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Full Length Research Paper

Biofertilization of micropropagated *Agave tequilana*: Effect on plant growth and production of hydrolytic enzymes

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Three beneficial bacterial strains [*Gluconoacetobacter diazotrophicus* (Pal5), the diazotrophs (11B) and Pachaz (008)] and an arbuscular mycorrhizal fungus [*Glomus intraradices* (AMF)] were evaluated for their ability to enhance plant growth and the production of hydrolytic enzymes in micropropagated *Agave tequilana* Weber var. Blue. Results show that the growth of the agave plants and the production of hydrolytic enzymes in their roots were influenced by the presence of these microorganisms. AMF + 11B treatment induced the greatest fresh weight, showing significant differences with respect to other combinations. Microscopic analysis showed dense root colonization in the AMF treated plants. Pal 5 treatment produced taller plants, indicating a better plant nitrogen nutrition and possibly phytohormone production by *Gluconoacetobacter*. Treatment Pachaz 008 presented the highest values of the most important agronomic variables, such as the diameter of the pseudo-stem. On another hand, differential catalytic activities of the enzymes β-glucosidase, cellobiohydrolase and endo-1,4-β-D-glucanase were detected in inoculated roots in comparison to the un-inoculated control . We offer explanations about those results based on nutritional and hormonal relationships between the microorganisms and the agave plantlets, as well as on the microbial mechanism to colonize the agave roots.

Key words: Bacterial and mycorrhizal inoculants, *Agave* plantlets, hydrolytic enzymes.

INTRODUCTION

Blue agave (*Agave tequilana* Weber *var.* Azul) is a crop of economical, social and cultural importance in Mexico because it is the raw material for the "tequila" production; tequila is a national and centenary alcoholic beverage (Granados, 1993). In the last decade, the volume of exportation of that drink showed a sustained increment (7.49% in average per year) (INEGI, 1997; Valenzuela, 2003; Macías and Valenzuela, 2009). For that reason, the tequila corporations have identified the availability of agave plants among their priorities. Several millions of agave are planted per year in the states possessing the denomination of origin of tequila. This necessity along with the restrictions in the use of pesticides in this crop have pushed the search for pest and disease-free propagules.Some corporations are using the micropropagation in order to achieve their goals of mass propagation of good quality agaves. Between 2000 and 2010, about ten millions of plantlets of *A. tequilana* were produced by means of plant tissue culture only for the new plantations of the firm SAUZA, S.A. (AGROMOD, S. A., *Pers. Comm.*).

Even though micropropagated plants have many advantageous characteristics, they have some limitations, such as lack of capacity for an adequate acclimatization to the field conditions (Hartmann et al., 1997), due to physiologic changes during the *in vitro* phase (Ovando et al., 2005; Ovando-Medina et al., 2007), and for the fact that their roots do not have

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symbiotic microorganisms. In agave *ex vitro* plants, poor performance have been observed during the first months in the field in comparison to the conventional plants produced by farmers (*A. tequilana* is a monocot propagated, principally through vegetative methods).

Biofertilization of micropropagated plants, using plant growth promoting microorganisms (PGPM's), such as diazotrophic bacteria and mycorrhizal fungi, produces an improved growth, development and increases the rate of *ex vitro* survival (Jaizme-Vega et al., 2004; Ovando-Medina et al., 2007).

In nature, interactions between PGPM's and plant growth promoting microorganism (PGPM's) and plant roots play an essential role in the plant health, through different mechanisms: 1) solubilization of nutrients and breakdown of the organic matter; 2) nitrogen fixation; 3) root zone extension by fungi hyphae; 4) production of phyto-hormones and 5) suppression of soil-borne pathogens (Klibansky and González, 1996; Azcón, 2000; Salvador et al., 2001).

