Biotechnol Lett (2010) 32:1071–1076
DOI 10.1007/s10529-010-0256-2

ORIGINAL RESEARCH PAPER

Colonization of sorghum and wheat by seed inoculation with *Gluconacetobacter diazotrophicus*

M. F. Luna · M. L. Galar · J. Aprea · M. L. Molinari · J. L. Boiardi

Received: 4 February 2010 / Accepted: 18 March 2010 / Published online: 2 April 2010

Abstract Colonization of sorghum and wheat after seed inoculation with *Gluconacetobacter diazotrophicus* strains PAL 5 and UAP 5541/pRGS561 (containing the marker gene *gus*A) was studied by colony counting and microscopic observation of plant tissues. Inoculum levels as low as 10^2 CFU per seed were enough for root colonization and further spreading in aerial tissues. Rhizoplane colonization was around 7 log CFU g^{-1} (fresh weight). *G. diazotrophicus* was found inside sorghum and wheat roots with populations higher than 5 log CFU g^{-1} (fresh weight). Stem colonization remained stable for 30 days post inoculation with endophyte concentrations from 4 to 5 log CFU g^{-1} (fresh weight) (in both plants). Population in leaves decreased continuously being undetectable after 17 days post inoculation.

Keywords Endophytes · *Gluconacetobacter diazotrophicus* · Inoculation · Sorghum · Wheat

M. L. Galar · J. Aprea · M. L. Molinari · J. L. Boiardi (✉)
Facultad de Ciencias Exactas, CINDEFI (UNLP; CCT-La Plata, CONICET), Universidad Nacional de La Plata, Calles 47 y 115 (1900), La Plata, Argentina
e-mail: boiardi@quimica.unlp.edu.ar

M. F. Luna
CIC PBA, La Plata, Argentina

Introduction

The use of Plant Growth Promoting Bacteria (PGPB) as biofertilizer and biocontrol organisms is being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture. Plant growth stimulation by microorganisms can be a consequence of nitrogen fixation, production of phytohormones, biocontrol of phytopathogens in the root zone or enhancement of minerals availability. PGPB must grow on or around the roots for further plant colonization, which is of primary importance for an effective plant–microbe association. After this initial colonization step, certain bacteria are able to enter roots by different mechanisms and establish endophytic populations (Rosenblueth and Martínez-Romero 2006). A N_2-fixing endophytic bacterium, *Gluconacetobacter diazotrophicus*, was described by Cavalcante and Döbereiner as associated with sugarcane (1988). This bacterium has also been found in natural association with other host plants. It can enhance plant growth independent of nitrogen fixation as has been shown in sugarcane and maize (Riggs et al. 2001; Saravanan et al. 2007). Its colonization behavior has been poorly characterized in plants others than sugar cane and it is not known what inoculum concentration is required for plant entry. This is not an important issue with sugarcane, which is vegetatively propagated, but should be important to extend inoculation with this microorganism to seed propagated crops.

In the present study an attempt was made to study the colonization behaviour of *G. diazotrophicus* in wheat (*Triticum aestevium*) and sorghum (*Sorghum bicolor*). Seeds were inoculated at different inoculum levels and plants grown under gnotobiotic conditions. Colony counting of homogenized tissues and microscopic observation of organ sections were performed in order to determine its colonization capability. It is shown that seed inoculation of *G. diazotrophicus* led to extensive root colonization of either plant followed by bacterial spreading to aerial tissues, without any symptom of plant growth inhibition irrespective of the inoculum level.

Materials and methods

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.

Culture conditions

Bacterial strains were grown in flasks (250 ml) containing 50 ml of LGI medium (Stephan et al. 1991) at pH 6, on a rotatory shaker at 200 rpm and 30°C for 48 h.

Inoculum preparation

Bacterial cultures with a final concentration of $\sim 1.10^9$ CFU ml^{-1} were centrifuged and resuspended in the same volume of buffer phosphate saline buffer (PBS) pH 6.0. This suspension was 10-fold serially diluted in PBS and 10 ml of the appropriate bacterial suspension used to inoculate 100 g of seeds.

