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Bacillus subtilis Bs006 FORMULATIONS: STORAGE STABILITY, BIOLOGICAL ACTIVITY AND ENDOPHYTISM IN VEGETABLES

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ABSTRACT

Solid and liquid formulations based on the plant growth-promoting bacteria *Bacillus subtilis* Bs006 were designed as biological inoculant for vegetable nursery production. Considering the importance of microbial survival from the production process to the soil application, spore viability (CFU) in each formulation was assessed during twelve months of storage at 20, 30, and 40°C. At the three temperature levels evaluated, survival was higher than 85 and 90% for solid and liquid formulations, respectively. The bacterial biological activity was evaluated as plant growth promotion on lettuce, broccoli, and tomato in nursery. The formulations were applied at three concentrations ($1x10^7$, $5x10^7$, $1x10^8$ CFU/mL) at sowing and 21 days after seeding. Root and aerial part length and dry weight were the response variables evaluated. A positive effect was observed, particularly with the liquid formulation at $1x10^8$ spores/mL, showing the longest length of root and aerial part and the highest dry weight values in the root and foliar parts. Regarding the endophytism, *B. subtilis* colonized roots, stems, and leaves, achieving concentrations between $8x10^2$ and $1x10^5$ CFU/g.

Keywords: Inoculant viability, plant growth promotion, beneficial bacteria, biological inoculant.

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INTRODUCTION

One of the fundamental stages of vegetable production systems is plant nursery multiplication, in which appropriate management results in healthy, vigorous, and disease-free seedlings. These characteristics will improve the tolerance of plants to environmental stresses and soil pathogens incidence.

Applying beneficial microorganisms to promote plant growth during the nursery phase results in an efficient and sustainable alternative. The rhizobacteria *Bacillus subtilis* has proven to be an efficient rhizosphere colonizer by mechanisms such as siderophore production and the iron chelation effect, which favors its competitiveness by limiting the growth of microorganisms that depend on iron (Scharf et al., 2014). This rhizobacterium interacts with plants as part of the colonization strategy, exhibiting an endophytic behavior in different crops and being capable of inhabiting plant tissues without causing plant diseases (Le Cocq et al., 2017). It promotes growth by the synthesis of hormones such as gibberelic acid and indole-3-acetic acid (Shahid et al., 2021). Additionally, *B. subtillis* could be an antagonist against some pathogens (Berg, 2009), and induce tolerance to biotic and abiotic stress (Abdallah et al., 2018; Miljaković et al., 2020).

The Corporación colombiana de investigación agropecuaria (AGROSAVIA) has developed a biological inoculant based on *B. subtilis* strain Bs006 due to its characteristics as an effective colonizer and plant growth promoter in different plant systems such as blackberry, gooseberry and banana (Zapata and Díaz, 2012; Pérez et al., 2015; Gámez et al., 2019). Furthermore, this strain presented endophytic capacity in all evaluated plant systems, which allows a synergistic interaction with the plant and contributes to phytostimulation, with positive effects on plant growth and pathogens control (Eid et al., 2019; Rai et al., 2020). Therefore, it is an interesting tool in plant propagation systems, allowing the improvement of seedling characteristics for commercial vegetable exploitation (Rojas-Badía et al., 2020).

B. subtilis must be viable when released in the soil to obtain the expected beneficial effects on plants. Consequently, stability studies evaluating viability over time are essential to define the maximum storage time in which its characteristics are maintained (Corrêa et al., 2015; Santos et al., 2012). In this case, viability and biological activity are critical and must be monitored under different storage conditions. Intrinsic factors of microorganisms and characteristics of the formulation process are involved in preserving viability from the bioproduct manufacture to the inoculation in the soil (Fernández-Sandoval et al., 2012). Based on this, we infer that differences in the growth of different vegetables in the nursery may occur when applying solid and liquid formulations containing plant growth-promoting bacteria. Therefore, this study aimed to evaluate the shelf-life of two formulations of *B. subtilis* Bs006 with different characteristics and to compare their biological activity and endophytism *in planta*.

