

PEROXIDASE AND POLYPHENOLOXIDASE ACTIVITIES IN TOMATO ROOTS INOCULATED WITH *Glomus clarum* or *Glomus fasciculatum*

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ABSTRACT. Roots of tomato plants cultivated using mycorrhizal inoculant-coated seeds were studied in order to evaluate the induction dynamics of defense mechanisms in the tomato-*Glomus clarum* and tomato-*Glomus fasciculatum* interaction through peroxidase and polyphenoloxidase behavior. Both enzymes kept low activity levels at early symbiosis stages; thereafter enzymatic activities increased in colonized roots reaching a maximum, which was higher in *G. clarum*-inoculated plants, then these activities declined. The isoenzymatic analysis was carried out the days of the dynamics when the studied enzymatic activities were relevant. In general, all the treatments showed four constitutive polyphenoloxidase isoforms with differences in their appearance on time and intensity, while three constitutive peroxidase isoenzymes and an additional one in colonized roots were detected. Our results indicated that arbuscular mycorrhizal fungi initially provoked a like-defense response in tomato roots, which was subsequently suppressed. Indeed, a higher compatibility between *G. fasciculatum* species and tomato plant was suggested because of a lower induction of both enzymes in *G. fasciculatum*-colonized plants.

RESUMEN. Se estudiaron las raíces de plantas de tomate cultivadas a partir de semillas recubiertas con inoculantes micorrizógenos, para evaluar la dinámica de inducción de mecanismos de defensa en la interacción tomate-*Glomus clarum* y tomate-*Glomus fasciculatum* a través del comportamiento de peroxidasa y polifenoloxidasas. La actividad de ambas enzimas se mantuvo baja durante el comienzo de la simbiosis; posteriormente se produjo un incremento en las plantas colonizadas, siendo más acentuado en las raíces inoculadas con *G. clarum* y al estabilizarse la simbiosis tienden a disminuir ligeramente. Se realizó el análisis isoenzimático los días de la dinámica donde fue mayor la actividad de las enzimas estudiadas; en general, se observaron cuatro bandas constitutivas con actividad polifenoloxidasas en todos los tratamientos aunque con diferencias en cuanto a su aparición en el tiempo y en la intensidad de estas. Mientras en el patrón de peroxidasas se observaron tres isoenzimas constitutivas y una adicional, específica de la simbiosis, en las raíces colonizadas por los hongos micorrizógenos arbusculares. Los resultados indican que estos hongos provocaron inicialmente una respuesta similar a la “defensiva” en las raíces de tomate, la cual fue subsecuentemente suprimida. Además, la menor inducción de las enzimas estudiadas en las plantas colonizadas por *G. fasciculatum* sugiere una mayor compatibilidad entre esta especie y el tomate.

Key words: arbuscular mycorrhizae, *Glomus*, isoenzymes, peroxidases, tomato, polyphenoloxidases

Palabras clave: micorrizas arbusculares, *Glomus*, isoenzimas, peroxidasas, tomate, polifenoloxidasas

INTRODUCTION

Mycorrhizas are among the most universal, intimate and important symbiosis in terrestrial ecosystems. The arbuscular mycorrhizal (AM) type, formed between plants and zygomycetous fungi of the Glomales order, is the most ancient and widespread mycorrhizal symbiosis (1).

Indeed, enhanced mineral nutrition, stress alleviation, microbial changes in the rhizosphere, competition with the pathogen for nutrients and infection sites, modifications in root system morphology, anatomical changes such as increased lignification of root endodermal cells and biochemical alterations in plant tissues are the most frequently evoked mechanisms (2). Qualitative and quantitative alterations in protein expression have been reported in various AM association (3, 4), but only weak, very local or transient induction of plant defense mechanisms seems to occur in AM symbiosis in comparison to that of the plant reaction to pathogens (5).

There is considerable evidence for the role of AM fungi in the control of root pathogens (2,6). However, the mechanisms of action are still poorly understood,

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especially if we consider that the observed effects more likely result from different factors acting together. The colonization of root by AM fungi induces biochemical changes within host tissues, these include stimulation of the phenylpropanoid pathway with consequent increment of phenylalanine ammoniolyase and chalcone isomerase activities (7,8), change in aliphatic polyamines levels (9), increase of certain hydrolase activities, mainly chitinases and β 1-3 glucanases (10,11), synthesis of proteins of unknown function (4,12) and activation of defense-related genes (13).

