

## Hydrolytic enzyme activities in maize (*Zea mays*) and sorghum (*Sorghum bicolor*) roots inoculated with *Gluconacetobacter diazotrophicus* and *Glomus intraradices*

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### Abstract

Two strains of *Gluconacetobacter diazotrophicus* (Pal 5, UAP5541) and the arbuscular mycorrhizal fungus *Glomus intraradices* increased both the shoot and root dry weight of sorghum 45 days after inoculation, whereas they had no effect on the shoot and root dry weight of maize. Co-inoculation (*Gluconacetobacter diazotrophicus* plus *Glomus mosseae*) did not increase the shoot and root dry weight of either plant. There was a synergistic effect of *Gluconacetobacter diazotrophicus* on root colonization of maize by *Glomus intraradices*, whereas an antagonistic interaction was observed in the sorghum root where the number of *Gluconacetobacter diazotrophicus* and the colonization by *Glomus intraradices* were reduced. Plant roots inoculated with *Gluconacetobacter diazotrophicus* and *Glomus intraradices*, either separately or together, significantly increased root endoglucanase, endopolymethylgalacturonase and endoxyloglucanase activities. The increase varied according to the plant. For example, in comparison with non-inoculated plants, there were higher endoglucanase (+328%), endopolymethylgalacturonase (+180%) and endoxyloglucanase (+125%) activities in 45-day old co-inoculated maize, but not in 45-day old sorghum. The possibility is discussed that hydrolytic enzyme activities were increased as a result of inoculation with *Gluconacetobacter diazotrophicus*, considering this to be one of the mechanisms by which these bacteria may increase root colonization by AM fungi.

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**Keywords:** Arbuscular mycorrhizal; Endoglucanase; Endopolymethylgalacturonase; Endoxyloglucanase; *Gluconacetobacter diazotrophicus*; *Glomus intraradices*

### 1. Introduction

*Gluconacetobacter diazotrophicus* (Yamada et al., 1997) is an associative nitrogen-fixing bacterium that is of great interest in sustainable agriculture. This is because it is able to fix nitrogen in the presence of KNO<sub>3</sub> at low pH values (<3.0) and high sugar concentrations (primarily derived from the plant) and can excrete approximately half of the fixed nitrogen in forms (e.g. ammonia) that can be assimilated by the plants (Stephan et al., 1991; Cojho et al., 1993). Arbuscular mycorrhizal (AM) fungi form symbiotic

associations with many higher plants in which the fungi receive photosynthetically-fixed carbon and, in turn, transport phosphate to the host (Cooper and Tinker, 1978). In many plants *Gluconacetobacter* and AM fungi form an intercellular association with the root, thereby contributing significantly to the N and P nutrition of the plant. These symbionts may even interact with each other to the additional benefit of the plant (Vessey, 2003). The synergistic effect of *Gluconacetobacter diazotrophicus* on AM colonization of plant roots has been described although the mechanisms involved in these interactions have not been studied (Paula et al., 1991; Isopi et al., 1995; Reis et al., 1999).

The mechanism by which *Gluconacetobacter diazotrophicus* penetrates the plant root is not known. However, it takes place via the young radical tissues with the probable generation of infection threads similar to the process

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of infection of leguminous plants by rhizobia (James and Olivares, 1998). It is known that rhizobia produce cell wall degrading enzymes to assist their penetration of the plant root (Mateos et al., 1992, 2001; Jimenez-Zurdo et al., 1996) and it is probable that *Gluconacetobacter diazotrophicus* also produces hydrolytic enzymes for this purpose. Hydrolytic enzymes are also involved in the penetration and development of AM fungi in plant roots. In particular, cellulase, pectinase and xyloglucanase activities have been reported in colonized roots and in the external mycelium of AM fungi (Garcia-Romera et al., 1991; Garcia-Garrido et al., 1992; Rejon-Palomares et al., 1996). It is possible that cell wall degrading enzymes produced by *Gluconacetobacter* and AM fungi can act together to improve the development of one or both symbionts within the root.

The possible synergistic involvement of cell wall degrading enzymes produced by the AM fungus *Glomus intraradices* and *Gluconacetobacter diazotrophicus* Pal 5 and UAP in plant colonization and growth was examined.

