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ORIGINAL PAPER

Exogenous systemin has a contrasting effect on disease resistance in mycorrhizal tomato (*Solanum lycopersicum*) plants infected with necrotrophic or hemibiotrophic pathogens

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Abstract A study was performed to determine the effect of the systemin polypeptide on the bio-protective effect of arbuscular mycorrhizal fungi (AMF) in tomato plants infected with *Alternaria solani*, *Phytophthora infestans* or *P. parasitica*. Before infection, tomato plants were colonized with two different AMF, *Glomus fasciculatum* or *G. clarum*. In addition, a group of inoculated plants was treated with systemin, just after emergence. The exogenous application of systemin marginally suppressed the resistance against *A. solani* leaf blight observed in *G. fasciculatum* mycorrhizal plants but significantly enhanced it in plants colonized with *G. clarum*. Systemin induced resistance to *P. parasitica* in leaves of *G. fasciculatum* mycorrhizal plants, in which AMF colonization alone was shown to have no protective effect. Conversely, none of the treatments led to resistance to root or stem rots caused by *P. infestans* or *P. parasitica*. The above effects did not correlate with changes in the activity levels of β-1,3-glucanase (BG), chitinase (CHI), peroxidase (PRX), and phenylalanine ammonium lyase (PAL) in leaves of infected plants. However, they corroborated previous reports showing that colonization by AMF can lead to a systemic resistance response against *A. solani*. Systemic resistance to *A. solani* was similarly observed in non-mycorrhizal systemin-treated plants, which, in contrast, showed increased susceptibility to *P. infestans* and *P. parasitica*. The results indicated that the pattern of systemic disease resistance conferred by mycorrhizal colonization was dependent on the AMF employed and could be altered by the exogenous application of systemin, by means of a still undefined mechanism.

Keywords *Glomus clarum* · *G. fasciculatum* · *Alternaria* · *Phytophthora* · Systemin

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Introduction

Tomato (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) is considered to be the most important horticultural crop worldwide, with almost 4.5 million cultivated hectares used for its production (Nuez et al. 1996; Food and Agriculture Organization 2004). However, tomato production is hampered by its susceptibility to numerous diseases, particularly those caused by fungal and oomycete pathogens. Among these, early blight of tomato caused by the necrotrophic fungal pathogen *Alternaria solani* can cause considerable reductions in yield (Jones et al. 1993). The disease is manifested by damping off in young seedlings, collar and fruit rot, leaf spots, and/or stem lesions in older plants. Early blight is a limiting factor for

the production of potato and tomato, most prominently, in high humidity conditions prevalent in tropical climates (Agrios 1997; Maiero et al. 1991). Furthermore, efforts to improve early blight resistance in tomato cultivars have been hindered by the lack of single resistance genes and the complex patterns of inheritance. Consequently, no commercial tomato cultivar possesses adequate levels of resistance to *Alternaria*, and no genetic source of early blight resistance is known within the cultivated species (Martin and Hepperly 1987; Nash and Gardner 1988; Foolad et al. 2000), although resistant accessions have been identified in undomesticated tomato species such as *Lycopersicon hirsutum* and *L. pimpinellifolium* (Barksdale and Stoner 1977; Martin and Hepperly 1987; Maiero et al. 1989, 1991; Kalloo and Banerjee 1993). The genus *Phytophthora* belongs to the oomycetes, a group of microorganisms that share unique metabolic, biochemical, and rRNA sequence similarities, many of which have the added characteristic of being devastating plant pathogens partly due to their difficult control and management. A notorious example is *P. infestans*, which causes the late blight diseases of potato and tomato. This pathogen is a recurrent phytosanitary problem due to the periodic development of resistance to the most widely employed systemic fungicides and the appearance of highly aggressive strains (Yamamizo et al. 2006; Kamoun et al. 1999; Judelson 1997).

More than 80% of terrestrial plant species form symbiosis with arbuscular mycorrhizal fungi (AMF), which belong to the phylum *Glomeromycota* (Schüssler et al. 2001). AMF are obligate biotrophs with plants, exchanging carbon provided by the plants for mineral nutrients extracted from the soil. The association with AMF has been shown to increase resistance to soil-borne pathogens and nematodes (Azcón-Aguilar and Barea 1996; Smith and Read 1997; Cordier et al. 1998; Dumas-Gaudot et al. 2000). AMF-related resistance has been attributed to several factors, including an improved plant nutrition and root biomass, changes in root system morphology and exudation pattern, reduction in abiotic stress, and modification of and/or competition with antagonistic microbial populations. An induced systemic resistance response (ISR), similar to that triggered by certain nonpathogenic soil bacteria and fungi, is also believed to lead to resistance in non-mycorrhizal roots and/or aerial tissue of mycorrhizal plants, although an increased plant vigor resulting from the symbiosis with the AMF could also be involved. A systemic resistance response against *P. parasitica* was observed in non-mycorrhizal root sections of mycorrhizal tomato plants (Cordier et al. 1998; Pozo et al. 2002). Moreover, a recent report indicated that colonization of tomato plants with AMF significantly reduced early blight disease symptoms (Fritz et al. 2006), believed to be caused in part by an ISR-like mechanism. The activation of systemic resistance responses in mycorrhizal plants suggests the involvement of a mycorrhiza-induced mobile signal. However, little is known concerning the signal(s) involved in the induction of ISR after formation of the AM symbiosis (Hause and Fester 2005), although jasmonic acid (JA), which has been found to be important for the establishment of this symbiotic association (Hause et al. 2002; Isayenkov et al. 2005), could be suitable candidate, considering its involvement in long-distance signaling in other phenomena, such as in the wound response. In this respect, systemin is a mobile 18 amino acid (aa) polypeptide associated with the wound response in tomato, which involves the systemic induction of the so-called systemic wound-responsive proteins or SWRPs, including several proteases, protease inhibitors, polyphenol oxidase, and signal pathway associated proteins and others, such as threonine deaminase, which could be involved in defense against insect herbivores (Bergey et al. 1996; Constabel et al. 1995; Ryan 2000; Ryan and Pearce 2003; Chen et al. 2005). Wound systemic signaling is believed to occur by means of a positive amplification loop in which systemin and JA, or a related oxylipin, are self induced through the vascular tissue (Li et al. 2002; Ryan and Moura 2002; Stenzel et al. 2003; Narváez-Vásquez and Ryan 2004; Schilmiller and Howe 2005). In addition, systemin over-expression was correlated with resistance to necrotrophic fungal pathogens (i.e., *Botrytis cinerea*; Díaz et al. 2002) in tomato and related solanaceous plants. Accordingly, an investigation was undertaken to analyze the possible role of systemin in the modulation of a local and/or systemic resistance response triggered in mycorrhizal tomato plants infected with *P. infestans*, *P. parasitica*, or *A. solani*. Changes in the accumulation of the pathogenesis-related (PR) proteins CHI, BG, PRX, and PAL were measured. These proteins have been shown to enhance resistance to certain fungal pathogens by hydrolyzing their cell wall components (Simmons 1994), by catalyzing the synthesis of reactive oxygen species (ROS), which can lead to cell wall fortification and pathogen containment or death (Hammond-Kosack and Jones 1996) or of salicylic acid (SA), an inducer of the expression of a variety of PR genes mostly effective against biotrophic pathogens (Mauch-Mani and Slusarenko 1996). There is evidence that the expression of these genes could be modulated by systemin in tomato, as shown by reports describing that the application of systemin to mycorrhizal tomato plants in the early stages of colonization induced root and leaf accumulation of BG and CHI activity (Noval et al. 2004) and that ROS production in tomato cultured cells, triggered by the addition of fungal oligosaccharide elicitors, was greatly enhanced by pre-incubation with systemin (Stennis et al. 1998). Moreover, SA accumulation derived from PAL expression could negatively interact with systemin/JA signaling (Doares et al. 1995), thereby having the potential

