PEROXIDASE AND POLYPHENOLOXIDASE EXPRESSION IN *Sorghum bicolor* ROOTS, INOCULATED WITH DIFFERENT ARBUSCULAR MYCORRHIZAL FUNGUS SPECIES

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ABSTRACT. Peroxidase and polyphenoloxidase isoenzymatic expression was studied in *Sorghum bicolor* roots, inoculated with six arbuscular mycorrhizal fungus species belonging to *Glomus* genus, two and three months after seed germination. Fungal colonization and visual density percentages were determined, obtaining high values in both cases, it confirming that symbiosis was adequately established. In general, different electrophoretic patterns were observed in both systems analyzed, taking into account the evaluation time and mycorrhizal species used, in which seven peroxidase and three polyphenoloxidase isoforms were detected. The three-month peroxidase system showed the highest polymorphism; *Glomus mossae* and *Glomus claroideum* species being remarkable.

Key words: arbuscular mycorrhizae, peroxidases, polyphenoloxidases, isoenzymes, sorghum

RESUMEN. Se estudió la expresión de isoformas de los sistemas enzimáticos peroxidasas y polifenoloxidasas, en raíces de *Sorghum bicolor* inoculadas con seis especies de hongos micorrízicos arbusculares pertenecientes al género *Glomus*, a los dos y tres meses después de germinadas las semillas. Se determinaron los porcentajes de colonización fúngica y densidad visual, obteniéndose elevados valores en ambos, lo cual confirma que la simbiosis se estableció adecuadamente. De forma general, se observaron diferencias en los patrones electroforéticos de los dos sistemas analizados, atendiendo a los factores tiempo de evaluación y especie de micorriza utilizada, donde se detectaron siete isoformas de peroxidasas y tres de polifenoloxidasas. El sistema peroxidasa a los tres meses mostró un elevado polimorfismo, destacándose los tratamientos con *Glomus mosseae* y *Glomus claroideum*.

Palabras clave: micorrizas arbusculares, peroxidasas, polifenoloxidasas, isoenzimas, sorgo

INTRODUCTION

Arbuscular mycorrhizas constitute the oldest symbiosis known, so that they prevail from the beginning of terrestrial plant evolution (1). This is not a specific association, because any arbuscular mycorrhizal (AM) fungus can colonize any symbiosis-forming susceptible plant. However, in spite of this non-specificity, some of these fungi benefit more a certain host than others and, mainly, they show different adaptation degrees to establish the micorrhiza and function under particular edaphoclimatic conditions (2).

All these are related to notable physiological and biochemical changes, which take place in the host plant after AM fungal colonization. Some investigations involve mechanisms related to pathogen resistance increase (3, 4), as well as rhyzosphere microbial changes, competition with pathogen for nutrients and infection zones, modifications on root system morphology, and anatomic changes, such as lignification increment in root endodermic cells (5, 6). One of the biochemical disorders reported is the occurrence of qualitative and quantitative changes on protein expression (7, 8, 9); particularly, the expression of new peroxidase isoforms has been detected in tomato (2, 10).

Among plant mechanisms involved in the recognition and defense against pathogens, one of the most notable facts is the fast production and accumulation of oxygen reactive species, such as superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), known as oxidative explosion (11). Oxygen reactive species were proposed to activate gene expression in defense processes, altering cell oxidation-reduction state and modulating the activity of transcription factors, sensitive to oxidation-reduction changes (12). All these processes require a fine regulation, for which the activity of several enzymes involved in the oxidative metabolism is needed, such as peroxidases and polyphenoloxidases.

Electrophoretic techniques, carried out under native conditions in polyacrilamide gels, can be used not only for separating fungal and host proteins but also to define genetic markers: isoenzymes, which allow to determine differences in the root colonization process among different AM fungi (13). In sorghum, specifically, differences in peroxidase expression patterns among cultivars, varieties and plant organs have been reported (14), which indicates that isoenzymatic patterns are specific for each genetic material. This way, it is confirmed that the method used is a valuable tool, for determining plant genetic variations.

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Taking all those aspects into account, this work has the objective to study the differential expression of peroxidase (EC 1, 11, 1,7) and polyphenoloxidase (EC 1, 10, 3,1 and EC 1, 10, 3,2) isoenzymes in *Sorghum bicolor* roots, inoculated with six species of AM fungi, belonging to *Glomus* genus.

