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Short term effects of *Glomus claroideum* and *Azospirillum brasilense* on growth and root acid phosphatase activity of *Carica papaya* L. under phosphorus stress

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ABSTRACT. Arbuscular mycorrhizal fungi (AMF) are able to increase root enzymatic activity of acid and alkaline phosphatases. However, the role of AMF on phosphatase activity has not been reported in papaya (*Carica papaya* L.), which is frequently established at places with soil phosphorus (P) deficiencies. The goals of this research were to determine the effect of *Glomus claroideum* (Gc), and plant growth promoting rhizobacterium *Azospirillum brasilense* strain VS7 [Ab] on root phosphatase activity and seedling growth of *Carica papaya* L. cv. Red Maradol under low P conditions. There were four treatments-colonization with: 1) Gc, 2) Ab, 3) Gc+Ab, and 4) non-inoculated seedlings. Plants were established in a coarse sand:sandy loam substrate under P-limitation ($11\mu\text{g P ml}^{-1}$), supplied with a modified Long Ashton Nutrient Solution. Seedling growth was severely reduced by low P. Gc+Ab inoculated plants had greater total dry matter and leaf area than non-colonized plants. Gc-inoculated plants had greater leaf area than non-colonized plants. Treatments did not differ in leaf area ratio, specific leaf area and, total chlorophyll content. There was a non-significant effect on stem relative growth rate with Gc and Gc+Ab plants. Mycorrhizal colonization enhanced the bacterial population 3.4-fold in the Gc+Ab treatment compared with the population quantified in Ab treatment. Soluble and extractable root acid phosphatase activity (RAPA) was higher in Gc inoculated plants. We discussed on the possible relation among both inoculated microorganisms and also with the P-limitation which plants were established.

Key words: Plant growth promoting microorganisms, microbial interaction, P-limitation

INTRODUCTION

Papaya is an important crop fruit for Mexico due to its nutritional value and, its economical value as an export crop. In Mexico, many of the commercial orchards are established in soils with low fertility. Thus, producers require to apply

RESUMEN. Algunas investigaciones han demostrado que los hongos micorrízicos arbusculares pueden modificar la actividad enzimática de la raíz (fosfatasa ácida o alcalina), sin embargo, ésta se desconoce en cultivos como papaya, la cual es frecuentemente establecida en suelos con problemas de limitación por fósforo. El objetivo del trabajo consistió en evaluar el efecto de la inoculación de *Glomus claroideum* (Gc) y de la cepa *Azospirillum brasilense* VS-7 (Ab) sobre el crecimiento y actividad enzimática de la fosfatasa ácida en raíz de *Carica papaya* cv. Maradol roja establecida bajo condiciones de limitación por fósforo. Se consideraron cuatro tratamientos 1) Inoculación con Gc, 2) Inoculación con Ab, 3) inoculación con Gc+Ab y, 4) testigo. Las plantas fueron trasplantadas en un sustrato que consistió de la mezcla de arena y suelo limo-arenoso, a la cual se aplicó solución nutritiva de Long Ashton con $11\text{ mg de P ml}^{-1}$. El crecimiento de las plantas fue limitado por la deficiencia de P. Las plantas inoculadas con ambos microorganismos presentaron mayor materia seca y área foliar en comparación con plantas testigo. Las plantas inoculadas con Gc mostraron mayor área foliar que las plantas testigo. No se observaron diferencias significativas en la relación área foliar, área foliar específica y contenido de clorofila. No se observaron diferencias significativas en la tasa de crecimiento del tallo entre las plantas inoculadas con Gc y Gc+Ab. Las plantas con Gc incrementaron 3.4 veces la población de las bacterias en comparación con la población cuantificada en las plantas inoculadas con Ab. La actividad enzimática de la fosfatasa ácida en raíz, tanto soluble como extractable, fue más alta en las plantas inoculadas con Gc. Se hace la discusión de las posibles interacciones que se tuvieron entre los microorganismos inoculados y la condición de limitación de P en las que fueron establecidas las plantas.

Palabras clave: Microorganismos promotores del crecimiento vegetal, interacción microbiana, limitación por fósforo.

