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Endoproteolytic activities in pea roots inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae* and/or *Aphanomyces euteiches* in relation to bioprotection

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SUMMARY

Arbuscular mycorrhizal (AM) symbioses are known to play a role in increased resistance of plants against soilborne pathogens. Mechanisms involved in this phenomenon are not yet well understood. This work investigates possible roles of endoproteolytic activities in bioprotection of *Pisum sativum* roots by *Glomus mosseae* against *Aphanomyces euteiches*. First, it is demonstrated that bioprotection occurs only in pre-mycorrhizal plants. Second, endoproteolytic activities were analysed qualitatively and quantitatively during AM symbiosis, in plants infected with either zoospores or mycelium of *A. euteiches*, and in mycorrhizal plants infected with the pathogen. In mycorrhizal symbiosis a progressive increase in endoproteolytic activities was observed following root colonization by *G. mosseae*. By contrast, in roots inoculated with *A. euteiches*, a drastic increase in endoproteolytic activities was observed which was correlated with the amount of pathogen occurring in roots. Qualitative differences were seen among the endoproteolytic activities detected in roots inoculated with zoospores or mycelium. The constitutive as well as mycorrhizal and pathogen-induced activities were further characterized as 'trypsin-like' serine endoproteases. Interestingly, in a situation of bioprotection, only low levels of the activities normally associated with the infection by *A. euteiches* were detected, suggesting that the synthesis of these proteins is directly linked to the growth or virulence of the pathogen.

Key words: endoproteolytic activities, arbuscular mycorrhizal fungi, *Pisum sativum*, *Aphanomyces euteiches*, bioprotection.

INTRODUCTION

Pea root rot (*Pisum sativum*) caused by *Aphanomyces euteiches* is the most serious pea disease in several countries (Papavizas & Ayers, 1974; Mauffras *et al.*, 1997). To date neither commercial resistant cultivars nor effective fungicides have been available (Rao *et al.*, 1995). Because the pathogen may survive in the soil for more than 10 yr, the only existing control is to avoid planting peas in infested fields for many years (Jones & Linford, 1925). The difficulties in controlling *Aphanomyces* pea root rot, together with the real need for more sustainable agriculture, have prompted the search for biological alternatives. Rosendahl (1985) was the first to report that infection with *A. euteiches* was suppressed by the arbuscular mycorrhizal (AM) fungus *Glomus fasciculatum* when pea plants were challenge-inoculated with the pathogen after 2 wk mycorrhization.

Mycorrhizas are mutualistic associations that occur between plant roots and fungi. The most common type, the AM symbiosis, is essentially non-specific and reflects an extreme compatibility between the two partners (Gianinazzi-Pearson, 1996).

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Reports of improved growth, health and resistance to biotic and abiotic stresses of mycorrhizal plants are widespread (Bethlenfalvay, 1992). Among these benefits, AM fungi can reduce plant diseases and especially damage caused by soilborne plant pathogens (Dehne, 1982; Zambolin & Schenck, 1983; Rosendahl, 1985; Caron, 1989; Linderman, 1994; Azcón-Aguilar & Barea, 1996). The disease reduction is the outcome of complex interactions between the plant, the pathogen, the AM fungi, the microbial community on and around the plant, and the physical environment. Despite its potential in agricultural practices, bioprotection by AM fungi is still a poorly understood area of this plant–microbe interaction (Azcón-Aguilar & Barea, 1996).

Until now, several potential mechanisms have been described concerning bioprotection by AM fungi, including improvement of plant nutrition (Hooker *et al*., 1994); damage compensation (Cordier *et al*., 1996; Pinochet *et al*., 1996); competition for photosynthates (Linderman, 1994); competition for colonization or infection sites (Dehne, 1982; Cordier *et al*., 1996); anatomical or morphological modifications of the root system (Atkinson *et al*., 1994), induction of changes in mycorrhizosphere microbial populations (Citernesi *et al*., 1996); and activation of plant defence (St Arnaud *et al*., 1995). The major inducible defence mechanisms in response to pathogen attacks are cell-wall modifications, enhancement of secondary metabolism, and accumulation of proteins including the so-called pathogenesis-related (PR) proteins (Dixon & Harrison, 1990; Collinge *et al*., 1994). Most investigations concerning previously identified plant defence genes in response to AM symbiosis showed minor and transient increases in expression following colonization, which are somehow insignificant in comparison to responses that occur following attack by pathogens (Gianinazzi-Pearson *et al*., 1992; Franken & Gnädinger, 1994; Lambais & Medhy, 1995; Blee & Anderson, 1996). However, it has been suggested that AM symbiosis could predispose the plant to respond more rapidly to pathogenic attacks of the roots (Dehne, 1982; Zambolin & Schenck, 1983; Rosendahl, 1985; Caron, 1989; Gianinazzi, 1991; Linderman, 1994; St Arnaud *et al*., 1995; Azcón-Aguilar & Barea, 1996; Gianinazzi-Pearson, 1996).

Among all proteins that can be produced in response to biotic or abiotic stresses, proteases were shown to be induced by wounding (Linthorst *et al*., 1993; Pautot *et al*., 1993; Schaller & Ryan, 1996); citrus exocortis viroid infection (Vera & Conejero, 1988, 1989; Tornero *et al*., 1996) and herbivorous insects (Schaller & Ryan, 1996). From these studies, protein degradation as well as protein processing and maturation appear as key events in plant defence, playing a role either directly in hydrolysing pathogenic proteins or indirectly in signal transduction pathways. Moreover, proteolysis in plants plays a fundamental role in developmental and physiological processes (Callis, 1995). However, at present little is known about the expression of protease genes in plant–pathogen interactions and nearly nothing in AM symbiosis.

In order to extend previous investigations on the molecular mechanisms governing bioprotection by AM fungi, the aim of the present study was to investigate the potential role of endoproteolytic activities in bioprotection conferred by the mycorrhizal fungus *Glomus mosseae* against *A. euteiches*.

MATERIALS AND METHODS

Chemicals

All reagents for gel electrophoreses were purchased from Bio-Rad, except acrylamide which was purchased from Boerhinger-Ingelheim. Corn meal agar (CMA) was from Difco, the alkaline phosphatase-conjugated antibody from Biosys, and nitrocellulose membrane from Schleicher & Schuell. All other reagents were supplied by Sigma.

Fungal material (Glomus mosseae *and* Aphanomyces euteiches)

A soil-based mycorrhizal inoculum of *Glomus mosseae* (Nicol and Gerd.) Gerdemann and Trappe (BEG 12) containing fungal propagules and chopped mycorrhizal *Allium porrum* L. roots was used as described previously by Dumas-Gaudot *et al*. (1994).

