Mycol. Res. **106** (4): 499–506 (April 2002). © The British Mycological Society
DOI: 10.1017/S0953756202005798 *Printed in the United Kingdom.*

Pre-penetration events in fungal parasitism of nematode eggs

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Received 6 July 2001; accepted 16 March 2002.

The present investigation deals with the main factors involved in early infection of nematode eggs by fungal parasites. We studied the effect of hydrophobicity on appressorium formation by germlings of *Pochonia rubescens* (syn. *Verticillium suchlasporium*), *P. chlamydosporia* (syn. *V. chlamydosporium*) and *Lecanicillium lecanii* (syn. *V. lecanii*). Appressoria were frequently formed on hydrophobic surfaces such as polyvinyl chloride or polystyrene and were infrequently formed on hydrophilic materials such as glass or aluminium. Infected eggs probed with the FITC-labelled lectin Concanavalin-A showed intense labelling corresponding to appressoria formed by fungal parasites on the eggshell surface. Proteolytic activity was found in extracts from conidia and germlings of fungal parasites (especially *P. chlamydosporia*) in the absence of nematode eggs. Addition of the serine proteinase inhibitors phenylmetylsulphonyl fluoride (PMSF) or diisopropyl fluorophosphonate (DFP) to the extracts reduced their proteolytic activity. PMSF was the most effective inhibitor. Zymography also revealed proteolytic activity in extracts from the three fungi tested. This activity mostly corresponded to bands of Rf's of substrate degradation similar to that of purified main protease (P32) from *P. rubescens*. Other bands with molecular weight higher than P32 (low Rf) were found especially for *P. chlamydosporia* extracts. For *L. lecanii* only bands of low Rf were found. Serum anti-P32 partially inhibited proteolytic activity of extracts from conidia and germlings. Application of PMSF and DFP to the inoculum, reduced egg penetration for the three species studied. PMSF caused the highest reduction in eggs infected by *L. lecanii*, while DFP significantly reduced egg infection by both *P. chlamydosporia* and *L. lecanii*. Our results therefore show hydrophobicity, appresorium formation and protease production as factors involved in early parasitism of nematode eggs.

INTRODUCTION

Fungal parasites of nematode eggs have great potential as biocontrol agents since they also infect females of sedentary nematodes and destroy the eggs they contain (Stirling 1991). The development of these antagonists as biocontrol agents requires a deep knowledge of the steps involved in their pathogenicity against nematodes. Prior to infection, egg parasitic fungi must adhere to the eggshell, then penetrate this barrier that protects the nematode from the environment (Jansson & Lopez-Llorca 2001). The mode of host penetration (enzymatic vs. mechanic) by fungal pathogens has been a matter of controversy for many years (Money 2001). Early host-pathogen interactions in fungal plant pathogens include involvement of both physical and chemical signals, which influence appressoria formation (Kolattukudy *et al.* 1995).

Before penetration, spores and penetration structures, such as appressoria, must adhere to the host surface. Appressoria can be defined as hyphal structures formed by fungal pathogens to adhere to their host's surface. These structures are an adaptation to concentrate mechanical force and enzymatic degradation in a small area thus facilitating host penetration (St Leger 1993). Adhesion and formation of appressoria are well known for several plant pathogens such as *Uromyces appendiculatus* (Kwon & Hoch 1991), and entomopathogenic fungi such as *Metarhizium anisopliae* (St Leger *et al.* 1989).

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Appressoria of the nematode egg parasite *Pochonia rubescens* (syn. *Verticillium suchlasporium*) have been studied using scanning electron microscopy (SEM) (Lopez-Llorca & Claugher 1990). Presence of mucilaginous material between the surface of appressoria and the eggshell was observed. This material could function as a fungal adhesive to assist in eggshell penetration by the fungus. Similar material was found in *Dactylella oviparasitica* appressoria infecting *Meloidogyne* spp. eggs (Stirling & Mankau 1979). The extracellular material of most fungi contains (glyco)proteins and carbohydrates (Xiao *et al*. 1994, Bircher & Hohl 1997, Jansson & Friman 1999, Apoga & Jansson 2000).

Here we describe the formation of appressoria on artificial surfaces by the nematophagous fungi *P. rubescens*, *P. chlamydosporia* (syn. *V. chlamydosporium*) and *Lecanicillium lecanii* (syn. *V. lecanii*). We also investigated the presence of carbohydrates on conidia, germlings and appressoria formed on nematode eggs upon infection. We have also detected proteolytic activity in ungerminated conidia and germlings in the absence of eggs. The effect of protease inhibitors on egg infection by these fungal parasites of nematode eggs is also reported.

MATERIALS AND METHODS

Fungi and nematodes

Three egg-parasitic nematophagous fungi were used for the experiments. *Pochonia rubescens* (CBS 464.88, ATCC 76547), *P. chlamydosporia* and *L. lecanii* were isolated from infected eggs of *Heterodera avenae* in Dundee (Scotland), Sevilla (south-west Spain) and Girona (north-east Spain) respectively, using a growth-restricting medium (Lopez-Llorca & Duncan 1986). All strains were kept on corn meal agar (CMA) at 4 °C.

