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Short communication
ANHYDRASE CARBONIC ACTIVITY IN ARBUSCULAR MYCORRHIZA

Comunicación corta
Actividad anhidrasa carbónica en micorrizas arbusculares

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ABSTRACT. The action of the metalloenzyme carbonic anhydrase (AC) in plants has been mainly associated to increase the CO_2 concentration inside chloroplast to enhance the carboxylation rate of *Rubisco* enzyme, being this reaction that integrates the CO_2 in carbohydrates during photosynthesis. However, the AC activity has been found in non-green tissues also, like roots, nodules, etiolated leaves, seeds, as well as in bacteria and fungi, which plays diverse and specific rolls. In the present study, the isoenzyme patterns of AC were analyzed in tomato (*Solanum lycopersicum* L.) roots inoculated in individual way with six different arbuscular mycorrhizal fungi (AMF) strains, taking the samples at two moments of symbiosis, and afterward they were compared among them. Results revealed that the AM fungus presence provoked the appearance of isoenzymes with AC activity in tomato roots cv. 'AMALIA', and the obtained isoenzymatic patterns showed a differential response dependent on inoculated strain, principally at early stages of symbiosis. The possible fungal origin of observed isoenzymes is discussed. The present work constitutes the first report about AC isoenzymes in AMF- colonized roots.

Key words: carbonic anhydrase, isoenzymes, mycorrhizae, tomato

RESUMEN. La acción de la metaloenzima anhidrasa carbónica (AC) en plantas se ha asociado fundamentalmente al incremento de la concentración de CO_2 dentro del cloroplasto, para incrementar la tasa de carboxilación de la enzima *Rubisco*; siendo esta la reacción que integra el CO_2 en carbohidratos durante la fotosíntesis. No obstante, la actividad AC también se ha encontrado en tejidos no verdes como raíces, nódulos, hojas etioladas, semillas, así como en bacterias y hongos; donde desempeña funciones diversas y específicas. En el presente estudio, se analizaron los patrones de isoenzimas AC en raíces de plantas de tomate (*Solanum lycopersicum* L.) inoculadas de forma individual con seis cepas de hongos micorrizógenos arbusculares (HMA) diferentes. Tomándose las muestras en dos momentos de la simbiosis y, posteriormente se compararon entre sí. Los resultados revelaron que la presencia del hongo MA provocó la aparición de isoenzimas con actividad AC en las raíces de tomate cv. 'AMALIA'. Los patrones isoenzimáticos obtenidos mostraron una respuesta diferencial en dependencia de la cepa inoculada, principalmente en estadios tempranos de la simbiosis. Se discute el posible origen fúngico de las isoenzimas observadas. Este trabajo constituye el primer informe de isoenzimas AC en raíces colonizadas por HMA.

Palabras clave: anhidrasa carbónica, isoenzimas, mycorrhizae, tomate

INTRODUCTION

Carbonic anhydrases (CA, E.C. 4.2.1.1) form a highly diverse family of enzymes distributed in three life dominions (bacteria, archaea and eukaryotes). Five different classes of CA (α, β, γ, δ and ε) have been described, whose isozymes are localized in distinct cell compartments, such as cytosol, mitochondria and chloroplasts. Some are secreted and others are associated to membranes (1).

This enzyme catalyzes the rapid interconversion of carbon dioxide and bicarbonate: $CO_2 + H_20 \text{ --->} H_2CO_3^- \text{ --->} HCO_3^- + (H^+)$. Carbon dioxide is a ubiquitous gas molecule and the final product of cell respiration.

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Where as bicarbonate is an important biological substrate involved in multiple metabolic reactions, such as lipid, uracil and arginine biosynthesis (2).

CA is involved in various physiological processes, for instance, ion exchange, acid/base balance, carboxylation/decarboxylation reactions and inorganic carbon diffusion between the cell and its environment as well as inside the cell (3). Particularly, two CA transcripts were detected in roots of *Flaveria bidentis* (L.) Kuntze and it is proposed that one isoform provides bicarbonate to anaplerotic reactions involving non-photosynthetic forms of phospho-enol-pyruvate carboxylase (PEPC), while the other, also found in flowers, is related to lipid biosynthesis and antioxidant activity (4).

In young soybean (*Glycine max* (L.) Merrill) nodules, CA activity can make CO_2 recycling easy, whereas in mature nodules, it can provide CO_2 diffusion towards outside nodule system (5); therefore, besides its role in carbon metabolism, another role for this enzyme has been suggested on symbiotic nitrogen fixation and its expression is informed either in nodules or a free-living bacterium of *Lotus japonicus-Mesorhizobium loti* model (6).

