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Enhanced molecular visualization of root colonization and growth promotion by *Bacillus subtilis* EA-CB0575 in different growth systems

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ABSTRACT

Bacillus subtilis EA-CB0575 is a plant growth-promoting bacterium (PGPB) associated with banana and tomato crops. Root colonization is an important trait for PGPB microorganisms and potentiates the bacterial effect related to the mechanisms of plant growth promotion. Therefore, detection of bacterial colonization of roots in different culture systems is important in the study of plant–microorganism interactions. In this study, fluorescent in situ hybridization (FISH) and catalyzed reporter deposition–FISH (CARD–FISH) were evaluated to determine the colonization ability of *B. subtilis* EA-CB0575 on banana and tomato roots planted on solid and liquid Murashige and Skoog medium ($MS_{(S)}$ and $MS_{(L)}$, respectively) and in soil for tomato plants. Results showed *B. subtilis* colonization 0–30 days post inoculation for banana and tomato plants in different culture systems with differential distribution of bacterial cells along tomato and banana roots. FISH and CARD–FISH methodologies were both successful in detecting *B. subtilis* colonies, but CARD–FISH proved to be superior due to its enhanced fluorescence signal. The presence of bacteria correlated with the promotion of plant growth in both plant species, providing clues to relate rhizospheric colonization with improvement in plant growth. FISH and CARD–FISH analysis results suggested the presence of native microbiota on the roots of in vitro banana plants, but not on those of tomato plants.

1. Introduction

Multiple bacterial species contribute to crop productivity and therefore to sustainable agriculture by plant growth promotion and biocontrol of phytopathogens (Beneduzi et al., 2012; Yuan et al., 2013). The ubiquitous nature of bacteria and their physiological traits such as resistant spore formation, secondary metabolite production, and signal peptide production contribute to their usefulness as active ingredients of bio-inoculants and biopesticides (Wu et al., 2015). *Bacillus* strains are known as plant growth-promoting bacteria (PGPB). These bacteria use direct mechanisms, such as phosphate solubilization, nitrogen fixation, and phytohormones and siderophores production, or indirect mechanisms, such as induction of systemic resistance to pathogens, production of antibiotic and lytic enzyme, inhibition of plant ethylene synthesis, or competence by nutrients or niche (Chen et al., 2016; Meng et al., 2016; Walia et al., 2014). These microorganisms are applied to a wide range of agricultural crops to improve plant development (Chen et al., 2016; Walia et al., 2014); however, their main application is as spores due to their resistance to adverse environmental conditions and ease of handling during the production of bio-inoculants (Meng et al., 2017). However, the success in plant growth promotion has been found to greatly vary between plant species; growth promotion can depend on the growth system (in vitro culture, greenhouse, or field), substrates, plant health, and pH, type, and moisture of the soil. In addition, growth promotion is related to the ability of these bacteria to germinate (when spore-forming bacteria are used) and colonize plant roots (De Souza et al., 2015; Kloepper et al., 1980; Ugoji et al., 2006).

In addition to anchoring the plant and supplying it with water and nutrients, roots perform other vital functions: they exude compounds such as ions, organic acids, amino acids, sugars, mucilage, enzymes,

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and carbon-containing primary and secondary metabolites into the rhizosphere and draw microorganisms toward the roots and create a hot spot for microbial activity (Bais et al., 2006; Lareen et al., 2016). The composition of root exudate is determined by plant species, plant age, soil type, soil moisture, and other edaphic factors, and its profile can be related to the presence and differentiation of microorganisms in the roots due to effect of chemotaxis and efficient motility of microorganisms (Steinauer et al., 2016). A range of studies have determined the effects of root exudate components, such as malic, fumaric, or citric acid (Rudrappa et al., 2008; Zhang et al., 2014) or plant polysaccharides (Beauregard, 2015) on the root colonization process of *Bacillus*. However, it is important to note that when inoculants based on *Bacillus* spores are applied to the plant, they must germinate to form metabolically active cells (Setlow, 2014). Once germination occurs, microorganisms could be attracted by chemotaxis and start the root colonization process (Rudrappa et al., 2008) and exert their PGPB potential (Rudrappa et al., 2008). Colonization of plant roots is a complex process that comprises different stages, attraction to the root being the first of these. The second stage is the establishment of bacteria on the root and utilizing plant-derived nutrients. For this process, bacteria can evolve a variety of mechanisms to enhance colonization ability, such as biofilm formation (Beauregard, 2015; Raaijmakers et al., 2010), surfactin production (Lopez et al., 2009), and metabolic enzyme production (Dutta et al., 2013). Some of these processes are regulated by quorum sensing, an important process whereby microorganisms improve their ability to survive, regulate their gene expression in a population density-dependent manner, adjust their physiology according to their environmental conditions, and coordinate the behavior of the entire cell population (Chin-a-Woeng et al., 2003). The next step is the generation of transcriptional and translational changes over microorganisms on the rhizosphere due to different effectors present in the plant (Fan et al., 2012). In addition, growth promotion mechanisms of beneficial bacteria are stimulated by environmental signals and some variations on plant processes such as metabolomic or transcriptomic profile (Beauregard, 2015; Fan et al., 2012; Vacheron et al., 2013).

The relationship between plant growth promotion and bacterial rhizospheric colonization is frequently reported in the soil science literature (Beauregard, 2015; Gao et al., 2016), but to our knowledge, no study till date has verified this relationship. On the other hand, some studies have determined the importance of maintaining a high population of PGPB on roots to obtain good plant growth and to activate PGPB potentials on roots (Chin-a-Woeng et al., 2003; Gao et al., 2013; Kloepper et al., 1980). Nevertheless, it is possible that inoculated populations of beneficial bacteria activate their direct/indirect mechanisms for growth promotion over a short period, generating changes in plant growth without colonizing the plant. A number of studies have demonstrated a non-uniform distribution of microorganisms on plant roots, reporting some areas of the root with low population and other areas with high population (Fan et al., 2012; Lugtenberg et al., 2001). Several studies have been conducted on the colonization of gram-negative microorganisms on plant roots; however, so far little is known about gram-positive bacteria (Fan et al., 2012). Nevertheless, some reports have garnered interest in the last few years and studies using methodologies such as green florescent protein insertion (Fan et al., 2012; Liu et al., 2006), electron microscopy (Ugoji et al., 2006), and traditional cultures (Ahmad et al., 2011) have been published. However, alternative methodologies such as fluorescent in situ hybridization (FISH) and catalyzed reporter deposition (CARD–FISH), which combine the precision of molecular techniques with the visual information from microscopy, are important for improving microorganism detection and in situ monitoring to permit visualization and identification of individual cells and colonies within their natural microhabitat for different media and substrates. *B. subtilis* visualization using these methodologies could be determined if inoculated PGPB spores germinate and colonize root tissues.

Spores have been isolated from different environmental samples by heat shock protocols and culture techniques. These procedures do not ensure that spores are actively inhabiting those niches, and therefore, determining active colonization can give more solid evidence of *B. subtilis* omnipresent nature (Earl et al., 2008). We hypothesized that *Bacillus subtilis* EA-CB0575 colonizes banana and tomato roots when vegetative cells or spores are applied to meristems or seeds respectively in different culture systems, and promotes plant growth. To test our hypothesis we conducted series of specific experiments inoculating *B. subtilis* strain on banana and tomato in different culture systems (in vitro and greenhouse experiments) to determine plant growth promotion and root colonization ability of this rhizobacteria using FISH and CARD-FISH methods, and evaluating which method produce a best visualization of these microorganisms on plant roots.

2. Materials and methods

2.1. Microorganism and culture conditions

B. subtilis EA-CB0575 (accession number KC170988), isolated from the rhizosphere of *Musa* AAA variety Valery in Urabá, Colombia (7°51′58.6″ N, 76°37′39.0″ W), was cultured at 30 °C in tryptic soy broth (105.459, Merck & Co, Kenilworth, NJ, USA) for vegetative cell production and in SBM medium (Posada-Uribe et al., 2015) for spore production. Cultures were incubated at 150 rpm and 30 °C for 12 h for vegetative cells and 72 h for spore production. Spores were subjected to heat shock at 80 °C for 20 min and cells were recovered by centrifugation at 3289 *g* and 4 °C for 20 min and the pellet resuspended in sterile distilled water to OD_{600} = 1.0, which is equivalent to 1×10^8 CFU/mL.

