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Plant cell-wall degrading hydrolytic enzymes of *Gluconacetobacter diazotrophicus*

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Abstract
The strains PAl5 and UAP5541 of *Gluconacetobacter diazotrophicus* were able to produce endoglucanase (EG), endopolymethylgalacturonase (EPMG) and endoxyloglucanase (EXG) enzymes using sucrose or the corresponding substrate as the sole source of carbon. The results indicate that the hydrolytic activity of the PAl5 strain was inducible, whereas that of the UAP5541 strain seems to be constitutive (regardless of the concentration of sucrose used). When the concentration of the enzymatic substrates was increased in the culture medium, the production of hydrolytic enzymes decreased. The different behaviour of the strains PAl5 and UAP5541 grown in the culture medium with different concentrations of sucrose and the enzymatic substrates demonstrates, that the microbial response and the control of the enzymatic expression of *G. diazotrophicus* strains were different and complex and may be important in their capacity to penetrate plant root as well as in their competitive capacity against other microorganisms.

Keywords: *Gluconacetobacter diazotrophicus*, endoglucanase, endopolymethylgalacturonase, endoxyloglucanase, hydrolytic enzymes

1. Introduction

Gluconacetobacter diazotrophicus (Yamada et al., 1997) is a nitrogen-fixing bacterium that has been found in *Saccharum* spp. (Cavalcante and Döbereiner, 1988), *Perkinsiella saccharicida* (Ashbolt and Inkerman, 1990), *Pennisetum purpureum*, *Ipomea batata* (Paula et al., 1991) and *Coffea arabica* (Jimenez-Salgado et al., 1997). The ability of these bacteria to colonize *Sorghum vulgare* and *Zea mays* has been demonstrated (Isopi et al., 1995; Caballero-Mellado et al., 1998). *G. diazotrophicus* is of great interest for sustainable agriculture because it is able to fix nitrogen in the presence of KNO_3 at low pH values and high sugar concentrations, and can excrete approximately half of the concentrated nitrogen on potential forms that can be assimilated by the plants (Stephan et al., 1991; Cojho et al., 1993).

The mechanism by which *G. diazotrophicus* penetrates the plant root is still not known. Preliminary works have demonstrated that the bacteria requires young radicular tissues and the probable generation of infection threads similar to the infection process of leguminous plants by *Rhizobium* (James et al., 1994). It is known that the cell-wall degrading enzymes cellulases, hemicellulases and pectinases are implicated in the penetration of roots by beneficial plant microorganisms such as *Rhizobium* (Mateos et al., 1992; Jimenez-Zurdo et al., 1996), *Frankia* (Igual et al., 2001), and arbuscular mycorrhizal fungi (Garcia-Romera et al., 1991; Garcia-Garrido et al., 2000). Therefore, *G. diazotrophicus* should have the aptitude to produce extracellular enzymes that allow the bacteria to penetrate the root cell in order for it to establish itself inside the plant. It is known that *Rhizobium* cellulases and pectinases are cell bound and associated with the cell envelope, whereas the cellulases produced by *Frankia* are of extracellular nature and these enzymes of *Rhizobium* and *Frankia* are of a constitutive nature (Mateos et al., 1992; Igual et al., 2001). However, the hydrolytic enzymes of the arbuscular mycorrhizal fungi seem to be of an inducible nature (Garcia-Romera et al., 1991; Garcia-Garrido et al., 2000). The aim of this work was to study whether *G.*

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diazotrophicus was able to produce cellulases, hemicellulases (xyloglucanases) and pectinases in the presence of sucrose and enzymatic substrates as carbon sources.