In this work, we studied the effect of inoculation of PGPM's on the growth of agave plantlets as well as on the production of hydrolytic enzymes in their roots.With the aim of increasing the probabilities of infection, PGPM's must be inoculated in the starting of the *ex vitro* phase, but is not possible to assure that the roots will be colonized; a thumb rule is that a second inoculation must be done just before the transplant to the field. There are several hypotheses to explain the mechanisms of microbial colonization of root tissues, including the production of hydrolytic enzymes by the PGPM's (García-Garrido et al., 2000) to degrade cell walls of epidermal and cortex cells of the root, in a process similar to the pathogenic infections. Therefore, in the programs of biofertilization of micropropagated plants, an increment in the activity of hydrolytic enzymes could be taken as an indicator of effective PGPM colonization.

MATERIALS AND METHODS

This study was carried out during 2004 in the Soconusco region (the most Southern site of Mexico), which has a typical tropical climate with an intense six-month rainy period.

Plant

4800 plantlets of *A. tequilana* Weber *var.* Azul micropropagated by the biotechnology firm AGROMOD, S. A. (Frontera Hidalgo, Chiapas, MEXICO) were used. Mother plants for the tissue culture procedure were sampled from fields of the company SAUZA, S. A. (Tequila, Jalisco, MEXICO). A pre-acclimatization stage was required, in which plantlets from laboratory were transferred to nursery trays containing a steam-sterilized substrate (1:1 w/w mix of peat moss and coconut fiber). Substrates were sterilized separately injecting steam (100°C) to piles of 1 m^3 during 40 min. Plants were placed during one month in a glass greenhouse with controlled humidity (90%) and temperature (25°C); photoperiod was provided by the daylight. The experiment of biofertilization was carried out in the hardening-off phase, using pre-acclimatized plants of 6 to 7 cm of height.

Microbial inoculants

PGPM's studied consisted of three bacterial and one fungal strains: *Gluconoacetobacter diazotrophicus* (PAL 5, a collection strain), the strain11B (a diazotrophic bacterium isolated from the rhizosphere of a banana crop) (Martínez, 2004), the strain Pachaz 008 (a diazotrophic bacterium isolated of the rhizosphere of a papaya crop) (Becerra, 2001), the arbuscular mycorrhizal fungus *Glomus intraradices* Schenk and Smith (AMF, a collection strain). Bacterial inocula were prepared in 1 L Erlenmeyer flasks with nutritive broth, incubating them during 12 h on a rotatory shaker (28°C, 200 rpm) adjusted to 1×10^8 cells/ml by dilution with sterilized distillated water. The AMF was produced in a system of co-cultivation fungal spores/transgenic roots of carrot (*Daucus carota*) in Petri dishes with minimal medium (Becard and Fortín, 1988); cultures were maintained during two months in darkness to 28°C.

Biofertilization trial

The experiment was done in a plant nursery during 10 months; plants were sowed in celled trays with a mixture of perlite, pulverized coconut fiber and coffee husks (1:1:1 weight based) as substrate, which was previously pasteurized. Roots of each plant was inoculated, at the start of the experiment period (day 0), with 3 ml of the bacterial suspension and/or one squared centimeter of AMF culture medium containing 50 spores (in average), carrot root fragments and AMF mycelia. A factorial experiment (2^4) was designed combining the presence/absence of the four inoculants, totalizing 16 treatments with 300 randomly distributed plants for each one (Table 1). Treatment 1 was the absolute control and treatments 2 to 16 contained the four inoculants.

After six months (185 days after the transplant, DAT), plants were transferred to 500 cm^3 pots containing the same substrate than that in the previous phase; at the same time, a second inoculation was realized with the same mix of microorganisms, doubling the inocula (6 ml of bacterial suspension and/or 100 AMF spores). All the treatments were irrigated by automated aspersion twice a day and fertilized each month with the Steiner's nutritive solution (Steiner, 1984). Variables registered monthly included: height (expressed as the length of the longest leaf), leaf number, leaf width, fresh and dry weights. The variable stem diameter was only measured 285 DAT. The presence of bacteria was determined in the roots of ten plants per treatment by the method of most probable number (MPN) and to verify the mycorrhizal colonization, roots were stained by the technique of Phillips and Hayman (1970) and observed under the light microscope.

