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How to cite

CORDIER, Christelle, GIANINAZZI, Silvio, GIANINAZZI-PEARSON, Vivienne. Colonisation patterns of root tissues by *Phytophthora nicotianae* var. *parasitica* related to reduced disease in mycorrhizal tomato. In: Plant and soil, 1996, vol. 185, n° 2, p. 223–232. doi: 10.1007/BF02257527

This publication URL: https://archive-ouverte.unige.ch/unige:172670
Publication DOI: 10.1007/BF02257527

Plant and Soil **185**: 223–232, 1996.

Colonisation patterns of root tissues by *Phytophthora nicotianae* var. *parasitica* related to reduced disease in mycorrhizal tomato

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Received 17 January 1996. Accepted in revised form 20 March 1996

Key words: biocontrol, *G. mosseae*, immunocytochemistry, *P. nicotianae* var. *parasitica*, tomato

Abstract

Tomato plants pre-colonised by the arbuscular mycorrhizal fungus *Glomus mosseae* showed decreased root damage by the pathogen *Phytophthora nicotianae* var. *parasitica*. In analyses of the cellular bases of their bioprotective effect, a prerequisite for cytological investigations of tissue interactions between *G. mosseae* and *P. nicotianae* v. *parasitica* was to discriminate between the hyphae of the two fungi within root tissues. We report the use of antibodies as useful tools, in the absence of an appropriate stain for distinguishing hyphae of *P. nicotianae* v. *parasitica* from those of *G. mosseae* inside roots, and present observations on the colonisation patterns by the pathogenic fungus alone or during interactions in mycorrhizal roots. Infection intensity of the pathogen, estimated using an immunoenzyme labelling technique on whole root fragments, was lower in mycorrhizal roots. Immunogold labelling of *P. nicotianae* v. *parasitica* on cross-sections of infected tomato roots showed that inter or intracellular hyphae developed mainly in the cortex, and their presence induced necrosis of host cells, the wall and contents of which showed a strong autofluorescence in reaction to the pathogen. In dual fungal infections of tomato root systems, hyphae of the symbiont and the pathogen were in most cases in different root regions, but they could also be observed in the same root tissues. The number of *P. nicotianae* v. *parasitica* hyphae growing in the root cortex was greatly reduced in mycorrhizal root systems, and in mycorrhizal tissues infected by the pathogen, arbuscule-containing cells surrounded by intercellular *P. nicotianae* v. *parasitica* hyphae did not necrose and only a weak autofluorescence was associated with the host cells. Results are discussed in relation to possible processes involved in the phenomenon of bioprotection in arbuscular mycorrhizal plants.

Introduction

There exist many reports about the potential of arbuscular mycorrhizal fungi as biological control agents against soil-borne root diseases (Dehne, 1982; Linderman, 1994; Liu, 1995; Schenck and Kellam, 1978), and in particular against species of *Fusarium* (Caron et al., 1986a; Hwang et al., 1992; Jalali and Jalali, 1991) or *Phytophthora* (Guillemin et al., 1994). In hypotheses that have been proposed to explain such bioprotection (Caron, 1989; Hooker et al., 1994), most mechanisms involve the accumulation of plant-derived compounds in response to the mycorrhizal infection like arginine (Baltruschat and Schönbeck, 1975), phenolics (Dehne et al., 1978) and isoflavonoids (Morandi et al., 1984). Another explanation proposed for the protective effect of arbuscular mycorrhiza is their role in improving the mineral nutrition of host plants, and more precisely phosphorus uptake (Davis and Menge, 1980; Graham and Menge, 1982). However, there have been other reports that such improved P nutrition is associated with an increase in disease severity (Davis et al., 1979) or has no effect on bioprotection (Caron et al., 1986b). In fact, virtually nothing is known about the cellular, molecular or physiological basis of the mechanisms of root pathogen control in mycorrhizal plants. Recent ultrastructural and immunocytochemical studies suggest that enhanced protection of mycorrhizal Ri T-DNA- transformed carrot roots against infection by *Fusarium oxysporum* f. sp. *chysanthemi* involves the accumulation of electron dense deposits, containing

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phenolics and hydrolytic enzymes, and which restrict pathogen progression (Benhamou et al., 1994).

The aim of our research is to characterise tissular and cellular root reactions induced against *P. nicotianae* var. *parasitica* by the presence of an arbuscular mycorrhizal fungus in roots of a variety of tomato (*Lycopersicon esculentum* cv. Earlymech) which is susceptible to this pathogen, in order to determine the contribution of modifications to biocontrol. In the present paper, we describe the effect of *Glomus mosseae* within tomato roots on the colonisation pattern of the pathogenic fungus, and compare some plant reactions to these two fungi. For this, an immunological approach similar to that previously described for arbuscular mycorrhizal fungi (Cordier et al., 1995) was developed to distinguish between the hyphae of each fungus outside and inside roots.

Materials and methods

Plant inoculation and growth conditions

Tomato cv. Earlymech seeds were surface-sterilised, pregerminated for a week in vermiculite under light and treated as follows: 1) control plants were transplanted into 400 g disinfected (γ-irradiated) clay loam soil (pH 8, 26 mg kg^{-1} Olsen P); 2) plants were inoculated with *G. mosseae* (BEG 12) using leek root inoculum placed in the planting hole at the time of transplanting into the disinfected soil; and 3) 3 week-old plants from 1) and 2) were inoculated with *Phytophthora nicotianae* var. *parasitica* by watering with a zoospore suspension.

