



Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to *Phytophthora* infection in tomato plants

Maria J. Pozo^{1,3}, Christelle Cordier¹, Eliane Dumas-Gaudot², Silvio Gianinazzi², Jose M. Barea¹ and Concepción Azcón-Aguilar¹

¹ Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, C.S.I.C., 18008 Granada, Spain

² UMR BDCE-IPM, INRA, BP 86510 Dijon Cédex 21065, France

Received 14 May 2001; Accepted 23 October 2001

Abstract

Development of biological control for plant diseases is accepted as a durable and environmentally friendly alternative for agrochemicals. Arbuscular mycorrhizal fungi (AMF), which form symbiotic associations with root systems of most agricultural, horticultural and hardwood crop species, have been suggested as widespread potential bioprotective agents. In the present study the ability of two AMF (*Glomus mosseae* and *Glomus intraradices*) to induce local or systemic resistance to *Phytophthora parasitica* in tomato roots have been compared using a split root experimental system. *Glomus mosseae* was effective in reducing disease symptoms produced by *P. parasitica* infection, and evidence points to a combination of local and systemic mechanisms being responsible for this bioprotector effect. The biochemical analysis of different plant defence-related enzymes showed a local induction of mycorrhiza-related new isoforms of the hydrolytic enzymes chitinase, chitosanase and β -1,3-glucanase, as well as superoxide dismutase, an enzyme which is involved in cell protection against oxidative stress. Systemic alterations of the activity of some of the constitutive isoforms were also observed in non-mycorrhizal roots of mycorrhizal plants. Studies on the lytic activity against *Phytophthora* cell wall of root protein extracts also corroborated a systemic effect of mycorrhizal symbiosis on tomato resistance to *Phytophthora*.

Key words: Arbuscular mycorrhizas, bioprotection, *Lycopersicon esculentum*, *Phytophthora*, systemic effect.

Introduction

Plants have developed a range of quite sophisticated defence mechanisms. They commonly react to pathogens with an integrated set of responses including reinforcement of cell walls by deposition of lignin-like polymers and structural proteins, formation of low-molecular-weight antimicrobial phytoalexins and accumulation of pathogenesis-related (PR) proteins with potential antimicrobial activity (Somssich and Hahlbrock, 1998). It is well known that these mechanisms can be activated by either non-pathogenic micro-organisms or environmental factors prior to disease development (Gianinazzi, 1984). Once activated, the natural resistance mechanisms of the plant maintain an enhanced defensive capacity for prolonged periods, and are effective against multiple pathogens. This state of enhanced defensive capacity developed by a plant when appropriately stimulated has been termed systemic acquired resistance (SAR) or induced systemic resistance (ISR) (van Loon *et al.*, 1998). The induction of resistance to diseases in plants by manipulation of the microbial populations naturally present in the plant environment is a promising research area. In fact, plant 'immunization' by micro-organisms can be a natural, safe, effective, persistent, and durable alternative to the use of pesticides in controlling plant diseases.

Beneficial micro-organisms that improve plant health through the enhancement of plant resistance/tolerance against biotic stresses include bacteria, such

³ Present address and to whom correspondence should be sent. Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843-2132, USA. Fax: +1 979 845 6483. E-mail: mpozo@ppserver.tamu.edu

as *Pseudomonas* spp. or *Bacillus* spp. and fungi such as *Trichoderma* sp., *Gliocladium* sp. or mycorrhizal fungi (van Driesche and Bellows, 1996; Azcón-Aguilar and Barea, 1996; Whipps, 1997; Ongena *et al.*, 1999). Arbuscular mycorrhizal (AM) fungi form symbiotic associations with the root systems of most agricultural, horticultural and hardwood crop species, thus, they are widespread potential biocontrol agents. Many authors have reported that the AM symbiosis can reduce root disease caused by several soil-borne pathogens including different *Phytophthora* species (Davis and Menge, 1980; Bartschi *et al.*, 1981; Mark and Cassels, 1996; Murphy *et al.*, 2000; Norman and Hooker, 2000). For example, colonization of tomato plants by *Glomus mosseae* has been demonstrated to reduce disease development in plants infected with *Phytophthora parasitica* (Cordier *et al.*, 1996, 1998; Pozo *et al.*, 1996, 1999; Trotta *et al.*, 1996; Vigo *et al.*, 2000). However, the mechanisms underlying this protective effect are still not well understood.

Efforts to develop an appropriate biotechnology to apply these and other beneficial organisms in agro-systems in order to optimize the effectiveness of biological control, depends on improving our knowledge of the mechanisms involved and their regulation (Azcón-Aguilar *et al.*, 2001).

Alterations in the isoenzymatic patterns and biochemical properties of some defence-related enzymes such as chitinases (Pozo *et al.*, 1996), chitosanases (Pozo *et al.*, 1998) and β -1,3-glucanases (Pozo *et al.*, 1999) have previously been shown during mycorrhizal colonization of tomato roots, with the induction of new isoforms. These hydrolytic enzymes are believed to have a role in defence against invading fungal pathogens because of their potential to hydrolyse fungal cell wall polysaccharides (Grenier and Asselin, 1990; Sela Buurlage *et al.*, 1993; Simmons, 1994). Thus, the induction of these activities in mycorrhizal symbiosis may be involved in the protector effect against fungal pathogens (Dumas-Gaudot *et al.*, 1996). In the present study a split-root experimental system was used to compare the ability of two different AM fungi (AMF) (*Glomus mosseae* and *Glomus intraradices*) to induce these enzymes either locally or systemically, as well as to induce resistance to *Phytophthora parasitica* in tomato roots. Additionally, the isoenzymatic pattern of superoxide dismutases, enzymes known to be involved in signalling and defence processes (Bowler *et al.*, 1992) has been investigated. Finally, the lytic activity of root protein extracts for the various plant treatments against *Phytophthora* cell wall has been analysed.

