European Journal of Plant Pathology **107:** 191–207, 2001.
© 2001 *Kluwer Academic Publishers. Printed in the Netherlands.*

Resistance reaction of conifer species (European larch, Norway spruce, Scots pine) to infection by selected necrotrophic damping-off pathogens

M. Kacprzak[1,3], F.O. Asiegbu[1,*], G. Daniel[2], J. Stenlid[1], M. Mañka[3] and M. Johansson[1]
[1]*Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Box 7026, Uppsala, Sweden;* [2]*Department of Wood Science, Swedish University of Agricultural Sciences, Box 7008, Uppsala, Sweden;* [3]*Department of Forest Pathology, University of Agriculture, Poznan, Poland;* [*]*Author for correspondence (Fax: +46 18 30 92 45; E-mail: Fred.Asiegbu@Mykopat.slu.se)*

Accepted 26 October 2000

Key words: damping-off, *Fusarium*, *Rhizoctonia*, conifer seedlings, necrosis, resistance

Abstract

Three conifer species (European larch, Norway spruce, Scots pine) were investigated for their resistance to five damping-off pathogens (*Rhizoctonia solani, Fusarium solani, F. oxysporum, F. culmorum, F. avenaceum*). Inoculation of the primary roots of seedlings with these pathogens caused host cell death which did not prevent the invasive growth of these fungi; seedlings that had formed secondary and tertiary roots could overcome the infection to a significant degree. Infections with *R. solani* caused significant mortality to all the conifer species. In contrast, the tree species expressed different levels of resistance when challenged with the *Fusarium* isolates, with Norway spruce being the most resistant compared to uninoculated controls. Some of the *Fusarium* isolates were more pathogenic to certain hosts than others; *F. oxysporum* for European larch, *F. avenaceum* for Scots pine, *F. solani* for European larch; only *F. culmorum* was significantly pathogenic to Norway spruce. No significant differences in disease severity were observed at different soil pH (4.3–7.5). Disease progression was delayed at lower (10–15 °C) rather than higher temperatures (20–25 °C).

Introduction

Damping-off disease of seedlings is caused by several fungal genera that are distributed world-wide. These pathogens affect seeds, seedlings, and older plants including many economically important vegetables and cereals, fruit crops and forest trees. For example, various strains of *Fusarium oxysporum* can cause wilt diseases in more than 100 plant species (Armstrong and Armstrong, 1981). Kolattukudy and Gamble (1995) have reported that at least 111 plant species belonging to 87 different genera of agricultural, horticultural or forestry importance were susceptible to *F. solani*. Damping-off disease has been reported in Europe on tree seedlings since the eighteenth century (Boyce, 1961). Generally, the disease is caused by a number of fungi that live saprotrophically in the upper layers of the soil, but which, under favourable conditions, can become pathogenic (Hartley, 1921; Warcup, 1950). The most important of these fungi for coniferous seedlings are *Fusarium* spp., *Rhizoctonia* spp., *Pythium* spp., *Phytophthora* spp. and *Alternaria* sp. (Lilja et al., 1993).

Damping-off of pine seedlings in Poland is caused mostly by *Fusarium* spp. and *Rhizoctonia solani* (Mañka, 1998) with symptoms of stunted growth, needle chlorosis, and browning beginning at the needle tips. Fine roots are killed in most of the fungal damaged root systems. The succulent tissues of the seedling are easily penetrated by the fungus, which invades and kills the cells. The major routes for penetration into host tissues reported were through wounds (Beyer-Ericson et al., 1991). Stress-induced nursery practices such as waterlogging and transplantation pre-dispose seedling roots to infection by soil-borne pathogens (Beyer-Ericsson et al., 1991). Griffin (1958) reported that

abiotic factors such as pH directly affects the vigour of the host rather than the growth of the pathogen.

In agricultural crop plants, instances where resistance can be attributed solely to a single defence mechanism are very few (Ji et al., 1998). Several responses may occur simultaneously or consecutively. It is therefore likely that more than one biochemical mechanism may be responsible for restricted growth of a pathogen. For example, the hypersensitive necrotic response (HR) is often associated with the accumulation of phytoalexins or with lignification (De Witt, 1987). A limited number of reports describing host–parasite interactions in perennial plants have documented conspicuous morphological responses of the plant cell to fungal invasion such as papillae formation, suberization and accumulation of electron dense materials (Popoff et al., 1975; Stenlid and Johansson, 1987; Farquhar and Peterson, 1989; Bonello et al., 1991; Asiegbu et al., 1993; 1994).