Peroxidase (E.C. 1.11.1.7) and polyphenoloxidase (E.C.1.10.3.1 and E.C.1.10.3.2) induction is apparently elicited in higher plants in response to physiological and environmental stress; they are considered defense-relative enzymes because of their activation in host plants against pathogen attack (14).

In the present work, the induction dynamics of peroxidase and polyphenoloxidase activities by AM fungi *Glomus clarum* and *Glomus fasciculatum* in tomato roots was studied during the colonization process, as well as their isoenzymatic patterns, with the aim of evaluating the elicitation of these defense- relative enzymes.

MATERIALS AND METHODS

Plant and fungal material. Tomato seeds (*Lycopersicon esculentum* Mill) INCA-17 variety were dressed with arbuscular mycorrhizal inoculum. Thus, a saturated saccharose solution was used as adherent. Mycorrhizal inoculants contained 26.30 and 22.06 spores/g of *Glomus clarum* and *Glomus fasciculatum* respectively.

The experimental design consisted of three different treatments: non-mycorrhizal plants (Nm) and plants inoculated with *Glomus clarum* (Gc) or *Glomus fasciculatum* (Gf). To accomplish this study, the plants were grown in metallic pots containing a sterile mixture of compacted Red Ferralitic soil (15) and filter cake (3:1 v/v). **Growth conditions and plant harvest.** Tomato plants were grown in a semicontrolled environment room (23°C +/- 2°C temperature, 80-85% relative humidity and natural photoperiod). Plants were harvested 3, 5, 7, 9, 15, 21 and 26 days after germination. Root systems were carefully washed in running water, rinsed in distilled water and weighed.

Approximately 200 mg of root systems were kept for quantification of arbuscular mycorrhizal colonization by clearing and staining the roots (16). Mycorrhizal colonization was expressed by the percentage of colonized cortex in the root system.

Protein extraction and quantification. Fresh roots were ground at 4°C in an ice-chilled mortar with liquid nitrogen and the resulting powder was suspended in 100mM (sodium acetate) extracting buffer, pH 5.2 (1:2, w/v) with polyvinylpyrrolidone 5 % and β -mercaptoethanol 0.05%. Crude homogenates were agitated in shaker for 45 min. in ice bath. After that, homogenates were filtered through

four gauze caps and centrifuged at 14000g for 25 min. at 4°C. The supernatant fractions were kept frozen at -20°C. Three replicates were processed in each case. Protein contents were determined by Bradford's method using bovine serum albumin (BSA) as standard (17).

Enzymatic assays. Peroxidase and polyphenoloxidase activities were analyzed in all tomato root extracts using colorimetric assays.

Peroxidase activity was carried out according to a continuous method described (18). Guayacol and hydrogen peroxide were used as substrates. The speed of guayacol oxidation for the enzyme in hydrogen peroxide presence was determined at 470 nm.

Polyphenoloxidase activity was determined by a continuous method described (19). Pirogallol was used as enzyme substrate. The speed of pirogallol oxidation for the enzyme was evaluated at 420 nm.

In both cases the optical density variation on time (DO.D)/(Dt) was studied during two min. for intervals of 15 seconds. The enzymatic activities were calculated according to the expression:

$$\text{Enzymatic activity} = \frac{\Delta OD}{\Delta t} \frac{1}{k} \frac{V_1}{V_2} \cdot \text{dilution}$$

Where:

k - guayacol or pirogallol molar extinction coefficient since peroxidase or polyphenoloxidase activity.

V_1 - assay volume

V_2 - enzyme volume.

The specific activities for these continuous methods were calculated as follows:

Specific activity (EAU/mg) = enzymatic activity / mg of total proteins
Statistical analysis. A complete randomized bifactorial variance analysis (ANOVA) of the specific activity values obtained and percentage of mycorrhizal infection was carried out; the evaluated factors were:

- Treatment (non-mycorrhizal plants, plants inoculated with *G. clarum* and plants inoculated with *G. fasciculatum*).
- Time (3, 5, 7, 9, 15, 21 and 26 days).

The analysis of Duncan's Multiple Range test with $p < 0.01$ and LSMEAN (Minimal cuadratic means) according to statistic package SAS* were included.