Preparation of enzymatic extracts

The roots sampled at random monthly from each of the treatments were kept cold during transport to the laboratory, and then were pulverized in a mortar with liquid nitrogen. The extraction was made by mixing 1 g of fresh powdered root, 15% (w/w)

Table 1. Treatment matrix resulting from the combination of four microbial strains inoculated to *ex vitro* plants of *A. tequilana* Weber *var*. Azul.

Treatment	Microbial strain			
	Diazotroph Pachaz 008	Diazotroph 11B	*G. diazotrophicus* PAL 5	*G. intraradices* AMF
1	-	-	-	-
2	+	-	-	-
3	-	+	-	-
4	+	+	-	-
5	-	-	+	-
6	+	-	+	-
7	-	+	+	-
8	+	+	+	-
9	-	-	-	+
10	+	-	-	+
11	-	+	-	+
12	+	+	-	+
13	-	-	+	+
14	+	-	+	+
15	-	+	+	+
16	+	+	+	+

Presence (+); absence (-) of the inoculants.

polyvinylpyrrolidone (Sigma-Aldrich™) with 3 ml of buffer B (Tris 12.11 g/L, $MgCl_2$ 2.03 g/L, $NaHCO_3$ 0.84 g/L, β - mercaptoethanol 700 µl/L, phenylmethylsulfonylfluoride (PMSF) 0.026 g/L, Triton X-100 3 ml/L, pH 7.0). The resulting suspension was filtered and centrifuged at 10,000 rpm for 5 min. The supernatant was frozen until use.

Enzyme assays

The extracts were used to determine cellulase activity comprising the following enzymes: endo 1,4-β-D-glucanase, cellobiohydrolase and β-glucosidase, using the methods of Burke et al. (1998) and Coughlan (1985), modified for each enzyme. The activity of endo 1,4-β-D-glucanase was measured using carboxylmethylcellulose as a substrate; to determine the activity of cellobiohydrolase, Avicel PH 101 was used as substrate ,and for the activity of β-glucosidase the substrate was ρ-nitrophenol-β-D-glucopyranoside (all reagents were from Sigma-Aldrich™).

Determination of protein

Total protein was determined in the extracts by the Bradford method (1976; Sigma-Aldrich reagent). For the calibration curve, a standard protein (bovine serum albumin from Sigma-Aldrich™ to 6 g/dL) was used.

Statistical analysis

The experiment was organized in a completely randomized design totaling 16 treatments; the final data of the morphological and biomass variables were processed by ANOVA and the averages were compared by the method of least significant difference (α=0.05).

RESULTS AND DISCUSSION

The results showed that the growth of agave plants and the production of hydrolytic enzymes in their roots were influenced by the presence of PGPMs, since all variables analyzed in the control treatment presented a different behavior.

Effect of biofertilization on plant growth

The fresh and dry weight had a tendency to rise throughout the study period; until the third month, the treatments had very similar values, with a gradual differentiation from the fourth month and became very different at sixth months. They showed a significant increase after the second inoculation (185 DAT), particularly in treatments 11 (11B and AMF), 9 (AMF), 8 (Pachaz 008, 11B, Pal 5), 13 (Pal 5, AMF) and 4 (Pachaz 008, 11B), although the un-inoculated treatment (1) had a moderate increase. The behavior described may have a double cause: the microbial re-inoculation and the change of plants into pots; the latter allowed more space, reducing the mechanical root stress and increasing the penetration of water. This explains the increase in fresh and dry weight in non-inoculated plants. However, the fact that in treatments 16, 14, 7, 10 and 15 no major changes were manifested between the sixth and eighth month, indicates that the change to the pots does not fully explain the weight gain.

The variables of height and width of the blade showed a clear distinction between treatments until the sixth month

Table 2. Growth data of *A. tequilana* vitro plants treated with biofertilizers 236 days after transplant.