MATERIALS AND METHODS

Microorganism

Rhizobacteria *B. subtilis* Bs006 isolated from cape gooseberry in Cómbita (Boyacá, Colombia) (Caviedes, 2010) was obtained from the Collection of Microorganisms with Interest in Biological Control of AGROSAVIA (Uribe et al., 2021), and kept in cryopreservation at -70°C. The initial reactivation was seeded on Luria Bertani agar (LB) and incubated at 28°C for five days.

Preparation of *B. subtilis* Bs006 inoculum

Petri dishes containing LB agar were seeded with *B. subtilis* Bs006 using the massive round loop technique and incubated at 28°C for 24 hours. A bacterial suspension was prepared in a standardized culture medium coded as JM (Díaz et al., 2015). The biomass was surface scraped from a Petri dish and placed in 200 mL of medium contained in 500-mL Erlenmeyer flask. The procedure was repeated three times to complete the 800 mL required to inoculate the bioreactor in the next stage. The inoculum was incubated at 28°C, with an agitation rate of 150 rpm, for 48 hours in a shaker (New Brunswick® Innova 40). Breed's staining was performed as immediate microbiological quality control, and viability was evaluated by plate count method using heat shock to quantify spores and bacilli as final microbiological quality control (after 24 hours of incubation).

Fermentation of *B. subtilis* Bs006

The inoculum from the preceding stage (800 mL) was added to an STR bioreactor (Sartorius® BiostatB) with temperature control, stirring, and aeration. Previously, 7.2 L of JM liquid culture medium was prepared, and all the logistical setup of the bioreactor was carried out (sterilization of the equipment, culture medium, peripherals connection, services, and software activation). For the fermentation process, standardized operating conditions were applied: a working volume of 8 L, a temperature of 28°C, application of specific aeration and agitation ramps, and a fermentation

time of 48 hours. The response variables were the total dry biomass, cell, and spore concentration of *B. subtilis* Bs006 in the fermentation broth. Cell growth was determined by spreading bacterial suspension on LB agar with heat shock for spores and without heat shock for bacilli. The dry weight was obtained using a moisture balance (Precisa® Pro Executive EM-120HR).

Formulation process and storage stability study

A food grade preservative derived from a sodium salt (0.1% w/v) was added to the fermentation broth obtained as described above for the liquid formulation. For the solid formulation, the fermentation broth was centrifuged at 5000 rpm (Hettich® Rotina 380) for 20 minutes. Subsequently, the biomass was washed with sterile water and mixed with selected excipients such as carbohydrates, inorganic diluents and disintegrants to obtain a granulate. Next, the mixture of biomass and excipients was granulated using a 2-mm mesh and dried in a fluidized bed dryer (Glatt® Uni-glatt 8512) using the top-spray configuration, an inlet temperature of 35 °C, and an air inlet angle of 90°. The drying ended when the humidity of the granulate was less than 5%.

The liquid formulation was stored in plastic vials tubes, and the solid formulation was stored in tri-laminated aluminum bags. The samples were stored at 20 ± 2°C, 30 ± 2°C, and 40 ± 2°C. Viability expressed as colony-forming units (CFU/mL) was evaluated at the beginning of the study (time zero) and every two months for up to one year of storage at the three temperature levels. For this, serial dilutions of each formulation (10^{-6} and 10^{-7} for the liquid formulation and 10^{-7} and 10^{-8} for the solid formulation) were made in saline solution (0.85% w/v) and Tween 80 (0.1% v/v) and subjected to heat shock for spore quantification and without heat shock for bacilli quantification. The survival percentage on a dry basis was calculated from the viability results over time in a logarithmic form (Equation 1).

$$\text{(Equation 1) Survival (\%)} = 1 - \frac{\text{Log viability t0} - \text{Log viability tx}}{\text{Log viability t0}} \text{x } 100$$

The microbial contaminants content of formulations was determined by the spread plate method. The formulations were diluted in a Tween 80 (0.1% v/v) solution and dilutions were plated in Nutrient-Agar and incubated at 30 ± 0.5 °C for 48 h for bacteria determination. For fungi content, 0.1 mL of 10^{-1} and 10^{-2} dilutions were plated in Potato Dextrose-Agar and incubated at 25 ± 0.5 °C for 72 h. Results were expressed as microbiological purity using Equation 2 (Corporación colombiana de investigación agropecuaria [Agrosavia], 2022).