## 2. Materials and methods

### 2.1. Growth of plants and inoculation procedures

Maize (*Zea mays* L. cultivar Calderon) and sorghum (*Sorghum bicolor* L. cultivar DK34) plants were grown from surface-sterilized seeds (15 min, 50% NaClO). Seeds were sown in moist sand and, after 10 days, uniform size seedlings were selected and transplanted to 300-ml capacity open pots containing a grey loam soil collected from the garden of the Estación Experimental del Zaidín (Granada, Spain). The soil (pH 8.1, 35.9% sand, 43.6% silt, 20.5% clay, 1.8% organic matter) contained (mg kg<sup>-1</sup>): 6.2 P (NaHCO<sub>3</sub>-extractable), 0.3 N and 132 K. Soil was steam sterilized at 100 °C for 1 h on each of three consecutive days and mixed with autoclaved (120 °C, 20 min) quartz sand (free of nutrients) in the proportion of 2:3 (vol./vol.). The pH of the sand/soil mixture after sterilization was 7.8.

The AM fungus chosen was *Glomus intraradices* (DAOM 197198). An in vitro monoxenic culture system, using Ri T-DNA transformed carrot roots (DC1 clone) and *Glomus intraradices* spores, was used as described by Chabot et al. (1992). *Glomus intraradices* spores were surface-sterilized (Mosse, 1962) and aseptically transferred to plates with 10 ml 10 mM 2-(*N*-morpholin) ethane sulphonic acid (MES) buffer (pH 7.0) plus 0.04 g of Gel-Gro (ICN Biochemicals, Aurora, Ohio, USA). The Petri plates were sealed to reduce dehydration and contamination risks and were incubated in the dark at 25 °C. After 14 days a plug of 2 cm<sup>2</sup> of Gel-Gro containing germinated spores was transferred to a Petri plate with transformed carrot roots growing in M medium (Bécard and Fortín, 1988). The monoxenic culture plates were incubated in the dark at 25 °C for 2–3 months and used as an inoculum source. Each seedling of maize and sorghum was inoculated with a 1 cm<sup>2</sup>

plug of culture containing approximately 50 *Glomus intraradices* spores and mycelium associated with the carrot roots by inserting it into a hole in the soil pot, 1 cm below the 10 days seedling root.

Two bacterial strains of *Gluconacetobacter diazotrophicus* Pal 5 (ATCC 49037), isolated from sugar cane in Brazil (Cavalcante and Dobereiner, 1988), and UAP 5541, isolated from sugar cane in Mexico (Fuentes-Ramirez et al., 1993) were used. The strains were maintained at 4 °C in LGI medium (pH 6.2) which contained (g<sup>-1</sup> l): 0.2 K<sub>2</sub>HPO<sub>4</sub>, 0.6 KH<sub>2</sub>PO<sub>4</sub>, 0.2 MgSO<sub>4</sub> 7H<sub>2</sub>O, 0.02 CaCl<sub>2</sub> 2H<sub>2</sub>O, 0.002 NaMoO<sub>4</sub> 2H<sub>2</sub>O, 0.01 FeCl<sub>3</sub> 6H<sub>2</sub>O, 100 sucrose and 20 agar. Five milliliter of bromothymol blue (9.5% in 0.2 M KOH) were added (Cavalcante and Dobereiner, 1988). Both bacterial strains were grown in Erlenmeyer flasks (125 ml) containing 50 ml SYP basal medium (pH 6.2) which contained (g<sup>-1</sup> l): 10 sucrose, 1 yeast extract, 1 K<sub>2</sub>HPO<sub>4</sub> and 3 KH<sub>2</sub>PO<sub>4</sub>. Cultures were shaken at 200 rpm and maintained at 28 °C (Caballero-Mellado and Martinez-Romero, 1994). Plants were inoculated with 1 ml of culture (1 × 10<sup>7</sup> cells ml<sup>-1</sup>) harvested at stationary phase (72 h).

Soil pots were inoculated with *Glomus intraradices* immediately prior to transplanting (10 days old plants). *Gluconacetobacter diazotrophicus* was inoculated immediately after transplanting by adding 1 ml of culture to the plant roots before covering them with soil.

The plants were kept in a controlled-climate glasshouse, and were watered with 10 ml Hewitt's nutrient solution per week (Hewitt, 1952). The solution used for AM-inoculated plants contained 25% of the P concentration used in the Hewitt solution and the solution used for *Gluconacetobacter diazotrophicus* did not contain N. Natural light was supplemented by Sylvania incandescent and cold-white lamps, 400 μmol m<sup>-2</sup> s<sup>-1</sup>, 400–700 nm; with a 16–8 h light–dark cycle. Air temperature was 19 °C night and 25 °C day and the relative humidity was 50%.

Three sampling dates and 4 treatments were used in the experiments for each of the two plant species and each of the two *Gluconacetobacter diazotrophicus* strains. The four treatments were: (1) uninoculated controls, (2) inoculated with *Glomus intraradices*, (3) inoculated with *Gluconacetobacter diazotrophicus*, and (4) inoculated with both *Glomus intraradices* and *Gluconacetobacter diazotrophicus*.

