment; **e** application 7 days after transferred to container media (7DAT); **f** application on the seeding substrate and 7 DAT; **g** seeds treatments and 7 DAT; **h** application on the container media; **i** application on the seeding substrate and container media; **j** seeds treatments and container media; **k** application on the seeding substrate, seeds treatment, container media, and 7 DAT

Table 2 Total bacteria in the tomato rhizosphere originating from plants that were treated with a mixture of *Bacillus subtilis* FMCH002 and *Bacillus licheniformis* FMCH001 (Quartzo®) at the concentration of 1×10^{10} colony forming units (UFC g^{-1} of root)

Treatments	UFC g^{-1} root
Control	2.0×10^5 c
Application on the seeding substrate	2.8×10^6 c
Seeds treatment	4.0×10^5 c
Application on the seeding substrate and seeds treatment	5.0×10^7 a
Application 7 days after transferred to container media (7 DAT)	5.0×10^7 a
Application on the seeding substrate and 7 DAT	6.0×10^7 a
Seeds treatments and 7 DAT	3.0×10^7 b
Application on the container media	5.7×10^7 a
Application on the seeding substrate and container media	5.8×10^7 a
Seeds treatments and container media	5.9×10^7 a
Application on the seeding substrate, seeds treatment, container media, and 7 DAT	6.0×10^7 a
CV (%)	29.05

Quartzo® (FMC Química do Brasil Ltda.) = *Bacillus subtilis* FMCH002 and *Bacillus licheniformis* FMCH001

Table 3 A number of *Bacillus subtilis* FMCH002 and *Bacillus licheniformis* FMCH001 g^{-1} of root determined by the quantitative real-time PCR technique in tomato roots originating from plants were treated with the mixture of *B. subtilis* FMCH002 and *B. licheniformis* FMCH001 (Quartzo®) at the concentration of 1×10^{10} colony forming units (UFC mL^{-1})

Treatments	UFC g^{-1} of root
Control	0
Application on the seeding substrate	1.78×10^4
Seeds treatment	1.17×10^6
Application on the seeding substrate and seeds treatment	9.20×10^3
Application 7 days after transferred to container media (7 DAT)	7.46×10^9
Application on the seeding substrate and 7 DAT	3.56×10^6
Seeds treatments and 7 DAT	8.35×10^9
Application on the container media	3.47×10^6
Application on the seeding substrate and container media	7.41×10^5
Seeds treatments and container media	5.60×10^6
Application on the seeding substrate, seeds treatment, container media, and 7 DAT	2.03×10^6

Quartzo® (FMC Química do Brasil Ltda.) = *Bacillus subtilis* FMCH002 and *Bacillus licheniformis* FMCH001

rhizosphere is directly related to its efficacy, and tracking the population of the introduced bacterium can be proposed as a strategy to assure more consistent results in the field. Chen et al. [53] reported that root colonization by *B. subtilis* B579 at less than 10^4 CFU g^{-1} of rhizosphere did not affect cucumber production, while concentrations higher than that have promoted the plant performance.

The tomato growth promotion can occur through a direct production of plant hormones or increasing nutrient uptakes. *B. subtilis* and *B. licheniformis* are reported to be hormone and siderophore producers, phosphate solubilizers, and nitrogen fixers [29, 41, 54–57]. The isolates of *B. subtilis* FMCH002 and *B. licheniformis* FMCH001 produced 9.60 and 16.88 µg of IAA mL^{-1}, which within the range of that hormone exogenous production by bacteria to promote plant growth [53]. Although the quantification of the IAA production was performed in situ and not in planta, the commercial product is made up of the cells and supernatant as active ingredients; therefore, at least in the supernatant fraction of the product, the plant hormone is likely to be encountered [49] and has respond at least in part to the root growth promotion observed activity [58]. Once in intimate association with the root, the bacterium can produce the hormone to sustain the hormone supply to the plant [27].

Among the benefits of exogenous IAA amendment to the rhizosphere, root elongation and shoot growth are the direct benefits, especially when the bacterium also interferes with the hormone transport within the plant [59]. Although the hormone is more implicated in plant growth, it also may play a role in stress tolerance, since defense- and cell wall–strengthening genes [60].

Noteworthy, for a plant growth promoter product to be commercially available to growers at an acceptable cost, it has to exert growth promoting benefits to multiple hosts. At least, one of the strains parts of the evaluated product (*B. licheniformis* FMCH001) has confirmed its growth promotion benefit to maize, particularly observed on roots, and such benefit was sustained even under drought stress [11]. Actually, the evaluated product is registered in Brazil for gall nematode management [61], and according to Brazilian legislation, it can be recommended for whatever crop for which the nematode is a problem. From our results, one of the mechanisms by which the product may act in the soil-borne disease control for which it is recommended is the compensatory or tolerance induced effect inferred from the growth promotion potential of the crop [62].

B. subtilis and *B. licheniformis* are widely marketed and used in agricultural systems as antagonists to various phytopathogens, such as nematodes, but it is also benefiting the plant as plant growth promoters. We observed that applications of *B. subtilis* FMCH002 and *B. licheniformis* FMCH001 through seed treatment, as well as applications on the seedling substrate, and container media substrate, promoted root system growth, and both isolates survived in the roots of treated plants.

B. subtilis and *B. licheniformis* are widely marketed and used in agricultural systems as antagonists to various phytopathogens, such as nematodes, but it is also benefiting the plant as plant growth promoters, and this can be a benefit to the plant in the absence of the pathogen and support a preventive-basis application of the product, which results in higher protection against the plant parasitic nematode and, in its absence, promotes plant growth. We observed that applications of *B. subtilis* FMCH002 and *B. licheniformis* FMCH001 through seed treatment, as well as bacterial amendment to the seedling substrate, and container media substrate, promoted root system elongation and sustained its population high in the roots of treated plants.

Acknowledgements Peterson Sylvio de Oliveira Nunes acknowledges Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship. Wagner Bettiol (CNPq 307855/2019-8)

and Flavio H V Medeiros (CNPq 317266/2021-7) acknowledge Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq for the productivity fellowship. The authors acknowledge Chr. Hansen for donating the commercial product and *Bacillus* isolates, as well as for performing the qPCR analyses.

Author contribution WB and PSON conceived, designed, and performed the greenhouse and laboratory experiments. TSO and JRAZ performed the qPCR analysis. PSON and FHVM analyzed the data. WB, JRAZ, TSO, and FHVM contributed with reagents/materials/analysis tools. PSON, WB, and FHVM wrote the paper. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

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