to modify the resistance against the necrotrophic (*A. solani*) or hemibiotrophic pathogens (*P. infestans* and *P. parasitica*) used in this study.

The results described here, which are further discussed, indicate that systemin shifted the ISR pattern observed in mycorrhizal plants by means of an unidentified mechanism not related to changes in PR protein accumulation.

Materials and methods

Plant material and treatments

The tomato (*S. lycopersicum* L., syn. *L. esculentum* Mill.) variety Amalia (Álvarez et al. 1997) was the cultivar employed in this study. It is susceptible to the three pathogens examined and was developed by the Department of Genetics and Plant Breeding of the National Institute of Agricultural Sciences (INCA, La Habana, Cuba). Seeds were surface sterilized with commercial sodium hypochlorite and were sown in trays filled with a standard germination mixture. After germination, plantlets having two fully expanded leaves were transplanted to 1-kg pots containing a 1:1 *w*/*w* mixture of red ferralitic soil and earthworm humus and were grown in a glasshouse covered with a polyethylene shade screen for a 50% reduction in available photosynthetically active radiance, under otherwise natural conditions.

Exogenous synthetic systemin (BQ SOS Laboratories, México) was added as a soil-drench solution to recently emerged plantlets in amounts (30 ml at 44 nmol/pot) shown previously to induce the accumulation of PR proteins in roots and leaves and also of two reported SWRPs in tomato and potato foliage: trypsin inhibitor and polyphenol oxidase activity (Noval et al. 2004; Tejeda-Sartorius et al. 2007, and unpublished data). All other treatments were watered with a similar volume of water containing no systemin. The accumulation of wound-responsive proteins in the foliage of systemin-treated plants is indicative that systemin can be absorbed by the root system to induce the systemic accumulation of SWRPs, presumably after its transport through the transpiration stream, similarly to the traditional method consisting of the uptake of a small volume of a buffered systemin solution through the cut hypocotyls of two-leaf plantlets (Pearce et al. 1991). Two species of AM fungi were used as inocula: *Glomus clarum* (Nicolson and Schenk) and *G. fasciculatum* (Walker and Koske). Both AM fungi are constituents of the EcoMic® bio fertilizer (INCA) and were previously certified to have an average titer of 25 spores/g. Mycorrhizal inoculation was performed by coating the seeds with the respective AM fungi spore preparation before sowing, as described by Fernández et al. (2000).

All pathogens employed in this study were isolated from diseased tomato plants cultivated in Cuba and were subsequently identified by standard procedures. *A. solani* (Ellis & Martin) Jones & Grout isolates were cultured in potato dextrose agar plates, whereas *P. infestans* (Mont.) de Bary and *P. parasitica* Dastur (=*P. nicotianae* Breda de Haan) were grown in maize agar (30 g maize flour and 15 g agar per l) and maize-wheat germ agar (20 g of freshly germinated wheat seedlings, 30 g maize flour, and 15 g agar per l), respectively. All pathogens were incubated at 25°C in the dark. Plants were infected 21 days after germination by spraying the pathogen-containing suspensions (5 ml/plant) directly on the surface of the leaves (*A. solani*) or by their application on the stem/root interface (*P. infestans* and *P. parasitica*). *A. solani* inocula were prepared from hyphae (≈12.3 g) scraped from the surface of four plates after a 15-day incubation period. At this time, the colonies had extended to a diameter of approximately 85 mm. Collected hyphae were macerated in a mortar with sterile water and taken to a final volume of 500 ml. The suspension was examined under a Neubauer chamber to assess the number of hyphal fragments and spores and was subsequently adjusted to density of approximately 1×10^5 fungal fragments and spores per ml. *P. infestans* and *P. parasitica* inocula were obtained as above, except that the suspensions were incubated at 4°C for 1 h to stimulate the liberation of zoospores before counting and adjustment of the final spore density to 10^5 zoospores/ml.

To ensure adequate spore germination and pathogen progression, all plants were maintained under high humidity conditions for the entire duration of the experiment (72 h). To score the disease severity in the foliage of pathogen-infected plants, an average value per plant was obtained after dividing the level of damage (necrotic or blighted areas) on the surface of each individual leaf by the total number of leaves examined. The severity of the infection was visually evaluated according to a scale in which infection was graded at six different levels: level 5 representing 75 to 100% damaged tissue; level 4, 50 to 75%; level 3, 25 to 50%; level 2, 10 to 25%; level 1, ≤10%; and level 0, healthy leaves with no visible damage. The severity of stem and root rots in *Phytophthora*-infected plants was evaluated also; damage was assessed using the above scale by measuring the length of necrotized-infected tissues on longitudinal sections of these tissues. After disease assessment, leaf and root samples were stored at −20°C for subsequent enzymatic assays (see below).

Plant growth was determined by measuring the total foliar and radical mass in treated 3-week-old plants not challenged by pathogens. Arbuscular mycorrhizal colonization was evaluated as the percentage of root system cortex with fungal structures

after KOH digestion and trypan blue staining (Phillips and Hayman 1970) as described by Trouvelot et al. (1986).