P-fertilizers to improve plant productivity. Nutrient availability is also limiting because soil fertility is low or some elements such as phosphorus is fixed by soil colloids.⁵³ In that case, plant nutrition depends on factors such as organic exudates to modify soil pH, kind and age of root system to improve the nutrimental absorption, enzyme releasing, and others nutrimental uptake mechanisms.^{16,26,45,53} Microbial activity is particularly important under limiting soil fertility so that they can release the necessary nutrients to plants. Rhizospheric beneficial microorganisms play an important role on plant nutrition.^{9,24} Plant growth promoting rhizobacteria and arbuscular mycorrhizal fungi (AMF) could be ap-

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plied in nursery plant management to improve vigor, nutrition and plant quality.^{1,35,40} The establishment of AMF in root allows the improving of nutrimental uptake by plants.³³ Mycorrhizal activity influences on soil pH modifications⁷ and beneficial bacteria populations^{4,39} which also contribute in plant growth promotion.^{9,10} Benefits of rhizospheric microorganisms to plant growth, nutrimental uptake, gas exchange, rooting enhancement of cuttings and, alleviation of stress after transplanting and drought stress have been reported.^{14,20,21,29,30,40,47,49} Reported phosphorus uptake mechanisms of plants include greater enzymatic excretion through the root systems which allow the enhancing of P-plant nutrition.⁴¹ With phosphorus uptake, the establishment of AMF in root system produce changes related with the enzymatic activity of either acid or alkaline phosphatases^{25,37,50} which contributes to greater soil nutrimental availability. The aims of this research were to determine the effect of *Glomus claroideum* and *Azospirillum brasilense* VS7 on root acid phosphatase activity and seedling growth of *Carica papaya* L. cv Red Maradol under low P conditions.

MATERIAL AND METHODS

Seed management. Commercial and certified seeds of *Carica papaya* cv. red Maradol were superficially disinfested with alcohol 70% (30s) and chloramine T 2% (1 min) and after they were sowed in containers containing steam-sterilized sand. Fifteen days-old seedlings were transplanted to plastic pots of 2 liters, containing a coarse sand:sandy loam as growth substrate whose chemical features were ($\mu\text{g g}^{-1}$) 0.9 NO₃-N, 2.1 NH₄-N, 1.5 P, 10.7 K, pH 7.7, EC 0.17 and textural analysis 85% sand, 10% clay and 5% silt. At this moment, plants were also inoculated, according to their treatments, with the two beneficial microorganisms.

Microbial strains. Seedlings were inoculated with *Azospirillum brasilense* VS-7 (Mexican strain isolated from Valle de Santiago, Guanajuato, Mexico and supplied it by the Chemistry Laboratory, UNAM) and *Glomus claroideum* Schenck and Smith (isolated identified from *Glomus* consortium collected from Zacatecas, Mexico.¹⁹) The bacterial inoculum was prepared on solid nutrient agar media incubated at 28°C. The bacterial colonies were rinsed with sterile distilled water. Seedlings were inoculated with three milliliters of the bacterial inoculum suspension. The bacterial population applied on root system was 14.6 UFC x 10⁹ ml⁻¹ plant⁻¹.

Spores of *Glomus claroideum* were collected from a pure culture in sand and superficially disinfested according to Becard and Fortin technique,¹² and then 500 spores per plant were inoculated applying them directly to the root system of each seedling. Simultaneously, the spore viability

percentage was determined by the tetrazolium bromide technique⁴³ which was 80.5%.

Cultural conditions. After 24 h to the transplanting and inoculation, the plants were fertilized and weekly watered with 200 ml of Long Ashton Nutrient Solution ($\mu\text{g ml}^{-1}$): 175.9 N, 156.2 K, 160.2 Ca, 98.4 S, 11.7 Na, 5.4 Cl, 5.0 Fe-quelate sequestrane, 0.54 Mn, 0.54 B, 0.10 Cu, 0.06 Zn and 0.006 Mo. Phosphorus was applied at 11 $\mu\text{g P ml}^{-1}$ for all the plants.

Plants were grown in a glasshouse for eight weeks under mean minimum/maximum temperature 16.8/34.8°C and mean minimum/maximum relative humidity 47.5/100% (May-July, 1999) determined by Hobo Data Logger Onset S/N 185122. Morphological parameters such as height, stem diameter and leaf number were evaluated. Total chlorophyll content was evaluated with the Spadmeter model Spad 502, Minolta Corp., using a standard curve for chlorophyll from previous destructively harvested papaya plants. The regression equation model estimated was $y = -18.695 + 6.0508 (x)$ which had a high correlation ($r^2 = 97.1$) and it was used to determine total chlorophyll content in leaves. Root acid phosphatase (soluble and extractable) activity was determined at 55 days after transplanting. It was estimated according to the modified p-nitrophenylphosphate method.⁵⁵ Briefly, 0.1 g of roots were gently washed with sterile deionized water and set in micrutubes added with 450 μl of a buffer solution (pH 5.5). Samples of roots were set in the buffer solution in order to determine the soluble enzymatic activity and also, another samples were powdered with a micropestle to evaluate the extractable enzyme. Enzymatic activity was measured by adding 150 μl of p-nitrophenylphosphate (3 mM) and, micrutubes were centrifuged at 10,000 rpm, 10 minutes and incubated at 37°C, 45 minutes. After incubation, 150 ml of Cl₂Ca (5M) and 400 μl of KOH (5M) were added, then samples were centrifuged at 10,000 rpm during 10 minutes. Standard curve was made by using p-nitrophenol (7 mM). Absorbance lectures (420 nm) were taken with UV HP-chemistation computing system.