Plants were harvested after 15, 30 and 45 days and the shoot and root dry weight determined after drying (70 °C, 48 h). The root system was washed and rinsed several times with sterile distilled water and cut into four. One part of the root was used to estimate the dry weight, the second part for enzymatic activity determinations, the third part for the evaluation of the bacterial population and the fourth was cleared and stained for microscope examination (Phillips and Hayman, 1970). The percentage of total root length that was colonized by AM fungi was measured (Giovannetti and Mosse, 1980). The infection by *Gluconacetobacter diazotrophicus* was evaluated in sterilized roots (5 min 1%

chloramine T) which were pulverized in a mortar with isotonic solution (0.089% NaCl). Serial dilutions ( $1 \times 10^{-2}$  to  $1 \times 10^{-7}$ ) were made and 0.1 ml from each dilution was inoculated in 5 ml of semi-solid LGI medium (Cavalcante and Dobereiner, 1988). After 5 days at 28 °C the presence of *Gluconacetobacter diazotrophicus* was identified by the distinct thick yellow–orange pellicle on the surface of the medium and by the clearing of the medium below the surface (Cavalcante and Dobereiner, 1988). *Gluconacetobacter diazotrophicus* was enumerated using the most probable number (MPN) method.

## 2.2. Preparation of extracts for enzyme assays

Roots (10 g fresh weight) were pulverized in a mortar under liquid nitrogen. The resulting powder was homogenized in 30 ml 100 mM Tris–HCl buffer (pH 7.0) containing 0.02 g polyvinyl-polypyrrolidone (PVPP), 10 mM MgCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub>, 10 mM β-mercaptoethanol, 0.15 mM phenylmethyl sulfonyl fluoride (PMSF) and 0.3% (wt/vol.) X-100 Triton. Sodium azide (0.03%) was added to all solutions to inhibit microbial growth. The liquid was filtered through several layers of cheesecloth and centrifuged at 20,000g for 20 min.

The supernatant fraction was dialyzed (Spectra/Por membrane, MWCO: 6000–8000) against several hundred volumes of the same diluted extracting solutions (1:9, vol./vol.) for 16 h at 4 °C. The samples were frozen until used.

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

## 2.3. Enzyme assays

The 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 seed as substrates. The viscosity method is the best test to measure hydrolytic activities of plant roots colonized by different symbiotic microorganisms (García-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 to 0.20 ml mark (between 1 and 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 root extract. Viscosity reduction was determined at 37 °C (Rejón-Palomares et al., 1996). One unit of enzyme activity was expressed as the specific activity ( $U = RA \text{ mg}^{-1} \text{ protein}$ ) where RA is the relative activity calculated by applying the

formula  $\%V = T_0 - T_A 100T_0$ ,  $T_{50} = 50T_A \%V^{-1}$  (Bateman, 1963).  $RA = T_{50} 10^3$  is the reciprocal of time in hour 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 controls for all enzyme assays were autoclaved enzyme supernatants and autoclaved buffers.

## 2.4. Statistical treatments

The data were subjected to one-way ANOVA. The mean values of ten replicate pots were compared using the LSD test ( $P=0.05$ ). Percentage data were subjected to arcsine transformation before analysis.

## 3. Results

### 3.1. Plant growth and root colonization by *Glomus intraradices* and *Gluconacetobacter diazotrophicus*

Table 1 shows that the inoculation of maize with either *Glomus intraradices* or *Gluconacetobacter diazotrophicus* Pal 5 did not increase shoot or root dry weights but both increased shoot and root dry weights of 30- and 45-day old sorghum plants. However, co-inoculation did not increase the shoot and root dry weight in either plant. The percentage of AM root length colonized following co-inoculation of maize plants was significantly higher at both 30 days (+127%) and 45 days (+86%) than for plants inoculated with *Glomus intraradices* alone. However, 30- and 45-day old sorghum inoculated with *Glomus intraradices* alone had a significantly higher AM colonization level than that co-inoculated *Gluconacetobacter diazotrophicus* Pal 5. The MPN of *Gluconacetobacter diazotrophicus* Pal 5 in the maize root was similar in all treatments. However, the MPN was higher in the root of 30- and 45-day old sorghum inoculated with *Gluconacetobacter diazotrophicus* Pal 5 alone than when this bacteria was co-inoculated with *Glomus intraradices*.

