

Full Length Research Paper

Endophytic bacteria isolated from ipê mirim (*Tecoma stans* Bignoniaceae) and its application for plant growth promotion

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Endophytes are usually protected from soil environment competitiveness and stress. However, the presence of heavy metals can negatively affect the structure and diversity of endophyte communities. The aim of the present study was evaluate the diversity of endophytic bacteria from ipê mirim (*Tecoma stans* Bignoniaceae) grown in an area of Atlantic rainforest contaminated with metals and evaluate the ability of these bacteria to promote plant growth and seed germination. Results show that endophytic bacterial density in plants was stable among sites with different level of metals; however, bacterial richness was lower in plants from sites with low level of metals. At least 28 genera were isolated, where *Methylobacterium* (21.32%), *Bacillus* (19.12%), *Pseudomonas* (11.03%) and *Curtobacterium* (7.35%) the dominant groups. Isolates were selected from Rhizobiales order and the capability of this dominant group in plant growth promotion was evaluated. Results showed that *Methylobacterium* spp. and *Rhizobium* sp. increased germination and improved seedling growth of tomato Santa Cruz Kada Gigante. Therefore, results show that the endophyte cultivable community is not influenced by the presence of low metal concentration, and plant growth promoter bacteria that can be used on tomato seedlings production were successfully selected and on future phytoremediation studies.

Key words: *Methylobacterium* spp., *Rhizobium* sp., *Tecoma stans*, microbial diversity, metals, plant growth promoting bacteria (PGPB), tomato, growth promotion.

INTRODUCTION

The endophytic bacteria colonize the inner plant tissues without causing disease and without visible external

structures (Hardoim et al., 2008, 2015). This relationship can be beneficial for both plant and microorganisms; studies

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have shown that these bacteria or fungi also can be used as a source for new drugs (Strobel et al., 2004), enzymes, antibiotics (Martinez-Klimova et al., 2016; Braga et al., 2016), and allow the development of new bioremediation and agricultural techniques (Kobayashi, 2000; Dourado et al., 2013).

The diversity of endophytic bacteria may be impacted by biotic (plant genotypes and microbial community) and abiotic (nutrients, water, temperature, pH and pollutants) factors (Hardoim et al., 2008). Although endophytes are protected of stress and competitiveness from soil environment (Gans et al., 2005), the presence of heavy metals may negatively affect the structure and diversity of microbial communities. Thus, it is important to understand the factors that affect the endophytic community and its impact on the biotechnological potential of this community.

Methylobacterium spp. has been isolated from several plant species (Omer et al., 2004), actively colonizing the leaf surface or the inner plant tissues, such as soybean, sugarcane, cotton, citrus, eucalyptus, bamboo, *Catharanthus roseus*, tobacco, strawberry, mangrove plants and different Fabaceae species (Madhaiyan and Poonguzhali, 2014; Dourado et al., 2015). Inside the host plant *Rhizobium* and *Methylobacterium* could produce phytohormones (IAA and cytokinin) or interact with the microbial community (endophytes and pathogens) (Madhaiyan et al., 2006; Dourado et al., 2015; Azevedo et al., 2016). In addition, *Methylobacterium* spp. can promote the seed germination and increase leaf area and plant height (Madhaiyan et al., 2006; Kumar et al., 2011; Bogas et al., 2016; Gopalakrishnan et al., 2015), the stomata number, chlorophyll and malic acid content (Cervantes-Martinez et al., 2004), and fixing N₂, inducing the formation of nodules on the host plant (Sy et al., 2001; Gopalakrishnan et al., 2015), suggesting that these bacteria can change the plant physiology during interaction.

The aim of this study was to isolate and characterize endophytic bacterial community from *T. stans*, known as Ipê-mirim, yellow trumpetbush or yellow bells, grown in an Atlantic Rain Forest with a history of heavy metal contamination; and evaluate the diversity and the application of these bacteria in promoting tomato plant (*Lycopersicon esculentum* Mill) growth.

MATERIALS AND METHODS

Plant and soil samples

Endophytic bacteria were isolated from Ipe-Mirim (*Tecoma stans*, Bignoniaceae) plants grown in an Atlantic Forest area of Nagib Najar Park (Mogi das Cruzes, São Paulo), with a history of metals contamination. The plants and soil were sampled randomly in four points in the Park: Site 1 (46°12'49.6''W-23°31'03.6''S), site 2 (46°2'49.0''W; 23°31'08.1''S), site 5 (46°12'44.2''W; 23°31'09.9''S) and site 6 (46°12'39.5''W; 23°31'19.1''S) in April of

2007 and May of 2008. For each sampling site, three plants were collected and immediately transported to the laboratory for isolation of the endophytic bacterial community.