$$\text{(Equation 2) Microbiological purity (\%)} = \frac{\frac{\text{CFU}}{\text{g}} \text{ active ingredient}}{\frac{\text{CFU}}{\text{g}} \textit{active ingredient} + \frac{CFU}{g} \textit{Contaminants}} \text{x } 100$$

Plant growth promotion

Plant growth promotion was evaluated on lettuce (*Lactuca sativa*) Batavia Grandes Lagos variety, broccoli (*Brassica oleracea*) Calabrese variety, and tomato (*Solanum lycopersicum*) Chonto hybrid Santa Clara. Seeds of each plant were placed in 50-cell seedling trays of 113 cm^3 per cell, with Canadian Sphagnum peat (PRO-MIX GTX) as substrate. Liquid and solid formulations were applied in drench (5 mL/plant) in three doses (at sowing, 7 and 21 days later). Non-uninoculated plants were used as a control treatment (Table 1).

The bioassays were established in a greenhouse using a randomized complete block design with three replicates; the experimental unit was a planting tray with 15 seedlings. Once the seedlings presented the optimal transplant size (10 to 15 cm, 50 days after transplant), five samples were taken from each experimental unit, and the roots were washed carefully to remove the peat. Next, root length and foliar areas were measured. Finally, the root and foliar tissues were dried in an oven at 60°C for four days to determine dry biomass weight.

Endophytism

After nursery, three seedlings were taken (experimental units), the tomato plants were sectioned into roots, stems, and leaves, and the lettuce and broccoli plants were sectioned into roots and leaves. Initially, the roots were washed. A sample of 0.3 g of each vegetable was disinfected by immersion in a 70% (v/v) ethanol solution for three minutes and rinsed with sterile type III water (Cordero et al., 2010). Subsequently, roots were placed in 27 mL of a saline solution (NaCl at 0.86% and Tween 80 at 0.1%) and crushed with an immersion blender

Table 1. Formulations and concentrations of *Bacillus subtilis* Bs006 tested in the plant growth promotion bioassay on lettuce, broccoli and tomato.

Treatment	Description	Concentration
T1	Liquid formulation	$1x10^8$ CFU/mL
T2	Liquid formulation	$5x10^7$ CFU/mL
T3	Liquid formulation	$1x10^7$ CFU/mL
T4	Solid formulation	$1x10^8$ CFU/mL
T5	Solid formulation	$5x10^7$ CFU/mL
T6	Solid formulation	$1x10^7$ CFU/mL
T7	Control	Water

(Kitchen Aid® ref. KHB1231ER) to obtain a homogeneous mixture (stock suspension) from which a serial dilution (10^{-1}, 10^{-2}) was made. Next, the stems and leaves were washed with water, forming a sample composed of 1 g of each plant tissue; these were disinfected by washing with 1% sodium hypochlorite solution (NaClO) for 5 min, with alcohol 70% for 3 min and finally rinsed with sterile type III water. Then, 9 mL of saline solution was added and crushed to obtain a homogeneous mixture, of which two serial dilutions (10^{-2}, 10^{-3}) were made for lettuce and broccoli and one dilution (10^{-2}) for tomato, they were subsequently subjected to heat shock at 80°C for 15 min and then at 10°C for 5 min; this process is selective for spores since the vegetative cells are eliminated. Then 100 µL of each suspension was seeded on NA agar in triplicate, incubating at 28°C for 48 hours. Finally, the colonies with morphology typical of *B. subtilis* strain Bs006 (irregular edge, umbelliform elevation, curly surface, and mucoid consistency) were counted, and the results were expressed as CFU/mL.

Statistical analysis

For the bioassay of storage stability, the means were compared using a two-way ANOVA test according to storage temperature and type of formulation. Comparisons were made with a significance level of $P < 0.05$. For plant promotion growth and endophytism, the means were compared using a one-way ANOVA test, and a Tukey means comparison test ($p \leq 0.05$) using the statistical software Statistix® v10.0.