A virulent strain of *Aphanomyces euteiches* SRSF 502, kindly provided by C. Richard (Centre de Recherche et de Développement, Agriculture et Agroalimentaire Canada, Ste-Foy Québec, Canada), was grown on potato dextrose agar (PDA) or CMA at 23°C in darkness and subcultured every month on Petri dishes. Zoospore suspensions were prepared as described by Beghdadi *et al*. (1992), except that rinsing steps were carried out using Volvic mineral water (Volvic, Puy de Dôme, France). Suspensions of 10^2, 10^3, 10^4 and 10^5 zoospores were used as inoculum. Inoculation of pea plants was also performed by inoculating the main root with two mycelial plugs from a 5-d-old culture.

Plant material and methods of inoculation

Seeds of pea (*Pisum sativum* L. cv. Frisson) compatible to mycorrhization were sterilized by subsequent immersions in 3.5% calcium hypochlorite and 96% ethanol for 10 min, thoroughly rinsed with sterile de-ionized water, and then germinated under sterile conditions on vermiculite at 24°C for 3 d.

Mycorrhizal plants were obtained by transplanting 3-d-old pea plantlets into *G. mosseae* inoculum soil layered (1:1) with sterile sand (one plant 200 ml^{-1})

whereas uninoculated plants were transplanted into γ-irradiated clay loam soil (26 ppm. Olsen P) layered with sterile sand. Plants were grown in a climatic room under a controlled environment: photon flux density was 300 μmol m^{-2} s^{-1} with a 16-h day photoperiod and r.h. was maintained at 60% under controlled temperature (23°C/18°C). After 15 d, plants were processed to challenge inoculation with *A. euteiches*. Respective control plants received sterile Volvic water. Pathogen infection was carried out by watering the plants with zoospores at different dilutions (10^2–10^5 zoospores per pot) or by carefully applying mycelial plugs into the soil. Plants from the different treatments were flooded every other day with Volvic mineral water. The treatments were: uninoculated (Nm), mycorrhized (G), *A. euteiches* zoospore-infected (Az), *A. euteiches* mycelium-infected (Am), simultaneously inoculated with *G. mosseae* and *A. euteiches* (SimGA), pre-mycorrhized and post-infected with *A. euteiches* zoospores (Gaz), pre-mycorrhized and post-infected with *A. euteiches* mycelium (Gam).

Experimental design

Experiment A. Plants were inoculated with *G. mosseae* and harvested after 4, 6, 10, 15, 20 and 25 d.

Experiment B. Plants were simultaneously inoculated with *G. mosseae* and *A. euteiches* zoospores at a dilution of 10^5 per pot and harvested 10 d after inoculation (d.a.i.).

Experiment C. After 15 d mycorrhization, plants were inoculated with increased zoospore dilutions (from 10^2 to 10^5 zoospores per pot). Plants were harvested 10 d.a.i.

Experiment D. After 15 d mycorrhization, plants were infected with pathogen mycelium and sampled 10 and 20 d.a.i.

Experiment E. After 15 d mycorrhization, plants were inoculated with 10^5 zoospores per pot. Plants were harvested 0, 1, 2, 4, 6 and 10 d.a.i.

All sets of experiments were repeated at least twice. Results are given for one representative experiment. For all experiments, roots from the different treatments were collected from three plants from each treatment in three replicates. Samples were immediately frozen in liquid nitrogen and stored at −65°C until protein extraction.

Production of antigen, immunization and preparation of antisera against Aphanomyces euteiches

A. euteiches was maintained on CMA at 4°C. Ten mycelial plugs from the edge of a CMA culture were transferred to glucose–peptone broth in Petri dishes. The Petri dishes were incubated for 12 d at 24°C and the mycelium was washed three times in phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM Na_2HPO_4, 3 mM KOH, 2 mM KH_2PO_4). Excess water was removed and the mycelium was dried and kept frozen at -20°C. The frozen mycelium was crushed in an ice-chilled mortar in PBS (2:1 v/v). The extracts were centrifuged for 20 min at 20000 ***g*** at 4°C. The supernatant was recovered and slowly mixed with a saturated solution of $(NH_4)_2SO_4$ to a 66% final solution and left for 18 h at 4°C under constant stirring. Proteins were pelleted by centrifugation at 15000 ***g*** for 30 min at 4°C and resuspended in a minimal volume of PBS. The solution was dialysed for 24 h against PBS. Protein concentration was determined by the method of Bradford (1976) and the extract was kept frozen at −65°C until needed.

Two 6-month-old female white rabbits were immunized. Before immunization, pre-immune serum was collected. At each immunization, rabbits were injected with 0.6 ml (containing 0.9 mg protein) extract emulsified with 0.6 ml adjuvant, once intramuscularly in each rear leg and twice subcutaneously in the neck. Immunizations were achieved by monthly injections for 4 months, the first three with Freund's incomplete adjuvant and the last one with Ribi adjuvant system (Ribi Immunochem System, Hamilton, Montana, USA). Blood was allowed to clot for 18 h, the serum was separated by centrifugation, and 0.02% sodium azide was added before storage at −20°C.

Quantification of arbuscular mycorrhizal colonization and pathogenic infection

Mycorrhizal colonization was evaluated microscopically. At the time of harvest, parts of root samples were randomly collected for staining with trypan blue as described by Phillips & Hayman (1970). Mycorrhizal colonization was expressed as the frequency of infected root samples (F%), the percentage of colonized cortex (M%) and the intensity of arbuscule development (A%) within the root system according to Trouvelot *et al*. (1986).

Except for experiment A, infection with *A. euteiches* by root-rot rating scores was estimated at the time of sampling, as described by Rao *et al*. (1995). The spread of *A. euteiches* into the root system was determined by an indirect ELISA using one of the polyclonal antisera produced against *A. euteiches*. At each step of the assay, wells of ELISA plates (NUNC-Immuno Plate maxisorb™ surface, NUNC Brand Products, Denmark) were filled with a volume of 0.1 ml. Calibration curves were established with appropriate dilutions of *A. euteiches* antigens prepared as follows. The mycelium of pathogen previously grown on PDA for 5 d at 23°C in darkness was carefully removed, weighed and

lyophilized. Mycelium (1 mg f. wt) was then triturated with a pestle and mortar, resuspended in 1 ml 100 mM McIlvaine extracting buffer pH 6.8 (McIlvaine, 1921), and centrifuged at 9000 ***g*** for 45 min at 4°C. The supernatant was diluted from 10^{-2} to 10^{-4} mg ml^{-1} in 20 mM carbonate buffer, pH 9.8.