Physical and chemical soil analysis

The physical and chemical soil analysis was performed at the Núcleo de Ciências Ambientais (NCA) at University of Mogi das Cruzes, under the responsibility of Prof. Dr. Andrew Fernando de Oliveira. The levels of metals were compared to soil quality reference values determined by CETESB (São Paulo State Environmental Company) (<http://www.cetesb.sp.gov.br>).

Isolation of endophytic bacteria from branches

Stems (5 mm of diameter) were surface disinfected (70% ethanol for 30 s; 2% sodium hypochlorite for 2 min and sterile distilled water for 30 s), macerated in PBS buffer (NaCl, 8 g l⁻¹; KCl, 0.2 g l⁻¹; Na₂HPO₄, 1.44 g l⁻¹; KH₂PO₄, 0.24 g l⁻¹) and appropriate dilutions (10⁻¹, 10⁻² and 10⁻³) were plated with the controls (triplicate of 200 µl water culture from the last disinfection step) on culture medium TSA (Tryptic Soy Agar - Oxoid) 5%, amended with benomyl (50 mg.mL⁻¹) and incubated at 28°C for 30 days. After growth, the colonies were counted and randomly picked out for further identification. Means were compared by analysis of variance (ANOVA) with Bonferroni test, comparison of independent samples and the Pearson correlation method using the Bioestat software (v.5.0, 2015).

Molecular identification and diversity analysis

The total DNA of the isolates were extracted, the gene 16S rRNA was amplified with primers 968F and 1401R (Araújo et al., 2002) and sequenced by HUG-CELL center (<http://genoma.ib.usp.br/en>). All 140 bacterial sequences presented in this study were submitted to GenBank (accession numbers KX914446 - KX914662).

The sequences were classified in RDPquery (<http://simo.marisci.uga.edu>) using only type-strain sequences from the RDP database (<http://rdp.cme.msu.edu/>). A phenetic tree was built using the MEGA 6.06 software (www.megasoftware.net) using the neighbor-joining method and Jukes-Cantor model, with a consistency test with 1000 bootstrap replicates. The resultant tree was edited with ITOL program (<http://itol.embl.de/>). The sequences were grouped into OTUs (Operations Taxonomic units), as 97% (species) and 95% (genus) similarity criteria with the Mothur program (Schloss et al., 2009). The richness (Ace and Chao1) and diversity indexes (Simpson and Shannon-H') were evaluated and the communities from each sampling sites were compared by β-Libshuff method.

In vitro Rhizobiales colonization assay

Since *Methylobacterium* and *Rhizobium* (Rhizobiales) were the most abundant group it was selected to proceed the analysis, testing its biotechnological potential in plant experiment.

Rhizobium sp. and *Methylobacterium* spp. tomato germination assay

The colonization of tomato Cherry and Santa Cruz Kada Gigante for 47 isolates of Rhizobiales (*Rhizobium* and *Methylobacterium*)

Table 1. Soil content of bioavailable metal and soil pH on the sampling placing of *T. stans* plants.

Metal	Soil content of bioavailable metal ($\mu\text{mol/g}$)			
	Site 1	Site 2	Site 5	Site 6
Soil pH	7.25	7.5	5.5	7.25
Ca	6.4	9.6	6.8	5.5
Mg	2.8	6.9	7.2	4.8
Fe	0.3	0.3	1.9	0.3
Mn	0.08	0.12	0.05	0.24
Al	0.3	-	7	-
Cu	0.05	0.08	0.02	0.95
Zn	0.1	0.2	0.5	0.4
Cd	-	-	-	0.01
Characteristics	Low metal	Low metal	Low pH, high Al	High metal (Cu, Cd)

was evaluated *in* plates. For this, the bacteria were cultured in TSB medium (Oxoid) for 72 h at 28°C under agitation (150 rpm), washed and suspended in PBS. Approximately 200 seeds were inoculated with 2 ml of this bacterial suspension (OD 600 nm = 0.8) for 60 min, and further rinsed in PBS and incubated in a humid chamber at 25°C for up to 6 days. The endophytic colonization was assessed by re-isolation of the inoculated isolates from surface disinfected plant tissues on TSA 5%.

Rhizobiales tomato germination assay

Experiment was performed as reported above only with the 29 *Rhizobiales* strains able to colonize tomato plants. These 29 strains were screened for direct plant growth promotion (PGP) abilities. They were inoculated in seeds of Cherry and Santa Cruz Kada Gigante tomato and plant germination rate was measure five days after planting.

