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Defence-related enzymes in pepper roots during interactions with arbuscular mycorrhizal fungi and/or *Verticillium dahliae*

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Abstract. Previous studies have described that arbuscular mycorrhizal fungi (AMF) can reduce the deleterious effect of *Verticillium dahliae* Kleb. on pepper growth and yield. In mycorrhizal plants, the bioprotection against soil-borne pathogens can result from the preactivation of defence responses that include some structural modifications and the accumulation of Pathogenesis-Related (PR) proteins. Our first objective was to study if *V. dahliae* induced defence mechanisms in roots before infected pepper developed visible symptoms of disease. The second aim was to determine if AMF induced defence-related enzymatic activities in pepper roots before or after pathogen's attack. Results showed that the colonization of pepper roots by *Glomus deserticola* (Trappe, Bloss and Menge) induced the appearance of new isoforms of acidic chitinases, superoxide dismutase (SOD) and, at early stages, peroxidases. In contrast, *V. dahliae* neither stimulated the phenylpropanoid pathway nor elicited hydrolytic activities in infected pepper roots. Only in mycorrhizal plants, the inoculation with *V. dahliae* slightly increased both phenylalanine ammonia-lyase (PAL) and peroxidase activities two weeks later. Mycorrhizal-specific induction of new isoforms of acidic chitinases and SOD together with enhanced peroxidase and PAL activities 2 weeks after pathogen inoculation could be involved in the biocontrol of *Verticillium*-induced wilt in pepper by AMF.

Key words: antioxidant enzymes, *Capsicum annuum*, *Glomus deserticola*, hydrolytic enzymes, mycorrhizal symbiosis, phenylalanine ammonia-lyase, *Verticillium dahliae*

Abbreviations: AMF – arbuscular mycorrhizal fungi; DM – dry matter; PAGE – polyacrylamide gel electrophoresis; PAL – phenylalanine ammonia-lyase; SOD – superoxide dismutase

Introduction

The defence mechanisms developed by plants include both the elaboration of structural barriers and the induction of active

host-specific responses that provide resistance (Singh et al., 2000). The active mechanisms imply the recognition of the pathogen by host plant. As a consequence of the union between elicitors released by the pathogen and specific receptors of the plant cell surface, a cascade of signalling processes is initiated and, finally, plant defences are activated. These defences can include activation of genes that are coding for enzymes of the phenylpropanoid pathway or for pathogenesis-related (PR) proteins, some of them with chitinase, chitosanase and β-1,3-glucanase activities (Pozo et al., 2002a) that degrade wall of many pathogenic fungi (Van Loon and Van Strien, 1999).

Verticillium dahliae Kleb. is a soil-borne pathogen that causes vascular wilt disease in several plant species, including pepper (Pegg, 1989). The fungus enters the root, and hyphae then move to vascular tissues (Schnathorst, 1981). Xylem colonization by the fungus increases the resistance to water flow within the plant thus resulting in leaf water deficits and reduced photosynthetic rates (Adams et al., 1987). Garmendia et al. (2004a) suggested that the arbuscular mycorrhizal fungus (AMF) *Glomus deserticola* (Trappe, Bloss and Menge) could reduce the deleterious effect of *V. dahliae* on pepper growth and yield. Arbuscular mycorrhizal fungi colonize the roots of most plant species to the mutual benefit of both host plant and fungus. The establishment of this mutualistic association involves a continuous cellular and molecular dialogue between both symbionts (Bonfante-Fasolo, 1984) that includes the production of plant defence related proteins (Lambais, 2000). The preactivation of these defence responses could contribute to enhance the resistance of mycorrhizal plants to soil-borne pathogens (Azcón-Aguilar et al., 2002).

The first aim of the present work was to determine if *V. dahliae* induced defence mechanisms in pepper roots before the development of any visible symptoms of the disease. Our second objective was to study if AMF induced defence related enzymatic activities in roots before or after the pathogen's attack. To this end, chitinase, chitosanase, β-1,3-glucanase, peroxidase, superoxide dismutase (SOD) and phenylalanine ammonia-lyase (PAL) activities were analysed at three different times: just before *V. dahliae* inoculation, seven and 14 days after pathogen inoculation.