Table 2 shows that the inoculation of maize with *Glomus intraradices* or *Gluconacetobacter diazotrophicus* UAP5541 or with both microorganisms together did not increase the shoot and root dry weights or the MPN. However, the percentage of AM root length colonization of maize co-inoculated with both *Glomus intraradices* and *Gluconacetobacter diazotrophicus* UAP5541 was higher at both 30 days (+413%) and 45 days (+338%) than that of the other treatments. The inoculation of sorghum with *Glomus intraradices* or *Gluconacetobacter diazotrophicus* UAP5541 increased the shoot and root dry weights of 45-days plants but the co-inoculation did not increase the shoot and root dry weights of sorghum. The percentage of root length colonization in 45-days sorghum plants inoculated

Table 1

Shoot and root dry weight, mycorrhizal colonization and bacterial MPN of maize (*Zea mays*) and sorghum (*Sorghum vulgare*) inoculated or not with *Glomus intraradices* in presence or in absence of the strain Pal 5 of *Gluconacetobacter diazotrophicus*

Plants	Treatments	Shoot dry weight (mg)			Root dry weight (mg) after (days)			Root length colonization (%) after (days)			MPN $\times 10^3$ g <sup>-1</sup> root after (days)		
		15	30	45	15	30	45	15	30	45	15	30	45
Maize	Control	242a	456a	622a	205a	371a	463a	0	0	0	0	0	0
	<i>Glomus intraradices</i>	244a	435a	604a	222a	332a	463a	1.1a	11.1a	23.2a	0	0	0
	Pal	261a	442a	621a	191a	381a	504a	0	0	0	0.4a	1.1a	2.1a
	<i>Glomus intraradices</i> + Pal	263a	450a	640a	243a	352a	505a	3.3a	25.3b	43.2b	0.2a	1.1a	2.1a
LSD		38	58	55	54	50	48	2.2	9.3	12.2	0.5	0.8	0.5
Sorghum	Control	40a	142a	422a	30a	125a	332a	0	0	0	0	0	0
	<i>Glomus intraradices</i>	36a	215b	533b	35a	196b	519b	2.2a	33.1b	42.3b	0	0	0
	Pal	42a	244b	564b	53b	230b	520b	0	0	0	2.4a	11.2a	12.4b
	<i>Glomus intraradices</i> + Pal	41a	175a	456a	50b	136a	391a	1.3a	10.5a	18.2a	2.6a	12.3a	0.8a
LSD		12	30	54	13	35	53	2.1	10.4	14.3	0.6	2.9	1.6

Column values followed by the same letter are not significantly different as determined by one-way ANOVA followed by LSD test ( $P=0.05$ ).

with *Glomus intraradices* was 61% higher than when the AM endophyte was co-inoculated together with *Gluconacetobacter diazotrophicus* UAP5541. The MPN was higher in the root of 45-days sorghum inoculated with *Gluconacetobacter diazotrophicus* UAP5541 alone than when this bacteria was co-inoculated with *Glomus intraradices*.

### 3.2. Hydrolytic enzyme activities

No significant differences in the hydrolytic activities EG, EPMG and EXG of 15- and 30-days maize and sorghum

plants were recorded in any of the treatments tested (Figs. 1–4).

Fig. 1 shows that the EG, EPMG and EXG activities of 45-days maize were higher (EG + 140%, EPMG + 60% and EXG + 50%) when the plants were inoculated with *Glomus intraradices* and the strain Pal 5 of *Gluconacetobacter diazotrophicus* together. However, the EG activity was higher and the EPMG and EXG activities were similar in 45-days maize inoculated with *Glomus intraradices* or *Gluconacetobacter diazotrophicus* Pal 5 than those of non-inoculated plants.

Table 2

Shoot and root dry weight, mycorrhizal colonization and bacterial MPN of maize (*Zea mays*) and sorghum (*Sorghum vulgare*) inoculated or not with *Glomus intraradices* in presence or in absence of the strain UAP5541 of *Gluconacetobacter diazotrophicus*

Plants	Treatments	Shoot dry weight (mg) after (days)			Root dry weight (mg) after (days)			Root length colonization (%) after (days)			MPN $\times 10^3$ g <sup>-1</sup> root after (days)		
		15	30	45	15	30	45	15	30	45	15	30	45
Maize	Control	152a	220a	433a	105a	237a	401a	0	0	0	0	0	0
	<i>Glomus intraradices</i>	113a	273a	421a	135a	254a	377a	0	8.2a	10.3a	0	0	0
	UAP	103a	245a	447a	143a	221a	410a	0	0	0	1.4a	1.1a	8.2a
	<i>Glomus intraradices</i> + UAP	104a	251a	472a	142a	221a	410a	0	42.1b	45.2b	2.1a	2.5a	8.1a
LSD		52	54	56	39	35	34		14.1	13.3	0.4	0.6	1.2
Sorghum	Control	45a	210a	604a	52a	262a	652a	0	0	0	0	0	0
	<i>Glomus intraradices</i>	58a	296b	716b	63a	270a	733b	0	30.6b	70.1b	0	0	0
	UAP	57a	254a	721b	61a	233a	722b	0	0	0	0.5a	2.4a	21.2b
	<i>Glomus intraradices</i> + UAP	66a	217a	541a	57a	231a	661a	0	14.2a	43.4a	0.3a	2.5a	0.4a
LSD		22	39	65	21	40	45		8.1	16.3	0.2	0.5	2.1