Pea roots corresponding to the different treatments from experiments B, C, D and E were extracted in 100 mM McIlvaine buffer (1 g ml^{-1}) (McIlvaine, 1921). Crude supernatants were diluted in 50 mM carbonate buffer (1 : 50 v/v) and loaded into ELISA plates. Plates were incubated 18 h at 4°C, washed three times with Tris–casein buffer pH 7.6 (Kenna *et al*., 1985) for 5 min each, and then incubated for 1.5 h with Tris–casein buffer. *A. euteiches* antiserum was diluted 1 : 5000 in Tris–casein and incubated for 2 h at 25°C. On each plate, antigens of *A. euteiches* and pea root extracts were also probed against pre-immune serum at the same dilution. After several washes as described above, the mouse anti-rabbit IgG alkaline phosphatase-conjugate diluted 1 : 4000 in Tris–casein buffer was incubated for 2 h and then washed three times with Tris–casein buffer and twice with substrate buffer (10% diethanolamine pH 9.8). Phosphatase substrate (Sigma 104™) was mixed with substrate buffer and pipetted into each well. Readings were made spectrophotometrically at 405 nm after 20 min incubation, and data were presented as an average of at least three wells. Absorbance values for non-sensitized wells were subtracted from mean absorbance values of sensitized wells. In this way, any non-specific background reaction was taken into account for further analyses.

The specificity of the antiserum against *A. euteiches* was also confirmed by western blotting. Root and mycelial extracts were separated in 15% SDS–PAGE. Following separation, proteins were electrotransferred to nitrocellulose membranes (porosity 0.1 μm) for 3 h at 200 mA and indirect immunological detection was carried out according to Tahiri-Alaoui *et al*. (1990). The polyclonal antiserum raised against the mycelium of *A. euteiches* was used at a dilution of 1 : 1000.

Protein extraction and quantification

Frozen roots from different treatments were ground at 4°C in an ice-chilled mortar with liquid nitrogen and the resulting powder was suspended in McIlvaine extracting buffer pH 6.8 (McIlvaine, 1921) to give a concentration equivalent to 1 g f. wt root ml^{-1}. Crude homogenates were centrifuged at 9000 ***g*** for 45 min at 4°C and the supernatant fractions were kept frozen at −20°C. The mycelium of *A. euteiches* previously grown on PDA for 5 d at 23°C in darkness was carefully removed and extracted under the same conditions. Protein content of the various extracts was determined by the method of Bradford (1976) using BSA as a standard.

Quantitative measurement of endoproteolytic activities

Endoproteolytic activities were assayed with azoalbumin as substrate. After clarifying the substrate by centrifugation at 12000 ***g*** for 10 min, 0.25 ml of a solution of 2% azoalbumin in 0.1 M sodium phosphate buffer pH 7 was mixed with 0.15 ml crude extract. The reaction mixture was incubated for 2 h at 37°C, then stopped by adding 1.2 ml 10% trichloroacetic acid, and allowed to stand for 30 min at 4°C to ensure the complete precipitation of the remaining azoprotein. After centrifugation at 10000 ***g*** for 10 min, 1.2 ml of the supernatant was transferred to a test tube containing 1.4 ml of 1 M NaOH. Absorbance values were determined at 440 nm after 30 min. Protease activity was expressed as the amount of proteins required to produce an absorbance change of one under the conditions of the assay.

Detection of endoproteolytic activities after electrophoresis

All extracts were subjected to discontinuous SDS–PAGE (15% acrylamide, 20 × 20 cm) copolymerized with 0.2% gelatin on a Bio-Rad system as described by Jameel *et al*. (1984). Total proteins of either root or *A. euteiches* mycelium extracts (45 μg) were loaded on SDS gels. In order to discriminate between fungal and plant endoproteolytic activities, co-electrophoresis was also carried out with 60 μl *A. euteiches*-infected root extracts mixed with *A. euteiches* mycelium extract in the respective proportions 1 : 4, 1 : 2 and 3 : 4. Extracts were separated on SDS–PAGE (10% acrylamide) and compared with the respective controls. Electrophoreses were performed at 4°C, at a constant current of 25 mA.

Renaturation of endoproteolytic activities was carried out using a modification of the method described by San Segundo *et al*. (1990). Gels were incubated in de-ionized 2% Triton™ X-100 at 37°C for 1 h. The optimum pH of proteolytic activities was determined with four renaturing buffers: 0.1 M glycine–HCl pH 3; 0.1 M sodium acetate pH 5; 0.1 M sodium phosphate pH 7; and 0.1 M Tris–glycine pH 8.9. Gels were then incubated in respective buffers containing 1% Triton™ X-100 for 18 h at 37°C. Endoproteolytic activities were revealed by staining the gels with 0.1% amido black in a mix of methanol–acetic acid–H_2O (30 : 10 : 60) and appeared as white regions against a dark blue background. All analyses were repeated at least twice. Gels were visualized under white light and photographed using Polaroid 665 film. One gel representative of all the others was chosen for illustration.

Inhibition of endoproteolytic activities

Extracts were pre-incubated for 30 min on ice with the following inhibitors: EDTA; 1, 10 phenanthrolin; phenylmethylsulphonylfluoride (PMSF); 3,4-dichloroisocoumarin (DCI); L-trans-epoxy-succinyl-leucylamide-(4-guanidino)-butane (E-64); iodoacetic acid; pepstatin A; leupeptin; tosyl-L-lysylchloromethyl ketone (TLCK); tosyl-L-phenyl-alanyl chloromethyl ketone and bestatin (Table 3). Extracts with or without inhibitors were then subjected to electrophoresis as previously described. Gels (15 × 10 cm) were incubated in the renaturating buffer with or without the respective inhibitors. After staining with amido black, the gels were scanned and then analysed using Kodak Digital Science 1 software. Inhibitory activity was expressed as the percentage of inhibition of each activity compared with the respective controls which were separated on the same gel.

Data analysis

Treatments of disease index, quantification by ELISA of the pathogenic fungus, and level of mycorrhization were compared for statistical significance using the Newman–Keuls test.

RESULTS

Mycorrhizal colonization

In experiment A, arbuscules were detected 6 d.a.i, and after 25 d the value of *F* was 93.1%, of *M* 46.6%, and of *A* 40.9% (Table 1). In experiment B where plants were inoculated simultaneously with the pathogen and the mycorrhizal fungus, the mycorrhizal colonization was reduced (Table 1). In experiments C–E where plants were post-inoculated with the pathogen, at the end of the experiments mycorrhizal plants were well colonized with *F* ranging from 75 to 97.8% (Table 1). These parameters were not significantly affected in plants post-inoculated with the pathogen.

