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Effect of some abiotic factors on the biological activity of *Gluconacetobacter diazotrophicus*

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ABSTRACT

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Aims: The effect of some abiotic factors, dryness, heat and salinity on the growth and biological activity of *Gluconacetobacter diazotrophicus*, and the influence of a salt stress on some enzymes involved in carbon metabolism of these bacteria is studied under laboratory conditions.

Methods and Results: Strain PAL-5 of *G. diazotrophicus* was incubated under different conditions of drying, heat and salinity. Cells showed tolerance to heat treatments and salt concentrations, and sensitivity to drying conditions. Higher NaCl dosage of 150 and 200 mmol l^{-1} limited its growth and drastically affected the nitrogenase activity and the enzymes glucose dehydrogenase, alcohol dehydrogenase, fumarase, isocitrate dehydrogenase and malate dehydrogenase.

Conclusions: *Gluconacetobacter diazotrophicus*, despite its endophytic nature, tolerated heat treatments and salinity stress, but its nitrogenase activity and carbon metabolism enzymes were affected by high NaCl dosage.

Significance and Impact of the Study: The investigation of the biological activity of *G. diazotrophicus* in response to different abiotic factors led to more knowledge of this endophyte and may help to clarify pathways involved in its transmission into the host plant.

Keywords: *Gluconacetobacter diazotrophicus*, abiotic factors, carbon metabolism, endophyte, enzymes, nitrogen fixation.

INTRODUCTION

Gluconacetobacter diazotrophicus is an obligatory aerobe capable of fixing atmospheric nitrogen (Attwood *et al.* 1991; Stephan *et al.* 1991; Alvarez and Martínez-Drets 1995). This bacterium is well adapted to extreme conditions such as high sugar concentrations and low pH (Stephan *et al.* 1991; James and Olivares 1997; Chanway 1998). Despite their capacity to survive in such extreme conditions, survival in soils, particularly in natural soils, is limited (Baldani *et al.* 1997).

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This endophyte possesses an active pentose-phosphate pathway, constituting the main operative pathway for the catabolism of glucose phosphate (Alvarez and Martínez-Drets 1995). The same authors detected a complete tricarboxylic acid cycle in this bacterium, and also respiratory chain-linked enzymes for the oxidation of glucose and keto-gluconates. Glucose dehydrogenase and cytocrome *ba* are the key components of the respiratory system of *G. diazotrophicus* during aerobic diazotrophy (Flores-Encarnación *et al.* 1999). Although it does not transport or metabolize sucrose *per se* (Alvarez and Martínez-Drets 1995), it grows on sucrose because of the extracellular saccharolytic enzyme activity that provides the bacteria with glucose or fructose for growth. In addition, *G. diazotrophicus* grows well on monosaccharides

such as glucose, fructose and galactose, and also on glycerol, ethanol and mannitol, but not on many other carbon compounds such as dicarboxylic acids or maltose (Gillis *et al.* 1989; Li and MacRae 1991; Ureta *et al.* 1995).

Gluconacetobacter diazotrophicus colonizes sugarcane roots, stems and leaves (Cavalcante and Döbereiner 1988; Li and MacRae 1991; Reis *et al.* 1994), where it is present in the intercellular space of parenchyma, and is considered an obligate endophyte. Some reports have shown that this micro-organism can maintain detectable populations in the sugarcane rhizosphere, especially in sugarcane trash (Li and MacRae 1992; Reis *et al.* 1994).

When cultivated in saline soils (semi-arid or arid regions) or under saline-water irrigation, yield and juice quality are reduced in sugarcane (Lingle *et al.* 2000). NaCl accumulates in sugarcane tissues (Chowdhury *et al.* 1997), thereby affecting the physiology and metabolism of the endophyte leading to a significant yield reduction.

Other dinitrogen-fixing bacteria (not obligate endophytes) that establish symbiotic association with legumes such as *Mezorhizobium ciceri* (Soussi *et al.* 2001) and *Rhizobium meliloti* (Breedveld *et al.* 1993) can tolerate 0·4 and 1·0 mol l^{-1} NaCl, respectively, during free-living growth. However, salt-stress responses lead to changes in lipopolysaccharides, protein-profile alterations and accumulation of compatible solutes that may constitute adaptative responses to the stress (Soussi *et al.* 2001).