The plant-parasitic nematode *Meloidogyne javanica* was grown monoxenically on excised tomato roots transformed with *Agrobacterium rhizogenes* in agar plates (Gamborg's B-5, 20 g l^{-1} saccharose, 1 % agar) in the dark at 25 ° (Verdejo-Lucas 1995). The nematodes were sub-cultured every 4–5 weeks.

Infection of nematodes

Egg-masses from monoxenic cultures of *Meloidogyne javanica* were dissected axenically. Nematode eggs (25–50) were axenically spread on a 1 % water agar (WA) plate using a glass rod. Each egg was inoculated with 10 µl of a 10^6 conidia ml^{-1} suspension of a fungal egg-parasite. After inoculation, 3 plates per fungal species were incubated at 25 ° in the dark, and were scored daily for fungal infection. Eggs surrounded by a dense fungal colony were classified as infected and the percentage of egg infection was then calculated. The final percentage of infection was the average of percentages scored for three plates per fungal strain. Ten infected eggs were sampled at random from plates and mounted on microscopy slides with water. The number of hyphae crossing the eggshell per individual egg was scored. Egg penetration was estimated as the average number of hyphae per egg, to indicate the severity of egg infection by individual fungal strains.

Effect of surface hydrophobicity on appressorium development

Conidia of either *Pochonia rubescens* or *P. chlamydosporia* were incubated at 25 ° for 24 h in 0.0125 % (w/v) yeast extract in water (YEM) as described by St Leger *et al*. (1989). Conidial germination was estimated by counting the number of conidia bearing a germ tube longer than the spore width, in three 100-conidia-aliquots at 4 h intervals.

Germination of conidia of *P. rubescens* or *P. chlamydosporia* in YEM medium showed that, although some differences in the time-course of germination were found between the fungi, all conidia had germinated after 20–24 h.

Drops (100 µl each) of conidial suspensions (10^6 conidia ml^{-1}) in YEM of either *P. rubescens*, *P. chlamydosporia* or *L. lecanii* were incubated on 15×15 mm squares of the test materials (surfaces). These surfaces included in order of increasing hydrophobicity (according to St Leger *et al*. 1989): cellophane (precut cellophan sheets, Hoefer-Pharmacia Biotech), aluminium foil (Freshmate, Vileda), glass coverslips (Menzel-Glaser, Germany), polystyrene (Petri dish, Bibby Sterilin, UK), polyvinyl chloride (PVC) and teflon (Biorad, USA). Squares of these materials were washed in sterile distilled water and blotted dry on to sterile filter paper before sterilizing. Cellophane, alumnium and glass squares were sterilised by autoclaving. PVC and teflon were rinsed in absolute ethanol and dried before use. Four squares of each material (with one drop of conidial suspension each) were placed in a 8.5 cm diam Petri dish. Petri dishes were placed in moist chambers and were incubated at 25 ° in the dark. Three replicate plates per surface and fungus combination were set up. Conidia on the surfaces were examined microscopically 20–24 h after inoculation on the surfaces to detect appressorium formation. Conidia on non-transparent surfaces were stained with 0.1–1 % Calcofluor white and observed with a fluorescence microscope (Olympus BH-2) with appropriate filters. The experiment was carried out three times.

Lectin labelling of fungal structures

Fluorescein isothiocyanate (FITC) conjugates of the following lectins were used in labelling experiments: Concanavalin A (Con A), *Ulex europaeus* agglutinin (UEA-I), soybean agglutinin (SBA) and wheat germ agglutinin (WGA). For tests of binding specificity, the following blocking sugars with their corresponding

lectins (in brackets) were used: D-methyl-mannopyranoside (Con A), L-fucose (UEA-I), *N*-acetyl-D-galactosamine (SBA) and *N*-acetyl-D-glucosamine (WGA). All chemicals were purchased from Sigma.

The following fungal structures were lectin-labelled as described by Forrest & Robertson (1986). Conidia and germlings were obtained by shaking (100 rpm) for 16 h at either 4 or 25 ° freshly extracted (ungerminated) conidia (10^6 conidia ml^{-1}) in water. Germlings on polystyrene were obtained after 24 h incubation as explained above. *M. javanica* infected eggs 7 d after inoculation with conidia of either *P. rubescens* or *P. chlamydosporia* (10^4 conidia per egg) at 25 ° on WA plates were also labelled. Samples were first washed three times in cold PBS, pH 7.4. Washed samples were then incubated in 200 µl of FITC-lectin conjugates (100 mg ml^{-1} in PBS) for 2 h at 4 °. As controls, the appropriate competing sugar hapten was included at a concentration of 200 mM. After 2 h the samples were washed three times in PBS, mounted on glass slides and observed with a fluorescence microscope.