2. Materials and Methods

Organism and culture conditions

The experiments were carried out with the bacterial strains of *Gluconacetobacter diazotrophicus* PAl5 (ATCC 49037), isolated from sugar cane in Brazil (Cavalcante and Döbereiner, 1988), and UAP5541, isolated from sugar cane in Mexico (Fuentes-Ramirez et al., 1993). The strains were maintained at 4°C in an LGI medium (pH 6.2) which contained (g^{-1} l): 0.2 K_2HPO_4, 0.6 KH_2PO_4, 0.2 $MgSO_4$ $7H_2O$, 0.02 $CaCl_2$ $2H_2O$, 0.002 $NaMoO_4$ $2H_2O$, 0.01 $FeCl_3$ $6H_2O$, 100 sucrose and 20 agar. Five ml of bromothymol blue (9.5% in 0.2 M KOH) were also added (Cavalcante and Döbereiner, 1988). All bacterial strains were grown in SYP basal medium (pH 6.2) which contained (g^{-1} l): 10 sucrose, 1 yeast extract, 1 K_2HPO_4 and 3 KH_2PO_4 (Caballero-Mellado and Martinez-Romero, 1994), using different sucrose and enzymatic substrate combinations as the carbon source. In the first assay, *G. diazotrophicus* strains were grown in 0, 0.5, 1, 2.5, 5, and 10 g^{-1} l of sucrose as the sole carbon source. In the second assay, *G. diazotrophicus* strains were grown in 0, 0.25, 0.5, 0.75 and 1 g^{-1} l of the enzymatic substrates carboxymethylcellulose (CMC from Sigma), pectin (citrus pectin from Sigma) or xyloglucan as the sole carbon source. Xyloglucan was extracted from nasturtium seeds (*Tropaeolum majus* L.) as has been described (McDougall and Fry, 1989). In the third assay, the concentration of 0, 0.5, 1, 2.5, 5, and 10 g^{-1} l of sucrose in presence of 0.25, 0.5, 0.75 and 1 g^{-1} l of each of the enzymatic substrates were used as the carbon source in the growth medium of *G. diazotrophicus* strains. The bacteria were grown at 28°C with orbital shaking at 200 rpm in Erlenmeyer flasks (125 ml) containing 50 ml of culture medium. Cultures harvested at the stationary phase (9×10^8 cells per ml) were centrifuged at 5,000 x g for 15 min and the supernatants were used as extracellular enzyme extracts.

Total proteins were measured (Bradford, 1976) using a Bio-Rad kit with BSA as the standard.

Enzyme assays

The extracellular enzyme extracts were assayed to determine the activities of endoglucanase (EG) (EC 3.2.1.4), endopolymethylgalacturonase (EPMG) (EC 3.2.1.15) and endoxyloglucanase (EXG) (3.2.1.151). All hydrolytic activities were assayed by the viscosity method (Rejón-Palomares et al., 1996) using CMC, citrus pectin and xyloglucan from nasturtium seeds as substrates to determine EG, EPMG and EXG, respectively. The EG, EPMG and EXG enzymes have high substrate specificity and the viscosity method is the best test to measure the different hydrolytic activities of plant roots colonized by different symbiotic microorganisms (Garcia-Garrido et al., 2000; Aranda et al., 2005).The reduction in viscosity was determined at 0–30 min intervals. Approximately 0.8 ml of the reaction mixture was sucked from a 2 ml tube into a 1-ml syringe, was then allowed to flow down to the 2 ml tube and the time taken for the meniscus to flow from the 0.70 ml to the 0.20 ml mark (between 1–3 min) was recorded. The reaction mixture in the 2 ml tube contained 1 ml of 0.5% substrate in 50 mM citrate-phosphate buffer (pH 5.0) and 0.2 ml of extracellular enzyme extracts. Viscosity reduction was determined at 37°C (Rejón-Palomares et al., 1996). One unit of the enzyme activity was expressed as the specific activity (U = RA mg^{-1} protein) where RA is the relative activity calculated by applying the formula: $\%V = T_0 - T_A \times 100 \times T_0^{-1}$, $T_{50} = 50 \times T_A \times \%V^{-1}$ (Bateman, 1963). RA = $T_{50} \times 10^3$ is the reciprocal of time in h for 50% viscosity loss. T_0 is the viscosity of the reaction mixture at 0 time, T_A is the viscosity of the reaction mixture at 30 min, V is the viscosity loss of the reaction mixture at 30 min and T_{50} is the time necessary to reach a 50% of viscosity loss of the reaction mixture at 30 min. The control for all enzyme assays were autoclaved enzyme supernatants and autoclaved buffers.