Materials and methods

Plant and fungal material

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Earlymech) were surface-sterilized with a commercial bleach solution

(10%, v/v), and sown in wet autoclaved vermiculite. Plantlets were transplanted when the first true leaf was expanded.

Two cylindrical plastic containers (250 ml) were taped together, side by side, forming the experimental unit. A nick in the upper part between both compartments allowed the root system to be split in two halves. Both compartments contained a sterile mixture of quartz sand and soil (9:1, v/v) and an additional quartz sand layer was added at the top of both compartments.

Isolates from two species of AM fungi were used as inocula: *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe (BEG 12) and *Glomus intraradices* Smith and Schenck (BEG 72). Mycorrhizal inoculation was carried out in one of the compartments by adding 10% of the final volume of a sand/soil-based inoculum enriched in fungal propagules and containing chopped *Allium porrum* L. roots colonized by the AM fungus. 'Nm' (non-mycorrhizal) was designated for each part of the root system of control plants (plants non-inoculated with AMF in any of the root compartments). '-Gm' was designated for the non-mycorrhizal half of the root system of plants inoculated with *G. mosseae*. The other half, which was mycorrhizal, was designated '+Gm'. Similarly, the non-mycorrhizal and mycorrhizal halves of plants inoculated with *G. intraradices* were designated '-Gi' and '+Gi', respectively (Fig. 1).

At potting, all plants received an aliquot of a filtrate (<20 μ m) of both AM inocula in order to provide the microbial populations accompanying the mycorrhizal inocula but free from AM propagules.

Development of mycorrhizal colonization was periodically assessed as indicated below. Absence of mycorrhizal colonization was confirmed in control plants (Nm) as well as in the non-inoculated compartments (-Gm, -Gi) of mycorrhizal plants. When the mycorrhizal colonization level was higher than 40% (usually 4 weeks after potting) in the AM inoculated half of the root system (+Gm, +Gi), the root pathogen *Phytophthora parasitica* var. *nicotianae* was inoculated in one of the compartments of a set of plants (Fig. 1).

Phytophthora parasitica var. *nicotianae* (isolate 201, kindly provided by P Bonnet, INRA, Antibes, France) was grown on a malt-agar medium at 25 °C in darkness for 3 weeks, and the inoculum was prepared by washing the growing mycelia with sterile water (16 ml plate⁻¹). The suspension obtained was used to inoculate the appropriate tomato plants by injecting 8 ml per plant close to the root system. Control plants were similarly supplied with 8 ml of sterile water.

The root halves growing in the compartment where the pathogen was inoculated were noted as '+Pp', and their corresponding counterpart halves non-inoculated with *Phytophthora* were noted as '-Pp'.

The experimental treatments are shown in Fig. 1.

Growth conditions and plant harvest

Tomato plants were grown in a controlled environment room (25/18 °C day/night temperature, 60% relative humidity, 16 h photoperiod with a photosynthetic photon flux of 400 μ mol photons m⁻² s⁻¹). They were watered three times per week with Long Ashton nutrient solution at one-quarter phosphorus strength. Plants were harvested 2 weeks after inoculation with the pathogen, except for a set of plants that remained growing for four additional weeks (10 weeks of growth) to study the time-related changes in chitinase activities as described earlier (Pozo *et al.*, 1998). Plants were then carefully washed in running tap water, rinsed in deionized water and weighed. The root halves inoculated with *Phytophthora* were evaluated for necrotic lesions by applying an arbitrary visual assessment scale from