2.2. Plant inoculation and sowing

Commercial banana seedlings of variety 'Williams', which were grown in vitro and obtained from Meristemos Colombia S.A.S (Rionegro, Colombia), were disinfected using 1.5% bleach for 20 min and 75% ethanol for 1 min, then washed with sterile distilled water three times and inoculated by soaking banana roots in a *B. subtilis* EA-CB0575 suspension. This suspension was prepared using spores or vegetative cells at 1×10^8 CFU/mL, and plant roots were soaked for 1 h in 250 mL of the bacterial suspension. Plants were then dried at room temperature under axenic conditions. Inoculated and non-inoculated plants were planted in three different systems: (i) solid $MS_{(S)}$ medium (Murashige and Skoog, 1962) prepared using basal salt medium (Phytotech Laboratories, M524) supplemented with 5% agar (BD, 214010); (ii) liquid $MS_{(L)}$ medium without agar and prepared using sterile expanded perlite (Antiguo mas Moderno, La Calera, Cundinamarca) as an inert support (9 g of perlite and 50 mL of MS liquid medium); and (iii) a commercial mixture of soil (sandy loam soil, sand, and rice husk in the ratio of 1:0.5:0.5, Vivero Tierra Negra, Antioquia). Seedlings were planted in each system and incubated at 30 °C for a photoperiod of 12/12 h light/dark using white light (60 μmol/m^2/s) for in vitro cultures for 30 days. Banana plants were kept under greenhouse conditions (29–33 °C, watered every other day) for 4 months in soil.

Tomato seeds of variety 'Chonto Santa Cruz' were disinfected using 5% bleach for 10 min and 70% ethanol for 1 min using a vacuum pump (Boeco, R-300) at 10 K Pa, washed with sterile distilled water between reagents, and inoculated as previously described for banana seedlings, soaking the seeds in bacterial suspension. Seeds were sown in the three systems ($MS_{(S)}$, $MS_{(L)}$, and soil) and kept under growth chamber or greenhouse conditions (22–30 °C, relative humidity 65%, and photoperiod 12/12 h) for 30 days (in vitro plants) or 60 days (soil plants). Banana and tomato plants were sown in commercial soil and fertilized using 1 g/plant of commercial fertilizer (N:P:K, 15-15-15, Anasac) and a foliar fertilizer (3 L/ha) (Wuxal Tapa negra, Bayer Crop Science) for 30 days post inoculation (dpi). Assays at in vitro level did not use addition of fertilizers.

2.3. Probes for fluorescentin situhybridization (FISH) and catalyzed reporter deposition (CARD–FISH)

Two types of probes were used for FISH: 1) a universal bacterial probe mix containing an equimolar mix of EUB338, EUB338-II, and EUB338-III (EUBmix) (Amann et al., 1990; Daims et al., 1999) and 2) Bsub-ss-0463-aA-22A, a design probe specific to *B. subtilis* strains (Posada et al., 2016). Specific oligonucleotide probes were labeled at the 5′-end with the fluorescent dyes 56-FAM, Alexa488 (wavelength absorption/emission 495 nm/520 nm) or Cy3 (Integrated DNA Technologies, Coralville, IA, USA; cyanine, wavelength absorption/emission 552 nm/565 nm), and Cy3 for the universal bacterial probe mix. We used the specific probe labeled with horseradish peroxidase (HRP; Biomers, Ulm, Germany) at the 5′-end for CARD–FISH. FISH probes were suspended in TE buffer (10 mM Tris-HCl, pH 7.2, 1 mM EDTA), and CARD–FISH probes were suspended in sterile distilled water at 50 ng/μL for FAM, Alexa, and HRP and at 30 ng/μL for Cy3. Probes were stored at −20 °C in the dark until use.

2.4. Sample fixation and preparation

Tomato and banana roots from the different growth systems ($MS_{(S)}$, $MS_{(L)}$, and soil) were fixed and prepared according to de-Bashan et al. (2010) modification of Daims et al. (2005) protocol. Roots from each growth system were cut and divided into three sections—upper, middle, and lower roots—to determine the presence of bacteria in the different sections. Roots were washed with 0.85% NaCl and fixed with NaCl and ethanol (ratio 1:1). Fixed samples were stored at 4 °C for 1 h, air-dried and placed on gelatinized slides (0.1% w/v gelatin [#G2500, Sigma-Aldrich] and 0.01% w/v $CrKO_8S_2*12H_2O$ [243361,Sigma-Aldrich]) for tomato roots, whereas banana root samples were placed on positive-charge slides. Samples were covered with molten 0.25% agarose and air-dried. Slides were dehydrated by rinsing with successive 50%, 80%, and 96% ethanol solutions (3 min each), then air-dried at 37 °C. Banana roots were additionally prepared using a tissue processor (TP1020, Leica) for tissue infiltration; roots were paraffin-embedded (Paraplast, 39601006, Leica) and sectioned using a microtome (RM2125 RTS, Leica). Banana samples were deparaffinized by immersing them in xylene (10 min, three washes), alcohols (96%, 70%, 60%, 40%, and 20% in succession, 3 min for each solution), and water. Lysozyme (#L7651, Sigma-Aldrich, 10 mg/mL) was applied over the tomato and banana samples, incubated at 37 °C for 20 min, dehydrated as described above, and air-dried. Slides were stored at 4 °C until hybridization. Tomato seeds, before and after inoculation, were embedded in resin (Technovit H8100) and cut using a semiautomatic ultramicrotome (EM UC7 LEICA), obtaining longitudinal cuts of 5 μm and later used for hybridization.

2.5. FISH

Hybridization buffer was prepared using 360 μL of 5 M NaCl, 40 μL of 1 M Tris-HCl (#10812846001, Sigma-Aldrich) at pH 8.0; 2 μL of 10% sodium dodecyl sulfate (SDS; #L3771, Sigma-Aldrich); 700 μL of deionized formamide (#F9037, Sigma-Aldrich); and 900 μL of ultrapure water. The amount of formamide and water depended on probe stringency. In this case, probe stringency was 35%. A solution made up of 40 μL of hybridization buffer, 4 μL of EUBmix probe, and 10 μL of the designed probe was added to each sample (specific and universal probes) or 40 μL of the hybridization buffer and 6 μL of the designed probe (Cy3-labeled-specific probe). This process was performed in the dark to reduce bleaching of the fluorophore. Slides were placed into 50-mL Falcon tubes with a piece of paper towel impregnated with the remaining hybridization buffer (hybridization chambers) and heated in the dark in a hybridization oven (model 5430, VWR International, Radnor, PA, USA) at 46 °C for 2 h. Later, the slides were rinsed with a washing buffer compound made of 1 mL of 1 M Tris-HCl at pH 8.0, 500 μL of 0.5 M EDTA at pH 8.0, 700 μL of 5 M NaCl (to meet 35% stringency), 50 mL of ultrapure water, and 50 μL of 10% SDS. Falcon tubes containing total volume of this buffer were preheated to 48 °C and slides were put inside the tubes in a water bath for 15 min. The slides were rinsed for a few seconds with deionized water at 4 °C and then air-dried at room temperature in the dark.