At plant harvest stage other growth parameters included dry mass (shoot and root), leaf area ratio (LAR=total leaf area/ total plant dry weight), specific leaf area (SLA=total leaf area/dry weight of leaves), and relative stem growth rate $[3.1416 (\text{stem diameter}/2)^2 (\text{eight}/\text{days})]$.³⁴ Bacterial establishment on surface root were evaluated using the serial dilutions technique on yeast extract and red Congo *Azospirillum* specific medium in petri dishes. The frequency of total AM colonization was determined through the root staining⁴⁸ and using the method of Biermann and Linderman.¹⁵

Experimental design and treatment. The experiment consisted in a completely randomly design with four treatments and 25 replications per treatment. Thirteen replica-

Table 1. Effect of the inoculation of arbuscular mycorrhizal fungus and plant growth promoting rhizobacterium on yield dry matter and morphological parameters of *Carica papaya* cv. Red Maradol established under low P conditions, at 55 days after transplanting.

Treatment	Total dry matter g	Relative stem growth rate cm ³ day ⁻¹	Leaf area cm ²	LAR cm ² g ⁻¹	SLA m ² g ⁻¹
Control	0.82 b	0.024 ab	71.7 b	93.2 a	307.7 a
<i>Glomus claroideum</i>	1.07 ab	0.035 ab	117.7 a	143.6 a	421.4 a
<i>Azospirillum brasilense</i>	0.70 b	0.020 b	65.2 b	107.1 a	287.4 a
<i>G. claroideum</i> + <i>A. brasilense</i>	1.40 a	0.039 a	115.8 a	85.9 a	279.4 a

LAR=Leaf area ratio; SLA=Specific leaf area. Means followed by the same letter are not significant (Tukey $\alpha=0.05$). n=13.

tions were used in growth analysis and the remained twelve plants were used for root phosphatase analysis and, mycorrhizal and bacteria analysis, using four plants with three replications in each sampling time (n=12). Five plants were used for plant growth evaluation and for determining the chlorophyll content considering two leaves per plant (n=10). The treatments consisted on 1) inoculation of spores of *Glomus claroideum* (Gc), 2) inoculation with *Azospirillum brasilense* VS7 (Ab), 3) inoculation with Gc+Ab, and 4) non-inoculated seedlings, all they established at 11 $\mu\text{g P ml}^{-1}$. Data were analyzed by the analysis of variance procedure⁵² and a test ($\alpha=0.05\%$) was used for the means separation.

RESULTS

Plant growth response. Plant growth was severely reduced by P stress. After 15 days, the Ab inoculation produced decreases on relative stem growth rate but it was observed that dual microbial inoculation promoted higher values in this parameter as well as total dry matter (Table 1); nevertheless, this benefit seems to be associated by the presence Gc which increased significantly the leaf area in comparison with Ab inoculation and non-inoculated plants (Table 1). Significant differences were not observed by the microbial inoculation on LAR and SLA. However, AMF inoculation showed the highest values but in the dual inoculation, this effect was notoriously diminished (Table 1) even when it was compared with control plants.

Effects on root acid phosphatase activity (RAPA). Gc-inoculated plants showed the highest values of soluble and extractable RAPA (Figure 1a-b). This effect was significant ($\alpha=0.01$) in comparison with treatments with the dual inoculation and control plants. *Azospirillum* inoculation reduced the soluble RAPA in comparison with mycorrhizal plants (Figure 1b).

Effects on chlorophyll. Significant differences between total chlorophyll content were not observed in all treatments (data non shown) but, the chlorophyll content was

higher (>300 mmol m⁻²) in all the treatments inoculated with the microorganisms in comparison with control plants.

