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Colonization and yield promotion of tomato by *Gluconacetobacter diazotrophicus*

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ABSTRACT

Gluconacetobacter diazotrophicus is a N_2-fixing bacterium originally associated with sugarcane and considered a Plant Growth Promoting Bacteria (PGPB) for diverse crops. Aiming to find PGPB for horticultural species, tomato seedlings were inoculated with *G. diazotrophicus* to test its ability to colonize and to evaluate whether it can enhance fruit production. Tomato seedlings were inoculated with *G. diazotrophicus* PAL 5 and UAP 5541/pRGS561 (containing the marker gene *gus*A) under gnotobiotic conditions. In greenhouse experiments tomato seedlings were only inoculated with *G. diazotrophicus* PAL 5. Colonization was monitored by plating bacterial suspensions from homogenized tissues and by microscopic localization of bacteria after staining with *gus* substrate. Tomato yields were determined quantifying total tomato production throughout the crop in two different seasons. Root and stems endophytic population was higher than 4.0 log CFU g^{-1} fresh weight. Microscopic localization showed colonizing bacteria in sites of emergence of lateral roots, root hairs, and stomata. Inoculated plants significantly increased both number and weight of fruit production as compared to non-inoculated controls. These results show the ability of *G. diazotrophicus* to stimulate fruit production of tomato plants.

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1. Introduction

Gluconacetobacter diazotrophicus is a N_2-fixing bacterium originally associated with sugarcane as described by Cavalcante and Döbereiner (1988), but it has also been found in natural endophytic association with other host plants such as sweet potato (Paula et al., 1991), cameron grass (Döbereiner et al., 1993), coffee (Jiménez-Salgado et al., 1997), finger millet (Loganathan et al., 1999) and pineapple (Tapia-Hernández et al., 2000). Its natural occurrence in the rhizosphere of different plants has also been documented (Jiménez-Salgado et al., 1997; Loganathan et al., 1999; Muthukumarasamy et al., 2005; Santos dos et al., 2006). The interaction of *G. diazotrophicus* with sugarcane represents a model system for monocot–diazotrophic association and even though this relation is not yet fully understood, different reports indicate that *G. diazotrophicus* seems to be able to promote plant growth (for a review see Pedraza, 2008). Inoculation with *G. diazotrophicus* may be beneficial for sugarcane plant growth by providing fixed N (Anitha and Thangaraju, 2010; Bastián et al., 1998; Sevilla et al., 2001). This response is dependent both on the genotype of *G. diazotrophicus* and the sugarcane variety (Muñoz-Rojas and Caballero-Mellado, 2003). Plant growth stimulation by this bacterium has been ascribed not only to N_2-fixation but also to phytohormones production, biocontrol of phytopathogens, mineral nutrient solubilization and disease resistance induction (Arencibia et al., 2006; Bastián et al., 1998; Sevilla et al., 2001). Inoculation experiments with *G. diazotrophicus* have been conducted in order to test whether this microorganism could colonize and enhance plant growth in plants other than sugar cane. Caballero-Mellado et al. (1998) observed the internal colonization of maize inoculated with *G. diazotrophicus*. Different strains of *G. diazotrophicus* were able to colonize rice (Cocking et al., 2006; Loganathan and Nair, 2003; Rouws et al., 2010; Sevilla and Kennedy, 2000) and wheat (Cocking et al., 2006; Luna et al., 2010; Sevilla and Kennedy, 2000; Youssef et al., 2004). Adriano-Anaya et al. (2006) found that inoculation with *G. diazotrophicus* increased sorghum dry matter but had no effect on maize. On the other hand, a synergistic effect of *G. diazotrophicus* on arbuscular mycorrizal colonization of plant roots has been described (Adriano-Anaya et al., 2006; Isopi et al., 1995; Paula et al., 1991; Reis et al., 1999). Considering all these features *G. diazotrophicus* is considered to be a PGPB (Saravanan et al., 2007; Pedraza, 2008). In this study tomato seedlings were inoculated with *G. diazotrophicus* to test its ability to colonize and to evaluate whether this bacterium can enhance fruit yield.