Biochemical assays

CHI, BG, PRX, and PAL activities were assayed in leaf and root protein extracts prepared as described elsewhere (Pérez et al. 2004). The release of *N*-acetyl-glucosamine from colloidal chitin (Fluka) by CHI activity was determined at 585 nm according to the discontinuous method described by Boller et al. (1983). BG activity was assayed colorimetrically at 450 nm by measuring the reduction in copper-based reagents by the glucose units released by BG from the laminarin substrate. The analysis was performed in a microplate format according to a modification (Noval et al. 2004) of Zheng and Wozniak's (1997) method. PRX activity was determined according to Fric (1976) using guaiacol and H_2O_2 as substrates. In this reaction, the PRX-catalyzed transfer of electrons from the electron donor guaiacol to the H_2O_2 acceptor generates a highly colored oxidation product. The reaction was thus followed by measuring the change in absorbance at 470 nm, at 15-s intervals, for a total reaction time of 2 min. PAL activity was analyzed according to Nagarathna et al. (1993), using phenylalanine as substrate. Activity was determined by measuring the PAL-catalyzed formation of *trans*-cinnamic acid from phenylalanine at 275 nm. All enzymatic activities [in nKat or pKat; Tipton (1993)] were calculated per milligram total protein. Protein content was measured according to the method of Bradford (1976), employing a commercial kit (Bio-Rad Laboratories, USA). All substrates and enzymes employed as controls were acquired from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Experimental design and statistical analysis

All experiments were conducted using a completely randomized block design with three blocks. Each block included four experimental groups (uninfected controls and plants infected with *A. solani*, *P. parasitica*, or *P. infestans*) and six treatments: untreated, systemin, *G. clarum*, *G. clarum* + systemin, *G. fasciculatum*, *G. fasciculatum* + systemin). Each block consisted of 48 plants (two plants per treatment × six treatments × four experimental groups). The experiments were repeated four times within a 2-year period (2004–2005). One-way analysis of variances (ANOVAs) were utilized to evaluate differences between treatments. For ANOVAs where the *F* test was significant at 0.01 or lower probability level, the least significant difference test was applied to detect differences among treatment means. All four independent experiments yielded similar results. Accordingly, the results described below represent those derived from one typical experiment.

Results

Plant growth and fungal colonization

The mycorrhizal colonization level detected in the inoculated plants at the time when they were challenged with the pathogens was 41.3 and 40.7% on average for roots inoculated with *G. clarum* and *G. fasciculatum*, respectively. Systemin application slightly, but significantly, reduced the level of *G. clarum* colonization (36%) but did not affect colonization with *G. fasciculatum* (37.6%; Table 1). No significant differences in foliar mass were detected between non-inoculated plants (untreated and systemin-treated plants) and mycorrhizal plants (±systemin). However, in plants inoculated with *G. clarum*, systemin treatment led to a significant increment in foliar mass (Fig. 1a). Radical mass was similarly unaffected by AM fungi colonization except by the significant increase observed, once again, in the *G. clarum*–systemin combination (Fig. 1b).

Alternaria is a necrotrophic pathogen that relies on hydrolytic activity and/or the production of host-specific toxins, and other substances such as a nontoxic susceptibility-inducing factor, for the development of the typical chlorotic and necrotic lesions observed in infected plants (Langsdorf et al. 1990; Thomma 2003). In this study, the induced resistance to *A. solani* in mycorrhizal plants ± systemin was evaluated by measuring the development of necrotic areas on the surface of the leaves. Colonization of tomato plants with *G. fasciculatum*, but not *G. clarum*, significantly reduced early blight disease symptoms. Interestingly, systemin treatment alone led to a significant resistance response against this pathogen, whereas in mycorrhizal plants, it had a contrasting effect, significantly reducing the severity of the infection in plants colonized with *G. clarum* on one hand and marginally increasing the susceptibility in those colonized with *G. fasciculatum* on the other (Fig. 2a).

Table 1 Mycorrhizal colonization (M%) in tomato plants inoculated with two AMF species ± systemin

Treatments	M%[a]
Untreated	–
Systemin	–
G. clarum	41.3 a
G. clarum + systemin	36.0 c
G. fasciculatum	40.6 ab
G. fasciculatum + systemin	37.6 bc
CV	3.83

[a] Data in the same column not sharing a letter in common differ significantly at $p<0.01$.
CV variance coefficient

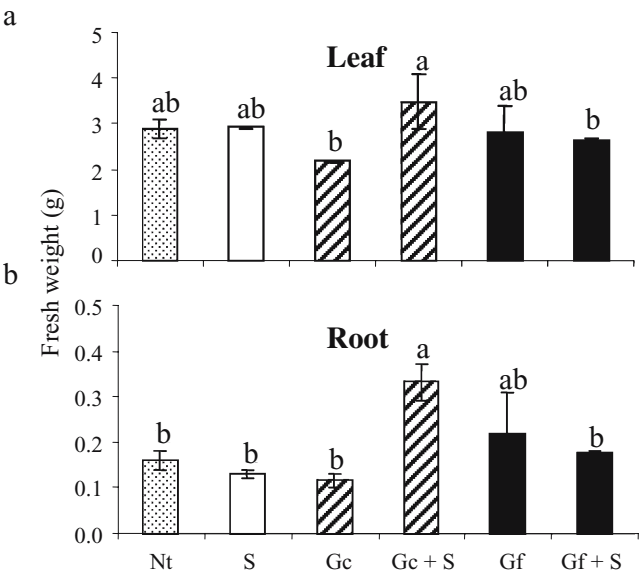

Fig. 1 Shoot (**a**) and root (**b**) fresh weights of tomato plants as affected by inoculation with AM fungi and/or systemin. The determination was performed prior to pathogen challenge, 21 days after germination. *Nt* untreated controls; *S* systemin; *Gf G. fasciculatum*; *Gc G. clarum*. *Bars with different letters* are significantly different at $p \leq 0.01$. *Error bars* are ±SE of the mean

Resistance against the two *Phytophthora* species tested in this study was evaluated by measuring the extent of diseased tissue produced in roots, stems, and leaves. No treatment was able to confer a significant protective effect against root and stem rots produced by *P. infestans* or *P. parasitica* infection (results not shown). Leaves of untreated and mycorrhizal plants were similarly susceptible to *P. infestans* and *P. parasitica*, although a marginal decrease in leaf blight was observed in mycorrhizal plants challenged with *P. infestans* (Fig. 2b and c). The application of exogenous systemin significantly increased the susceptibility to both *Phytophthora* species tested, which contrasted with the protective effect observed in plants infected with *A. solani* (Fig. 2b and c). However, it had a significant effect on resistance against *P. parasitica* when it was applied in combination with *G. fasciculatum* (Fig. 2b).

