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FULL RESEARCH PAPER

Mycorrhizal protection of chili plants challenged by *Phytophthora capsici*

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Abstract Hydrogen peroxide (H_2O_2) has been implicated in many stress conditions. Control of H_2O_2 levels is complex and dissection of mechanisms generating and relieving H_2O_2 stress is difficult, particularly in intact plants. Here the role of the mycorrhizal inoculation in chili plants challenged with *Phytophthora capsici* was investigated to study the effect on hypersensitive response. In the treatment without mycorrhiza (treatment T3) and with mycorrhiza (considered treatment T4) visible disorders were detected two days after inoculation with *P. capsici*, but in the next days T3 plants rapidly developed 25% more necrotic lesions on the leaves than T4 plants. Leaf necrosis correlated with H_2O_2 accumulation and the greater damage observed in T3 plants coincided with larger accumulation of H_2O_2 after 12 h of inoculation accompanied with an increase in POX (peroxidase) and SOD (superoxide dismutase) activity. T4-infected and mycorrhizal plants exhibited an earlier accumulation of H_2O_2 starting 6 h after inoculation with lower levels compared to T3 plants. Correlated with observed damage, POX and SOD activity measured in T4 plants indirectly suggest a smaller accumulation of ROS (reactive oxygen species) leading to a decrease in the wounds observed and slightly diminishing the advance of the pathogen. According to these findings, we conclude that mycorrhizal colonization contributes significantly in maintaining the redox balance during oxidative stress, but the exact mechanism is still uncertain.

Keywords H_2O_2 · Antioxidant enzymes · Peroxidase · Reactive oxygen species · Superoxide dismutase

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Introduction

Plants, constantly exposed to microorganisms, have evolved different strategies to defend themselves against pathogen infection. They commonly react

with a wide range of defence responses against pathogens that may include biochemical compounds. A general and early defence mechanism triggered off by plants after a microbial attack is the production of reactive oxygen species (ROS) (Lamb and Dixon 1997). Several reports (Mehdy 1994; Wojtaszek 1997; Park et al. 1998) indicate that ROS give origin to many important changes related to signalling and induction of other local and systemic defence mechanisms that plants use for protection. Furthermore, some reports have indicated modifications in ROS as well as in antioxidative enzymatic activity as a result of plant symbiosis in association with beneficial microorganisms such as mycorrhizal fungi (García-Garrido et al. 2002). Plant-mycorrhizal interactions would not be limited only to the exchange of nutrients; the response of plants to arbuscular mycorrhizal colonization may involve a differential temporal and spatial induction of defence mechanisms (García-Garrido and Ocampo 2002). Many reports indicate that the establishment of symbiosis in host-mycorrhizal interactions triggers similar host defence responses upon pathogen infection (Gianinazzi-Pearson 1996; Gianinazzi-Pearson et al. 1996). A differential and systemic alteration in the expression of four defence genes was observed in all tissues only during the pathogenic interaction of arbuscular mycorrhizal beans (Guillon et al. 2002). Some mycorrhizal plants have shown minor susceptibility to pathogen attack such as tomato plants against *Phytophthora nicotianae* var. *parasitica* (Pozo et al. 1999). How mycorrhizas may affect the tolerance against plant pathogens is unclear, but some compounds and enzymes involved during the first stages of the symbiosis establishment may be responsible in the protection of subsequent infections. Thus, ROS detected in mycorrhizal-pretreated plants (Fester and Hause 2005), may be involved in the plant protection observed and some antioxidant enzymes may change in their activity level. An increase in superoxide dismutase (SOD) activity has been detected in plant-mycorrhizal interactions (Lanfranco et al. 2005) and root mortality caused by infection of *Phytophthora capsici* was completely eliminated by pre-inoculation with antagonistic *Glomus intraradices*. Peroxidase (POX) activity increased by 116.9% in pathogen-treated roots but by only 21.2% in mycorrhiza +pathogen-treated roots, compared with the control, thereby alleviating root mortality. These results suggest that *G. intraradices* is a potentially effective protection agent against *P. capsici* (Zheng et al. 2005). However, the exact mechanism by which mycorrhizal fungal colonization confers the protective effect is not completely understood; a greater understanding of these beneficial interactions is necessary for the exploitation of mycorrhizal fungi within organic and/or sustainable farming systems.