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2. Materials and methods

2.1. Organisms and maintenance

G. diazotrophicus PAL 5 (ATCC 49037) (Cavalcante and Döbereiner, 1988) and a β-glucuronidase marked *G. diazotrophicus* strain UAP5541/pRGS561 (Fuentes-Ramírez et al., 1999) were maintained at 4 °C on potato medium (Stephan et al., 1991) with nalidixic acid (Nal) (15 μg ml^{-1}) and Nal and streptomycin (Sm) (45 μg ml^{-1}), respectively.

2.2. Culture conditions

Bacterial strains were grown in flasks (1 l) 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.

2.3. Inoculum preparation

To study the colonization pattern of inoculated bacteria, cultures of *G. diazotrophicus* PAL 5 or UAP 5541/pRGS561 were centrifuged and resuspended in the same volume of pH 6.0 phosphate saline buffer (PBS) and used as inocula (Luna et al., 2010). For greenhouse experiments bacterial cultures (approximately 1.10^9 CFU ml^{-1}) were used as inocula.

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 *G. diazotrophicus* PAL 5 or UAP 5541/pRGS561 under gnotobiotic conditions; and the other one to test fruit yield by inoculation with *G. diazotrophicus* PAL 5 in greenhouse.

2.4.1. Colonization 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 on semisolid medium (0.5% agar). Seedlings were immersed in an appropriate volume of the bacterial inoculum (*G. diazotrophicus* PAL 5 or UAP 5541/pRGS561) with constant shaking for 10 min. Negative controls were immersed in an appropriate volume of sterile pH 6.0 PBS. Inoculated seedlings were placed into flasks containing 100 ml of semi solid Fahräeus 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 three treatments each (non-inoculated controls, inoculated with *G. diazotrophicus* PAL 5 and inoculated with *G. diazotrophicus* UAP 5541/pRGS561), and 50 plants/treatment, were performed.

Plants were harvested two days after seedling inoculation (when it is possible to separate roots and stems) and then at 2–3 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 three minutes with constant agitation for roots and 2 min for stems. Samples were then washed four 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, containing Nal, Sm and X-Gluc (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt, 40 μg ml^{-1}) for *gus*A-marked strain or Nal for strain PAL 5. Colony forming units were counted after incubation at 28 °C for 3 days. As previously described by Luna et al. (2010) three control procedures were performed to ensure the efficiency of the surface disinfection method: (1) disinfected plant tissue samples taken 96 h postinoculation (P.I.) (with the *gus*A-marked strain) were observed by optical microscopy after staining, (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).

Plants inoculated with strain UAP 5541/pRGS561 were harvested and separated into roots and stems. GUS activity was tested daily during the first week after inoculation and at three-day intervals thereafter. The plants were carefully removed from the growth medium and roots were gently washed with sterile water in order to wash the remaining agar away. The staining procedure was carried out as described by Jefferson et al. (1987). Non-inoculated plants were analyzed at the same time intervals. Samples were observed after staining and photographed using a Carl Zeiss Photomicroscope. Multiple samples were examined either directly or by using hand-cut sections of plant tissues immersed in agarose blocks.

2.4.2. Greenhouse experiments

Non-disinfected tomato seeds were germinated as indicated for colonization experiments. Seedlings without visible contamination were transferred to speedling trays (100 cells per tray of 25 ml each) previously filled with pH 6.0 sterile vermiculite and placed in a controlled growth chamber under the same conditions indicated above. Sterile modified Hoagland's solution with KNO_3 as nitrogen source (Hoagland and Arnon, 1950) was weekly added to each cell throughout the experiment period in the growth chamber. Four week-old seedlings were inoculated with 5 ml of a *G. diazotrophicus* PAL 5 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 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 greenhouse with natural daylight. Experiments comprised two treatments: non-inoculated control and inoculated with *G. diazotrophicus* PAL 5. 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. 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; available 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 determined by measuring total tomato production throughout the season. Fruit number and weight were examined once a week since two months after inoculation and during the following two months of the experiments. Tomato yield production was expressed as means of the total measurements per replicate during two months of evaluation. Statistical analysis was performed using one way analysis of variance (ANOVA)

followed by Duncan's test. *P*-values ≤0.05 were considered as significant.

