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ORIGINAL ARTICLE

## The decrease in the population of *Gluconacetobacter diazotrophicus* in sugarcane after nitrogen fertilization is related to plant physiology in split root experiments



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### KEYWORDS

Nitrogen fertilization;  
Bacterial population;  
*G. diazotrophicus*;  
Sugarcane;  
Plant growth  
promoting bacteria

**Abstract** It has been established that a decrease in the population of *Gluconacetobacter diazotrophicus* associated with sugarcane occurs after nitrogen fertilization. This fact could be due to a direct influence of  $\text{NH}_4\text{NO}_3$  on bacterial cells or to changes in plant physiology after fertilizer addition, affecting bacterial establishment. In this work, we observed that survival of *G. diazotrophicus* was directly influenced when 44.8 mM of  $\text{NH}_4\text{NO}_3$  (640 mg N/plant) was used for *in vitro* experiments. Furthermore, micropropagated sugarcane plantlets were inoculated with *G. diazotrophicus* and used for split root experiments, in which both ends of the system were fertilized with a basal level of  $\text{NH}_4\text{NO}_3$  (0.35 mM; 10 mg N/plant). Twenty days post inoculation (dpi) one half of the plants were fertilized with a high dose of  $\text{NH}_4\text{NO}_3$  (6.3 mM; 180 mg N/plant) on one end of the system. This nitrogen level was lower than that directly affecting *G. diazotrophicus* cells; however, it caused a decrease in the bacterial population in comparison with control plants fertilized with basal nitrogen levels. The decrease in the population of *G. diazotrophicus* was higher in pots fertilized with a basal nitrogen level when compared with the corresponding end supplied with high levels of  $\text{NH}_4\text{NO}_3$  (100 dpi; 80 days post fertilization) of the same plant system. These observations suggest that the high nitrogen level added to the plants induce systemic physiological changes that affect the establishment of *G. diazotrophicus*.

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**PALABRAS CLAVE**

Fertilización nitrogenada;  
Población bacteriana;  
*G. diazotrophicus*;  
Caña de azúcar;  
Bacterias promotoras del crecimiento de plantas

**La disminución de la población de *Gluconacetobacter diazotrophicus* en caña de azúcar, después de la fertilización nitrogenada, está relacionada con la fisiología de las plantas en experimentos de raíz dividida**

**Resumen** La población de *Gluconacetobacter diazotrophicus* asociada a la caña de azúcar disminuye después de la fertilización nitrogenada, lo cual podría ocurrir por la influencia directa del  $\text{NH}_4\text{NO}_3$  sobre la supervivencia bacteriana, o por cambios en la fisiología de las plantas, que impiden el establecimiento bacteriano. En el presente trabajo se observó que en experimentos *in vitro* la supervivencia de *G. diazotrophicus* fue influenciada por 44,8 mM de  $\text{NH}_4\text{NO}_3$  (640 mg N/plant). Además, *G. diazotrophicus* fue inoculado en plántulas micropropagadas de caña de azúcar, que fueron usadas para realizar experimentos de raíz dividida, en las que ambos extremos de los sistemas se fertilizaron con un nivel basal de  $\text{NH}_4\text{NO}_3$  (0,35 mM; 10 mg N/planta). A los 20 días posteriores a la inoculación (dpi), la mitad de plantas fueron fertilizadas en uno de sus extremos con una dosis elevada de  $\text{NH}_4\text{NO}_3$  (6,3 mM; 180 mg of N/plant). Este nivel fue menor al que afectó directamente a las células de *G. diazotrophicus*; sin embargo, provocó una disminución de la población bacteriana en comparación con plantas testigo fertilizadas con niveles basales de nitrógeno. La disminución de la población fue mayor para raíces fertilizadas con un nivel basal de nitrógeno en comparación con las raíces fertilizadas con altos niveles del mismo sistema de plantas (100 dpi; 80 días posfertilización). Estas observaciones indican que el alto nivel de nitrógeno añadido a las plantas inducen cambios fisiológicos sistémicos que afectan el establecimiento de *G. diazotrophicus*.

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## Introduction

*Gluconacetobacter diazotrophicus* is a gram-negative bacterium, initially isolated as endophyte from Brazilian sugarcane plants<sup>8</sup> and subsequently from sugarcane plants in other countries<sup>6,34</sup>. In addition, *G. diazotrophicus* has also been isolated from the inner tissue of diverse hosts<sup>13</sup>, such as *Ipomoea batatas*, *Pennisetum purpureum*<sup>12</sup>, *Saccharococcus sachari*<sup>3,31</sup>, *Eleusine coracana*<sup>18</sup>, pineapple<sup>37</sup>, and also from the rhizosphere of *Coffea arabica*.<sup>17</sup> More recently this bacterium was isolated from wetland rice<sup>27</sup>, carrot, radish and beetroot<sup>19</sup> and was related to a dominant phylotype detected as endophyte from needles of *Pinus flexilis* and *Picea engelmannii* using 16S rRNA pyrosequencing<sup>9</sup>.