Material and methods

Biological material, growth conditions and experimental design

Pepper seeds (*Capsicum annuum* L. cv. Piquillo) were germinated on washed sand. Before transplanting, seedlings were irrigated with Long Ashton Nutrient Solution (LANS; Hewitt, 1966) and received deionized water on alternate days. When 1-month-old, 32 seedlings were transplanted to 1 l plastic containers filled with a mixture of vermiculite-sand-soil (2.5:2.5:1 v/v/v). Soil poor in available P was chosen. It had a pH (H_2O) of 8.9, 0.3% organic matter, 0.08% nitrogen, 2.0 mg kg^{-1} phosphorus, 58.8 mg kg^{-1} potassium and 41.98% $CaCO_3$. Before preparing the mixture, the soil was sieved (2 mm) and steam sterilised at 100 °C for 1 h on three consecutive days. When transplanted to pots, seedlings were divided into two groups: (a) Non-mycorrhizal plants (NM) (16 plants) and (b) Plants inoculated with *G. deserticola* (M) (16 plants). Mycorrhizal inoculum was supplied by the Estación Experimental del Zaidín (Granada). Arbuscular mycorrhizal fungus was applied as a soil-based inoculum (20 g per pot) and included root fragments, spores and hyphae from a 3-months-old culture of leek and alfalfa. Plants were fertilized and irrigated as explained by Garmendia et al. (2004a).

When 2 months old, during the vegetative phase of plants and once AMF was established, six non-mycorrhizal and six mycorrhizal plants were inoculated with *V. dahliae*. Inoculation was performed by adding a suspension of 3.6×10^7 conidia to the substrate of each pot (Hoyos et al., 1993). *Verticillium* was isolated from diseased pepper grown in field and maintained on Messiaen culture medium prior to inoculation. Twelve plants (six non-mycorrhizal and six mycorrhizal) were kept as uninoculated healthy controls. Therefore, four treatments were compared: non-mycorrhizal plants inoculated (NM + V) or not (NM−V) with *V. dahliae*, and mycorrhizal plants inoculated (M + V) or not (M−V) with the pathogen.

Plants were grown in a greenhouse at 25/15 °C day/night and received natural daylight supplemented with irradiation from fluorescent lamps Son-T-Agro (Philips Nederland B.V., Eindhoven) that provided a minimum photosynthetic photon flux (PPF) of 300 μmol m^{-2} s^{-1} during a 14 h photoperiod.

Three plant harvests were performed: (a) just before *V. dahliae* inoculation, (b) seven days and (c) 14 days after *V. dahliae* inoculation.

Disease assessment, estimation of AMF colonization and growth parameters

In order to assess if *V. dahliae* had progressed from root to shoot, surface-disinfected cross-stem sections were cut and plated on Messiaen culture medium for the fungus isolation and identification. Plates were incubated in darkness at 25 °C for 10–15 days. The disease severity was non-destructively estimated by calculating a disease index as the sum of wilted, chlorotic and necrotic leaves related to the total leaves per plant, expressed as percentage (Goicoechea et al., 2000).

Root samples were cleared and stained (Phillips and Hayman, 1970) and the percentage of root colonization was determined by examining a minimum of 80–90 1 cm-root segments under the microscope. Results are expressed as percentage of root colonization (Hayman et al., 1976). Shoot and root DM were determined after drying at 80 °C for 2 days.

Phenylalanine ammonia-lyase (PAL) assay

Phenylalanine ammonia-lyase (EC 4.1.1.5) activity was assayed according to Dunn et al. (1998) with slight modifications. Frozen roots (1 g) were ground in a mortar under liquid nitrogen and homogenized in 10 ml 0.1 M sodium borate buffer (SBB) (pH 8.8) containing 10 mM β-mercapto-ethanol and 10% (w/w) PVPP. Samples were homogenized by shaking in an ice bath for 1 h. Then crude extracts were centrifuged at 12,000 rpm for 20 min at 4 °C. Reaction mixtures containing 750 µl enzyme extract, 1.25 ml SBB and 500 µl SBB plus 0.05 M L-phenylalanine were incubated in a 40 °C water bath for 3 h. Phenylalanine ammonia-lyase activity was determined by measuring absorbance at 290 nm. Enzyme activity was defined as the amount of *trans*-cinnamic acid formed from the substrate L-phenylalanine per hour. Root soluble proteins were quantified by the protein dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Electrophoresis, hydrolytic and antioxidant enzymatic assays

Frozen root samples (1 g) were crushed to a fine powder in a mortar under liquid nitrogen. Soluble proteins were extracted in 2 ml McIlvaine (citric acid/Na_2HPO_4) buffer (pH 6.8) with 8% PVPP (w/v), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl (PMSF) and 0.1% Triton X-100. Crude extracts were homogenized for 10 min by

shaking in an ice bath, and centrifuged at 11,500 rpm for 30 min at 4 °C. The supernatant was divided into aliquots and stored at −80 °C for further analysis. Root soluble proteins were quantified by the protein dye-binding method of Bradford (1976) using BSA as a standard.