The isolate of *P. nicotianae* var. *parasitica* (isolate 201, subsequently referred to as *P. nicotianae* v. *parasitica*) was kindly provided by P Bonnet (INRA, Antibes, F.). Zoospore production was induced by growing on sterile V8 medium, pH 7.0 (300 g L^{-1} V8 frozen Findus 'minestrone', 2 g L^{-1} $CaCO_3$, 16 g L^{-1} Bacto Agar) for 7 days in the dark at 24 °C. Pieces of mycelium were cut out and put into Petri dishes containing 10 mL of sterile soil suspension, pH 6.5 (200 g clay loam soil per litre H_2O). Zoospores were obtained 1 week later by chilling the dishes for 1 hour at 4 °C with shaking. The culture was then incubated for 30 minutes at 24 °C, zoospores collected, their concentration estimated and 20000 used to inoculate each plant.

Plants from the different treatments were grown in a constant environment (18/23 °C, 16 h photoperiod, 300 μmol photons m^{-2} s^{-1}, 60% relative humidity) and harvested at 4, 5, 7 and 9 weeks after planting (equivalent to 1, 2, 4 and 6 weeks after *P. nicotianae* v. *parasitica* inoculation). Fresh shoot and root mass was recorded, and mycorrhizal colonisation and pathogen infection estimated.

Estimation of mycorrhizal colonisation and pathogen infection

Arbuscular mycorrhizal roots were observed after KOH digestion (10%) at 90 °C during 45 min and trypan blue staining (Phillips and Hayman, 1970). Colonisation was determined microscopically by the procedure described by Trouvelot et al. (1986) and evaluated as the percentage of root cortex with fungal structures (M%). The spread of *P. nicotianae* v. *parasitica* was estimated as the percentage of root length with necrotic zones in tomato plants harvested at 1, 2, 4 and 6 weeks after pathogen inoculation.

Statistical analyses for plant growth and root colonisation were realised on 5 plants for each treatment and time point, and the experiment was repeated 4 times. Data were analysed with the Stat-ITCF program and differences were calculated by the Newman-Keul's test. Results are given for one representative experiment.

Root systems of non-mycorrhizal and mycorrhizal tomato plants inoculated 4 weeks with *P. nicotianae* v. *parasitica* were also stained using 2, 3, 5 triphenyltetrazolium chloride (TPTC) as described by Klotz and De Wolfe (1965). Living tissues stain red with this vital stain whilst roots killed by the pathogen do not change colour. Unstained and stained root regions were selected and half were pre-fixed in PBS (10 m*M*) /0.1% Tween 20 with 4% paraformaldehyde / 10% DMSO for 30 min and dehydrated through a graded ethanol series for immunocytochemical studies. The other half of the unstained and stained regions of roots was used to estimate mycorrhizal colonisation as described above.

Polyclonal antibodies raised against *P. megasperma* were tested for their cross-reaction with *P. nicotianae* v. *parasitica* on pre-fixed infected tomato roots. Root samples were washed in PBS 10 m*M* / 0.1% Triton × 100 for 10 mn followed by PBS for the same time and were lightly digested with cellulase and pectinase as described by Tisserant et al. (1993). They were then incubated at 4 °C overnight with the polyclonal antibodies diluted 1:1000 in PBS. Two revelation procedures were used to detect hyphae associated with roots. In the first, samples were washed in PBS and antibody binding visualised in the light microscope by indirect immunofluorescence after a 1 hour incubation at room temperature in the dark with fluorochrome FITC conjugated secondary antibodies (Sigma) diluted 1:400. Samples were washed with PBS and mounted in citifluor medium (Oxford Instruments) for observation by light microscopy under blue light (Leica I3, excitation filter : 450–500 nm). In the second procedure, samples were incubated 2 hours at room temperature in alkaline phosphatase conjugated secondary antibodies (Biosys) (diluted 1:100), after washing in PBS and TBS (100 m*M* Tris/HCl, 150 m*M* NaCl, pH 8), then washed with TBS pH 8 and Tris-HCl (100 m*M*), pH 9.5. To detect antibody binding, samples were incubated 30 min in the dark in a solution containing Tris-HCl pH 9.5, 50 m*M* $MgCl_2$, 68 m*M* α naphtyl phosphate and 116 m*M* 'Fast blue RR salt', in the presence of levamisole (Sigma) (1 m*M*) to inhibit endogenous alkaline phosphatases. Visualisation of staining in the light microscope was improved by clearing samples in a small volume of sodium hypochlorite (3 °) for 1 hour. Statistical analyses were carried out on 72 TPTC-stained and unstained root pieces from mycorrhizal and non-mycorrhizal root systems.