Column values followed by the same letter are not significantly different as determined by one-way ANOVA followed by LSD test ( $P=0.05$ ).

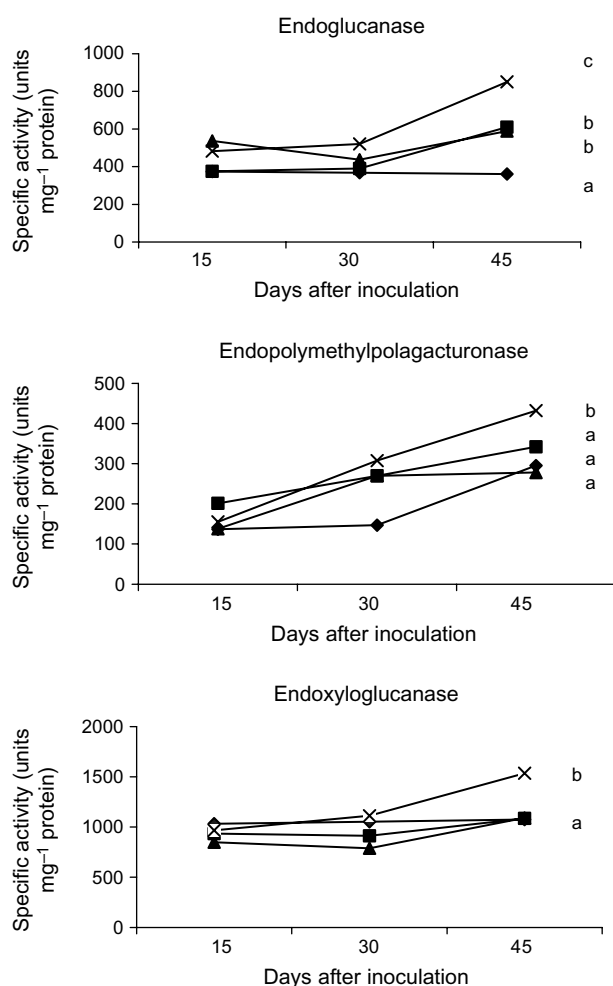


Fig. 1. Endoglucanase, endopolymethylpolagacturonase and endoxyloglucanase activities of maize inoculated or uninoculated with *Glomus intraradices* in presence or in absence of *Gluconacetobacter diazotrophicus* Pal 5. The 45 days values followed by the same letter are not significantly different as determined by one-way ANOVA followed by LSD test ( $P=0.05$ ). ◆, Uninoculated control; ■, plant inoculated with *Glomus intraradices*; ▲, plant inoculated with *Gluconacetobacter diazotrophicus*; ×, plant inoculated with *Glomus intraradices* and *Gluconacetobacter diazotrophicus*.

The EG, EPMG and EXG activities of 45-day old sorghum inoculated with *Gluconacetobacter diazotrophicus* Pal 5 alone were higher (EG +160%, EPMG +60% and EXG +185%) than the non-inoculated controls (Fig. 2). The EPMG and EXG activities of plants inoculated with *Glomus intraradices* were also higher (EPMG +38% and EXG +42%) than the controls but EG activity was similar to non-inoculated plants. Plants inoculated with both microorganisms had similar EPMG or lower EG and EXG activities than the controls.

Significant increases in EG (+328%), EPMG (+180%) and EXG (+125%) activities of 45-days maize inoculated with *Glomus intraradices* or *Gluconacetobacter diazotrophicus* UAP5541 were observed, and these activities were increased further when both microorganisms were inoculated together (Fig. 3).