All extracts were analysed by 15% (w/v) polyacrylamide gel electrophoresis (PAGE) under native conditions, at pH 8.9 (according to Davis, 1964) and at pH 4.3 as described by Reisfeld et al. (1962). Proteins (7 μg per sample) were loaded for acidic and basic electrophoresis systems (Mighty Small II SE 250, Hoefer Scientific Instruments, San Francisco, USA). After staining, gels were photographed (Polaroid film No. 665) (Pozo et al., 1998) and scanned (HP ScanJet 3c). Each electrophoresis was repeated three times.

Detection of chitinase (EC 3.2.1.14) activity after PAGE

Glycol chitin was embedded in the gels at 0.01% (w/v) and used as substrate for chitinase activity (Michaud and Asselin, 1995). Anodic electrophoresis gels were run in Tris–glycine buffer at room temperature at a constant voltage of 15 mA per gel. Acrylamide gels were incubated for 4 h at 37 °C in 50 mM sodium acetate (pH 5). Basic PAGE was carried out in gels without chitin in 0.3 M *β*-alanine and 0.8% (v/v) acetic acid. Chitin was incorporated in a 7.5% (w/v) polyacrylamide overlay gel and protein transfer to the overlay gel was done by blotting for 14 h at 37 °C. Afterwards, acrylamide gels were incubated for 4 h at 37 °C in 50 mM sodium acetate (pH 5). Staining of chitinase isoforms was performed by calcofluor white M2R (0.01%, w/v) in 0.3 M Tris–HCl (pH 8.9) and visualised under UV light (365 nm) (Pozo et al., 1996, 1998).

Detection of β-1,3-glucanase (EC 3.2.1.39) activity after PAGE

For *β*-1,3-glucanase gel analysis, a soluble fraction of purified *β*-glucans from *Saccharomyces cerevisiae* was used as substrate (Grenier and Asselin, 1993). Acidic PAGE were run in Tris–glycine buffer at room temperature at a constant voltage of 15 mA per gel and incubated for 3 h at 37 °C in 50 mM sodium acetate (pH 5). Catodic PAGE was carried out in gels without *β*-glucans in 0.3 M *β*-alanine and 0.8% (v/v) acetic acid. *β*-glucans were incorporated in a 7.5% (w/v) polyacrylamide overlay gel and protein transfer to the overlay gel was done by blotting for 14 h at 37 °C. Then, acrylamide gels were incubated for 3 h at 37 °C in 50 mM sodium acetate

(pH 5). Staining of β-1,3-glucanase isoforms was performed by aniline blue fluorochrome (0.025%, w/v) in 150 mM K_2HPO_4 (pH 8.6) and visualised under UV light (365 nm) (Pozo et al., 1999).

Detection of chitosanase (EC 3.2.1.99) activity after PAGE

Chitosanase activity was determined in Davis system gels with 0.01% (w/v) glycol chitosan as substrate. Gels were run in Tris–glycine buffer at room temperature at a constant voltage of 15 mA per gel. Then, they were incubated for 4 h at 37 °C in 10 mM sodium acetate (pH 5). Staining of chitosanase isoforms was performed by calcofluor white M2R (0.01%, w/v) in 0.3 M Tris–HCl (pH 8.9) and visualised under UV light (365 nm) (Pozo et al., 1998).

Detection of peroxidase (EC 1.11.1.7) activity after PAGE

Detection of peroxidase isoforms in PAGE was performed in Tris–glycine (pH 8.3) buffer at room temperature at a constant voltage of 15 mA per gel. After protein separation, gels were incubated in 0.1 M Tris–HCl (pH 7.6) with 4-chloro-1-naphthol and H_2O_2 according to Ye et al. (1990) with slight modifications. Coloured bands corresponding to H_2O_2 hydrolysis appeared against the clear background.

Detection of superoxide dismutase (SOD) (EC 1.15.1.1) activity after PAGE

Superoxide dismutase isoforms were detected after PAGE by using the technique of Beauchamp and Fridovich (1971), based on the inhibition of nitroblue tetrazolium (NBT) reduction. Davis system gels were run in Tris–glycine (pH 8.3) buffer at room temperature at a constant voltage of 15 mA per gel. After that, gels were incubated in darkness for 20 min in 2.45 mM NBT with 50 mM potassium phosphate buffer (KPB) (pH 7.8) and for 15 min in 28 μM of riboflavin and 28 mM TEMED with 50 mM KPB (pH 7.8). Bands appeared colourless against the blue background corresponding to the reduced NBT (Palma et al., 1993).

All chemicals used for electrophoresis were from Bio-Rad (Prat de Llobregat, Barcelona, Spain). Other products were from Sigma Chemical Co. (Alcobendas, Madrid, Spain).