In this study, we studied the reaction of three conifer tree species, *Pinus sylvestris* L. (Scots pine), *Picea abies* L. (Karst.) (Norway spruce) and *Larix europaea* (*Larix decidua* Mill.) (European larch) to infection by *F. solani* (Mart.) Sacc., *F. avenaceum* (Fr.) Sacc., *F. culmorum* (W.G. Smith) Sacc, *F. oxysporum* Schlecht. and *R. solani* Kuhn.

Materials and methods

Fungal pathogens

F. solani, *F. avenaceum*, *F. culmorum*, *F. oxysporum* and *R. solani* were isolated from diseased Scots pine seedlings from the following nurseries: Podlesie (Forest District Oborniki Wlkp., western Poland in 1996), Grodziec (Forest District Siewierz, southern Poland in 1997), Kukawy (Forest District Kowal, central Poland in 1996 and in 1997). *Rhizoctonia* isolates were identified (by M. Mañka and M. Kacprzak) based on standard taxonomic procedures as outlined by previous authors (Gilman, 1957; Kronland and Stanghellini, 1988; Sneh et al., 1991).

Inoculum preparation

Micro- and macro-conidia of *Fusarium* spp. maintained on synthetic nutrient poor agar (SNA) medium (Nirenberg, 1981) at 22 °C for 3–4 weeks, were harvested, washed three times in sterile distilled water, centrifuged between washes and finally diluted to provide spore suspensions of 10^3 and 10^7 spores ml^{-1} which were used for inoculations. *R. solani* was cultured on SNA liquid medium at 22 °C for 3–4 weeks, the mycelium homogenized in a sterile Waring blender, and 1 ml of the mycelium-medium slurry used for inoculations.

Hosts

Seeds of *Pinus sylvestris* (Scots pine), *Picea abies* (Norway spruce) and *Larix europaea* (European larch) were supplied by courtesy of Forest District Siewierz, Poland. Seeds were surface sterilized in 30% H_2O_2 for 15 min under agitation and then washed thoroughly with sterile distilled water (SDW). The seeds were sown on sterile 1% (w/v) water agar in Petri dishes (ca. 25 in each 9.0 cm dish) and allowed to germinate at 22 °C in the dark to provide seedlings for the pathogenicity tests.

Pathogenicity test on Petri dishes

Fourteen days after sowing, seedlings were aseptically transferred into a second set of Petri dishes (three replicate dishes of 10 seedlings/dish) containing sterile 1% (w/v) water agar (Asiegbu et al., 1993). Half of each Petri dish was overlaid with moist sterile filter paper prior to the transfer of seedlings, with the root region placed on the filter paper; 1 ml mycelial or spore suspension was then applied. A second moist sterile filter paper was laid over the roots after fungal inoculation. The root region (half of each plate) was covered with aluminium foil and the Petri dish was sealed with parafilm. The seedlings were then incubated at 20 °C with a photoperiod of 16 h. Control plant roots were inoculated with SDW. After 5, 10 and 15 days, the degree of necrotic browning response was assessed visually.

Degree of necrosis response and seedling mortality

The degree and rate of necrosis response (Asiegbu et al., 1993; 1999b) on root surfaces was measured visually at 5, 10 and 15 days post-inoculation (p.i.). This was measured as the extent of root browning (0 = no browning; 1 = slight browning (not dense); 2 = moderate browning; 3 = strong browning; 4 = very strong browning). Mortality was recorded as number of dead seedlings after 5, 10 and 15 days.

Pathogenicity test in pots

Seedlings were infected with test fungi on Petri dishes as described above. After 24 h, seedlings were transferred into pots, 9.0 cm × 9.0 cm (10 plants/pot) containing sterile sand with pH pre-adjusted to 4.5 (3 replicate pots per treatment). Transplanted seedlings were watered every 3–5 days with SDW enriched with Ingestad's nutrient solution (Asiegbu et al., 1999a). Each litre of water was supplemented with 1 ml solution A and 1 ml solution B of Ingestads nutrient solution.

Effect of temperature on pathogenicity of the fungal isolates to Scots pine seedlings

In the first set of experiments, seedlings infected in Petri dishes, as described above, were incubated at 10–15 and 20–25 °C. In the second set of experiments, 24 h p.i. with fungi in Petri plates, seedlings were transferred into pots containing sterile sand with the pH adjusted to 4.5, using HCl. Transplanted seedlings received SDW supplemented with Ingestads nutrient solution adjusted to pH 4.5, every 3–5 days. The plants in pots were incubated either at 10–15 or 20–25 °C under a photoperiod of 16 h.