RESULTS

The *B. subtilis* Bs006 broth used as an active ingredient for the formulations had a concentration (expressed as viability) of $3.97x10^9$ CFU/mL of bacilli and spores. The dry biomass weight was 1.1 g/L.

Storage stability of formulations

The survival of *B. subtilis* Bs006 over time in the liquid and solid formulations after storage was calculated considering the viability (CFU) of the microorganism in the formulation (Fig. 1). After 12 months of storage, the survival percentage was higher than 85% for solid formulation and greater than 90% for liquid formulation at the three temperature levels evaluated. Comparing the two types of formulations, the survival decreased more sharply in the solid formulation than in the liquid formulation, particularly at 30 and 40 °C.

At the end of the stability study (after 12 months of storage), the survival of solid and liquid formulations was not significantly different at 20 and 30 °C; the survival was higher in the liquid formulation only at 40°C (Fig. 2). The statistical analysis showed that the type of formulation (solid or liquid) did not affect the survival percentage of *B. subtilis* Bs006 significantly (formulation $F_{1,12}=2.891$, P0.1148). Contrastingly, the effect of storage temperature was significant ($F_{2,12}=4.990$, $P=0.0265$).

The initial microbial purity (Equation 2) of the formulations reached 99.998% and 99.999% for the liquid and solid formulations, respectively. These values of microbial purity are higher than the limit stablished for bacterial inoculants, which is 95% according to the Colombian Regulatory Agency (Instituto Colombiano de Normas Técnicas [ICONTEC], 2018); Instituto Colombiano Agropecuario [ICA], 2020). The concentration of bacterial contaminants increased after twelve months of storage compared with the concentrations at the beginning of the assay ($1.33x10^4$ CFU/mL for the liquid formulation and $1.93x10^5$ CFU/g for the solid formulation). For the liquid formulation, the final contaminant concentration was $-6.33x10^7$ CFU/mL, increasing 47% at 20 °C, 46% at 30 °C, and 45% at 40 °C compared with the initial concentration of contaminants. With these values, the microbial purity of the liquid formulation remained higher