Many studies have been conducted to identify and physiologically characterize *G. diazotrophicus* (Stephan *et al.* 1991; Sievers *et al.* 1998). However, there is a lack of information about the response of this bacterium to abiotic stresses such as salinity, drying and heat, and of the mechanisms involved in tolerance.

The aim of the present study is to examine how abiotic factors such as drying, heat and salinity can affect the growth and biological activity of *G. diazotrophicus*. We therefore evaluated the metabolic changes produced by a salt stress on fumarase, isocitrate dehydrogenase, malate dehydrogenase, glucose dehydrogenase, gluconate dehydrogenase, 6-phosphogluconate dehydrogenase and alcohol dehydrogenase enzymes.

MATERIALS AND METHODS

Strain

The bacteria used in these experiments were *G. diazotrophicus* strain PAL-5. This micro-organism was isolated from sugarcane (Alagoas, Brazil) by Cavalcante and Döbereiner (1988) and maintained in solid LGIP medium (Reis *et al.* 1994) supplemented with 20 mg l^{-1} yeast extract and stored at −70°C in 20% glycerol (v/v).

Media

G. diazotrophicus was cultured in LGIP medium containing $(NH_4)_2SO_4$ 0·8 mmol l^{-1} and sucrose at 1 or 10% w/v (to determine the effect of salt concentration on growth). The NaCl concentrations of the culture media were prepared within the range of 0–200 mmol l^{-1} at 0, 25, 75, 100, 150 and 200 mmol l^{-1} concentrations.

LGIP liquid medium amended with 10 and 1 mmol l^{-1} $(NH_4)_2SO_4$ was used in the experiments to find out the effect of desiccation and temperature, respectively. The pH values of the media (5·5) were adjusted with acetic acid after heat sterilization.

Growth estimation

The viability of cultures was determined qualitatively by streaking on LGIP agar containing sucrose at 1 and 10% (w/v) amended with 0, 50, 100, 200, 300 and 600 mmol l^{-1} NaCl, or by inoculation into LGIP liquid medium in which the O.D. at 560 nm was used as an index of growth. Viable counts were made by the surface drop-plating method of Miles and Misra (1938) on LGIP agar medium containing sucrose (1% w/v) using five replicate drops (0·02 ml) for each dilution. Incubation was at 30°C for 6 days.

Resistance of cells to desiccation

G. diazotrophicus was grown in LGIP medium containing 10 mmol l^{-1} $(NH_4)_2SO_4$ on a shaker to maintain aerobic conditions for 72 h at 30°C. After three successive transfers of the culture to fresh medium, 50 ml samples were centrifuged at 12 000 × *g* for 10 min. The cells were washed once with sterile saline solution (0·9%), resuspended in a volume of 5 ml saline solution (A_{560}, 0·6–0·8), and then collected on membrane filters (Millipore, 0·45 μm). The filters were then transferred to a dry absorbent pad in a Petri dish and placed in a drying chamber at 30°C. After 0, 1, 2, 3, 5, 7, 14 and 28 days, the dried cells were removed from the filters by shaking vigorously and sonicating for 2 s in sterile saline solution. Measured portions of the resulting suspensions were used for the determination of viable cells in the preparations examined. Viable counts were made by plate counts using LGIP agar medium. Five replicates were prepared from each filter membrane examined.

Effect of temperature

Cells suspensions (A_{560}, 0·6) were grown in LGIP medium containing 1 mmol l^{-1} $(NH_4)_2SO_4$ for 72 h, then injected into capillary tubes and heated to 28, 40, 50 and 60°C, for 2 or 3 h. Following thermic treatment, the tubes were cooled to 4°C and the viable cells detected on LGIP agar medium.

Assay for sensitivity to salt concentration

Gluconacetobacter diazotrophicus was grown in LGIP medium on a shaker with gentle agitation for 72 h at 30°C. After three successive transfers of the culture to fresh medium, cells were harvested by centrifugation (12 000 × *g*, 10 min) and resuspended in sterile saline solution (0·9%) to yield an O.D. of 0·6 at 560 nm. Aliquots (1 ml) of the cell suspensions were transferred to 250 ml Erlenmeyer flask containing 50 ml of the fresh medium to which NaCl was added at concentrations of 0, 25, 75, 100, 150 and 200 mmol l^{-1}. The growth estimation after 36 h was determined as described above.