Proteolytic activity in conidia and germlings

Conidial suspensions of *Pochonia rubescens*, *P. chlamydosporia* and *Lecanicillium lecanii* (0.7×10^7 conidia ml^{-1}) in sterile distilled water were prepared. Aliquots (1.5 ml) were either kept at 4 ° to prevent germination or incubated with gentle shaking at room temperature for 16 h. Then, ungerminated conidia and germling suspensions were centrifuged at 13200 ***g*** for 5 min. Supernatants were kept on ice. Proteolytic activity was estimated using 100 mM N-Succinyl-Ala-Ala-Pro-Leu *p*-Nitroanilide (Sigma) in dimethyl sulphoxide as substrate. A typical assay consisted of 250 µl of supernatant, 250 µl sterile distilled water, 490 µl 20 mM Tris/HCl buffer pH 8.0 and 10 µl substrate. Assay reaction mixtures were incubated at 37 ° for 0.5, 3, 8 and 20 h. Proteolytic activity was estimated as absorbance at 410 nm (A_{410}). Since the majority of the proteases of *P. rubescens* and *P. chlamydosporia* are serine proteases (Lopez-Llorca 1990; Segers *et al.* 1994), two specific inhibitors for such activities, phenylmethylsulphonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP), were also included in the proteolytic assays. PMSF was added at 0.2, 0.5 or 1 mM, whereas DFP was included at 1, 2.5 or 5 mM in the assay mixture. Inhibitors were from Sigma. Rabbit polyclonal antisera anti *P. rubescens* P32 (anti-P32) protease (Lopez-Llorca & Robertson 1992) were also used to block, at 1:200 dilution, proteolytic activity of extracts instead of chemical inhibitors. Rabbit preimmune serum was used for controls.

Proteolytic activity of fungal extracts was also examined electrophoretically (zymography). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli (1970), using 0.75 mm width polyacrylamide gels (12% in separation gel and 3.9% in stacking gel) in a Mini-Protean II Cell (Bio–rad, USA). SDS–PAGE gels also contained 0.1% gelatin. Samples were applied to gels in buffer with no β-mercaptoethanol and unboiled (semi-native conditions). After electrophoresis, gels were incubated shaking (100 rpm) at 35 ° for 1 h in 50 mM Tris/HCl (pH 8.0) buffer with 2.5% Triton X-100. Electrophoresis gels were then incubated at 30 ° for 1–2 h in 50 mM Tris/HCl (pH 7.5) buffer. Gels were stained with 0.5% Coomassie brilliant blue (Sigma) in 40% methanol and 10% acetic acid. Destaining was carried out in the same solvent mixture but with no stain. Proteolytic activity was revealed as unstained white bands on a blue background of non-digested gelatin.

Effect of serine protease inhibitors on egg-infection

Primarily, the effect of PMSF (1, 2 or 5 mM) and DFP (2 or 5 mM) on viability (as percentage of conidial germination on CMA) of the fungi *Pochonia rubescens*, *P. chlamydosporia*, and *Lecanocillium lecanii* was estimated. Two to three CMA Petri dishes per fungus were each inoculated with approximately 100 conidia resuspended in inhibitor solutions at the concentrations described above. Control plates were inoculated with conidia in sterile distilled water. After 5 days incubation, fungal colonies on the plates were scored.

Nematode eggs were inoculated with conidia of each fungus as described above. Ten-µl-drops of either PMSF (0.2, 0.5, 1, 2 or 5 mM) or DFP (2 or 5 mM), were placed on each egg at the time of fungal inoculation. Inhibitors were reapplied every 12 h during the 48 h incubation of eggs on the plates. Controls were eggs inoculated with fungi and with drops of sterile distilled water. After 48 h incubation, the percentages of egg infection and egg penetration were determined as described above.

Statistical analyses

SPSS package version 10.0 was used for data analyses using one way ANOVA and Duncan's multiple range test.

RESULTS

Induction of appressoria

The three fungi formed appressoria less often on hydrophilic materials such as aluminium foil (Figs 1–2) or glass (Fig. 3) than on hydrophobic surfaces such as polystyrene (Fig. 4) or PVC (Fig. 5). These trends were similar for all three species studied. The capacity to differentiate and form appressoria varied with the species. *Pochonia chlamydosporia* was the most effective fungus forming most appressoria on all materials except PVC (Fig. 1). *Lecanocillium lecanii* formed the least appressoria on most materials.