Regarding mycorrhized plants, CA activity was mainly determined in leaves (7-9), as the least affected indicator in plants, compared to non-inoculated ones under salt stress and metal toxicity conditions, among others. From the above mentioned, the objective of this study was to determine CA isozyme patterns in tomato (*S. lycopersicum*) roots mycorrhized with six different AMF strains at two times of symbiosis.

MATERIALS AND METHODS

Plant material: tomato (*S. lycopersicum*) plants cv. 'AMALIA' (obtained at Plant Breeding Department from the National Institute of Agricultural Sciences – INCA-) were used as hosts. Seeds were disinfected by a sodium hypochlorite solution at 10 % for 10 min, followed by successive washings with abundant distilled water. Seeding was performed in pots of 300 mL capacity, containing a sterile substrate of Lixiviated Red Ferralitic soil mixed with filter cake at 3:1 (v/v) and sterilized by autoclaving at 121 °C for two hours in cycles of three successive days.

Plants were grown in a glasshouse at an average temperature of 25 ± 3 °C, 75-80 % relative humidity and natural photoperiod.

Fungal material: six AMF strains from INCA collection were used as inoculum. Two ecotypes of *Funneliformis mosseae* (Nicol. & Gerd.) Walker & Schüßler (10, 11), *Rhizoglomus intraradices* (Schenck & Smith) Sieverding *et al*. (12), *Acaulospora scrobiculata* Trappe, *Glomus cubense* (Y. Rodr. & Dalpé) (13) and *Glomus* sp. Inocula were obtained under controlled conditions using *Sorghum bicolor* L. as a trap plant. Spores were poured into petri dishes for its counting and selection by means of a stereo 70x (Stemi 2000-C) microscope (14). Afterwards, they were disinfected using Chloramine T solutions at 2 and 5 %, successively, for 10 min each. Tomato plants were inoculated by adding 200 spores per pot at planting time. A randomized complete design with ten replicates and two replications was used.

Sampling and evaluation: samples were taken at 18 and 32 days after germination in five plants per treatment within each period. Roots were carefully washed; a portion was destined to determine mycorrhizal colonization whereas the other for determining isozyme patterns through poly-acrylamide gel electrophoresis (PAGE).

Mycorrhizal colonization: about 200 mg of secondary roots were taken in each sample, which were oven dried at 70 °C and stained according to a methodology modified by Rodriguez *et al*. (15). Evaluations were performed by means of a stereo 70x (Stemi 2000-C) microscope, determining the percentage of mycorrhizal colonization. All data were transformed by arcsen$\sqrt{x}$ function and statistically processed using ANOVA single classification, where means were compared according to Tukey for $p<0,05$.

Preparation of protein extracts and separation conditions: root proteins were extracted according to a methodology proposed by Solórzano[A] with the modified buffer solution of extraction by Tris-HCl 50 mmol L^{-1} (pH 7,2) and processing five replicates per each case. Protein extracts obtained were lyophilized and then re-suspended in a solution containing Tris-HCl 50 mmol L^{-1} (pH 6,8), glycerol and blue bromophenol, so as to adjust protein concentrations to 1 ug uL^{-1}. For protein separation by PAGE, a polyacrylamide concentrating gel at 4 % and a separating gel at 8,5 % were used, applying 20 uL of each sample and repeating the process three times[A].

Electrophoresis was developed through vertical minigel electrophoresis (BIORAD) equipment and the buffer solution Tris-Glycine 19 mmol L^{-1} (pH 8,3).

[A] Solórzano, E. *Proteínas de defensa y estudio enzimático en la interacción tomate-Alternaria solani*. [Tesis de Doctorado], Universidad Agraria de La Habana, La Habana, Cuba, 2002, 100 p.

Runs were performed at the rate of 25 mA for 90 min at 4 °C.

CA staining: bands were stained using β-naphthyl acetate (0,5 mg), fast blue RR salt and the buffer solution Tris-HCl 0,5 mol L^{-1} (pH 7,19). The gel was incubated in the dark at 37 °C for four hours until the appearance of reddish bands[B].