Statistical treatments

Each datum is the average of five replicate samples. The data were analysed by using the one-way ANOVA followed by the standard errors of means test (P=0.05).

3. Results

When the strains UAP5541 and PAl5 of *G. diazotrophicus* grew in presence of sucrose as the only carbon source, the hydrolytic activities EG, EPMG and EXG were detected in the culture media of both strains. Fig. 1 shows that the specific activities of the above enzymes, found in the supernatant, were increased as the concentration of sucrose increased in the culture medium of the strain PAl5, whereas the hydrolytic activities of UAP5541 strain were not significantly increased. Under the same culture conditions, the strain PAl5 produced a higher quantity of hydrolytic enzymes than the strain UAP5541, especially when bacteria were grown in a higher concentration than 5 g^{-1} l of sucrose.

Production of EG, EPMG and EXG activities by the strains of *G. diazotrophicus* that grew in the culture medium and contained the substrate of each enzyme (cellulose, pectin or xyloglucan) as sole carbon source, was detected (Fig. 2). In the UAP5541 strain the highest enzymatic activity of the three enzymes was observed when 0.25 g^{-1} l of the

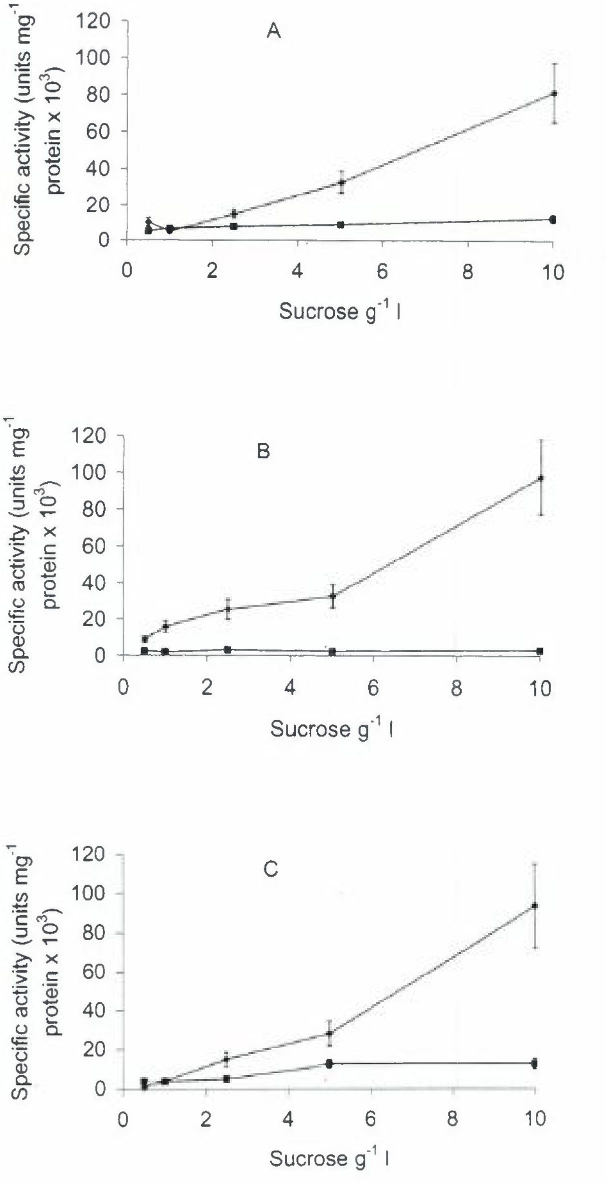

Figure 1. Endoglucanase (A), endopolymethylpolygacturonase (B) and endoxyloglucanase (C) activities of PAl5 (◆) and UAP5541 (■) of *Gluconacetobacter diazotrophicus* grown in presence of sucrose in the culture medium. Data are the means ± standard errors of mean of five replicate samples.