Enzyme activities related to carbon metabolism

G. diazotrophicus was grown in an LGIP medium on a shaker with gentle agitation (180 rev min^{-1}) for 48 h at 30°C. After three successive transfers of the culture to fresh medium, cells were harvested by centrifugation (12 000 × *g*, 10 min) and resuspended in sterile saline solution to yield an O.D. of 0·9 at 560 nm. Aliquots (1 ml) of the cell suspensions were transferred to 250 ml Erlenmeyer flasks containing 50 ml of LGIP medium containing 0·8 or 10 mmol l^{-1} $(NH_4)_2SO_4$ and NaCl at concentrations of 0, 75, 150 and 200 mmol l^{-1} and incubated at 30°C for 36 h under aerobic conditions (180 rev min^{-1} in a shaker).

After being grown under different conditions indicated as previously, the cells were harvested by centrifugation at 12 000 × *g* for 10 min, washed twice with 50 mmol l^{-1} sterile phosphate-buffer (pH 7·0) and resuspended in a volume of 1 ml of buffer. The cells were stored at 4°C until disruption by sonication at 60 W with 5-s intervals for 7 min. Intact cells and debris were removed by centrifugation at 12 500 × *g* for 15 min. Clear supernatants were separated for enzyme evaluation. All operations were carried out at 4°C, and enzyme activities were monitored between 2 and 4 h.

The following enzymatic activities related to carbon metabolism were assayed: fumarase (EC 4.2.1.2; Kanarek and Hill 1964), isocitrate dehydrogenase (EC 1.1.1.42; Chen and Cullimore 1988), malate dehydrogenase (EC 1.1.1.37; Alvarez and Martínez-Drets 1995), NAD-glucose dehydrogenase (EC 1.1.1.47; Levering *et al.* 1988), NAD-gluconate dehydrogenase (EC 1.1.1.69; Levering *et al.* 1988), NAD-6-phosphogluconate dehydrogenase (EC 1.1.1.42; Kochetov 1982) and alcohol dehydrogenase (EC 1.1.1.1; Thynn and Werner 1996). The evolution of the enzymatic activity was followed by O.D. (340 nm) at 30°C except malate dehydrogenase that was measured with an artificial electron acceptor (DCIP; Panreacquimica, S.A., Barcelona, Spain) to 590 nm, and fumarase that was measured at 240 nm at 25°C. NADH dehydrogenase interference was avoided by adding Triton X-100 (2%) to the reaction.

Nitrogenase activity assay

For the assay of nitrogenase activity the procedure reported by Burris (1974) was used. Bacteria were grown in 250 ml Erlenmeyer flasks at 30°C, containing 50 ml of LGIP medium supplemented with 0·8 mmol l^{-1} $(NH_4)_2SO_4$. After 48 h, aliquots (2 ml) were transferred to 12 ml serum vials containing 100 and 200 mmol l^{-1} of NaCl concentrations, and then incubated at 30°C for 2 h under gentle agitation (180 rev min^{-1}). The cotton plugs were carefully replaced by suba-seal and acetylene was injected to a final concentration of 10% (v/v). Gas samples (0·2 ml) were assayed for ethylene at 30, 60 and 90 min on a Konic gas chromatograph (Konik Instruments, Inc., Miami, FL, USA) fitted to a 200 cm Porapak-R column (Konik Instruments) and a hydrogen flame ionization detector. The results were obtained calculated by using a standard ethylene (0·2 nmol) and expressed as nmol C_2H_4 (mg prot.)$^{-1}$.

Protein determination

Protein content was determined by the Lowry method (Lowry *et al.* 1951) with Folin Ciocalteu reagent and using bovine serum albumin as a standard.

Statistical design and analysis

In all experiments, the values were the mean of three replicates per treatment, except for the enzyme activity assays and protein content in which five replicates were performed. The results were subjected to a one- or two-way analysis of variance with a least significant difference (LSD) test between mean values.