2.6. CARD–FISH

CARD–FISH was developed using the procedure reported by Teira et al. (2004) with modifications. Permeabilization mix (lysozyme 10 mg/mL, proteinase K 10.9 mg/mL, 0.1 M Tris-HCl, 0.05 M EDTA, and sterile distilled water 8 mL) was applied to the samples, incubated at 37 °C for 1 h and rinsed with water, 0.01 M HCl, and ethanol before inactivation of peroxidases with 0.1% H_2O_2 as per Teira et al.'s protocol. Hybridization buffer, dextran sulfate (10% D8906, Sigma), 900 mM NaCl, 20 mM Tris-HCl, 0.05% Triton X-100, 35% formamide, 1% blocking reagent (10% Boehringer Mannheim blocking reagent 1096196, Roche, dissolved with maleic acid buffer [100 mM maleic acid and 150 mM NaCl, pH = 7.5]), and 3500 μL of water were applied to the samples and mixed with HRP–Bsub-ss-0463-aA-22A in the ratio 20:1. Samples were then hybridized for 15 h in the dark and slides were washed with a buffer of 1 mL of 1 M Tris-HCl at pH 8.0, 500 μL of 0.5 M EDTA at pH 8.0, 700 μL of 5 M NaCl solution (to meet 35% stringency), 50 mL of ultrapure water, and 50 μL of 10% SDS. Falcon tubes with this buffer were preheated to 37 °C and slides were put inside the tubes in a water bath for 15 min. The slides were rinsed for a few seconds with deionized water at 4 °C, then air-dried at room temperature in the dark, and then amplified. Samples were incubated in PBS-T for 15 min, and 30 μL of one mixture compound by 493 μL of amplification buffer (10% dextran sulfate, 2 M NaCl, 0.1% blocking reagent and PBS 1 ×), 5 μL of 200:1 solution amplification buffer and 30% H_2O_2, 3.4 μL of green emission (Atto 488 NHS, Sigma), or 2.5 μL of red emission (CF 555-SE, Biotium) dye tyramide were applied over the samples. The samples were then incubated at 37 °C for 1 h in the dark. Slides were washed with water, then with 96% ethanol, and dried at room temperature. Some slides were stained with DAPI (4′,6-diamidino-2-phenylindole, R37606, Invitrogen), incubated at 20 °C during 1 h and then dried at room temperature for visualization.

2.7. Visualization

CARD–FISH slides were stained using DAPI (R37606, Life Technologies) and dried for 30 min. Citifluor (AF1 mountant, Electron Microscopy Sciences, Hatfield, PA, USA) was applied to dried hybridized slides (FISH and CARD–FISH slides) and visualized under oil immersion with the 100× objective of a fluorescence microscope (Olympus BX41 Tokyo, Japan) or an Axioscope A1 (Carl Zeiss, Oberkochen, Germany). Microscopes had filters Cy3 and FITC for samples hybridized with the fluorochrome 56-FAM or Alexa 488. Photographs were taken (AxiocamCC5 or Evolution V) and processed with an imaging software (ImagePro Plus 6.3, Media Cybernetics, Silver SpringMD or ZEN2012 Blue edition, Carl Zeiss).

2.8. Scanning electron microscopy

Root samples were fixed using 2.5% glutaraldehyde in phosphate buffer at pH 7.4 for 2 h. Then, samples were washed with phosphate buffer and gradually dehydrated twice with 20%, 40%, 60%, 70%, 80%, and anhydrous ethanol (E7023, Sigma) for 40 min each. Samples were subjected to a critical-point-drying process (Tousimis modelo Samdri-PVT 3B) and coated with gold–palladium in a sputter coater before scanning electron microscopy (SEM) visualization (Hitashi 300 HT77 10 120 KV at La Paz, Mexico or FEI Quanta 250 of Universidad de Caldas, Manizales, Colombia).

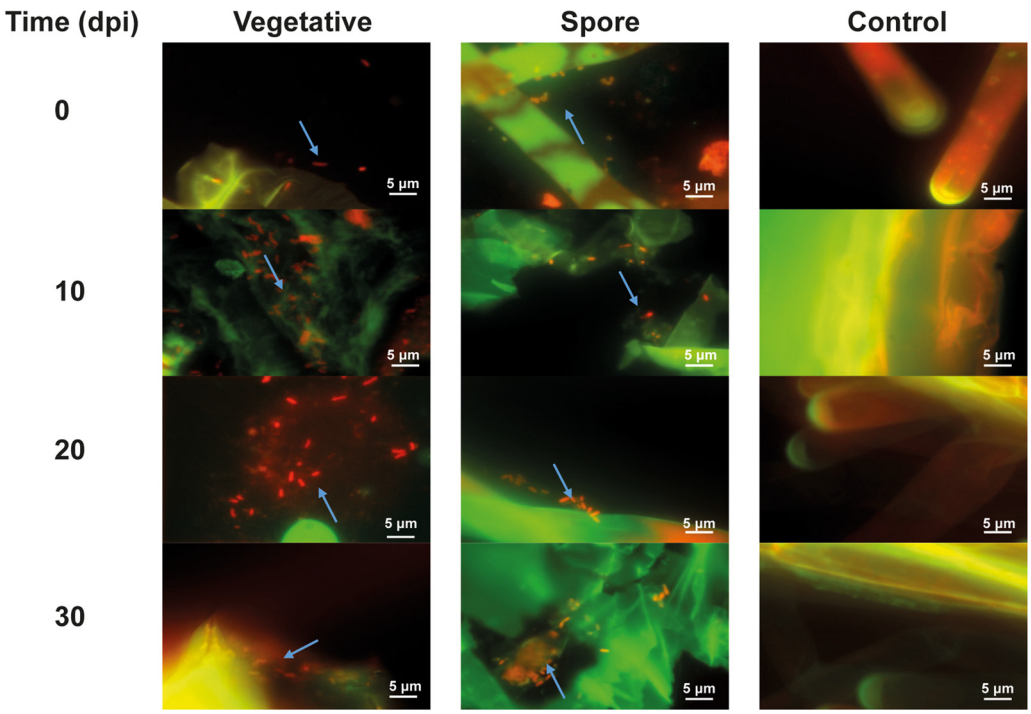

Fig. 1. Evaluation of *Bacillus subtilis* EA-CB0575 colonization on banana roots grown on $MS_{(s)}$ by fluorescent in situ hybridization (FISH) using the specific probe Bs575. Blue arrows indicate zones with presence of microorganisms. Seedlings of banana var. Williams were disinfected and planted on $MS_{(s)}$ medium with 5% agar. Shown roots are primary roots where root hairs can be found. The /Cy3/ probe was used for these hybridizations with *B. subtilis* due to the autofluorescence of banana roots when FITC when FITC filter (green emission) was used. Photographs correspond to overlapping images of fluorescence microscopy on FITC and Cy3 filters. Red cells correspond to *B. subtilis* cells that hybridized with specific probe for *B. subtilis*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.9. Culturable bacterial populations on roots

Total culturable bacterial populations on banana roots were evaluated every 10 days. Five grams of roots from each group (bacterial spore-treated, vegetative cell-

treated or non-treated control) were homogenized using an Ultraturrax IKA 20 W with 45 mL of phosphate buffer (pH 7.4) for 10 min at 2000 rpm. The resulting homogenate was used for surface culture using serial dilutions (10^{-3} to 10^{-6}) on TSA × 0.5, and results were reported in CFU/mL.

2.10. Growth promotion evaluations on banana plants

The effect of *B. subtilis* EA-CB0575 on banana plant growth promotion was evaluated with a completely randomized design with 15–24 experimental units per treatment, depending on the trial. Trial 1 used plants inoculated since the stage of in vitro plant culture on MS ($MS_{(S)}$ or $MS_{(L)}$), and trial 2 used plants inoculated at the greenhouse stage in commercial soil.

2.10.1. Trial 1

In vitro banana plants were inoculated with vegetative or spore *B. subtilis* EA-CB0575 cells (1×10^8 CFU/mL), sown on $MS_{(S)}$ or $MS_{(L)}$, and incubated for 30 days at 30 °C with a 12/12 h photoperiod. Later, plants were transferred to trays containing a 1:1 mixture of peat (Anasac) and a commercial mixture of soil (sandy loam soil, sand, and rice husk in the ratio of 1:0.5:0.5, Vivero Tierra Negra, Llanogrande, Antioquia). Plants were kept under greenhouse conditions with a 12/12 h photoperiod, at 22–30 °C, and 65%–85% relative humidity for 30 days, then transferred to 3 kg of commercial soil for 120 days under the same conditions. Plants were fertilized using 15-15-15 fertilizer (1 g/plant, Anasac) and the foliar fertilizer Wuxal Tapa Negra (Cropscience 1 cc/L).

The response variables evaluated were shoot length, plant diameter, number of leaves, and total, shoot, and root dry weight (TDW, SDW, and RDW, respectively). These trials were repeated twice at separate times.

2.10.2. Trial 2

Banana plants were inoculated with *B. subtilis* EA-CB0575 under greenhouse conditions by submerging the roots into *B. subtilis* EA-CB0575 bacterial suspensions containing spores or vegetative cells (1×10^7 CFU/mL or 1×10^8 CFU/mL). The plants were then incubated for 1 h and planted into 3 kg of commercial soil mixture. Evaluations were taken for 120 days while the plants were kept under the same conditions as specified for trial 1. These evaluations were independently repeated three times.