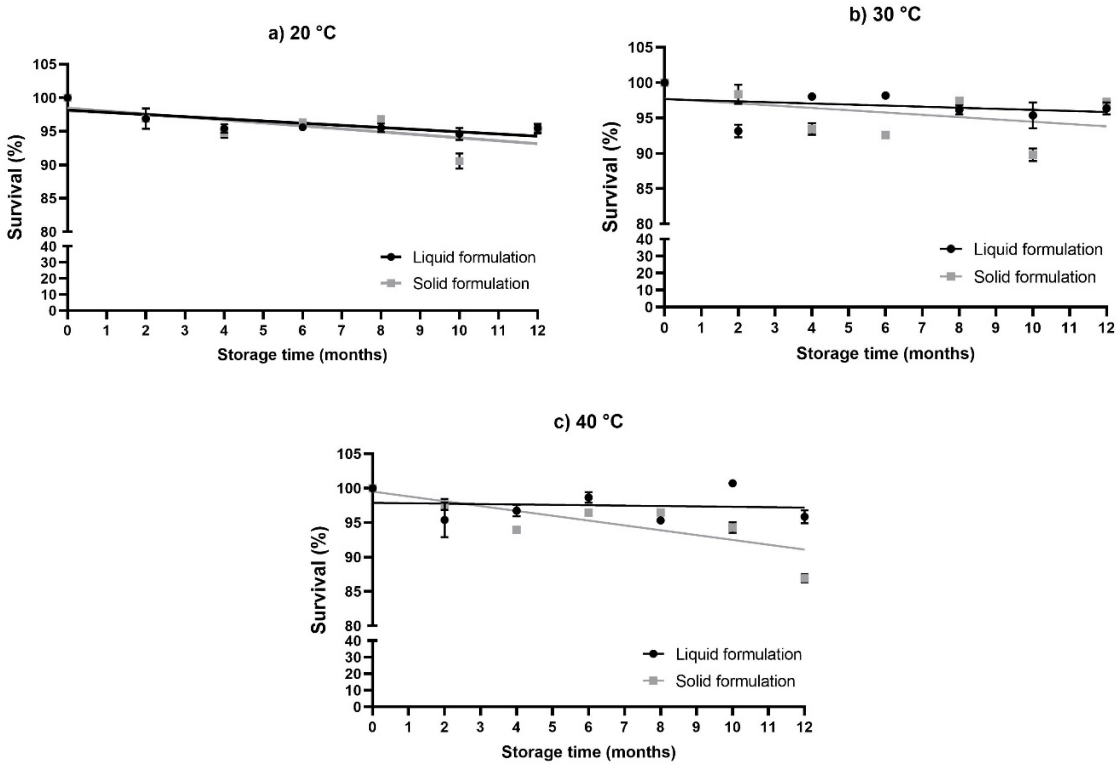

Fig. 1. Survival of *Bacillus subtilis* Bs006 (CFU) in solid and liquid formulations during 12 months of storage at a) 20 °C, b) 30 °C and c) 40 °C.

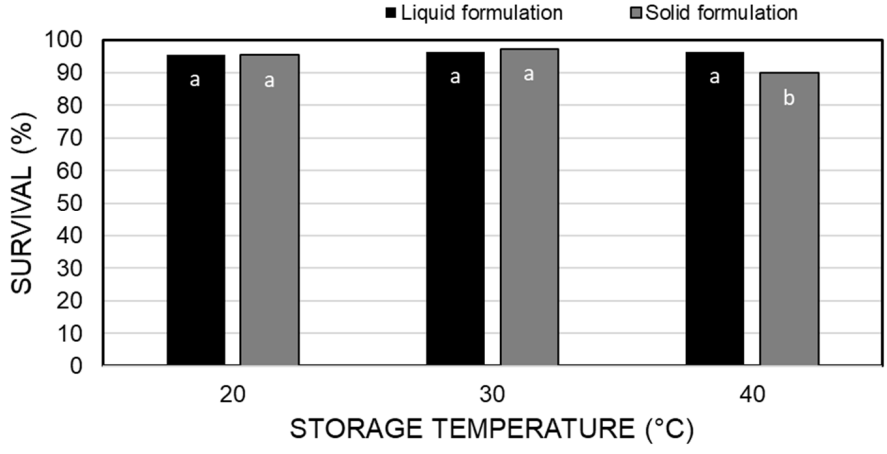

Fig. 2. Survival of *Bacillus subtilis* Bs006 in solid and liquid formulations after 12 months of storage at 20, 30 and 40 °C. Columns with the same letter are not different according to the Tukey test (p≤0.05).

than 98% for 8 months. For the solid formulation, the increment in contaminants concentration was lower than in the liquid formulation, with values of 37% at 20 °C, 38% at 30 ° C, and 38% at 40 °C. The microbial purity of this formulation remained higher than 99% for 10 months of storage at the three temperature levels evaluated. In contrast, fungal contaminants were not detected because they were below the limit of quantification of the assay (100 CFU/g) for the two formulations at the three temperature levels evaluated.

Plant growth promotion

A positive effect on plant height due to the

application of *B. subtilis* Bs006 was observed with liquid formulations at the highest concentrations (T1, T2) in the three systems evaluated, reaching values 30.8%, 31.3%, 27.5% higher than the control for T1 and 18.5%, 26.4%, 3.7% for T2 in lettuce, broccoli and tomato, respectively (Table 2). With the three solid formulations of *B subtilis* Bs006 (T4, T5, T6), there were no significant differences compared to the control.

For root length, there were no statistically significant differences between treatments for broccoli and tomato at the end of the bioassay. However, lettuce presented a more incipient radical system with the application of the liquid formulation of *B. subtilis*, with T1 showing significant difference with respect to the other treatments, and reaching a value of 11.59 mm that was 32% higher than the control treatment (Table 2).