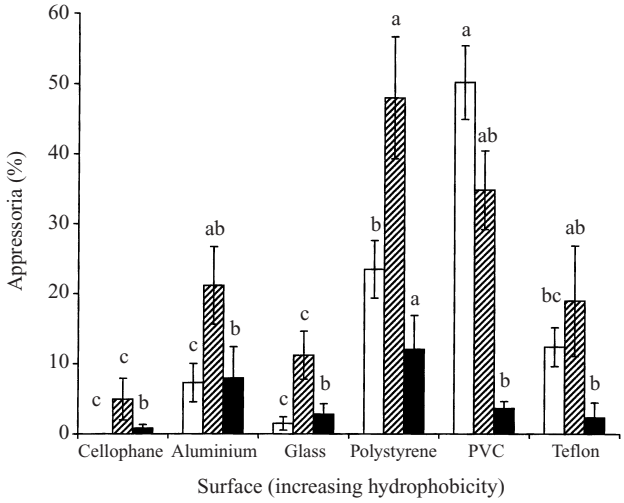


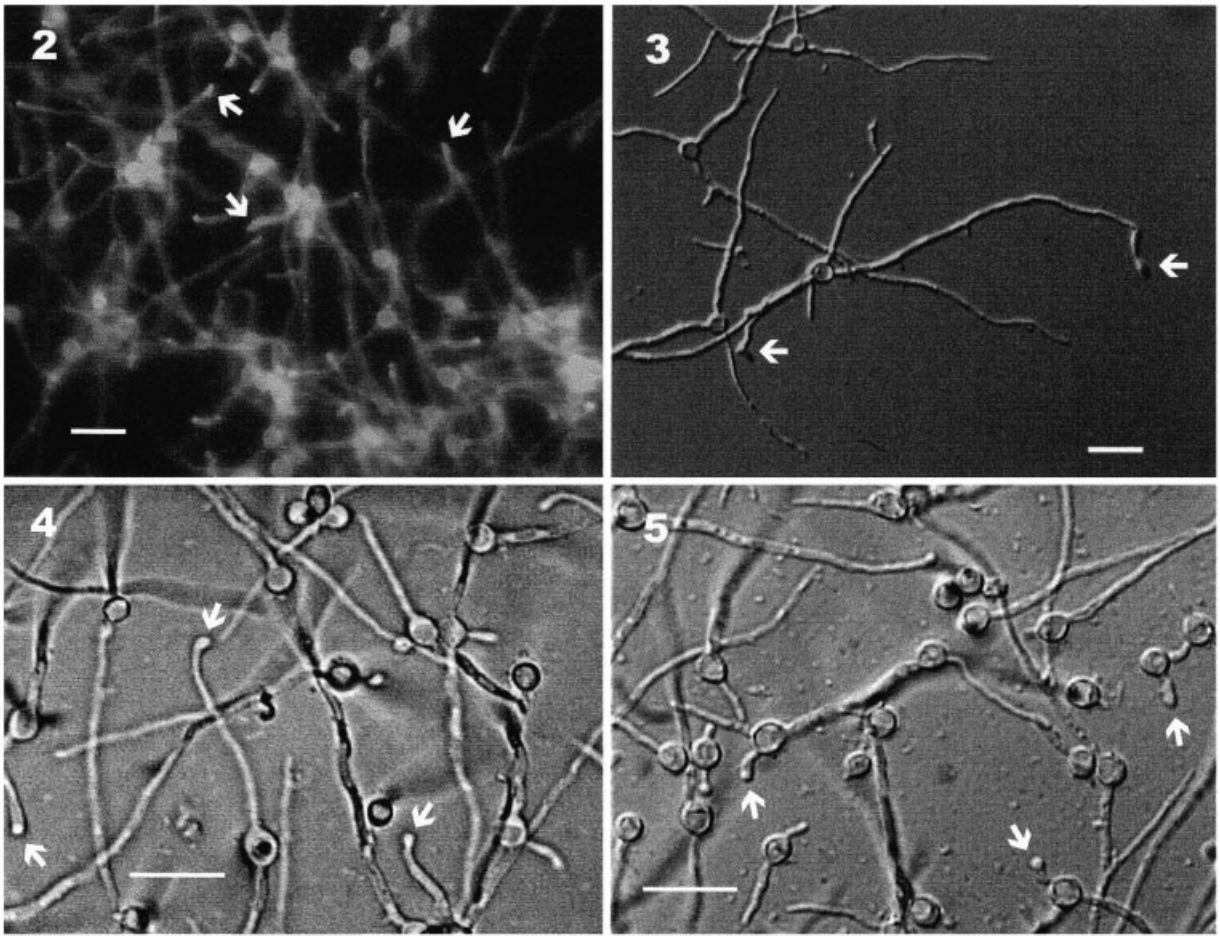
Fig. 1. Effect of surface hydrophobicity of various materials on appressorium formation by germlings of *Pochonia rubescens* (open bar), *P. chlamydosporia* (hatched bar) and *Lecanocillium lecanii* (solid bar) in YEM medium, 24 h after incubation at 25 °C in the dark. Abbreviation: PVC (Polyvinyl chloride). Values are averages of three similar experiments. For each fungus, same letter indicates homogeneous group according to Duncan's Multiple range test. Error bars are standard errors of the means.