Biochemical assays showed that exogenous systemin application significantly increased BG activity in roots of uninfected plants. BG activity was also significantly increased in uninfected mycorrhizal plants colonized with both symbionts. However, when systemin was added in combination with AMF, the induction of BG activity in roots was suppressed (Fig. 3a). Foliar BG activity in uninfected plants was approximately fivefold lower than that in roots of equivalent plants. Moreover, most treatments led to a significant reduction in activity, particularly in systemin- and *G. clarum*–systemin-treated plants, although in the *G. fasciculatum*–systemin combination, the activity levels were significantly higher than in untreated plants (Fig. 3b). In general and irrespective of the treatment applied, BG activity levels in leaves of *A. solani*-infected plants were generally lower than those detected in uninfected plants. Systemin again appeared to have a suppressive effect (Fig. 3c). BG levels in leaves of treated plants infected with *Phytophthora* did not differ significantly from those detected in the untreated, infected controls, except for the reduction in activity observed in the *G. clarum*–systemin combination infected with *P. parasitica* (Fig. 3d and e).

CHI activity in roots of uninfected plants was clearly induced by systemin. Activity levels in roots of mycorrhizal plants colonized by *G. clarum*, although much lower than the above, were still significantly higher than in untreated controls (Fig. 4a). In leaves of uninfected plants, all treatments, except *G. clarum* mycorrhizal plants, led to a significant reduction in CHI activity levels (Fig. 4b). Irrespective of the treatment, CHI activity levels in *A. solani*-infected plants were two- to fourfold higher than those in uninfected plants, although only the *G. clarum*–systemin combination had significantly higher levels than untreated controls (Fig. 4c). In contrast, a suppressive effect

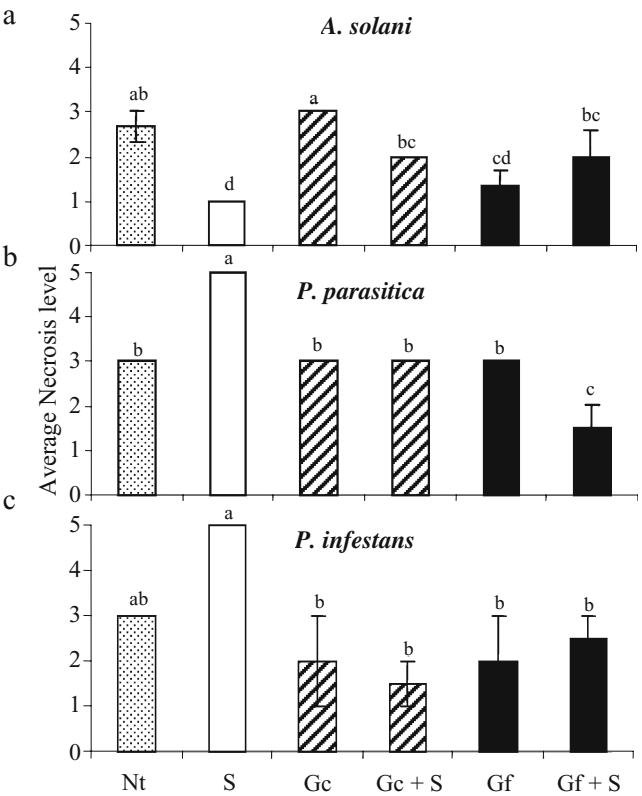

Fig. 2 Average foliar damage in tomato plants treated with AM fungi and/or systemin and subsequently challenged with **a** *A. solani*, **b** *P. parasitica*, and **c** *P. infestans*. Control plants remained untreated (*Nt*; *stippled bars*) or were supplied with exogenous systemin (*S*; *white bars*). Exogenous systemin was also added to mycorrhizal plants previously colonized with *G. fasciculatum* (*Gf* and *Gf* + *S*; *black bars*) or *G. clarum* (*Gc* and *Gc* + *S*; *striped bars*). *Bars with different letters* are significantly different at $p \leq 0.01$. *Error bars* are ±SE of the mean