The most concentrated liquid formulation of *B. subtilis* Bs006 (T1) had a remarkable response since the dry biomass of the seedlings was higher than that observed in the control and other treatments ($p \leq 0.05$) in the three plant systems evaluated (Table 3). For example, biomass values in T1 in lettuce, broccoli, and tomato were higher than the control at 172%, 86%, and 93%, respectively.

Regarding aerial biomass of the seedlings, the most concentrated liquid formulations of *B. subtilis* Bs006 (T1) recorded highest values, showing statistically significant differences ($p \leq 0.05$) with the formulations of *B. subtilis* Bs006 (T2, T3, T4, T5 and T6) and with the control (T7) (Table 3). The plant growth-promoting effect of liquid formulations of *B. subtilis* Bs006 T1 and T2 was more noticeable in broccoli, resulting in statistically significant differences with respect to the control (T7) (Table 3).

Endophytism

B. subtilis Bs006 was recovered from the roots and leaves of broccoli and lettuce, and from the roots, leaves and stems of tomato. In the roots of broccoli and lettuce, populations higher than $2x10^4$ CFU/g were counted (except in T6 for broccoli with $3x10^3$ CFU/g). In tomatoes,

Table 2. Root and aerial length of lettuce, broccoli and tomato seedlings treated with *Bacillus subtilis* Bs006 30 days after sowing.

Treatment	Root length (mm)			Aerial length (mm)		
	Lettuce	Broccoli	Tomato	Lettuce	Broccoli	Tomato
T1	11.59 a*	16.29 a	17.27 ab	14.17 a	15.79 a	16.25 a
T2	8.97 b	15.59 a	18.15 ab	12.83 ab	15.21 a	13.22 b
T3	9.06 b	15.59 a	17.72 ab	11.68 bc	13.13 bc	12.05 bc
T4	9.44 b	14.26 a	15.60 b	10.93 c	12.74 bc	11.6 c
T5	9.84 ab	12.54 a	16.96 ab	10.29 c	12.05 c	11.76 c
T6	8.51 b	13.44 a	15.29 b	10.71 c	13.93 b	11.73 c
T7	8.79 b	14.49 a	19.33 a	10.83 c	12.03 c	12.75 bc

*Means followed by the same letter in each column are not different according to the Tukey test ($p \leq 0.05$).

Table 3. Root and foliar dry weight of lettuce, broccoli and tomato seedlings treated with *Bacillus subtilis* Bs006 30 days after sowing.

Treatment	Root dry weight (mg)			Foliar dry weight (mg)		
	Lettuce	Broccoli	Tomato	Lettuce	Broccoli	Tomato
T1	9.0 a*	10.8 a	42.4 a	79.1 a	104.1 a	207.3 a
T2	4.8 b	11.5 a	27.3 b	57.0 b	92.3 a	130.5 b
T3	3.9 b	7.2 b	20.5 c	37.2 c	67.9 b	102.5 c
T4	2.9 b	6.2 b	14.0 d	32.7 c	56.0 bc	84.3 c
T5	2.9 b	4.1 b	17.0 cd	35.0 c	51.4 c	86.5 c
T6	3.6 b	5.9 b	20.0 c	34.7 c	66.6 b	93.7 c
T7	3.3 b	5.8 b	22.0 bc	35.1 c	53.3 c	127.6 b

*Means followed by the same letter in each column are not different according to the Tukey test ($p \leq 0.05$).

populations fluctuated from $6x10^3$ CFU/g to $8x10^4$ CFU/g (Fig. 3 A, B, and C). For broccoli and lettuce leaves, populations between $3x10^3$ CFU/g and $1x10^5$ CFU/g were recovered. For tomato, the most significant population was recovered from the solid formulation treatments, with values between $2x10^3$ CFU/g and $1x10^4$ CFU/g, while T6 was the treatment that recorded the highest value. In contrast, for the liquid formulation, the concentration was not higher than $8x10^2$ CFU/g (Figs. 3 A, B and C). In tomato stems, populations between $1x10^3$ CFU/g and $5x10^4$ CFU/g were recovered, and the highest values from the solid formulation treatments showed significant differences ($p \leq 0.05$) (Fig. 3C).