Table 1. *Mycorrhizal colonization roots* (*G*), *mycorrhizal roots co-inoculated with the pathogen* (*simGAz*), *mycorrhizal roots post-infected with increasing amounts of pathogen zoospores* (*GAz*) *or pathogen mycelium* (*GAm*)

Experiments	Treatments	*F*(%)	*M*(%)	*A*(%)
A	G_4	0 a	0 a	0 a
	G_6	26.7 b	0.7 b	0.4 b
	G_{10}	66.7 c	5.4 c	2.8 b
	G_{15}	65.5 c	16 d	11.9 c
	G_{20}	73.3 c	17.6 d	14.1 c
	G_{25}	93.1 d	46.6 e	40.9 d
B	G	68.5 f	14.1 f	10.5 f
	simGAz	43.1 g	5.3 g	4.1 g
C	G	78 h	33.5 g	27.5 h
	GAz 10^2	76.7 h	24.8 h	18.4 h
	GAz 10^3	79.8 h	31.8 h	26.5 h
	GAz 10^4	84 h	32.7 h	25 h
	GAz 10^5	80 h	36.1 h	27.9 h
D	G	97.8 i	53.9 i	44.2 i
	GAm	93.3 i	36.1 i	30.6 i
E	G_0	68.2 j	22.6 j	11.5 j
	G_1	68.8 j	22.2 j	10.4 j
	G_2	71.7 j	22.3 j	18.2 j
	G_4	70.5 j	26.4 j	21.4 j
	G_6	71.1 j	28.3 j	25.2 j
	G_{10}	75 j	28 j	24.2 j
	GAz_1	66.3 j	24.8 j	14.7 j
	GAz_2	67.6 j	22.3 j	20 j
	GAz_4	67.5 j	22.3 j	19.6 j
	GAz_6	72.5 j	26.7 j	20.7 j
	GAz_{10}	69.8 j	25.6 j	20.6 j

G, mycorrhized; sim GAz, simultaneously inoculated with *Aphanomyces euteiches* zoospores and *Glomus mosseae*; GAz, pre-mycorrhized and post-infected with *A. euteiches* zoospores; GAm, pre-mycorrhized and post-infected with *A. euteiches* mycelium. In experiment A, mycorrhizal parameters were evaluated from 4 to 25 d after inoculation with *G. mosseae*. They were determined at time of sampling, i.e. after 10 d for experiment B and after 25 d for experiments C and D. In experiment E, mycorrhizal parameters were estimated 0, 1, 2, 4, 6 and 10 d.a.i. with 10^5 *A. euteiches* zoospores. *F*% represents the frequency of colonization, *M*% the intensity of colonization and *A*% the arbuscule intensity. Results are expressed as the mean of three replicates. Statistical analyses were carried out independently for each experiment. Results with the same letter are not significantly different at $P = 0.05$ (Newman–Keuls test).

Pathogenic infection

Pathogenic infection was evaluated by both root-rot rating scores and quantification of the amount of pathogen in roots by ELISA. The latter was done using a polyclonal antiserum raised against *A. euteiches*. Specificity of this polyclonal antiserum was tested by both western blotting and ELISA. By western blotting, after separation by SDS–PAGE, no cross-reaction occurred between the antiserum and proteins from non-inoculated or mycorrhizal roots, whereas this antiserum reacted strongly with antigens in protein extracts of either *A. euteiches* mycelium or non-mycorrhizal pea roots infected with *A. euteiches* (not shown). By ELISA, the antiserum from all bleeds showed a linear response with higher absorbance values to the increased concentrations of antigens of *A. euteiches* (data not shown). The polyclonal antiserum was tested against uninoculated, *G. mosseae*-inoculated and *A. euteiches*-infected pea roots. Only extracts of *A. euteiches* infected roots gave strong absorbance values (Table 2).

The protection against *A. euteiches* may depend on the pre-establishment of the mycorrhizal symbiosis.

Table 2. Aphanomyces euteiches *infection, as determined by root rot score rating and ELISA, in pea roots co-inoculated with* A. euteiches *and* Glomus mosseae (B) *or in mycorrhizal roots post-inoculated either with increased amounts of zoospores* (*C*) *for 10 d or with mycelium* (*D*) *for 10 and 20 d*

Experiments	Treatments	Root rot score rating	*A. euteiches* quantification (mg ml^{-1})
B	Nm	1 a	0 a
	G	1 a	0 a
	Az	3.4 b	0.081 b
	sim GAz	3.2 b	0.068 b
C	Nm	1 g	0 f
	G	1 g	0 g
	Az		
	10^2 zoospores	1.6 ef	0.038 def
	10^3	2.2 de	0.093 d
	10^4	2.6 cd	0.155 c
	10^5	3 c	0.155 c
	GAz		
	10^2 zoospores	1 g	0 f
	10^3	1.3 fg	0.029 ef
	10^4	2 de	0.043 def
	10^5	2.2 de	0.071 de
D	Nm_{10}	1 l	0 i
	G_{10}	1 l	0 i
	Am_{10}	2.7 i	0.186 g
	GAm_{10}	1.5 k	0.025 i
	Nm_{20}	1 l	0 i
	G_{20}	1 l	0 i
	Am_{20}	3.3 h	0.121 h
	GAm_{20}	2.1 j	0.021 i

Nm, uninoculated; G, mycorrhized; Az, *A. euteiches* zoospores infected; sim Gaz, simultaneously inoculated with *A. euteiches* zoospores and with *G. mosseae*; GAz, pre-mycorrhized and post-infected with *A. euteiches* zoospores; Am, *A. euteiches* mycelium-infected; GAm, pre-mycorrhized and post-infected with *A. euteiches* mycelium. Results are expressed as the mean of nine replicates for disease index and of three replicates for ELISA. Statistical analyses were carried out independently for each experiment. Results with the same letter are not significantly different at $P = 0.05$ (Newman–Keuls test).

Fig. 1. Photograph of pea plants 10 d after *Aphanomyces euteiches* infection with 10^5 zoospores per pot. Left to right: *G. mosseae*-inoculated; uninoculated; pre-inoculated with *G. mosseae* for 15 d and *A. euteiches*-infected; and plants infected with *A. euteiches* alone.

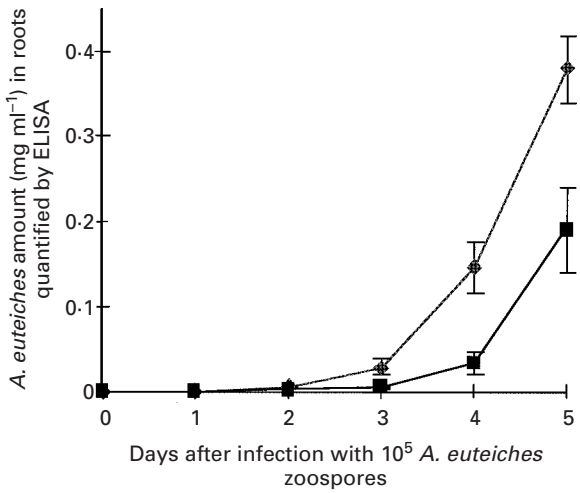

Fig. 2. Kinetics of *Aphanomyces euteiches* development in roots either infected with *A. euteiches* alone (diamonds), or pre-inoculated with *G. mosseae* and then *A. euteiches*-infected (squares) (Experiment E). Amount of *A. euteiches* in roots was quantified by ELISA 0, 1, 2, 4, 6 and 10 d.a.i.