For studies of tissue colonisation and host responses, three mm long root pieces infected by *P. nicotianae* v. *parasitica* or colonised by *G. mosseae*, with and without pathogen inoculation, were fixed and embedded in LR White resin as described by Gianinazzi and Gianinazzi-Pearson (1992). Indirect immunogold labelling with silver enhancement (IGSE) was used to visualise antibody binding in the light microscope. Semi-thick sections (0.5 μm) of embedded root tissues were treated as described by Gianinazzi and Gianinazzi-Pearson (1992) with the primary antibody against *P. megasperma* diluted 1:1000 in TBS / 0.05% Tween / 1% BSA and antibody binding detected using 5 nm gold-labelled secondary antibodies (Biocell) diluted 1:20 followed by silver enhancement of the signal. Controls were performed by omitting primary antibody. Sections were counter stained with 2% basic fuchsin and the site of antibody fixation, indicated by a deposit of silver grains, was observed in epipolarized light. The number of *P. nicotianae* v. *parasitica* hyphae in different root tissues was estimated from 20 serial cross sections taken in 4 different positions in each of 25 root fragments of mycorrhizal and non mycorrhizal tomato roots infected by the pathogen. Statistical analyses of data was carried out as described above.

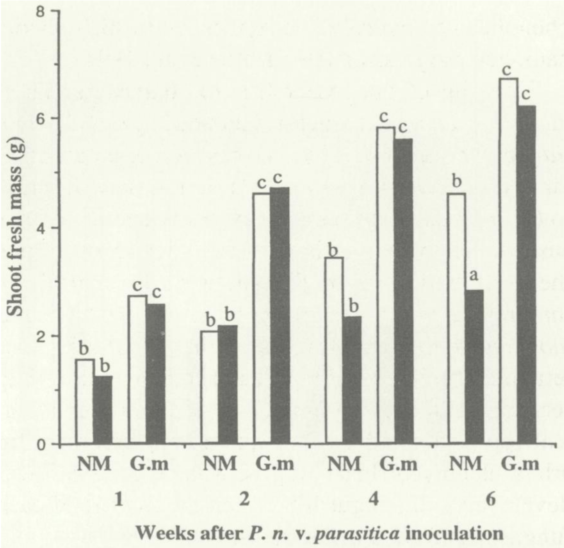


Figure 1. Fresh shoot mass (g) of non-mycorrhizal (NM) and mycorrhizal (+ G. m) tomato cv *Earlymech* plants uninoculated (□) or inoculated (■) with *P. nicotianae* v. *parasitica* 3 weeks after *G. mosseae* inoculation, and harvested 1, 2, 4 and 6 weeks after *P. nicotianae* v. *parasitica* inoculation. For each week, values followed by the same letter are not significantly different ($p = 0.05$).

Cytochemical studies

The response of tomato root tissues to invasion by *G. mosseae* and *P. nicotianae* v. *parasitica* alone or together, was investigated in semi-thick sections by toluidine blue staining (Feder and O'Brien, 1968; Stössel et al., 1980) and autofluorescence (Fernandez and Heath, 1986) to detect phenolics accumulation. The infection pattern of *P. nicotianae* v. *parasitica* in tomato roots was studied at the ultrastructural level after reaction of thin sections with periodic acid-thiocarbohydrazide-silver proteinate (PATAg) to localise 1,4 and 1,6 polysaccharides (Thiery, 1967).

Results

Effects of G. mosseae *and/or* P. nicotianae *v.* parasitica *infection on tomato growth*

When tomato plants were inoculated with *P. nicotianae* v. *parasitica* three weeks after *G. mosseae* inoculation, the degree of mycorrhizal colonisation was 56.2% (± 3.9%) of the root system cortex.

Results for fresh shoot mass (Figure 1) showed that there were no significant differences between non-mycorrhizal controls and non-mycorrhizal, *P. nico-*

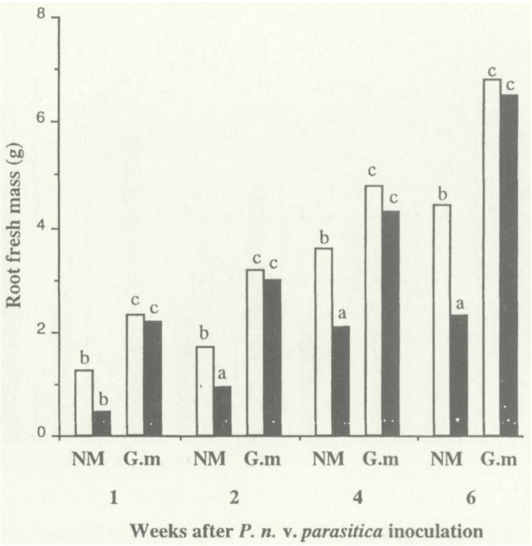


Figure 2. Fresh root mass (g) of non-mycorrhizal (NM) and mycorrhizal (+ G. m) tomato cv Earlymech plants uninoculated (☐) or inoculated (■) with *P. nicotianae* v. *parasitica* 3 weeks after *G. mosseae* inoculation, and harvested 1, 2, 4 and 6 weeks after *P. nicotianae* v. *parasitica* inoculation. For each week, values followed by the same letter are not significantly different ($p = 0.05$).

tianae v. *parasitica* -infected tomato plants at 1 and 2 weeks after pathogen inoculation, but a growth depression appeared in the latter at 4 weeks and this became significant at 6 weeks. The pathogen had no effect on the growth of tomato plants pre-colonised by *G. mosseae*. The first indication of this bioprotective effect was observed at the level of root development (Figure 2). Comparisons of the fresh root mass for non-mycorrhizal roots infected by *P. nicotianae* v. *parasitica* with non-mycorrhizal controls showed a significant decrease in the former already 2 weeks after pathogen inoculation, whilst there was no effect of the pathogen on fresh root mass of plants inoculated first with *G. mosseae*, even after 6 weeks.