Lectin labelling of extracellular material associated with fungal structures

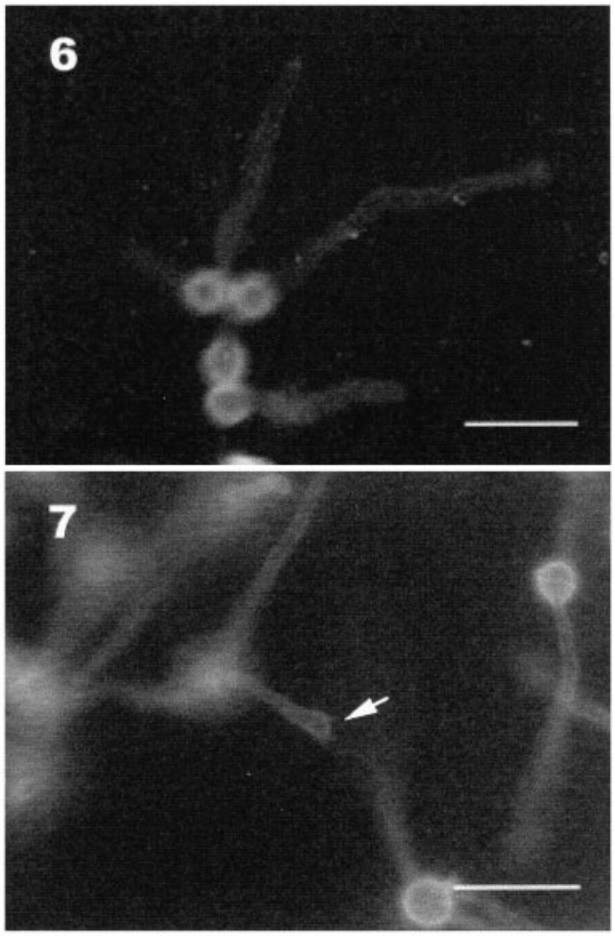
FITC-labelled lectins (Con A, SBA, UEA and WGA) were used to probe conidia, germlings or infected eggs. Of the four lectins tested, only Con A specifically labelled fungal structures. Walls of ungerminated conidia as well as those of 16 h germlings in water (no surface) were strongly labelled (Fig. 6). Labelling was less intense for *Pochonia rubescens* than for *P. chlamydosporia*. The conidial wall of germlings on polystyrene was also labelled by Con A (Fig. 7, arrow). Con A labelling of germ tubes and appressoria walls was fainter than that of conidia walls. When infected eggs (Fig. 8) were probed with lectins, ConA showed specific labelling in the hyphal wall (Fig. 9). However, fluorescence was brightest in appressoria formed by the fungal parasites on the eggshell.

Extracellular proteolytic activity in conidia and germlings of egg-parasites in absence of inducing substrate

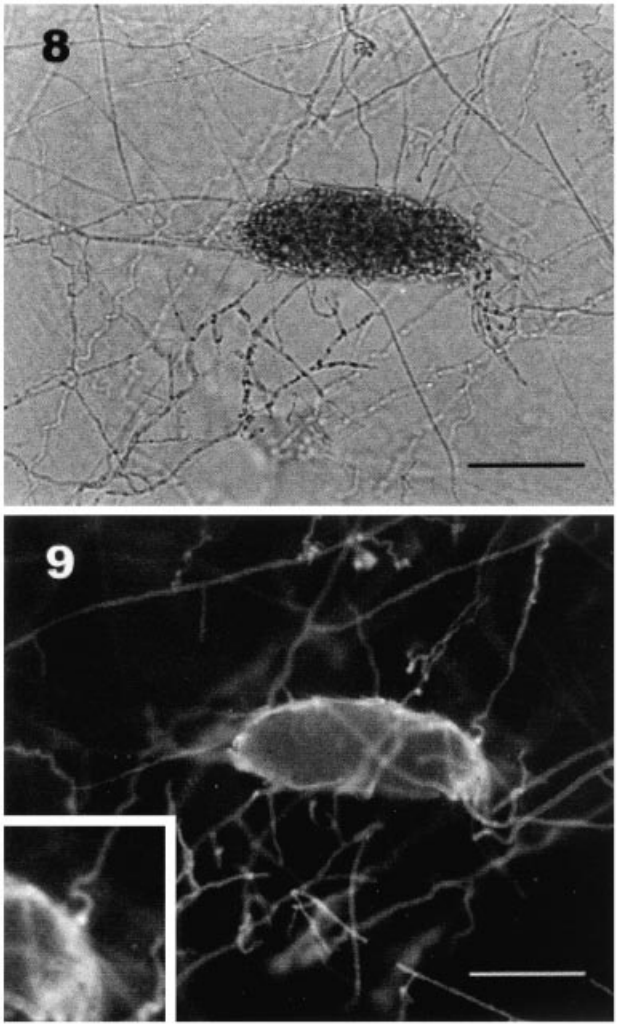
Aqueous extracts from both conidia and germlings showed proteolytic activity (Figs 10–12). *Pochonia*



Figs 2–5. Micrographs of 24 h germlings of *Pochonia chlamydosporia* on several materials. Hydrophilic surfaces: **Fig. 2.** Aluminium foil. **Fig. 3.** Glass. Hydrophobic surfaces: **Fig. 4.** Polystyrene. **Fig. 5**. PVC. Arrows indicate appressoria in germ tubes. Bars = 5 μm.



Figs 6–7. Staining of *Pochonia chlamydosporia* germlings with Concanavalin A lectin fluorescently labelled (Con A-FITC). **Fig. 6.** 16 h germlings in water (no surface). **Fig. 7.** 24 h germlings on polystyrene. Note intense labelling in conidial walls and fainter in germ tubes and appressoria (arrow). Bars = 5 μm.



Figs 8–9. Con A-FITC staining of *Pochonia rubescens* infecting *Meloidogyne javanica* egg. **Fig. 8.** Normal illumination. **Fig. 9.** UV light. Note intense labelling in appressoria on egg shell (insert). Bars = 50 μm.