Rhizobiales- tomato plant greenhouse assay

One *Rhizobium* sp. and two *Methylobacterium* spp. strain able to colonize the inner plants tissues were evaluated for their effect on the germination rate and plant growth promotion. For this, bacteria were inoculated into tomato seeds, as described previously, and sown in trays (expanded polystyrene with 128 cells at a depth of 0.5 cm) containing PlantMax® HT substrate. Ten seeds were sown by tray cell, and five cells (repetitions) per treatment, with completely randomized blocks. The control group was sown with distilled water (without bacteria). After 20 days in the greenhouse (temperature average was 25°C and relative humidity average was 80%), the germination rate was evaluated and 5 seedlings of each replication/ treatment were transferred to plastic pots with PlantMax® HT substrate and evaluated after 10 days according to seedling height, size of root system, leaves number. For all analysis, two independent experiments were performed.

Phosphate solubilization and nitrogen fix testes in plant growth *Rhizobiales* strains

Three *Rhizobiales* bacteria (two *Methylobacterium* and one *Rhizobium*), used in the previous experiment, were grown in plates with culture medium with inorganic phosphate (glucose 10 g l⁻¹,

NH₄Cl 5 g l⁻¹, NaCl 1 g l⁻¹ and MgSO₄.7H₂O 1 g l⁻¹, pH 7.2) for 48 h at 28°C and after growth, the presence of clear halo around the colony indicated the phosphate solubilization (Verma et al., 2001). The same three *Rhizobiales* isolate were screened for the ability to fix N₂, inoculated into tubes containing Nfb (Döbereiner et al., 1995) and incubated at 28°C for 10 days. Isolates that grew in this medium were re-inoculated in the same condition for three consecutive times. Isolates that grew in all inoculated tubes were considered positive.

RESULTS

Physical and chemical soil analysis

The pH and bioavailability of Ca (calcium), Mg (magnesium), Fe (Iron), Mn (manganese), Al (aluminum), Cu (cuprum), Zn (zinc) and Cd (cadmium) was assessed in an Atlantic Rain Forest soil. The pH was similar for sites 1, 2 and 6, ranging from 7.25 to 7.5, but was significantly different for site 5, which present acid soil with pH 5.5 (Table 1).

The concentration of bioavailable Mg, Fe and Zn ranged from 2.8, 0.3 and 0.1 for site 1 to 7.2, 1.9 and 0.5 $\mu\text{mol.g}^{-1}$ for site 5, respectively. The higher concentration of Mn, Cu and Cd was observed in site 6 (Table 1). The bioavailability of Ca ranged from 5.5 to 9.6 $\mu\text{mol.g}^{-1}$ in site 6 and site 2, respectively.

Isolation of endophytic bacteria from branches

Isolation of cultivable endophytic bacteria from *T. stans* revealed a large discrepancy in bacteria counts. Plants grown in sites 5 and 6 showed the highest bacterial density, while plants from site 2 presented the lowest value (Table 2). However, Pearson analysis showed no correlation between the metal concentration and bacterial counts ($p = 0.47^{\text{ns}}$ for Fe; $p = 0.13^{\text{ns}}$ for Zn; $p = 0.73^{\text{ns}}$ for Cu; $p = 0.97^{\text{ns}}$ for Mn).

Table 2. Richness and diversity analyzes of endophytic bacteria of *T. stans* at similarity level of 97%.

Sampling site	Log ₁₀ CFU.g steam ¹	# OTUs	Richness		Diversity	
			CHAO 1	ACE	Shannon H'	Simpson
1	2.4	21	56 (30.6-147.5)	70.1 (33.7-211.2)	2.4 (2.1-2.7)	0.1209 (0.0748-0.167)
2	1.8	22	79 (39.1-212.7)	99.8 (43.2-307.7)	3.0 (2.7-3.3)	0.0154 (-0.0047-0.0355)
5	3.7	28	203.5 (83.6-581.3)	406 (96.1-2125.9)	3.3 (3.0-3.5)	0.0025 (-0.0039-0.0088)
6	3.1	26	118 (55.2-315.2)	182 (37.1-2210.4)	3.2 (2.9-3.5)	0.0053 (-0.0039-0.0145)