This was tested in our experimental system by inoculating plants simultaneously with *G. mosseae* and 10^5 *A. euteiches* zoospores (Table 2, experiment B). After 10 d infection neither the root-rot rating scores nor the ELISA readings were significantly different between non-mycorrhizal and mycorrhizal plants. For pathogenic infection with *A. euteiches* zoospores, the root-rot rating score was always significantly higher in non-mycorrhizal than in pre-mycorrhizal plants (Table 2, experiment C). The different aspects of the root system corresponding to plants of experiment C inoculated with 10^5 zoospores are illustrated in Fig. 1. Inoculation of non-mycorrhizal plants with increased *A. euteiches* zoospore concentrations led to higher *A. euteiches* ELISA readings, although a plateau seemed to be reached following inoculation with 10^5 zoospores. In mycorrhizal plants post-infected with *A. euteiches*, the amounts of *A. euteiches* antigens were always significantly reduced compared with non-mycorrhizal plants (Table 2, experiment C). In plants infected with the *A. euteiches* mycelium (Table 2, experiment D), the root-rot rating score was higher in non-mycorrhizal plants compared with mycorrhizal plants post-infected with the pathogen. This result was supported by the quantification of *A. euteiches* antigens in the roots by ELISA.

The kinetics of *A. euteiches* development in mycorrhizal and non-mycorrhizal roots was followed (Fig. 2). In non mycorrhizal roots, a progressive increase in ELISA readings was observed from 2–10 d.a.i. In pre-mycorrhizal roots, this increase was

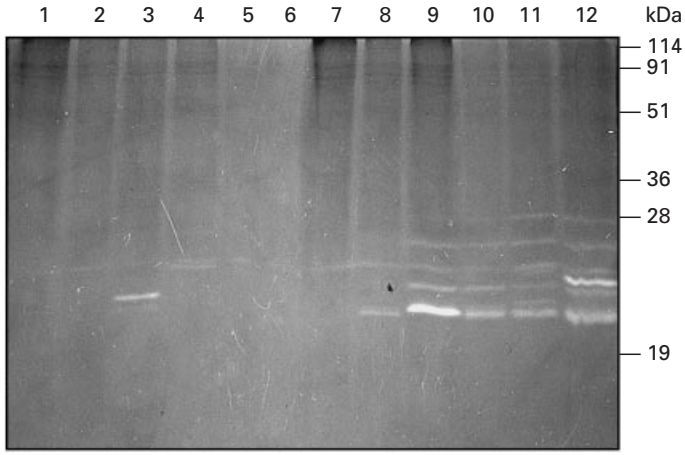

Fig. 3. Protease staining following SDS–PAGE of protein extracts from mycorrhizal or non-mycorrhizal roots of *Pisum sativum* cv. Frisson. 45 μg of total proteins were loaded per well. Samples corresponding to extracts from non-mycorrhizal (lanes 1–6) and mycorrhizal (lanes 7–12) roots after 4 (lanes 1 and 7), 6 (lanes 2 and 8), 10 (lanes 3 and 9), 15 (lanes 4 and 10), 20 (lanes 5 and 11), and 25 (lanes 6 and 12) d. 12 μl pre-stained low-molecular-mass markers from Bio-Rad were loaded, and their molecular mass is indicated in the right margin.

delayed and started between 4 and 6 d.a.i. At 10 d.a.i., ELISA readings were higher in non-mycorrhizal plants than in pre-mycorrhizal roots. This was supported by the root-rot rating score (data not shown).

Endoproteolytic activities

Following electrophoretic separation of proteins in gelatin-containing SDS–PAGE gels, maximum enzyme activity was obtained at neutral pH, but with a broad optimum pH from 5 to 9 (data not shown). According to their electrophoretic mobility, proteolytic bands were observed in two main regions, one for MW ranging from 91 to 114 kDa, and the other for MW between 19 and 28 kDa.

Faint proteolytic activities were detected in non-mycorrhizal roots aged from 6–25 d (Fig. 3, lanes 1–6, experiment A). Development of mycorrhizal symbiosis led to a progressive increase of endoproteolytic activities (Fig. 3, lanes 7–12). At 6 d.a.i. (Fig. 3, lane 8), when the first arbuscules were detected, two faint bands were observed between 19 and 28 kDa. At 10 and 15 d after inoculation with *G. mosseae*, i. e. when the colonization had reached an *F* of at least 65.5%, and an *A* of 2.8% and 11.9%, respectively (Fig. 3, lanes 9 and 10), two new bands appeared. Finally, after 20 and 25 d the endoproteolytic profile was more complex, with the induction of two other bands (Fig. 3, lanes 11 and 12).

The global changes in endoproteolytic activity after mycorrhizal or pathogenic infection were quantified spectrophotometrically. In experiment C, where mycorrhizal and non-mycorrhizal plants were inoculated with increasing amounts of zoospores, the endoproteolytic activities in mycorrhizal roots were twice as high as in control roots (Fig. 4, lane 1). In

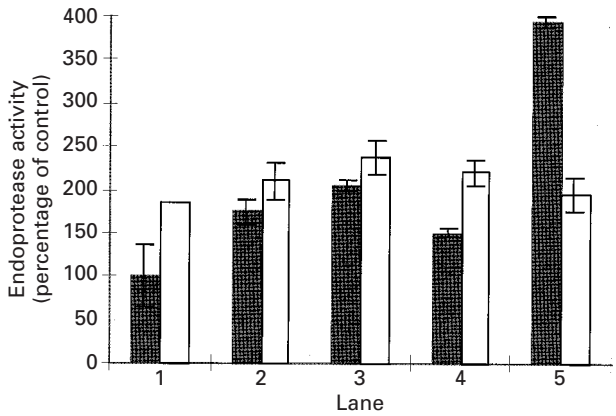

Fig. 4. Quantification by spectrophotometry of global endoproteolytic activities after infection of non-mycorrhizal (grey bars) or mycorrhizal (white bars) roots of *Pisum sativum* cv. Frisson with increasing amounts of *Aphanomyces euteiches* zoospores. Endoproteolytic activity was quantified in roots from control (lane 1) and inoculated with 10^2 (lane 2), 10^3 (lane 3), 10^4 (lane 4) and 10^5 (lane 5) zoospores. Changes in endoproteolytic activity were expressed relative to the activity detected in the control (100%). Values are the mean of three replicates (±SD).

non-mycorrhizal roots infected with zoospores, the endoproteolytic activity increased. The activity was 4-fold higher in roots infected with 10^5 zoospores, compared with control roots (Fig. 4, lane 5). In mycorrhizal roots post-infected with zoospores, no drastic increase in endoproteolytic activity was observed.