2.11. Growth promotion evaluations on tomato plants

Evaluations on tomato plants included two types of trials, each of them arranged in a completely randomized design with 8–10 experimental units per treatment, depending on the trial.

2.11.1. Trial 1

Stratified tomato seeds (−20 °C for 24 h) were inoculated with *B. subtilis* EA-CB0575 at 1×10^7 or 1×10^8 CFU/mL, sowed in a commercial soil mixture and kept under greenhouse conditions for 60 days. Conditions for growth were 30 ± 2 °C, 70% relative humidity, 12/12 h photoperiod, and fertilization and variable responses were the same as those for the banana assays. These assays were independently repeated three times.

2.11.2. Trial 2

Stratified and disinfected tomato seeds were used for experiments at in vitro conditions. These seeds were inoculated with *B. subtilis* EA-CB0575 (1×10^8 CFU/mL), sowed in $MS_{(S)}$ or MS_L, incubated at 30 °C for 30 days and then harvested. Each plant was fertilized for 30 days using 15-15-15 fertilizer (1 g/plant, Anasac) and the foliar fertilizer Wuxal Tapa Negra (Cropscience 1 cc/L). The growth promotion effect was determined by evaluating shoot length, number of leaves, and SDW, RDW, and TDW. These experiments were repeated three independent times.

2.12. Statistical analysis

Analysis of variance by fitting a linear model was used to analyze trials for plant growth promotion (StatGraphics Centurion XVI Version 16.1.18, Statpoint Technologies Inc., Virginia; USA). Normality and

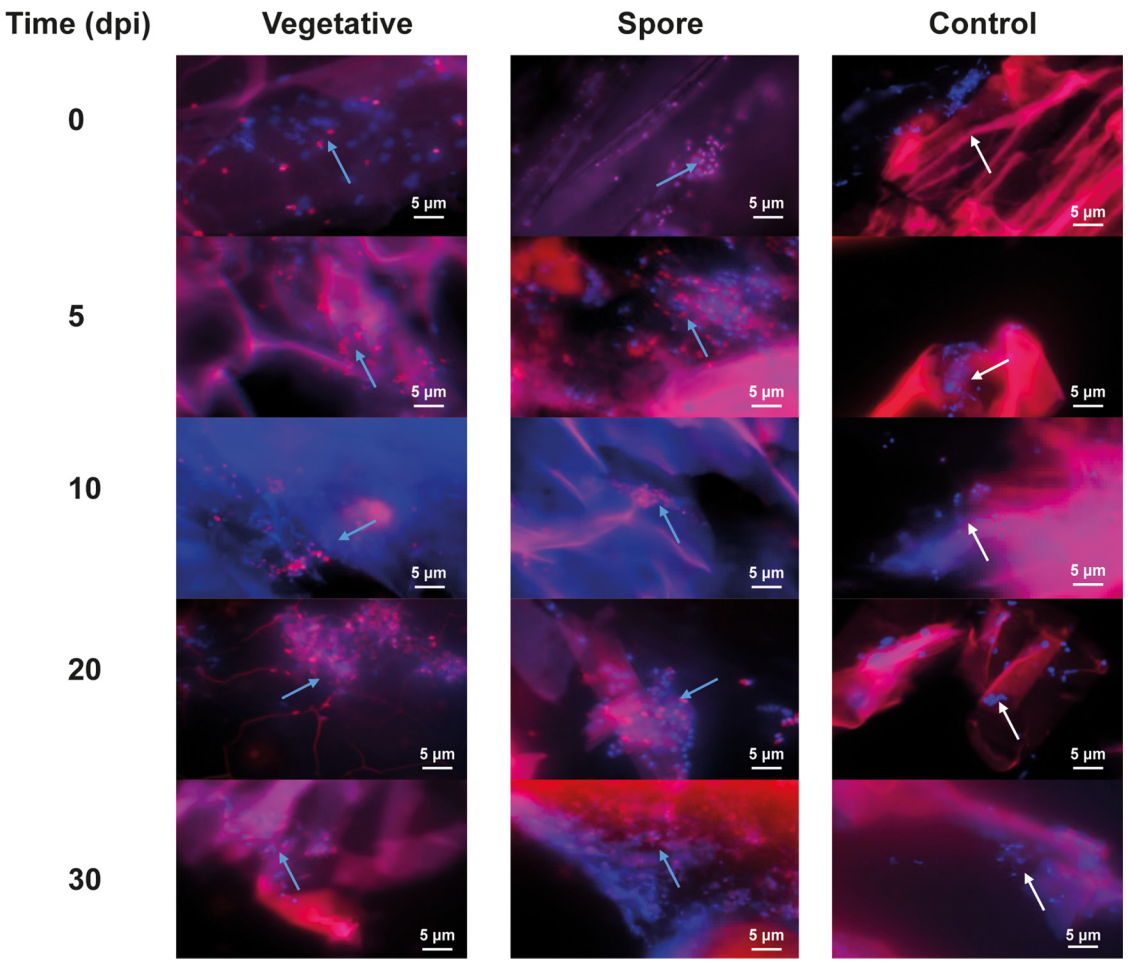

Fig. 2. Evaluation of *B. subtilis* EA-CB0575 colonization on banana roots by Catalyzed Reporter Deposition-Fluorescent In situ Hybridization (CARD-FISH) using the specific probe HRP-Bs575. Blue arrows indicate zones with presence of hybridized microorganisms. White arrows indicate zones with non-hybridized microorganisms on control roots. Seedlings of banana var. Williams were disinfected and planted on $MS_{(s)}$ medium with 5% agar. Shown roots are primary roots where root hairs can be found. The /HRP-Bs575/ probe was used for these hybridizations with *B. subtilis* cells and amplification was developed using CF-555-SE-tyramide (red emission). Photographs correspond to hybridizations with the specific probe. Fuchsia cells correspond to hybridized *B. subtilis* cells. Blue cells correspond to native microorganisms of banana plant that did not hybridize with specific probe. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

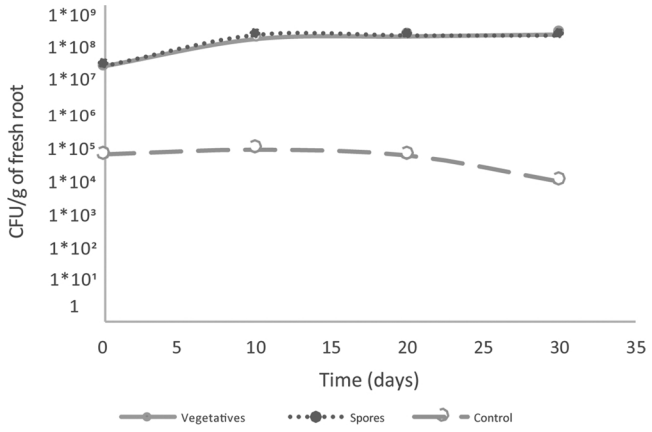

Fig. 3. Microbial population of similar colonies to *B. subtilis* EA-CB0575 morphology present in banana roots in $MS_{(s)}$ between 0 and 30 dpi. Colonies are beige, irregular, entire, matt and crateriform.

homoscedasticity of residuals from all response variables were ensured by testing them using Kolmogorov ($P > 0.05$) and Levene's tests ($P > 0.05$) and checking their graphical distributions by R function plot. In case of a significant P value ($P < 5\%$ for in vitro or 10% for greenhouse assays), means were compared using Dunnett and least significance difference multiple comparison tests.

3. Results

3.1. *B. subtilis* EA-CB0575 colonize banana roots in different growth systems

To determine root colonization of *B. subtilis* EA-CB0575 on in vitro banana plants grown in different systems ($MS_{(s)}$ and $MS_{(L)}$), FISH and CARD–FISH were performed during 30 dpi. With FISH evaluations, both vegetative and spore cells of the inoculated strain were detected on banana roots on different dpi, with an increase in bacterial population (Figs. 1–3; Supplementary material Fig. S1). The bacterial population size was also determined by culturable methods, finding an increase of one order of magnitude for vegetative and spores cells in $MS_{(s)}$ between days 0 and day 10, no changes for treatment control were identified (Fig. 3), indicating that *B. subtilis* EA-CB0575 cells colonized the banana roots. The results suggest that *B. subtilis* spores germinated into vegetative cells, according to the presence of rod-shaped cells with size of approximately 1.85 ± 0.31 µm at different times post inoculation. Similar results were also found for plants growing on $MS_{(L)}$.