Molecular identification and diversity analysis

A total of 140 endophytes were isolated, being 58 from Site 1, 25 from Site 2, 30 from site 5 and 27 from site 6. The endophytic cultivable bacterial community associated of *T. stans* steam was composed by at least 28 genera: *Actinoplanes*, *Aquabacterium*, *Bacillus*, *Brevibacterium*, *Citrobacter*, *Curtobacterium*, *Enterobacter*, *Hymenobacter*, *Kineococcus*, *Klebsiella*, *Massilia*, *Methylobacterium*, *Microbacterium*, *Mycobacterium*, *Pandora*, *Pantoea*, *Patulibacter*, *Plantibacter*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Sphingobium*, *Sphingomonas*, *Staphylococcus*, *Stenotrophomonas*, *Terracoccus*, *Williamsia*, *Xanthomonas* (Figure 1). The genus *Methylobacterium* (21.32%), *Bacillus* (19.12%), *Pseudomonas* (11.03%) and *Curtobacterium* (7.35%) were the dominant groups, and represented 58.82% of the total culturable bacterial community inside the *T. stans* stems. However, it was observed that both *Methylobacterium* and *Bacillus* were predominantly isolated from site 1 (with low metal content) and *Pseudomonas* presented higher number in plants from site 5 (with low pH).

At 97% similarity level, the bacterial community contained 92 OTUs. The highest richness appeared in plants from Site 5 followed by Site 6 (Table 2). For diversity (described by *H'*), the highest value was obtained in plants from site 5 too and the lowest value appeared in plants from site 1. Although the Pearson correlation test showed no correlation, the metal accumulated in site 5 (Mg, Fe, Al and Zn) and P6 (Mn, Cu and Cd) seems to result in higher bacterial density (Table 1), richness and diversity (Table 2) in cultivable endophytic bacterial community isolates from *T. stans* steam. In addition, using β -Libshuff analysis with the Bonferroni correction as significance criterion, it was observed that the endophytic bacterial communities of *T. stans* present in site 1 and 2 are similar ($p = 1.00$), but are significantly different from sites 5 and 6 ($p < 0.0001$), and Site 5 is different from site 6 (Figure 2A and B); site 5 and 6 present higher metal concentrations, where site 5 present lower pH and higher Al content while Site 6 present toxic metals: Cu and Cd. Although the bacterial community isolated from plants growth in sites 1 and 2

exhibit greater similarity, these points differ significantly, each being composed of a low specific community overlapping (Figure 2A and Figure B -red bars), probably due to the low concentration of metal.

Screening of *Rhizobiales* strains for induction of tomato seed germination

From the total of 47 tested isolates, we were able to re-isolate from tomato only 29 isolates classified as *Methylobacterium* and *Rhizobium*, which were used to proceed the studies. To select *Rhizobiales* strains that directly promote plant growth, one *Rhizobium* sp. and two *Methylobacterium* spp. isolates were screened for direct plant growth promotion (PGP) abilities. These *Rhizobiales* were inoculated in seeds of *Cherry* and *Santa Cruz Kada Gigante* tomato, resulted in a germination rate that ranged from 90 to 98% and from 30 to 84%, respectively. However, as the germination rate was high for seeds of tomato *Cherry*, the effect of *Methylobacterium* spp. inoculation was not observed (Figure 3). In tomato *Santa Cruz Kada Gigante*, four isolates (A40, A59, B74 and B76) significantly reduced the germination rate, while six isolates (A21, A76, B40, B61, C2 and LGM86) increased the germination rate (Figure 3).

Rhizobiales- tomato plant greenhouse assay

Based on results of seed germination, strains A76, B61 and C2 were selected for evaluation of effect on tomato *Santa Cruz Kada Gigante* growth (germination rate, number of leaves, shoot and root length) in greenhouse experiments.

In the tomato *Santa Cruz Kada Gigante* plants inoculated with strain C2, the germination rate increased statistically ($p < 0.05$) up to 13.64% compared to control. The strains A76 and B61 did not exert any significant difference in the evaluated conditions. For plant growth promotion, the highest values were obtained with the inoculation of C2 strain, which promoted a significant ($p < 0.05$) increasing in shoot (17.3%) and root (17.8%) length (Figure 4).

