Scientia Horticulturae 191 (2015) 113–120

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Colonization and plant growth-promotion of tomato by *Burkholderia tropica*

Pamela R. Bernabeu[a], Mariano Pistorio[b], Gonzalo Torres-Tejerizo[b], Paulina Estrada-De los Santos[c], María L. Galar[a], José L. Boiardi[a], María F. Luna[a,d,*]

[a] Centro de Investigación y Desarrollo en Fermentaciones Industriales (CINDEFI), UNLP, CCT-La Plata-CONICET, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calles 50 y 115 (1900), La Plata, Argentina
[b] Instituto de Biotecnología y Biología Molecular, UNLP, CCT-La Plata-CONICET. Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina
[c] Instituto Politecnico Nacional, Escuela Nacional de Ciencias Biologicas. Prol. Carpio y Plan de Ayala, Col. Santo Tomas, D.F. CP 11340, Mexico
[d] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-PBA), Argentina

ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 30 April 2015
Accepted 5 May 2015
Available online 23 May 2015

Keywords:
Burkholderia
Inoculation
Tomato
PGPB
Colonization

ABSTRACT

Several diazotrophic *Burkholderia* species have been described to exhibit some activities involved in plant growth promotion and biological control. In this work seedlings of tomato plants were inoculated with this bacterium in order to study colonization of different vegetal tissues and plant growth promoting ability under greenhouse conditions. Tomato seedlings inoculated with *Burkholderia tropica* strain MTo-293 and two derivative strains containing the marker genes *gus*A and *gfp*, respectively (constructions described in this work), were grown under gnotobiotic conditions. Colonization was monitored both by colony counting of bacterial suspensions from homogenized tissues with or without previous surface disinfection and by microscopic observation of entire plant tissues. In another set of experiments tomato seedlings were inoculated with *B. tropica* MTo-293 for evaluation of tomato production under greenhouse conditions. Tomato yields were determined by quantifying total tomato production throughout the crop in two different seasons. *B. tropica* could be isolated from root surfaces (>7.0 log CFU g^{-1} fresh weight) and from surface-disinfected and disrupted roots (>5.0 log CFU g^{-1} fresh weight) and stems (>4.0 log CFU g^{-1} fresh weight) of inoculated plants. Microscopic studies showed colonizing bacteria on root hairs, root tips, lateral root emergence sites, and stomata. In greenhouse experiments inoculated plants showed a consistent increase of both number and weight of fruits as compared to uninoculated controls. Although this enhancement in fruit production was only statistically significant for fruit weight in the first crop season, our results show a consistent tendency to a higher yield (5–15%) for the inoculated treatments also in the second year. These results show that seedling inoculation with *B. tropica* led to effective root colonization of tomato plants followed by bacterial spreading to aerial tissues. This significant colonization was accompanied by an enhancement of tomato production in two different crop seasons.

1. Introduction

Green biotechnology is a branch of biotechnology applied to agricultural processes to provide environmentally friendly solutions as an alternative to traditional agriculture, mainly directed to reduce dependence on fertilizers, pesticides and other agrochemical products (Sanghi and Singh, 2012). In this way, the use of plant growth promoting bacteria (PGPB) has gained an important place as it leads to a sustainable agriculture (Glick, 2012). PGPB is a group of microorganisms able to confer beneficial effects on plant growth and development, without causing damage either to the host or the environment. These microorganisms stimulate plant growth as a consequence of different mechanisms, such as atmospheric nitrogen fixation, production of phytohormones, enhancement of mineral availability and biocontrol of phytopathogens, among others. PGPB have to colonize and grow on or around the roots for the establishment of an

* Corresponding author at: Centro de Investigación y Desarrollo en Fermentaciones Industriales (CINDEFI), UNLP, CCT-La Plata-CONICET, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calles 50 y 115 (1900), La Plata, Argentina. Tel.: +54 221 4833794; fax: : +54 221 4833794.
E-mail address: mafla@quimica.unlp.edu.ar (M.F. Luna).

http://dx.doi.org/10.1016/j.scienta.2015.05.014
0304-4238/

effective plant–microbe interaction (Bloemberg and Lugtenberg, 2001). After this necessary step some of them are able to enter roots and establish endophytic populations (Compant et al., 2005a). PGPB include a variety of bacterial genera such as *Azoarcus*, *Azospirillum*, *Burkholderia*, *Erwinia*, *Bacillus*, among others (Bashan and de Bashan, 2005). Over the last years an increasing interest in the genus *Burkholderia* has been shown. This bacterium has been described as a genus rich in plant-associated nitrogen fixers with a wide environmental and geographic distribution (Estrada-De los Santos et al., 2001) and, since its description in 1992, more than 70 different species have been recognized as members of this bacterial genus (www.bacterio.cict.fr/). The first *Burkholderia* species described with nitrogen-fixing ability was *B. vietnamiensis* isolated from the rhizosphere of rice plants (Gillis et al., 1995). *B. vietnamiensis* belongs to the *Burkholderia cepacia* cluster and harbors opportunistic pathogenic strains. Since then, some environmental saprophytic species belonging to this genus have been identified as rhizospheric and endophytic diazotrophic bacteria such as *Burkholderia xenovorans* (Estrada-De los Santos et al., 2001), *Burkholderia unamae* (Caballero-Mellado et al., 2004), *Burkholderia tropica* (Reis et al., 2004), *Burkholderia silvatlantica* (Perin et al., 2006a,b) and *B. australis* (Paungfoo-Lonhienne et al., 2014). Additionally, nitrogen-fixing nodulating species have been described, being *Burkholderia phymatum* and *Burkholderia tuberum* the first ones reported (Moulin et al., 2001; Vandamme et al., 2002), to which 10 more have been added nowadays. Caballero-Mellado et al. (2007) revealed the occurrence of nitrogen-fixing *Burkholderia* species associated with tomato plants cultivated in different locations in Mexico. They found that the rhizosphere of tomato is a reservoir of different diazotrophic *Burkholderia* species including *B. unamae*, *B. xenovorans* and *B. tropica,* and more recently *Burkholderia caballeronis*, which interestingly is also able to nodulate *Phaseolus vulgaris* (Martínez-Aguilar et al., 2013). These species are able to exhibit some in vitro activities involved in bioremediation, plant growth promotion and biological control (Caballero-Mellado et al., 2007; Tenorio-Salgado et al., 2013). Onofre-Lemus et al. (2009) observed that *B. unamae* promotes tomato plant growth through 1-amino-cyclopropane-1-carboxylate (ACC) deaminase activity. In addition, some *B. tropica* and *B. unamae* strains tested were beneficial to maize plant growth, increasing up to 30% the dry weight (Castro-Gonzalez et al., 2011). There are few studies on plant colonization by *B. tropica*, and these refer to the association of *B. tropica* with sugar cane (Govindarajan et al., 2006; Perin et al., 2006a,b; Oliveira et al., 2009). Oliveira et al. (2009) suggest that *B. tropica* shows a higher competitiveness and colonization efficiency compared with other PGPB.