Isoform detection. Isoenzymatic patterns were determined in extracts from 21 and 26d for peroxidases and from 15, 21 and 26d for polyphenoloxidases. These extracts were concentrated in dialysis membranes using polyethylenglycol (PEG) 6000 and they (10 mg of proteins per sample) were analyzed by polyacrylamide gel electrophoresis (PAGE) under native conditions (20). Two gels were used: concentrating gel (upper gel)-5% and separating gel (lower gel)-12.5 %.

Electrophoreses were run with TRIS-Glycine buffer pH 8.3 at 40 mA for 2h at 25°C +/- 2°C.

*Cary, N. S. SAS Institute INC., 1987.

Peroxidase activity on gel was revealed by staining the gel for 10 min. with hydrochloric benzydin solution and hydrogen peroxide solution (1/1, v/v) (21).

Polyphenoloxidase activity on gel was revealed by staining the gel for 30 min. with L-proline solution and dihydroxyphenylalanin (22). These reactions were stopped with 10 % acetic acid.

RESULTS AND DISCUSSION

Fungal colonization. Mycorrhizal colonization with both *G. clarum* and *G. fasciculatum* began the first day after germination (data not shown) because seeds were dressed with inoculum and a well established symbiosis could be observed after 15 days of growth. More than 20 % of root length became mycorrhizal (Table I). After 26 days the percentage of root colonization reached 28-30 % for inoculated plants. These percentages were similar for colonized plants at three harvests (15, 21 and 26 days); but the percentages of visual density (Table II) were higher for *G. fasciculatum*- colonized plants, reaching values between 0.56 and 1.28% at 15 and 26 days respectively, whereas *G. clarum* colonized plants reached up to 0.56% at 26 days only. This variable indicates better the symbiotic efficiency. These results agree with the previous report (23), it confirming that *G. fasciculatum* is a more aggressive species than *G. clarum* because of its different colonization levels.

Table I. Percentage (%) of mycorrhizal colonization inside root tissues in 15, 21 and 26 day-old tomato plants inoculated with *Glomus clarum* (Gc) or *Glomus fasciculatum* (Gf) and non-mycorrhizal plants (Nm)

Treatments	Days after germination		
	15	21	26
Nm	1f	4e	7d
Gc	19c	28b	28b
Gf	21c	29ab	30a
S.E		0.62**	
V. C. (%)		5.71	

Table II. Percentage (%) of visual density inside root tissues in 15, 21 and 26 day-old tomato plants inoculated with *Glomus clarum* (Gc) or *Glomus fasciculatum* (Gf) and non-mycorrhizal plants (Nm)

Treatments	Days after germination		
	15	21	26
Nm	0.01 e	0.04 d	0.07 d
Gc	0.45 d	0.54 c	0.56 c
Gf	0.56 c	1.03 b	1.28 a
S.E.		0.31**	
V.C. (%)		3.61	

Peroxidase and polyphenoloxidase activities. Peroxidase specific activity studied in tomato roots (Figure 1a) showed values around zero until 9 days. On 21 days, a statistically significant increment ($p < 0.001$) was observed in inoculated plants relative to uninoculated control roots, which was markedly high in tomato-*G. clarum* interaction, thereafter enzymatic activity decreased below the control roots, which tended to increase at 26 days. At the end of the dynamics, *G. fasciculatum*-inoculated plants showed very low values, even less than control plants.