Treatment	Fresh weight (g)	Dry weight (g)	Height (cm)	Width of leaf (cm)	Number of leaf	Diameter of stem* (mm)
1	107.72^{ab}	9.69^{abc}	26.28^{ab}	2.97^{cd}	8.55^{a}	32.50^{bc}
2	112.73^{ab}	8.89^{abc}	27.55^{ab}	3.02^{cd}	7.25^{abc}	39.85^{a}
3	102.09^{ab}	7.83^{abc}	26.08^{ab}	3.26^{bcd}	6.75^{bc}	35.25^{abc}
4	126.94^{ab}	10.41^{abc}	29.08^{ab}	3.08^{bcd}	8.00^{abc}	36.10^{abc}
5	107.96^{ab}	8.12^{abc}	26.41^{ab}	3.44^{abc}	7.25^{abc}	35.70^{abc}
6	112.10^{ab}	9.28^{abc}	30.90^{a}	3.31^{bcd}	7.50^{abc}	31.75^{bc}
7	83.98^{b}	7.93^{abc}	24.54^{b}	2.94^{cd}	6.875^{abc}	35.55^{abc}
8	136.84^{ab}	11.50^{abc}	28.12^{ab}	3.09^{bcd}	8.00^{abc}	33.40^{bc}
9	129.93^{ab}	12.43^{a}	29.06^{ab}	2.72^{cd}	7.75^{abc}	32.74^{bc}
10	91.08^{b}	7.70^{abc}	28.34^{ab}	4.60^{a}	6.75^{bc}	37.60^{ab}
11	157.13^{a}	12.85^{a}	31.10^{a}	3.35^{bc}	8.12^{abc}	31.35^{c}
12	112.59^{ab}	9.35^{abc}	27.52^{ab}	3.24^{bcd}	7.37^{abc}	34.80^{abc}
13	124.20^{ab}	11.13^{abc}	26.31^{ab}	3.27^{bcd}	8.25^{ab}	33.00^{bc}
14	85.48^{b}	6.44^{c}	24.22^{b}	3.81^{b}	6.37^{c}	36.80^{abc}
15	108.93^{ab}	9.18^{abc}	27.04^{ab}	3.40^{bc}	7.25^{abc}	36.37^{abc}
16	76.20^{b}	6.90^{bc}	27.06^{ab}	2.60^{cd}	7.37^{abc}	34.78^{abc}

* This variable was measured 285 days after transplant. The data are averages of 50 randomly selected repetitions. Different letters mean statistical difference (DMS, $\alpha = 0.05$). Treatments are combinations of four microbial strains: Diazotroph Pachaz 008, Diazotroph 11B, *G. diazotrophicus* and *G. intraradices*.

month, with a substantial increase at the end, although this was not immediately after the second inoculation. The number of leaves and appearance had irregular kinetics throughout the study, and therefore, were not considered reliable variables for evaluating the effect of biofertilization on the growth of micropropagated agave.

Final data of the growth variables, including the diameter of the stem are shown in Table 2.

Treatment 11 had the highest fresh weight at the end of the experiment (a 35-fold increase), been statistically different from all the other combinations of strains. Plants inoculated with *G. intraradices* (treatment 9) had a 28.9-fold fresh weight increase, whilst those treated with individual 11B (treatment 3) had a 22.7-fold fresh weight increase. These data suggest that, in the interaction, the main effect was caused by the AMF. The AMF-induced increase is explained by an enhanced effective root zone and root mass of the plant, facilitating the entry of water; similar findings have been reported previously for different mycorrhizal systems (Bago et al., 2000).

Treatments 11 (*G. intraradices* + diazotroph 11B) and 9 had the greatest dry weight data, so again the AMF can be a promoter of increased biomass of agave plant micropropagated in the phase of acclimatization. Some authors report that arbuscular mycorrhizal fungi, as well as transporting phosphorus and other minerals to the roots, act as stimulants for greater efficiency in photosynthesis, so that relative fresh weight to dry weight is usually increased in mycorrhizal plants (Gianinazzi-Pearson et al., 1991; Bago et al., 2000). Microscopic analysis revealed that the roots of the plants of the treatments 11 and 9 were densely colonized by mycelium, vesicles and spores at the end of the experiment.