DISCUSSION

The results obtained after storage of *B. subtilis* Bs006 formulations may be related to the intrinsic capacity of the microorganism to produce spores. Spores are resistance structures that can withstand stress conditions and subsequently allow the survival of microorganisms (Setlow, 2014). This characteristic is fundamental in bioproduct storage and in the establishment of microorganisms in the soil where adverse conditions must be faced. Regarding the formulation process, unit operations can affect the microorganism; in the case of the liquid formulation, the exposure to stress conditions is minimum, which favors the viability of the microorganism. On the other hand, in the solid formulation, the microorganism is subjected to unit operations such as separation, mixing, granulation, and drying, which can affect the microbial viability. The adverse effects of this process at the cellular level have been mainly related to damage to the lipid conformation of the cell membrane and the oxidation of essential molecules for the cell; the damage can be accentuated with storage time (Fernández-Sandoval et al., 2012). The fluidized bed drying process was used to reduce exposure of the microorganism to high temperatures; heat transfer is more efficient, allowing the drying process to be carried out faster (Berninger et al., 2018).

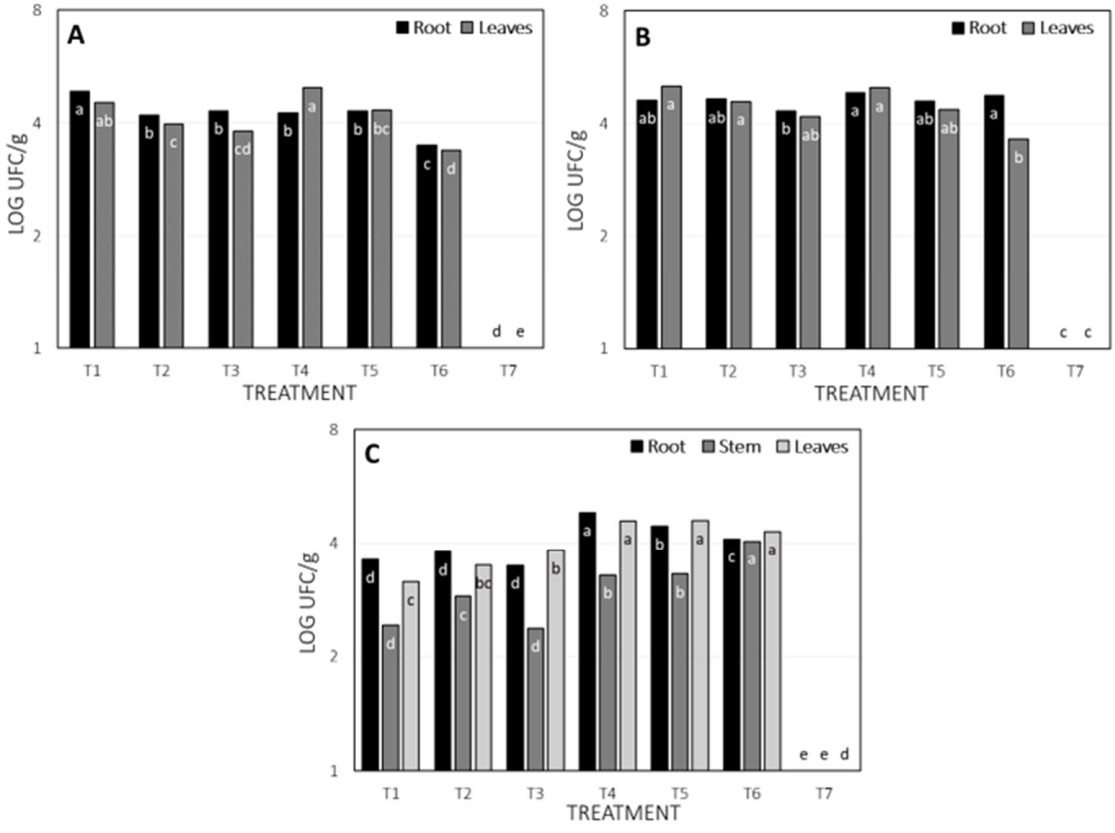

Fig. 3. Endophytic population of *Bacillus subtilis* Bs006 recovered from A. Broccoli, B. Lettuce, and C. Tomato seedling tissues. Columns with the same letter for each tissue (root, stem, leaves) are not different according to the Tukey test ($p \leq 0.05$).