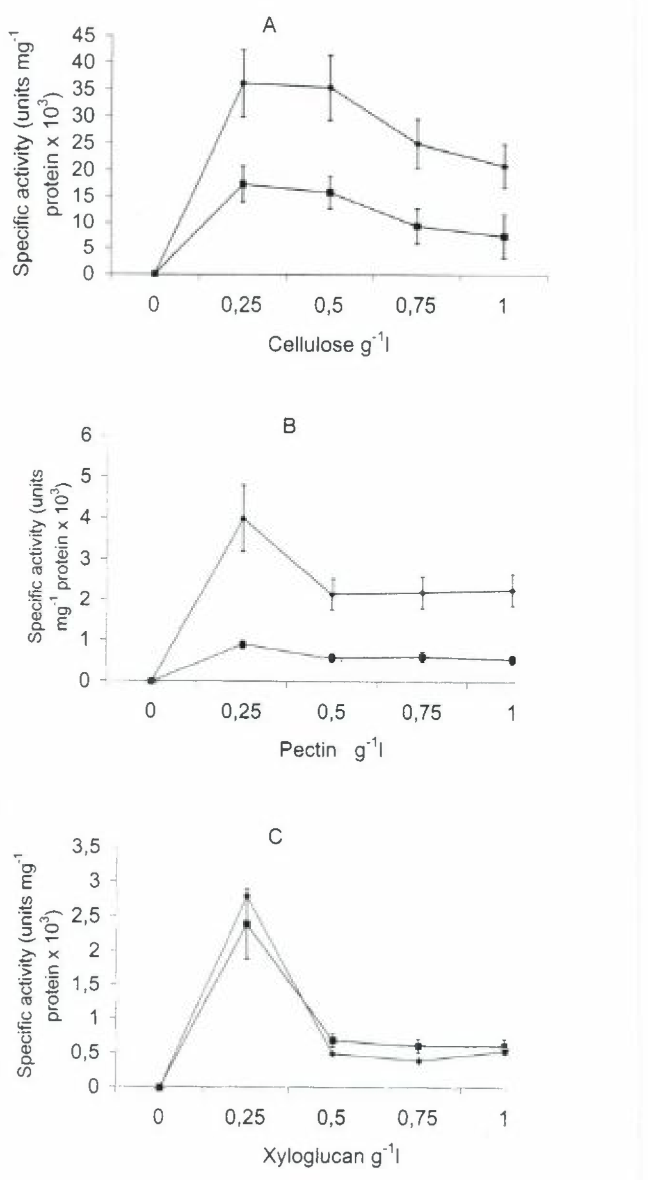

Figure 2. Endoglucanase (A), endopolymethylpolygacturonase (B) and endoxyloglucanase (C) activities of PAl5 (◆) and UAP5541 (■) of *Gluconacetobacter diazotrophicus* grown in presence of the corresponding enzymatic substrate, CMC, citrus pectin and xyloglucan, in the culture medium. Data are the means ± standard errors of mean of five replicate samples.

substrates were added to the culture medium, but when the concentration of substrates was higher than 0.25 g^{-1} l, the activities of the three enzymes decreased. Higher EG and PMG activities in UAP5541 than in PAl5 strains was detected. However, non-significant differences in EXG activities between UAP5541 and PAl5 strains grown in the presence of whichever substrate concentration were observed (Fig. 2).