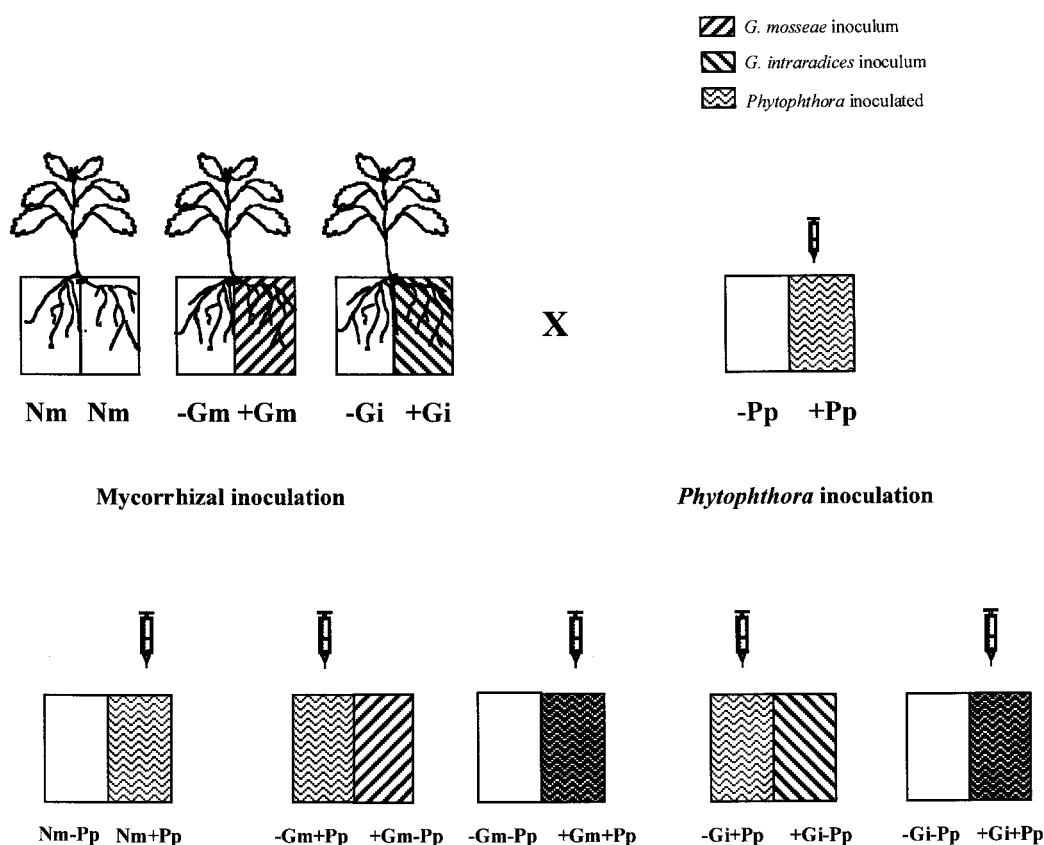


Fig. 1. Schematic model of the compartmental system and fungal inoculation treatments. Nm, non-mycorrhizal; -Gm/+Gm, non-mycorrhizal/mycorrhizal with *G. mosseae*; -Gi/+Gi, non-mycorrhizal/mycorrhizal with *G. intraradices*; -Pp/+Pp, non-inoculated/inoculated with *P. parasitica* (see Materials and methods).

0 (no symptom) to 5 (fully necrotic root). Quantification of *P. parasitica* in tomato root extracts was carried out by using a commercial ELISA kit (Agriscreeen, Adgen Diagnostic Systems, Auchincruive, Ayr, Scotland, UK) following the supplier's instructions. Root systems were immediately frozen in liquid nitrogen and stored at -80°C until protein extraction. A representative sample of each root system was kept for the determination of mycorrhizal colonization by clearing and staining the roots using trypan blue (Phillips and Hayman, 1970). Mycorrhizal colonization, determined by using the gridline intersection method (Giovannetti and Mosse, 1980), was expressed as the percentage of root length colonized by the AM fungi.

Protein extraction and quantification

Frozen roots were ground at 4°C in an ice-chilled mortar with liquid nitrogen and the resulting powder was suspended in 100 mM MacIlvaine (citric acid/ Na_2HPO_4) extracting buffer, pH 6.8 (1:1, w/v). Crude homogenates were centrifuged at 15000 g for 30 min at 4°C and the supernatant fractions were kept frozen at -20°C . Protein content was determined using BSA as a standard (Bradford, 1976).

Electrophoresis and enzymatic assays

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 previously

(Reisfeld *et al.*, 1962). All chemicals for electrophoresis were from Bio-Rad (Prat de Llobregat, Barcelona, Spain). Other compounds were from Sigma Chemical Co. (Alcobendas, Madrid, Spain). In all cases, after staining, gels were photographed (Polaroid film No. 665) and scanned (HP ScanJet 3c).

Detection of β -1,3-glucanase activity after PAGE: A soluble fraction of purified β -glucans from *Saccharomyces cerevisiae* was used as substrate for β -1,3-glucanase activity. Electrophoresis, transfer of proteins, incubations, and staining of the gels were performed as previously described (Pozo *et al.*, 1999).

Detection of chitinase and chitosanase activity after PAGE: Glycol chitin, glycol chitosan or a mix of both were embedded in the gels at 0.01% (w/v) and used as substrate for chitinase, chitosanase or bifunctional chitinase-chitosanase activities, respectively, as described previously (Pozo *et al.*, 1998).

Detection of superoxide dismutase (SOD) after PAGE: SOD isoforms were detected directly on the gel after electrophoresis by the method described by Beauchamp and Fridovich, based on the inhibition of the nitro-blue tetrazolium (NBT) reduction by superoxide radicals generated photochemically (Beauchamp and Fridovich, 1971). Bands appear colourless against the blue background corresponding to the reduced NBT. Gels were incubated as described earlier (Palma *et al.*, 1993). Characterization of the different isoforms was carried out using the same method but with a previous incubation of

the gel (30 min, 25 °C) in potassium phosphate buffer (50 mM, pH 7.8, containing 2 mM KCN or H₂O₂ as inhibitors (Palma *et al.*, 1993).