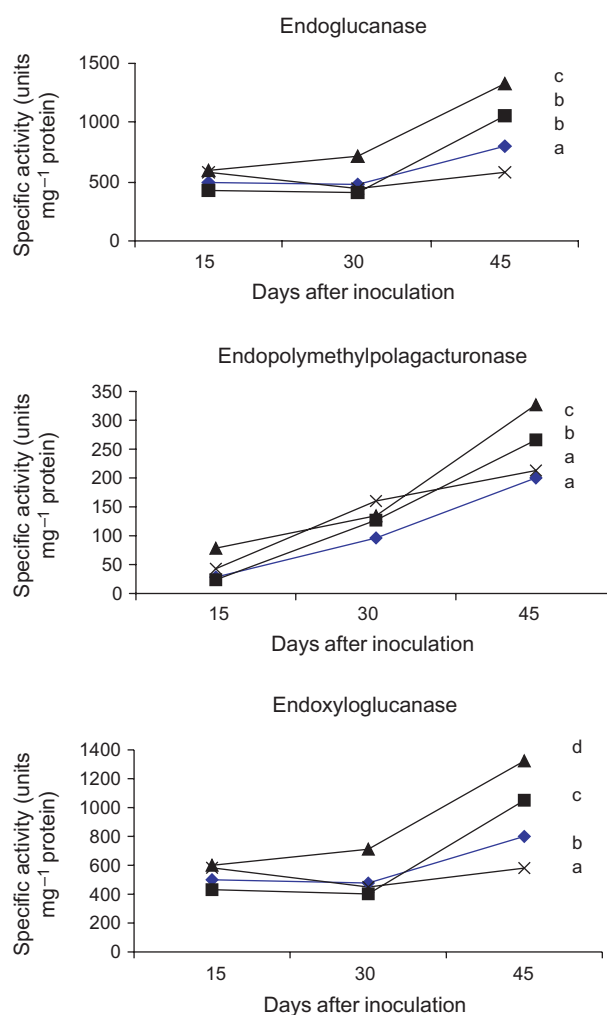


Fig. 2. Endoglucanase, endopolymethylpolagacturonase and endoxyloglucanase activities of sorghum inoculated or uninoculated with *Glomus intraradices* in presence or in absence of *Gluconacetobacter diazotrophicus* Pal 5. The 45 days values followed by the same letter are not significantly different as determined by one-way ANOVA followed by LSD test ( $P=0.05$ ). ◆, Uninoculated control; ■, plant inoculated with *Glomus intraradices*; ▲, plant inoculated with *Gluconacetobacter diazotrophicus*; ×, plant inoculated with *Glomus intraradices* and *Gluconacetobacter diazotrophicus*.

There were no significant differences in EG activity of 45-days sorghum roots in any of the treatments (Fig. 4). However, the EPMG and EXG activities of 45-days sorghum inoculated with *Glomus intraradices* (EPMG +55% and EXG +108%) or *Gluconacetobacter diazotrophicus* UAP5541 (EPMG +88% and EXG +33%) were higher than the non-inoculated controls, but plants inoculated with both microorganisms together had a significantly lower enzymatic activity (EPMG –55% and EXG –25%) than the controls (Fig. 4).

#### 4. Discussion

*Gluconacetobacter diazotrophicus* increased the growth of several plants but the response varied with the plant type