On the other hand, Castro-Gonzalez et al. (2011) observed that genes related to transmissibility of bacterial pathogenicity mainly found in clinical isolates of *B. cepacia* complex could not be detected in any of the analyzed plant-associated nitrogen-fixing *Burkholderia* isolates. Recent analyses have shown the existence of two distinct groups of *Burkholderia* species (Estrada-De los Santos et al., 2013). The first one (A group) includes many nonpathogenic environmental and plant-associated species, and the other one (B group) diverse human, plant and animal pathogenic species, as well as, opportunistic pathogens and some environmental species. Based on their findings, Angus et al. (2014) give support to the separation of the group A and B *Burkholderia* species into two distinct genera which open the potential for a safe application of the plant-associated group A *Burkholderia* species in an agricultural context.

Despite the attention given to these diazotrophic species in the last years, plant colonization and the possible beneficial role of these *Burkholderia* species on plant growth are still little known. In order to find PGPB for horticultural species, the aim of the present work is to characterize the colonization pattern of *B. tropica* MTo-293 (Reis et al., 2004) when tomato seedlings are inoculated under gnotobiotic conditions and to determine whether this colonization leads to an improvement of fruit yield under greenhouse conditions.

2. Materials and methods

2.1. Organisms and maintenance

Bacterial strains and plasmids used in this study are listed in Table 1. *B. tropica* MTo-293 (ATCCBAA 569) (Reis et al., 2004), isolated from surface-sterilized maize stems, was kindly provided by Dr. Jesús Caballero-Mellado (Centro de Ciencias Genómicas, Cuernavaca, Morelos, México). This organism and their derivatives strains were maintained at 4 °C in LB medium (Sambrook et al., 1989) for monthly subcultures and in LGI medium (Stephan et al., 1991) with glycerol 20% at −80 °C. Glucuronidase (*gus*) and green fluorescent protein (*gfp*) marked *B. tropica* strains (called hereafter *Burkholderia-gus* and *Burkholderia-gfp*, respectively) were grown on solid medium containing Tetracycline (Tc) (15 μg ml^{-1}), for maintenance of the plasmids.

2.2. DNA manipulation and genetic constructs

Procedures to obtain total DNA, plasmid purification, restriction-enzyme analysis, cloning, and *Escherichia coli* transformation, were performed according to previously established techniques (Sambrook et al., 1989).

2.2.1. Construction of a constitutive gusA-marked Burkholderia

In order to obtain the *Burkholderia-gus* derivative strain, the pFs*7p-gusA* plasmid (pFAJ1700 derivative containing a constitutive promoter fused to the *gusA* gene, Tc^r) (Onofre-Lemus et al., 2009), was introduced by conjugation into *B. tropica* MTo-293. The plasmid stability was tested with stationary-phase cultures of *B. tropica*. These were diluted to obtain an optical density (OD_{600}) of 0.02 in 6 ml of fresh BSE liquid medium (Estrada-De los Santos et al., 2001) without antibiotics and cultivated for 8 h. One hundred microliter aliquots of these cultures were inoculated into fresh BSE liquid medium and incubated for 24 h. This procedure was repeated once, but the culture was incubated for 48 h, and then samples were diluted and plated on BAc agar (Estrada-De los Santos et al., 2001) without antibiotics. Two hundred colonies were picked and transferred to plates with Tc or without the antibiotic. The plasmid stability frequency in *B. tropica* derivatives was based on the total number of recovered colonies on medium without an antibiotic compared to the number of colonies resistant to Tc.

2.2.2. Burkholderia-gfp construction and plasmid stability

In order to obtain the *Burkholderia-gfp* derivative strain, first a stable plasmid bearing *gfp* gene was constructed (pFAJ1708::GFP). For the construction of pFAJ1708::GFP, pFAJ1708 (Dombrecht et al., 2001) was digested with *EcoRI*, and ligated to a 0.77 Kpb fragment containing the GFP gene from pGreenTir (Miller and Lindow, 1997). *B. tropica* MTo-293 electrocompetent cells were prepared by the procedure described by Tung and Chow (1995) for *E. coli*. The electrocompetent cells were transformed with the plasmid pFAJ1708::GFP by electroporation. *B. tropica* MTo-293 carrying pFAJ1708::GFP were selected according to the Tc resistance and the green fluorescent phenotype. The pFAJ1708 was selected because it carries the *par* locus of RK2 giving the plasmid a stable maintenance in bacteria (Dombrecht et al., 2001). The stability of the markers was analyzed after extensive cultivation of strain *Burkholderia-gfp* in LGI medium without any selective pressure (no antibiotics were added). The Tc resistance and the green fluorescence under ultraviolet (UV) illumination were analyzed over the course of 7 serial batch cultures that spanned approximately 9 generations each one.

Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Description	Reference/source
B. tropica MTo-293	Wild type strain	Reis et al. (2004)
E. coli DH5α	*recA*, Δ*lacU169*, F80d*lac* ZDM15	Bethesda Res. Lab.
Burkholderia-gus	MTo-293 carrying pFs*7p-gusA*, *gus* tagged	This study
Burkholderia-gfp	MTo-293 carrying the pFAJ1708::GFP, *gfp* tagged	This study
pFAJ1708	Broad host cloning vector, Tc^r	Dombrecht et al. (2001)
pGreenTir	Ap^r, gfp-P64L/S65Tcassette	Miller and Lindow (1997)
pFAJ1708::GFP	pFAJ1708 derivative containing a constitutive promoter fused to the *gfp* gene, Tc^r	This study
pFs*7p-gusA*	pFAJ1700 derivative containing a constitutive promoter fused to the *gusA* gene, Tc^r	Onofre-Lemus et al. (2009)

Tc^r: Tc resistant.

The pFAJ1708::GFP stability frequency in *B. tropica* derivatives was assessed by plating appropriate dilutions of the cultures in LGI agar plates with and without Tc and comparing the total number of recovered colonies on medium without an antibiotic compared to the number of colonies resistant to Tc (Pistorio et al., 2002).

2.3. Growth kinetic of strains

Growth patterns of wild type and derivative strains of *B. tropica* were compared in LGI liquid medium. Culture media were inoculated with an appropriate volume of centrifuged, washed and resuspended inoculum to an initial OD_{600} of 0.05. Cultures were incubated at 28 °C with reciprocal shaking (250 rpm) and samples were taken every two hours for 48 h. Growth parameters such as specific growth rate values (μ) were determined by the slope of bacterial counting (CFU ml^{-1}) in solid LGI medium versus time. Growth kinetic parameters were determined at least three times.

2.4. Plant experiments

Two types of experiments were performed inoculating tomato plants (*Lycopersicum esculentum* cv. "superman", Seminis): one to evaluate the colonization pattern of *B. tropica* under gnotobiotic conditions using *gusA* or *gfp*-labeled srains; and the other to test fruit yield by plants inoculated with *B. tropica* MTo-293 in greenhouse conditions.