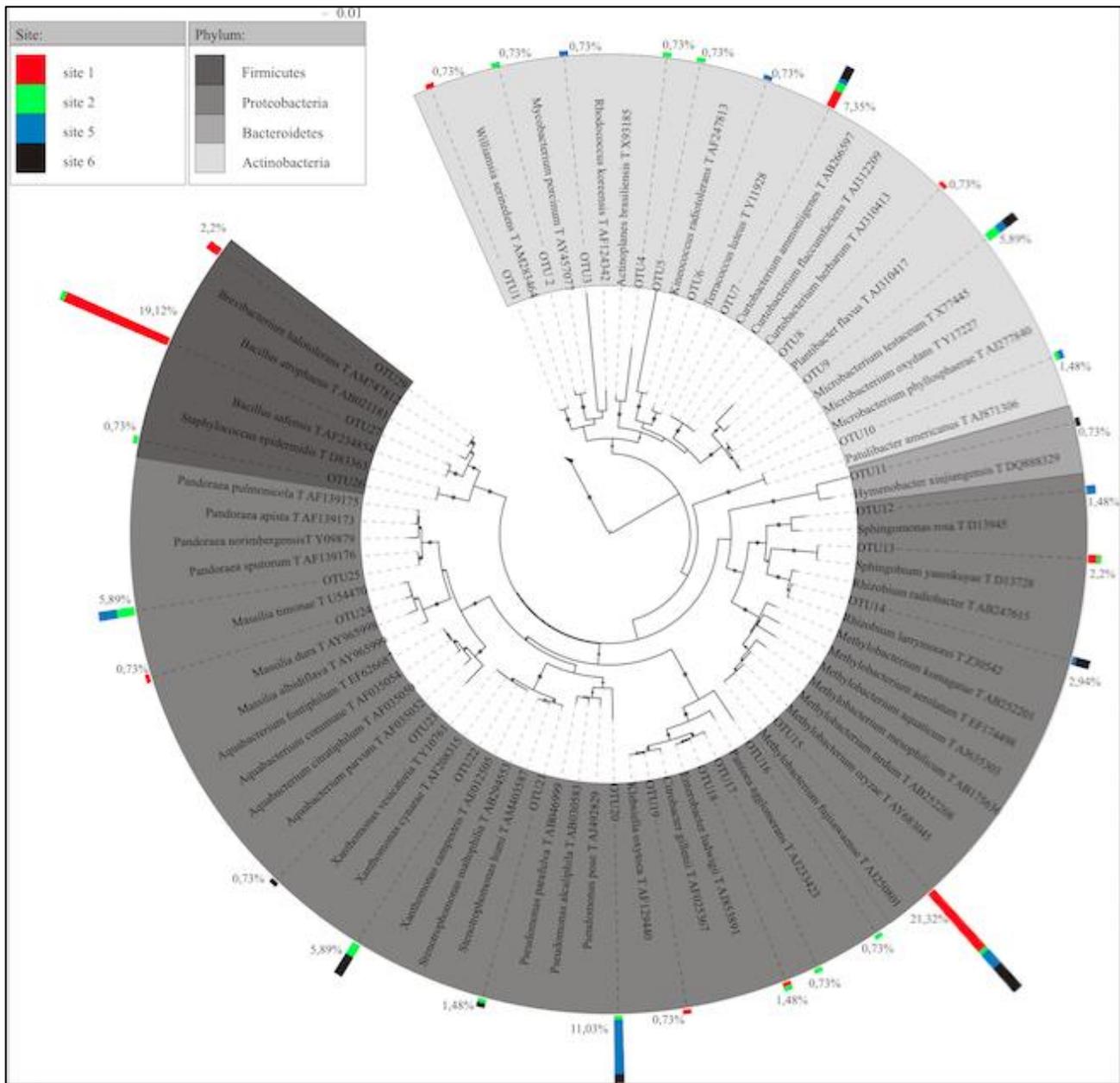


Figure 1. Phylogenetic tree of endophytic bacteria found in *T. stans*. Built by Neighbor-Joining method, Jukes-Cantor model. With Bootstrap of 1000. Colored bars indicate the isolation place and its height is proportional to the OTU frequency.

In contrast, a negative effect on root length was observed in plants inoculated with strain A76. The effect of bacteria inoculation on number of leaves was also evaluated and ranged from 8 to 12 leaves. However, no bacteria showed a statistical difference when compared with the control plants (Figure 4).

Considering that, the isolated C2 significantly increased the germination rate and shoot and root length after seed inoculation for tomato *Santa Cruz Kada Gigante*, this isolate was also inoculated in seeds of Tomato *Cherry*.

Although an increase in germination rate was not observed in tomato seedling *Cherry* resulting from inoculated seeds, the shoot (13.57%) root (21.91%) length and leaves number (8%) increased statistically ($p < 0.05$) compared with the control seedlings.

Phosphate solubilization and nitrogen fix assays in plant growth *Rhizobiales* strains