This study focuses on H_2O_2 production, POX and SOD activity as a result of the interaction between chili plants (*Capsicum annum*) and the mycorrhizal fungus *Glomus fasciculatum* after the infection of *Phytophthora capsici*.

Materials and methods

Plant material and mycorrhizal inoculation

Ancho Chili plants (*Capsicum annuum*) were germinated in trays of sterile soil with peat moss (*Sphagnum*), vermiculite, slime and germinase in a ratio of (4:3:8:2) and replanted after 45 days in pots with a sterile sand/slime soil medium. During transplanting, they were inoculated with *Glomus fasciculatum* by placing 25 g of inoculum-soil with 150 spores in the middle of the pot. After 30 days, all plants were inoculated with the corresponding treatments using an agar-mycelium disc of *Phytophthora capsici* (Pc8A isolate, kindly supplied by L. Pérez, ICA from Guanajuato University, Mexico) on top of each one of the 10 leaves. Trial treatments were: T1: without mycorrhiza; T2: with mycorrhiza; T3: without mycorrhiza, with pathogen and T4: with mycorrhiza, with pathogen. Sampling was carried out at different times (0, 1, 3, 6, 12 h, 2 and 5 days after the inoculation of *P. capsici*). Extra plants with mycorrhiza were grown under the same condition for the colonization assay. Roots from plants with mycorrhiza were washed with distilled water, cleared and stained through with Phillips and Hayman's (1970) procedure. Mycorrhizal colonization was determined with the gridline intersection method (Giovanetti and Mosse 1980). Data presented are % of infected root length.

Chili plant infection

The production of inoculum was based on the Xu and Ko (1998) method, with some modifications. *Phytophthora*

capsici was grown in V8–agar in the dark at room temperature, until 90% of the Petri dish was covered with mycelium. Agar-mycelium discs 6 mm diam were used as inoculum. A disc was placed on each leaf before introducing all the plants (infected and non-infected) into the greenhouse chamber with a photoperiod of 14 h light and 10 h dark, at 23–26°C and a RH of 95–98%. These conditions were maintained during 72 h after the infection.

Disease severity range assay

After inoculating the leaves with the pathogen, the disease severity was visually monitored according to a scale of 0–5 with:

0. No visible lesion or damage
1. Scarce presence of small discoloured leaves lesions
2. Minor small discoloured lesions covering 25% of the leaves
3. Moderate brown lesions in the 50% of the leaves and 25% defoliation
4. Mild wilt, 50% defoliation and initial small discoloured lesions in stems
5. Stems and leaves severely affected

In situ H_2O_2 determination

H_2O_2 detection technique reported by Thordal-Christensen et al. (1997) was used. Method of H_2O_2 detection by DAB staining was developed as follows. Cut leaves (clipped at their base) were placed inside a solution of 3,3-diamine-benzidine (DAB) at 1 mg ml^{-1}, pH 3.8, for 8 h under white light at 25°C. The samples were then boiled in ethanol at 96% for 10 min. After cooling, the samples were kept at room temperature (RT) in fresh ethanol for 4 h, before being photographed and preserved in ethanol at RT.

H_2O_2 densitometry analysis

The leaves were stained to detect H_2O_2, before being submitted to a densitometry analysis. WinRhizo 2004[a] software (Régent Instruments Inc.) was used to determine their optical density, according to manufacturer's directions. The leaves were carefully scanned and the images were recorded for further analysis. The calibration was done by selecting three areas of interest from each image: background, healthy tissue and tissue with H_2O_2 accumulation. Three areas of intensity were marked in each area. The number of pixels was read, considering only those areas where H_2O_2 had accumulated.

POX activity

The extraction process was done using the method described by Sulman et al. (2001). A glass micro-pestle was used to crush 0.3 g of foliar tissue inside microcentrifuge tubes filled with liquid nitrogen, homogenized with 500 µl of buffer extraction (40 mM Na_2PO_4 pH=7.0, 1% Triton x-100, 0.5 M KCl) and stirred for 5 min. The foliar tissue was centrifuged at 10,000 rpm in an Eppendorf microcentrifuge for 5 min at 4°C. A second tube was used to recover the supernatant to perform a second extraction on the tablet. The enzyme activity was determined using this supernatant. All the samples were placed inside an ice bath. The total enzymatic activity of peroxidase was determined by spectrophotometry according to the procedure described by Thaler et al. (1996), adapted to microtitrating plates. 50 µl of the extract were added to 200 µl of phosphate buffer at 10 mM and a pH of 7.0, using 20 mM guaiacol (Sigma) and 4.4 mM H_2O_2 (Sigma) as substrate. Changes in absorbance due to guaiacol oxidation were followed at 25°C with a microplate reader (Microplate Bio-Rad, Inc.) at 470 nm after 30 sec of the extract addition. Peroxidase activity was expressed as the increase in absorbance at 470 nm $min^{-1}mg^{-1}$ of protein.