MATERIALS AND METHODS

Plant and fungal material. Six AM fungus species from *Glomus genus* belonging to the National Institute of Agricultural Sciences were studied (Table I).

Table I. Treatments and AM fungus species evaluated

Treatments	Species
Gf	*Glomus fasciculatum* (Gerd. & Trappe emend. Walker & Koske)
Gc	*Glomus clarum* (Nicolson & Schenck)
Gm	*Glomus mossae* (Nicol. & Gerd.) Gerdeman & Trappe
Gcd	*Glomus claroideum* (Schenck & Smith)
Ga	*Glomus sp*1(yellow) Güira
Gi	*Glomus intraradices* (Schenck & Smith)

This research was carried out in September, for which *Sorghum bicolor* was selected as host plant and two seeds were put per plastic container (5L) on a sterile kaolin clay substrate (15). Eight containers were used per treatment, to which 75 g of inoculum from each studied species were added. Mycorrhizal inoculants contained 250 spores.g^{-1} (16).

Growth conditions and plant harvest. Sorghum plants were grown under protected conditions, at environmental temperature and relative humidity, as well as natural photoperiod. Plants were harvested at two stages of symbiosis development: two and three months after germination, because this could be considered a cyclic process and during each root reinfection, changes in plant response take place. Root systems were carefully washed and weighed.

During the second evaluation period, about 200 mg of roots per treatment were selected, in order to evaluate fungal colonization and visual density percentages, following Herrera's methodology (17). Three replications were processed in each case.

Protein extraction. Fresh roots were macerated in mortar with liquid nitrogen, and the resulting powder was homogenized in 50 mM (Tris-HCl) extracting buffer, pH 7.2 (1:1 w/v) with sucrose 10 % and β mercaptoethanol 0.1 %. Crude extracts were agitated in shaker for 45 minutes in ice bath. Then, they were filtered through four gaize caps and centrifuged at 14 000 g for 25 minutes, at 4ºC. The obtained supernatants were kept frozen at -20°C. In each case, three replications were processed.

Electrophoresis. Electrophoretic analyses were performed in polyacrylamide gels (PAGE) (Fluka quality reagent), under native conditions, using 20 µL of sample in each case, according to Maribona's method (18), with the following modification: concentrating gel (upper) 4 %; and separating gel (lower) 7.5 %.

Electrophoreses were performed in a vertical minigel chamber (BIORAD), run at 25mA and 4°C, during 90-120 min. For this purpose, Tris-Glicine 19mM pH 8.3 buffer was used.

For peroxidase staining, gel was steeped in hydrochloric benzidine and hydrogen peroxide, until blue bands appeared within 5-10 min. (19). Polyphenoloxidase activity in gel was revealed with the use of L-Proline and dihydroxiphenyl-L-alanine (DOPA), dissolved in a buffer sodium phosphate 0.1M pH 6.5 solution (20). Both reactions were stopped with HAc 10 %.

An F90X Nikon camera and a Konica (100 asa) film were used to take pictures from gel, which were afterwards scanned (Acer Slim6). The pictures, as well as the corresponding zymograms to each electrophoresis, are shown in the results, where the relative mobility value of each isoform (Rf) appears.

Statistic analysis. Data of mycorrhizal colonization percentage were transformed by arcsen$\sqrt{x}$ function; and a variance analysis (ANOVA) was applied to them, as well as to visual density percentages, using a randomized complete design. Means were compared, following Duncan's Multiple Range test with $p \leq 0.05$.

RESULTS AND DISCUSSION

Fungal colonization. Table II shows mycorrhyzal colonization (%C) and visual density (%VD) percentages in root tissues of sorghum plants inoculated with different AM fungus species.

Tabla II. Mycorrhizal colonization and visual density percentages

Treatments	%C	%VD
Gf	57 ± 0.012 b	6.55 ± 0.67
Gc	68 ± 0.025 a	6.27 ± 0.50
Gm	58 ± 0.018 b	5.73 ± 0.87
Gcd	51 ± 0.006 c	4.67 ± 0.67
Ga	56 ± 0.017 b	5.08 ± 0.58
Gi	61 ± 0.006 b	5.98 ± 0.57
Sig.	***	n.s.