In order to understand the mechanisms involved in

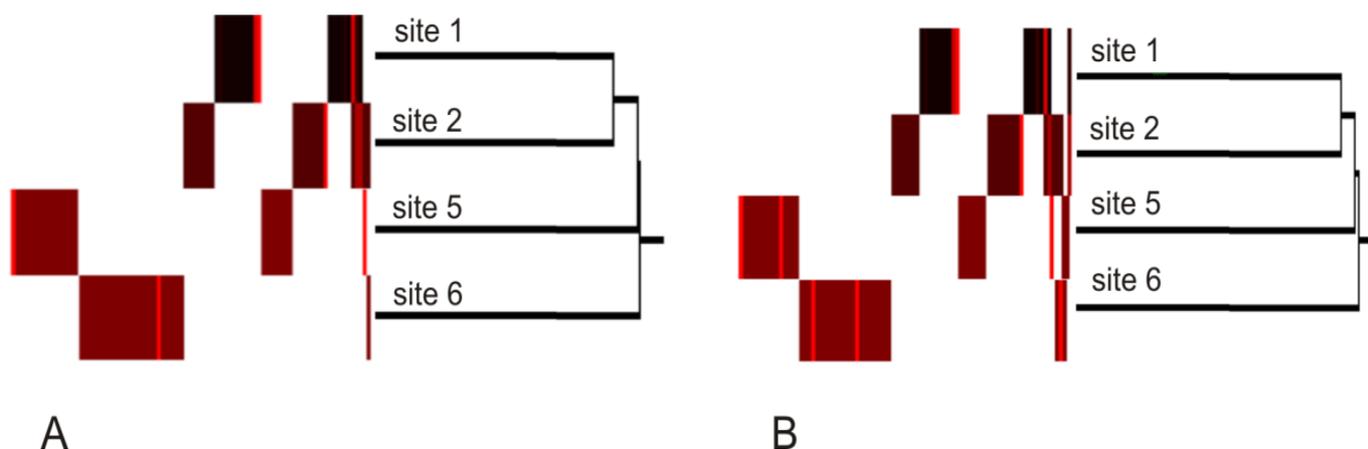


Figure 2. Heatmap and Cluster based on Jaccard index built from sequences of isolate from contaminated places. (A) Sequences at 97% similarity level (species); (B) Sequences at 95% similarity level (genus). The red color indicates the highest frequency of OTUs and black indicates the lowest.