*G. diazotrophicus* is a nitrogen fixing bacterium that produces phytohormones, such as indol acetic acid<sup>15,32,33</sup> and gibberellins<sup>4</sup>. This bacterial species is able to stimulate the growth of sugarcane after its inoculation<sup>25,35,36,40</sup>. The principal mechanism for stimulating plant growth occurs through the auxinic via<sup>32,35</sup> and depends on the sugarcane variety and the genotype of *G. diazotrophicus*<sup>25</sup>.

Isolation of *G. diazotrophicus* from sugarcane plants depends on the amount of nitrogen fertilization applied to the crops: the higher the level of nitrogen applied to the crops, the lower the probability to isolate *G. diazotrophicus*<sup>14,28,31</sup>. In addition, seven genotypes of *G. diazotrophicus* associated with sugarcane plants fertilized with low levels of nitrogen were identified in Brazilian fields and the diversity between them seemed to be affected by the high levels of nitrogen applied to sugarcane crops<sup>7</sup> while only one genotype was detected in sugarcane plants fertilized with high levels of nitrogen in Mexican fields.

Moreover, *G. diazotrophicus* colonization is reduced in plants fertilized with high doses of nitrogen<sup>14,22,25</sup>.

The decrease in the population of *G. diazotrophicus* associated with sugarcane plants could be due to pleomorphic changes that occur while culturing bacteria in the presence of high nitrogen concentrations<sup>29</sup>. Additionally, it has been proposed that the decrease in the population of *G. diazotrophicus* associated with sugarcane could be due to physiological changes that the plant suffers in the presence of high nitrogen fertilization<sup>7,14</sup>.

Split root experiments have been developed to evaluate the systemic effect of a specific substance on plants, when this substance is supplied only on one end of the plant, while the other end could be used as control<sup>42</sup>, but also to evaluate the systemic effect on plant pathogens due to the action of the induced systemic resistance produced by rhizobacteria<sup>1</sup>.

In this work we show a statistical analysis of the behavior of the population of *G. diazotrophicus* present inside the roots and in the rhizosphere using split root experiments, both at high or low nitrogen levels in the form of  $\text{NH}_4\text{NO}_3$ . In accordance with our results, the negative effect of nitrogen on the population of *G. diazotrophicus* is influenced by the plant.

## Materials and methods

Bacterial strains used for *in vitro* studies were *G. diazotrophicus* PAL 5<sup>T</sup>, PAL 3 and UAP 5560, each one corresponding to a different genotype. PAL 5<sup>T</sup> represents the predominant genotype isolated from different Brazilian sugarcane varieties (ET 3), PAL 3 corresponds to

**Table 1** Bacterial number of three strains of *G. diazotrophicus* grown in solid LGI media supplemented with different  $\text{NH}_4\text{NO}_3$  concentrations

[ $\text{NH}_4\text{NO}_3$ ] mM	Log CFU/ml (SD)		
	PAL 5T	PAL 3	UAP 5560
0	8.43 ( $\pm 0.63$ )A	9.00 ( $\pm 0.62$ )A	9.09 ( $\pm 0.30$ )A
0.35	8.79 ( $\pm 0.17$ )A	9.09 ( $\pm 0.35$ )A	9.16 ( $\pm 0.15$ )A
0.7	9.05 ( $\pm 0.31$ )A	9.09 ( $\pm 0.36$ )A	8.79 ( $\pm 0.17$ )A
1.4	9.31 ( $\pm 0.15$ )A	9.44 ( $\pm 0.08$ )A	8.89 ( $\pm 0.17$ )A
2.8	9.01 ( $\pm 0.27$ )A	9.20 ( $\pm 0.17$ )A	9.16 ( $\pm 0.27$ )A
5.6	8.95 ( $\pm 0.24$ )A	9.31 ( $\pm 0.28$ )A	8.95 ( $\pm 0.24$ )A
11.2	9.29 ( $\pm 0.35$ )A	9.33 ( $\pm 0.05$ )A	9.05 ( $\pm 0.35$ )A
22.4	8.72 ( $\pm 0.30$ )A	9.41 ( $\pm 0.12$ )A	8.85 ( $\pm 0.11$ )A
44.8	0 C	0 C	5.02 ( $\pm 0.25$ )B
89.6	0 C	0 C	5.07 ( $\pm 0.05$ )B
179.2	0 C	0 C	0 C

Values correspond to the media of five independent samples determined by the DPSM method. Mean values with equal letters are not statistically different at  $p \leq 0.05$ , using the *t*-Student test. SD: standard deviation.

a rare genotype not frequently isolated (ET 5) and UAP 5560 is the predominant genotype isolated from different Mexican sugarcane varieties (ET1)<sup>7</sup>.