2.4.1. Bacterial culture conditions

Bacterial strains were grown in flasks (1l) containing 250 ml of LGI medium (Stephan et al., 1991) with glycerol (10 g l^{-1}) and yeast extract (1 g l^{-1}) at pH 6.0, on a rotatory shaker at 200 rpm and 30 °C for 48 h.

*Inoculum preparation***.** To study the colonization pattern of the inoculated bacteria, cultures of *B. tropica* were centrifuged and resuspended in sterile pH 6.0 phosphate saline buffer (PBS) at a final concentration of approximately 1.10^9 CFU ml^{-1} to be used as inoculum for all the experiments (Luna et al., 2012). For greenhouse experiments the complete bacterial culture (without centrifugation) was used as inoculum also at a final concentration of approximately 1.10^9 CFU ml^{-1}.

2.4.2. Gnotobiotic experiments

Tomato seeds were surface disinfected with 70% ethanol for 5 min followed by one wash with sterile water and immersion in 2% sodium hypochlorite for 10 min followed by three washes with sterile water. Seeds were germinated for 4 days at 28 °C in the dark between wet filter paper. Seedlings (50 per treatment) were immersed in 10 ml of the bacterial inoculum, volume enough to submerge them fully, with constant shaking for 10 min. Negative controls were immersed in the same volume of sterile pH 6.0 PBS. Inoculated seedlings were placed into flasks (5 per flask) containing 100 ml of semisolid Fähraeus (Fähraeus, 1957) medium (0.5% agar). Plants were grown in a controlled growth chamber under a light/dark cycle of 16 and 8 h, respectively, at 28 °C for 25 days. Three independent experiments with four treatments each (uninoculated control, inoculated with *B. tropica* MTo-293, *Burkholderia-gus* and *Burkholderia-gfp*), and 50 plants/treatment, were performed. The colonization pattern was quantitatively evaluated by *culture dependent techniques* and qualitatively, using *microscopic assays.*

2.4.2.1. Culture dependent techniques. Plants were harvested two days Post Inoculation (PI) (when it was possible to separate roots and stems) and then at 4–5 days intervals. Roots and stems were then separated. For each tissue three samples were combined and three replicates of tissue samples collected (9 plants each time) to determine the average colonization and fresh weight (fw). To determine endophytic population, plant tissues were rinsed with sterile distilled water and disinfected with 2% sodium hypochlorite for 3 min with constant agitation for roots and 2 min for stems. Samples were then washed 4 times with sterilized water and manually crushed using a mortar and pestle. The homogenates were resuspended in 1 ml of PBS and vortexed. This suspension was 10-fold serially diluted and plated on LGI agar plates. Colony forming units were counted after incubation at 28 °C for 2 days. In order to confirm the identity of the inoculated strain the suspensions were grown on the selective medium once every 4–5 days. As previously described by Luna et al. (2012), 3 control procedures were performed to ensure the efficiency of the surface disinfection method: (1) disinfected plant tissue samples taken 96 h PI with *Burkholderia-gus* or *Burkholderia-gfp* were observed by optical microscopy after staining or directly by fluorescence microscopy, respectively, (2) disinfected tissues were placed for 1 min onto plates containing LGI, removed and plates were incubated at 28 °C, (3) the wash solution from the last rinse was cultured on LGI plates.

To determine the extent of total colonization (rhizoplane and endophytic population) of inoculated seedlings, another set of plantlets were removed from the agar. Roots were rinsed with sterile distilled water and processed as above, without surface disinfection. Rhizoplane population was determined by subtracting the inside population from the total bacterial counts determined without surface disinfection (Gyaneshwar et al., 2001).

2.4.2.2. Microscopic assays. Plants inoculated with *gusA* or *gfp*-labeled strains were carefully removed from the growth medium and roots were gently washed with sterile water in order to wash the remaining agar away and were harvested and separated into roots and stems.

Bacterial *gus* and *gfp* activities were tested daily during the first week after inoculation and at three-day intervals thereafter. Bacterial *gus* activity was also tested on samples of the greenhouse experiment 20 days PI (see below). The expression of the *gusA* reporter gene was monitored in plants inoculated with the *gusA*-marked strain. The staining procedure to determine GUS activity was carried out as described by Jefferson et al. (1987) using X-Gluc (5-bromo-4-chloro-3-indolyl-D-glucuronic acid) as substrate.

Samples were observed after staining and photographed using a Carl Zeiss Photomicroscope. Bacterial *gfp* qualitative detection was made using an epifluorescent microscopic Leica DM 2500 with Hg lamp. Uninoculated plants were analyzed at the same time intervals. Multiple samples of plant tissues were examined directly, with or without superficial disinfection.

2.4.2.3. Co-inoculation experiments. Co-inoculation experiments were carried out to determine whether derivative strains behaved as the wild-type strain when these were inoculated on tomato seedlings under gnotobiotic condition (see above). Equal parts of each strain at the same final concentration, were used as inoculum as follows: (1) *B. tropica* MTo-293 and *Burkholderia-gus*; (2) *B. tropica* MTo-293 and *Burkholderia-gfp*; and the controls for each strain: (3) *B. tropica* MTo-293; (4) *Burkholderia-gfp* and (5) *Burkholderia-gus*. Roots samples were analyzed 7 days PI by quantitative assay (Culture dependent techniques) as described below. Bacterial counting (CFU ml^{-1}) was determined in solid LGI medium with and without the appropriate antibiotic.

2.4.3. Greenhouse experiments

Non-disinfected tomato seeds were germinated as indicated for gnotobiotics experiments. Seedlings without visible contamination were transferred to speedling trays (100 cells per tray of 25 ml each) filled with sterile vermiculite (previously washed with sterile phosphoric acid (10% w/v) to obtain a final pH 6.0) and placed in a controlled growth chamber under the same conditions indicated above. Sterile modified Hoagland's solution with KNO_3 as the nitrogen source (Hoagland and Arnon, 1950) was weekly added to each cell throughout the experiment period in the growth chamber, as described previously by Luna et al. (2012). Four week-old seedlings were inoculated with 5 ml of a *B. tropica* MTo-293 suspension (approximately 1.10^9 CFU ml^{-1}) directly in each cell and incubated overnight. Control plants received the same volume of sterile water. Plants were then transplanted to the soil and put into the greenhouse. Two independent experiments, from December to April (4 months) in two consecutive seasons (2008–2009 and 2009–2010), were carried out in the greenhouse with natural daylight. Experiments were comprised of two treatments: uninoculated control and inoculated with *B. tropica* MTo-293. The experimental design was a randomized complete block design with four replicates per treatment and two plots per block with 20 plants each. The plots were divided in 2 rows separated by a 1 m wide gap and plants were placed at every 50 cm (5 m^2 per plot). Plants were not fertilized. All treatments received water daily by overhead irrigation.