RESULTS

The data in Fig. 1 show that strain PAL-5 survives in dry conditions for a period of 28 days. Although, >95% of the total vegetative cells were killed within 1 day of drying, only a very slight further drop in the viability of the cells was noted in the subsequent days of drying (Fig. 1).

Preliminary experiments showed that strain PAL-5 grew well in LGIP medium at 28°C but failed to grow at 65°C. For this reason, as the bacteria were suspected to be sensitive to a particular incubation temperature, the effect of temperature on growth in LGIP medium was examined between 28 and 60°C. The results are shown in Fig. 2.

Gluconacetobacter diazotrophicus strain PAL-5 shows different pattern of growth when cultured in LGIP solid media containing different concentrations of NaCl

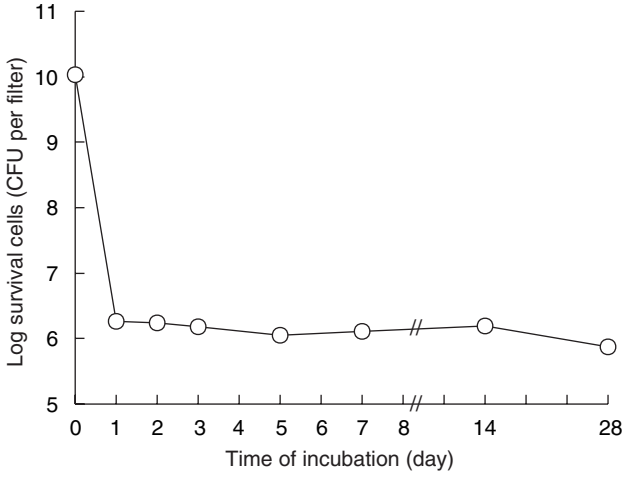

Fig. 1 Effect of desiccation on vegetative cells of *G. diazotrophicus*. The cells were collected on membrane filters and incubated at 30°C

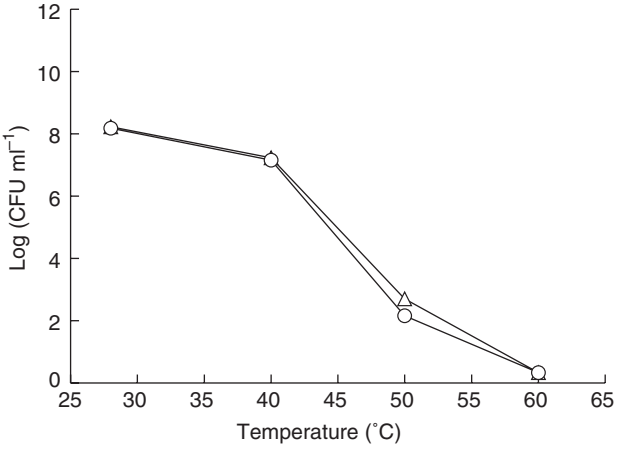

Fig. 2 Effect of temperature after 2 (△) or 3 h (○) of heat treatment on *G. diazotrophicus* PAL-5 strain at 36 h growth

(0–600 mmol l^{-1}) and sucrose (1 and 10% w/v). The results obtained in these experiments showed (Table 1) that the tolerance of strain PAL-5 to salt concentration, under free-living conditions, could be affected by the concentration of sucrose in the culture media. In this sense, strain PAL-5 did not grow (colony formed units) on LGIP media containing 100 mmol l^{-1} NaCl and 1% sucrose. However, *G. diazotrophicus* grew in LGIP media with 200 mmol l^{-1} NaCl and 10% sucrose.

The growth of *G. diazotrophicus* in LGIP liquid media (Fig. 3) confirmed that the highest tolerance of these bacteria to salt concentrations were obtained in culture media amended with 10% (w/v) sucrose. Thus, the presence of 150 and 200 mmol l^{-1} of NaCl in LGIP medium with 1% (w/v) sucrose inhibited growth of these

Table 1 Growth of *G. diazotrophicus* PAL-5 in LGIP solid media containing different NaCl and sucrose concentration after incubation at 30°C for 6 days

	NaCl concentration (mmol l^{-1})					
Sucrose %	0	50	100	200	300	600
1	+++	+++	+++	–	–	–
10	+++	++	++	+	–	–

Growth (colonies) after 6 days rated as (–), (+), (++) and (+++).