Statistics

Growth parameters (Table 1), root protein concentrations (Table 2) and PAL activities (Figure 1) just before *V. dahliae* inoculation were evaluated by Student's *t*-test. Root protein concentrations (Table 2) and PAL activities (Figure 1) on days 7 and 14 after pathogen inoculation were analysed with one-way analysis of variance (ANOVA); means ± SD were calculated and, when the *F*-ratio was significant, least significance differences were evaluated by the Tukey-b-test. Significance levels were always set at 5%.

Results

Non-mycorrhizal and mycorrhizal plants had similar shoot and root biomass just before *V. dahliae* inoculation and the extent of root cortex colonized by AMF in pepper inoculated with *G. deserticola*

Table 1. Shoot DM (g plant^{-1}), root DM (g plant^{-1}) and mycorrhizal colonization (%) in non-mycorrhizal (NM) and mycorrhizal (M) plants just before inoculating *Verticillium dahliae*

Treatments	Shoot DM (g plant^{-1})	Root DM (g plant^{-1})	Mycorrhizal colonization (%)
NM	0.87 ± 0.26 a	0.47 ± 0.14 a	–
M	0.90 ± 0.29 a	0.43 ± 0.13 a	25.32 ± 6.76

Means ± SD ($n = 3$ plants) were compared with Student's *t*-test within each column. Values followed by a common letter are not significantly different ($p \leq 0.05$).

Table 2. Total soluble proteins (mg g^{-1} DM) in roots of non-mycorrhizal (NM) and mycorrhizal (M) plants inoculated (+V) or not (−V) with *Verticillium dahliae*

	Soluble proteins (mg g^{-1} DM) Days after pathogen inoculation		
Treatments	0[1]	7[2]	14[2]
NM−V	6.77 ± 0.53 b	5.66 ± 0.57 d	5.89 ± 0.19 b
M−V	13.70 ± 1.19 a	8.26 ± 1.00 c	7.33 ± 0.17 ab
NM+V	–	9.69 ± 0.60 b	6.36 ± 1.22 b
M+V	–	11.14 ± 0.20 a	8.70 ± 0.30 a

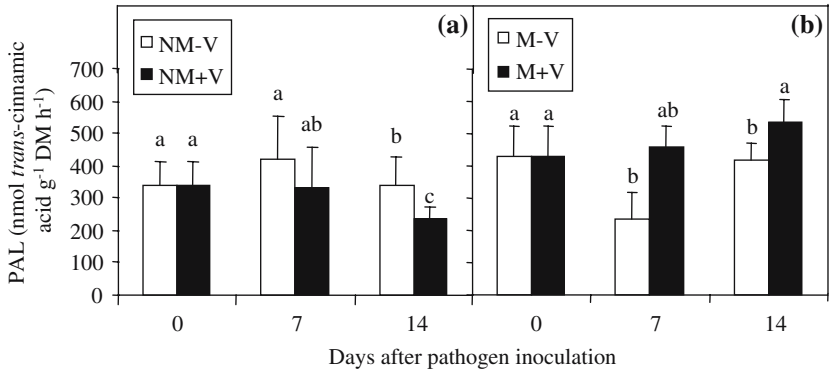

Figure 1. Phenylalanine ammonia-lyase (PAL) activities (nmol *trans*-cinnamic acid g^{-1} h^{-1} DM) in roots from non-mycorrhizal (NM) (a) and mycorrhizal (M) (b) plants inoculated (+V) or not (−V) with *Verticillium dahliae*. Means ± SD ($n = 3$ plants) just before inoculating *Verticillium* were compared with Student's *t*-test and data on days 7 and 14 after pathogen inoculation were analysed by Tukey-b-test. Histograms followed by a common letter are not significantly different ($p \leq 0.05$).

reached around 25% at that moment (Table 1). Although *Verticillium* -infected plants did not show any disease symptom throughout the two first weeks after pathogen inoculation, *V. dahliae* was isolated from around 40% of cross-stem sections taken from both non-mycorrhizal and mycorrhizal pepper on day 14 (data not shown), which indicates that plants were systemically infected.

Phenylalanine ammonia-lyase activity achieved comparable levels in roots of both non-mycorrhizal and mycorrhizal plants before pathogen inoculation (Figure 1a, b). The presence of *G. deserticola* never induced PAL activity in healthy pepper. In fact, such activity was lower in mycorrhizal than in non-mycorrhizal roots on day 7. Conversely, the highest PAL activity on day 14 was measured in mycorrhizal plants inoculated with *V. dahliae*.

Roots colonized by *G. deserticola* initially showed higher amount of proteins than non-mycorrhizal ones (Table 2). The inoculation with the pathogen produced a transient enhancement in protein content in both non-mycorrhizal and mycorrhizal roots in comparison with their respective healthy controls, but these differences disappeared one week later.