3. Results

3.1. Colonization experiments. G. diazotrophicus

PAL 5 or UAP 5541/pRGS561 were isolated from surface disinfected and non-disinfected plant tissues and enumerated in specific media to estimate the epi- and endophytic colonization. *In situ* microscopic localization of *G. diazotrophicus* UAP 5541/pRGS561 allowed detection of colonization sites. Tomato plants colonization was not accompanied by inhibition of plant growth or other macroscopically visible disease symptom.

3.1.1. Control of disinfection treatment of plant tissues

(1) No zones of blue staining were observed in disinfected samples from plants previously inoculated with the *gus*A-marked strain, (2) no bacterial growth was observed in LGI plates mounted with disinfected tissues, (3) bacterial growth was rarely observed when the wash solution from the last tissue rinse was plated on LGI. Similar to the root tests, no bacterial colonies were found on aerial parts neither in post disinfection wash tests nor in 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 Dong et al. (2003).

3.1.2. Root colonization

Colony counting of plants inoculated with either *G. diazotrophicus* PAL 5 or UAP 5541/pRGS561 showed a similar behavior for both strains (Fig. 1).

The rhizoplane of tomato plantlets was rapidly colonized by *G. diazotrophicus* after seedling inoculation: bacterial concentrations of 7.0–7.5 log CFU g^{-1} (fw) were observed since 2 days P.I. and remained almost constant along the 25 days period of observation (Fig. 1).

Endophytic population remained at 5.0–6.0 log CFU g^{-1} (fw) for the first 7 days (Fig. 1). After that, the population showed fluctuations varying between 4.0 and 6.0 log CFU g^{-1} (fw) along the observation period. No bacteria could be isolated from the control plants (non-inoculated).

Tomato roots recovered at different times revealed the presence of *G. diazotrophicus* after GUS technique application. Seedlings inoculated with strain UAP 5541/pRGS561 showed intense blue staining in root-stem junctions and in sites of emergence of lateral roots after 2 days P.I. (Fig. 2). Microscopic observations showed blue color on root hairs, sub-stomatal cavities of root–stem junctions, epidermal regions and on the external cell layer as shown in root transversal sections (Fig. 2). Root tip cells were rarely stained. Non-inoculated roots did not develop blue zones after GUS staining, confirming the lack of endogenous β-glucuronidase activity and providing the specificity of the visualization procedure (data not shown).

3.1.3. Stems colonization

Surface disinfected stems from inoculated tomato seedlings showed significant bacterial populations after 2 days P.I., varying around 3.5–5.0 log CFU g^{-1} (fw) throughout the experiment (Fig. 1). Stem endophytic bacterial population, although significant, was lower than the one found in roots (Fig. 1).

Table 1
Tomato fruit yields of plants inoculated with *G. diazotrophicus* PAL 5.[a]

		FN	FW	% FN	% FW
1st year	Non-inoculated	996	149	7.33	13.83
	PAL5	1069*	170*		
2nd year	Non-inoculated	1128	148	17.77	13.85
	PAL5	1325*	168*		

FN, total fruit number; FW, total fruit weight (kg).

[a] Results are expressed as means of the total measurements per treatment replicate (20 plants) during the evaluation period. The data were subjected to statistical analysis performed using one way analysis of variance (ANOVA) followed by Duncan's test and the means compared. *P*-values ≤0.05 were considered as significant.

* Indicate values that were significantly different from the non-inoculated control at 5% level.

3.2. Greenhouse experiments

Fruit yields both number and weight in both experiments were markedly affected by the inoculation with *G. diazotrophicus* PAL 5 (Table 1).

4. Discussion

Although there are some reports describing associations of tomato with different PGPB (Caballero-Mellado et al., 2007; Gamalero et al., 2004; Gravel et al., 2007; Poonguzhali and Madhaiyan, 2008), as far as we are aware this is the first report showing tomato colonization by *G. diazotrophicus*.