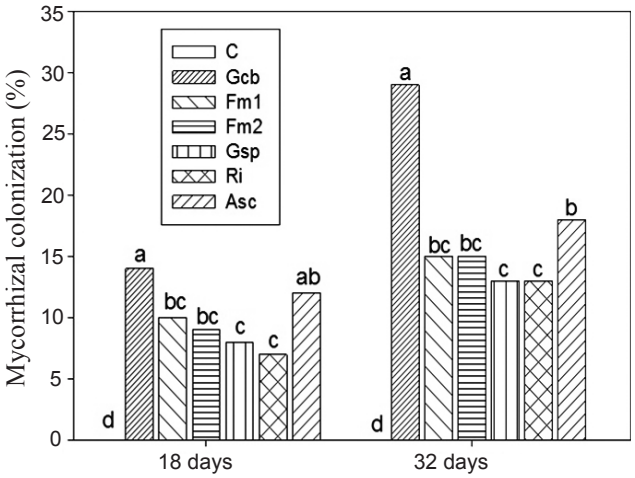
The relative position of each band was settled in the gels obtained according to its relative mobility (Rf), resulting from dividing average migration distance between migration distance of run front and characterizing each band by a numeric value. F90X Nikon camera was used to take documentary evidence of isozyme patterns.

RESULTS AND DISCUSSION

Mycorrhizal colonization: fungal presence was not observed in roots of control plants (Figure 1). Tomato (*S. lycopersicum*) roots inoculated showed percentages of mycorrhizal colonization between 7 and 14 % at 18 days and from 13 to 29 % at 32 days, with significant differences among some treatments in both periods. *G. cubense* treatment was notable with statistically higher values, while *Glomus* sp and *R. intraradices* showed lower values of colonization, without significant differences with *F. mosseae* strains.

The efficient functioning of *G. cubense* strain was demonstrated in previous works for the soil conditions studied (16), which is confirmed in this study. It is remarkable to state the low values of mycorrhizal colonization obtained and few differences between the strains tested, which can be attributed to the short experimental period (one month) during which young plants were evaluated.

CA isoenzymes: AC activity was not observed in roots of control plants (Figure 2). In mycorrhized treatments, at 18 days, three isoforms were observed: No. 2 with Rf 0,33 appeared in all of them but with very low intensity in the treatment with *R. intraradices*; No. 1 with Rf 0 35 was detected in roots inoculated with *Glomus* sp. and *A. scrobiculata*, while in colonized roots by *G. cubense* strains and *F. mosseae,* the isoenzyme No. 3 with Rf 0,25 was observed. At 32 days, only the isoenzyme No. 3 was detected in all mycorrhized treatments, except the one inoculated with *A. scrobiculata*.



C-control Gcb-*Glomus cubense*
Fm1-*Funneliformis mosseae1* Fm2-*Funneliformis mosseae2*
Gsp-*Glomus* sp. Ri-*Rhizoglomus intraradices*
Asc-*Acaulospora scrobiculata*
Columns with unequal letters differ significantly according to Tukey, p<0,05

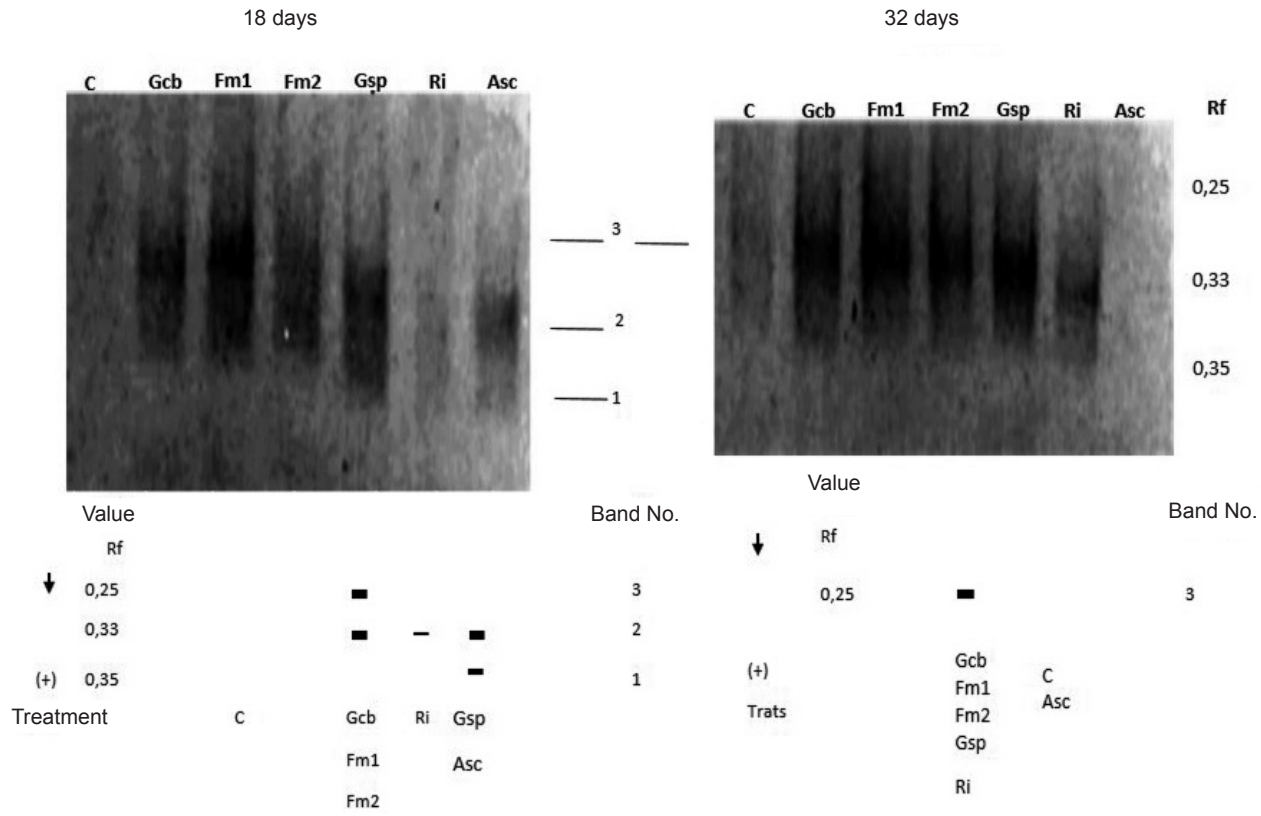
Figure 1. Percentage of mycorrhizal colonization in tomato roots inoculated with six AMF strains at 18 and 32 days after germination