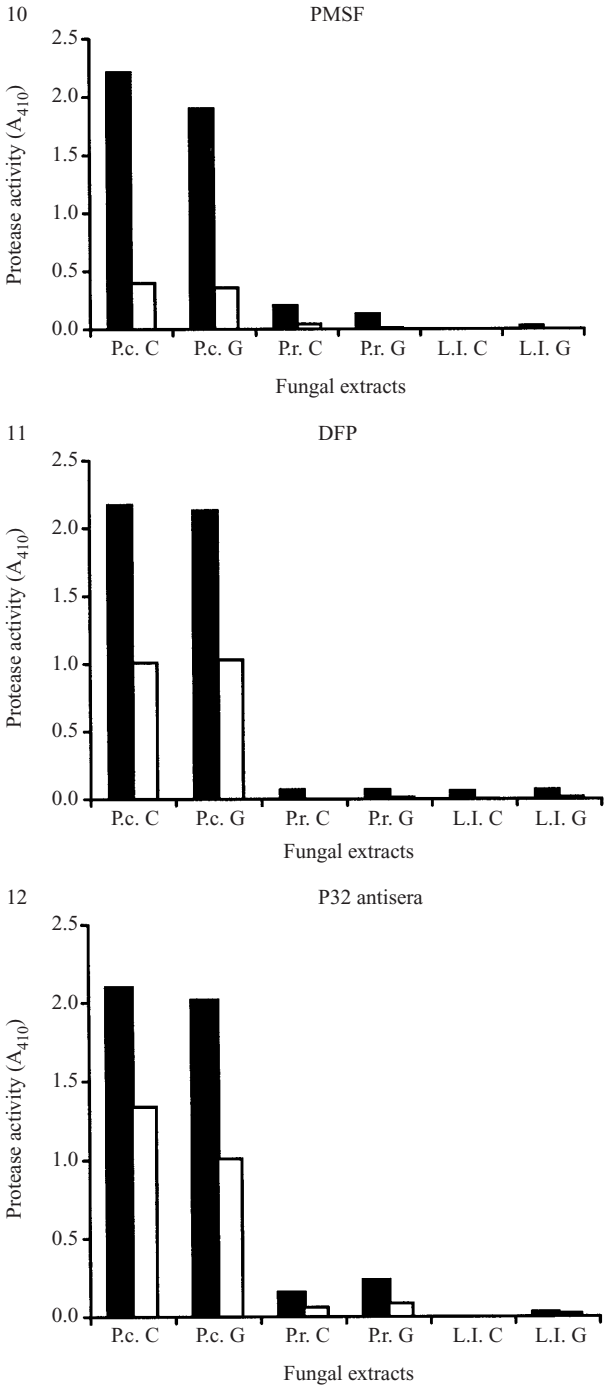
chlamydosporia had the highest activity of the three species tested (nearly six times more activity than *P. rubescens*). Extracts from *Lecanocillium lecanii* had the lowest proteolytic activity. Addition of serine proteinase inhibitors (PMSF or DFP) to the extracts reduced their proteolytic activity. PMSF virtually abolished proteolytic activity of *P. rubescens* extracts, even at 0.2 mM (Fig. 10). PMSF had the same inhibitory effect on extracts of *P. chlamydosporia* (Fig. 10). DFP also reduced the proteolytic activity of fungal extracts (Fig. 11) but to a lesser extent than PMSF.

Serum anti-P32 partially inhibited proteolytic activity of extracts from conidia and germlings (Fig. 12). A reduction was found for extracts of conidia and germlings of *P. chlamydosporia* and *P. rubescens*. Controls (treated with preimmune serum) showed similar proteolytic activity (Fig. 12) to that found for extracts with no chemical inhibitors (Figs 10–11).

Proteolytic activity of aqueous extracts from conidia and germlings was also analysed electrophoretically. Zymography revealed proteolytic activity in extracts from the three fungi tested (Fig. 13). As in the previous assays, *P. chlamydosporia* and *P. rubescens* showed the highest activity. This activity corresponded to bands of Rf's of substrate degradation similar to that of purified P32 (0.29). For extracts from *P. rubescens* these bands were 0.30 (conidia) or 0.26–0.32 (germlings). For *P. chlamydosporia* these were: 0.27 (conidia) and 0.29–0.33 (germlings). Other bands with molecular weight higher than P32 (low Rf) were found especially for *P. chlamydosporia* extracts. These were 0.24 for extracts of condia and germlings of *P. chlamydosporia* and for germlings of *P. rubescens*. Germlings and conidia of *P. chlamydosporia* also showed another very clear band of Rf 0.14 and 0.12, respectively. For *L. lecanii* only bands of low Rf were found (0.15 for both conidia and germling extracts).

