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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

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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).

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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.