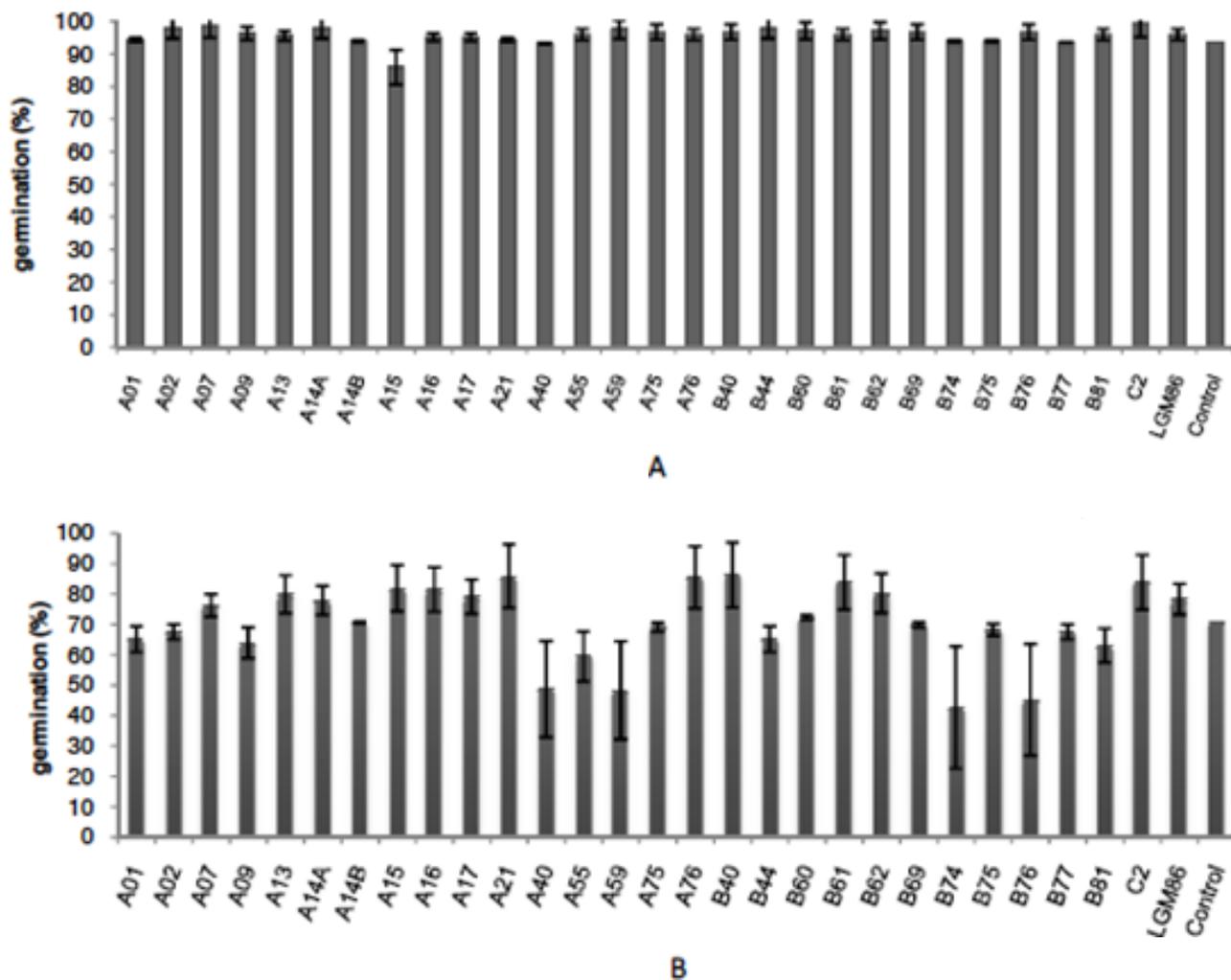


Figure 3. Influence of endophyte Rhizobiales inoculation in tomato germination. (A) Cherry; (B) Santa Cruz Kada Gigante.

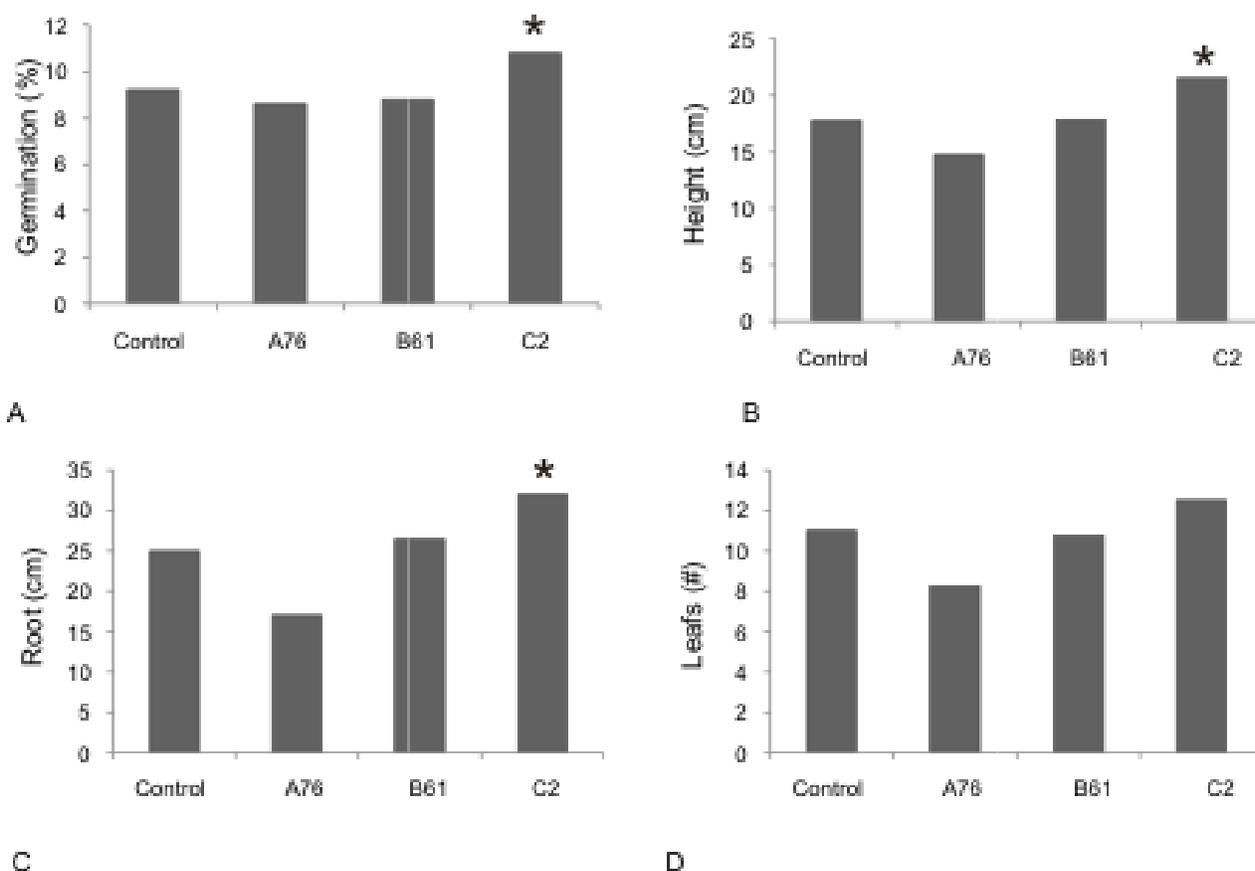


Figure 4. Response of tomato plant *Santa Cruz Kada Gigante* in greenhouse with *Methylobacterium* sp. (strains A76, B61) and *Rhizobium* sp. (C2) inoculation. (A) Germination; (B) Shoot height; (C) Root length and (D) number of leaves (*statistically different at $p < 0.05$).