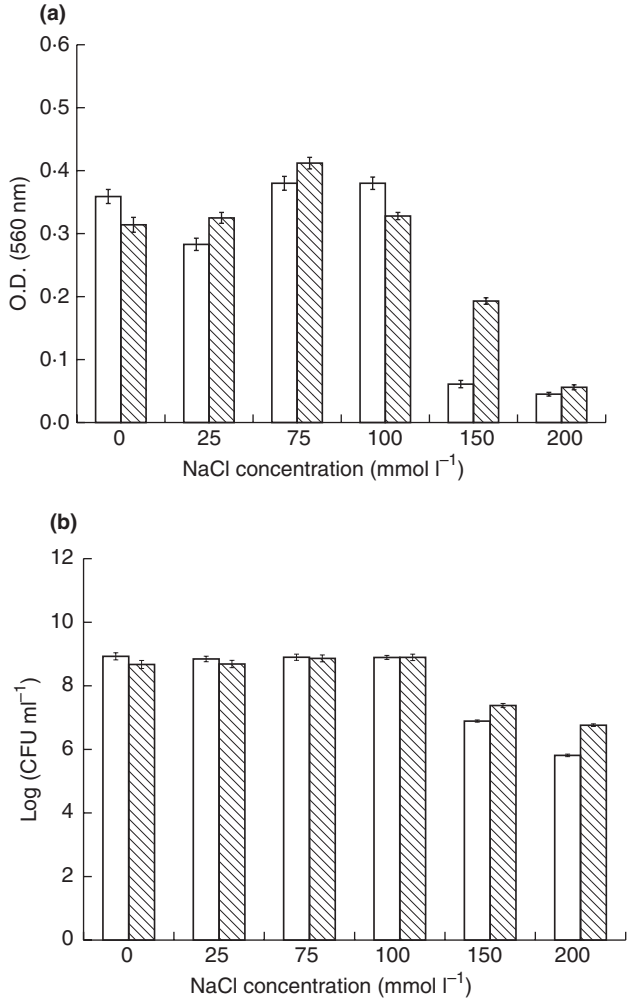

Fig. 3 Growth of *G. diazotrophicus* incubated at 30°C for 36 h in LGIP liquid medium amended with different NaCl and sucrose concentrations: (a) O.D. 560 nm; (b) log colony forming units ml^{-1}. Symbols: □ sucrose 1%; ▨ sucrose 10%

micro-organisms when compared with the growth obtained in LGIP medium containing 10% (w/v) sucrose. This data show that concentration of sucrose could affect salt tolerance when *G. diazotrophicus* was cultured in LGIP media. It can