Microbial colonization. At 15 days, the establishment of Ab was affected by the inoculation of AM fungus. The plants solely inoculated with Ab had a high population

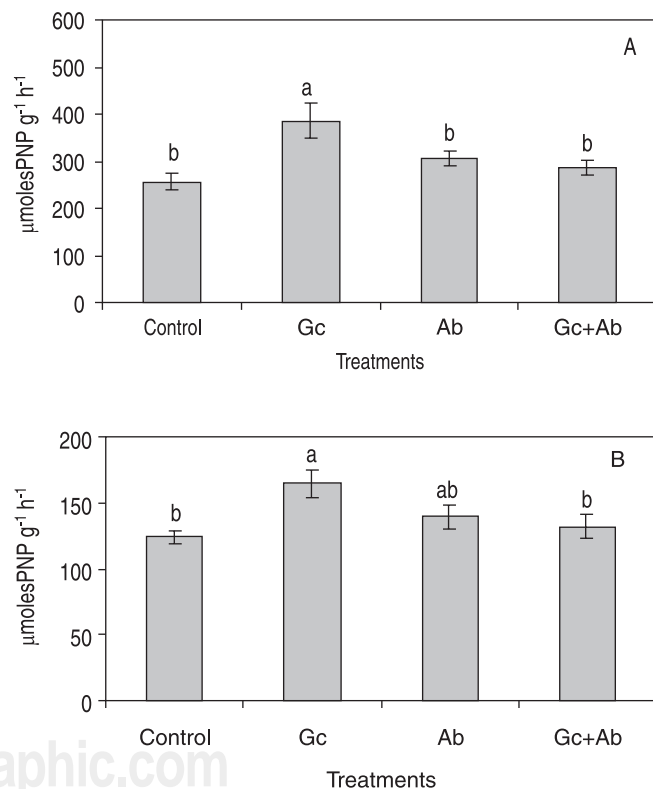


Figure 1. Soluble (A) and extractable (B) root acid phosphatase activity of *Carica papaya* L. cv. Red Maradol plants grown under low P conditions, after 55 days. Gc=*Glomus claroideum*; Ab=*Azospirillum brasilense*. PNP=p-nitrophenol released. Identical letters on the columns in each graph are not significantly different (Tukey $\alpha=0.05$). n=12.

Table 2. Effect of the inoculation of arbuscular mycorrhizal fungus and plant growth promoting rhizobacterium on the bacterial population and mycorrhizal colonization of *Carica papaya* L. cv. Red Maradol established under low P conditions, at two dates after transplanting.

Treatment	Mycorrhizal colonization %	Bacterial population	Mycorrhizal colonization %	Bacterial population
		CFU x 10 ⁷ g ⁻¹ root fw		CFU x 10 ⁷ g ⁻¹ root fw
	- - - - - 15 days - - - - -		- - - - - 55 days - - - - -	
Control	0.0 b	0.0 c	0.0 b	0.0 c
<i>Glomus claroideum</i>	2.2* b	0.0 c	12.3 a	0.0 c
<i>Azospirillum brasilense</i>	0.0 b	200.0 a	0.0 b	45.0 b
<i>G. claroideum</i> + <i>A. brasilense</i>	4.2* a	70.0 b	3.9 ab	151 a

fw=Fresh weight; *Appresoria basically were observed. Means followed by the same letter are not significant (Tukey $\alpha=0.05$). n=12.

(> 200 x 10⁷ CFU g⁻¹ of dry root), but when Gc was inoculated, the bacterial population significantly decreased at 70 x 10⁷ UFC g⁻¹ (Table 2). In contrast, AM colonization with both microorganisms was higher than the solely fungus inoculation, although mainly apresoria and extramatrical mycelium were observed in both treatments. At 55 days, AMF colonization was non-significantly affected by Ab (Table 2) but, the bacterial colonization increased when dual inoculation was done in comparison with the first sampling. AMF-colonization was not observed in control and Ab-inoculated plants.