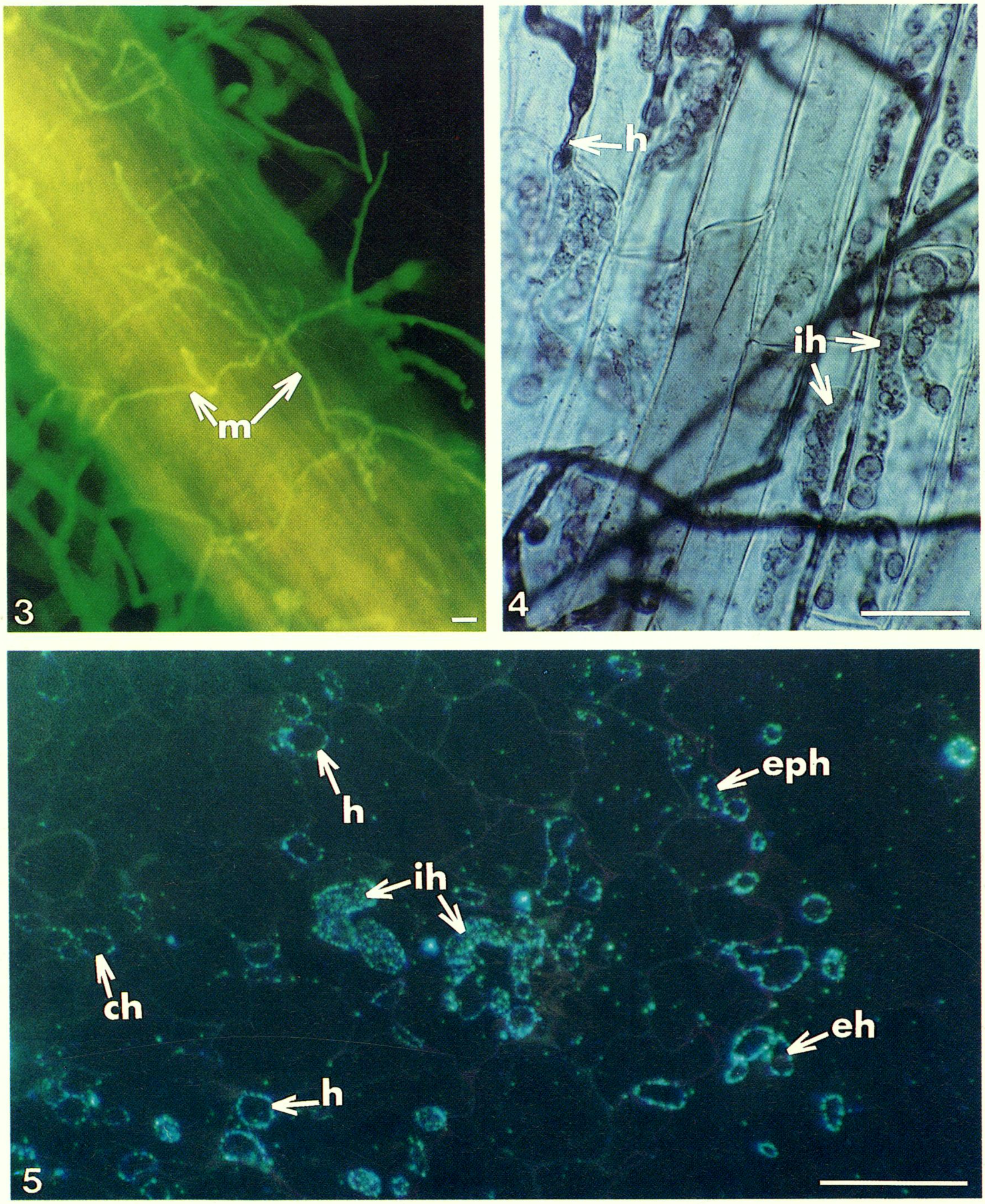
Necrosis development in non-mycorrhizal or mycorrhizal P. nicotianae *v.* parasitica *infected tomato roots*

Inoculation of non-mycorrhizal tomato with *P. nicotianae* v. *parasitica* resulted in rapid development of necroses in large parts of the root system (Table 1). After 2 weeks, 18.2% of the root system was necrosed and this increased to 39.0%, 6 weeks after inoculation. Mycorrhizal root systems challenged with *P. nicotianae* v. *parasitica* showed significantly less necroses at 2, 4 and 6 weeks after pathogen inoculation, with necroses frequency being reduced by more than 50% compared to non-mycorrhizal, *P. nicotianae* v. *parasitica* - infected roots.