The microbiological stability of this type of inoculant is critical to preserve the expected activity from production to application in plants. Solid formulations tend to favor the microbiological stability of the product since low water activity decreases the proliferation of contaminating microorganisms, which may affect the viability of the microorganism of interest (Laconelli et al., 2015). Although the risk of contamination over time is lower in the solid formulation, the drying process is a critical stage that can affect cell structures, reflected over time. The origin of the contamination could be related to the raw materials or containers employed in the fermentation and formulation process, even though aseptic conditions are maintained. However, the concentration of contaminants should not exceed the limit for microbial purity of 95% established for bacterial inoculants in Colombia (Instituto Colombiano de Normas Técnicas [ICONTEC], 2018). Considering the differences of survival under storage, the liquid formulation is more stable at the highest storage temperature (40 °C). However, factors such as the volume of liquid for transportation and the contamination are disadvantages of liquid formulations that should be considered for the selection of the type of formulation.

Regarding biological activity, the best results were obtained with the liquid formulations T1 and T2, demonstrating that *B. subtilis* Bs006 has growth-promoting activity in lettuce, broccoli, and tomato. On the one hand, the biomass of *B. subtilis* can efficiently colonize the rhizosphere of plants, occupy spaces, and compete for nutrients with soil pathogens (Santoyo et al., 2021). This bacterial species produces phytohormones such as auxins (indole-3-acetic acid), which interact with plants as part of the colonization strategy with the additional benefit of promoting growth, giving plants tolerance against biotic and abiotic stress (Abdallah et al., 2018; Miljaković et al., 2020). Notably, *B. subtilis* Bs006 has shown its ability to colonize the root system of blackberry, banana, and cape gooseberry plants during the nursery phase, promoting their growth with similar results to those obtained with conventional chemical fertilization schemes (Gámez et al., 2019; Díaz-García et al., 2015; Pérez et al., 2015; Zapata and Díaz, 2012). Liquid formulations of *B. subtilis* Bs006 based on the complete fermentation broths contain biomass and metabolites produced during fermentation, which could be involved in the growth promotion activity. In contrast, the solid formulation only contains the separated biomass, which would explain why the values obtained for length and dry weight in the three vegetables were similar to the control at all the concentrations evaluated.

As observed in other plants (Gámez et al., 2019), *B. subtilis* Bs006 showed endophytism in the vegetables evaluated, allowing the recovery of a concentration differential that demonstrated an "upward dilution" effect within broccoli and lettuce tissues, depending on the type of formulation applied. Although recovered concentrations were lower in tomatoes, these values could be considered normal since these are endophytic populations. In general, endophytic bacterial populations range from $1x10^3$ to $1x10^7$ CFU/g of fresh vegetable weight (Compant et al., 2010; Jha et al., 2018; Afzal et al., 2019) and particularly for tomatoes, the lower population of *B. subtilis* did not present a negative effect on plant promotion growth activity. The endophytic colonization implies a complex communication between microorganisms and the plant; several environmental and genetic factors are involved in this process, allowing bacteria to enter the plant endosphere, colonize and migrate to different organs.

CONCLUSIONS

The liquid formulation with the highest concentration of *B. subtilis* Bs006 (T1) showed a significant growth-promoting effect on broccoli, lettuce, and tomato seedlings. This formulation also had a survival higher than 90% after 12 months of storage at the three temperature levels evaluated (20°C, 30°C, 40°C), maintaining the microbial purity for 8 months. The endophytism assay showed that the microorganism could colonize the roots and leaves of broccoli, lettuce, and tomato regardless of the type of formulation. In conclusion, this inoculant showed a growth-promoting effect and could be integrated into plant propagation systems to improve the characteristics of plants for commercial exploitation.

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Author contribution

It is declared that each of the authors participated actively in the bibliographic review,

in the development of the methodology, in the discussion of the results, and review and approval of the final version of the article.

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