Detection of lytic activity against *Phytophthora* cell wall: *Phytophthora* mycelium was collected from malt-agar plates. Several washes were performed to eliminate the remaining media. The mycelium was ground in an ice-chilled mortar with liquid nitrogen and the resulting powder suspended in MacIlvaine extracting buffer. Crude homogenates were centrifuged at 15 000 g for 30 min at 4 °C as previously described for roots. The pellet was resuspended in buffer, sonicated for 5 min to break the remaining hyphae and cell walls and then centrifuged. Resuspension of the pellet, sonication and centrifugation was repeated twice. The final pellet, consisting of a crude cell wall fraction, was mixed by using a homogenizer with the gel buffer (Tris-HCl, pH 8.8) before being directly incorporated into the PAGE gel and used as substrate. After electrophoresis, the gel was washed for 20 min in sodium acetate 50 mM pH 5.0 at 37 °C, and then incubated for 24 h at 37 °C in a new bath of the same buffer. For detecting possible basic activities of root extracts able to break down crude extracts of *Phytophthora* cell walls, proteins separated by the Reisfeld electrophoresis method were transferred by blotting to an overlay gel containing the pathogen cell wall extracts. Lysis zones appeared as clear bands against the translucent cell wall-containing matrix when the gels were visualized with indirect light. Bands of lysis were photographed against a black background provided by a cloth, as described previously (Grenier and Asselin, 1990).

Statistical analysis

Three independent experiments were carried out, each with nine replicate plants per treatment. Data were subjected to ANOVA, followed by Fisher's Protected Least Significant Difference test when appropriate. Since trends were similar in the different experiments, results from only one of them are reported here. All electrophoreses were repeated at least three times, and

results on alterations in isoenzyme activities were confirmed in the root extracts from the different experiments.

Results

Plant growth and fungal colonization

The split root compartmental system allowed the colonized (+Gm, +Gi) and non-colonized (–Gm, –Gi) parts of the root system of mycorrhizal plants to be considered independently, and to be compared with the roots of non-mycorrhizal plants (Nm). Growth of the tomato plants, mycorrhizal colonization and quantification of *Phytophthora* inside the root as well as the level of disease symptoms are shown in Table 1.

The mycorrhizal colonization level was 50% on average for roots inoculated with *G. mosseae* and about 56% for those inoculated with *G. intraradices*. In all cases the absence of colonization of the non-AM inoculated part of the root system of these plants was confirmed. AM colonization levels were not significantly affected by the inoculation with the pathogen.

No significant differences were observed in the fresh weight of plants inoculated with *G. mosseae*. However, colonization by *G. intraradices* resulted in a growth depression, mainly at the shoot level.

Non-mycorrhizal plants and, to a lesser extent, *G. intraradices*-colonized plants, were considerably affected by inoculation with *P. parasitica*. Losses in the weight of the root half inoculated with the pathogen were 46% for non-mycorrhizal and 40% or 35% in *G. intraradices*-inoculated plants, depending on whether the pathogen was inoculated in the mycorrhizal root half

Table 1. Weights of tomato plants as affected by the inoculation with AM fungi and/or *Phytophthora parasitica* in the same or different halves of the root system when grown in a root compartmental system

Mycorrhizal colonization, levels of the pathogen inside the roots and an estimation of the disease symptoms are presented.

Root system		Fresh weight (g) ^a			M (%) ^{ac}	Disease index ^{ad}	Phyt ^e	
Half 1	Half 2	Shoot	Root system ^b		Total biomass			
			Half 1	Half 2				
Nm	Nm	11.35 ab	1.81 abc	1.84 abc	14.99 a	0 b	0 c	–
–Gm	+Gm	11.03 abc	1.68 bcde	1.71 bcd	14.42 ab	50 a	0 c	–
–Gi	+Gi	10.04 cd	1.63 bcde	1.88 ab	13.55 bc	56 a	0 c	–
Nm–Pp	Nm+Pp	10.59 bcd	1.84 abc	0.99 f	13.42 bc	0 b	3.5 a	1.98
–Gm–Pp	+Gm+Pp	11.73 a	2.00 a	1.46 bcdef	15.19 a	47 a	1.8 b	0.97
+Gm–Pp	–Gm+Pp	11.30 ab	2.32 a	1.73 bcd	15.35 a	49 a	2.0 b	1.07
–Gi–Pp	+Gi+Pp	10.07 cd	1.57 bcde	1.13 ef	12.77 c	53 a	2.8 ab	1.72
+Gi–Pp	–Gi+Pp	9.63 d	1.98 ab	1.23 def	12.84 c	57 a	3.0 ab	1.56

^aData in the same column not sharing a letter in common differ significantly at $P \leq 0.05$.

^bData corresponding to fresh weight of root halves 1 and 2 were analysed together providing comparisons among the treatments for both root halves.

^cPercentage of root length colonized by the mycorrhizal fungus.

^dVisual estimation of the disease symptoms from 0 (no symptom) to 5 (fully necrotic root).

^eQuantification by ELISA of *Phytophthora* levels inside the roots, expressed in absorbance units.

(+Gi) or in the other half (−Gi). However, in *G. mosseae*-colonized plants there was no significant reduction. In each of the three experiments, *Phytophthora* infection consistently, although not significantly, decreased shoot growth of non-mycorrhizal plants and of *G. intraradices*-colonized plants when the pathogen was inoculated in the non-mycorrhizal half of the root system. However, growth of *G. mosseae* mycorrhizal plants was not reduced by inoculation with *Phytophthora*. Thus, *G. mosseae* exerted a protective effect against *P. parasitica*. It is remarkable that plants colonized by both *G. mosseae* and *P. parasitica* (whether inoculated in combination or separately) produced larger roots in the half not infected by the pathogen (+Gm−Pp, −Gm−Pp) than plants colonized by *G. mosseae* alone. *G. intraradices* did not confer any protection in terms of plant growth.

