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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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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 NH_4NO_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 NH_4NO_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 NH_4NO_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 NH_4NO_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 NH_4NO_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
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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 NH_4NO_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 NH_4NO_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 NH_4NO_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 NH_4NO_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*.

Introduction

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

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

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*[14,28,31]. 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[7] 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[14,22,25].

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[29]. 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[7,14].

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[42], but also to evaluate the systemic effect on plant pathogens due to the action of the induced systemic resistance produced by rhizobacteria[1].

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 NH_4NO_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^T, PAl 3 and UAP 5560, each one corresponding to a different genotype. PAl 5^T represents the predominant genotype isolated from different Brazilian sugarcane varieties (ET 3), PAl 3 corresponds to