The nutrient composition of soil was: organic matter 3.45%; organic carbon (Walkley and Black, 1974) 2.10%; total nitrogen (Bremner, 1960) 0.24%; phosphorous (Bray and Kurtz, 1945) 40 ppm; nitrates 130 ppm; potassium 124 ppm; sodium 190 ppm; pH 6.2; conductivity 2.78 mho cm^{-1}.

Crop yield was evaluated by measuring fruit number and weight of mature tomatoes throughout the season. Fruit number and weight were determined once a week since two months after inoculation and during the following two months of the experiments. Tomato yield was calculated by adding the number of fruit or weight per week per plot ($n = 20$) during two months of evaluation and results expressed per m^2. It was expressed as the average of the 4 independents replicates of total production for each treatment.

A set of seedlings, of the same plants used for the greenhouse experiment, were inoculated with 5 ml of a *Burkholderia-gus* suspension instead of the wild type strain and grown in vermiculite containing pots to be analyzed only by microscopy. These plants were grown for 20 days in a controlled growth chamber as described above.

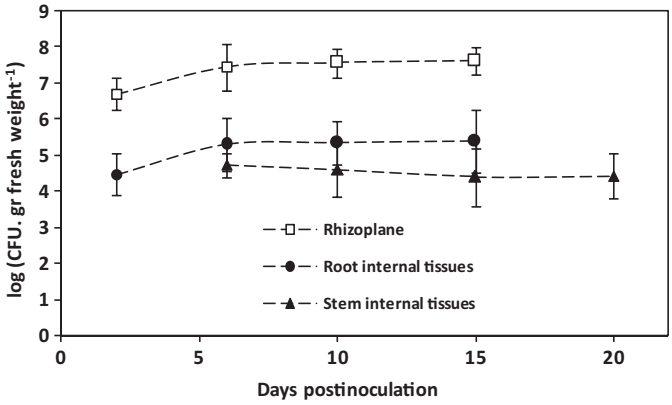

Fig. 1. Root and stem populations of tomato plants after seedling inoculation with *Burkholderia-gfp* derivative strain. Data are the means of three independent experiments and bars represent the standard errors of the means.

2.4.4. Statistical data analysis

Fruit number and weight data were statistically analyzed using Studentís *t* test. *P*-values ≤ 0.05 were considered significant.

3. Results

3.1. Vector stability of Burkholderia-gus

Plasmid analysis showed that there was 100% stability of the plasmid in *B. tropica* without selective pressure; the results were identical for all of the cultures analyzed.

3.2. Vector stability of Burkholderia-gfp

The Tc^r phenotype was maintained during the whole period of analysis showing a high stability of the marker. All colonies were also fluorescent under UV light either in the presence or absence of the antibiotic selection.

3.3. Growth kinetic

Growth patterns for wild type strain MTo-293 and their derivative strains (*gfp* and *gus*) in LGI liquid medium were very similar, reaching concentrations at about 2.10^9 CFU ml^{-1} after 30 h. The specific growth rate values (μ) were not significantly different, reaching values of 0.293 ± 0.04, 0.288 ± 0.05 and 0.279 ± 0.07 h^{-1}, for *B. tropica* MTo-293, *Burkholderia-gus* and *Burkholderia-gfp* derivative strains, respectively.

3.4. Co-inoculation experiments

Epiphytic and endophytic populations of tomato plants inoculated with any of the *B. tropica* strains reached similar values 10 days PI (averaging for each strain 7.0–7.5 and 5.0–5.5 log CFU g^{-1} (fw), respectively) showing that wild type and derivative strains expressed very similar behavior on tomato root colonization.

From the above described results we have assumed that wild type and derivative strains used in this work can be considered equivalent.

3.5. Gnotobiotic experiments

The colonization of tomato plants by *B. tropica* strain MTo-293 was not accompanied by inhibition of plant growth or other macroscopically visible disease symptom.