A visual estimation of disease symptoms in the root system indicated a maximum value of 3.5 in non-mycorrhizal plants (Nm+Pp). The minimum rates, 1.8 and 2.0, were found in plants colonized by *G. mosseae* when the pathogen was inoculated in the mycorrhizal or in the non-mycorrhizal half of the root system, respectively. It can be concluded that colonization by *G. mosseae* confers a significant reduction in disease development, regardless of whether *Phytophthora* were inoculated in the mycorrhizal or in the non-mycorrhizal part of the root system. Plants colonized by *G. intraradices* (−Gi+Pp, +Gi+Pp) showed an intermediate level of disease, with no significant differences between non-mycorrhizal (Nm+Pp) or *G. mosseae*-mycorrhizal plants, as shown in Table 1.

The levels of pathogen inside the roots, as estimated by the ELISA test, correlated well with the visual estimation of root symptoms. Maximum levels were detected in non-mycorrhizal plants, followed by those in *G. intraradices*-colonized plants and the minimum level of the pathogen was detected in plants colonized by *G. mosseae* (+Gm+Pp). The reduction in both disease and *Phytophthora* level in plants with half of their root system colonized by *G. mosseae* is remarkable since it occurs even when both fungi were inoculated in different compartments, and consequently, did not share the same roots. However, the degree of protection was higher when *G. mosseae* and *P. parasitica* shared the same root half, as deduced from the consistent, although small, differences found in the three experiments concerning shoot growth, disease index and the amount of pathogen inside the root.

Isoenzyme analysis

Protein content of the root extracts is shown in Table 2. The general tendency is that protein content in non-mycorrhizal roots of mycorrhizal plants (−Gm or −Gi) was similar to that of non-mycorrhizal plants (Nm), while it was higher in mycorrhizal roots (+Gm, +Gi).

Inoculation with *Phytophthora* (+Pp) did not appear to alter root protein content. Protein content in extracts from mycorrhizal root halves was not higher when the other half of the root system was infected by *Phytophthora*.

Chitinase/chitosanase activity: Figure 2 shows lytic activities detected in gels containing both glycolchitin and glycolchitosan as substrate, after calcofluor (panel A) or Coomassie blue (panel B) staining. Calcofluor staining allows detection of chitinases and chitosanases,

Table 2. Protein content in root extracts as affected by fungal treatments

Treatment ^a		Root proteins (mg g ⁻¹ fresh weight) ^b	
Half 1	Half 2	Half 1	Half 2
Nm	Nm	0.74 abc	0.75 abc
−Gm	+Gm	0.76 abc	1.12 d
−Gi	+Gi	0.80 bc	1.11 d
Nm−Pp	Nm+Pp	0.58 a	0.70 abc
−Gm−Pp	+Gm+Pp	0.81 bc	1.10 d
+Gm−Pp	−Gm+Pp	0.74 abc	0.69 ab
−Gi−Pp	+Gi+Pp	0.66 ab	1.14 d
+Gi−Pp	−Gi+Pp	0.87 c	0.80 bc

^aSee Fig. 1 for treatment nomenclature.

^bAll data were analysed together providing comparisons among the treatments for both root halves. Those data not sharing a letter in common differ significantly at $P < 0.05$.

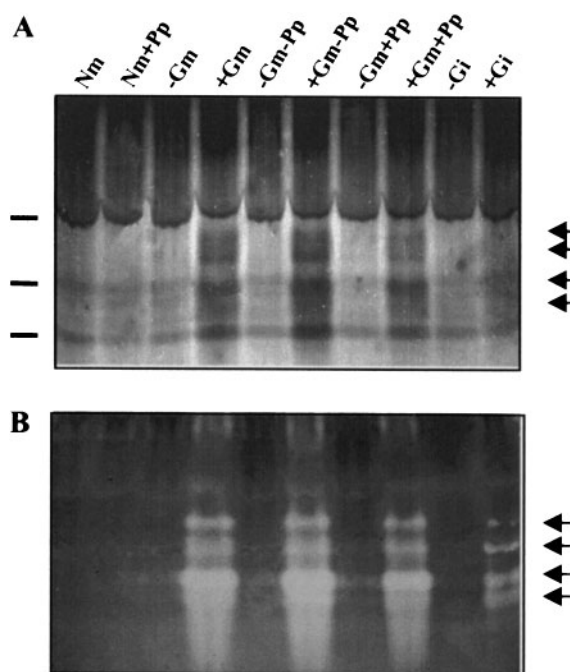


Fig. 2. Lytic activities detected in gels containing glycolchitin and glycolchitosan as substrate. (A) Gel stained with Calcofluor to detect both chitinase and chitosanase activities. (B) Gel stained with Coomassie blue to detect chitosanase activity only. See Fig. 1 for abbreviations.

and Coomassie blue only chitinase activities. The artificial nature of the experimental model did not affect significantly the chitinase or chitosanase activities since the isoenzyme pattern of non-mycorrhizal plants (Nm) is equivalent to that found for tomato plants in non-compartmented systems (Pozo *et al.*, 1998). This consists of three main constitutive chitinase isoforms (Fig. 2, bars) and no chitosanase activity. No differences were observed between non-mycorrhizal parts of the root system of AM inoculated plants (-Gm, -Gm-Pp, -Gm+Pp, -Gi) and control non-mycorrhizal plants (Nm). However, in mycorrhizal roots (+Gm, +Gi) with or without the pathogen, there was induction of new acidic chitinase and chitosanase isoforms, as well as better renaturation ability of chitinases in mycorrhizal halves (data not shown). The presence of bifunctional chitinase/chitosanase enzymes (Fig. 2, arrows) were only observed in the mycorrhizal part of the root system, showing the localized character of the induction.