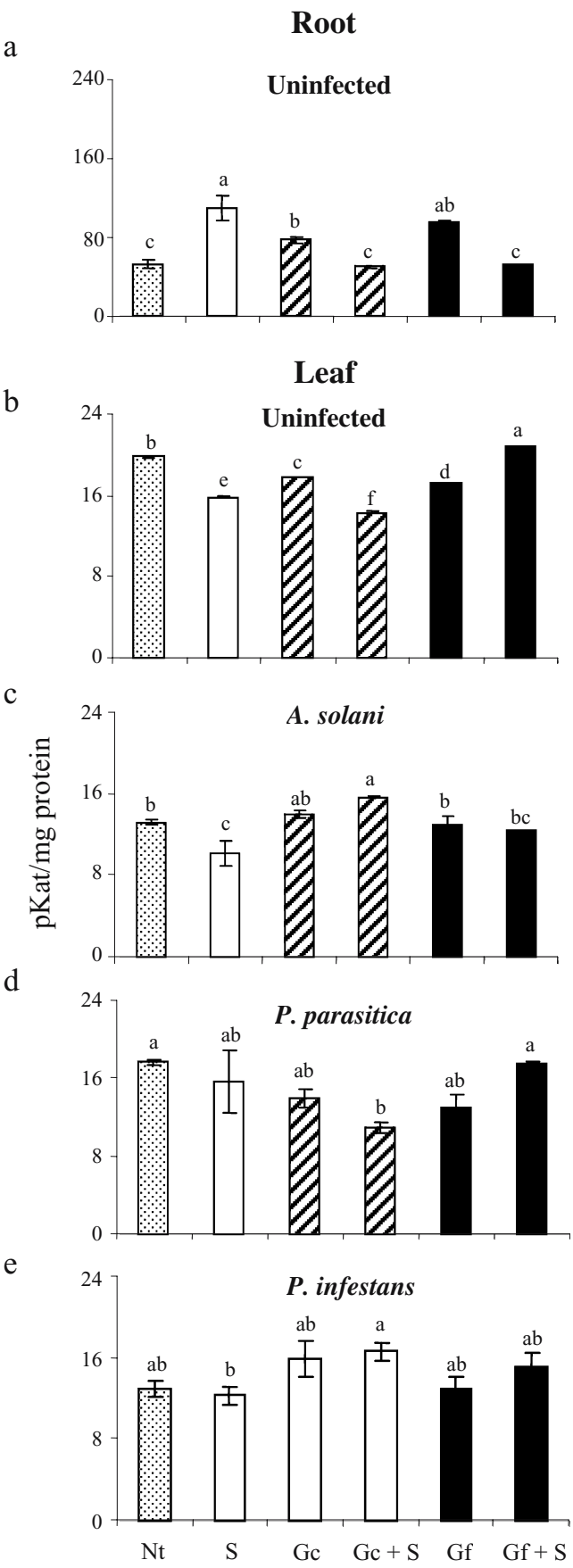

Fig. 3 BG activity levels detected in roots and leaves of mycorrhizal ▶ tomato plants treated with systemin and infected with three different pathogens. BG activity was analyzed in root and leaf protein extracts obtained from uninfected plants (**a** and **b**) or in leaf protein extracts obtained from plants infected with *A. solani* (**c**), *P. parasitica* (**d**), or *P. infestans* (**e**). Infected and uninfected controls remained untreated (*Nt*; *stippled bars*) or were supplied with exogenous systemin (*S*; *white bars*). Exogenous systemin was also added to mycorrhizal plants previously colonized with *G. fasciculatum* (*Gf* and *Gf* + *S*; *black bars*) or *G. clarum* (*Gc* and *Gc* + *S*; *striped bars*). *Bars with different letters* are significantly different at $p \leq 0.01$. *Error bars* are ±SE of the mean

on CHI activity was observed in systemin-treated plants infected with *P. parasitica* (Fig. 4d). Also relevant was the generalized reduction in CHI activity in all plants infected with *P. infestans*, which was pronounced in *G. clarum*- and *G. clarum*–systemin-treated plants (Fig. 4e).

Irrespective of the treatment applied, no local accumulation of PRX activity was observed in roots of uninfected plants (Fig. 5a). Similarly, no change in PRX activity in leaves of uninfected plants was observed except for the significant reduction detected in the *G. fasciculatum*–systemin combination (Fig. 5b). Conversely, increased PRX activity levels were detected in *G. clarum* and *G. fasciculatum*–systemin mycorrhizal plants and in systemin-treated plants subsequently infected with *A. solani* (Fig. 5c). Only in the latter plants did this effect coincide with the disease resistance detected against this pathogen, whereas an inverse correlation between PRX activity levels and resistance to *A. solani* was observed in *G. clarum*–systemin and *G. fasciculatum* mycorrhizal plants (compare Figs. 2 and 5c). Also relevant was the observation that, contrary to *G. clarum*–systemin mycorrhizal plants, systemin appeared to compensate for the suppressive caused by *A. solani* infection in *G. fasciculatum* mycorrhizal plants (Fig. 5c). These results were suggestive of a species-dependent effect regarding the systemic changes in PRX activity in response to *A. solani* infection in mycorrhizal plants, which was differentially affected by exogenous systemin. Although infection with *P. parasitica* produced a generalized increased in PRX activity levels in comparison to unchallenged plants, all further treatments, except the *G. clarum*–systemin combination, showed a significant decrease in activity with respect to untreated controls. Curiously, the effect in *G. clarum* and *G. clarum*–systemin mycorrhizal plants infected with *P. parasitica* was contrary to that observed in equivalent *A. solani*-infected plants (Fig. 5d). No obvious effect on PRX activity was detected in *P. infestans*-infected plants irrespective of the treatment applied (Fig. 5e).

Similar to BG and CHI, a local induction of PAL activity was detected in the roots of uninfected systemin-treated plants (Fig. 6a), whereas a significant systemic induction of PAL activity was detected in the foliage of all treated,