Surface sterilization and inoculation of seeds

Wheat (*Triticum aestevium* cv. "baguette") and sorghum (*Sorghum bicolor* cv. "energy") seeds were surface sterilized 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 inoculated and immediately placed into flasks containing 100 ml of Fahraeus-semisolid medium (0.5% agar).

Plant culture conditions

Plants were grown in gnotobiotic conditions in a controlled-climate glasshouse under a day and night cycle of 16 and 8 h, respectively, at 28°C for 30 days. Seven treatments (in each of three independent experiments), with around 50 plants/treatment, were used for each plant species: uninoculated controls, inoculated with *G. diazotrophicus* PAL 5 (10^6 CFU - $seed^{-1}$) and inoculated with *G. diazotrophicus* UAP 5541/pRGS561 with inoculums levels of 10^6, 10^5, 10^4, 10^3 and 10^2 CFU $seed^{-1}$.

Determination of microbial populations in rhizoplane and inside surface-sterilized plant tissues

The third day post (seed) inoculation (P.I.) it was possible to separate roots and shoots and plants were harvested thereafter at 2 day intervals and separated into roots and shoots (or leaves and stems). For each plant species three samples were combined and three replicates of tissue samples were collected (nine plants each time) to determine the average colonization value and fresh weights. To determine endophytic population plant tissues were sterilized with 2% sodium hypochlorite for 3 min with constant vortex agitation for sorghum roots, 10 min for wheat roots and 2 min for either shoots. Tissues were washed four times with sterilized water. Samples were crushed manually with mortar and pestle. The homogenates were resuspended in 1 ml of PBS and vortexed. This suspension was 10-fold serially diluted and cultured on LGI agar plates, containing Nal, Sm and X-Gluc (40 μg ml^{-1}) for *gus*A-marked strain or Nal for strain PAL 5, at 28°C for 3 days and colonies enumerated. Three control procedures were performed to ensure the efficiency of the surface sterilization method: (i) sterilized plant tissue samples of 96 h P.I. (with *gus*A-marked strain) were observed by optical microscopy after staining, (ii) sterilized vegetal tissues were placed for 1 min onto plates containing LGI, tissues removed and plates incubated at 28°C, (iii) the wash solution from the last rinse was cultured on LGI plates.

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

Optical microscopy observations

Plants inoculated with strain UAP 5541/pRGS561 (with 10^6 CFU $seed^{-1}$) were harvested and separated into roots and shoots. GUS activity was tested daily during the first 7 days P.I. and from then at 3-day intervals. The staining procedure using X-Gluc was done as described by Jefferson et al. (1987). Uninoculated plants were analyzed at the same interval times. After staining, samples were observed and photographed using a Carl Zeiss Photomicroscope. Multiple samples were examined either directly or by using hand-cut sections.

Results and discussion

Control of sterilization treatment of plant tissues

(1) No zones of blue staining were observed in sterilized samples from plants previously inoculated with the *gus*A-marked strain. (2) No bacterial growth was observed at 3 days after mounting of surface-sterilized roots on LGI plates. (3) Bacterial growth was rarely observed when the wash solution from the last tissue rinse was plated on LGI. Colonies were observed in less than 5% of these washes. Similar to the root tests, no bacterial colonies were found on stems and leaves neither in post sterilization wash tests nor in incubations of whole aerial tissues. Based on these results, we can assume that the sterilization procedure employed in this work was sufficient to kill and/or wash away bacteria from plant surfaces.

Tissues colonization by *G. diazotrophicus*

Colony counting of plants inoculated with either *G. diazotrophicus* PAL 5 or UAP 5541/pRGS561 showed a similar behavior for both strains (data with strain PAL 5 are not shown). *G. diazotrophicus* efficiently colonized wheat and sorghum tissues after seed inoculation. It was not accompanied by inhibition of plant growth or other macroscopically visible disease symptoms.