### Experiment 1. *In vitro* assays

The ability of *G. diazotrophicus* strains to grow at different nitrogen concentrations was explored in two *in vitro* conditions: one using LGI solid Plates<sup>8</sup> supplemented with different concentrations of  $\text{NH}_4\text{NO}_3$  and the other using semisolid LGI medium<sup>8</sup> supplemented with different concentrations of  $\text{NH}_4\text{NO}_3$  (Sigma–Aldrich A3795). In the first case, bacteria were grown until stationary phase (five independent growth tubes by strain) in MESMA liquid medium<sup>14</sup> for 48 h at 30 °C and 200 rpm. Cells were washed twice by centrifugation and resuspended in  $\text{MgSO}_4$  10 mM (Sigma–Aldrich M7506). Each bacterial suspension was serially diluted (factor 1:10) and dilutions were placed in plates at different  $\text{NH}_4\text{NO}_3$  concentrations. Bacterial population was quantified by the DPSM method<sup>10</sup>. For the second condition, bacterial strains were grown until stationary phase (five independent growth tubes by strain), in 150 ml of MESMA liquid medium, for 48 h at 30 °C and 200 rpm. Each washed bacterial suspension was serially diluted (factor 1:10) and 100  $\mu\text{l}$  of each dilution were placed in series of semisolid LGI tubes in triplicate<sup>25</sup> containing the amount of  $\text{NH}_4\text{NO}_3$  assessed (data observed in Tables 1 and 2). Quantification was carried out by the most probable number method (MPN) using a McCrady table with three replicate vials for each dilution.

### Experiment 2. Plant assays

The effect of  $\text{NH}_4\text{NO}_3$  on bacterial association with sugarcane was assessed with the use of split root experiments. For this purpose, sugarcane plantlets variety MEX 57-473 were obtained by micropropagation as described previously<sup>25</sup>. Micropropagated plantlets were free from bacteria. Forty plantlets were inoculated with *G. diazotrophicus* PAL 5T strain by immersion of roots for 1 h in the bacterial

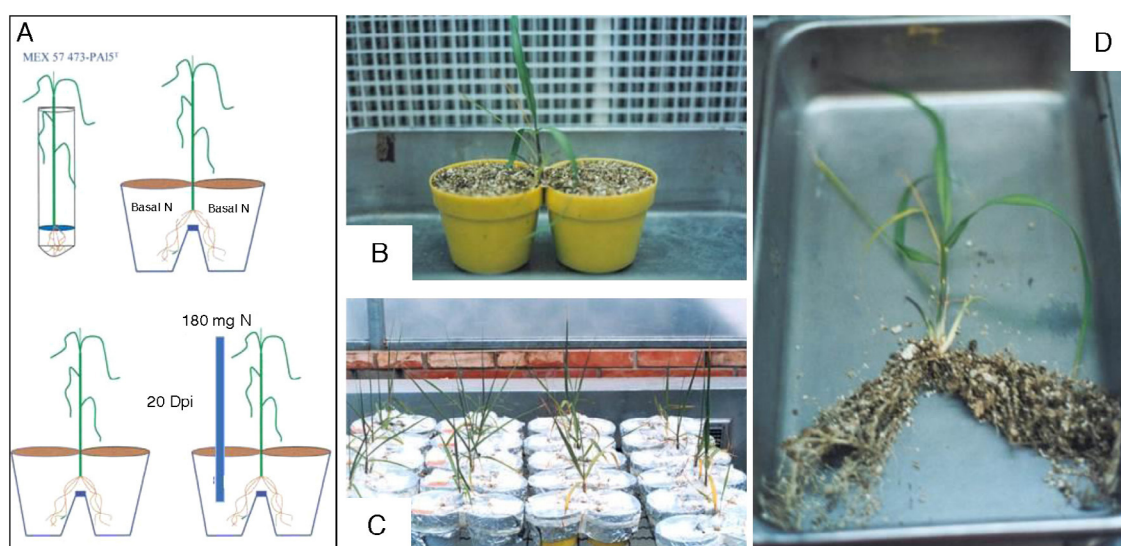
suspension ( $5 \times 10^8$  UFC/ml). Forty plantlets were used as non-inoculated controls and were only immersed in distilled sterile water. To obtain the bacterial suspension, *G. diazotrophicus* -draft PAL 5T was grown in 10 flasks containing 35 ml of TESMA medium until stationary phase, cells were washed by centrifugation twice; after, the pellet was resuspended in 35 ml of  $\text{MgSO}_4$  (10 mM) and mixed to obtain 350 ml of bacterial suspension. Ten milliliters of this suspension were dispensed in tubes of 25 cm  $\times$  2.5 cm and a single tube was used to inoculate each sugarcane plantlet.