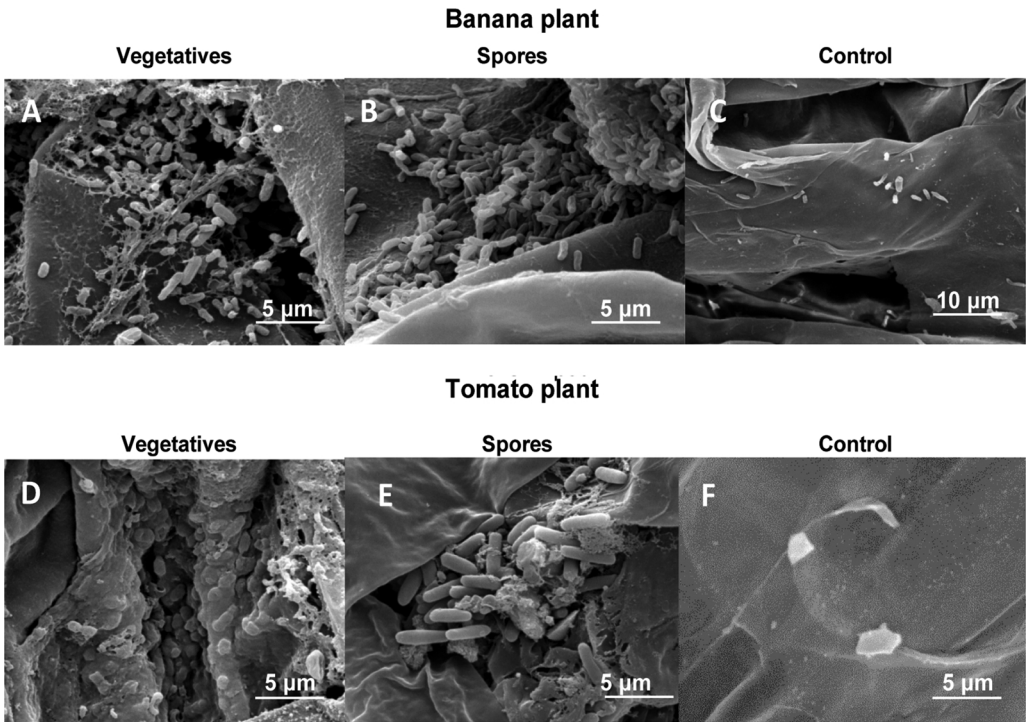

Fig. 4. Scanning electron microscopy (SEM) images of banana and tomato roots at 30 dpi with *B. subtilis* EA-CB0575. (A) inoculated banana roots with vegetative cells of *B. subtilis* EA-CB0575 (B) inoculated banana roots with spores of *B. subtilis* EA-CB0575 (C) Control banana roots without inoculation. (D) inoculated tomato roots with vegetative cells of *B. subtilis* EA-CB0575 (E) inoculated tomato roots with spores of *B. subtilis* EA-CB0575 (F) Control tomato roots without inoculation.

Due to high root autofluorescence, CARD–FISH was used to enhance fluorescent signal intensity. This methodology produced pictures with higher intensity of light and reduced exposure time of UV light needed for the fluorescence. We determined that the exposure time needed with CARD–FISH was one-eighth of that needed to obtain the same pictures with FISH (Fig. 2 and Supplementary material Table S1). As found with FISH, both vegetative and spore cells of the strain EA-CB0575 were detected on banana roots on different dpi, with an apparent increase of bacterial population. Control samples did not hybridize with the specific probe, suggesting the absence of native species of *B. subtilis*; however, some cells were stained with DAPI (blue cells), suggesting the presence of a native microbiota that could come from the meristems used for in vitro banana propagation. Treatment with vegetative cells showed rod-shaped cells and some spore-like structures (Fig. 2, 5–30 dpi), indicating that many cells remain active and only a portion goes into the sporulation process. Additionally, the formation of cellular aggregates was observed on inoculated roots, suggesting the production of a biofilm-type structure by *B. subtilis* EA-CB0575 (Figs. 1 and 2). SEM was used as a complementary analysis, showing dense rod-shaped cells colonizing the root tissues after 30 dpi (Fig. 4A–C), reaffirming that EA-CB0575 bacteria colonize banana roots.

B. subtilis EA-CB0575 colonization pattern was determined in the upper, middle, and lower parts of the banana roots. However, no particular colonization pattern was observed in these zones. EA-CB0575 cells were detected bordering the main and adventitious roots and at the intersections between these two types of roots (Fig. S2). This high colonization ability of *B. subtilis* EA-CB0575 vegetative and spore cells on banana roots suggests a growth promotion effect on banana plants. This effect was observed on plants inoculated under in vitro (Fig. 5A and B) and greenhouse conditions (Fig. 5C).

3.2. *B. subtilis EA-CB0575 spores or vegetative cells promote growth of banana plants*

Banana plants inoculated with spore or vegetative cells of *B. subtilis* EA-CB0575 in vitro on growth system $MS_{(L)}$ showed differences in TDW and shoot length when vegetative cells were applied 2 months post inoculation (Supplementary material Fig. S3). Furthermore, significant differences in TDW and SL were found in plants treated with *B. subtilis* spore application during the last month of the evaluation (6 months post inoculation) (Fig. 5A). The increases were 83.6% for TDW and 15.7% for SL. Evaluations on $MS_{(S)}$ showed increases in NL and SL at 2 months post inoculation when both types of cells were introduced (Supplementary material Fig. S3). However, for the last month of the evaluation, only plants that had been inoculated with vegetative cells showed a significant increase in SL and TDW, with increases by 17.1% and 25.1%, respectively (Fig. 5B). Banana plants under greenhouse conditions showed successful results for plant growth promotion, specifically for TDW and RDW when they were inoculated with both types of cells. For TDW, 10^7 CFU/mL was the best concentration and for RDW, it was 10^8 CFU/mL, with increases of 7.4%–16.7% for both variables (Fig. 5C).

3.3. *B. subtilis EA-CB0575 colonizes tomato roots in different culture systems at in vitro level and in soil*

Evaluations were performed to determine if *B. subtilis* rhizosphere colonization was different in tomato roots compared with that in banana roots. We encounter the presence of *Bacillus* on tomato roots in different culture systems (Figs. 6 and S1) at 15 dpi and root colonization at 0–30 dpi (Fig. 7). Results did not show the presence of *B. subtilis* EA-CB0575 in the control treatment (Figs. 6, 7 and S1). FISH and CARD–FISH were used for detecting vegetative cells and spores on roots grown in $MS_{(S)}$ and $MS_{(L)}$ and results indicated that the strain EA-CB0575 colonized the tomato roots. SEM was used to complete the evaluation of *B. subtilis* EA-CB0575 colonization, finding similar results to banana root evaluations due to the presence of rod-shaped cells on inoculated roots with spores and vegetative cells of the microorganisms and reinforcing the idea of *B. subtilis* presence and formation of biofilm-like structures on root surfaces (Fig. 4D–F). When *B. subtilis* cell distribution was evaluated, we established a differential distribution on tomato roots, with a predominant presence in the upper and lower zones of roots and a poor colonization in the middle zone (Fig. S2).