DISCUSSION

Carica papaya has been considered as a dependent plant of mycorrhizal inoculation,³⁶ however little is known about the fungal interaction with plant growth promoting rhizobacteria (PGPR) fruit crops. Our results was less significant due to the source of inoculum so that mycorrhizal spores require specific conditions for germinating and establishing root colonization. Another sources of inoculum such as soil with hyphae, spores and colonized roots have been successfully used, showing earliest beneficial effects,^{3,22,38,57} that kind of inoculum might make sure off the mycorrhizal effectiveness. Nevertheless, plant growth was mainly dependent on mycorrhizal condition and the solely inoculation of Ab produced negative effects plant dry weight and leaf area. There are some reports about the synergistic effect of AMF and PGPR^{5,6,38} however, some negative effects could be obtained⁵⁴ due to the physiological bacteria activity could show inhibition to the fungal establishment and its effectiveness.^{46,57} Our results indicate that *A. brasilense* combined with *G. claroideum* produced some benefits on total dry matter and leaf area (Table 1) showing a synergistic effect as it has been mentioned by Bashan and Levanony.⁸ In that sense, the knowledge of the possible interaction between AMF and PGPR must be measured so that it could affect the

beneficial effects of both microorganisms. *G. claroideum* inoculation stimulated plant growth in comparison with the control. The negative response of bacterial inoculation could be associated with the apparently specificity of this bacteria so that it are often associated with C4 and some C3 plants⁸ and the inoculation success could be related with that specificity⁵⁷ even it could show inconsistency in plant response to inoculation.¹¹

In some studies, the benefits of AMF on RAPA are attributed at specific soil conditions^{57,38} and plant genotype.²⁸ Under our plant growth conditions, the soluble and extractable RAPA significantly increased by the presence of *G. claroideum* (Figure 1). P-limitation seems to induce secretion of soluble RAPA as a natural plant response,^{27,28,61} however dual inoculation showed a contrary effect possibly to the microbial compatibility and this could represent a specific sink of energy from plant before expressing their beneficial effect, particularly by *G. claroideum*. The extractable RAPA was increased by effect of Gc and Ab, it might suggest that plant could have required more P in the microbial symbiotic phase in order to supply the C-sources required by both microorganisms in order to satisfy their nutrition and beneficial activity. In that way, AMF have the ability to stimulate the RAPA^{23,38,50,56} and that response was highly significant under P-limitations for plant growth. These benefits let the plant to express high relative stem growth rate. Microbial inoculated plants did not show nutrimental deficiencies symptoms and the sufficiency nitrogen application in the nutrient solution could be related with the non significant effects on total chlorophyll content obtained; however, inoculated plants with both microorganisms had higher chlorophyll content. In this case, AMF activity could have participated on the N uptake even under P-limitation which favored increases in chlorophyll. It represents benefits to the plant because it maintains certain nutrimental balance in plants grown under these specific culture conditions. Although a some significant benefits by AMF were observed on plant

growth, it could be related with the low root colonization percentage observed. The low mycorrhizal colonization could have caused as a result of P-limitation in soil. It is known that plants under P-starvation produce a high ethylene releasing^{2,18,32} as a consequence of adventitious root formation.⁵¹ This ethylene could have not only affected the microbial establishment but also mediated the plant response through root architecture and morphology modifications.¹⁸ Some volatile exudates can promote or inhibit the AMF-colonization and internal growth in roots.¹³ In that sense, the ethylene as a product of high level of root auxins which could have acted as indirect delaying agent of AMF and bacteria establishment in roots and then, avoid the expression of their benefits. Boller¹⁷ mentioned that ethylene is a plant signal to activate protective mechanisms against pathogenic fungi and also on AMF-spore germination⁶⁰ which are inhibited by the presence of enzymes whose activity produce degradation of fungal wall cell (chitin and β -1,3-glucane) and this effect could have also delayed the AMF-establishment in roots exposed under P-limitation. However, this hypothesis needs to be studied and confirmed.

The beneficial effects of microbial inoculation on plant growth was observed at 55 days and it is possible that more significant benefits might be observed a few days later. It is emphasized according to the observed AMF-establishment in root system. The incipient effects of both microorganisms on plant growth might be related with the competence of both symbionts by C-compounds⁵⁸ and, it could have produced certain disequilibria on plant physiology so that the plant had not only to satisfy its physiological requirements but also the energy required by both symbiotic microorganisms.⁵ However, utilizing dual microbial inoculation (AMF and bacteria) generally show synergistic effects^{31,38} and some studies have had negative effects on plant growth and mycorrhizal effectiveness.⁴⁶ In some cases *Azospirillum* bacteria besides of having the ability of fixing atmospheric nitrogen, they are able to produce some antibiotic effects on fungal growth,^{9,24} this effect could affect to AMF. Nevertheless, bacterial population seemed to be stimulated by AMF so that it has been discussed that mycorrhizal fungi could modify its environment in order to induce a selective influence on composition and density of rhizobacteria.^{31,42,44} Further studies must be conducted in order to know the possible physiological effects of PGPR on AMF effectiveness not only under substrates with low nutrimental fertility but also under optimum conditions to plant growth.

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