The presence of low mycorrhizal colonization in the control plants at the end of the experiment can be explained by 'contamination' with atmospheric dust; as from 185 DAT, the plants were potted in nursery conditions. Another possible explanation is that the substrate, based on coffee husks, may contain mycorrhizal fungi spores that survived the pasteurization process and that functioned as a natural inoculum.

Treatment 6 (diazotrophs Pachaz 008 + Pal 5) and 11 had the highest height and showed significant differences with the other treatments. Since the length of the third leaf represented the height of the plant, biofertilizers can be said to induce the elongation of the leaves, which may be due to better plant nutrition and production of active metabolites of phytohormones by microorganisms.

In this regard, several studies have shown that biofertilizers, either bacterial or fungal, improve the plant nutrition by phosphate, nitrogen and trace elements. For example, Johansen et al. (1992, 1993) showed, using radioactive labeled phosphorus and/or nitrogen (^{15}N and ^{32}P), that those elements can be mobilized by AMF hyphae into roots of *Trifolium subterraneum* and other plants. It was noticed that in treatment 10 (diazotroph Pachaz 008 + *G. intraradices*) plants had wider leaves, having statistically significant differences with the other

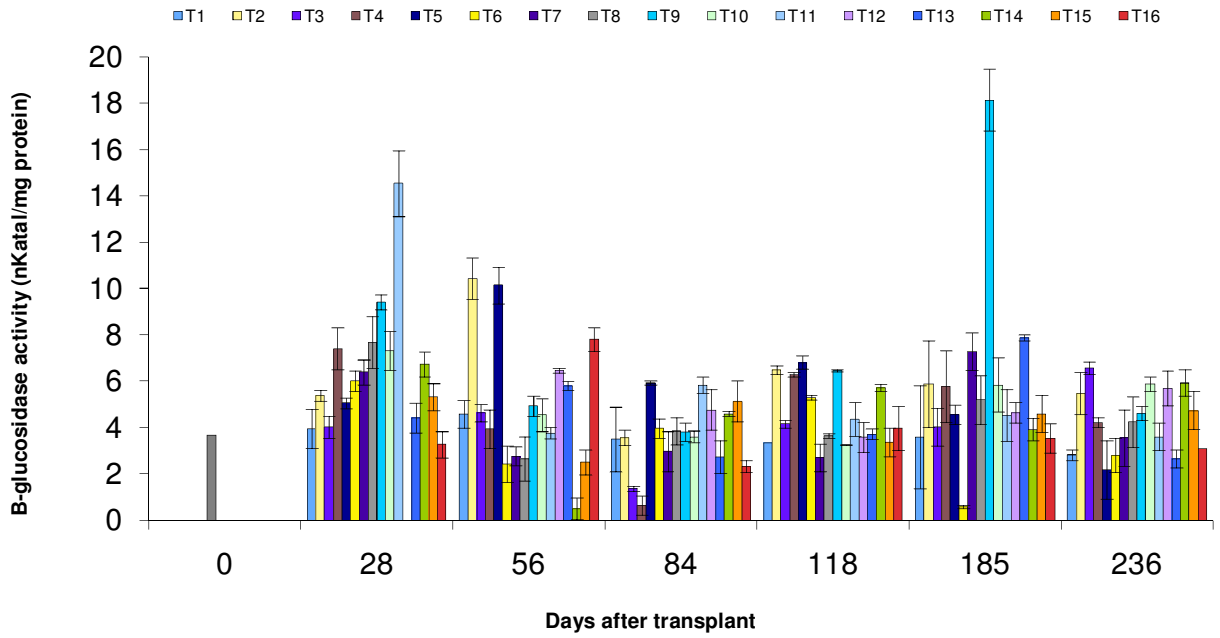

Figure 1. Biofertilization effect on the activity of the enzyme β-glucosidase in vitroplants of *A. tequilana* Weber var. Blue. Treatments are combinations of four microbial strains: Diazotroph Pachaz 008, Diazotroph 11B, *G. diazotrophicus*, *G. intraradices*.

treatments. The possible explanation is that such microbial strains could be producing plant growth regulators (phytohormones) of cytokinin type, since these promote leaf expansion (Salisbury and Ross, 1995).