After inoculation, both inoculated and non-inoculated plantlets were placed in sterile split root systems (Fig. 1), each consisting of two pots joined by the upper part. Each pot had 500 ml capacity and contained sterile vermiculite. For each plant, half of the roots were placed in pot 1 and the other half in pot 2, approximately 3 roots in each pot. The split root systems were watered in both pots with enough water and low doses of  $\text{NH}_4\text{NO}_3$  (10 mg N/plant equivalent to 0.35 mM) and mineral salts, according to Muñoz-Rojas and Caballero-Mellado<sup>25</sup>. The pots were covered with aluminum foil, and the zone where the plants emerged was protected with sterile cotton. The plantlets were maintained under greenhouse conditions with controlled temperature (26–30 °C) with a light/dark photoperiod of 16/8 h. Twenty days after inoculation (dpi) the bacterial number was determined for five plants both in their rhizospheres and inside the roots. At 20 dpi, 15 plants of each treatment (inoculated and non-inoculated) were supplemented with a high dose of  $\text{NH}_4\text{NO}_3$  in one of the pots (180 mg of N/plant equivalent to 6.3 mM) (Fig. 1) under sterile conditions. Plants were again maintained under greenhouse conditions and watered periodically with distilled sterile water. Rhizospheric and endophytic bacteria were recovered from the two pots for each plant system and the population was determined at 35, 55 and 100 dpi, equivalent to 15, 35 and 80 days post fertilization (dpf). Bacterial number was determined as described previously by the most probable number method using a McCrady table with three replicate vials for each dilution<sup>25</sup>. For this purpose, five independent plants or systems for each treatment (inoculated and non-inoculated) and treatments

**Table 2** Bacterial number of three strains of *G. diazotrophicus* grown in semisolid LGI media supplemented with different  $\text{NH}_4\text{NO}_3$  concentrations

[ $\text{NH}_4\text{NO}_3$ ] mM	Log of cell number/ml (SD)		
	PAL 5 <sup>T</sup>	PAL 3	UAP 5560
0	8.57 ( $\pm 0.43$ )A	8.51 ( $\pm 0.43$ )A	8.49 ( $\pm 0.30$ )A
0.35	8.43 ( $\pm 0.37$ )A	8.47 ( $\pm 0.35$ )A	8.27 ( $\pm 0.13$ )A
0.7	8.43 ( $\pm 0.31$ )A	8.44 ( $\pm 0.41$ )A	8.43 ( $\pm 0.31$ )A
1.4	8.46 ( $\pm 0.35$ )A	8.29 ( $\pm 0.36$ )A	8.28 ( $\pm 0.32$ )A
2.8	8.40 ( $\pm 0.27$ )A	8.33 ( $\pm 0.38$ )A	8.21 ( $\pm 0.42$ )A
5.6	8.38 ( $\pm 0.34$ )A	8.26 ( $\pm 0.37$ )A	8.25 ( $\pm 0.33$ )A
11.2	8.32 ( $\pm 0.35$ )A	8.13 ( $\pm 0.25$ )A	8.13 ( $\pm 0.38$ )A
22.4	8.32 ( $\pm 0.44$ )A	8.17 ( $\pm 0.32$ )A	8.15 ( $\pm 0.40$ )A
44.8	2.56 ( $\pm 0.32$ )B	2.62 ( $\pm 0.44$ )B	2.57 ( $\pm 0.35$ )B
89.6	0 D	0 D	1.03 ( $\pm 0.45$ )C
179.2	0 D	0 D	0 D

Values correspond to the media of five independent samples determined by the MPN method using a McCrady table with three replicate vials for all dilutions of each sample. Mean values with equal letters are not statistically different at  $p \leq 0.05$ , using the *t*-Student test. SD: standard deviation.



**Figure 1** Scheme representing the system used for the split root experiments (A). After *G. diazotrophicus* inoculation, the root of each plant was divided and placed in 2 pots. At 20 dpi half of the plants were supplemented with high doses of  $\text{NH}_4\text{NO}_3$  on one side of the system. Images from plants in the split root experiment (B), in the greenhouse (C) and one plant at 35 dpi before the bacterial count (D).

at different nitrogen levels were analyzed in each time. The plants were carefully removed from the vermiculite, each side of the root system was placed in an independent sterile container, and the root was shaken to discard vermiculite that was not adhered. The resultant root-vermiculite was submerged in enough sterile water (covering the root) and vortexed to maximal velocity for 40 s: the suspension was used to perform rhizospheric bacterial quantification. Vermiculite weight was obtained by drying samples without the roots. Furthermore, for endophytic bacteria quantification, each root was placed in a sterile bottle, washed to discard vermiculite and disinfected with 70% ethanol for 30 s. Then the roots were rinsed with distilled water and the surface was sterilized with a 1.5% sodium hypochlorite solution (Sigma-Aldrich 425044) for 20 min. Later, the roots

were rinsed six times with sterile distilled water under sterile conditions. Fresh roots were macerated in water in 1:10 (w/v) proportion. Each sample used for bacterial quantification was diluted (factor 1:10) until dilution 1:1,000,000 and after, 100  $\mu\text{l}$  of each dilution were placed in a tube containing semisolid LGI medium (without nitrogen for the growth of diazotrophic bacteria), three tubes were dispensed. After bacterial growth, positive and negative tubes were registered; the presence of a yellow pellicle at the top of the LGI semisolid medium, was recorded as a positive tube<sup>8,25,27</sup>. In addition, acidification of the media was observed with color changes from green to yellow, which is characteristic of *G. diazotrophicus* growth; furthermore, after 7 days the yellow color was absorbed by bacteria<sup>8</sup>. The estimation of the bacterial number of each sample was carried out by the