Proteolytic activity in root extracts from experiment C were also analysed after electrophoretic separation and compared to the pattern of endoproteolytic activities of the *in vitro*-produced mycelium of *A. euteiches*. Two faint bands between 91 and 114 kDa, and a strong endoproteolytic activity between 19 and 28 kDa, were detected for *in vitro*-produced mycelium of *A. euteiches* (Fig. 5a, lane 11;

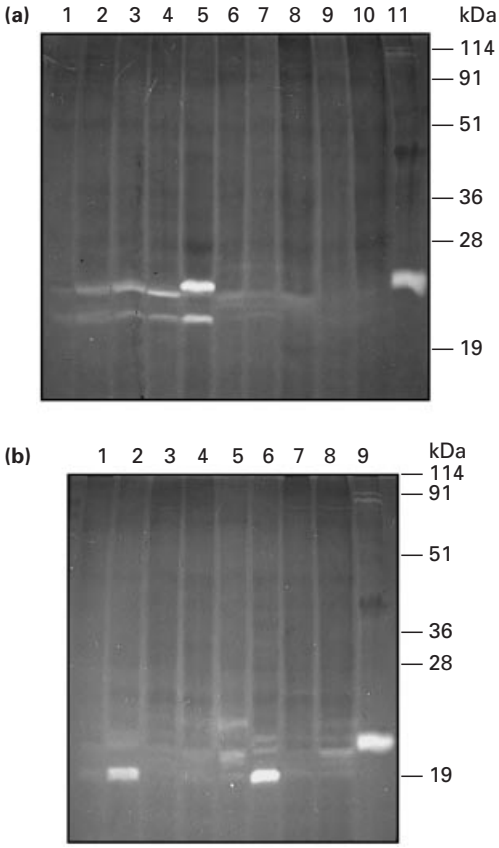

Fig. 5. Protease staining following SDS–PAGE of protein extracts of mycorrhizal or non-mycorrhizal roots of *Pisum sativum* cv. Frisson after inoculation either with increasing amounts of zoospores (a) or with mycelium (b) of *Aphanomyces euteiches*. 45 µg total proteins were loaded per well. (a) Samples correspond to extracts of non-mycorrhizal (lanes 1–5) or mycorrhizal (lanes 6–10) roots, and of *A. euteiches* mycelium (lane 11). Pea plants were infected with 10^2 (lanes 2 and 7), 10^3 (lanes 3 and 8), 10^4 (lanes 4 and 9) and 10^5 (lanes 5 and 10) zoospores. (b) Samples of extracts of non-mycorrhizal (lanes 1, 2, 5, 6) or mycorrhizal roots (lanes 3, 4, 7, 8) and of *A. euteiches* mycelium (lane 9). Pea plants infected with mycelium of pathogen were harvested 10 (lanes 1–4) and 20 (lanes 5–8) d.a.i. In both cases, 12 µl pre-stained low-molecular-mass markers from Bio-Rad were loaded, and their molecular mass is indicated in the right margin.

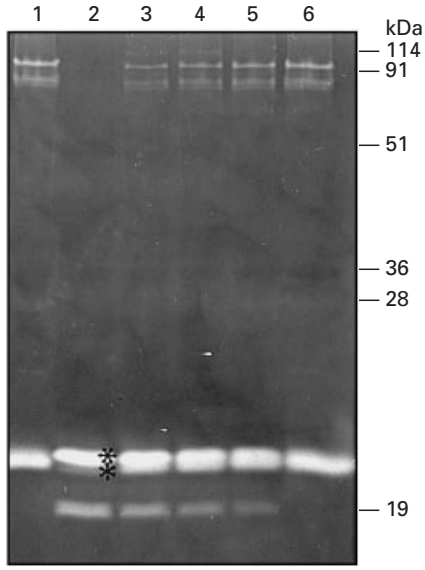

Fig. 6. Protease staining following co-electrophoresis in gelatin-containing SDS gels, of protein extracts of non-mycorrhizal roots of *Pisum sativum* cv. Frisson inoculated for 10 d with 10^5 zoospores, and of *in vitro*-produced mycelium of *Aphanomyces euteiches*. 45 µg total proteins were loaded per well. Samples loaded were of extracts of *A. euteiches* mycelium (lanes 1 and 6); non-mycorrhizal *A. euteiches*-infected roots (lane 2); three-quarters root extract mixed with one quarter mycelium extract (lane 3); half root extract mixed with half mycelium extract (lane 4); one quarter root extract mixed with three-quarters mycelium extract (lane 5). 12 µl pre-stained low-molecular-mass markers from Bio-Rad were loaded, and their molecular mass is indicated in the right margin. Stars in lane 2 indicate the presence of two isoforms.

Fig. 5b, lane 9). Endoproteolytic activities were strongly stimulated in response to increased amounts of zoospores, the strongest signal of the two bands (between 19 and 28 kDa) being obtained in plants inoculated with 10^5 zoospores (Fig. 5a, lane 5). In pre-mycorrhizal roots infected with zoospores (Fig. 5a, lanes 7–10), no modification of the endoproteolytic patterns was observed compared with mycorrhizal roots. On the contrary, in experiment B in which plants were co-inoculated with *G. mosseae* and *A. euteiches* and where no bioprotection was induced, endoproteolytic activities were as high in mycorrhizal roots as in control roots in response to pathogenic infection (data not shown). According to data from Fig. 4., global endoproteolytic activity was always higher in mycorrhizal roots than in non-mycorrhizal roots, except for plants infected with 10^5 *A. euteiches* zoospores. However, irrespective of the number of *A. euteiches* zoospores used, in gelatin-containing polyacrylamide gels we observed that endoproteolytic activity was higher in non-mycorrhizal than in mycorrhizal roots. Even if the inclusion of gelatin in polyacrylamide gels provides a sensitive way of detecting multiple proteolytic activities in crude extracts of plant origin (Michaud *et al.*, 1993), some endoproteases may not be revealed by this method. They might have more specific substrates or could be difficult to renaturate following SDS treatment (Michaud *et al.*, 1993).

In experiment D, where plants were infected with mycelium of *A. euteiches*, after 10 d infection the constitutive band with the higher mobility increased (Fig. 5b, lane 2). This proteolytic activity was strongly reinforced after 20 d infection (Fig. 5b, lane 6). In pre-mycorrhizal roots infected with *A. euteiches* mycelium (Fig. 5b, lanes 4 and 8), the endoproteolytic activity was only faintly modified as compared to mycorrhizal roots. Endoproteolytic activity which increased in response to mycelium infection differed from that reinforced in response to zoospore infections. Particularly between 19 and 28 kDa, the band which was strongly induced in response to the higher number of zoospores (Fig. 5a, lane 5) was only faintly expressed in roots infected

Table 3. *Effect of inhibitors or effectors on the activity of proteases in crude extracts*

Reagent	Concentration	Effect on protease activity
MCE	2 mM	No activation
DTT	2 mM	No activation
E-64	10 μM	No inhibition
Iodoacetic acid	1 mM	No inhibition
Bestatin	0.2 mM	Weak inhibition
Pepstatin A	0.24 mM	Weak inhibition
1, 10-phenanthrolin	2 mM	Weak inhibition
DCI	1 mM	Strong inhibition
Leupeptin	15 μM	Strong inhibition
TPCK	1 mM	No inhibition
TLCK	1 mM	Strong inhibition
PMSF	1 mM	Weak inhibition
EDTA	5 mM	Weak inhibition

with mycelium (Fig. 5b, lanes 2 and 6). This endoproteolytic band had a similar apparent MW to that expressed by *A. euteiches* mycelium (Fig. 5a, lane 11; Fig. 5b, lane 9). When the electrophoresis was repeated with 10% acrylamide gels, the band induced in pea roots infected with *A. euteiches* zoospores appeared to be composed of two separated bands migrating closely (Fig. 6, lane 2, stars). The upper band was interpreted as being of plant origin (Fig. 6, lane 2) while the lower one may originate from the fungus (Fig. 6, lanes 1–2). This result was confirmed by the co-electrophoresis of root and mycelium extracts mixed in different proportions (Fig. 6, lanes 3–5).