Several authors stated that *Gluconoacetobacter* produce phytohormones; Fuentes-Ramirez et al. (1993) stated that *Acetobacter diazotrophicus* (later renamed *G. diazotrophicus*) is a species with high production of auxins; Albores (2003) reports that the beneficial effect of several *Azospirillum* strains on banana plants was due in part to the production of indole-3-acetic acid auxin. Several other microorganisms associated with plants are capable of producing auxins, cytokinins, gibberellins and abscisic acid (Costacurta and Vanderleyden, 1995); however, it has not yet been verified that the strains used in this study produce cytokinins.

In the variable number of leaves, only the whole leaves were taken into account while throughout the experimental period (285 days), the plants renovated their leaves; in this case, the non-inoculated treatment had the highest number of leaves at the end. Since the increase of leaves in plants of the class Liliopsida (monocots) is the result of apical growth of the stem, it follows that the bacterial strain Pachaz 008 (T2) induced the growth of agave stalk through the production of metabolites of the auxin family. The total production of leaves (few at the end and lost throughout the period) was correlated with the diameter of the stem. With respect to the variable diameter of the stem, treatment 2 (diazotroph Pachaz 008) presented the highest values. The main variable in the selection of agave plants for planting in the field is the diameter of the stem, due to the fact that tequila beverage is prepared from sugars extracted from the stem. For the later reason, it is possible that the best inoculant is that based on diazotroph Pachaz 008. Again the most likely explanation lies in the production of phytohormones by the microbial strain and improved nitrogen nutrition of the agave plant.

Effect of biofertilization on the production of enzymes

Figure 1 shows the pattern of activity of β-glucosidase enzyme. Treatment-dependent differential activity is shown.

Un-inoculated control plants (treatment 1) showed no significant variation in enzyme activity during the eight months of monitoring. Microbial inoculated treatments 4, 6, 7, 8, 9, 10, 11, 13, 14 and 15 showed increased activity during the first month after inoculation and subsequently, the activity decreased, and became, in some cases, similar to the control plants. The activity of β-glucosidase in treatments 2, 3 (11B), 5 (*G. diazotrophicus)* and 16 (all microorganisms) increased more slowly, because its maximum was observed two months after inoculation and, as in the other treatments, then declined.

The results indicate that endophytic beneficial microorganisms penetrate the root cortical cells probably through a generic mechanism and that the speed depends on the type and composition of population, since