Roots colonization

Rhizoplane population, analyzed 3 days P.I., was 7.0–7.5 log CFU g^{-1} (fresh weight) for both plant species, independently of the inoculum level. This population remained essentially steady with slight fluctuations for over 2 weeks (data not shown), suggesting that seedling and root exudates provided nutrients enough to maintain this population.

Endophytic population remained at 6–6.5 log CFU g^{-1} (fresh weight) for more than 7 days (Fig. 1a, b). After that, endophytic population decreased to a level of 5.5 log CFU g^{-1} (fresh weight) and remained constant until the end of the experiment (30 days). Although these observations were made from plants inoculated with a concentration of $\sim 1.10^6$ CFU - $seed^{-1}$, endophytic colonization was observed over a range of inoculum levels (from 10^2 to 10^6 CFU per plant) without significant differences in the extent of colonization (Fig. 1c, d), indicating that few cells were enough for plant entry and further interior colonization. In uninoculated controls no bacteria could be detected by plate counting of homogenized tissues that have been or not previously sterilized.

G. diazotrophicus could be reisolated from homogenated surface-sterilized roots and shoots indicating internal colonization and spreading within the plants. Furthermore, uninoculated roots of wheat and sorghum plants did not develop blue zones after GUS staining, confirming the lack of endogenous β-glucuronidase activity and providing the specificity of the visualization procedure (Fig. 2b, d). Roots inoculated with strain UAP 5541/pRGS561 showed intense blue staining for both crops: the root surface just emerging from the seeds was always intensively colonized from 24 h P.I. onward, as well as the root hairs (Fig. 2a, c, e, f). Several root colonization sites were detected by GUS expression as possible routes of entry for this bacterium: (1) via lateral root emergence sites (Fig. 2g, arrows), suggesting a colonization route similar to that of sugar cane (James and Olivares 1997) could occur in sorghum and wheat plantlets; (2) via root hairs (Fig. 2e, f), that would indicate that