SOD activity

Total protein extraction was done using the Beyer and Fridowich (1987) method, with some modifications, after adapting the method to titration microplates. 0.3 g of tissue were homogenized in phosphate buffer (50 mM pH 7.0 with 0.1% Triton X-100 and 1% PVP), using an Eppendorf microcentrifuge with 15,000 g during 15 min, at 4°C. After recovering the supernatant, a second extraction was performed on the tablet. Both supernatants were mixed in order to determine SOD activity in the following hours, according to Beyer and Fridowich (1987) modified method. 50 µl of extract were added to the reaction mixture (phosphate buffer 10 mM pH 7, NBT

0.112 mM). They were incubated for 10 min at 25°C with constant white light, determining their absorbance at 570 nm. Their total activity was expressed as the inhibition of NBT photo reduction caused by the increase in absorbance at 570 nm min^{-1} mg^{-1} protein.

Experimental design and statistical analysis

A factorial design of 2×2 with four main treatments was used (T1 – without mycorrhiza, T2 – with mycorrhiza, T3 – without mycorrhiza – with pathogen and T4 – with mycorrhiza – with pathogen), at different sampling times after the inoculation of the pathogen *P. capsici.* Each treatment was replicated nine times. Every individual plant was considered a replicate. The data were analyzed using NOVA versus media of minimum significant difference at a significance level of 0.05. The densitometry analysis was expressed as the total number of pixels in the area of interest, treated statistically according to their significance.

Results

Disease severity in chili plants

Total colonization with mycorrhizal fungus was established in pepper roots at a 65.2% level two days before the pathogen was inoculated. Assessment of disease damage involved the use of a six-level scale (0= no visible lesions; 5= stem and leaves severely affected); 100% damage was considered to be 5. The rest of the corresponding damage % was calculated according to the six-level scale. No lesions were observed during the first samples. The lesions became visible after 2 days following inoculation, confirming the reports of Hwang et al. (1997). After 2 days post-infection, cell death in leaves of treatment T3 – without mycorrhiza and T4 with mycorrhiza represented 20% of the damage. One day after, there was a significant increase in lesion size. Damage in T3 was almost two-fold, reaching 38% while T4 damage was slightly less at 35%. In T3 plants without mycorrhiza, the damage progressed very rapidly. After days 4 and 5, T3 plants showed 64% and 84% damage respectively; whereas T4 plants with mycorrhiza experienced lower damage of 51% after 4 days and 62% after 5 days. T3 plants without mycorrhiza showed leaf damage of almost 70% of the total leaf surface area. Figure 1 shows a graph summarizing all the data collected on lesions of the infected plants.

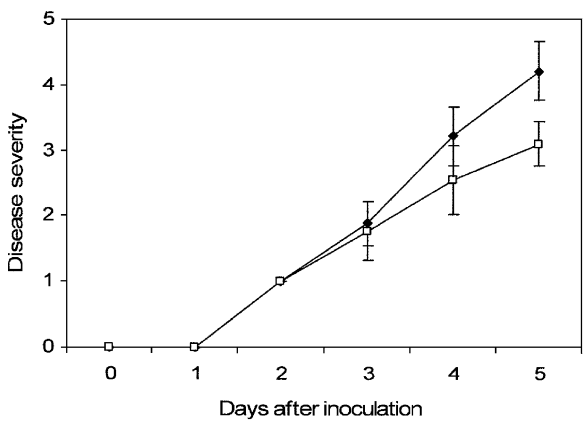

Fig. 1 Disease severity in chili plants. Disease range of damage caused by *P. capsici* after inoculation of leaves of chili plants *C. annuum* with (T3=♦) and without (T4=□); mycorrhizal infection was visually determined at different times according to the scale 0–5 (0 = no visible lesions; 5 = stem and leaves severely affected) as described in Materials and Methods. Each *bar* represents a mean ± standard error of n=9