The effect of carbon sources was studied by adding different amounts of sucrose together with different concentrations of enzymatic substrates to the culture medium. Fig. 3A shows that the PAl5 strain grown in basal medium plus sucrose and cellulose produced less endoglucanase activity than when the bacteria were grown in a culture medium with sucrose as the sole carbon source. The combined application of 0.25 g^{-1} l cellulose and 5 g^{-1} l sucrose induced higher endoglucanase activity than the other treatments in which sucrose and cellulose were added together. The endoglucanase activity of PAl5 strains decreased as the concentration of cellulose increased (Fig. 3A). The application of sucrose and cellulose to the culture medium of the UAP5541 strain increased endoglucanase activity to a higher level than when sucrose alone was

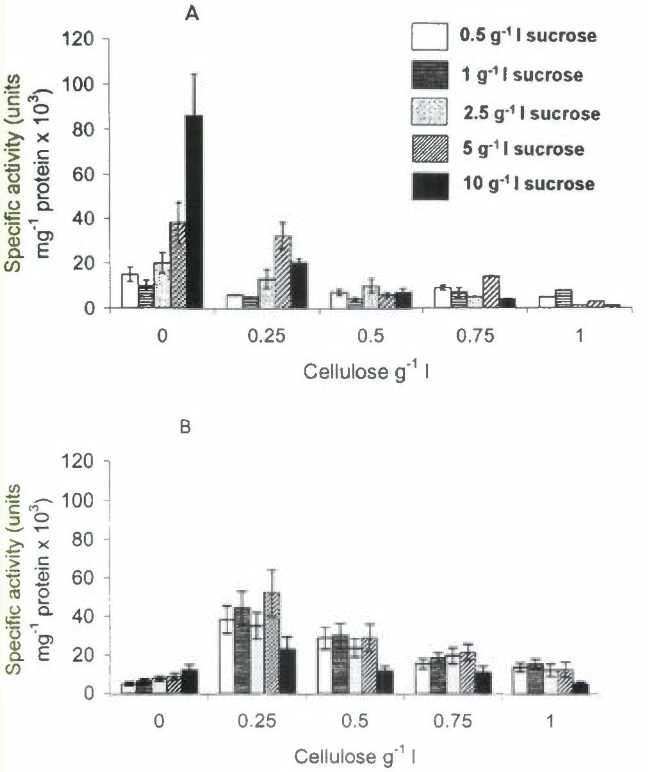

Figure 3. Endoglucanase activity of PAl5 (A) and UAP5541 (B) of *Gluconacetobacter diazotrophicus* grown in presence of sucrose and CMC in the culture medium. Data are the means ± standard errors of mean of five replicate samples.

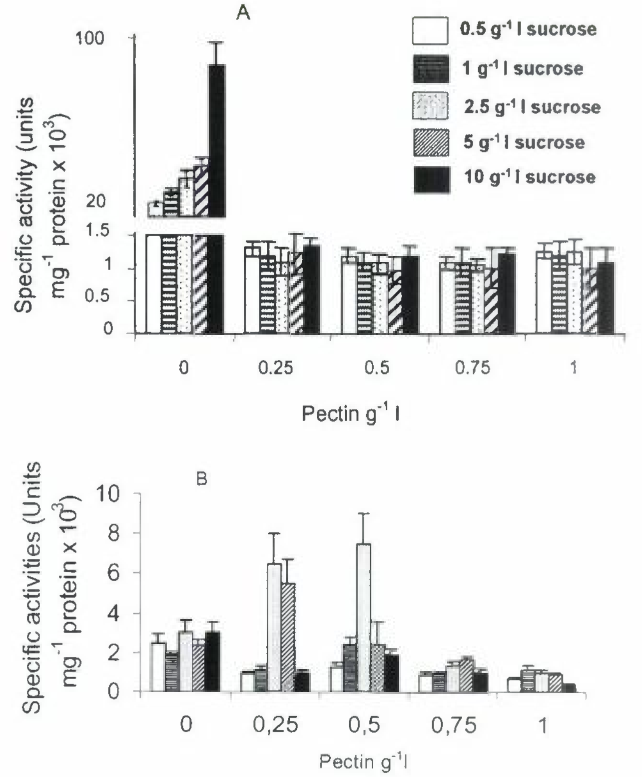

Figure 4. Endopolymethylpolygacturonase activity of PAl5 (A) and UAP5541 (B) of *Gluconacetobacter diazotrophicus* grown in presence of sucrose and citrus pectin in the culture medium. Data are the means ± standard errors of mean of five replicate samples.