There are not significant differences among values of each indicator, followed by common letters, according to Duncan's test ($p \leq 0.05$)

Every treatment showed high values in determined fungal indicators, mycorrhizal colonization ranging between 51-68 % and visual density between 4.67-6.55 %. Significant differences were not found among treatments for visual density. Nevertheless, roots inoculated with *G. clarum* obtained the highest value of mycorrhizal colonization, whereas the lowest value corresponded to *G. claroideum*-colonized roots. Similar values of mycorrhizal colonization and visual density percentages, to those obtained in sorghum roots through this work have been reported reaching between 46-68 % for mycorrhizal colonization, as well as between 4.30-11.97 % for visual density colonization (21). Our results prove the high quality

of inocula and the optimal conditions of the experiment to guarantee symbiosis establishment.

Electrophoresis analysis. Figure 1 shows peroxidase (Pox) zymogram and isoenzymatic pattern determined in mycorrhized sorghum roots, at two months after seed germination. There is a band with a mobility of 0.04 in all treatments, except for *G. intraradices* (Gi) inoculated roots. However, there are differences concerning band intensity, *G. fasciculatum* (Gf) providing the highest one, followed by *G. claroideum* (Gcd) and *Glomus sp*1 (Ga). Plants colonized by *G. clarum* (Gc) and *G. mossae* (Gm) showed the lightest bands.

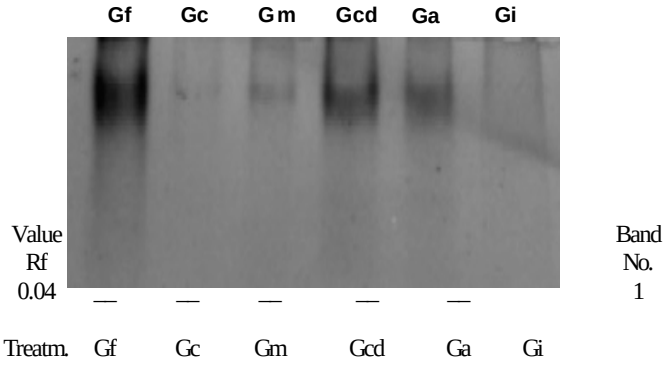

Figure 1. Isoenzymatic pattern and zymogram of peroxidases expresed in sorghum roots inoculated with six AM fungus species at two months after germination

Figure 2 shows Pox isoenzymatic pattern and zymogram at three months after germination, in which seven electrophoretic bands appeared. Band no. 7 was monomorphic and presented a relative mobility of 0.05, its higher intensity was observed in Gm treatment. The other six bands are polymorphic.

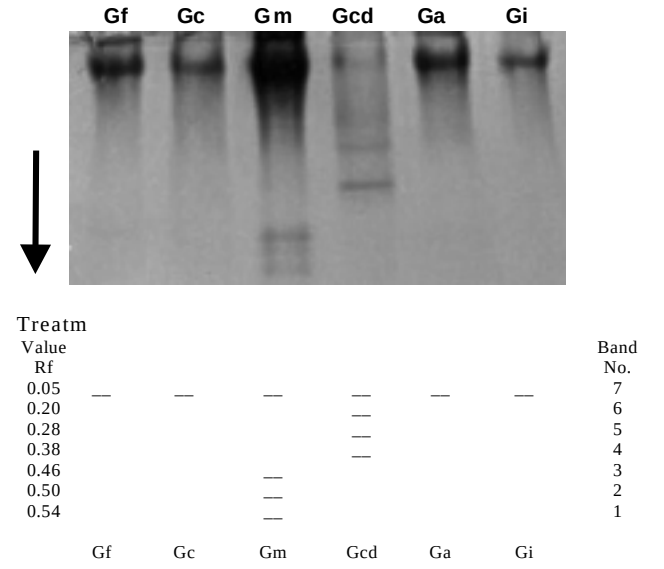

Figure 2. Isoenzymatic pattern and zymogram of peroxidase expressed in sorghum roots inoculated with six AM fungus species at three months after germination

Comparing Pox electrophoretic patterns in both times studied, the expression of one isoform (no. 7) in all treatments were observed at three months after seed germination, which presented a similar Rf value to that detected two months after germination with the difference that, during the first evaluation period, this isoenzyme was not observed in Gi treatment. These isoforms could be the same, if the little difference between its migrations (0.04 and 0.05) is taken into account. Besides, an intensity increment of this band in Gm-inoculated plants was detected at three months, compared to that shown at two months. An intensity reduction in treatments with Gf and Gcd was also observed.