Healthy non-mycorrhizal plants showed one acidic chitinase isoform in their roots (Figure 2, lanes NM−V) and the presence of *G. deserticola* induced three new acidic chitinase isoforms (Figure 2, lanes M−V). In contrast, we never observed new isoenzymes as a consequence of *V. dahliae* inoculation (Figure 2, lanes +V). On the other

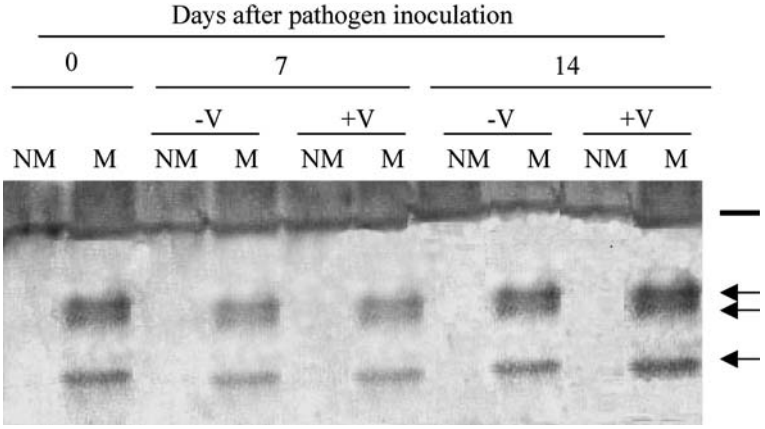

Figure 2. Acidic (a) and basic (b) chitinase activities after separation of proteins under native conditions using the Davis and the Reisfeld systems, respectively, in 15% (w/v) polyacrylamide gels containing 0.01% (w/v) glycol chitin as substrate. Roots extracts in McIlvaine buffer (7 μg of protein per sample) from non-mycorrhizal (NM) and mycorrhizal (M) plants inoculated (+V) or not (−V) with *Verticillium dahliae* were analysed just before inoculating the pathogen as well as 1 and 2 weeks later. Bars indicate constitutive isoforms and arrows mark additional isoforms.

hand, two constitutive isoforms of basic chitinase were detected in all pepper roots (data not shown) and neither *G. deserticola* or *V. dahliae* induced new isoforms. No time related differences were found.

Polyacrylamide gel electrophoresis results concerning β-1,3-glucanases in roots of non-mycorrhizal healthy pepper demonstrated the existence of three constitutive isoforms, one acidic and two basic (Figure 3a, b, lanes NM−V). An equivalent banding pattern was observed in plants inoculated with AMF (Figure 3a, b, lanes M) and/or *V. dahliae* (Figure 3a, b, lanes +V). The expression of both acidic and basic β-1,3-glucanases did not change along time.

No signal corresponding to chitosanase activity was detected in any treatment, regardless the presence or absence of *G. deserticola* and/or *V. dahliae* (data not shown).

Analysis of peroxidase isoenzymatic patterns in roots from healthy pepper provided evidence of five constitutive isoforms in non-mycorrhizal plants on day 0 (Figure 4, lane NM). A new isoform was detected in mycorrhizal plants on that day (Figure 4, lane M), but it disappeared on days 7 and 14 (Figure 4, lanes M−V). On the other hand, when *Verticillium*-inoculated plants were compared on day 14, the activity of peroxidase isoforms seemed to be greater in mycorrhizal than in non-mycorrhizal roots (Figure 4, lanes NM + V, M + V, day 14).