Concerning that, in the germ tube of *R. intraradices,* a highly efficient CO_2 fixation was observed during the dark (17), which was associated with the cycles of urea and tricarboxylic acids. However, the authors did not link this process with CA enzyme action. Then, numerous studies reporting a high metabolic activity with respect to carbon and nitrogen in AMF structures were performed (18-20), which apparently is directly related to plant-fungus nutrient exchange.

Recently, genes coding for CA were identified in a study on *R. irregularis* genome (21). In addition, transcripts with CA putative function were discovered in *Rhizoglomus-Medicago* symbiosis, whose expression is higher at intra-radical mycelium compared with spores, which are characterized as secreted proteins induced by the fungus (22).

These research results reveal two main aspects: 1) roots from tomato cv. 'AMALIA' show CA activity only in response to AM fungal colonization; 2) the appearance of CA isozymes showed a differentiated pattern depending on AMF strain inoculated, mainly within early stages of symbiosis.

[B] Iglesias, L. *Estudio de la variabilidad morfoagronómica y bioquímica en soya* (*Glycine max* L. Merrill). [Tesis de Doctorado], INCA, La Habana, Cuba, 1986, 100 p.



C-control Gcb-*Glomus cubense* Fm1-*Funneliformis mosseae*1 Fm2-*Funneliformis mosseae*2
Gsp-*Glomus* sp. Ri-*Rhizoglomus intraradices* Asc-*Acaulospora scrobiculata*
Lines represent AC isoforms Rf indicates relative mobility of each isoform PAGE, 25 mA

Figure 2. Images and zymotypes of CA isozymes in tomato roots inoculated with six AMF strains at 18 and 32 days after germination

Although these aspects do not allow establishing fungal or vegetable nature of isoenzymes detected, the foregoing evidence, together with the fact that no activity band was observed in control roots at the times analyzed, suggest its fungal origin. Despite that its vegetable origin should not be remarked, particularly or specifically induced by different AMF strains, as a response of tomato plants to molecular dialogue or signal exchange between symbionts. This fact shows the complexity of plant-fungus interaction, which determines compatibility and, most of all, symbiotic efficiency.

CA isozymes detected in mycorrhized roots could be involved in various biochemical and physiological processes, such as nitrogen fixation/assimilation and CO_2 metabolism, similarly to what is reported for *Rhizobium*-legumes interaction (6, 23). It is also important to highlight the role of this enzyme on pH regulation and acid-base balance in cells, so that its activity in mycorrhized roots probably affects AMF functioning, considering the relationship between soil pH and efficient or non-efficient fungus operation (24), as well as the best antioxidant response observed in inoculated plants (25, 26).

In this sense, it should be noted that a higher soil respiration and CO_2 production caused by AMF results in a weakly acid solution of carbonic acid, a reaction that may involve CA enzyme action that leads to rhizosphere acidification (27).

CONCLUSIONS

This scientific paper is the first study dealing with the activity of such enzyme system in mycorrhized roots; thus, it is interesting to deepen on further investigations to clarify its relationship with the symbiotic process and elucidate the fungal or vegetable nature of isoenzymes found.

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