Detection of P. nicotianae *v.* parasitica *within root tissues*

Trypan blue, which stains arbuscular mycorrhizal fungi in roots, has also been used to detect *Phytophthora* species (Phillips and Hayman, 1970), but in preliminary studies we found that it does not discriminate between external mycelium of *G. mosseae* and *P. nicotianae* v. *parasitica* and that it is extremely difficult to detect *P. nicotianae* v. *parasitica* hyphae inside roots using this staining technique. It was however possible to distinguish hyphae of *P. nicotianae* v. *parasitica* using the immunocytochemical approach with polyclonal antibodies against *P. megasperma*. Immunofluorescence showed that *P. megasperma* antibodies clearly recognised mycelium of *P. nicotianae* v. *parasitica* on the surface of roots (Figure 3), but no immunolabelling could be observed inside roots. *P. nicotianae* v. *parasitica* hyphae could be detected specifically inside roots by indirect immunolabelling using alkaline phosphatase conjugated secondary antibodies, and intercellular and intracellular hyphae of the pathogen were easily located (Figure 4).

After TPTC staining of roots 4 weeks after pathogen inoculation (Figure 2), the number of white root fragments in non-mycorrhizal root systems was estimated to be 29.4 for a root fresh mass of 2.08 g (14.1 per g) whereas in mycorrhizal root systems the number was 34.6 for a root fresh mass of 4.37 g (7.9 per g). With TPTC staining, white root fragments are considered to be infected by *P. nicotianae* v. *parasitica* whilst red-stained regions should be uninfected (Klotz and De Wolfe, 1965), indicating again a reduction in pathogen development in mycorrhizal roots. The frequency of *P. nicotianae* v. *parasitica* colonisation was estimated in roots using immunodetection coupled with TPTC staining. In non-mycorrhizal roots, hyphae of *P. nicotianae* v. *parasitica* were in fact not observed in red-stained root fragments but less than 50% of white root segments were colonised by the pathogen (Table 2). The frequency of *P. nicotianae* v. *parasitica* infection in white mycorrhizal roots was lower, but not significantly, than that of unstained non-mycorrhizal roots and, likewise, red-stained mycorrhizal roots were not infected by the pathogen. *G. mosseae* was present in both unstained and TPTC-stained root fragments



Figures 3–5. Immunodetection by light microscopy of *P. nicotianae* v. *parasitica* structures outside (Figures 3, 5) and inside (Figures 4, 5) tomato cv *Earlymech* roots using *P. megasperma* antibodies. Detection in pre-fixed infected root fragments of external mycelium (m) by immunofluorescence (Figure 3), and of intercellular (h) and intracellular hyphae (ih) by indirect immunoenzyme labelling (Figure 4). Detection of *P. nicotianae* v. *parasitica* hyphae by IGSE in a cross section of infected tomato roots and observed in epipolarised light (Figure 5). (eh) external hyphae, (eph) hyphae in epidermal cell, (h) intercellular and (ih) intracellular hyphae in the cortex, (ch) hyphae in central cylinder tissues. *Bar* = 25 μm.

Table 1. Percentage root length with necrosis in non mycorrhizal (NM) and mycorrhizal (+ *G. mosseae*) tomato cv. Earlymech plants 2, 4 and 6 weeks after *P. nicotianae* v. *parasitica* inoculation

Treatment	Weeks after *P. nicotianae* v. *parasitica* inoculation 2	4	6
NM + *P. nicotianae* v. *parasitica*	18.2(b)	27.7(b)	39.0(b)
G. mosseae + *P. nicotianae* v. *parasitica*	6.3(a)	11.2(a)	15.3(a)

For each column, values followed by different letters are significantly different ($p = 0.05$).

Table 2. Estimation of the frequency of TPTC-stained and unstained segments colonised by *P. nicotianae* v. *parasitica* and *G. mosseae* of non-mycorrhizal (NM) and mycorrhizal (+ *G. mosseae*) tomato cv. Earlymech roots 4 weeks after pathogen inoculation. Fungal pathogen structures were visualised after immunoenzyme labelling with *P. megasperma* antibodies and *G. mosseae* by trypan blue staining

	Percentage unstained white root segments with:		Percentage red-stained root segments with:	
	P. nicotianae v. *parasitica*	*G. mosseae*	*P. nicotianae* var. *parasitica*	*G. mosseae*
NM *P. nicotianae* v. *parasitica*	46.2 (a)	0 (c)	0 (c)	0 (c)
G. mosseae + *P. nicotianae* v. *parasitica*	29.5 (a)	72.0 (b)	0 (c)	69.4 (b)

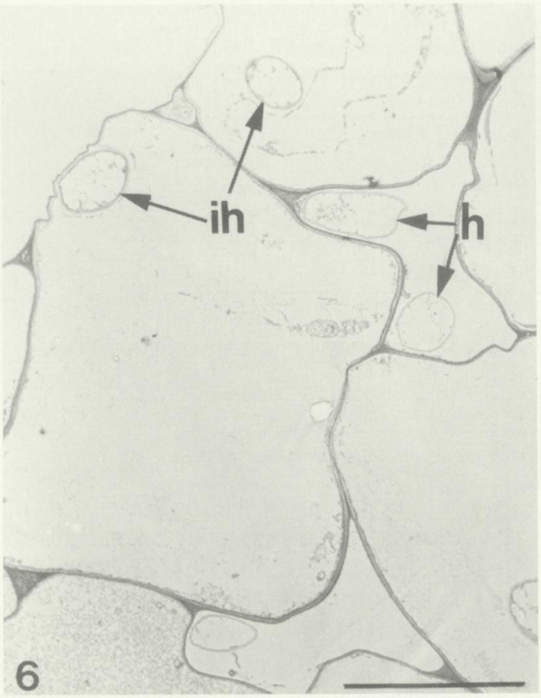
For each column, values followed by the same letter are not significantly different ($p = 0.05$).