Quantitative differences were observed in chitosanase activities of low relative mobility (RM) appearing in the upper part of the gel in 10-week-old plants (Fig. 3). The activity indicated by a black arrow was detected in all extracts. The activity of this isoform was higher in *Phytophthora*-infected roots (+Pp), except when the plants were colonized by *G. mosseae*. The presence of the pathogen induced the formation of at least another isoform (Fig. 3, white arrow). This isoform was more pronounced in non-mycorrhizal plants and in non-mycorrhizal roots of *G. intraradices*-colonized plants, while it was not detected in the +Gm+Pp half of the root system. *G. intraradices*-colonized roots showed an additional isoform (pointed line). At this stage of plant development isoforms with higher RM, typical of well-established symbiosis (Pozo *et al.*, 1998) were only detected in *G. mosseae*-colonized roots (Fig. 3, arrowheads).

β-1,3-glucanase activity: Analysis of β-1,3-glucanase isoenzymatic patterns provided evidence for the presence of two constitutive isoforms in non-mycorrhizal plants

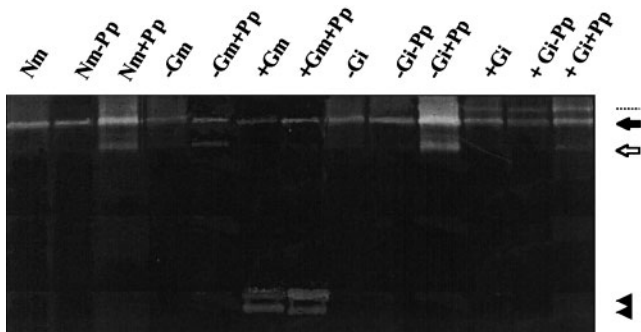


Fig. 3. Acidic chitosanase activities after separation of root proteins from 10-week-old plants. See Fig. 1 for abbreviations.

(Nm). A similar pattern was obtained for non-colonized roots of mycorrhizal plants (-Gm, -Gi) (data not shown). The new acidic isoforms described in tomato roots colonized by *G. mosseae* (Pozo *et al.*, 1999) were detected only in roots colonized by this fungus (+Gm, +Gm-Pp, +Gm+Pp), showing a localized induction (data not shown). *Phytophthora*-infected roots showed a general increase in the glucanase activities together with a weak band corresponding to an isoform with higher RM. Thus, induction of acidic glucanases by *Phytophthora* infection is also localized and not systemic.

One constitutive basic glucanase isoform was found in roots corresponding to all treatments. No changes were detected among non-colonized roots of mycorrhizal (-Gm, -Gi) and non-mycorrhizal plants (Nm). However, two additional isoforms were found in roots colonized by *G. mosseae* and infected with the pathogen (+Gm+Pp). These isoforms were not detected when both fungi were inoculated in the same plant, but in different halves of the root system (-Gm+Pp, +Gm-Pp).

Superoxide dismutase activity: Electrophoretic analysis of SOD isoforms showed a main constitutive isoform and a weaker one of higher RM in root extracts of all plants regardless of the treatment (Fig. 4, bars). A new isoform with low RM was detected in extracts of roots colonized by *G. mosseae* and *G. intraradices* (Fig. 4, arrow). No induction of new isoforms was observed after inoculation with *Phytophthora*.

Treatments with hydrogen peroxide or potassium cyanide, inhibitors of specific types of SOD isoforms, allowed the characterization of the detected isoforms. The constitutive isoforms were inhibited by both treatments, indicating that they are Cu-Zn SOD isoforms, while the mycorrhiza-induced one was not inhibited by any of the treatments, suggesting that it is a Mn-SOD

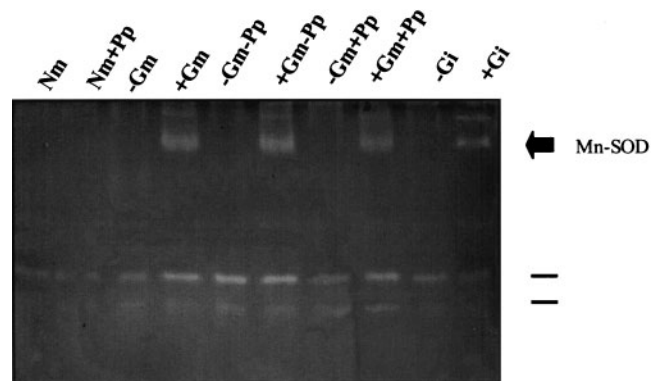


Fig. 4. Superoxide dismutase activities after PAGE in root protein extracts of the different treatments. See Fig. 1 for abbreviations. Constitutive isoforms are marked with bars. The mycorrhiza induced isoform, further characterized as a Mn-SOD, is marked with an arrow.