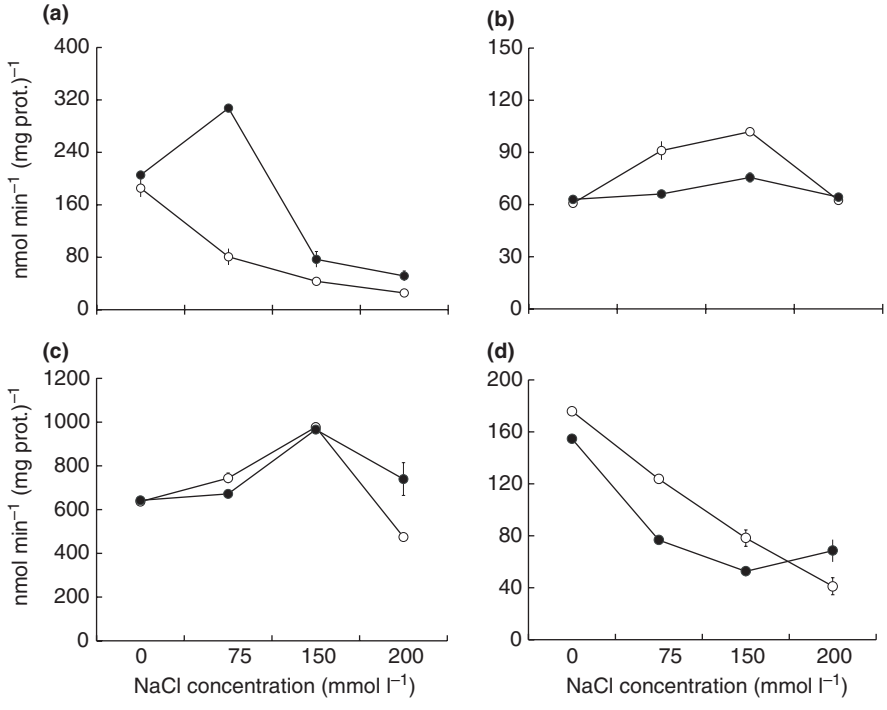

Fig. 4 Enzyme activities in *G. diazotrophicus* growth in LGIP liquid medium with different NaCl and ammonium concentrations after 36 h of growth. (a) Glucose dehydrogenase; (b) gluconate dehydrogenase; (c) 6-phosphogluconate dehydrogenase; and (d) alcohol dehydrogenase. Symbols: ○ ammonium 0·8 mmol l^{-1}; ● ammonium 10 mmol l^{-1}

be concluded that high concentrations of sucrose caused an increased tolerance of strain PAL-5 to NaCl concentration, probably as a consequence of adaptation to the stronger osmotic conditions imposed for the carbon source (sucrose) in the culture media.

Enzymatic activities of *G. diazotrophicus* strain PAL-5 were affected by the NaCl and ammonium concentrations added to the culture media (Figs 4 and 5). Thus, glucose dehydrogenase and alcohol dehydrogenase activities were drastically reduced when the concentration of NaCl was increased in the growth media. However, these inhibitory effects were also influenced by the ammonium concentrations. In this context, for example, when strain PAL-5 was grown as a non-fixer bacteria [10 mmol l^{-1}, $(NH_4)_2SO_4$], glucose dehydrogenase was less affected by the salt stress, than when these micro-organisms grew as nitrogen fixers [0·8 mmol l^{-1}, $(NH_4)_2SO_4$]. In addition, highest values of alcohol dehydrogenase and gluconate dehydrogenase activities were observed when *G. diazotrophicus* was grown under nitrogen-fixing conditions. Finally, gluconate dehydrogenase and 6-phosphogluconate dehydrogenase activities were increased by 75 and 150 mmol l^{-1} of NaCl, suggesting that these biological activities may be stimulated by certain NaCl concentrations.

Tricarboxylic acid cycle enzymes assayed (Fig. 5) decreased with the increasing concentrations of NaCl. In cells cultivated with the highest salt dosage, the inhibition produced in fumarase, isocitrate dehydrogenase and malate dehydrogenase were 85, 60 and 90%, respectively. These results prove that these enzymes were very sensitive to salt concentrations. However, these activities were not affected by the concentrations of ammonium added to the growth media and consequently with or without nitrogen fixation activity in the cell cultures.

Sodium chloride produced a partial or almost total inhibition of the nitrogenase activity (acetylene–ethylene assay) of *G. diazotrophicus* at 100 and 200 nmol l^{-1} NaCl, respectively (Fig. 6). Under these culture conditions, up to 150 and 0·8 mmol l^{-1} ammonium concentration a lack of growth was observed (Fig. 3). However, lower concentration of NaCl did not affect bacterial growth.

DISCUSSION

The results presented show that *G. diazotrophicus* PAL-5 strain can grow well in free-living conditions with salt dosage until 100 mmol l^{-1}. The best tolerance found with the high salt concentrations (150–200 mmol l^{-1}) was in presence of 10% sucrose, and it seems to be mediated by a bacterium regulatory process. Experimental results obtained in the last few years proved that the response of *G. diazotrophicus* to sucrose concentration in the medium could be interpreted as osmotolerance (Boddey *et al.* 1991). The nitrogenase activity [acetylene reduction activity (ARA)] of *G. diazotrophicus* grown in 10% sucrose with a final pH of 2·3 is less inhibited by 5 mmol l^{-1} NH_4Cl than when grown in medium with 1% sucrose (Reis *et al.* 1990). Under these same conditions, the $^{15}NH_4^+$ assimilation by