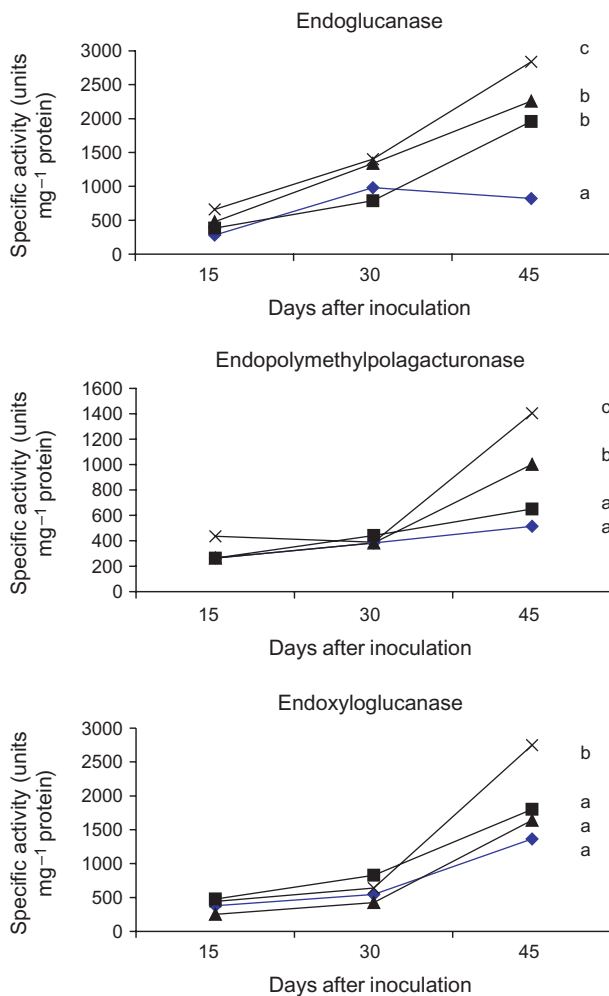


Fig. 3. Endoglucanase, endopolymethylpolagacturonase and endoxyloglucanase activities of maize inoculated or uninoculated with *Glomus intraradices* in presence or in absence of *Gluconacetobacter diazotrophica* UAP5541. The 45 days values followed by the same letter are not significantly different as determined by one-way ANOVA followed by LSD test ( $P=0.05$ ). ♦, Uninoculated control; ■, plant inoculated with *Glomus intraradices*; ▲, plant inoculated with *Gluconacetobacter diazotrophica*; ×, plant inoculated with *Glomus intraradices* and *Gluconacetobacter diazotrophica*.

(Reis et al., 2000). In this study, we observed that the strains Pal 5 and UAP5541 of *Gluconacetobacter diazotrophica* increased the dry matter of sorghum but had no effect on the dry maize matter. The type of plant also was important in the effectiveness of the AM fungi on plant growth (Vierheilig and Ocampo, 1991). In our assays, *Glomus intraradices* increased the growth of sorghum but had no effect on the growth of maize, in spite of the fact that maize is a mycotrophic plant and is considered to be dependent on the AM symbiosis for its optimal development and growth. Nevertheless, it is known that different plant species (even cultivars) have a different sensitivity to the AM symbiosis (Azcon and Ocampo, 1981; Vierheilig and Ocampo, 1991). On the other hand, close relationships between the percentage of AM colonization and their effect on plant

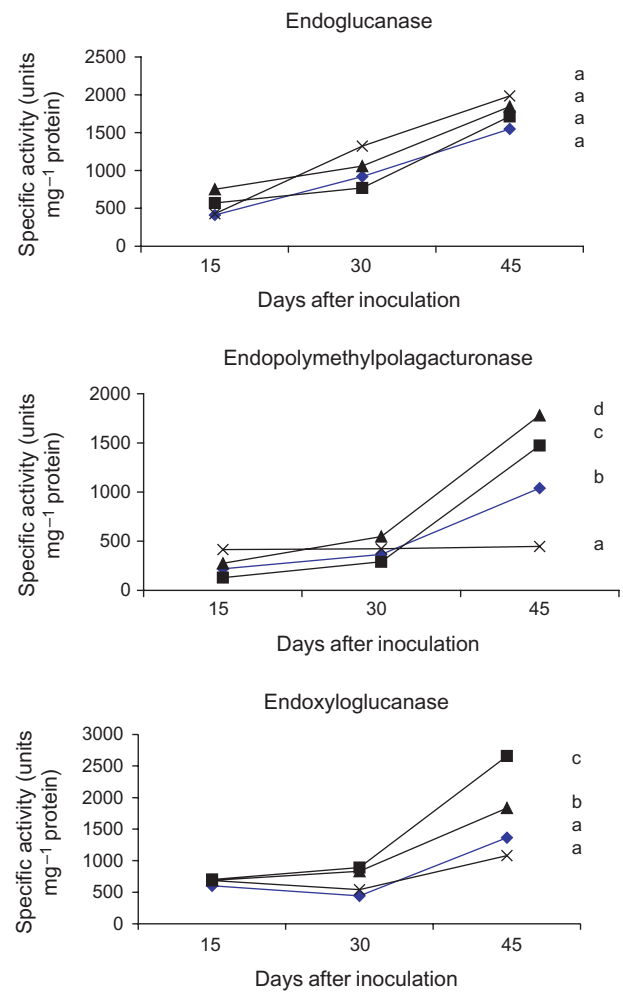


Fig. 4. Endoglucanase, endopolymethylpolagacturonase and endoxyloglucanase activities of sorghum inoculated or uninoculated with *Glomus intraradices* in presence or in absence of *Gluconacetobacter diazotrophica* UAP5541. The 45 days values followed by the same letter are not significantly different as determined by one-way ANOVA followed by LSD test ( $P=0.05$ ). ♦, Uninoculated control; ■, plant inoculated with *Glomus intraradices*; ▲, plant inoculated with *Gluconacetobacter diazotrophica*; ×, plant inoculated with *Glomus intraradices* and *Gluconacetobacter diazotrophica*.

growth are not commonly found (Vierheilig and Ocampo, 1991; Camprubi et al., 1995; Garcia-Garrido et al., 2000).