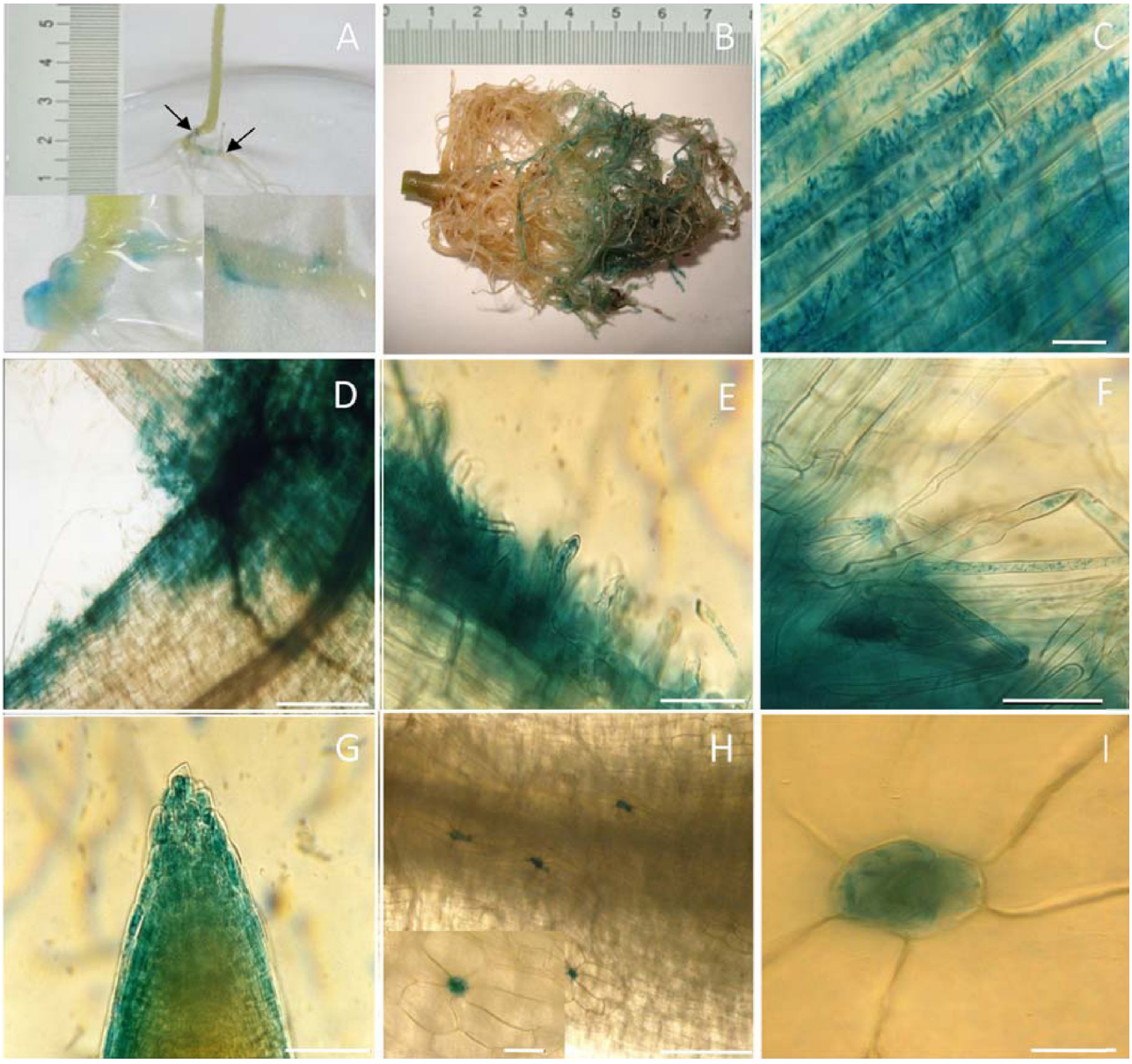

Fig. 2. Localization of *gus*A gene expression in tomato roots after seedling inoculation with *Burkholderia-gus* derivative strain.
Photographs of tomato roots (**A**–**B**) showing blue zones due to colonization of *Burkholderia-gus* on the root–stem junction and at the site of emergence of lateral roots (**A**, arrows) in plants growing under gnotobiotic conditions 5 days PI and on the rhizoplane (**B**) in plants growing in greenhouse under non sterile conditions 20 days PI. Light microscope images of tomato roots (**C**–**G**) growing under gnotobiotic conditions harvested at different days PI showing blue staining on epidermal tissues (24 h PI) (**C**), at the site of emergence of lateral roots (2 days PI) (**D**), on root young hairs near the tip and on mature hairs (2 days PI) (**E** and **F**), on cells of root cap (4 days PI) (**G**), and on stomatal cavities of root–stem junctions (7 days) (**H** and **I**). Scale bars: (**C**) 20 μm, (**D**) 200 μm, (**E**) 50 μm, (**F**) 50 μm, (**G**) 50 μm, (**H**) 50 μm and 200 μm, (**I**) 20 μm.

3.5.1. Effectiveness of disinfection treatment of plant tissue surfaces

(1) No zones of fluorescence were observed in disinfected samples from plants previously inoculated with *Burkholderia-gfp*, (2) no zones of blue staining were observed in disinfected samples from plants previously inoculated with *Burkholderia-gus*, (3) no bacterial growth was observed in LGI plates mounted with surface-disinfected roots, (4) bacterial growth was rarely observed when the wash solution from the last tissue rinse was placed in LGI. Similar to the root tests, no bacterial colonies were found on aerial parts either in the post disinfection wash solution or after incubations of whole aerial tissues. Based on these results, we can assume that the disinfection procedure employed in this work was sufficient for the elimination of the surface-associated bacteria, in agreement with that described by Dong et al. (2003).

3.5.2. Quantitative assays of root colonization by culture dependent techniques

Colony counting performed on non disinfected roots derived from seedlings inoculated with the *gfp*-marked strain revealed the presence of *Burkholderia-gfp* as the only colonizing microorganism, checked by growth with and without Tc in the culture medium (data not shown). Epiphytic population densities of around 7.5 log CFU g^{-1} (fw) at 7 days PI were detected, indicating adequate seed disinfection and significative rhizoplane colonization. This population remained stable during the 15 day period of observation (Fig. 1).

Burkholderia-gfp colonies were isolated from homogenates of surface disinfected root samples, indicating its endophytic localization. Endophytic population showed densities of around 5.0 log CFU g^{-1}(fw) as found for other endophytic bacteria (Compant