Moreover the Pox pattern at three months shows the induction of six additional Pox isoforms with different electrophoretic mobility: three of them corresponded to Gm-inoculated roots, and the other three isoforms to Gcd-inoculated roots. In this last treatment, the expression of a higher number of isoforms (4), with low intensity, coincided with the lowest fungal colonization and visual density values: 51 and 4.67 %, respectively. However, there were not significant differences ($p \leq 0.05$) for visual density between treatments. It is important to remark that the isoform expressed in Gcd treatment at two months showed a mean intensity. Unlike to Gm-inoculated plants, mean values of both analyzed fungal indicators were obtained, but the intensity of the isoform expressed at two months was low. Whereas at three months, although four electrophoretic bands were observed, the intensity of band no. 7 was markedly higher.

Figure 3 shows the isoenzymatic pattern and zymogram of polyphenoloxidase (Ppo) evaluated in sorghum roots inoculated with six AM fungus species, two months after plant germination. Two bands were detected: no.1 was only found in Ga treatment with a mobility of 0.33; whereas no. 2 was monomorphic with a migration of 0.17. No differences concerning isoform intensity were observed.

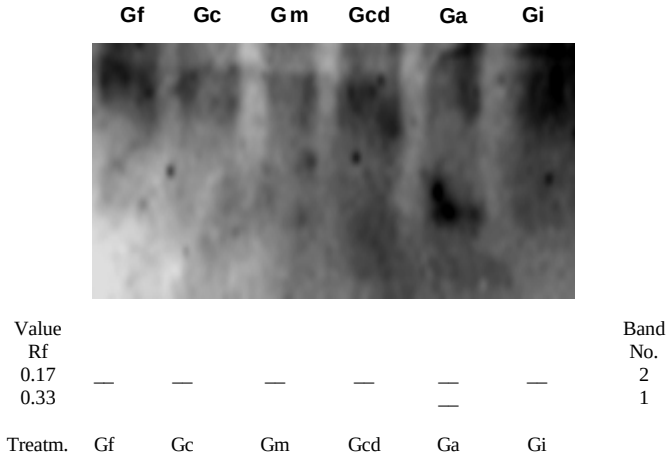

Figure 3. Isoenzymatic pattern and zymogram of polyphenoloxidases expressed in sorghum roots inoculated wit six AM fungus species at two months after germination

Figure 4 shows isoenzymatic pattern and zymogram of Ppo at three months. Two polymorphic bands were found: no.1 was only detected in Gi treatment with a mobility of 0.26; whereas no.2 appeared in plants inoculated with Gf, Gc, Gm, Ga, and Gi, presenting a migration of 0.15. The band intensity in this system was similar; however, the treatments with Ga and Gm were the most remarkable ones, followed by Gc.

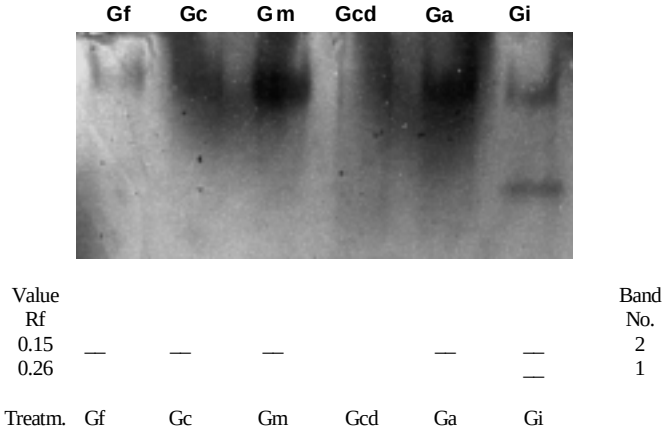

Figure 4. Isoenzymatic pattern and zymogram of polyphenoloxidases expressed in sorghum roots inoculated wit six AM fungus species at three months after germination

Ppo isoenzymatic patterns showed differences. In both cases, the corresponding isoform to band no.2 seems to be the same, due to the little difference between Rf values (0.02). Nevertheless, the other isoforms expressed in both gels (band no. 1) resulted different isoenzymes, because of differences observed in their migrations (Rf values of 0.33 and 0.26). In contrast with the Pox results, in this case, Ga and Gi treatments presented a higher number of bands, but not at the same period. These two treatments showed mean values in both fungal indicators studied; among them, the lowest values were from plants inoculated with Ga.