In our study, we found that co-inoculation with *Gluconacetobacter diazotrophica* and *Glomus intraradices* did not increase shoot dry weight in either of the plants tested. However, a synergistic effect of *Gluconacetobacter diazotrophica* on AM root colonization of maize (but not sorghum) by *Glomus intraradices* was recorded. Beneficial effects of *Gluconacetobacter diazotrophica* on AM colonization of plant roots have been reported previously (Paula et al., 1991; Isopi et al., 1995; Reis et al., 1999). However, the existence of soil microorganisms that increased the AM colonization but did not increase plant growth has also been reported (McAllister et al., 1997; Godeas et al., 1999). On the other hand, competition

between *Gluconacetobacter diazotrophicus* and AM fungi has been observed (Gryndler and Hrselova, 1998). In our experiments there was antagonism between *Glomus intraradices* and *Gluconacetobacter diazotrophicus* in the sorghum root which resulted in a decline in the number of *Gluconacetobacter diazotrophicus* and a decrease in the percentage of AM root colonized.

The level of hydrolytic enzyme activities varied according to the plant inoculated. Both strains of *Gluconacetobacter diazotrophicus* increased EPMG and EXG activities of sorghum, whereas there was no increase in either EPMG or EXG activities in maize inoculated with the strain Pal 5 nor in the EXG activity of the same plant inoculated with the strain UAP5541. These results indicated that the increases of hydrolytic enzyme activities were produced by plants in response to the bacterium. As plant cell wall degrading enzymes are involved in plant cell wall formation and growth (Fry, 2004), the increase that *Gluconacetobacter diazotrophicus* produces on the growth of the sorghum root might, in part, explain the increase of the hydrolytic activities observed in this plant. On the other hand, *Gluconacetobacter diazotrophicus* when grown in pure culture produced  $2 \times 10^3$ ,  $10 \times 10^3$  and  $5 \times 10^3$  units  $\text{mg}^{-1}$  protein of EG, EPMG and EXG, respectively, (data not presented). The fact that the strain Pal 5 increased the EG activity of maize and the strain UAP5541 increased the EG and EPMG activities, independently of their effect on the growth of the plant, suggests that these bacteria can contribute directly to increases in the hydrolytic activity of the root.

On the other hand, the hydrolytic activity produced and/or induced by *Glomus intraradices* differed according to the plant species (García-Garrido et al., 1999; García-Garrido et al., 2000) In fact, *Glomus intraradices* increased the EPMG and EXG activities of sorghum, whereas it did not increase these enzymatic activities of maize. However, EG activity was increased in AM colonized maize root but not in AM sorghum root. It is known that the AM fungi increase the hydrolytic activity of the root (García-Romera et al., 1991; García-Garrido et al., 1992). The induction of the different root cell degrading enzymes by the AM fungi is complex and the plant type was decisive in this process (García-Garrido et al., 2002).

The level of the hydrolytic enzyme activities in maize and sorghum inoculated with both *Gluconacetobacter diazotrophicus* and *Glomus intraradices* also changed according to the type of plant. In fact, the level of enzymatic activity in co-inoculated sorghum roots decreased to below the levels measured in the non-inoculated controls. The antagonism observed between *Gluconacetobacter diazotrophicus* and *Glomus intraradices* in the sorghum root may contribute to the drop in the enzymatic activities observed in the roots of these plants. On the other hand, the hydrolytic activities of maize roots inoculated with both *Glomus intraradices* and *Gluconacetobacter diazotrophicus* were higher than the treatments in which both microorganisms

were inoculated separately. However, the co-inoculation with *Glomus intraradices* and *Gluconacetobacter diazotrophicus* did not increase the root dry weight of maize indicating that the increase of hydrolytic activities observed in this plant was not mainly due to the cell wall degrading enzymes involved in the root cell formation and growth. The fact that *Gluconacetobacter diazotrophicus* increased the hydrolytic activities and the percentage of AM colonization of maize, independently of the effect this bacterium has on the growth of the root, suggests that the hydrolytic activities of *Gluconacetobacter diazotrophicus* could be one of the mechanisms by which this bacterium increased the colonization of maize root by *Glomus intraradices*.

To investigate the activities of hydrolytic enzymes, this study has been carried out using sterilized soil, so as to avoid the interference of other soil microorganisms. It is known that soil microorganisms can be important in the interaction between AM fungi and *Gluconacetobacter diazotrophicus*, since most of the processes that occur in soil are microbe-mediated and are carried out by enzymes (Nannipieri et al., 2002). Further research will investigate the implications of hydrolytic enzymes in the interactions between *Gluconacetobacter diazotrophicus* and AM fungi in non-sterilized soils.

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