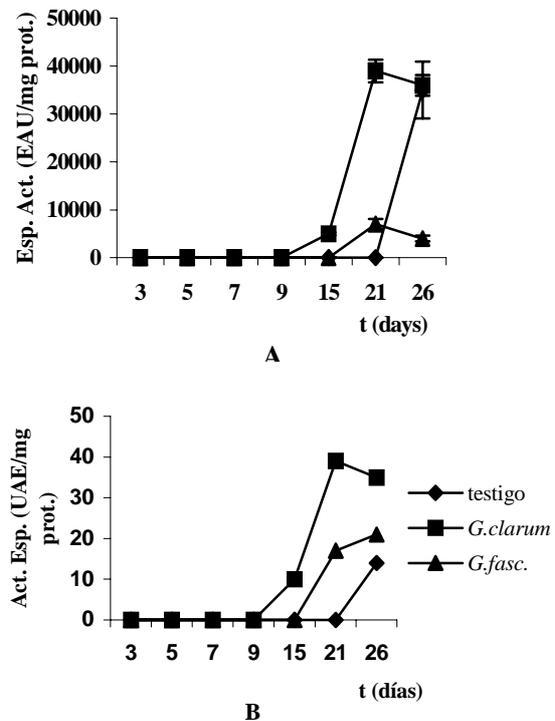


Figure 1. Specific activities of peroxidases (A) and polyphenoloxidases (B) in tomato roots inoculated with *Glomus clarum* (Gc), *Glomus fasciculatum* (Gf) and non-mycorrhizal plants (Nm)

Polyphenoloxidase specific activity in studied roots (Figure 1b) had similar behavior to peroxidase activity until nine days, showing very low values nearly to zero. *G. clarum*- inoculated roots activity increased at 15 days, reaching a maximum statistically significant ($p < 0.001$) at 21 days, then tended to decline slowly. On 21 days, the activity in *G. fasciculatum*-inoculated roots increased keeping high activity levels at 26 days, but these values were lower than enzymatic activity of colonized plants with *G. clarum*. Control plants kept low activity levels during the dynamics and only at the end it had a light increment, but always below colonized roots.

The low levels of peroxidase and polyphenoloxidase activities on early symbiosis stages suggest that a successful plant-microorganism recognition was produced and fungi penetration took place, taking into account the role of these enzymes in cell wall reinforcement of lignin

and suberine deposition on it (24) and as arbuscular mycorrhizas do not penetrate reinforced walls with these compounds because they are unable to degrade them; furthermore, such enzymatic reaction products are highly reactive and toxic since they can inhibit fungal growth or activity of hydrolytic enzymes secreted by them (25).

Results obtained by Llonín (23) under field conditions showed a higher percentage of infection and yield in *G. fasciculatum*-inoculated tomato than *G. clarum*-inoculated tomato plants. The notable increase in both enzymatic activities in treated plants with *G. clarum*, obtained in our experiment as well as a previous report (23), suggest that *G. fasciculatum* is the best physiologically prepared species for tomato colonization. Therefore we can suppose that it would possess an inhibitory mechanism of these enzymatic activities and its defense role in host plants, the compatibility between *G. fasciculatum* species and tomato being higher. According to figures 1 and 2, peroxidase and polyphenoloxidase activities increased when the symbiosis was established; these enzymes are related with plant defense mechanisms, coinciding with studies accomplished in *Allium porrum* (26) and in tobacco roots (27).

The defense mechanisms developed subsequent to microorganism penetration are difficult to be studied, since all plants have a similar repertoire against pathogen attack; if this constitutes a defense mechanism also depends on microorganism capacity to tolerate or not the reaction products.

Isoform analysis. Isoenzymatic study was carried out over the latest days of the dynamics according to the enzymatic activity results previously described. Figures 2 and 3 show acidic peroxidase and polyphenoloxidase isoforms present in evaluated treatments. In both cases four isozymes with different electrophoretic mobility were revealed; they have differences in their appearance on time and intensity.

Peroxidase electrophoresis (Figure 2) of crude extract from control tomato roots at 21 days showed one faint band (2) while at 26 days three main bands appeared (2, 3 and 4), they corresponding to constitutively expressed isoforms. Extract from *G. clarum*-inoculated roots displayed the constitutive isoforms at both sampling times, the last one with less intensity. One additional band (1) with higher mobility was also induced, but at 26 days it had a higher intensity. Plants colonized with *G. fasciculatum* revealed the bands 1 and 2 at 21 days, corresponding to new and constitutive isoforms respectively, while at 26 days only two constitutive isozymes were observed (2 and 3); all of these appeared as faint bands.