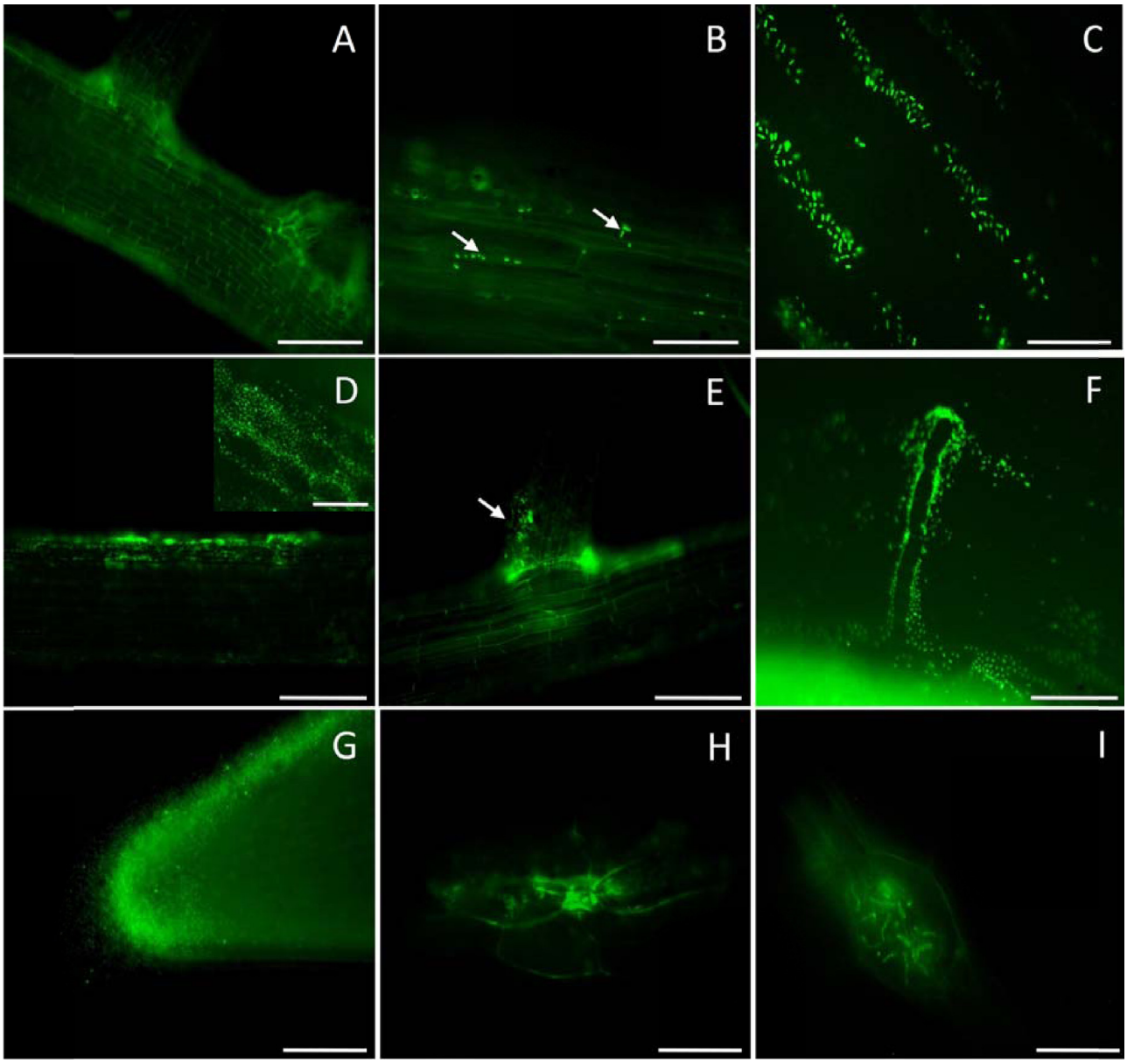

Fig. 3. Localization of *gfp* gene expression in tomato roots after seedling inoculation with *Burkholderia-gfp* derivative strain.
Fluorescence microscope images of tomato roots of plants growing under gnotobiotic conditions showing green fluorescence due to colonization of *Burkholderia-gfp*. The photograph (**A**) shows the auto fluorescence in tomato roots of uninoculated plants used as negative control. The following photographs show single fluorescent cells (**B**, arrows) on the surface of tomato roots (24 h PI), fluorescent bacteria along the junction (3 days PI) (**C**) and on the whole outline of epidermal cells (5 days PI) (**D**), at the site of emergence of lateral roots (3 days PI) (**E**, arrow), on root young hairs near the tip (24 h PI) (F), on cells of root cap (24 h PI) (**G**) and on stomatal cavities of root–stem junctions (3 days PI) (**H** and **I**). Scale bars: (**A**) 200 μm, (**B**) 50 μm, (**C**) 20 μm, (**D**) 200 μm and 50 μm, (**E**) 200 μm, (**F**) 50 μm, (**G**) 50 μm, (**H**) 50 , (**I**) 20 μm.

et al., 2010) that remained stable up to the end of the experiment. No bacteria could be isolated from the controls (uninoculated plants) both from disinfected and non-disinfected homogenate roots indicating the absence of endophytic native bacteria, at least under the conditions employed in this study.

3.5.3. Quantitative assays of stem colonization by culture dependent techniques

Homogenates from surface disinfected stems obtained from inoculated tomato seedlings showed bacterial populations of approximately 4.5 log CFU g^{-1} (fw) at the beginning of the experiment (6 days PI) and remained at 4.0 and 4.5 log CFU g^{-1} (fw) until the end of the experiment (Fig. 1). Although significant, the degree of stem endophytic population was lower than the one found in roots, as normally occurred with other endophytic bacteria (Compant et al., 2010; Luna et al., 2010, 2012).

3.5.4. Root colonization-microscopic assays

Burkholderia-gus and *Burkholderia-gfp* could be tested for *in situ* microscopic localization, specifically for surface colonization and entry sites. Direct observation of whole roots of tomato plants inoculated with *Burkholderia-gus* showed blue GUS staining in the root-stem junctions and in sites of emergence of lateral roots since 2 days PI and during the rest of the experiment after staining by GUS technique (Fig. 2**A**). Rhizoplane colonization of *B. tropica* was also detected in tomato plants inoculated with *Burkholderia-gus* under non sterile conditions 20 days PI (Fig. 2**B**). Microscopic observations of tomato roots recovered at different stages of plant growth revealed the presence of *Burkholderia-gus* strain on

Table 2
Tomato fruit yields of plants inoculated with *B. tropica* Mto-293[a].

		FN/m^2	FW/m^2	% FN	% FW
1st year	Uninoculated	47.05	7.12	5.10	17.00
	B. tropica	49.45	8.33[b]		
2nd year	Uninoculated	56.20	7.40	13.52	6.15
	B. tropica	63.80	7.85		

FN: fruit number.
FW: fruit weight (kg).
[a] Results are expressed as means of 4 independents replicates ($n = 20$) of total production per m^2 for each treatment during the evaluation period.
[b] Indicate values that are significantly different from the uninoculated control at 5% level.

epidermal regions (Fig. 2**C**), at sites of emergence of lateral roots (Fig. 2**D**), on root hairs (Fig. 2**E** and **F**), root tip cells (Fig. 2**G**) and on stomatal cavities of root–stem junctions (Fig. 2**H**). As expected, no blue color was detected after GUS staining on roots of uninoculated plants or plants inoculated with the wild type strain, confirming the lack of endogenous β-glucuronidase activity and providing the specificity of the visualization procedure (data not shown).

Plant roots that were uninoculated or inoculated with *B. tropica* Mto-293 had no fluorescent organisms when they were observed by fluorescence microscopy but presented high auto fluorescence (Fig. 3**A**). Single fluorescent cells could be sparsely observed on the surface of tomato roots at 24 h PI in samples of plants inoculated with *Burkholderia-gfp* (Fig. 3**B**). After that, the root surface was heavy colonized by fluorescent bacteria that formed a linear string along the junction of epidermal cells (Fig. 3**C**). As time progressed, bacteria colonized the whole outline of rhizodermal cells (Fig. 3**D**) and, as observed with the *gus* technique, numerous fluorescent bacteria were seen at sites of emergence of lateral roots (Fig. 2**E**), on root hairs (Fig. 2**F**), root tips (Fig. 2**G**) and stomatal cavities of root-stem junctions (Fig. 3**H** and **I**).

3.6. Greenhouse experiments

Inoculation of tomato plants with *B. tropica* MTo-293 affected crop yields under our experimental conditions. Number and weight of harvested fruits of inoculated plants increased as compared to uninoculated controls (Table 2). Although this enhancement in fruit production was only statistically significant in one case (fruit weight in the first crop season), our results show a consistent tendency to a higher yield (5–15%) for the inoculated treatments also in the second year.

4. Discussion

Effective root colonization by PGPB is considered critical to achieve successful plant–microbe–interaction and promotion of plant growth (Bloemberg and Lugtenberg, 2001). There are some reports in the literature that describe associations between different PGPB and tomato (Caballero-Mellado et al., 2007; Gamalero et al., 2004; Gravel et al., 2007; Luna et al., 2012; Onofre-Lemus et al., 2009; Poonguzhali et al., 2008) but, as far as we know, this is the first report describing tomato colonization by *B. tropica*. Using tagged strains it was possible to determine that *B. tropica* was able to colonize efficiently the rhizoplane of tomato plants not only under gnotobiotic (Figs. 1, 2 **C** and 3 **B**–**D**) but also under greenhouse conditions after seedling inoculation (Fig. 2**B**). *B. tropica* colonized different root sites that had been already reported as common entry points for other endophytic PGPB, such as root hairs and tips, sites of emergence of lateral roots (Compant et al., 2010; Luna et al., 2012; Prieto et al., 2011) and stomata. Stomata are not considered a common entry site for bacteria (Compant et al., 2005b; James et al., 2001, 2002). However, dense stomata colonization has been observed in rice and sugarcane for the endophytes *Herbaspirillum sp*. and *G. diazotrophicus*, respectively (James et al., 2001, 2002), in *Vitis* vinifera by *Burkholderia phytofirmans* (Compant et al., 2005b) and in tomato plants by *Gluconacetobacter diazotrophicus* (Luna et al., 2012). Further studies are required to determine whether stomata colonization could be an additional site of plant colonization for endophytic bacteria. We have observed a high root population, both epi- and endophytic, of *B. tropica* MTo-293 (Fig. 1) similar to those reported for *Azoarcus sp*. in association with gramineous plants (Hurek et al., 1994), *B. phytophirmans* in *Vitis vinifera* (Compant et al., 2005b), *B. unamae* in tomato plants (Onofre-Lemus et al., 2009), *B. tropica* and *B. unamae* in sorghum (Wong-Villarreal et al., 2012) and others (Compant et al., 2010). Finally, *B. tropica* could also be isolated from stems suggesting that *B. tropica* MTo-293 could spread from the roots to aerial tissues. Other techniques such as confocal laser scanning microscopy and transmission electron microscopy (James et al., 2002) will be necessary to determine the specific colonization sites inside the plants.