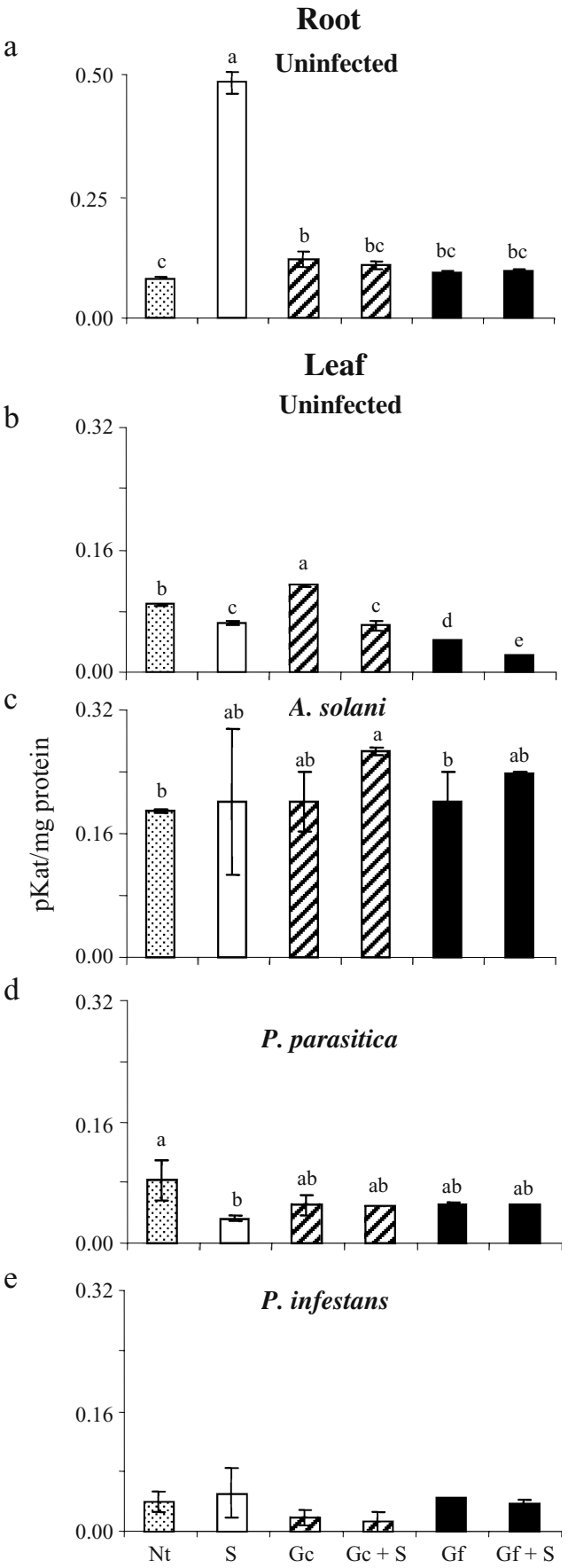

Fig. 4 CHI activity levels detected in roots and leaves of mycorrhizal ▶ tomato plants treated with systemin and infected with three different pathogens. CHI activity was analyzed in root and leaf protein extracts obtained from uninfected plants (**a** and **b**) or in leaf protein extracts obtained from plants infected with *A. solani* (**c**), *P. parasitica* (**d**), or *P. infestans* (**e**). Infected and uninfected controls remained untreated (*Nt*; *stippled bars*) or were supplied with exogenous systemin (*S*; *white bars*). Exogenous systemin was also added to mycorrhizal plants previously colonized with *G. fasciculatum* (*Gf* and *Gf* + *S*; *black bars*) or *G. clarum* (*Gc* and *Gc* + *S*; *striped bars*). *Bars with different letters* are significantly different at $p \leq 0.01$. *Error bars* are ±SE of the mean

uninfected plants (Fig. 6b). Infection with *A. solani* raised PAL activity levels in untreated plants but did not cause any further changes in treated plants, except in *G. clarum* mycorrhizal plants, in which an approximate threefold increase in activity was observed (Fig. 6c). Infection with both *Phytophthora* pathogens caused a generalized increase in PAL activity levels that was, in most cases, independent of the treatment applied (Fig. 6d and e). Curiously, the increased PAL activity produced in *G. clarum* mycorrhizal plants infected with *A. solani* was reversed in equivalent plants infected with both *Phytophthora* pathogens.

In summary, systemin treatment was observed to induce a strong local accumulation of CHI, BG, and PAL in the absence of pathogens, an effect that was suppressed in uninfected *G. clarum* and *G. fasciculatum* mycorrhizal plants (Figs. 3a, 4a, and 6a). A generalized decrease in PR protein activity was also observed in roots of *Phytophthora*-infected plants, irrespective of the treatment applied (results not shown). This coincided with the lack of resistance against *Phytophthora* root rot detected in this study. Moreover, the changes detected in PR protein activity levels in leaves had no obvious correlation with the significantly greater resistance to *Alternaria* or *Phytophthora* leaf blights observed in systemin-treated (vs *A. solani*), *G. fasciculatum* mycorrhizal (vs *A. solani*), and the *G. fasciculatum*–systemin combination plants (vs *P. parasitica*) nor with the significant increase in susceptibility to both *Phytophthora* species detected in leaves of systemin-treated plants.

Discussion

The colonization of plants by AMF leads to biochemical, physiological, and structural changes that are believed to modify the resistance responses to potential invaders, both locally and systemically. These changes can be beneficial or detrimental to the plant, depending on various factors (Cordier et al. 1996; Dugassa et al. 1996; Gange and West 1994; Slezak et al. 2000; Elsen et al. 2001).

As shown in Table 1, mycorrhizal plants had reached a reasonable level of colonization at the time of pathogen

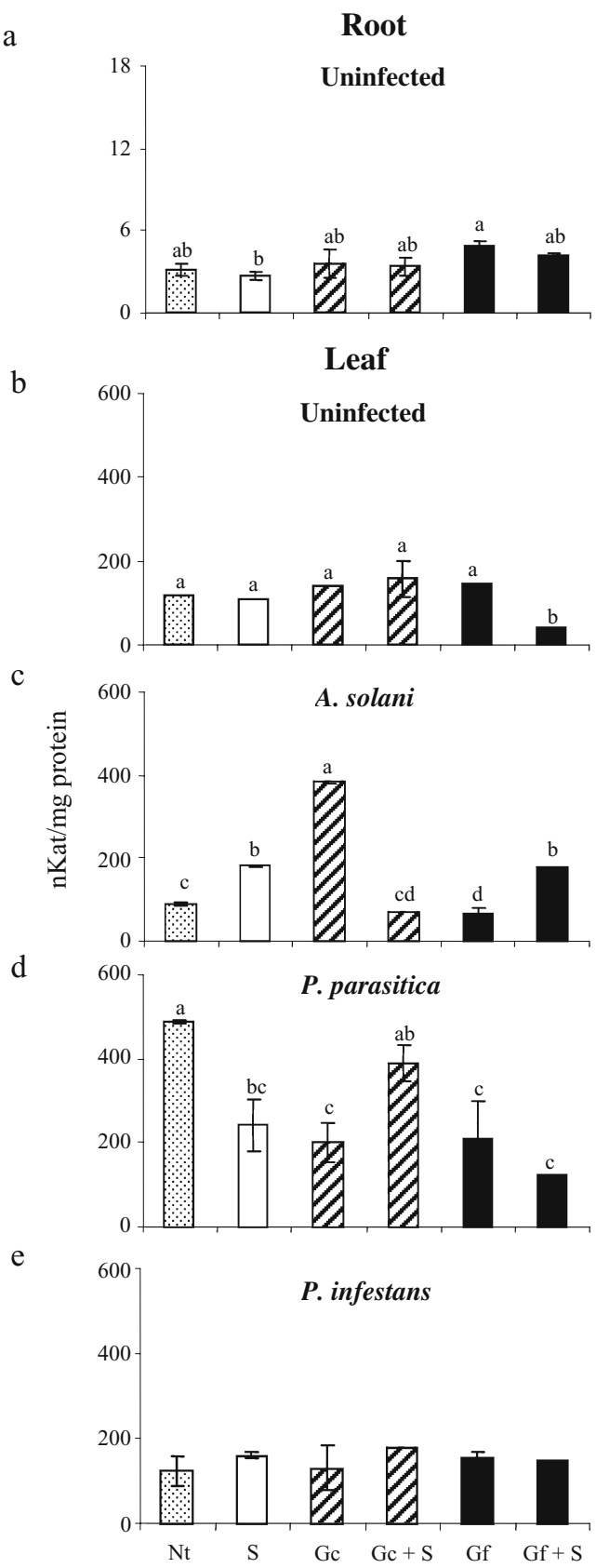

Fig. 5 PRX activity levels detected in roots and leaves of mycorrhizal tomato plants treated with systemin and infected with three different pathogens. PRX activity was analyzed in root and leaf protein extracts obtained from uninfected plants (**a** and **b**) or in leaf protein extracts obtained from plants infected with *A. solani* (**c**), *P. parasitica* (**d**), or *P. infestans* (**e**). Infected and uninfected controls remained untreated (*Nt*; *stippled bars*) or were supplied with exogenous systemin (*S*; *white bars*). Exogenous systemin was also added to mycorrhizal plants previously colonized with *G. fasciculatum* (*Gf* and *Gf* + *S*; *black bars*) or *G. clarum* (*Gc* and *Gc* + *S*; *striped bars*). *Bars with different letters* are significantly different at $p \leq 0.01$. *Error bars* are ±SE of the mean