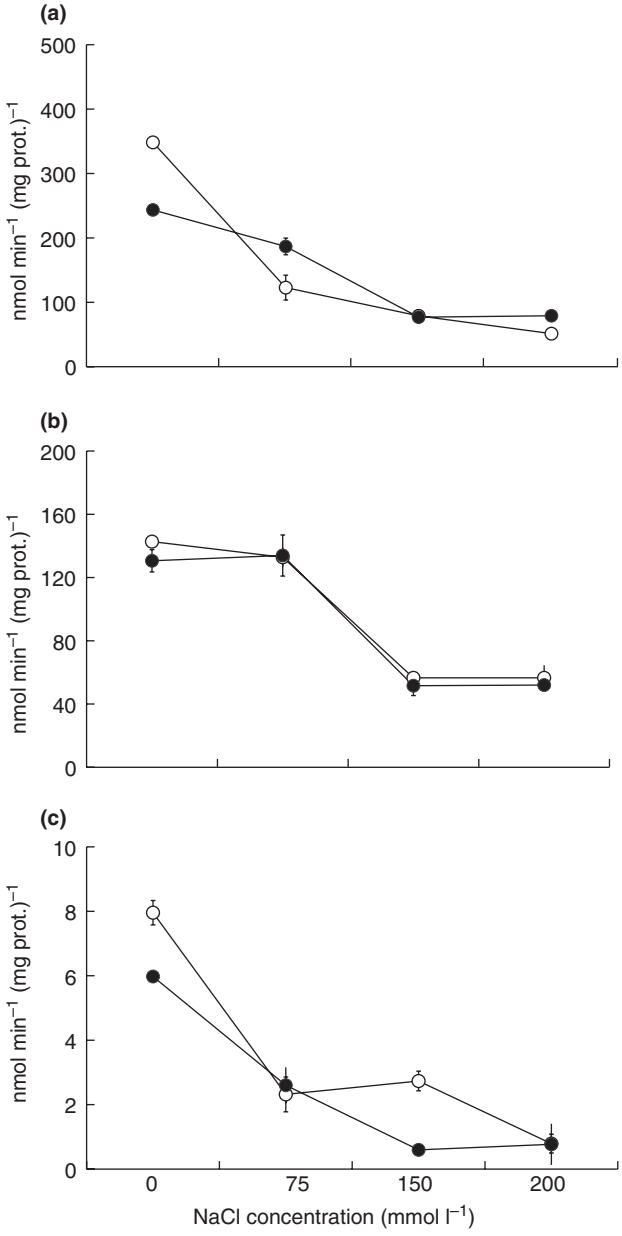

Fig. 5 Enzyme activities of tricarboxylic acid cycle: (a) fumarase; (b) isocitrate dehydrogenase; and (c) malate dehydrogenase in *G. diazotrophicus* growth in LGIP liquid medium with different NaCl and ammonium concentrations. Symbols: ○ Ammonium 0·8 mmol l^{-1}; ● ammonium 10 mmol l^{-1}

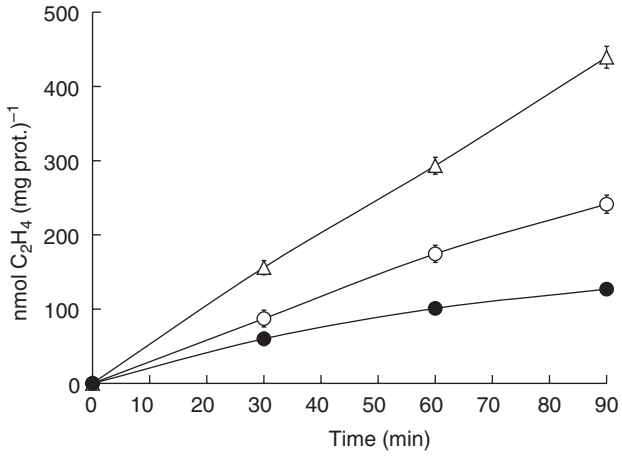

Fig. 6 Acetylene reduction activity (ARA) in *G. diazotrophicus* growth in LGIP liquid medium at 30°C for 48 h. The activity was measured after 2 h in the presence of several NaCl concentrations. Symbols: △ control; ○ NaCl 100 mmol l^{-1}; ● NaCl 200 mmol l^{-1}

whole cells is slower with 10% sucrose than in cells grown in 1% sucrose (Boddey *et al.* 1991). In addition, these authors found that cells grown in 10% sucrose were more tolerant to 1% NaCl (171 mmol l^{-1}) in semisolid medium. The process of *G. diazotrophicus* tolerance to salt could involve the production of different compatible organic metabolites such as glutamine, proline and glycine betaine or inorganic solutes (Na^+, K^+ and Mg^{2+}), as has been reported in other bacteria that live in saline soils (Sleator and Hill 2001).