Effect of serine protease inhibitors in egg-infection

Conidial germination was similar on control plates (with no inhibitors) and on those with either PMSF or



Figs 10–12. Proteolytic activity of aqueous extracts from conidia (C) and germlings (G) of *Pochonia rubescens* (P.r.), *P. chlamydosporia* (P.c.), and *Lecanocillium lecanii* (L.l.) and effect of serine protease inhibitors. **Fig. 10.** 0.2 mM phenyl-methyl-sulphonyl-fluoride (PMSF, open bar); **Fig. 11.** 5 mM Diisopropyl fluoro phosphate (DFP, open bar). Values are averages of two estimates per sample. **Fig. 12.** Antisera anti *P. rubescens* protease (anti-P32) at 1:200 (open bar) dilution. Controls are solid bars.

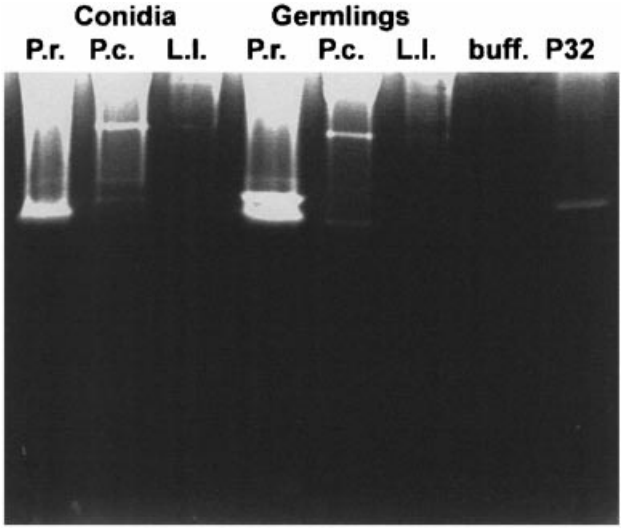
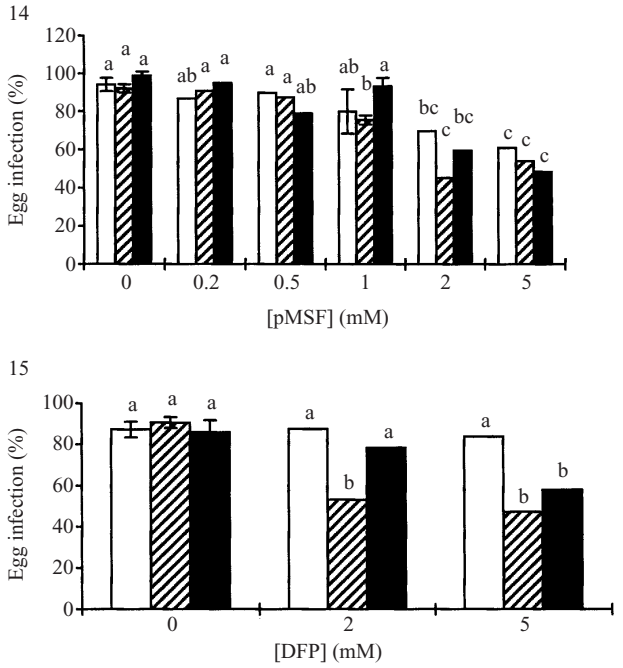


Fig. 13. Zymogram for proteolytic activity of aqueous extracts from conidia or germlings of *Pochonia rubescens* (P.r., conidia, P.r., germlings), *P. chlamydosporia* (P.c., conidia, P.c., germlings) and *Lecanocillium lecanii* (L.l., conidia; L.l., germlings). Buff.: sample buffer, P32: Purified serine protease from *P. rubescens*.



Figs 14–15. Effect of serine protease inhibitors on the pathogenicity (% of *Meloidogyne javanica* eggs infected) of three fungal parasites (*Pochonia rubescens*, open bar; *P. chlamydosporia*, hatched bar, and *Lecanocillium lecanii*, solid bar). **Fig. 14.** Eggs treated with PMSF; **Fig. 15.** Eggs treated with DFP. For each fungus, same letter indicates homogeneous group according to Duncan's Multiple range test.

DFP, for the three fungi tested (data not shown). Inhibitors were therefore considered non-toxic to the fungi and were used at the concentrations tested. Inhibitors did however affect pathogenicity for the three fungi considered. From 2 mM onwards PMSF significantly reduced egg infection by all fungi (Fig. 14). At the highest PMSF concentration there were reductions in egg infection compared to non-treated controls of 35%, 42% and 50% for *Pochonia rubescens*, *P. chlamydosporia*, and *Lecanocillium lecanii* respect-

ively. DFP (5 mM) significantly reduced egg infection by *P. chlamydosporia* and *L. lecanii* (Fig. 15).

PMSF also reduced the number of hyphae per egg for the three species studied. Although values were not significantly different, egg penetration by *P. chlamydosporia* (13.4 ± 12.8) was reduced by 2 mM PMSF, compared to controls (26.4 ± 10.8). *P. chlamydosporia* behaved similarly when DFP was used instead of PMSF (not shown).