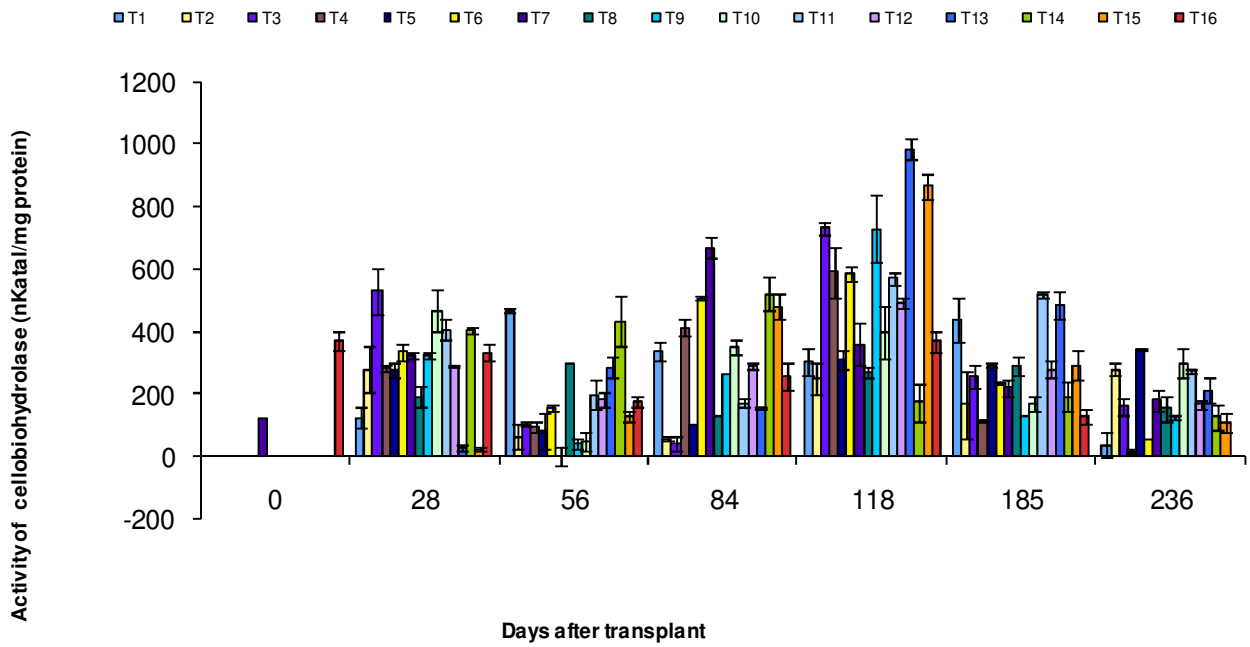

Figure 2. Biofertilization effect on cellobiohydrolase enzyme activity in vitroplants of *A. tequilana* Weber var. Blue. Treatments are combinations of four microbial strains: Diazotroph Pachaz 008, Diazotroph 11B, *G. diazotrophicus*, *G. intraradices*.