Tomato seedlings inoculated with *G. diazotrophicus* under gnotobiotic growth conditions resulted in efficiently colonized roots. These results suggest that both seedling and root exudates provided enough nutrients to sustain the growing population. Different root colonization sites were detected by GUS expression as possible entry routes of this microorganism into tomato plants (Fig. 2). These results are similar to those previously observed for sugarcane (James et al., 1994), maize (Cocking et al., 2006), sorghum and wheat (Luna et al., 2010) inoculated with the same bacterium. It has been reported that this is a common pattern for internal root colonization by other endophytic organisms (Compant et al., 2010). Although substomatal chambers are not frequently colonized by plant-beneficial bacteria (Compant et al., 2005), James et al. (2001, 2002) observed dense colonization of rice and sugarcane stomata by *Herbaspirillum* sp. and *G. diazotrophicus* respectively. Our results show *G. diazotrophicus* colonization of substomatal chambers in tomato plants. Therefore, as suggested by James et al. (2002) stomata could be another entry point allowing bacteria to spread throughout the internal tissues. *G. diazotrophicus* could be recovered from an homogenate of surface-disinfected roots and also from stems. The decrease of stem bacterial density in comparison to root colonizing populations has been already reported for this and other endophytic organisms (Luna et al., 2010; Zakria et al., 2008). Although our colonization experiments were based on early events in agarized plates, the colonization process seems to be maintained during the plant life cycle because, as observed in greenhouse conditions, it produced plant growth promotion. Plant roots and stems colonization lead to a significant increase in tomato production.

This study shows that inoculation of tomato with *G. diazotrophicus* could confer beneficial effects to this crop after efficient plant colonization. Further studies are required to determine whether this effect is through BNF, hormones production or any other growth promotion mechanism that conduce to enhance tomato fruit yield.

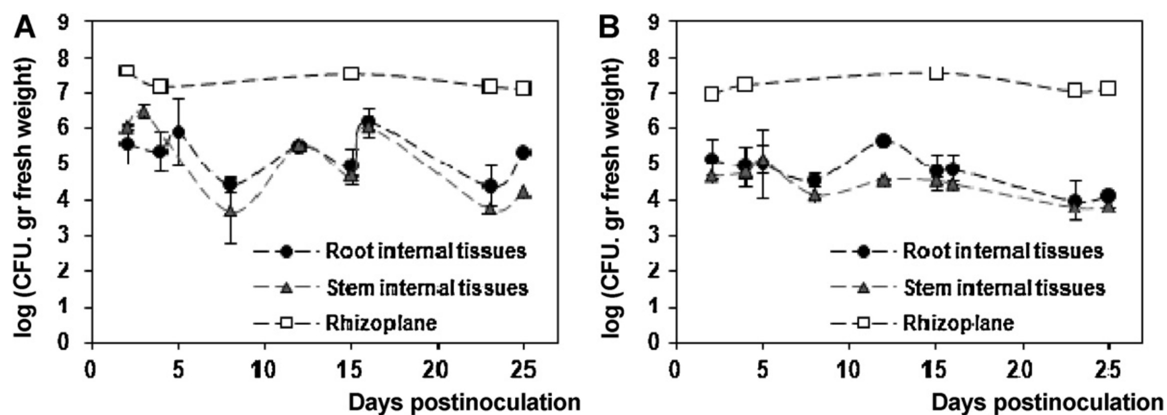

Fig. 1. Root and stem populations of tomato plants after seedling inoculation with strains UAP 5541/pRGS561 (A) and PAL 5 (B) of *G. diazotrophicus*. The data points are the means of three independent experiments and the bars represent the standard errors of the means.