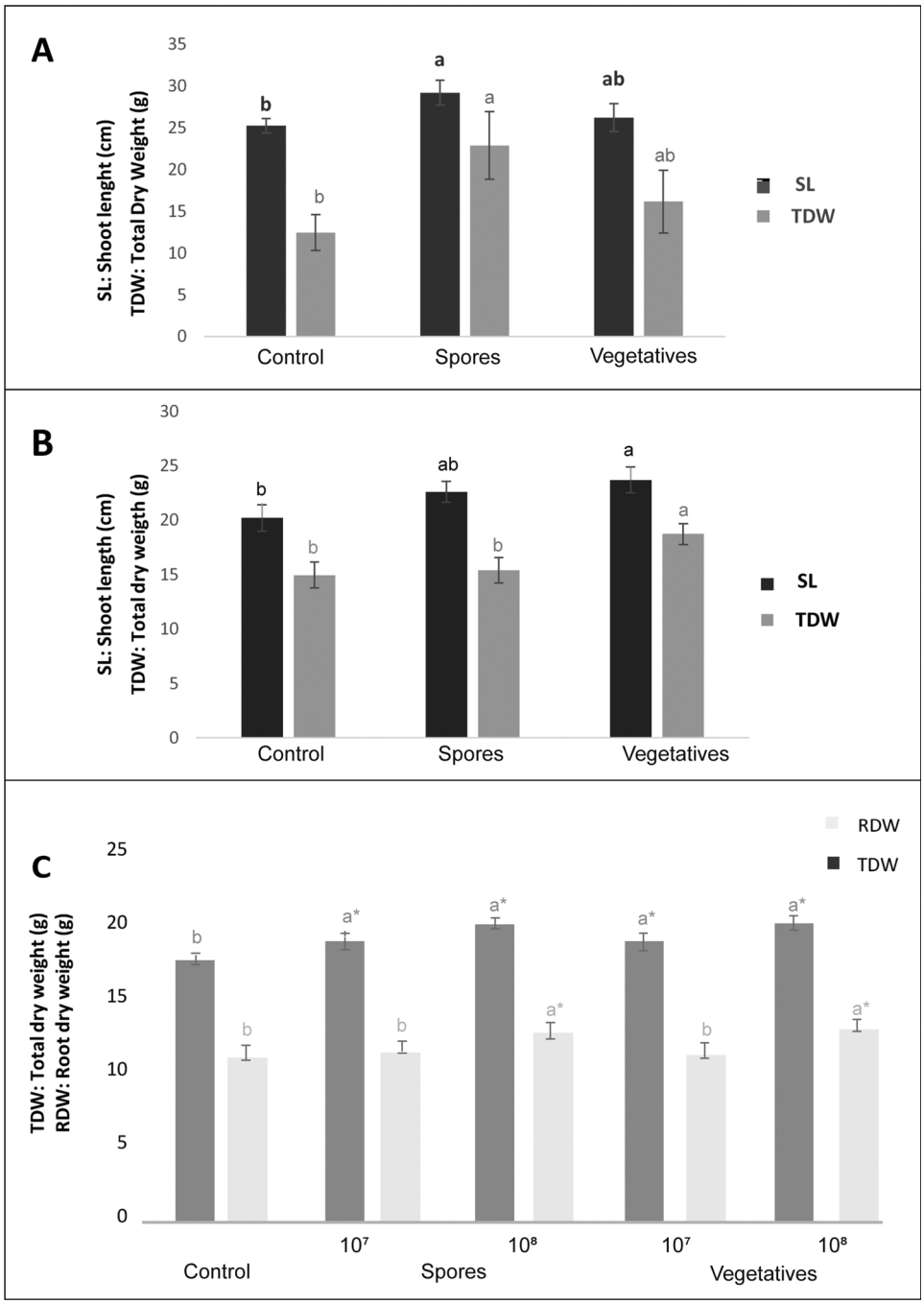

Fig. 5. Effect of *B. subtilis* EA-CB0575 inoculation on growth of banana seedlings at in vitro and greenhouse level. Data are averages of three evaluations at different times (each with n = 8 for in vitro level and n = 10 for greenhouse level). Different letters correspond to treatments with significant differences according to the LSD test (A) Banana plants inoculated with spores or vegetative cells on $MS_{(L)}$ in vitro, SL *p-value* = 0,0907,TDW *p-value* = 0.0524. (B) Banana plants inoculated with spores or vegetative cells on $MS_{(s)}$ in vitro, SL *p-value* = 0.0245, TDW *p-value* = 0.004. C. Banana plants inoculated under greenhouse conditions, SL *p-value* = 0.0001, TDW *p-value* = 0.0257. Asterisks correspond to treatments with significant differences with the control by Dunnett test, both with confidence of 95%. The results correspond to data obtained 180 dpi for in vitro level and 120 dpi for greenhouse level.

3.4. *B. subtilis EA-CB0575 promotes growth of tomato plants*

Growth promotion of tomato plants was evaluated for plants inoculated with spores and vegetative cells of *B. subtilis* under in vitro and greenhouse conditions. We found an increase in growth, specifically TDW and SL, when EA-CB0575 in vegetative cell form was applied to tomato seeds grown on $MS_{(L)}$, with increases of 10.7% and 69.3%, respectively (Fig. 8A). Plants propagated in the growth system $MS_{(S)}$ showed a higher TDW when seeds were inoculated with spores or vegetative cells of *B. subtilis* in all the evaluated concentrations (increases between 28.6% and 44.07%). But the shoot length was promoted only for treatments inoculated with the lower concentration of *B. subtilis* (13.7%–16.7%) (Fig. 8B). Assays at greenhouse level resulted in an increase in TDW when plants were inoculated with vegetative cells of *B. subtilis* at 1×10^8 CFU/mL with an increase of 82.4% compared with the control (Fig. 8C).

4. Discussion

B. subtilis is a species of interest for the development of bio-inoculants based on PGPB (De Souza et al., 2015). However, its effect can be limited for the execution of some mechanisms of plant growth promotion due to poor colonization of the rhizosphere (Gao et al., 2016). Colonization studies of PGPB, specifically with *B. subtilis*, are limited (Fan et al., 2012). However, the development of new methods for detecting and monitoring microorganisms in different environments and the improvement of existing methods is allowing scientists to establish the behavior of rhizobacteria in roots: their biofilm formation, their location in rhizodeposits, and their response to molecules secreted by plant or cell wall components. Some of this research has begun to

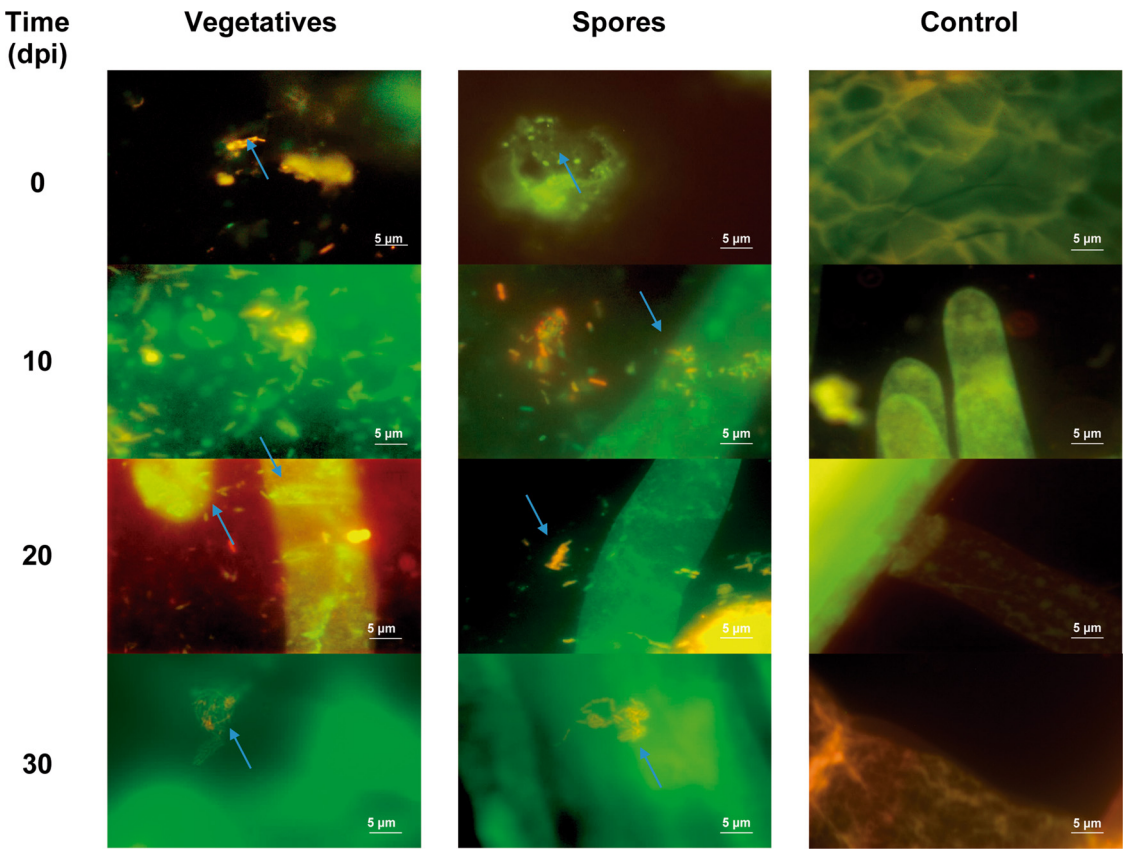

Fig. 6. Evaluation of *B. subtilis* EA-CB0575 colonization on tomato roots by fluorescent in situ hybridization (FISH) using the specific probe Bs575. Blue arrows indicate zones with presence of microorganisms. Seeds of tomato var. Chonto were disinfected and sown on MS(s) medium with 5% agar. Shown roots are primary roots where root hairs can be found. Seeds germinated 3 dpi. The /56-FAM.Bs575/ probe was used for these hybridizations with *B. subtilis* cells and EUBmix probe was used for Eubacteria hybridization. Photographs correspond to overlapping images with each probe. Yellow-orange cells correspond to *B. subtilis* cells that hybridized with both probes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