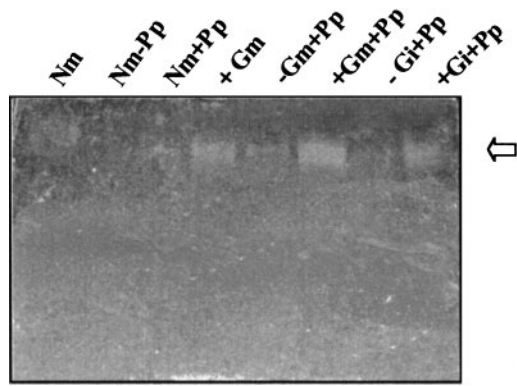


Fig. 5. Lytic activity of different root protein extracts against *Phytophthora* cell walls. See Fig. 1 for abbreviations.

isoform, according to the method described previously (Palma *et al.*, 1993).

Antifungal activity of the root protein extracts

In order to determine the lytic activity of the different root protein extracts against *Phytophthora* cell walls, an homogenate of *Phytophthora* mycelium cell wall was embedded uniformly in the gel matrix to serve as a potential substrate. Lysis was observed by the appearance of translucent haloes through the opaque suspension (Fig. 5, arrow). No lytic activity was detected in root extracts from non-mycorrhizal plants not inoculated with the pathogen (Nm), nor in root extracts corresponding to the non-infected half of the root system of a *Phytophthora* inoculated plant (Nm-Pp). A very faint signal was observed in roots infected by the pathogen (Nm+Pp). However, a clear signal of lytic activity was detected in extracts of mycorrhizal roots (+Gm, +Gm+Pp, +Gi+Pp). The highest intensity was produced in roots colonized by *G. mosseae* and infected by the pathogen (+Gm+Pp). When the pathogen was inoculated in the compartment with non-mycorrhizal roots of a mycorrhizal plant (-Gm+Pp, -Gi+Pp), the signal corresponding to lytic activity was higher than in the pathogen-inoculated non-mycorrhizal roots (Nm+Pp), although it was weaker than in mycorrhizal roots (+Gm, +Gm+Pp, +Gi+Pp). Whatever the plant treatment, no lysis band was detected when the root extracts were separated with the Reisfeld electrophoresis method.

Discussion

Tomato plants responded differently to inoculation with *G. mosseae* or *G. intraradices*. In the absence of *Phytophthora*, colonization by *G. mosseae* did not significantly affect plant development compared to non-mycorrhizal controls. However, tomato development was inhibited by inoculation with *G. intraradices*. The root

half colonized by *G. intraradices* was similar to the root halves in the controls. In spite of that, the non-mycorrhizal half of its root system, and the shoots in particular, showed a lower fresh weight. In general, the plant growth response to AMF colonization depends on the balance between a depressor effect due to the fungal requirements, mainly carbon for the production and maintenance of the fungal biomass (symbiosis cost), and the benefits of the interaction concerning a better nutritional status of the plant and other secondary effects (Buwalda and Goh, 1982; Graham, 2000). In previous studies using compartmental models, an increase in carbon translocation to the mycorrhizal part of the root system has been demonstrated (Koch and Johnson, 1984; Wang *et al.*, 1989). When symbiosis cost exceeds its benefits, the plant-AMF relationship can go from mutualism to parasitism (Smith and Smith, 1996; Johnson *et al.*, 1997). In fact, some studies have shown no growth stimulation, and even depression, during plant interactions with *G. intraradices* (Marschner and Crowley, 1996). This suggests that the fungus can act as an important carbon drain, as it is quite aggressive in its colonization ability and requires a high amount of plant photosynthates for the large number of vesicles and intraradical spores it produces (Peng *et al.*, 1993).

In plants inoculated with *Phytophthora*, no significant effect on disease development were found in *G. intraradices*-colonized plants, irrespective of whether the pathogen was inoculated in the mycorrhizal or non-mycorrhizal part of the root system. Nevertheless, disease development in plants colonized by *G. mosseae* was significantly lower. The levels of *Phytophthora* inside the roots and the disease symptoms were consistently lower when the pathogen was inoculated in the root half colonized by the AM fungus. However, damage was also significantly reduced when *Phytophthora* was inoculated in the non-colonized part of a *G. mosseae*-mycorrhizal plant. Thus, the bioprotection exerted by *G. mosseae* appears to be the result of a combination of local and systemic mechanisms. The same conclusion was reached by immunocytochemical studies (Cordier *et al.*, 1998). These studies showed that arbuscule-containing cortical cells of *G. mosseae*-mycorrhizal plants were immune to the pathogen and exhibited a localized resistance with the formation of cell wall appositions reinforced by callose adjacent to the intercellular hyphae. The systemically induced resistance in non-mycorrhizal root parts was characterized by elicitation of host wall thickenings containing non-esterified pectins and PR-1a proteins in reaction to the intercellular hyphae of the pathogen, and by formation of callose-rich encasement material around *P. parasitica* hyphae that were penetrating root cells.

A compensation mechanism could also be occurring, since plants colonized by both *G. mosseae* and

P. parasitica (whether inoculated in combination or separately) showed larger roots in the half not infected by the pathogen (+Gm–Pp, –Gm–Pp) than plants colonized by *G. mosseae* alone (+Gm). Consequently, plants colonized by *G. mosseae* could respond to attack by *P. parasitica* by producing larger roots in the parts of the root system that were not infected by the pathogen. These roots would then help sustain growth by absorbing nutrients that the damaged roots could not.