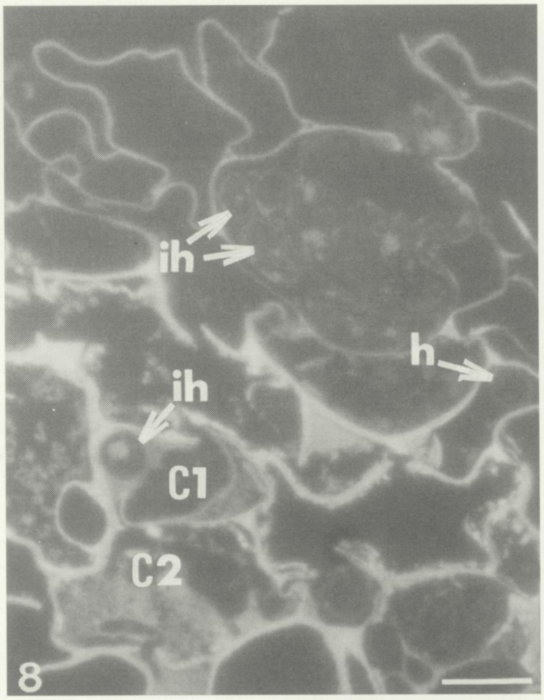
and the frequency of mycorrhizal colonisation was not affected by the presence of *P. nicotianae* v. *parasitica*.

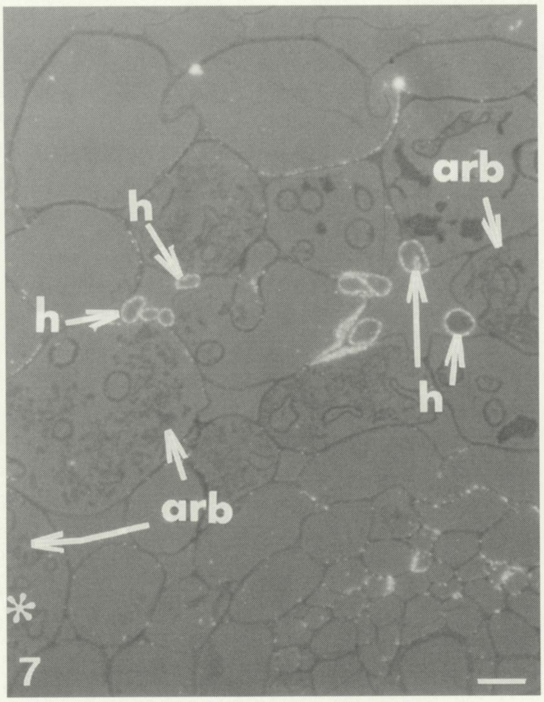
Light and electron microscope observations of root sections

P. megasperma antibodies were used to detect hyphae of *P. nicotianae* v. *parasitica* in cross sections of non-mycorrhizal tomato roots 2 weeks after pathogen inoculation (Figure 5). Hyphae of the pathogen at the root surface penetrated outer tissues inter or intracellularly and colonised epidermal, cortical and central cylinder tissues. Root colonisation in the cortex was essentially intercellular (see also Table 3) although some hyphae also penetrated host cells. Electron microscope observations revealed that most hyphae of the pathogen, whether intercellular or intracellular, were dead. In fact it was rare to observe living hyphae of *P. nicotianae* v. *parasitica* (Figure 6) in host tissue, suggesting that a large part of the biomass of the pathogen was inactive. *P. megasperma* antibodies specifically distinguished pathogen hyphae from those of *G. mosseae* in cross sections of mycorrhizal tomato roots infected by *P. nicotianae* v. *parasitica* (Figure 7). Living arbuscules were frequently observed in cells around intercellular spaces containing hyphae of the pathogen in mycorrhizal root fragments (Figure 7) but hyphae of *P. nicotianae* v. *parasitica* were never observed in the same cells as those containing *G. mosseae*. However, hyphae of *P. nicotianae* v. *parasitica* and *G. mosseae* could also separately colonise distinctly different parts of root systems. *P. parasitica*, like *G. mosseae*, preferentially infected the cortical tissue and far fewer hyphae were observed in epidermal tissue and the central cylinder (Table 3).