Several works correlate increments in these enzymatic activities with pathogen resistant (22, 23), and during mutualistic interactions. For instance, in eucalyptus, Pox induction was found by ectomycorrhizal formation, this increment was higher and most continuous in time, during interaction with the most aggressive ectomycorrhizal strain used (24). In arbuscular mycorrhizal studies, transient Pox activity increments have been shown, during the early stages of colonization, which were associated with fungus development control in surface root tissues (25, 26). Other authors have described an increment in total Pox activity, as well as the presence of two additional isoforms in advanced symbiosis stages, which were related to plant phosphorus level increment (27). Similarly, four isoform induction and high Ppo activity in tomato mycorrhized roots at advanced stages of colonization were reported, reaching different values depending on the AM fungus species present (21).

According to our results, there are certain differences in the establishment of symbiosis among the species studied, existing highly significant differences among fungal colonization percentages ($p \leq 0.05$), as well as in Pox and Ppo electrophoretic patterns, in both times evaluated.

There is a correspondence between the treatment, showing the highest fungal colonization value (Gc), and the few isoform appearance in electrophoresis, in which only one band was observed in the analyzed Pox and Ppo patterns. This issue is reaffirmed with a low intensity of the isoforms expressed.

In general, roots inoculated with Gf, Gm, Ga, and Gi, showed mean values of evaluated fungal indicators and presented a moderate number of isoforms in electrophoresis. However, there were certain differences in the number and intensity of all bands detected. In the case of Gf treatment, an isoform was expressed in all patterns studied, except for Pox pattern at two months, that presented the main intensity band. In this sense, it is important to consider that Gf treatment reached the highest visual density percentage. Despite this indicator did not show significative differences, it is very important because it describes more accurately fungus occupation level in roots.

Gm-inoculated plants presented a low intensity band at two months, in both enzymatic systems. Nevertheless, at three months, Ppo isoform with the higher intensity was expressed, as well as the number of bands (4) detected in Pox pattern and the intensity of band no.7 were higher. Ga and Gi treatments presented a similar behavior, with the difference that Pox expression at two months was not observed in Gi-inoculated plants and the time of appearance of two Ppo isoforms did not coincide either.

Results are logical if the involvement of both enzymes in different physiological processes are taken into account, such as lignification, cell wall suberization, polyphenol deposition, papilar structure formation, wall reinforcement by protein intercrossing, phenolic compound oxidation, and production of free radicals (2). The lowest induction of these isoenzymes is favorable to a successful symbiotic establishment and, therefore, high values of fungal indicators can be obtained. On the contrary, a higher number of isoenzymes is not suitable for mycorrhizal association, because lignification and cell wall reinforcement processes avoid arbuscular formation (28, 29). In this sense, it is important to emphasize that, peroxidase activity has not been detected in arbuscular cells (30).

Changes in Pox and Ppo expression patterns seem to be caused, specifically, by sorghum-AM fungus symbiosis and not by nonspecific reactions against a foreign organism, due to certain differences that appeared, according to the interaction established with each particular species. The time also contributed to detect differences in electrophoretic patterns. This was expected,

if it is considered that the establishment and functioning process of plant-AM fungus symbiosis is not rigid; on the contrary, it is characterized by a continuous exchange of signals, essential metabolites, nutritive substances, water, photosynthates, among others (31). The fact that a major polymorphism was not observed could be because AM fungus species studied belong to a common genus. Sorghum roots inoculated with six AM fungus species expressed different isoforms, which are differentially regulated and have probably complementary functions. So, peroxidase and polyphenoloxidase enzymes will play a role between plant defense, regulation of its development, control of the establishment and/or functioning of symbiotic interactions, since diverse genotypes are involved in this association (32).

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