Effect of class-specific inhibitors on endoproteolytic activity

Endoproteolytic activity was further characterized by sensitivity to various protease inhibitors. All activities between 19 and 28 kDa showed the same response whatever the inhibitors used.

Thiol reagents such as β-Mercaptoethanol (MCE) and DTT had no stimulatory effect on protease activity (Table 3), and E-64 or iodoacetate had no inhibitory effect, suggesting that these activities do not belong to cysteine proteases. Other protease inhibitors, such as bestatin (an inhibitor of leucine aminopeptidase), pepstatin A (an inhibitor of acidic aspartyl protease), and 1,10 phenanthrolin (an inhibitor of metallo protease), had weak inhibitory activities of 17, 26 and 15%, respectively.

Endoproteolytic activities between 19 and 28 kDa were strongly inhibited by serine protease inhibitors such as DCI and leupeptin (93 and 91%, respectively), as illustrated for leupeptin (Fig. 7a, b, lanes 1–4). Interestingly, endoproteolytic activities between 91 and 114 kDa were only faintly inhibited. TLCK also gave good inhibition (82%), suggesting that these endoproteases could be trypsine-like serine proteases while they were just faintly affected

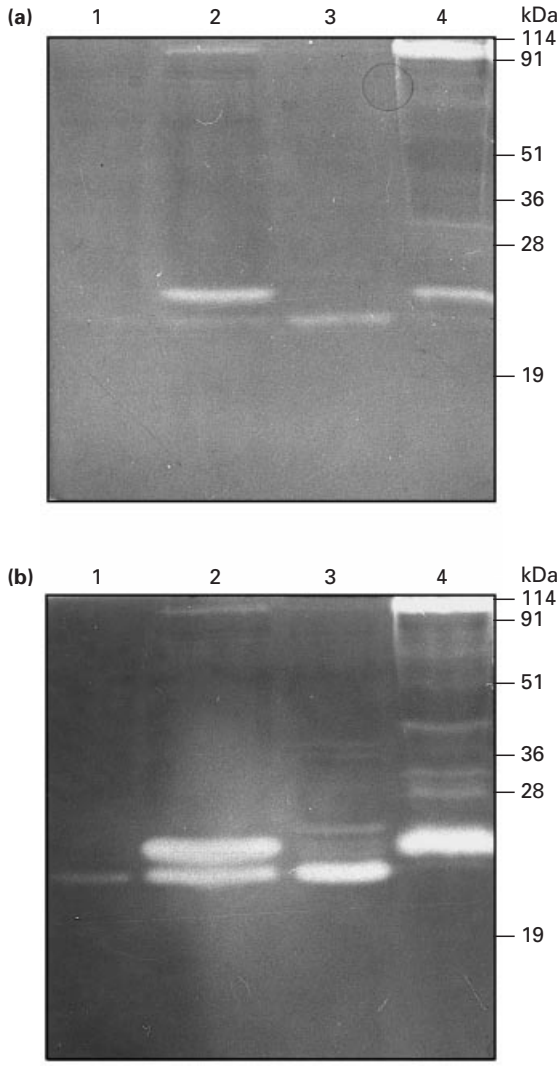

Fig. 7. Protease staining following SDS–PAGE (10 × 15 cm) of protein extracts (a) treated with leupeptin or (b) untreated. In both cases, samples loaded were of root extracts infected with mycorrhiza (lane 1), with *A. euteiches* zoospores at 10^5 (lane 2), and with *A. euteiches* mycelium (lane 3), and of *in vitro*-produced *A. euteiches* mycelium (lane 4). 12 μl pre-stained low-molecular-mass markers from Bio-Rad were loaded, and their molecular mass is indicated in the right margin.

by TPCK, an inhibitor of chymotrypsine-like serine proteases (21% inhibition). PMSF had a weaker inhibitory activity (70%) at the concentration tested (1 mM). These activities were also faintly affected by the removal of calcium ions with EDTA, which is required for the activity of subtilisin-like serine proteases (22% inhibition).

DISCUSSION

Quantification of Aphanomyces euteiches

The ELISA showed that the serum directed against *A. euteiches* mycelium recognized antigens produced by *A. euteiches* in pea roots. The severity of symptoms was positively correlated with the amount of pathogen detected *in planta*. This has been reported previously using another antiserum raised against *A. euteiches* (Kraft, 1994). Thus, in agreement with other reports (Werres & Steffens, 1994), the ELISA technique developed here offers an accurate alternative to quantification methods based on microscopical examinations of pea roots (Rosendahl, 1985).

Disease protection by Glomus mosseae

A protective effect expressed as a significant reduction in disease index was clearly demonstrated in mycorrhizal roots post-infected with either zoospores or mycelium. This protective effect was independent of the quantity or quality of the pathogenic inoculum. In agreement with other reports (Rosendahl, 1985; Kjøller & Rosendahl, 1997) the protective effect was maintained at the highest number of zoospores. This result is in agreement with Rosendahl (1985), who observed that *G. fasciculatum* and *G. intraradices* mycorrhizal pea roots were not damaged at the same degree as *A. euteiches*-infected roots. In parallel, the amount of pathogen was reduced by 50–100% in mycorrhizal roots post-infected with *A. euteiches* zoospores, and by 80% in mycelium-infected roots, as compared with the respective controls. This result is in good agreement with a previous report on pea root interactions with *G. intraradices* and *A. euteiches* (Rosendahl, 1985). This protective effect was obtained in plants for which the frequency of AM colonization reached about 70%. It required good root colonization by AM fungi. In fact, when plants were co-inoculated with *G. mosseae* and *A. euteiches*, no protective effect was observed. In that case, all parameters of mycorrhization were significantly affected by pathogenic inoculation and the frequency of mycorrhization only increased up to 43.1%. This result implies that (i) some competition exists for either infection sites or colonization, as suggested by Hooker *et al.* (1994) and Azcón-Aguilar & Barea (1996); and (ii) that low levels of mycorrhizal colonization are not sufficient to induce protection. The latter point was reported by Cordier *et al.* (1998) who studied the interaction between mycorrhizal tomato roots and the Oomycete fungus *Phytophthora parasitica*.