evaluate gram-positive bacteria (Beauregard et al., 2013; Dietel et al., 2013). To detect microorganisms in biological samples, some traditional culture methods, such as plate methodology (Moter and Gobel, 2000), molecular methods such as hybridization or reporter gene detection (Baldan et al., 2015; Cardinale, 2014; Dietel et al., 2013) or NanoSims (Chen et al., 2015), and new-generation methods, such as metagenomics (Leveau, 2007), have been used and are applicable to cultivable or non-cultivable microbiota (Mendes et al., 2013; Moter and Gobel, 2000).

In this study, FISH and CARD–FISH methods were used to determine *B. subtilis* EA-CB0575 colonization on banana and tomato roots in different culture systems using a specifically designed probe for *B. subtilis* species (Posada et al., 2016), with some variations at the 5′-end (56-FAM or Cy3 labeled for FISH and HRP labeled for CARD–FISH). We obtained successful results, detecting and monitoring microorganisms on both banana and tomato roots between inoculation time and 30 dpi.

One of the disadvantages of methods such as FISH is the difficulties in hybridization due to the low number of target genes in the samples (Zwirglmaier et al., 2005). However, *Bacillus* is a genus with a high number of copies of the 16S gene (Větrovský and Baldrian, 2013); therefore, this method can be successful for *Bacillus* detection (as presented in this research). Another failure attributed to the FISH method is the autofluorescence of the sample, which can interfere with the fluorescent signal of the hybridization. This can be overcome using confocal laser microscopy, which reduces sample noise (Cardinale, 2014), or the use of modified FISH methods such as CARD–FISH, RING–FISH (recognition of individual genes), DOPE–FISH (double-labeling of oligonucleotide probes), or GOLD–FISH (gold-facilitated in situ hybridization), which improve signal detection (Zwirglmaier et al., 2005). In this study, the increase of fluorescence was verified when using CARD–FISH respect to FISH, with an increase of 8.1 times related to FISH, result according to previous reports (Eickhorst and Tippkötter, 2008; Zwirglmaier et al., 2005).

The evaluation of colonization allowed us to determine the presence of *B. subtilis* on banana and tomato roots in different culture systems ($MS_{(L)}$, $MS_{(S)}$,or soil) under in vitro or greenhouse conditions. Colonization was detected at different times for different treatments (application of spores or vegetative cells of *B. subtilis* EA-CB0575). An increase in the microbial population was found visually and corroborated by plate methodology using banana roots with and without inoculation. The results indicate colonization on the rhizosphere and the differential distribution of *B. subtilis* EA-CB0575 on roots was determined. *B. subtilis* appeared predominantly in the upper and lower root sections and around the tip of the tomato plants. In banana plants, a similar distribution on roots was found between the three zones without apparent differences in cell distribution. However, it was difficult to determine *Bacillus* colonization around the tip in the banana plants due to the high autofluorescence of the tissues in the root zone. These results are consistent with those reported by Fan et al. (2012), who indicated the differential colonization patterns of *B. amyloliquefaciens* FZB42 in comparative plant species. These findings are also confirmed in other studies (Rudrappa et al., 2008; Saile and Koehler, 2006). However, there are no previous studies on banana or tomato rhizosphere colonization using FISH or CARD–FISH.

A relation has been found between the presence of biofilm-like structures and *B. subtilis* aggregation on roots for different samples and times. One main reason for this aggregation and matrix production could be the abundant exudates produced by specific zones, which has

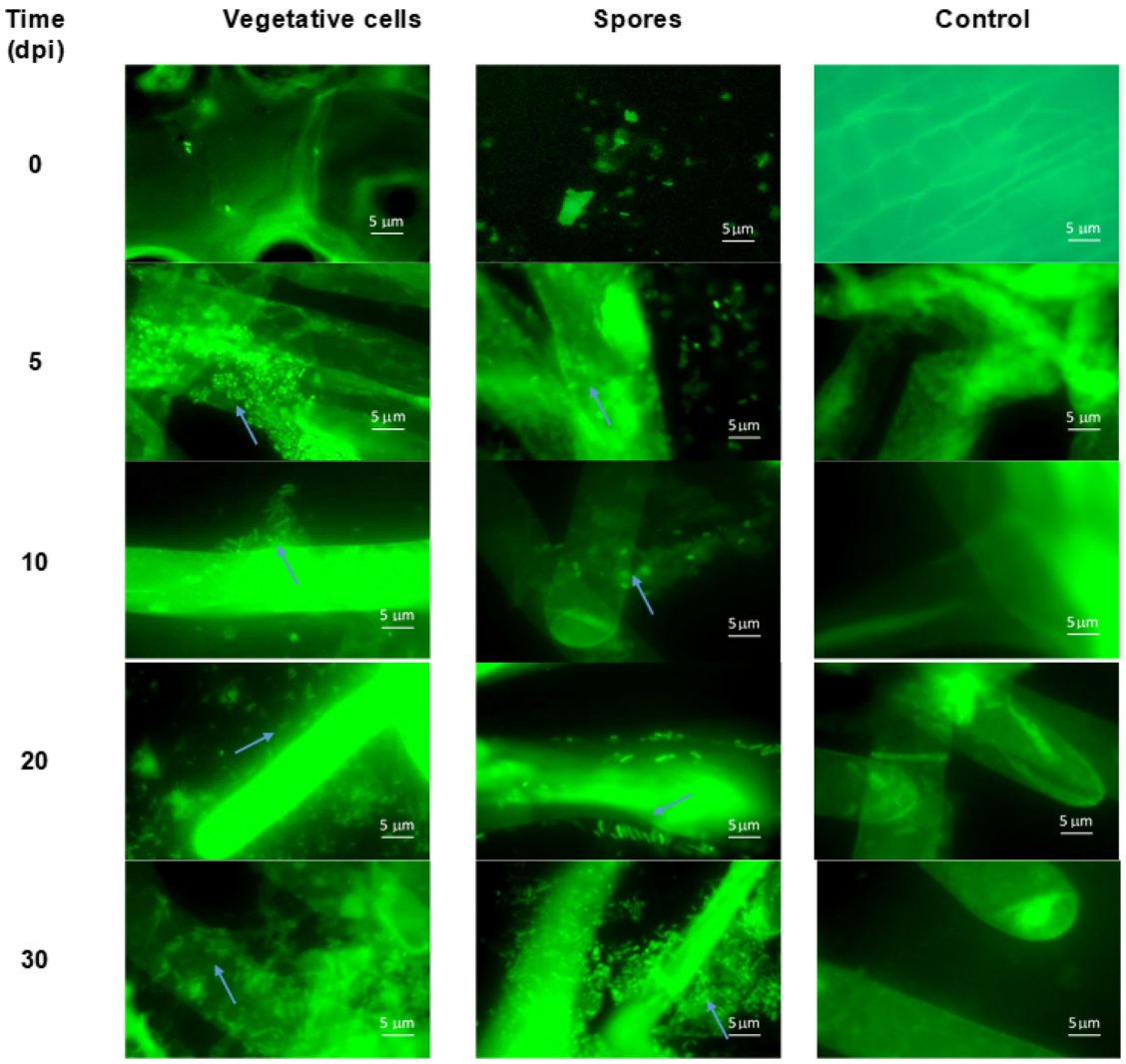

Fig. 7. Evaluation of *B. subtilis* EA-CB0575 colonization on tomato roots on $MS_{(s)}$ by Catalyzed Reporter Deposition-Fluorescent In situ Hybridization (CARD-FISH) using the specific probe HRP-Bs575. Blue arrows indicate zones with presence of microorganisms. Seeds of tomato var. Chonto were disinfected and sown on MS(s) medium with 5% agar. Shown roots are primary roots where root hairs can be found. Seeds germinated 3 dpi. The /HRP-Bs575/ probe was used for these hybridizations with *B. subtilis* cells and amplification was developed using Atto 488-NHS-tyramide (Green emission). Photographs correspond to hybridizations with the specific probe. Green cells correspond to hybridized *B. subtilis* cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