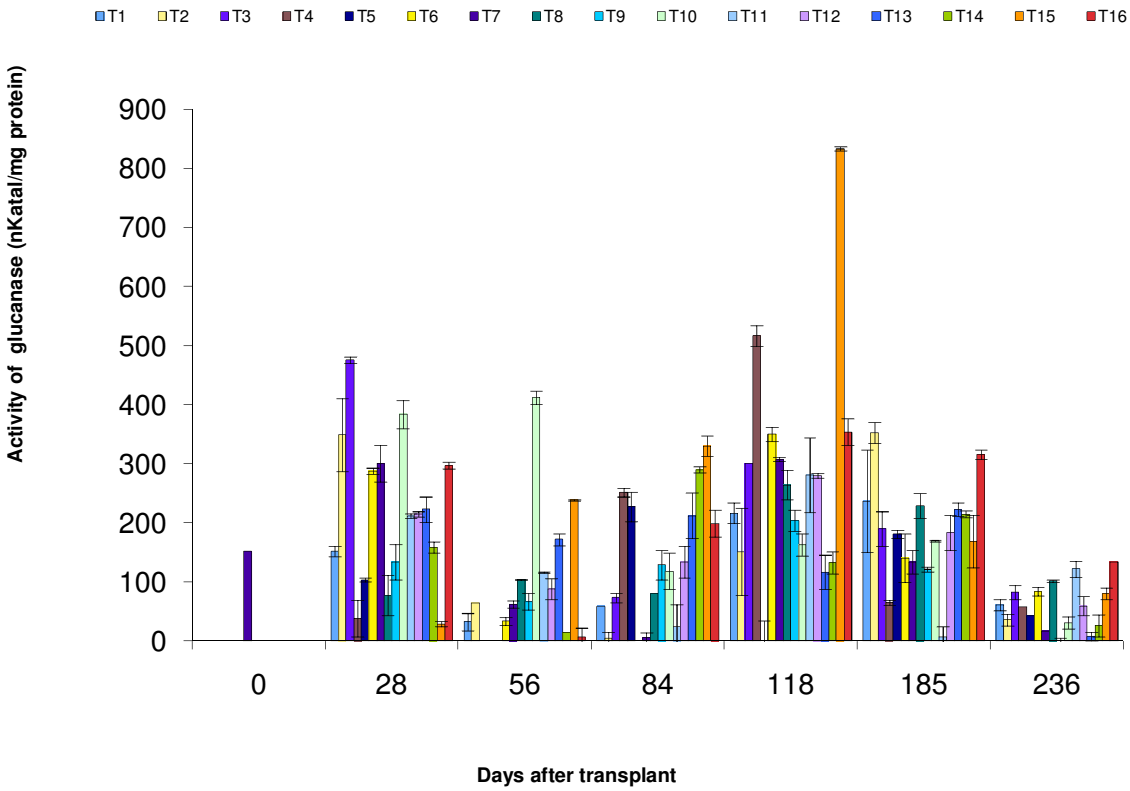

Figure 3. Effect of biofertilization on glucanase enzyme activity in vitroplants of *A. tequilana* Weber var. Blue. Treatments are combinations of four microbial strains: Diazotroph Pachaz 008, Diazotroph 11B, *G. diazotrophicus*, *G. intraradices*.

it was observed that treatments with individual bacteria or with all the microorganisms express the maximum hydrolytic activity.

Figure 2 shows the pattern of activity of the enzyme cellobiohydrolase during the experimental period. Un-inoculated control plants (treatment 1) had an increase at 56 DAT, stabilized during the experiment and declined to almost basal levels at 236 DAT. Treatments 8 and 13 (also had a peak of cellobiohydrolase activity up to 56 DAT. For its part, treatment 15 (11B, *G. diazotrophicus* and *G. intraradices*) had a significantly higher value up to 84 DAT, with its peak intensity at 118 DAT. The rest of the treatments significantly increased the production of the enzyme in the first month of culture.

In general, cellobiohydrolase activity increased in biofertilized plants after inoculation (28 DAT) and later showed a second increase (around 118 DAT), which is not associated with inoculation. This may be due to endogenous production of the enzyme by the plant for the generation of new roots.

Figure 3 shows the pattern of activity of the enzyme cellobiohydrolase during the experimental period. Un-inoculated control plants (treatment 1) showed variation in enzyme activity during the eight months of monitoring, although with low values, when compared with bio-fertilized treatments. The plants of treatments 2, 3, 6, 7, 10, 11, 13 and 16 had a significant increase in the first month, while other treatments had their maximum around 118 DAT.

The microorganism-plant interaction mediated by the hydrolytic enzyme production of cell wall polymers depends on the type of microorganism and/or composition of the population of inocula. However, no correlation was found between the morphological variables and the production of hydrolytic enzymes.

It can be seen that at the end of the experimental period, the increased activity of the enzymes β-glucosidase and cellobiohydrolase occurred in the roots inoculated with *Gluconoacetobacter*, 11B and PACHAZ 008, either alone or combined, however, activity of both enzymes was minimal when the two bacteria were inoculated together so that there was perhaps an antagonism that does not allow the development of both microorganisms and decreased the production of enzymes. Adriano-Anaya et al. (2006) found that *G. intraradices* and *G. diazotrophicus* population decreased when inoculated on roots of sorghum.

As for the glucanase enzyme activity, higher values were obtained in treatments where the diazotrophic bacterium 11B was present, while in treatment 9, which contained only *G. intraradices*, there was no activity of this enzyme, indicating that the fungus penetrates the roots of the agave using hydrolytic enzymes in the cell wall other than the glucanase, as throughout the study it had very low activity values (Figure 3). According to Garcia-Garrido et al. (1999, 2000) and Adriano-Anaya et al. (2005, 2006) hydrolytic activity produced and/or induced by *G. intraradices* differs according to plant species.

This study demonstrates for the first time that the PGPMs use enzymes that degrade the primary wall to colonize the roots of agave, since most of the treatments induced an activity of cellulases above that of the control treatment, which represents the hydrolytic activity produced by the plant cells *per se.*

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