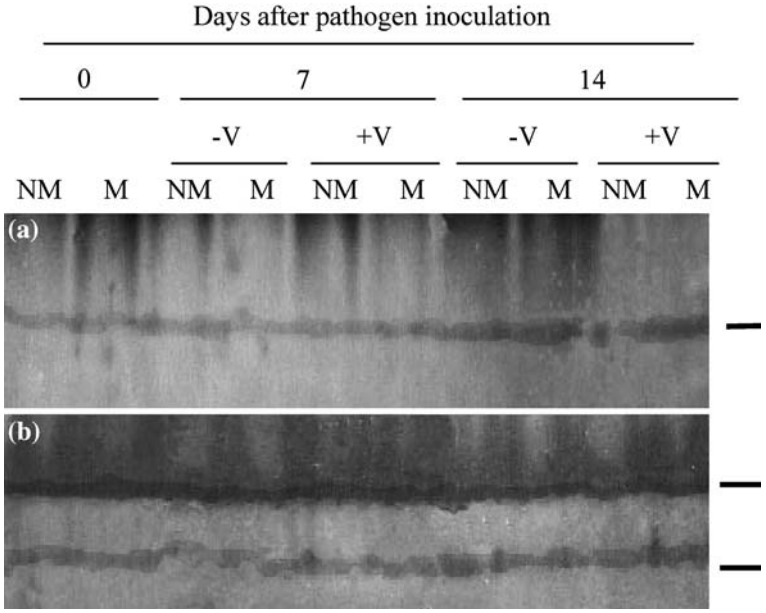

Figure 3. Acidic (a) and basic (b) β-1,3-glucanase activities after separation of proteins under native conditions using the Davis and the Reisfeld systems, respectively, in 15% (w/v) polyacrylamide gels containing β-glucans as substrate. Root extracts in McIlvaine buffer (7 μg of protein per sample) from non-mycorrhizal (NM) and mycorrhizal (M) plants inoculated (+V) or not (−V) with *Verticillium dahliae* were analysed just before inoculating the pathogen as well as 1 and 2 weeks later. Bars indicate constitutive isoforms and arrows mark additional isoforms.

Superoxide dismutase (SOD) activity in roots was analysed by using electrophoretic systems (Figure 5). Three constitutive isoforms were found in non-mycorrhizal plants (Figure 5, lanes NM−V). In addition, mycorrhizal association induced two new isoforms with similar mobility (Figure 5, lanes M−V). No induction of new isoforms was detected after inoculation with *V. dahliae* (Figure 5, lanes +V), and no changes in the expression of SOD isoforms were observed along time.

Discussion

Colonization of roots by AMF can stimulate the phenylpropanoid pathway (see Morandi, 1996 for review) and/or elicit plant chitinase (Pozo et al., 1996, 2002b), chitosanase (Pozo et al., 1998, 2002b), β-1,3-glucanase (Pozo et al., 1999, 2002b), peroxidase (Gianinazzi and Gianinazzi-Pearson, 1992) and SOD (Palma et al., 1993; Pozo et al., 2002b; Lambais et al., 2003) activities.

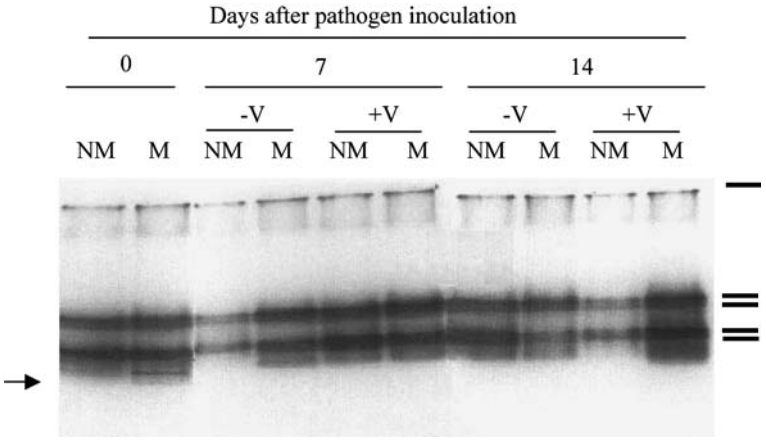

Figure 4. Peroxidase activity after separation of proteins under native conditions using the Davis system in 15% (w/v) polyacrylamide gels. Root protein extracts in McIlvaine buffer (7 μg of protein per sample) from non-mycorrhizal (NM) and mycorrhizal (M) plants inoculated (+V) or not (−V) with *Verticillium dahliae* were analysed just before inoculating the pathogen as well as 1 and 2 weeks later. After protein separation, gels were incubated in 0.1 M Tris–HCl (pH 7.6) with 4-chloro-1-naphthol and H_2O_2. Bars indicate constitutive isoforms and arrows mark additional isoforms.

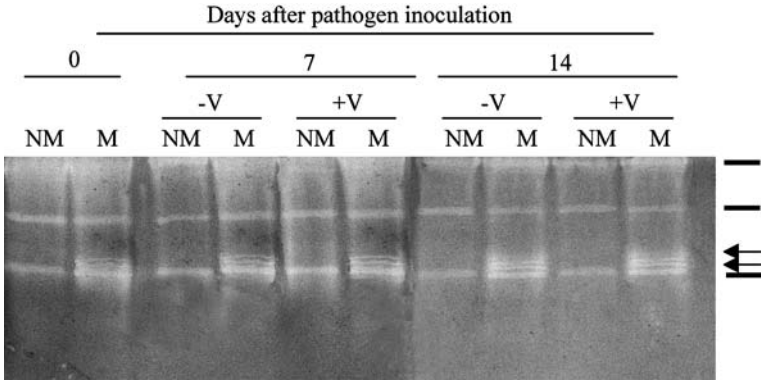

Figure 5. Superoxide dismutase (SOD) isoenzymes after separation of proteins under native conditions using the Davis system in 15% (w/v) polyacrylamide gels. Root protein extracts in McIlvaine buffer (7 μg of protein per sample) from non-mycorrhizal (NM) and mycorrhizal (M) plants inoculated (+V) or not (−V) with *Verticillium dahliae* were analysed just before inoculating the pathogen as well as 1 and 2 weeks later. Bars indicate constitutive isoforms and arrows mark additional isoforms.