been reported by several authors (Bais et al., 2006; Beauregard, 2015). It has been established that biofilm presence is required for cell fixation and colonization of plant tissues (Aleti et al., 2016). This biofilm gives advantages to bacterial cells because it gives them protection from predators and helps them retain nutrients (Dervaux et al., 2014). Using SEM, we determined the presence of this matrix that could keep the cells together, which was observed in inoculated tomato and banana roots. Note that this matrix was not present in the control treatment, where independent cells were detected for banana roots (Fig. 4). *Bacillus subtilis* EA-CB0575 produces lipopeptides, such as surfactin and fengycin. These compounds have been demonstrated to have an important role in the formation of a stable biofilm and to facilitate cell-spreading of *B. subtilis* by reducing surface tension (Fan et al., 2012; Raaijmakers et al., 2010). These compounds could be related to the formation of the observed biofilm, according to the proposed mechanism by Bloom-Ackermann et al. (2016) and we determined this strain produces biofilm at in vitro level in MSgg and TSB media (data not shown).

Treatment with initial spore inoculation showed the presence of vegetative cells at different times, indicating the germination of these spores. Germinating spores have also been reported for different species of *Bacillus* in *Arabidopsis* spp. and pasture plants, germinating 24 h after application (Reva et al., 2004; Saile and Koehler, 2006). The presence of vegetative cells on inoculated plants suggests that cells could be activating the mechanisms of growth promotion such as hormone, siderophore, and lipopeptide production, phosphate solubilization, and nitrogen fixation; genomic and biochemical potentials found for this strain in previous studies by biochemical and genomic analysis (data not shown). Plant growth promotion was evaluated for the different growth systems $MS_{(S)}$, $MS_{(L)}$, and soil (for tomato plants), and we determined the potential of *B. subtilis* EA-CB0575 to promote plant growth of banana and tomato plants in different systems under in vitro and greenhouse conditions. These evaluations showed successful results similar to previous evaluations under greenhouse and field conditions using this strain for banana plants and under greenhouse conditions for tomato plants (Posada et al., 2016; other data unpublished). Our results indicated reproducible responses of these plants to *B. subtilis* inoculation at different times and production scales. This is very important because one reported disadvantage of PGPB application is the non-reproducibility of results at different time points because of the effect of

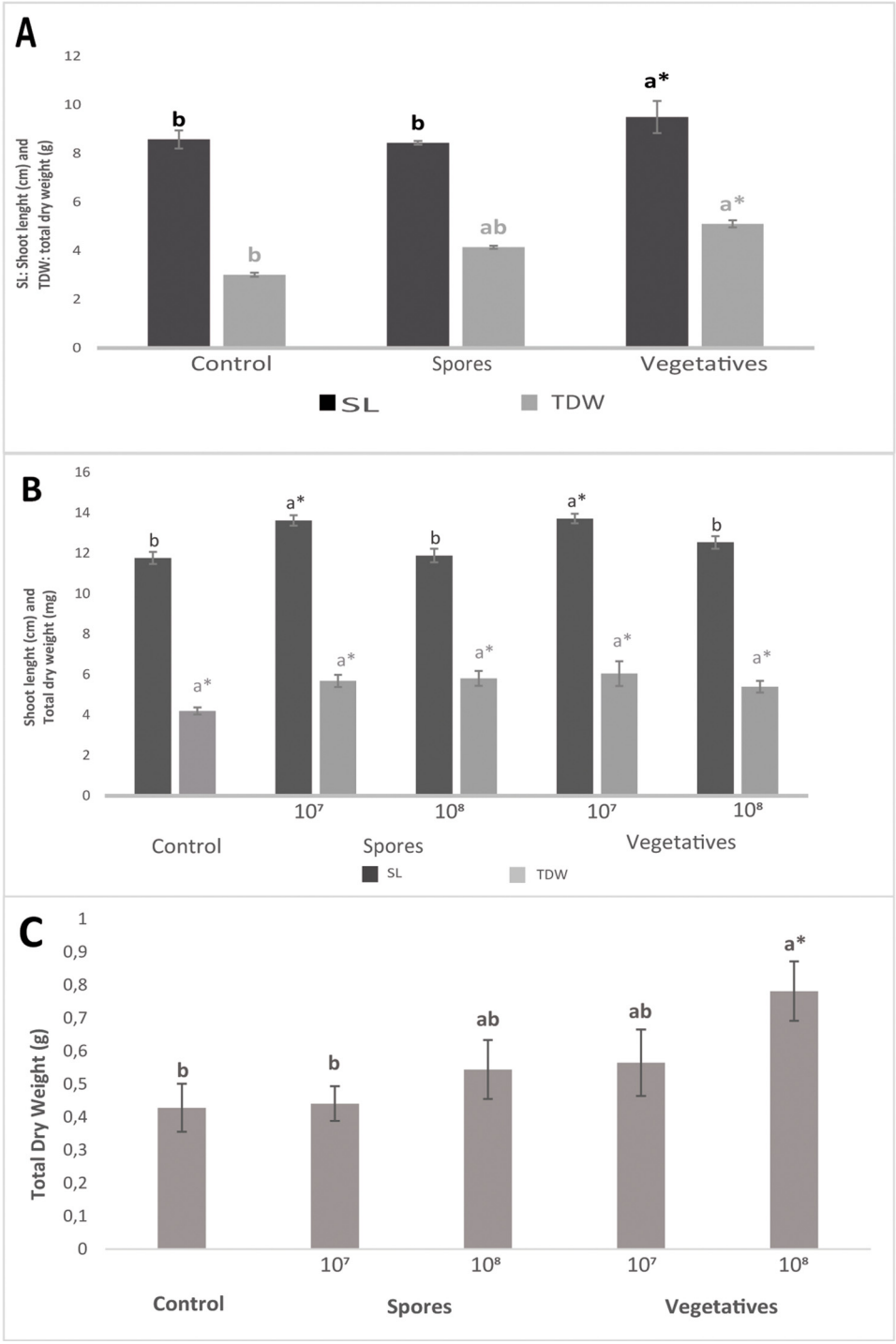

Fig. 8. Effect of *B. subtilis* EA-CB0575 inoculation on tomato seeds at greenhouse and in vitro level. Data are averages of 3 evaluations at different times (each with n = 8 for in vitro level and n = 10 for greenhouse level). Different letters correspond to treatments with significant differences according to the LSD test (A) Tomato seeds grown on $MS_{(L)}$, SL *p-value* = 0.0001, TDW = 0.0257. (B). Tomato seeds grown on $MS_{(s)}$,TDW *p-value* = 0.0524. (C) Tomato plants grown on soil at greenhouse level, SL *p-value* = 0.0245, TDW = 0.004. Asterisks correspond to treatments with significant differences with the control by Dunnett test, both with confidence of 95%. The results correspond to data obtained 30 dpi for in vitro level and 60 dpi for greenhouse level.

different uncontrollable factors under greenhouse and field conditions (Buddrus-Schiemann et al., 2010). The inoculation improved TDW and shoot length with significant statistical differences compared with the control for both banana and tomato plants.

In conclusion, FISH and CARD–FISH methods were successful for detecting and monitoring bacterial colonization on banana and tomato roots in different culture systems under in vitro and greenhouse conditions and, complemented with SEM, could give enough information to determine root colonization of the PGPB. We found different colonization distribution for the plant species evaluated and the presence of a matrix similar to biofilm was observed in different sections of the root. These microorganisms were maintained for 30 dpi: many of them as vegetative cells, and plants inoculated with spores showed vegetative cells in time, indicating the germination of the spores in the root area. We determined that plant growth promotion occurred in the evaluated plant species when *B. subtilis* EA-CB0575 was applied.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.micres.2018.08.017.

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