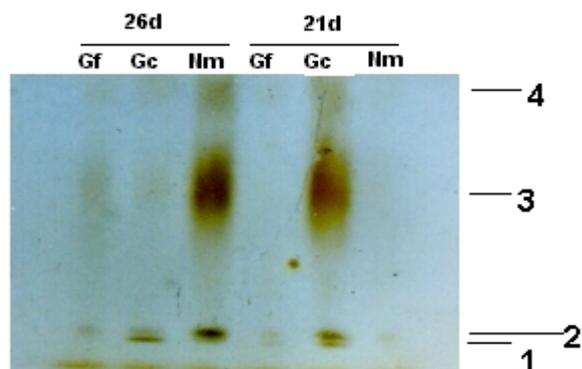


Figure 2. Peroxidase isozyme patterns on a 15% polyacrylamide gels. Root extracts (10 mg of proteins/sample) from non-mycorrhizal controls (Nm), *Glomus clarum*-colonized (Gc) and *Glomus fasciculatum*-colonized (Gf) tomato plants were electrophoresed at 21 and 26 days. 2,3 and 4 bands correspond to constitutive isoforms and band #1 is a mycorrhiza-related isozyme

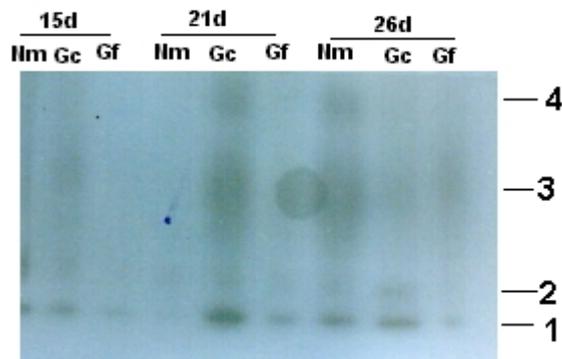


Figure 3. Polyphenoloxidase isozyme patterns on 15% polyacrylamide gels. Root extracts (10mg of proteins/sample) from non-mycorrhizal controls (Nm), *Glomus clarum*-colonized (Gc) and *Glomus fasciculatum*-colonized (Gf) tomato plants were electrophoresed at 15, 21 and 26 days. All bands correspond to constitutive isoforms.

Isoenzymatic study of polyphenoloxidases (Fig. 3) in non-mycorrhizal roots showed at 15 days two faint bands (1 and 2) and at 26 days four main bands were detected. Extract from roots colonized with *G. clarum* revealed three faint bands (1, 2 and 3) at 15 and 26 days, while at 21 days four main bands appeared. In *G. fasciculatum*-treated plants one faint band was detected (1) at 21 days and at the end of the dynamics two faint bands were observed (1 and 3). All of these bands correspond to constitutively expressed isoforms; specific symbiosis isozymes were not detected.

The activity of these isozymes appears to increase with plant age; that is why observed behavior in non-mycorrhizal plants in this study. In addition to defense

role of peroxidase and polyphenoloxidase (14), they are very related to development of other physiological states of the plant (28), such as respiration, different oxidative reactions, lipid peroxidation and abiotic stress.

Electrophoretic results (Figures 2 and 3) are in correspondence with induction dynamics of peroxidase and polyphenoloxidase (Figure 1a and b), coinciding the number of bands as well as their intensity with the activity values observed in each treatment. *G. clarum* species elicited higher levels of both enzymes in tomato roots, inducing a new peroxidase isoform, which corroborates that *G. fasciculatum* presents higher compatibility in the interaction with tomato plants (23).

Peroxidase and polyphenoloxidase activities and their isozymes were repressed at the end of the dynamics (Figures 1, 2 and 3). When colonization by AM fungi is successful, some fungal strategies of self-camouflage may occur such as important wall modifications during the colonization process, or repression of the induced plant defense mechanisms (29). In fact, the host plants influence wall morphology and composition of AM fungi as they develop within the root tissues.

Peroxidase and polyphenoloxidase induction is generally considered to be a part of a non-specific defense response initiated in plants after pathogen attack, but also a consequence of various physical, chemical and environmental stresses (14). However, the induction of one new root peroxidase isoform during AM symbiosis seems to be a specific response, since differential induction of chitinase and β 1-3 glucanase isoforms after symbiotic or pathogenic fungal interactions has been reported in various plants (11, 30, 31, 32, 33). In the present work, a similar induction of a specific peroxidase isoform in tomato roots has been shown to be a consequence of interactions with two different AM fungal species. In this context, it can make emphasis in the fact that *G. clarum* elicited the additional peroxidase isozyme and all constitutive peroxidase and polyphenoloxidase isoforms stronger than *G. fasciculatum*. It remains to be clarified if this can be related to the different colonization dynamics of both fungi, or to differences in their wall composition and/or structure.

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