In contrast with previous studies (see Morandi, 1996 for review), induction of PAL did not occur in pepper roots colonized by *G. deserticola*. Moreover, when healthy pepper were compared, we found that the level of PAL activity on day 7 was lower in mycorrhizal than in non-mycorrhizal roots. In line with these results, Guillon et al. (2002) observed no induction or even suppression of genes coding for the phenylpropanoid pathway in response to mycorrhizal colonization.

Three acidic chitinase isoforms related to mycorrhizal colonization were detected in pepper plants. Several authors (see Mukerji, 1999; Singh et al., 2000 for review) have observed that the increased chitinase activity during early stages of root colonization by AMF can be followed by suppression in later stages. In our study, the percentage of mycorrhizal colonization reached around 25% before the inoculation of plants with the pathogen. As such percentage can achieve at least 50% once the symbiosis is fully developed (Garmendia et al., 2004a, b), chitinase activity may have declined in later phases not studied in the present work. However, high levels of chitinase activity can persist in some plant-fungus combinations (Dehne and Schonbeck, 1978). On the other hand, the presence of *G. deserticola* in pepper roots did not induce the appearance of new chitosanase and β-1,3-glucanase isoenzymes. Pozo et al. (1999), working with tomato, found induction of new glucanase isoforms by *G. mosseae* but not by *G. intraradices* and suggested that this dissimilar behaviour could be due to different colonization dynamics of *Glomus* species and/or to differences in their wall composition and/or structure.

We also found a peroxidase isoform induced by *G. deserticola* on day 0, but it disappeared 1 week later. The role of peroxidases in arbuscular mycorrhizal symbiosis remains unclear (Lambais et al., 2003). However, it is known that they catalyse the oxidative polymerization of phenylpropanols to produce lignin and the cross-linking of cell wall proteins, contributing to enhanced cell wall reinforcement during fungal penetration, and might also be involved in scavenging H_2O_2 (Mittler, 2002). Transient induction in peroxidase activities followed by suppression has also been observed in roots from mycorrhizal leek and maize (Spanu and Bonfante-Fasolo, 1988; Fries et al., 1996). The fact that high phosphorus (P) levels can attenuate the suppression of specific peroxidase activities in mycorrhizal roots suggests that P nutrition is crucial for the control of peroxidase expression (Lambais et al., 2003). In our study, pepper plants were grown under moderate P nutrition.

Glomus deserticola induced two new SOD isoenzymes in pepper roots. The induction of new SOD isoforms by AMF depends on fungal species, stage of root colonization and phosphorus nutrition of host plant (Lambais et al., 2003) and can be a protective response of the plant cell against the entrance of foreign organisms (Palma et al., 1993). When comparing non-mycorrhizal and mycorrhizal lettuce plants, Ruiz-Lozano et al. (1996) did not observe changes in the isoenzyme pattern, but SOD activity increased in roots associated with *G. mosseae*.

In contrast with the enhancement in PAL activity reported in cotton hypocotyls treated with a protein-lipopolysaccharide secreted by *V. dahliae* (Dubery and Smit, 1994), we found that PAL activity remained unchanged or even slightly decreased in non-mycorrhizal pepper plants when they were inoculated with *V. dahliae*. Guillon et al. (2002) observed that the highest PAL transcript occurred quite late in the infection process of bean plants with *Rhizoctonia solani* (5 days after inoculation) and it took place when lesions on the hypocotyls were very evident. In our study, pepper plants had not still shown visible symptoms of the disease two weeks after pathogen inoculation. However, we cannot discard the possibility of a transient induction of PAL at early stages of infection. Moreover, the reduction in PAL activity can coincide with high expression of *pal* genes. In fact, Kervinen et al. (1998) observed that the strong expression of *pal* genes in barley at 32 h after the inoculation of plants with the fungus *Bipolaris sorokiniana* was concomitant with the decline of the first activity peak and such gene expression was related to a second activation of PAL at 40 h. In addition, the later authors found differences in expression between four *pal* genes in barley. While two of them were associated with rapid activation of defence responses, the strong expression of other two genes in roots and internodes seemed to be related to development-associated lignin formation. Since changes in stem lignins are related with the maintenance of leaf photosynthetic integrity during *Verticillium* wilt in *C. annuum* (Pomar et al., 2004), the study of the expression of *pal* genes could provide crucial information to better understand the mechanisms involved in the defence response of pepper plants against that pathogen.