DISCUSSION

Initial steps of host infection by fungal pathogens include host recognition. In the plant pathogen *Colletotrichum gloeosporioides*, ethylene, a phytohormone involved in maturation of climateric fruits, stimulates appressorium formation (Kolattukudy *et al.* 1995). Perception of a wide array of signals, mediated by receptors, initiates signal transduction pathways, which allows appressorium differentiation mechanisms (Dean, 1997). Appressorium formation may also be a thigmotropic response of the fungus after sensing the surface of the host (Kwon & Hoch 1991). Therefore, physical conditions of the host surface, such as hardness or hydrophobicity, influence appressorium formation (Staples & Hoch 1997). Our results show that fungal parasites of nematode eggs formed more appressoria on hydrophobic than on hydrophilic surfaces. This agrees with many plant (Lee & Dean 1994) and insect (St Leger *et al.* 1989) pathogenic fungi.

Adhesion of fungal parasites to host substrates is vital for the pathogenesis. Adhesion is often a process mediated by glycoproteins that 'glue' a fungus and its host together (Epstein & Nicholson, 1997). Studies on nematode-trapping and endoparasitic fungi have shown adhesive proteinaceous substances in spores and infection structures, which bind to nematode surfaces (Tunlid, Jansson & Nordbring-Hertz 1992). A thick adhesive pad, containing both proteins and carbohydrates, has been found on the infective conidia of the nematophagous fungus *Drechmeria coniospora* (Jansson & Friman 1999). Appressoria of *Pochonia rubescens* on nematode eggs have mucillaginous substances that seem to act as adhesives (Lopez-Llorca & Claugher 1990). Similar substances have been found in appressoria formed by *Dactylella oviparasitica* infecting *Meloidogyne* eggs (Stirling & Mankau 1979). Lectins with several types of labels have been used to probe conidia of fungal pathogens as a diagnostic tool to locate carbohydrate moieties that might participate in the adhesion process (O'Connell 1991, Xiao *et al.* 1994, Shaw & Hoch 1999). In our studies, we have found that the lectin Con A specifically labelled the wall of conidia and hyphae. Since Con A recognizes α-mannoside and α-glucoside groups, (most common carbohydrates present in glycoproteins), lectin labelling experiments suggest early secretion of substances containing these sugars and accumulation after appressorium differentiation on nematode eggs. Since no accumulation of lectin-bound material was found in appressoria differentiated on polystyrene, host factors (unknown) may also be influencing production of extracellular matrix (ECM) of fungal parasites of nematode eggs. Like appressorium formation, ECM production (Carver *et al.* 1999), as well as adhesion and spore germination (Apoga, Jansson & Tunlid 2001) is affected by surface hydrophobicity. Furthermore, ECM of fungal pathogens often contains proteases and other lytic enzymes (Clement, Butt & Beckett 1993, Jansson & Friman 1999).

There is increasing evidence that proteases of fungal parasites of nematode eggs could play an important role in infection. P32, a major serine protease produced by *P. rubescens*, was immunolocalized in appressoria of the fungus infecting *Heterodera schachtii* eggs (Lopez-Llorca & Robertson 1992). Segers *et al.* (1996) found that eggs of *Meloidogyne incognita* and *Globodera rostochiensis* were more susceptible to infection by *P. chlamydosporia* after pre-treatment with purified VcP1 serine protease of this fungus.

Early secretion of cuticle degrading enzymes has been found for fungal plant pathogens (Kolattukudy 1985, Mendgen, Hahn & Deising 1996), and a role of these enzymes in preparation of the infection court has been proposed. In the present work, enzymatic assays and zymography of extracts of conidia and germlings of *P. chlamydosporia* and *P. rubescens* have shown proteolytic activity. Specific inhibition of proteolytic activity of these extracts by serine protease inhibitors and by specific anti-P32 serum suggest that similar enzymes are present in pre-penetration stages (conidia and germlings) as well as appressoria of the fungi. Zymography strongly suggests that proteases of very similar Rf to that of purified P32 are found in extracts of conidia and germlings of *P. chlamydosporia* and *P. rubescens*. VcP1, from *P. chlamydosporia*, and P32 are both serine proteases with similar molecular mass (Lopez-Llorca 1990, Segers *et al.* 1994). Anti-P32 serum cross-reacts with VcP1 (Segers *et al.* 1995). We can therefore conclude that the main proteases for *P. chlamydosporia* and *P. rubescens* are present constitutively in conidia and germlings in the absence of nematode eggs.

In this study, we have found reductions in the infection of *M. javanica* eggs by three species of fungal parasites (*P. rubescens*, *P. chlamydosporia*, and *Lecanocillium lecanii*) upon addition of PMSF or DFP, two serine protease inhibitors. Egg infection was not abolished even when higher concentrations of the inhibitors were used. Pathogenesis is a complex phenomenon involving a wide range of determinants, not only extracellular enzymes. The signalling between the environment (soil, roots and nematode host) and nematophagous fungi leading to appressorium formation and infection is largely unknown. Increased knowledge in this area will help us to understand the pathogenicity of nematophagous fungi and to develop them for biocontrol of nematodes.

ACKNOWLEDGEMENTS

Help of I. Abrantes (University of Coimbra, Portugal) with identification of *Meloidogyne javanica* cultures was greatly appreciated. This work was partially funded with the Research Project Grant 'Rhizosphere colonizacion and Biological control' (AGL2001-0734) from the Spanish Ministry of Science and Technology.

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