challenge. Colonization was not negatively affected by systemin, although a tendency toward a reduction in M%, which became significant in mycorrhizal *G. clarum* plants treated with systemin, was observed in both AMF–systemin combinations. Curiously, it was precisely in these plants in which a tendency toward a greater foliar mass and a significant increase in radical biomass was observed (Fig. 1a and b). This result was in agreement with previous findings, made in mycorrhizal tomato plants colonized with several AMF, showing that the abundance of intracellular fungal structures within the root was not correlated with beneficial mycorrhizal effects, including root and shoot growth promotion (Burleigh et al. 2002). It also suggests that some systemin–AM fungi combinations may somehow lead to growth promotion. The mechanism(s) responsible for this synergistic effect on growth remain to be determined. It is tempting to speculate, however, that changes in the nitrogen content of the roots, resembling the unusual accumulation of nitrogen in tubers of prosystemin over-expressing potato plants, could have been involved in the observed promotion of root growth in mycorrhizal *G. clarum*–systemin plants (Narváez-Vásquez and Ryan 2002).

As shown in Fig. 2a, colonization with *G. fasciculatum* led to a significant reduction in necrotic lesions in leaves of plants infected with *A. solani*. On the other hand, no protection was provided by *G. clarum*. The differential pathogen resistance observed between these two AMF species was expected, considering that *G. fasciculatum* has consistently provided better protection under our experimental conditions (not shown). These results were in agreement with a recent study showing that colonization of tomato plants with *G. intraradices* significantly reduced early blight disease symptoms in conditions (e.g., low phosphorus concentrations in soil) that allowed extensive mycorrhiza formation (Fritz et al. 2006). Interestingly, the systemin treatment alone led to a clearly significant level of protection against this necrotrophic pathogen and appeared to interact positively with *G. clarum*, but not *G. fasciculatum*, as the combined treatment with the former AMF reduced the severity of the infection. The contrasting results obtained with these two AMF species coincided with the often

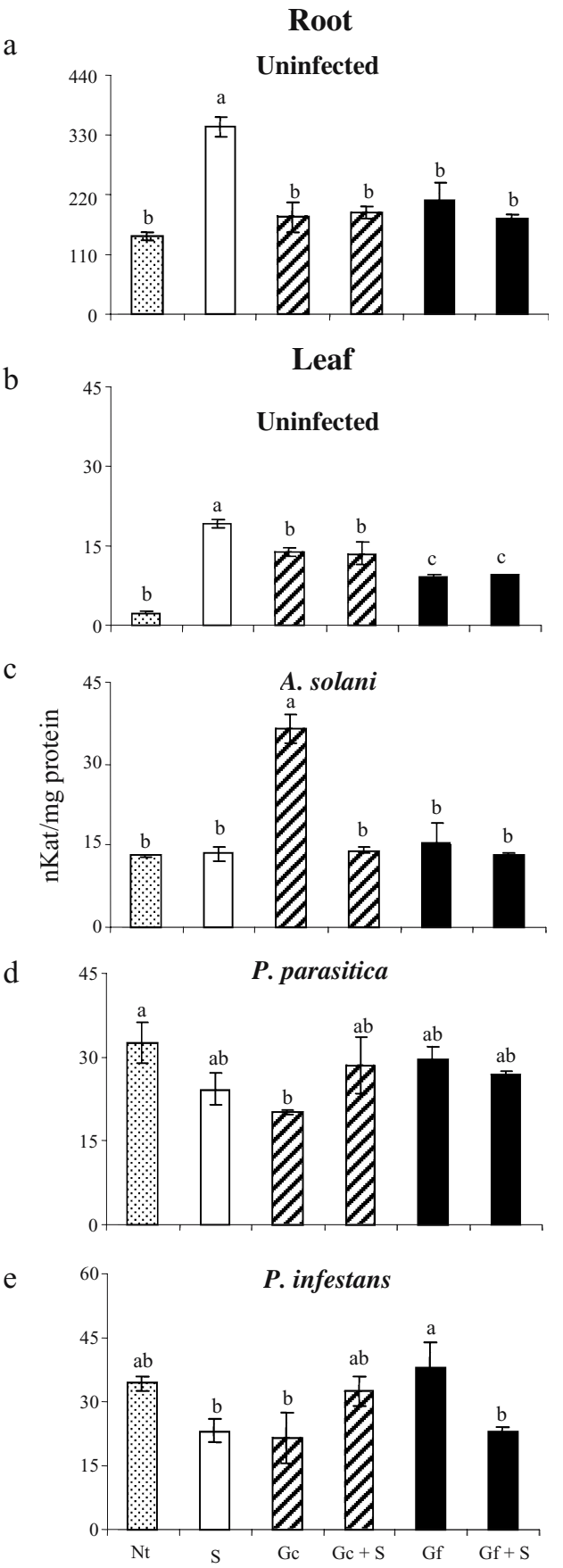

Fig. 6 PAL activity levels detected in roots and leaves of mycorrhizal ▶ tomato plants treated with systemin and infected with three different pathogens. PAL activity was analyzed in root and leaf protein extracts obtained from uninfected plants (**a** and **b**) or in leaf protein extracts obtained from plants infected with *A. solani* (**c**), *P. parasitica* (**d**), or *P. infestans* (**e**). Infected and uninfected controls remained untreated (*Nt*; *stippled bars*) or were supplied with exogenous systemin (*S*; *white bars*). Exogenous systemin was also added to mycorrhizal plants colonized with *G. fasciculatum* (*Gf* and *Gf* + *S*; *black bars*) or *Glomus clarum* (*Gc* and *Gc* + *S*; *striped bars*). *Bars with different letters* are significantly different at $p \leq 0.01$. *Error bars* are ±SE of the mean

observed variation in the plant's response to different mycorrhizal fungal species and even to isolates of the same species, including changes in the level of gene expression (see below; Smith and Smith 1997; Burleigh et al. 2002). Conversely, mycorrhizal colonization did not lead to a local or systemic resistance response against the two *Phytophthora* species examined. Except in the *G. fasciculatum*–systemin combination that generated an ISR against *P. parasitica*, the observed inability to induce a resistance response against these pathogens was not altered by the addition of exogenous systemin, which when applied in the absence of mycorrhizal fungi, increased the susceptibility to these pathogens in leaves. Therefore, it was evident that mycorrhizal colonization in tomato led to an ISR against a necrotrophic foliar pathogen that was species-dependent in relation to the level of resistance conferred and also in its interaction with systemin, whereas the effect was neutral against hemibiotrophic *Phytophthora* pathogens. Furthermore, systemin treatment alone had a clearly contrasting effect, inducing an ISR against *A. solani* in tomato, but increasing its susceptibility to *P. parasitica* and *P. infestans*.