In situ detection and H_2O_2 densitometry in chili infected leaves

A hypersensitive response increases the presence of reactive oxygen species, including an increase in H_2O_2 concentration (Thordal-Christensen et al. 1997). Figure 2 shows *in situ* determinations of hydrogen peroxide. The dark colour represents H_2O_2 accumulation after pathogen inoculation. T3 treatment involved a more intensive accumulation 12 h after infection. On the other hand, the accumulation was lower in T4, but appeared earlier, since the presence of this compound was detected after only 6 h. Both treatments showed a decline in the accumulation after reaching a peak; however, it was noteworthy that T4 plants kept an intermediate steady level that declined only at the end of the experiment. No significant accumulation was observed in T1 and T2 plants. A densitometry analysis was performed in order to accurately quantify the accumulation of H_2O_2. Results of the densitometry analysis are presented in Fig. 3a, showing that although during the first hours some of the leaves in the four treatments suffered discolouration, such discolouration was not due to *P. capsici* infection and was not significant, since the production stage began after 6 h. In T4 treatment, the production

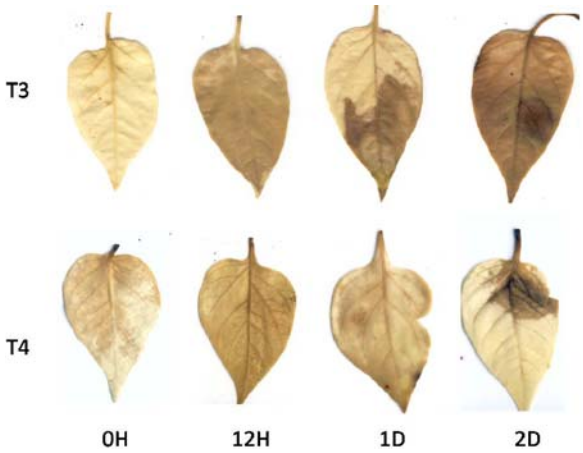

Fig. 2 *In situ* H_2O_2 accumulation. Accumulation of hydrogen peroxide *in situ* in chili plant leaves was followed at different times 2 days post-infection using DAB from two infected treatments (T3 and T4). T3 – without mycorrhizal and *P. capsici* and T4 – with mycorrhizal and *P. capsici*

started 6 h after the infection and even though it showed a slight decline, it remained almost at the same level for 3 days. On day 5, no accumulation was detected in this treatment. On the other hand, those leaves that were treated only with the pathogen (T3 treatment) showed a high accumulation after 12 h, declining 24 h after the infection (1 day). In the following two days, these T3 plants had a slight increment in the level of H_2O_2 (Figs. 3a, 2D and 3D), but this diminished again at the end of the study. The leaves of control T1 plants without mycorrhiza and without pathogen showed a basal accumulation of H_2O_2 that increased slightly after 24 h. In the leaves of mycorrhizal T2 plants without the pathogen, no H_2O_2 accumulation was observed.

Determination of the POX activity

Figure 3b presents a graph of the activity recorded in all treatments. This assay showed that during the first 2 h (0 and 1 h) after infection, the levels of enzymatic activity of peroxidase was very similar in the four treatments, remaining at a very low level in treatments T1 and T2. Treatment T3 showed an increase in enzymatic activity after 3 h, reaching a maximum transitory activity after 6 h; T4 treatment showed lesser activity with gradual increase, reaching a maximum transitory activity after 12 h, almost at the same level as that of T3 treatment. Although an increase in activity was observed in both treatments (T3 and T4), a rapid activity decline was also observed. Nevertheless, after 24 h (ID), the activity in both treatments began increasing gradually, until reaching its maximum level, 4 days (4D) after the inoculation. T3 treatment always showed greater activity. T3 experienced a rapid decline in activity after 5 days, contrary to what was observed in T4 treatment, where activity remained at almost the same level as day 4.

SOD activity

Figure 3c shows the results of SOD enzymatic activity. T3 plants showed two large progressive increases. Total activity of SOD in T1 and T2 treatments (without pathogen) remained almost at the same basal levels throughout the entire sampling time, contrary to what was observed in T3 and T4 treatments (with pathogen). The first increase was the highest and it occurred during the first 6 h, with a drastic decline in activity after 12 h of *P. capsici* inoculation. The second gradual increase in activity started in the 12 h period, 3 days after the inoculation of *P. capsici*; however, this activity declined in the following sampling days (days 4 and 5). T4 treatment in mycorrhizal plants showed a significant activity from 0 to 3 h followed by a slight activity increase at 12 h, remaining almost steady until 24 h after pathogen inoculation. The level of activity decreased after 2 days; however, from day 2 to day 5 (last determination) the plants showed an important activity recovery in the last stage, slightly greater than T3 plants without mycorrhiza.