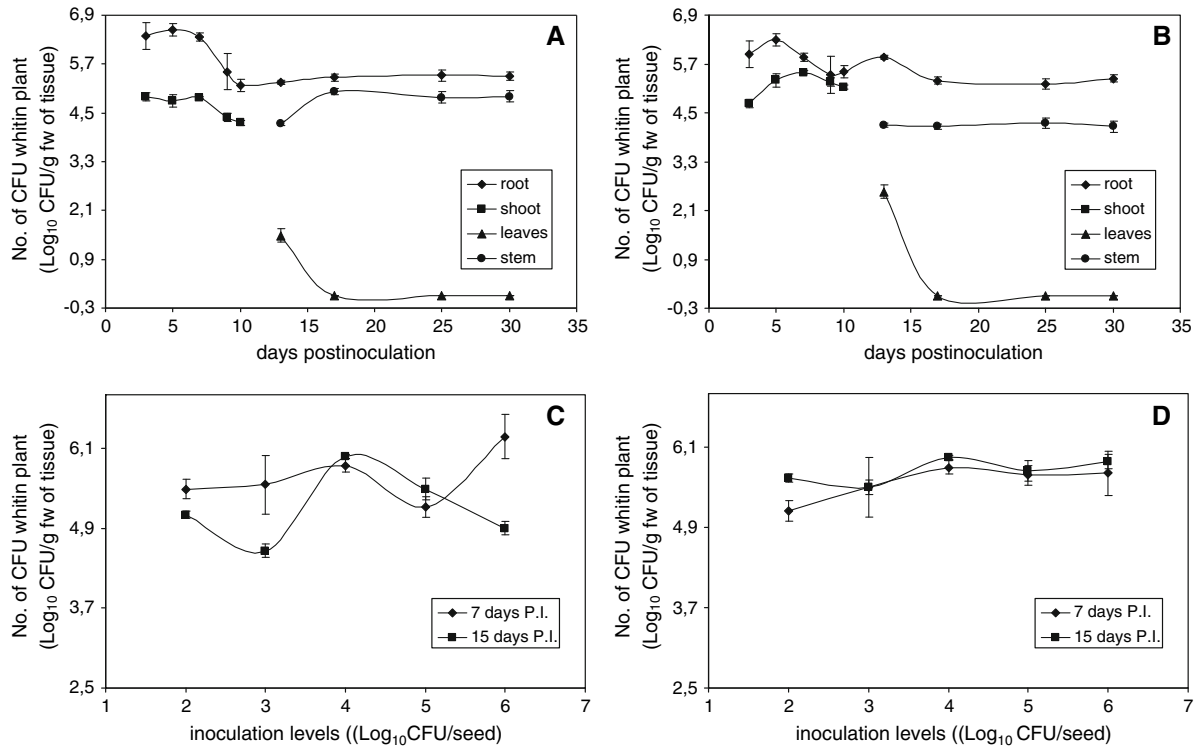

Fig. 1 Endophytic population of sorghum and wheat plants after seed inoculation with *G. diazotrophicus* UAP 5541/pRGS561. **a** and **b** Endophytic population in sorghum (**a**) and wheat (**b**) inoculated with 10^6 CFU seed^{-1}. **c** and **d** Endophytic population in sorghum (**c**) and wheat (**d**) roots with different inoculum levels. The standard error of the mean represented by each data point is indicated in the figures. *fw* fresh weight

G. diazotrophicus could also enter plants through them, as has been reported for some endophytic *Azospirillum sp.* (Hallmann et al. 1997); and (3) (only for wheat) from the root tips in cells of the root cap and meristem, since 24 h P.I. and maintained along the experiment (Fig. 2c arrows, h), which is considered a possible way of entry for other endophytic microorganisms like *Azoarcus* sp. (Hurek et al. 1994). Longitudinal view of plants roots recovered at different times revealed the presence of *G. diazotrophicus* in epidermal regions of roots (Fig. 2i) and in many of the intercellular spaces in the portion of roots just emerging from seeds (Fig. 2j). Transverse sections of wheat roots 3 days P.I. showed blue staining on internal tissues (Fig. 2k, l).

Shoots colonization

G. diazotrophicus was able to colonize aerial parts of sorghum and wheat plants, as already reported for other endophytic organisms (Zakria et al. 2008), but the concentration of this endophytic population was always lower than that found in roots (Fig. 1a, b). The highest level of bacterial population was reached at early stages of plant growth and remained around 4.5–5.5 log CFU g^{-1} (fresh weight) throughout. In 13 days old plants, when it was possible to separate stems from leaves, stem population remained at a level of 4.0–5.0 log CFU g^{-1} (fresh weight) whereas leaves population decreased continuously with time and no bacteria could be detected in plants examined around 17 days P.I. for both crops (Fig. 1a, b). Figure 2m, n show aerial tissues colonization. No blue zones were observed on samples of shoots taken from uninoculated plantlets after incubation with the GUS substrate (data not shown).

We have shown that *G. diazotrophicus* seed inoculation leads to extensive root colonization of sorghum and wheat followed by bacterial colonization of aerial tissues. Differences in the extent of colonization between this study and others (Sevilla