In general, the results derived from the in vitro activity analysis of four PR proteins showed poor or no correlation between modified levels of activity and a resistance to the challenging pathogens. This suggests that other mechanisms might be involved in the establishment of the resistance/susceptibility responses observed, a view supported by a microarray analysis reporting the identification of 168 genes induced by *A. brassicicola* infection in *Arabidopsis* (Schenk et al. 2000). In addition, some authors have postulated that a mechanism akin to the JA-dependent ISR produced by nonpathogenic rhizobacteria is responsible for the increased resistance to foliar pathogens observed in mycorrhizal plants (Fritz et al. 2006). In this respect, the systemic resistance to *A. solani* detected in systemin-treated plants could be related to the finding, also reported in *Arabidopsis*, that one of the recognized defense signaling pathways leading to resistance against *A. brassicicola* is known to be JA-dependent (Penninckx et al. 1996; Thomma et al. 1998, 2000; Thomma 2003; van Wees et al. 2003). Therefore, similar to the systemin-JA feed-forward loop leading to the amplification of the systemic wound response

described above, systemin application could have led to increased JA levels in the treated tomato plants and, consequently, to a JA-dependent ISR against *A. solani*.

The lack of a correlation between *A. solani* resistance and increased PR protein activity contradicts previous reports (Lawrence et al. 2000; Mora and Earle 2001). However, the in vitro assays were not designed to identify subtle differences in activity that could have arisen due to the induction of putative pathogen-specific isoforms in these usually polymorphic protein families. In support of this possibility, a recent study reported the presence of at least six basic and six acidic isoforms of BG in tomato roots and/or leaves, most of which showed constitutive activity. However, one acidic isoform (pI 3.8) was shown to be up-regulated in roots and, to a lesser degree in leaves, of tomato plantlets treated with systemin at the initial stages of colonization with *G. clarum* (Noval 2000). Another possible scenario that might explain the systemin-related ISR against *A. solani* observed in this study could be similar to the one proposed to explain the increased resistance to the necrotrophic pathogen *B. cinerea* reported in prosystemin over-expressing tomato plants (Díaz et al. 2002). These authors postulated that the induction of the putative protective genes, deemed to be different from PRs, was probably due to H_2O_2 accumulation occurring in response to oligogalacturonides (OGAs) released by the systemic activation of a wound-inducible polygalacturonase (Orozco-Cárdenas et al. 2001). The work by Stennis et al. (1998), cited above, which describes an augmented oxidative burst in tomato cell suspension cultures pretreated with systemin and subsequently induced with OGAs, is also in agreement with this possibility. However, further studies will be required to establish the precise mechanism(s) leading to an ISR against *A. solani* in mycorrhizal tomato plants and the roles played in it by systemin, JA, and/or ROS.

Resistance to *Phytophthora* spp. has been attributed to the plant's ability to mount a strong and rapid oxidative burst capable of blocking the progression of the pathogen (Kamoun et al. 1999; Vleeshouweres et al. 2000; Yoshioka et al. 2003; Yamamizo et al. 2006). Other factors have been described in mycorrhizal tomato plants showing resistance against pathogenic strains of *P. parasitica*, including the systemic induction, in non-colonized sections of mycorrhizal roots, of PAL, possibly related to the accumulation of toxic phenolic compounds and the reinforcement of the cell wall (Cordier et al. 1998) and the expression of novel glucanases and CHIs as well as superoxide dismutase (Pozo et al. 2002). However, in this work, no ISR against *P. parasitica* and *P. infestans* was observed in mycorrhizal tomato plants. A lack of induction above control levels in challenged untreated plants and, in some cases, a strong pathogen-dependent suppression of the in vitro activity of the PR defense-related proteins analyzed in this study, could have explained the lack of resistance observed. A similar down-regulation of PR protein expression was observed in tobacco plants rendered more susceptible to foliar pathogens by mycorrhizal colonization (Shaul et al. 1999). Moreover, systemin-treated plants showed an increased susceptibility to infection, possibly reflecting a negative cross-talk with either the SA and ethylene-dependent signaling pathways believed to be activated during compatible *P. infestans*–tomato interactions (Jeun et al. 2000; Niderman et al. 1995), or the ethylene-, SA-, and JA-independent pathways reported in tomato (Smart et al. 2003) and *Arabidopsis* (Roetschi et al. 2001).

Also interesting was the finding that systemin was able to induce a local increase in root activity of three PR proteins in uninfected plants, including the unlikely induction of PAL, which was also induced systemically, in leaves. This pattern confirmed previous reports indicating that systemin can be absorbed by the roots of tomato and potato plants to induce the local and systemic expression of defensive genes. However, the inductive systemin-related effect was suppressed in mycorrhizal plants. The negative effect on systemin-related induction of PR protein activity, which was probably as a manifestation of the down-regulation of defensive responses that usually accompanies colonization by AMF (David et al. 1998; Lambais 2000), contrasted with a previous report showing that BG and CHI activities were significantly increased in mycorrhizal *G. clarum* plants treated with systemin (Noval et al. 2004). A possible explanation of this discrepancy could be attributed to differences in the experimental conditions employed, including the growing conditions and age of the plants at which the activities were determined.

In conclusion, the results derived from this study were partially in agreement with several other reports indicating that the colonization by AMF can lead to a systemic pathogen resistance. The effects observed were dependent on the AMF and pathogens employed. Systemin was found to have a neutral or positive effect on pathogen resistance when interacting with mycorrhizal fungi. However, the mechanism(s) by which systemin interacts with AMF to modify pathogen resistance and the patterns of PR protein activity in mycorrhizal plants remain(s) to be determined.

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