most probable number method using a McCrady table with three replicate vials for each dilution (with a confidence limit of 95%). For rhizospheric bacteria quantification, the value obtained from the McCrady table was multiplied per 10 and the dilution factor was considered in order to obtain the number of bacterial cells/ml of liquid suspension (sample). This value was multiplied per the initial water volume where the root was vortexed and divided by the amount in grams (g) of vermiculite (V) present in the suspension (considered as the adhered soil to the roots)<sup>25</sup>. The final quantified values obtained were stated as the number of cells/g V. To assess endophytic bacteria quantification, the value obtained from the McCrady table, was multiplied by 10 and the dilution factor was considered in order to obtain the number of bacterial cells/ml of liquid suspension (sample); later this value was multiplied by 10 due to initial dilution (w/v) of fresh roots for each side of the root systems. Each bacterial number value obtained was transformed to logarithmic form for statistical purposes. All treatments explored in the present work had five bacterial number values which were used to calculate the standard deviation and the statistical analysis. To ensure that quantified bacteria corresponded to *G. diazotrophicus* PAL 5<sup>T</sup>, its ability to inhibit a sensitive strain (PAL 3) was checked and electrophoretic mobility patterns of 12 metabolic enzymes were compared with a reference strain<sup>6,7,17</sup>. To achieve this goal, some positive tubes of semisolid LGI media with characteristic growth of *G. diazotrophicus*, were used to streak the bacteria pellicle on solid plates of LGI media and selected colonies were assessed for their ability to inhibit a sensitive strain by the double agar layer method<sup>20,26</sup>. All selected isolates were able to inhibit the growth of *G. diazotrophicus* PAL 3 (an antagonistic characteristic of strain PAL 5<sup>T</sup>), and they also had the same pattern of electrophoretic mobility of the metabolic enzymes explored, as the reference strain *G. diazotrophicus* PAL 5<sup>T</sup> (data not shown).

## Statistical analysis

Data corresponding to each treatment for the different experiments were statistically compared in pairs with the *t*-Student test, using Sigma Plot of the Jandel Scientific Software. Results of comparison were used to generate a matrix of differences and similarities between treatments for assignment of letters (data not shown).

## Results

### Effect of NH<sub>4</sub>NO<sub>3</sub> on *G. diazotrophicus* strains *in vitro*

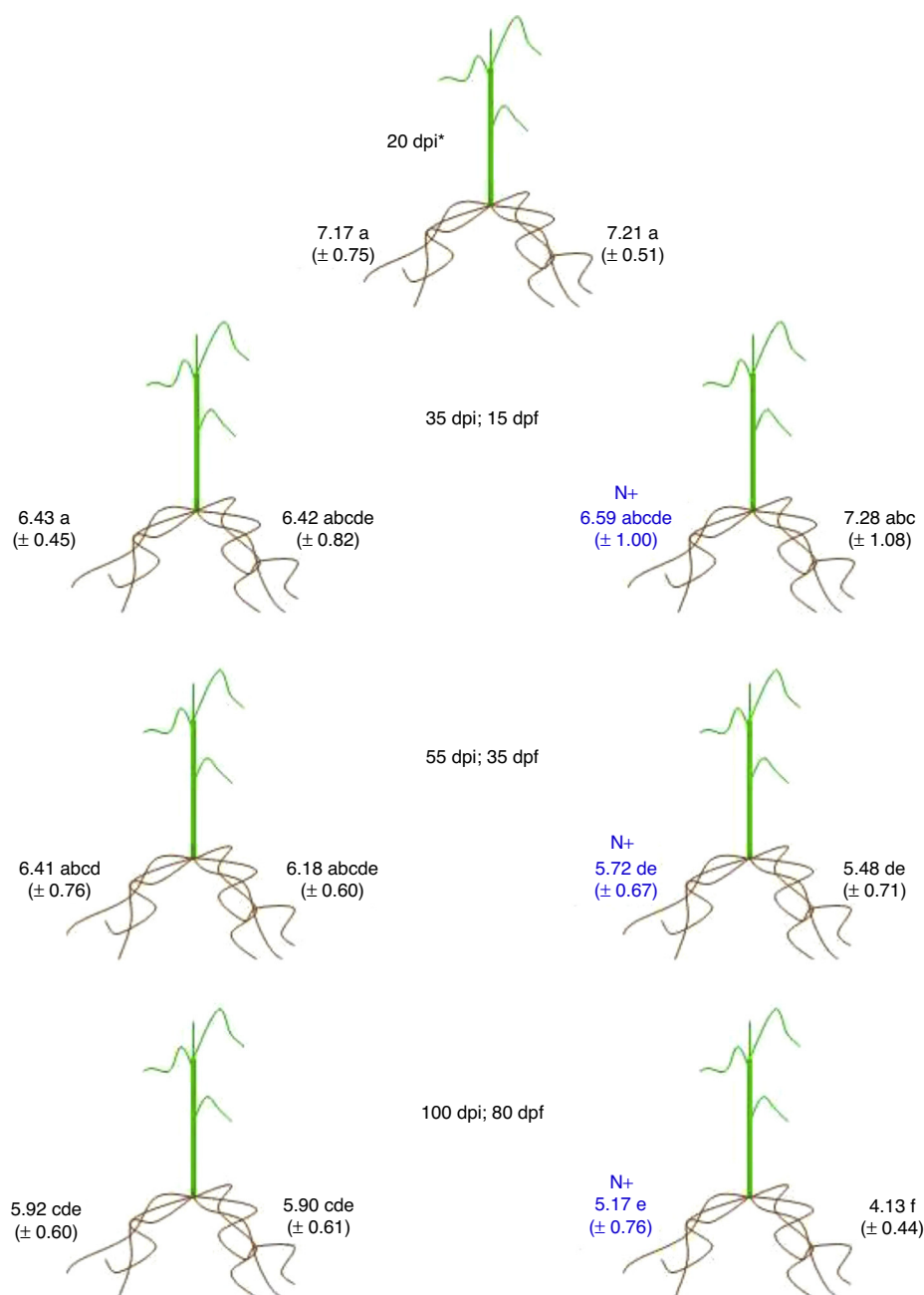
The three strains of *G. diazotrophicus* explored (PAL 5<sup>T</sup>, PAL 3 and UAP 5560) were able to grow in solid media until 22.4 mM of NH<sub>4</sub>NO<sub>3</sub> (Table 1), which is a high nitrogen level and corresponds to 640 mg of N/plant. The statistical analysis showed no differences between bacteria grown in the presence of 22.4 mM of NH<sub>4</sub>NO<sub>3</sub> in comparison with normal LGI. *G. diazotrophicus* UAP 5560 tolerated a concentration of 89.6 mM in solid LGI, but the bacterial number was reduced from 10<sup>8</sup> to 10<sup>5</sup> CFU/ml (Table 1). In semisolid LGI medium it was also observed that the three strains of *G. diazotrophicus*