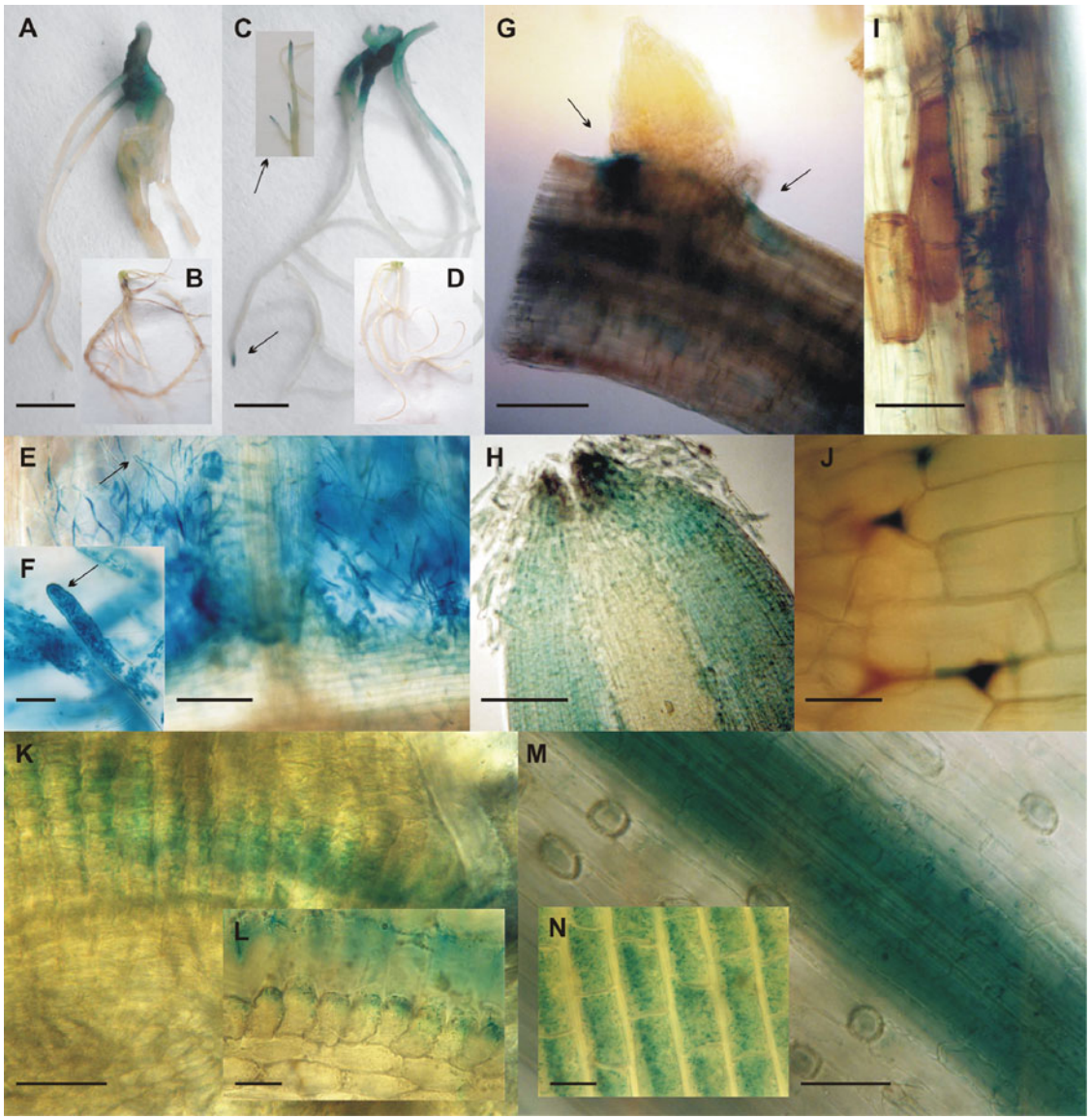

Fig. 2 Histochemical localization of *gus*A gene expression in sorghum and wheat plants after seed inoculation with *G. diazotrophicus* strain UAP5541/pRGS561. Photographs of sorghum (**a**) and wheat (**c**) roots showing *blue* zones due to *gus*A-marked cells at the root tips (*arrows*) and in roots just emerging from seeds. Not inoculated roots of sorghum (**b**) and wheat (**d**) show no *blue* zones. Light microscope images of roots showing *blue* staining in sorghum root hairs (**e** and **f**), at the site of emergence of lateral wheat roots (**g**) (*arrows*), in cells of wheat root cap and meristem (**h**), in epidermal tissues (**i**) and intercellular spaces of emerging sorghum roots (**j**). Transverse sections of wheat roots show *blue* staining on cortical tissues (**k** and **l**). Blue staining of wheat stems show colonization of aerial tissues (**m** and **n**). (**a** and **c**) bars = 3 mm; (**e**, **g** and **h**) bars = 200 μm; (**i**, **j**, **k** and **m**) bars = 50 μm; (**f**) bar = 20 μm; (**l** and **n**) bar = 10 μm

and Kennedy 2000; Cocking et al. 2006) may depend, not only on the methodology of inoculation, but also on the combination of host cultivar and strain bacterial endophyte. This study provides further evidence that *G. diazotrophicus* can colonize plants other than its original host (sugarcane) and it can establish stable endophytic associations with wheat and sorghum plants, at least under our experimental conditions. Future studies will be directed to know whether this significant colonization is accompanied by stimulation of plant growth as suggested elsewhere (Adriano-Anaya et al. 2006).

References

Cavalcante VA, Döbereiner J (1988) A new acid tolerant nitrogen-fixing bacterium associated with sugar cane. Plant Soil 108:23–31

Cocking EC, Stone PJ, Davey MR (2006) Iintracellular colonization of roots of *Arabidopsis* and crop plants by *Gluconacetobacter diazotrophicus*. In Vitro Cell Dev Biol Plant 42:74–82