Greenhouse experiments showed that, although the increase in tomato yields (number and weight of fruits) was not significantly different for all treatments, especially in the second year, inoculation with *B. tropica* MTo-293 led to consistent increases of fruit production between 5 and 15%. This is a very promising response for their use as a potential PGPB, similar to the responses reported for other PGPB inoculated under similar conditions (Hassen and Labuschagne, 2010; Lee et al., 2008; Luna et al., 2012). This higher production of tomato plants inoculated with *B. tropica* MTo-293 could not be attributed to a decrease in the levels of ethylene by the action of the ACC deaminase since it was previously reported that no ACC deaminase activity could be detected, and the *acdS* gene was absent, in all *B. tropica* examined, including the strain MTo-293 used in this study (Onofre-Lemus et al., 2009). Therefore, another plant growth-promoting mechanism had to be expressed by *B. tropica* MTo-293 to increase tomato production. This beneficial effect could be due to its ability to fix nitrogen, to solubilize insoluble mineral phosphorus, or to phytohormones production already described for *B. tropica* (Caballero-Mellado et al., 2007) or to some other unknown growth promotion mechanism.

5. Conclusions

This work shows that seedling inoculation with *B. tropica* MTo-293 led to effective root colonization of tomato plants followed by bacterial spreading to aerial tissues. This significant colonization was accompanied by a consistent increase of tomato production under greenhouse conditions in two different crop seasons.

References

Angus, A.A., Agapakis, C.M., Fong, S., Yerrapragada, S., Estrada-De los Santos, P., Yang, P., Hirsch, A.M., 2014. Plant-associated symbiotic *Burkholderia* species lack hallmark strategies required in mammalian pathogenesis. PloS One 9, e83779.
Bashan, Y., de Bashan, L.E., 2005. Bacteria/plant growth-promotion. In: Hillel, D. (Ed.), Encyclopedia of Soils in the Environment, 1. Elsevier, Oxford, U.K, pp. 103–115.
Bloemberg, G.V., Lugtenberg, B.J., 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. 4, 343–350.
Bray, R.H., Kurtz, L.T., 1945. Determination of total organic and available forms of phosphate in soils. Soil Sci. 59, 39–45.
Bremner, J.M., 1960. Determination of nitrogen in soil by the Kjeldahl method. J. Agric. Sci. 55, 1–23.
Caballero-Mellado, J., Martínez-Aguilar, L., Paredes-Valdez, G., Estrada-De los Santos, P., 2004. *Burkholderia unamae* sp nov.: an N_2-fixing rhizospheric and endophytic species. Int. J. Syst. Evol. Microbiol. 54, 1165–1172.
Caballero-Mellado, J., Onofre-Lemus, J., Estrada-De los Santos, P., Martínez-Aguilar, L., 2007. The tomato rhizosphere: an environment rich in nitrogen-fixing *Burkholderia* species with capabilities of interest for agriculture and bioremediation. Appl. Environ. Microbiol. 73, 5308–5319.

Castro-Gonzalez, R., Martinez-Aguilar, L., Ramirez-Trujillo, A., Estrada-De los Santos, P., Caballero-Mellado, J., 2011. High diversity of culturable *Burkholderia* species associated with sugarcane. Plant Soil 345, 155–169.

Compant, S., Clément, C., Sessitsch, A., 2010. Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42, 669–678.

Compant, S., Duffy, B., Nowak, J., Clément, C., Ait Barka, E., 2005a. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action and future prospects. Appl. Environ. Microbiol. 71, 4951–4959.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C., Ait Barka, E., 2005b. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Appl. Environ. Microbiol. 71, 1685–1693.

Dombrecht, B., Vanderleyden, J., Michiels, J., 2001. Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in gram-negative bacteria. Mol. Plant-Microbe Interact. 14, 426–430.

Dong, Y., Iniguez, L.A., Ahmer, B.M.M., Triplett, E.W., 2003. Kinetics and strain specificity of rhizosphere and endophytic colonization by enteric bacteria on seedlings of *Medicago sativa* and *Medicago truncatula*. Appl. Environ. Microb. 69, 1783–1790.

Estrada-De los Santos, P., Bustillos-Cristales, R., Caballero-Mellado, J., 2001. *Burkholderia*: a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl. Environ. Microbiol. 67, 2790–2798.

Estrada-De los Santos, P., Vinuesa, P., Martínez-Aguilar, L., Hirsch, A.M., Caballero-Mellado, J., 2013. Phylogenetic analysis of *Burkholderia* species by multilocus sequence analysis. Curr. Microbiol. 67, 51–60.

Fähraeus, G., 1957. The infection of clover root hairs by nodule bacteria studied by simple glass slide technique. J. Gen. Microbiol. 16, 347–381.

Gamalero, E., Trotta, A., Massa, N., Copetta, A., Martinotti, M.G., Berta, G., 2004. Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. Mycorrhiza 14, 185–192.

Gillis, M., Van Van, T., Bardin, R., Goor, M., Hebbar, P., Willems, A., Fernandez, M.P., 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. Int. J. Syst. Bacteriol. 45, 274–289.

Glick, B.R., 2012. Plant growth-promoting bacteria: mechanisms and applications. Scientifica 2012, http://dx.doi.org/10.6064/2012/963401

Gravel, V., Antoun, H., Tweddell, R.J., 2007. Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with Pseudomonas putida or Trichoderma atroviride: possible role of indole acetic acid (IAA). Soil Biol. Biochem. 39, 1968–1977.

Govindarajan, M., Balandreau, J., Muthukumarasamy, R., Revathi, G., Lakshminarasimhan, C., 2006. Improved yield of micropropagated sugarcane following inoculation by endophytic *Burkholderia vietnamiensis*. Plant Soil 280 (1–2), 239–252.

Gyaneshwar, P., James, E.K., Mathan, N., Reddy, P.M., Reinhold-Hurek, B., Ladha, J.K., 2001. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. J. Bacteriol. 183, 2634–2645.

Hassen, A.I., Labuschagne, N., 2010. Root colonization and growth enhancement in wheat and tomato by rhizobacteria isolated from the rhizoplane of grasses. World J. Microbiol. Biotechnol. 26, 1837–1846.