Biochemical analysis of root protein extracts was performed in order to determine local or systemic changes in some defence-related enzymes. The artificiality of the experimental model did not affect significantly the activity of hydrolytic enzymes since the isoenzyme pattern of non-mycorrhizal plants (Nm) was equivalent to that found for tomato plants in non-compartmented systems (Pozo *et al.*, 1996, 1998, 1999). The electrophoretic analysis showed that the previously described mycorrhiza-related new isoforms of chitinase, chitosanase and β -1,3-glucanase were induced locally as they were detected only in the AM colonized part of the root system. It was also shown that the better renaturation ability of chitinase activities (Pozo *et al.*, 1996) and the bifunctional chitinase-chitosanase capability of certain isoforms observed in mycorrhizal root extracts from non-split systems (Pozo *et al.*, 1998) occurs only in the mycorrhizal part of the root system. Thus, these properties are only locally affected.

Beside these localized responses, some effects in non-mycorrhizal root parts of AM plants were observed, pointing to certain systemic effects. This is supported by results on lytic activity against *Phytophthora* cell wall. A stronger lysis signal was found in roots colonized by *G. mosseae* and then infected with the pathogen (+Gm+Pp), although it was also detected in *G. mosseae* colonized roots in plants not inoculated with the pathogen (+Gm). It is remarkable that lytic activity against *Phytophthora* cell walls was found in extracts of non-colonized roots of *G. mosseae*-mycorrhizal plants when infected by the pathogen (–Gm+Pp). This activity was barely found in non-mycorrhizal plants inoculated with the pathogen (Nm+Pp). The lytic activity was also observed in pathogen-infected mycorrhizal and non-mycorrhizal roots of plants colonized by *G. intraradices* (+Gi+Pp, –Gi+Pp), although the lysis signal was higher when both fungi shared the same root half.

Lambais and Mehdy have already provided evidence for localized and systemic effects of mycorrhizal colonization on the expression of defence enzymes (Lambais and Mehdy, 1998). These authors described chitinase and β -1,3-glucanase coding mRNA accumulation in arbuscule-containing and adjacent cells, and repression of β -1,3-glucanase messenger accumulation some millimetres distant from the AMF colonized zone. These differences

in local and systemic patterns of gene expression for defence-related enzymes points to the possibility of multiple signalling pathways (Lambais and Mehdy, 1995; Lambais, 2000). Shaul *et al.* also suggest the existence of systemic regulatory processes that, initiated in the mycorrhizal roots, modify disease-symptom development and gene expression in their leaves (Shaul *et al.*, 1999). Plant hormones such as auxins, cytokinins and abscisic acid, known to act as long-distance signals, have altered levels in mycorrhizal plants (Allen *et al.*, 1980, 1982; Danneberg *et al.*, 1992; Dugassa *et al.*, 1996; Hirsch *et al.*, 1997). It is already known that changes in the hormonal balance of plants can modulate the expression of defence-related genes (Petruzelli *et al.*, 1999).

The experimental evidence presented in this study suggest that AM fungi are able to induce systemic protection against root pathogens. These results and those described in previous reports of plant protection by arbuscular mycorrhiza were then examined according to the criteria defined by van Loon *et al.* for verification of ISR (van Loon *et al.*, 1998). The overall conclusion based on this analysis is the existence of arbuscular mycorrhiza-mediated ISR.

By contrast to SAR, rhizobacteria-mediated ISR has been shown to be independent of salicylic acid accumulation and not to induce activation of PR genes (Hoffland *et al.*, 1996; Pieterse *et al.*, 1996; van Wees *et al.*, 1999). However, certain metabolic changes (increase in peroxidase activity, phytoalexin accumulation, PR accumulation, etc.) have been related to ISR but none of them were consistently associated to the induced resistance status in the different biological systems assayed. In spite of that, structural modifications (cell wall reinforcements, phenolic compound accumulation, and papilla formation) have been described extensively in ISR expressing plants. Parallel aspects to these described for rhizobacteria-mediated ISR have been found for the mycorrhiza-induced defence response in plants. It is known that salicylic acid accumulation during AM colonization is weak and transient, occurring only in early stages of the symbiosis establishment (Blilou *et al.*, 1999). Thus, it is unlikely to be involved in the observed systemic resistance. On the other hand, the main changes in the isoenzyme patterns of defence-related enzymes shown in the present study occur locally. Moreover, the structural modifications described at the cytological level using the split root system (Cordier *et al.*, 1998) are similar to those described in plants colonized by ISR-inducing rhizobacteria.

In view of the discussion above, it is considered that colonization by the AM fungus *Glomus mosseae* of tomato roots leads to ISR, similar to that widely described in rhizobacteria-induced plants, that is effective against *Phytophthora parasitica*. Activation of ISR in tomato has been demonstrated previously by inoculation

with certain rhizobacteria strains: *Pseudomonas fluorescens* WCS417, *Pseudomonas fluorescens* 89B-27 and *Serratia marcescens* 90-166 (Raupach *et al.*, 1996; Duijff *et al.*, 1997). It will be useful to compare both states of induced resistance in order to clarify common and different features. Finally, studies on ISR by co-inoculation with AM fungi and beneficial rhizobacteria and the possible pathogen spectrum affected is a promising research area, with important consequences for the rational exploitation of biological resources in order to achieve more sustainable plant production systems.

Acknowledgements

This work was sponsored by the projects AIR CT 94-0809 and TMR FMRX-CT96-0039 of the EU. MJ Pozo was supported by a post-doctoral fellowship from the Spanish Council of Scientific Research (CSIC). We thank Mrs Custodia Cano for excellent technical support and Dr Charles M Kenerley for critical reading of the manuscript.

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