Döbereiner J, Reis VM, Paula MA, Olivares FL (1993) Endophytic diazotrophs in sugar cane, cereals and tuber plants. In: Palacios R, Moor J, Newton WE (eds) New horizons in nitrogen fixation. Kluwer Academic Publisher, Dordrecht, pp 671–676

Fuentes-Ramírez LE, Caballero-Mellado J, Sepúlveda J, Martínez-Romero E (1999) Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization. FEMS Microbiol Ecol 29:117–128

Gyaneshwar P, James EK, Mathan N, Teddy PM, Reinhold-Hurek B, Ladha JK (2001) Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. J Bacteriol 183:2634–2645

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can J Microbiol 43:895–914

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

James EK, Olivares FL (1997) Infection and colonization of sugarcane and other graminaceous plants by endophytic diazotrophs. Crit Rev Plant Sci 17:77–119

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Riggs PJ, Chelius MK, Iniguez AL, Kaeppler SM, Triplett EW (2001) Enhanced maize productivity by inoculation with diazotrophic bacteria. Aust J Plant Physiol 28:829–836

Rosenblueth M, Martínez-Romero E (2006) Bacterial endophytes and their interactions with hosts. Mol Plant Microbe Interact 19:827–837

Saravanan VS, Madhaiyan M, Osborne J, Thangaraju M, Sa TM (2007) Ecological occurrence of *Gluconacetobacter diazotrophicus* and nitrogen-fixing *Acetobacteraceae* members: their possible role in plant growth promotion. Microb Ecol 55(1):130–140. doi:10.1007/s00248-007-9258-6

Sevilla M, Kennedy C (2000) Colonization of rice and other cereals by *Acetobacter diazotrophicus*, an endophyte of sugarcane. In: Ladha JK, Reddy PM (eds) The quest for nitrogen fixation in rice. IRRI press, Phillipines, pp 151–165

Stephan MP, Oliviera M, Teixeira KRS, Martínez-Drets G, Döbereiner J (1991) Physiology and dinitrogen fixation of *Acetobacter diazotrophicus*. FEMS Microbiol Lett 77:67–72

Zakria M, Udonishi K, Ogawa T, Yamamoto A, Saeki Y, Akao S (2008) Influence of inoculation technique on the endophytic colonization of rice by *Pantoea sp.* isolated from sweet potato and by *Enterobacter* sp. isolated from sugarcane. Soil Sci Plant Nutr 54:224–236