Discussion

Mycorrhizal infection and H_2O_2 accumulation

Biochemical and molecular changes taking place in plants during the development of mycorrhizal symbiosis can be very important in the subsequent interactions with other microorganisms, such as pathogens. Mycorrhizal inoculation before a pathogen attack has been reported to provide plant biological protection. Since the beginning of last decade, many strategies were employed in an attempt to make plants more resistant against pathogens and many of them included mycorrhizal symbiosis (Hwang et al. 1992; Mark and Cassells 1996; Norman et al. 1996; Cordier

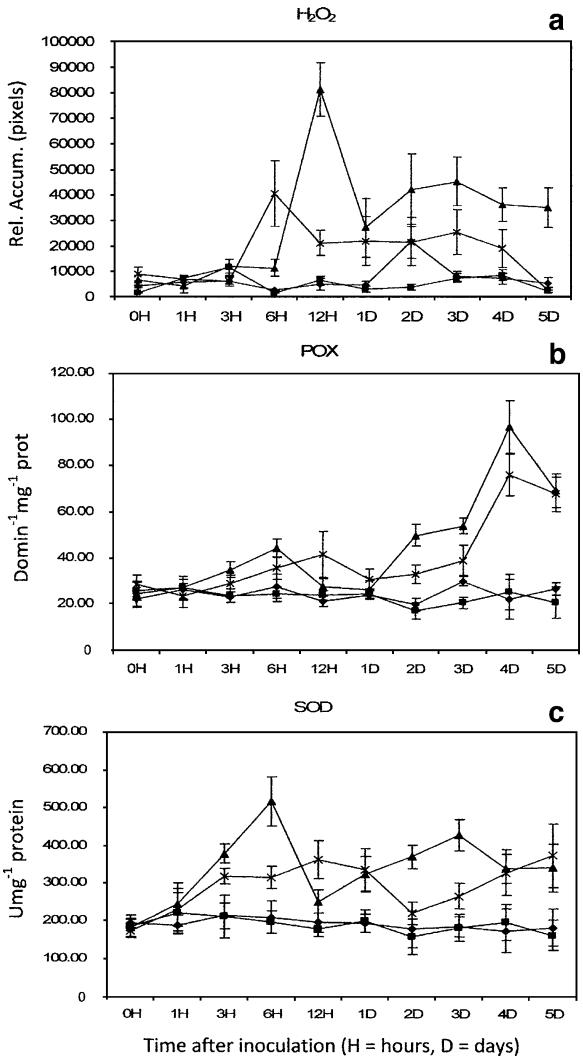

Fig. 3 Densitometry analysis of H_2O_2 and antioxidant enzymes activities. After inoculation of chilli plants with *P. capsici*, all plants were harvested at different times and the following analyses were run: (**a**) relative accumulation of H_2O_2 determined in pixels; (**b**) peroxidase activity determined as OD 470 nm $min^{-1}mg^{-1}$ protein; (**c**) total SOD activity measured as U mg^{-1} protein. Treatments applied were as follows: T1 – without mycorrhiza and *P. capsici* (■); T2 – with mycorrhiza and without *P. capsici* (♦); T3 – without mycorrhiza and with *P. capsici* (▲) and T4 – with mycorrhiza and *P. capsici* (*x*). Each *bar* represents a mean ± standard error of $n=9$

et al. 1996). In the current study, mycorrhizal presence seems to have a positive influence on plant resistance, since the damage caused by the pathogen was not as severe as the damage caused to plants without mycorrhiza (Fig. 1) and damage reduction was estimated at 25%, compared to non-mycorrhizal infected plants. Cordier et al. (1998) found that *Glomus mosseae* was effective in reducing disease symptoms produced by *P. parasitica* infection. Evidence suggests a combination of local and systemic mechanisms responsible for this bioprotector effect. Greater damage shown by plants infected with the pathogen, coincided with a larger accumulation of H_2O_2 in the leaves. Since H_2O_2 participates as an important component of the hypersensitive response in cell death surrounding the infection site, an increase in this compound is likely to cause a highly sensitive area. Due to the aggressiveness and fast infection rate of *P. capsici*, only plants infected with the pathogen showed more severe damage. The densitometry analysis showed that although pathogen-infected T3 plants were the only plants with larger H_2O_2 accumulation than T4 plants which had been colonized with mycorrhiza before being challenged with *P. capsici*, the accumulation appeared late (12 h after pathogen inoculation), while in T4 mycorrhizal plants the response became apparent after 6 h. These observations suggest the existence of a local and systemic signalling process in mycorrhizal plants, perhaps preceded by the transferring of H_2O_2 activity into the adjacent contact cells and also through distant sites. In tomato, *G. mosseae* induced local and systemic resistance to *P. parasitica,* and was effective in reducing symptoms produced by this pathogen (Pozo et al. 2002).