Hoagland, D.R., Arnon, D.I., 1950. The Water-culture Method for Growing Plants without Soil. Circular, 2nd ed. California Agricultural Experiment Station, 347.

Hurek, T., Reinhold-Hurek, B., van Montagu, M., Kellenberger, E., 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. J. Bacteriol. 176, 1913–1923.

James, E.K., Gyaneshwar, P., Manthan, N., Barraquio, W.L., Reddy, P.M., Ianetta, P.P.M., Olivares, F.L., Ladha, J.K., 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Mol. Plant Microb. Interact. 15, 894–906.

James, E.K., Olivares, F.L., Oliviera de, A.L.M., dos Reis, F.B., da Silva, L.G., Reis, V.M., 2001. Further observations on the interaction between sugar cane and *Gluconacetobacter diazotrophicus* under laboratory and greenhouse conditions. J. Exp. Bot. 52, 747–760.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907.

Lee, K.H., Koh, R.H., Song, H.G., 2008. Enhancement of growth and yield of tomato by *Rhodopseudomonas* sp: under greenhouse conditions. J. Microbiol. 46, 641–646.

Luna, M.F., Aprea, J., Crespo, J.M., Boiardi, J.L., 2012. Colonization and yield promotion of tomato by *Gluconacetobacter diazotrophicus*. Appl. Soil Ecol. 61, 225–229.

Luna, M.F., Galar, M.L., Aprea, J., Molinari, M.L., Boiardi, J.L., 2010. Colonization of sorghum and wheat by seed inoculation with *Gluconacetobacter diazotrophicus*. Biotechnol. Lett. 32, 1071–1076.

Martínez-Aguilar, L., Salazar-Salazar, C., Méndez, R.D., Caballero-Mellado, J., Hirsch, A.M., Vásquez-Murrieta, M.S., Estrada-De los Santos, P., 2013. *Burkholderia caballeronis* sp. nov., a nitrogen fixing species isolated from tomato (*Lycopersicon esculentum*) with the ability to effectively nodulate *Phaseolus vulgaris*. A. Van Leeuw. J. Microb. 104, 1063–1071.

Miller, W.G., Lindow, S.E., 1997. An improved GFP cloning cassette designed for prokaryotic transcriptional fusions. Gene 191, 149–153.

Moulin, L., Munive, A., Dreyfus, B., Boivin-Masson, C., 2001. Nodulation of legumes by members of the beta-subclass of *Proteobacteria*. Nature 411, 948–950.

Oliveira, A.L.M., Stoffels, M., Schmid, M., Reis, V.M., Baldani, J.I., Hartmann, A., 2009. Colonization of sugarcane plantlets by mixed inoculations with diazotrophic bacteria. Eur. J. Soil Biol. 45, 106–113.

Onofre-Lemus, J., Hernández-Lucas, I., Girard, L., Caballero-Mellado, J., 2009. ACC (1-aminocyclopropane-1-carboxylate) deaminase activity a widespread trait in *Burkholderia* species, and its growth-promoting effect on tomato plants. Appl. Environ. Microbiol. 75, 6581–6590.

Paungfoo-Lonhienne, C., Lonhienne, T.G., Yeoh, Y.K., Webb, R.I., Lakshmanan, P., Chan, C.X., Hugenholtz, P., 2014. A new species of *Burkholderia* isolated from sugarcane roots promotes plant growth. Microb. Biotechnol. 7, 142–154.

Perin, L., Martinez-Aguillar, L., Castro-Gonzalez, R., Estrada de los Santos, P., Cabellos-Avelar, T., Guedes, H.V., Reis, V.M., Caballero-Mellado, J., 2006a. Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. Appl. Environ. Microbiol. 72, 3103–3110.

Perin, L., Martínez-Aguilar, L., Paredes-Valdez, G., Baldani, J.I., Estrada-De Los Santos, P., Reis, V.M., Caballero-Mellado, J., 2006b. *Burkholderia silvatlantica* sp. nov., a diazotrophic bacterium associated with sugar cane and maize. Int. J. Syst. Evol. Microbiol. 56, 1931–1937.

Pistorio, M., Balagué, L.J., Del Papa, M.F., Pich-Otero, A., Lodeiro, A., Hozbor, D.F., Lagares, A., 2002. Construction of a *Sinorhizobium meliloti* strain carrying a stable and non-transmissible chromosomal single copy of the green fluorescent protein GFP-P64L/S65T. FEMS Microbiol. Lett. 214, 165–170.

Poonguzhali, S., Madhaiyan, M., Yim, W.J., Kim, K.A., Sa, T.M., 2008. Colonization pattern of plant root and leaf surfaces visualized by use of green-fluorescent-marked strain of *Methylobacterium suomiense* and its persistence in rhizosphere. Appl. Microbiol. Biotechnol. 78, 1033–1043.

Prieto, P., Schilirò, E., Maldonado-González, M.M., Valderrama, R., Barroso-Albarracín, J.B., Mercado-Blanco, J., 2011. Root hairs play a key role in the endophytic colonization of olive roots by *Pseudomonas* spp. with biocontrol activity. Microb. Ecol. 62, 435–445.

Reis, V.M., Estrada-De los Santos, P., Tenorio-Salgado, S., Vogel, J., Stoffels, M., Guyon, S., Caballero-Mellado, J., 2004. *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. Int. J. Syst. Evol. Microbiol. 54, 2155–2162.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, 2nd ed. Cold Spring Harbor, New York.

Sanghi, R., Singh, V., 2012. Green Chemistry for Environmental Remediation. John Wiley & Sons, New Jersey.

Stephan, M.P., Oliveira, M., Teixeira, K.R.S., Martinez-Drets, G., Döbereiner, J., 1991. Physiology and dinitrogen fixation *Acetobacter diazotrophicus*. FEMS Microbiol. Lett. 77, 67–72.

Tenorio-Salgado, S., Tinoco, R., Vazquez-Duhalt, R., Caballero-Mellado, J., Perez-Rueda, E., 2013. Identification of volatile compounds produced by the bacterium *Burkholderia tropica* that inhibit the growth of fungal pathogens. Bioengineered 4, 236–243.

Tung, W.L., Chow, K.C., 1995. A modified medium for efficient electrotransformation of *E. coli*. Trends Genet. 1, 128–129.

Vandamme, P., Goris, J., Chen, W.M., De Vos, P., Willems, A., 2002. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. Syst. Appl. Microbiol. 25, 507–512.

Walkley, A., Black, J.A., 1974. A critical examination of rapid method for determining organic carbon in soils. J. Soil Sci. 63, 251–254.

Wong-Villarreal, A., Velazquez-Méndez, A.M., Rosado- Zarrabal, T.L., Vite-Vallejo, O., Caballero-Mellado, J., 2012. Low diversity diazotrophic of culturable *Burkholderia* species associated with sorghum. Afr. J. Microbiol. Res. 6, 3058–3064.