explored were able to grow until 22.4 mM of NH<sub>4</sub>NO<sub>3</sub> (Table 2); the growth of the strains was affected at 44.8 mM in the order of 10<sup>2</sup> cells/ml and the growth of UAP 5560 also tolerated better than others the presence of high levels of NH<sub>4</sub>NO<sub>3</sub> (89.6 mM); however, under this condition bacterial numbers diminished in the order of 10<sup>1</sup> cells/ml.

### Effect of NH<sub>4</sub>NO<sub>3</sub> on the colonization of sugarcane testing *G. diazotrophicus* PAL 5<sup>T</sup> in split root experiments

The analysis of the population of *G. diazotrophicus* strain PAL 5<sup>T</sup> was carried out using the sugarcane variety MEX-57473 with split root experiments both in rhizospheres as endophytically. Bacteria were not detected in non-inoculated control plants. Rhizospheric population was similar in both sides of the root systems at 20 dpi, about 1 × 10<sup>7</sup> cells/g vermiculite (V) when basal levels of NH<sub>4</sub>NO<sub>3</sub> were present. No statistical differences were observed at 35 dpi (15 dpf) in the rhizospheric population between both sides of the system, neither between treatments fertilized with high levels of NH<sub>4</sub>NO<sub>3</sub> in comparison to those fertilized with basal levels. Furthermore, there were no differences when comparing the same treatments 20 dpi (Fig. 2). In accordance with our data, bacterial rhizospheric population decreased at 55 dpi (35 dpf) in plants fertilized with high levels of NH<sub>4</sub>NO<sub>3</sub> when compared to the initial population observed in plants at 20 dpi. However, no differences were observed between the bacterial numbers recovered from each side of the split root systems neither for the plants fertilized with high levels of NH<sub>4</sub>NO<sub>3</sub> nor for the plants treated with basal levels. Interestingly, at 100 dpi (80 dpf), fertilized plants with high levels of nitrogen had differences in rhizospheric population, detecting low bacterial numbers (around 1 × 10<sup>4</sup> cells/g V) in the pot fertilized with basal nitrogen in comparison with the bacterial numbers detected in the other pot of the system and also when compared to the bacterial numbers of both pots from plants fertilized with low levels of nitrogen, in the order of 10<sup>5</sup> cells/g V (Fig. 2).

The effect of nitrogen on the endophytic population of *G. diazotrophicus* PAL 5<sup>T</sup> was more evident (Fig. 3). Inside the roots, the population of *G. diazotrophicus* was detected in the order of 10<sup>3</sup> cells/g of root at 20 dpi. After the addition of high levels of NH<sub>4</sub>NO<sub>3</sub> in one pot of the split root systems corresponding to the treated plants containing high levels of nitrogen, no changes were observed in the bacterial population measured 35 dpi (15 dpf) when comparing pots of the same system or systems with basal nitrogen. However, 55 dpi (35 dpf), the bacterial population of roots from pots with basal nitrogen showed a decrease (around 50 cells/g of root) in comparison with the bacterial population of roots from pots added with high level of NH<sub>4</sub>NO<sub>3</sub> (around 6 × 10<sup>2</sup> cells/g of root) of the same plant system, and also when compared to the bacterial numbers observed in roots from plants fertilized with low levels of nitrogen (Fig. 3). Similar results were observed 100 dpi (80 dpf) in plants fertilized with high levels of nitrogen. In this case, the population of *G. diazotrophicus* was not detected inside the roots from pots fertilized with basal levels of NH<sub>4</sub>NO<sub>3</sub>, but bacteria were detected in roots from pots fertilized with high levels of nitrogen (around 10 cells/g of root) of the same