added. The UAP5541 strain produced less endoglucanase activity when grown in the presence of 0.5, 0.75, and 1 g^{-1} l cellulose than in the presence of 0.25 g^{-1} l cellulose. Within each cellulose concentration used the endoglucanase activity was only decreased in the presence of 10 g^{-1} l sucrose (Fig. 3B).

The PAl5 strain grown in basal medium plus sucrose and pectin produced less EPMG activity than, when the bacteria were grown in a culture medium with sucrose as the sole carbon source. The different concentration of sucrose and pectin assayed did not affect the EPMG activity of the PAl5 strain (Fig. 4A). The EPMG activity of the UAP5541 strain grown in presence of 0.25 g^{-1} l pectin combined with 2.5 and 5 g^{-1} l sucrose, and 0.5 g^{-1} l pectin combined with 2.5 g^{-1} l sucrose was higher than when grown in the presence of sucrose alone and than when grown in the presence of the other combined concentrations of sucrose and pectin (Fig. 4B).

Fig. 5 shows that the PAl5 and UAP5541 strains grown in basal medium plus sucrose and xyloglucan produced less EXG activity than when the bacteria were grown in a medium with sucrose as the sole carbon source. Non-significant differences in the EXG activity of the PAl5 strain grown at any concentration of the combined sucrose and xyloglucan were observed (Fig. 5A). The EXG of the UAP5541 strain grown in the presence of 0.25 g^{-1} l xyloglucan combined with 2.5 g^{-1} l sucrose was higher than when grown in the presence of the other combined concentrations of sucrose and xyloglucan (Fig. 5B).

4. Discussion

The results of our work demonstrate that the strains PAl5 and UAP5541 of *G. diazotrophicus* were capable of producing EG, EPMG and EXG enzymes. This fact opens the possibility that the penetration of these bacteria into plant roots and their subsequent mobility inside the plant (James et al., 1994; Reis Jr et al., 1995) may be the result of the hydrolytic activity of the bacteria, as happens with other beneficial endophytic microorganisms (Garcia-Romera et al., 1991; Mateos et al., 1992; Jimenez-Zurdo et al., 1996; Garcia-Garrido et al., 2000; Igual et al., 2001).

These bacteria produced EG, EPMG and EXG enzymes using sucrose as the sole source of carbon. The activity profiles of the hydrolytic enzymes found when the strains of *G. diazotrophicus* were grown in sucrose as the sole carbon source indicates that the hydrolytic activity of the PAl5 strain was inducible, whereas that of the UAP5541 strain seems to be constitutive (regardless of the concentration of sucrose used). It has been found that the PAl5 strain of *G.*