bacteria plant interaction, the *Methylobacterium* sp. strains A76, B61 and C2 were screened for direct PGP traits. The N_2 -fixing ability of endophytic strains was screened by growth on N_2 -free media. The strains C2 and B61 showed the capacity to grow in nitrogen-free conditions, while strains A76 and B61 formed a halo around the colonies on medium containing inorganic phosphate and were considered positive for phosphate solubilizing. In the present conditions, only the strain B61 was able to fix N_2 and solubilize inorganic phosphate.

DISCUSSION

Abiotic stress can select the adapted microbiome and consequently decrease bacteria diversity, indicating negative effects of the environment on the host plant and in endophytic bacterial community (Hardoim et al., 2015; Truyens et al., 2016). Previous reports show that the diversity and richness of endophytic community in *Arabidopsis thaliana* seeds decreased in plants exposed

to cadmium (Truyens et al., 2016). In the present study, low metals levels did not affect the density of endophytic bacteria within the *T. stans* stems grown in the contaminated soil.

The analysis also did not demonstrate variation in bacterial density in endophytic *T. stans* isolates, suggesting that the plant must adjust the bacterial density inside, independent of the effects of soil conditions. Plant parts in direct contact with the ground (roots and rhizosphere) suffer more influence because of the higher concentration of metals from soil. Moreover, endophytes were reported to tolerate higher heavy metal levels than rhizosphere bacteria (Idris et al., 2004).

In addition, using J-Libshuff analysis with the Bonferroni correction as significance criterion, it was observed that the endophytic bacterial communities of *T. stans* present in site 1 and 2 are similar ($p = 1.00$), but are significantly different from sites 5 and 6 ($p < 0.0001$). These differences in bacterial community may be associated with soil variations and non-environmental study of the area, which are located in the same park, with similar

humidity, temperature and rainfall. Sites 1 and 2 also exhibit similar levels of metals (both presented low metal concentration), although it can be suggested that there is correlation between the levels of individual metals and bacterial densities, as well as richness.

Furthermore, previous studies show that after heavy metals application, the microbial community decreases, but after a period of adaptation, it can be restored to previous levels (Rajapaksha et al., 2004) due to the selection of metal tolerant species (Baath, 1989), and also due the resilience of this microbial community under transitory impact. Endophytic bacteria present a key role on phytoremediation since it is able to enhance heavy metal phytoextraction (Rajkumar et al., 2009; Muehe et al., 2015). In this context, a higher density of endophytic bacteria of *Methylobacterium* genera can be explained by its heavy metal tolerance. Dourado et al. (2012) observed that *Methylobacterium* isolated from mangrove with oil contamination and high levels of heavy metal, showed high tolerance to cadmium, arsenic and lead. This high tolerance can reduce metal toxicity and promote tomato plant growth (Madhaiyan et al., 2007).

Methylobacterium and *Rhizobium* strains promoted tomato plants (Cherry and Santa Cruz Kada Gigante) growth and germination. Which was previously reported by other authors (Sy et al., 2001; Madhaiyan et al., 2006, 2007; Bogas et al., 2016). The mechanisms involved are: (1) phytohormone production: Mainly auxin and cytokinines (Dourado et al., 2015; Kwak et al. 2014); (2) Stress decrease: Heavy metal tolerance (Dourado et al., 2015) and ACC deaminase production (Bogas et al., 2016). Bacterial ACC deaminase uses the ethylene precursor: ACC (Aminocyclopropane-1-carboxylic acid) as source of nitrogen, decreasing the production of ethylene, increasing plant growth (Hardoim et al., 2008, 2015); (3) Nutrient uptake: Phosphate solubilization (Glick, 1995), nitrogen fixation and plant nodulation (Sy et al., 2001).

Rhizobium sp. strain C2 was able to fix N₂ and promote tomato seedlings Santa Cruz Kada Gigante and Cherry growth, indicating that it can be used to increase tomato production. Nitrogen fixer can help plant and mycorrhiza to improve vegetation of heavy metal-rich industrial sites (Ogar et al., 2015).

Finally, the present study shows that the presence of low metal concentration does not influence in endophyte cultivable community due to the niche of endophyte and well as its great plasticity. Furthermore, we were able to select plant growth promoter bacteria that can be used on tomato seedlings production and on future phytoremediation studies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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