An estimation of the number of *P. nicotianae* v. *parasitica* (Table 3) in different root tissues showed that these were overall less in mycorrhizal roots as compared to non-mycorrhizal roots. It is in the cortex of mycorrhizal roots that the number of hyphae of *P. nicotianae* v. *parasitica* were most significantly decreased (by 72.3%) in comparison with non-mycorrhizal *P. nicotianae* v. *parasitica*-infected roots. There was lower, but not significant, colonisation of *P. nicotianae* v. *parasitica* in the epidermis and central cylinder of mycorrhizal roots. The reduction in hyphal development of the pathogen was observed both in root tissues which were simultaneously colonised by *G. mosseae* (5.8 ± 1.4 hyphae per section) and in non-mycorrhizal root regions (33.11 ± 4.7 hyphae per section) of the mycorrhizal root system, as compared to a non-mycorrhizal root system (64.4 ± 9.2 hyphae per section).







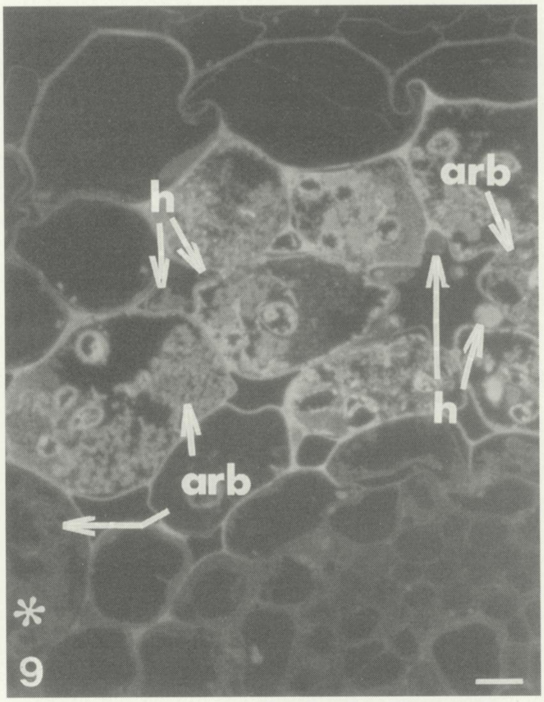


Figure 6. Micrograph of cortical tomato root tissue colonised by *P. nicotianae* v. *parasitica*. (h) intercellular hyphae, (ih) intracellular hyphae. Bar =10 μm. *Figure 7.* Immunodetection of *P. nicotianae* v. *parasitica* hyphae in a cross section of mycorrhizal *P. nicotianae* v. *parasitica*-infected tomato root, using *P. megasperma* antibodies. Observation in epipolarized light. (arb) living arbuscules, (h) intercellular *P. nicotianae* v. *parasitica* hyphae. *Bar* = 10 μm. *Figures 8 and 9.* Autofluorescence under blue light of cross-sections of non-mycorrhizal (Figure 8) and mycorrhizal (Figure 9, serial section of Figure 7) tomato roots infected with *P. nicotianae* v. *parasitica*. Strong autofluorescence and necrosis of pathogen-infected cells (C1) and uninfected cells (C2) are observed (Figure 8). Arbuscule-containing cells around the *P. nicotianae* v. *parasitica* hyphae weakly autofluoresce whereas those outside this region do not (asterisk) (Figure 9). (arb) living arbuscules, (h) intercellular and (ih) intracellular *P. nicotianae* v. *parasitica* hyphae. *Bar* = 10 μm.

Table 3. Estimation of the number of *P. nicotianae parasitica* hyphae per cross section of roots in different tissues of non-mycorrhizal (NM) and mycorrhizal (+ *G. mosseae*) tomato cv. Earlymech roots 2 weeks after inoculation with *P. nicotianae* v. *parasitica*

	Root tissue			
Treatment	Epidermis	Cortex		Central cylinder
		47.6 (b)		
NM + *P. nicotianae* v. *parasitica*	11.4 (a)	intercellular 36.0	intracellular 11.6	5.4 (a)
		13.2 (a)		
G. mosseae + *P. nicotianae* v. *parasitica*	5.8 (a)	intercellular 10.2	intracellular 3.0	3.2 (a)

For each column, values followed by different letter are significantly different ($p = 0.05$).

Light microscope observations of plant reactions to root infection by *P. nicotianae* v. *parasitica* (Figure 8) showed that the presence of *P. nicotianae* v. *parasitica* resulted in death and necrosis, not only of invaded cells but also of adjacent uninfected cortical cells in non-mycorrhizal roots. This was clearly associated with increased autofluorescence of the plant cell wall and contents, indicating an accumulation of phenolic components. In mycorrhizal roots, cells containing arbuscules were less necrosed in presence of *P. nicotianae* v. *parasitica* in the root cortex. Accumulation of phenolics was also observed in mycorrhizal *P. nicotianae* v. *parasitica*-infected root tissues (Figure 9), but this was limited to the arbuscule-containing cortical cells around the *P. nicotianae* v. *parasitica* hyphae.

Discussion

The results obtained here showed that the mycorrhizal fungus *G. mosseae* has a protective effect against the root pathogen *P. nicotianae* v. *parasitica* in tomato cv Earlymech plants. Significant positive effects of a preliminary mycorrhizal infection are observed on fresh root weight 2 weeks after pathogen inoculation and necrosis development in the root system is greatly reduced. Decreases in fresh shoot weight are observed later, 6 weeks after *P. nicotianae* v. *parasitica* inoculation, in non-mycorrhizal plants whereas there is no negative effect of the pathogen on mycorrhizal plant growth. Estimations of necroses and TPTC staining of non-mycorrhizal and mycorrhizal root systems clearly showed that mycorrhizal plants have more healthy roots, with an important decrease in root disease development. These results are comparable to those described for different mycorrhiza/pathogen interactions in the roots of other plants (Caron et al., 1986c; Davis, 1981; Guillemin et al., 1994).