POX and SOD activity in mycorrhizal chili plants

Mycorrhiza may provoke several changes in plants. Some reports have shown that SOD and POX activity in mycorrhizal plants increases as a result of the colonization process (Pozo et al. 2002; Wang et al. 2002). Our results indicated that although both treatments inoculated with the pathogen (T3 and T4) had an increase of POX and SOD, it was better regulated by T4 plants, since the changes in their activities was less variable compared to the variations founds in T3 plants (Fig. 3b and c). Even though treatments T3 and T4 showed peaks in the activity of both enzymes, T3 plants reached the highest activity levels. After 2 days, SOD activity increased gradually until reaching its peak 5 days after the pathogen inoculation. This greater consistency or stability in enzyme activity might be due to a better regulation of damage caused by the compounds produced during the defence reactions, leading to a more controlled cell death or

greater resistance. A direct and proportional relationship can be established between POX and SOD level of activity and pathogen damage. T3 plants with the highest level of enzymatic activity and H_2O_2 accumulation, also showed the most severe damage in leaves and stems. The higher POX and SOD activity and H_2O_2 accumulation detected in T3 plants may be due to the pathogen *P. capsici.* This is a pathogen that infects live host tissues then kills cells causing necrosis in a similar way to other *Phytophthora* species such as *P. sojae*, *P. parasitica* and *P. infestans*, that are reported as hemibiotroph microorganisms with biotrophic and necrotrophic phases (Drenth and Goodwin 1999, cited by Qutob et al. 2002; Erwin and Ribeiro 1996). In other kinds of pathogens with a necrotrophic lifestyle, some antioxidant enzymes activities might increase at the site of infection through extracellular enzymes provided by the pathogen (Mayer et al. 2001). The high concentrations of antioxidant enzymes and decreasing levels of ROS observed after 5 days of pathogen inoculation in T3 plants, suggest that *P. capsici* might have an external antioxidant activity. Three polypeptides with manganese superoxide dismutase (MnSOD) activity have previously been found in mycelium, zoospores and germinated cysts of *Phytophthora nicotianae* (Blackman et al. 2005) in which mycorrhizal plants challenged with the pathogen (T4) with sustained levels of POX and SOD activity showed less foliar damage, as well as a rapid and more localized production of H_2O_2.

Can mycorrhiza induce systemic resistance?

Although the assessment of damage and the biochemical determination of defence responses were carried out in a localized manner at the infection sites, it is important to highlight that the results obtained in T4 plants indicate a potential systemic response, since pathogen infection took place in foliar areas far removed from the mycorrhizal colonization of the roots. Similar systemic protection has been reported by Lingua et al. (2002). Mycorrhizal tomato plants infected with a phytoplasma have shown a reduction in foliar disease symptoms. Phenolic compounds (Cordier et al. 1998; Zhu and Yao 2004) and PR-1a proteins (Cordier et al. 1998) were systemically induced in non-mycorrhizal roots separated in a split-root system and an acid phosphatase activity was reported in foliar tissues (Selvaraj and Chellappan 2006). The contribution of mycorrhiza to plant protection against soil–borne pathogens is well supported, but foliar pathogen reports suggest that these are sometimes stimulated by mycorrhizal symbiosis. According to results from this investigation, it is possible that the presence of mycorrhiza enhances crucial plant defence mechanisms, since the first recognition events determine resistance or susceptibility; in addition, many secondary or intermediate responses depend on mechanisms of induction, as well as on the signalling molecules originated in early responses. Our results suggest that mycorrhizal colonization can increase chili plant tolerance against *P. capsici* infection, as the severity of symptoms was reduced. However, the specific mechanisms responsible for improved tolerance have yet to be elucidated.

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