Pathogenic mycelium was also able to infect non-mycorrhizal roots, but failed to infect mycorrhizal roots. A similar result has been reported in tomato roots infected with *P. parasitica* (Dassi *et al.*, 1998). The mycelium of *A. euteiches* may not directly penetrate roots, but produces zoospores which could in turn infect roots. Consequently, there is a delay between the inoculation time and the root infection process. M. Giovannetti *et al.* (unpublished) reported that the presence of *G. mosseae* drastically affected the percentage of zoospore germination in *P. parasitica*. In plants infected with zoospores, we observed a delay in the development of the pathogen in pre-mycorrhizal roots as compared with plants infected with *A. euteiches* alone. Therefore, we can speculate that the presence of *G. mosseae* could on the one hand, reduce the amount of zoospores produced by *A. euteiches* mycelium, and on the other hand, reduce the percentage of zoospore germination when plants were infected with increasing amounts of zoospores. Recently, Wilarso Budi *et al.* (1998) reported that bacteria isolated in sporocarps of *G. mosseae* could have an *in vitro* antagonistic activity against *A. euteiches*.

Endoproteolytic activities in AM versus pathogenic interactions

We have shown that colonization of pea roots by the AM fungus *G. mosseae* leads to an increase in global protease activities measured spectrophotometrically. This increase results from both a weak stimulation of some constitutive endoproteolytic activities, and the induction of additional ones. These activities were further characterized for their sensitivity to protease inhibitors and our results strongly suggest that they are trypsin-like serine proteases. To our knowledge, this is the first report of the presence of endoproteolytic activities in AM symbiosis. Moreover, we demonstrate here an increase in endoproteolytic activity during the development of mycorrhizal symbiosis, especially during the enrichment in arbuscules. Different reports have dealt with the implication of proteases in actinorrhizal (Ribeiro *et al.*, 1995) and nitrogen-fixing symbioses (Pladys *et al.*, 1986, 1991; Pladys & Vance, 1993; Kardailsky & Brewin, 1996). In particular, two classes of proteases, characterized as serine and cysteine proteases, respectively, have been suggested to play a role in the development and senescence of nodules (Pladys *et al.*, 1986; Manen *et al.*, 1991; Pladys & Vance, 1993; Ribeiro *et al.*, 1995; Kardailsky & Brewin, 1996). Several reports have previously described the differential expression of defence genes throughout the development of the mycorrhizal symbiosis

(Spanu *et al.*, 1989; Blee & Anderson, 1996; Gianinazzi-Pearson, 1996). They concluded that the localization of defence gene transcripts is compatible with the transient nature of arbuscules. Therefore, proteolytic enzymes may participate in the development and/or senescence of arbuscules.

Endoproteolytic activities were strongly stimulated in plants infected with the pathogen alone, and we found a positive relationship between the enhancement of the activity and the number of zoospores used to inoculate pea plants. This result is in good agreement with previous reports dealing with plant–pathogen interactions. An accumulation of a so-called PR protein characterized as an alkaline protease in response to citrus exocortis viroid infection of tomato leaves was reported by Vera & Conejero (1989), while an increase in protease activities occurred in tobacco leaves inoculated with tobacco mosaic virus (Lusso & Kuc, 1995). To further understand the role of endoproteolytic activity in the pathogenic process, we qualitatively analysed activity following separation by SDS gel electrophoresis. We confirmed the strong induction of an endoproteolytic activity which was further characterized as a trypsin-like serine protease and which could be of plant origin. Surprisingly, a distinct trypsin-like serine protease was shown to be stimulated in response to mycelium infection. These two endoproteolytic activities could therefore be new PR proteins, defined by van Loon *et al.* (1994) as plant proteins that are induced in pathological situations. In that case, pathological situations correspond to all types of infected states and not just to resistant, hypersensitive responses in which PR proteins are most common. It is now well established that PR proteins are produced during plant responses to infection by pathogenic microorganisms. In our case, the induction of endoproteolytic activities in *A. euteiches*-infected pea roots evolved in parallel to the development of symptoms and spread of the pathogenic fungus through the roots. This activity may be related to the senescing process as reported by Mohanty & Sridhar (1986) and Vera & Conejero (1989) in other plant–pathogen interactions. As underlined by Schaller & Ryan (1996), they could be implicated in the breakdown of proteins in the senescent parts of the roots to allow transport of amino acids into the growing parts. However, we cannot exclude the possibility that activity induced in response to *A. euteiches* infection could be implicated in a more specific mechanism, as recently suggested in another interaction (Lusso & Kuc, 1995). The fact that endoproteolytic activity was differentially expressed in response to infection with either zoospores or mycelium of *A. euteiches* could indicate a more specific role in pathogenesis. These may be enzymes with a unique activity against specific fungal proteins.

We have also shown that one of the induced endoproteolytic activities could be of fungal origin. In a pathogenic interaction, North (1982) has suggested that proteolytic enzymes may be needed by the pathogen to penetrate the host tissue or to utilize host proteins for nutrition. Several authors reported a correlation between pathogenicity and proteolytic activities (Pladys & Esquerré-Tugaye, 1974; Dobinson *et al.*, 1997; Gunnlaugsdottir & Gudmundsdottir, 1997; Rodier *et al.*, 1997). As reported by Kjøller & Rosendahl (1998), infection of pea roots by *A. euteiches* is initiated by rapid colonization by metabolically active hyphae, followed by oospore formation. This rapid colonization suggests the involvement of hydrolytic enzymes during invasion of the host tissue (Papavizas & Ayers, 1974), and fungal endoproteolytic enzymes could therefore participate in this process.

Regulation of endoproteolytic activities in plant protection

Plant endoproteolytic activities induced in response to *A. euteiches* infection were not induced in mycorrhizal plants. This lack of induction was correlated with a significant reduction of symptoms in roots of infected mycorrhizal plants, suggesting a possible role of these activities in the process of senescence. At the same time, only low levels of *A. euteiches* endoproteolytic activity, which may play an important role in the colonization of the host tissues, could be seen in these roots. This could be simply a consequence of the reduction of the amount of pathogen in mycorrhizal roots, but could also result from an inhibition of the synthesis of proteases originating from the pathogen in these roots. It is also possible that these proteases are actually synthesized but inactivated by specific protease inhibitors. Several authors have suggested that a low priming of defence genes following the establishment of AM symbiosis could lead to a stronger and quicker response of the plant to subsequent attack by a pathogen (Dehne, 1982; Rosendahl, 1985; Caron, 1989; Gianinazzi, 1991; Linderman, 1994; St Arnaud *et al.*, 1995; Azcón-Aguilar & Barea, 1996; Gianinazzi-Pearson, 1996). From this point of view it would be interesting to determine whether such protease inhibitors can be demonstrated in mycorrhizal roots post-infected with *A. euteiches*. This kind of experiment could assist in better understanding of the biochemical basis of bioprotection by AM fungi against *A. euteiches* in pea roots.

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