Using the whole tissue immunodetection technique developed here, it was possible to estimate the intensity of *P. nicotianae* v. *parasitica* in whole root fragments. When immunodetection was coupled with TPTC staining, we were able to confirm that red-stained tomato root fragments were indemn of the pathogen. However, white unstained roots did not always contain *P. nicotianae* v. *parasitica* hyphae, underlining the limit of this staining technique for estimation of root damage directly attributable to the pathogen.

Hyphae of *P. nicotianae* v. *parasitica* were also immunolocated in the rhizosphere and in cross sections of pathogen-infected tomato roots. The number of *P. nicotianae* v. *parasitica* hyphae in root tissues of non-mycorrhizal and *G. mosseae*-colonised tomato plants estimated after immunolabelling, showed that pathogen proliferation was significantly less in mycorrhizal root systems. Preliminary studies on the infection pattern of *P. nicotianae* v. *parasitica* in non-mycorrhizal tomato roots showed that infection is initiated by hyphae growing in the rhizosphere which penetrate outer root tissues inter and intracellularly to invade epidermal tissue. The pathogen preferentially colonises the root cortex with a predominance of intercellular hyphae. Surprisingly, most of the hyphae observed in the cortical root tissue were dead whatever the extent of the infection; further studies of this phenomenon are necessary in order to determine whether it is a normal aspect of pathogen development or if hyphal death is prematurely induced by the plant tissues. This infection pattern is similar to that observed for *P. parasitica* var. *nicotianae* in tobacco root tissues (Benhamou and Côté, 1992), where pathogen hyphae grew into epidermal cells followed by mainly intercellular fungal growth in the cortex. The process by which *P. parasitica* var. *nicotianae* infected tobacco roots involved zoospore encystment, germination on the root surface and formation of dense mycelium from which intense root colonisation occurred. It would appear from our studies of non-mycorrhizal tomato roots that *P. nicotianae* v. *parasitica* infection is also established by rhizosphere hyphae penetrating the root. These presumably resulted from mycelium developing after germination of zoospores that encysted at the root tip, as described also for strawberry infection by *P. fragariae* (Goode, 1966; Hickman, 1940).

Estimations of the number of hyphae of *P. nicotianae* v. *parasitica* in non-mycorrhizal and mycorrhizal root systems showed that pathogen proliferation was significantly less in the latter, whether *G. mosseae* was present or not in the same root tissue. Consequently, the bioprotective effect in mycorrhizal root systems not only results in an overall reduction in necroses development but it is also directly linked to reduced pathogen development within colonised cortical tissues. In mycorrhizal roots, hyphae of both *G. mosseae* and *P. nicotianae* v. *parasitica* could be found together in the same part of the root, indicating that pathogenic and symbiotic fungi are not mutually exclusive in root tissues. However, pathogen hyphae never invaded arbuscule-containing cells. This may be linked to the expression of certain defence-related genes reported to be induced by the presence of the symbiotic fungus in these cells (Gianinazzi-Pearson et al., 1992, 1996; Harrison and Dixon, 1994; Lambais and Mehdy, 1995). When the fungi were present in different parts of the root system, as in most cases, pathogen development was still reduced suggesting that bioprotection is not only due to direct interactions between the two fungi in the same root tissue, but also to a long-distance effect on pathogen infection.

A difference was observed in host reaction between non-mycorrhizal and mycorrhizal tissues. During pathogen infection of non-mycorrhizal tissues, the host cell showed a necrotic reaction and an increase in autofluorescence of both cell wall and contents, indicating an accumulation of phenolic components, a phenomenon that does not occur during mycorrhizal colonisation of cortical tissues (Gianinazzi-Pearson et al., 1996). In pathogen-infected mycorrhizal tissues, necrosis did not occur in arbuscule-containing cortical cells around intercellular *P. nicotianae* v. *parasitica* hyphae and phenolic components only weakly accumulated. This suggests that mycorrhizal effects on pathogen disease are not only to reduce the spread of overall pathogen development, but also to specifically limit host tissue damage caused by the pathogen where the mycorrhizal fungus is present. In mycorrhizal tissues, fewer plant cells necrose and the mycorrhizal association may continue to function, and so compensate for the damaged parts of the root. However, it is not possible to determine whether there is a difference in the rapidity of reaction between mycorrhizal roots and non mycorrhizal roots. Further detailed studies are necessary to better define plant cell reactions during pathogen interactions in mycorrhizal tomato roots in order to determine whether induced defense responses play a role in bioprotection and whether limited spread of the pathogenic fungus is related to alterations in its activity in mycorrhizal tissues.

Acknowledgements

This work was supported by an European AIR-Project no 3-CT94-0809. The authors are very grateful to Elmon Schmelzer (Max-Planck-Institute, Cologne, D) for his invaluable collaboration in providing the *P. megasperma* antiserum.

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Section editor: H Lambers