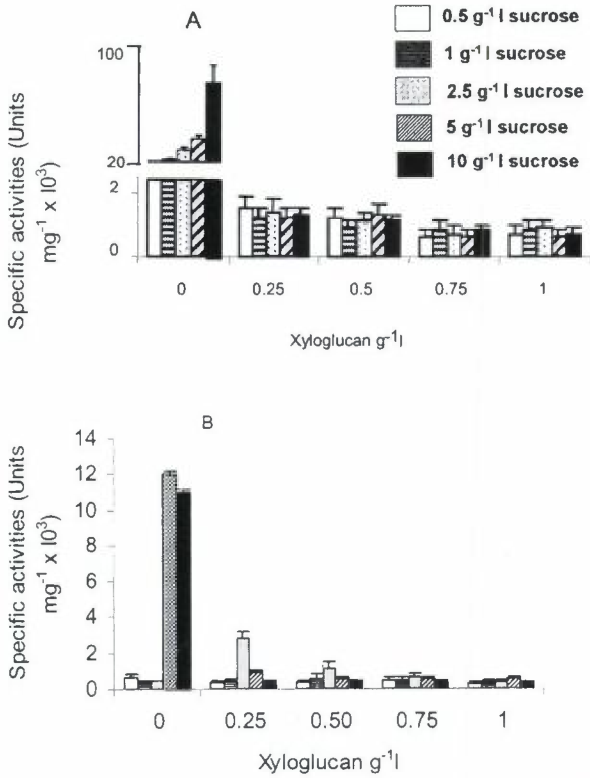

Figure 5. Endoxyloglucanase activity of PAl5 (A) and UAP5541 (B) of *Gluconacetobacter diazotrophicus* grown in presence of sucrose and xyloglucan in the culture medium. Data are the means ± standard errors of mean of five replicate samples.

diazotrophicus was not able to transport into the interior molecules of sucrose and that enzymes related to the sucrose metabolism are inducible and not constitutive (Alvarez and Martinez-Drets, 1995; Hernandez et al., 1995).

Both strains of *G. diazotrophicus* were able to produce EG, EPMG and EXG enzymes when grown in the medium with the corresponding substrate as the only carbon source. Nevertheless, when the concentration of the enzymatic substrates, CMC, pectin or xyloglucan was increased in the culture medium, the production of hydrolytic enzymes was decreased. The inhibition of enzymatic activity by an excess of substrate has been shown in symbiotic microorganisms such as *Rhizobium* and arbuscular mycorrhizal fungi. This fact has been suggested as a mechanism of the plant to control the development of these microorganisms inside the root (Martinez-Molina and Olivares, 1982; Garcia-Romera et al., 1990).

On the other hand, the different behaviour of the strains PAl5 and UAP5541 grown in the culture medium with different concentrations of sucrose and the enzymatic substrates demonstrates that the microbial response and the control of the enzymatic expression of the two *G. diazotrophicus* strains were different and complex. Plant cell-wall degrading micro-organisms use a wide variety of carbohydrates as carbon and energy sources, and have therefore developed mechanisms to modulate the synthesis of polysaccharidases. The reduction of EG, EPMG and EXG activities expressed when the strain PAl5 was grown in the presence of the structural polysaccharides suggests that these enzymes could be subject to catabolite repression by readily metabolizable sugars such as sucrose (Evans and Hedger, 2001). However, as happens with the hydrolytic enzymes detected in the strain UAP5541, some extracellular cellulases, hemicellulases and pectinases have been shown to be constitutively expressed at very low levels by some bacteria and fungi (Rixon et al., 1992; Torigoi et al., 1996; Zeilinger et al., 1996).

In fungi, it is well established that these constitutive enzymes are crucial for triggering the expression of cellulases, hemicellulases and pectinases; an initial attack on the cell wall by these plant cell-wall hydrolases results in the absorption of the hydrolysis products by the organism and the consequent general induction of a polysaccharidase expression by a mechanism which remains to be elucidated (Carle-Urioste et al., 1997). The observed increases of EG and EPMG activities of the strain UAP5541 in the presence of sucrose and the corresponding enzymatic substrate suggest that these mechanisms could be happening in this *G. diazotrophicus* strain. The different sensitivity of both bacteria to the combination of sucrose and enzymatic substrates can be decisive in their competitive capacity against other microorganisms (Tate, 1995). On the other hand, the higher capacity of the UAP5541 strain to produce hydrolytic enzymes in the presence of enzymatic substrates either in presence or in absence of sucrose may be a potential mechanism of adaptation of these bacteria to colonize plant roots.

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