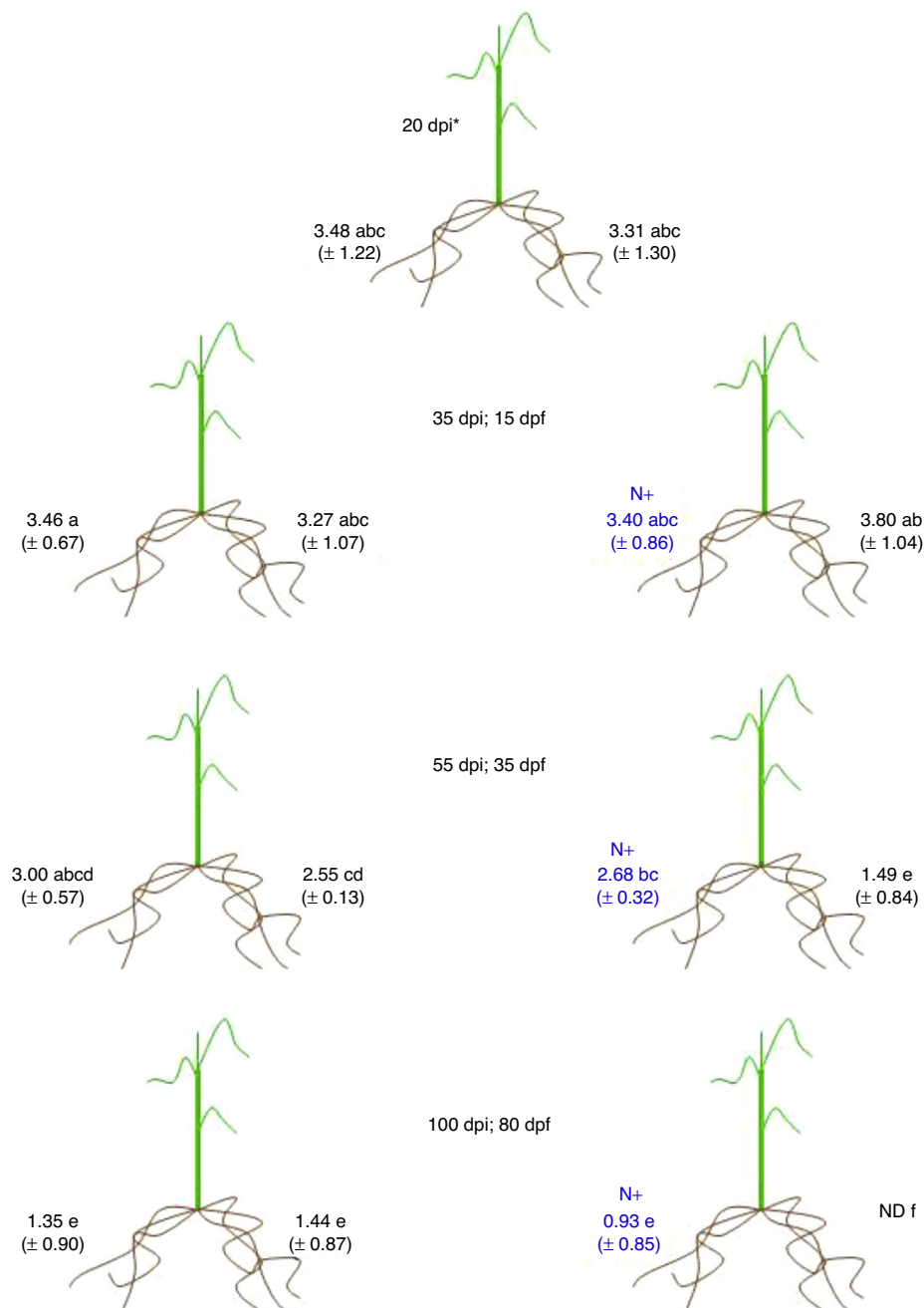
**Figure 2** Rhizospheric bacterial populations in split root experiments. Each value represents the media of data for five independent plants (Log of cell number/g V) with the respective standard deviation. Mean values with equal letters are not statistically different at  $p \leq 0.05$ , using the  $t$ -Student test. dpi: days post inoculation; dpf: days post fertilization; N+: addition of 180 mg of nitrogen/plant.

split root system. These fertilized pots are statistically similar to the bacterial numbers detected in pots from plants fertilized with basal levels of nitrogen (around 40 cells/g of root) (Fig. 3).