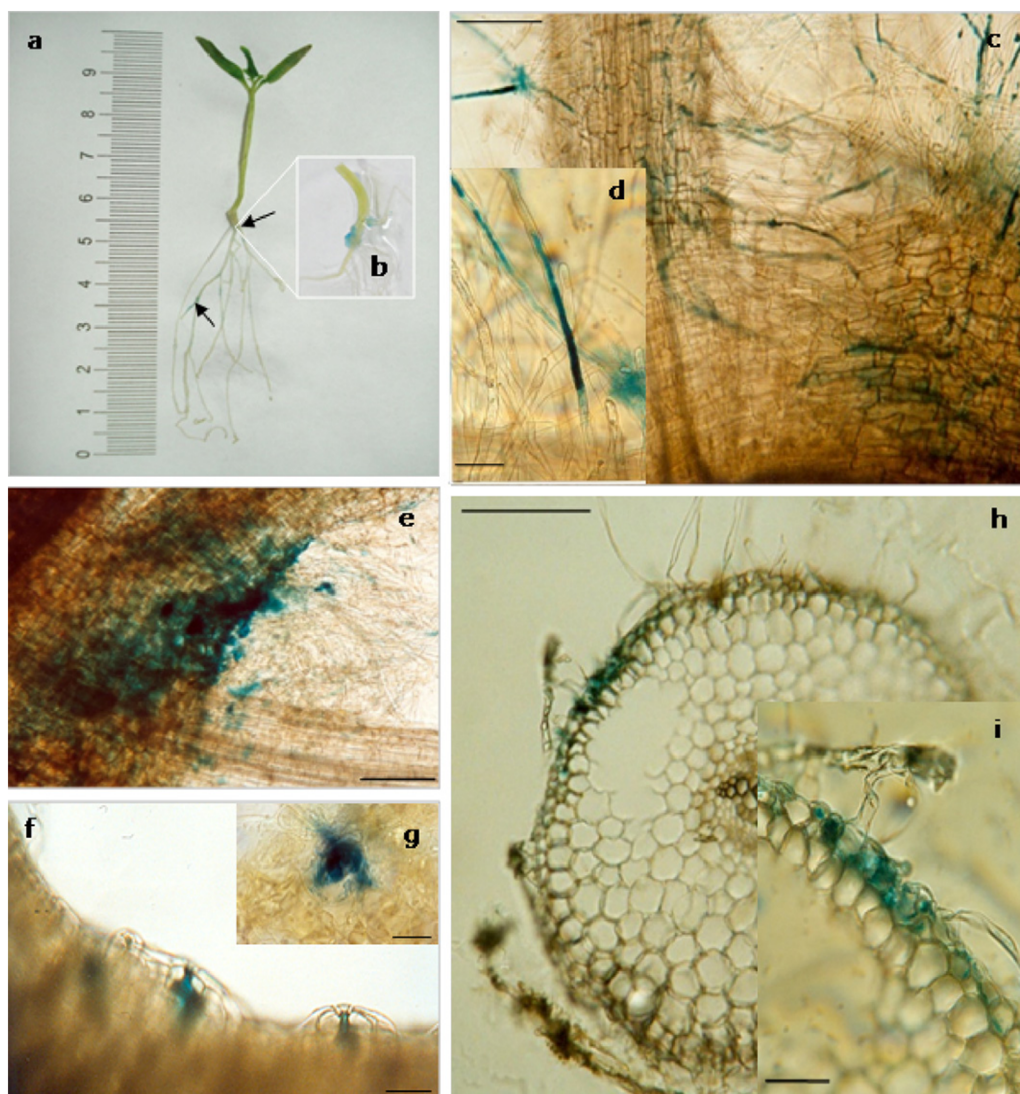

Fig. 2. Images of tomato tissues stained by GUS technique showing blue color due to *gus*-marked cells, after seedling inoculation with *G. diazotrophicus* UAP 5541/pRGS561. (a) and (b) Photographs of tomato plants showing blue zones in the root–stem junction and at the site of emergence of lateral roots (arrows) in plants harvested 8 days P.I. (c)–(i) Light microscope images of tomato roots showing blue staining on root hairs (c and d, day 7 P.I.), at the site of emergence of lateral roots (e, day 10 P.I.), in substomatal chamber (f, day 3 P.I.), in stomata (g, day 8 P.I.) and on the external cell layer of root transversal sections (h and i, day 20 P.I.). Bars (c, e and h) 200 μm; (f) 100 μm; (d and i) 50 μm; (g) 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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