In our study, the greatest PAL induction by *V. dahliae* was observed in plants associated with AMF on day 14. Similarly, Dehne and Schönbeck (1979) found that the simultaneous infection of tomato with *G. mosseae* and *Fusarium oxysporum* increased PAL activity of roots more than the mycorrhizal fungus alone or the pathogen alone.

Dubery and Slater (1997) observed new chitinase and β-1,3-glucanase isoenzymes in leaf discs of cotton following elicitation with cell-wall fragments from *V. dahliae*. Similarly, Cui et al. (2000) found an early induction of genes encoding for basic chitinase in a resistant cotton cultivar infected with this pathogen and Pozo et al. (1999) detected increased activities of β-1,3-glucanases in tomato roots 6 weeks after the inoculation with *Phytophthora parasitica*. In contrast, we did not detect any induction of chitinases, chitosanases and β-1,3-glucanases as a consequence of *Verticillium* infection, regardless whether pepper plants were associated with AMF or not. These contrasting results could be due to differences in the type of tissue analysed, the methodology used to inoculate the pathogen, and the time span between inoculation of *V. dahliae* and determination of enzymatic activities. In agreement with our results, Cui et al. (2000) did not find *Verticillium*-induced glucanases in cotton plants, and Pozo et al. (1998) did not either observe induced chitosanases in tomato roots after infection with *P. parasitica*.

The induction of Reactive Oxygen Species (ROS) scavenging enzymes, such as SOD and peroxidases, is the most common mechanism for detoxifying ROS synthesised during plant-pathogen interactions (Mittler, 2002). However, in our study, no induction of new SOD isoforms was observed after the inoculation of both non-mycorrhizal and mycorrhizal pepper plants with *V. dahliae*, which is in accordance with Pozo et al. (2002b) working with *Phytophthora*-infected tomato. On the other hand, only pepper roots associated with *G. deserticola* slightly increased peroxidase activity 2 weeks after *V. dahliae* inoculation. In a recent work, Pomar et al. (2004) did not find enhanced root peroxidase activity in roots of pepper inoculated with *V. dahliae* until day 21. However, in contrast with our results, Guenoune et al. (2001) observed that *G. intraradices* suppressed the induction of peroxidase activity in roots of alfalfa inoculated with *Rhizoctonia solani* and suggested that the suppression of defence-related properties was associated with the successful establishment of the mycorrhizal symbiosis. This finding reinforces the idea that differences in colonization dynamics of *Glomus* species could explain their dissimilar behaviour as plant bioprotectors. In fact, our research group has demonstrated that the effectiveness of AMF as biocontrol agents against *Verticillium*-induced wilt in pepper varies among *Glomus* species. The severity of the disease in plants colonized by *G. intraradices* was even higher than that observed in non-mycorrhizal plants in terms of plant growth and fruit yield (Garmendia et al., 2004b).

In summary, the colonization of pepper roots by *G. deserticola* induced the appearance of new isoforms of acidic chitinases, SOD and, at early stages, peroxidases. In contrast, *V. dahliae* alone neither stimulated the phenylpropanoid pathway nor elicited hydrolytic activities in infected pepper roots. However, this apparent maintenance of enzymatic activities could be due to the time interval – 1 week – between plant harvests, which may possibly be too long to detect temporal changes related to the defence response. Recently, Pomar et al. (2004) have suggested that through changes in stem lignins (monomer composition and crosslinking) and peroxidase activities in roots, stems and leaves the tolerant cultivars of *C. annuum* can retard the penetration by *V. dahliae* hyphae. In our study, the inoculation with *V. dahliae* slightly increased both PAL and peroxidase activities only in mycorrhizal roots. Taken together, the results suggest that the mycorrhizal-specific induction of new isoforms of acidic chitinases and SOD as well as the enhanced peroxidase and PAL activities could be involved in the biocontrol of *Verticillium*-induced wilt in *C. annuum* cv. Piquillo – a susceptible cultivar – by AMF. However, as previously reported by Guillon et al. (2002), a clear trend is not evident on how the AM symbiosis affects the plant's defence responses temporally and spatially during the pathogenic interaction. For example, the possible induction of enzymatic activities in both non-mycorrhizal and mycorrhizal roots few hours after *V. dahliae* inoculation should be analysed. Moreover, the possible presence of some microorganisms in the rhizosphere of mycorrhizal plants could also contribute to enhance the beneficial effect of *G. deserticola* as biocontrol agent against *Verticillium*-induced wilt.

In addition, another metabolic pathway to target in pepper-pathogen interactions might be the mevalonate pathway involved in the isoprenoid biosynthesis. In this sense, it has been found that the expression of different genes encoding enzymes implied in the biosynthesis of defence-related sesquiterpene phytoalexins is strongly induced following the infection of pepper roots by *Phytophthora capsici* (Ha et al., 2003).

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