## Discussion

Plants require nitrogen for their development. The addition of this component produces key molecules that increase plant growth or the accumulation of metabolic compounds<sup>2,5,11</sup>. Rhizospheric bacteria population could be

modified in response to nitrogen fertilization. It has been shown that ammonium nutrition increased root colonization by *Pseudomonas fluorescens* 2-79RLI at the root tip and in the lateral root zone when the pH of the nutrient solution was allowed to change according to the nitrogen form provided<sup>21</sup>. In contrast, the population of *G. diazotrophicus* associated with sugar cane diminishes after nitrogen fertilization<sup>14,25</sup>, regardless of the form of nitrogen supplemented<sup>22</sup>. This decrease could be explained by pleomorphic changes observed in *G. diazotrophicus* cells when they grow in the presence of high levels of  $\text{NH}_4\text{NO}_3$  (25 mM)<sup>29</sup>. However, in accordance with this study, bacterial cells



**Figure 3** Bacterial population inside roots in split root experiments. Each value represents the media of data for five independent plants (Log of cell number/g root) with the respective standard deviation. Mean values with equal letters are not statistically different at  $p \leq 0.05$ , using the  $t$ -Student test. dpi: days post inoculation; dpf: days post fertilization; ND: not detected; N+: addition of 180 mg of nitrogen/plant.

survive under this nitrogen concentration in culture media. On the other hand, it has also been suggested that the reduction in the number of bacterial cells associated with sugarcane occurs by stimulating changes in plant physiology after nitrogen fertilization<sup>14,31</sup>; however, this hypothesis has not been confirmed yet. In this work, the survival of *G. diazotrophicus* was influenced by 44.8 mM of  $\text{NH}_4\text{NO}_3$  (equivalent to 640 mg N/plant) irrespective of the genotype of *G. diazotrophicus* used (*in vitro* experiments). However, strain UAP5560 was more tolerant to nitrogen

concentrations, showing survival with a decreased number of cells. Nitrogen concentration affecting bacterial survival (44.8 mM) was higher than the level of nitrogen that affects the population associated with sugarcane (6.3 mM equivalent to 180 mg N/plant). Based on the results presented in this work and data previously published<sup>25</sup>, it was reasonable to propose that nitrogen fertilization induces changes in the physiology of plants that prevent *G. diazotrophicus* colonization. To verify this hypothesis, we carried out split root experiments to evaluate the effect of high levels of



$\text{NH}_4\text{NO}_3$  when applied on one side of system, while the population of *G. diazotrophicus* was measured on the other side. For this experiment, strain UAP5560 was applied to the sugarcane variety MEX 57-473, given that this interaction is very stable according to previous data<sup>25</sup>. As we had expected, *G. diazotrophicus* population diminished in the plants fertilized with high levels of  $\text{NH}_4\text{NO}_3$ . This decrease was more evident inside the roots exposed to basal levels of nitrogen, in comparison with the roots fertilized with high nitrogen levels, both in the same plant system. Moreover, this decrease in bacterial population was also evident in plant systems fertilized with low levels in both pots. These observations suggest that the effect of  $\text{NH}_4\text{NO}_3$  on the population of *G. diazotrophicus* occurs through systemic changes in the plant, affecting the establishment of *G. diazotrophicus* in the roots on the other side. This observation was more evident 100 dpi or 80 dpf, when a decrease in the population of *G. diazotrophicus* was observed, due to the age of the plant<sup>25</sup>. Supplementing high levels of  $\text{NH}_4\text{NO}_3$  to sugarcane plants induce a decrease in the sucrose content in stalks in early growth<sup>24</sup> and sucrose has been proposed as the principal carbon source to *G. diazotrophicus*<sup>12</sup>. Furthermore,  $\text{NH}_4\text{NO}_3$  produces changes in the components of the apoplastic sap of sugarcane plants, including aminoacids, proteins, and sugars<sup>38</sup>. Those changes could increase during plant growth and could be related to bacterial diminution. Moreover, exopolysaccharide production is required for biofilm formation and plant colonization by *G. diazotrophicus*<sup>23</sup>, and the changes occurring in the plant could inhibit biofilm formation and bacteria establishment. Finally, some chemical compounds are responsible for inducing a resistance to disease in plants<sup>30</sup>, but could also induce a systemic resistance (ISR) similar to that produced by rhizobacteria<sup>39,41</sup>, making it conceivable that  $\text{NH}_4\text{NO}_3$  could elicit ISR and prevent the colonization of *G. diazotrophicus* in sugarcane<sup>16</sup>. The nitrogen effect over the population of *G. diazotrophicus* is more evident inside the plant than in the rhizosphere (Figs. 2 and 3). This could be due to physiological changes occurring inside the plant and directly affecting the bacterial population; however, in the rhizosphere, firstly the metabolites have to be exported to provoke changes in the environment.

Taken together, the results in this study show that the decrease of *G. diazotrophicus* associated with sugarcane occurs due to changes in the physiology of the plant rather than by the direct effect that  $\text{NH}_4\text{NO}_3$  could exert on bacterial cells.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

## Conflict of interest

The authors declare that they have no conflicts of interest.

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