Gluconacetobacter diazotrophicus is found to be very sensitive to other abiotic factors such as heat and dryness, but these results confirm that a temperature of 50°C for 2–3 h, usually used for pathogen control by cane growers, does not completely destroy this endophyte (Ortega *et al.* 2001). Therefore the partial inhibition in the nitrogenase activity by heat treatment (50°C, 2 h) detected by Ortega *et al.* (2001), could, in part, be explained by the decrease in the endophyte population. However, despite the sensitivity of the PAL-5 strain to dryness, the remaining live cells detected at 28 days of incubation support the presence of *G. diazotrophicus* in the sugarcane trash (Reis *et al.* 1994).

The different activities shown by the enzymes responsible for the direct oxidation of glucose and gluconate, under salt stress, prove that the enzymes, glucose dehydrogenase and gluconate dehydrogenase are sensitive and salt tolerant, respectively. It was expected that both enzymes activities increase under nitrogen fixation, supported by its role in the respiratory protection mechanism of nitrogenase (Alvarez and Martínez-Drets 1995; Flores-Encarnación *et al.* 1999). In PAL-3 strain, higher O_2 tolerance of nitrogenase in the presence of glucose and gluconate (substrates of glucose dehydrogenase and gluconate dehydrogenase) was observed by Stephan *et al.* (1991). Besides, the increase of gluconate dehydrogenase activity under nitrogen fixation and with salt dosage of 75 and 150 mmol l^{-1}, support the result obtained by Flores-Encarnación *et al.* (1999) who detected higher respiratory-

specific activities in membranes of bacteria growth in medium with low ammonium, in PAL-5 strain. However despite this, the authors found that glucose dehydrogenase was affected by the high NH_4^+ concentration of the medium. This effect was not found in this study, perhaps because of low ammonium levels (10 mmol l^{-1} and not 40 mmol l^{-1}). The activation of 6-phosphogluconate dehydrogenase by NaCl could be the result of two effects combined, its salt tolerance, and presumably by the inhibition of glucose dehydrogenase. The increase of gluconate dehydrogenase, despite the inhibition of glucose dehydrogenase, suggests that considerable quantity of gluconate should have been available in the culture medium from the beginning of growth. The high values of 6-phosphogluconate dehydrogenase activity presented here confirm the substantial contribution of pentose phosphate pathway to glucose catabolism as in PAL-3 strain (Alvarez and Martínez-Drets 1995). However, the inhibition of glucose dehydrogenase by NaCl, could explain the partial effect detected in nitrogenase activity (ARA) in 100 mmol l^{-1} of NaCl, taking into account its role in the respiratory protection mechanism of nitrogenase (Flores-Encarnación *et al.* 1999).

The enzyme of the fermentative pathway, alcohol dehydrogenase is sensitive to NaCl as glucose dehydrogenase. The highest values in alcohol dehydrogenase activity obtained under nitrogen fixation seem to be related to the increase of gluconate dehydrogenase activity in this condition, that led to a slower intercellular oxygen concentration and therefore induced the activation of fermentative pathways. The enzymes of tricarboxylic acid cycle assayed were present in this strain, despite its incapacity to grow in dicarboxylic acid (Li and MacRae 1991; Ureta *et al.* 1995). These enzymes play a small role in this bacterium but were very sensitive to NaCl.

In summary, the results presented here showed that of the abiotic factors tested *G. diazotrophicus* was very sensitive to dryness conditions, by the contrary the cells expressed a higher tolerance to the heat treatments and salt concentrations in the culture media. In addition, the sodium chloride concentration drastically affected glucose dehydrogenase, alcohol dehydrogenase, tricarboxylic acid cycle enzymes and nitrogenase activity of this endophyte suggesting that this enzymes could be sensitive to saline stress.

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