Effect of sand pH on pathogenicity of the fungal isolates to Scots pine seedlings

Roots were infected on Petri dishes as described above. Seedlings, one day p.i., were transferred into pots with sterile sand adjusted to different pH values (i.e. 4.3, 5.8, 7.5) using either HCl or NaOH to maintain pH. The seedlings were incubated at 20–25 °C with a 16 h photoperiod. After planting, seedlings received SDW supplemented with nutrient solution every 5 days. The pH of the sand was checked over the course of 5 days between watering, during which the pH of the added nutrient solution was adjusted with either HCl or NaOH, as needed. After 10, 20, 30, 40 days, seedlings and roots were examined for damping-off symptoms.

Tissue preparation for fluorescence microscopy (determination of cell death)

Seedlings were harvested 3, 5, 10 days p.i. Nuclear staining was carried out as described by Henry and Deacon (1981). Briefly, excised root regions (first 10 mm from root tip) were hydrolysed in 3% HCl in 95% ethanol (5 min at room temperature), washed twice in phosphate–citrate buffer, pH 3.8, stained with 0.001% acridine orange in phosphate buffer for 15 min at room temperature and rinsed twice in phosphate-citrate buffer. Root pieces were examined under a Leitz Orthoplan fluorescence microscope with excitation filter I_2: BP 450–490. The number of fluorescent nuclei within a microscope field of view using a ×40 objective was counted for both infected and control roots (3 fields of view per root in a total of 10 roots per sample). Images were recorded using Kodak 135 film at 1600 ASA.

Determination of pH changes in infected roots

Infected Scots pine and Norway spruce seedlings were harvested 10 days p.i. from Petri-dish experiments. Excised roots (10 per sample) were macerated with 3 ml of deionized water using a mortar and pestle; roots of uninfected control seedlings were treated similarly. The pH was measured using an Orion pH meter (model 420 A). Three replicates were used for each treatment.

Tissue preparation for light and transmission electron microscopy (TEM)

Samples were collected at defined time intervals, fixed in glutaraldehyde and embedded in London Resin (London Resin co., Basingstoke, UK) as described by Asiegbu et al. (1993; 1994). For light microscopy, semi-thin sections (4 μm thick) were stained with toluidine blue (general stain for lignin and phenolic-like compounds) (Asiegbu et al., 1993; 1994). Ultra-thin sections were collected on nickel grids and observed without staining in a Philips CM 12 transmission electron microscope (TEM).

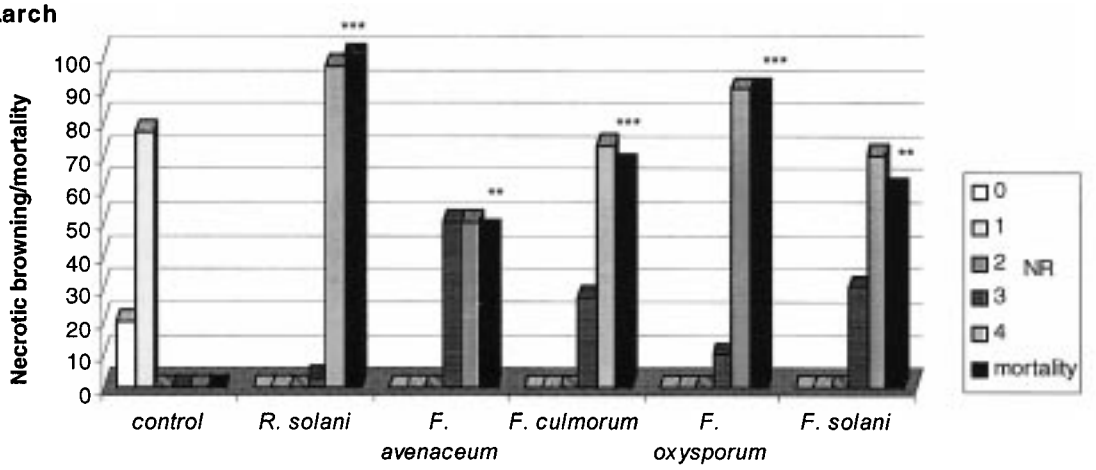
Preparation of samples for scanning electron microscopy (SEM)

The procedure used was as described by Asiegbu et al. (1993) except that roots were observed using an Hitachi S-4500 SEM operated at 15 kV.

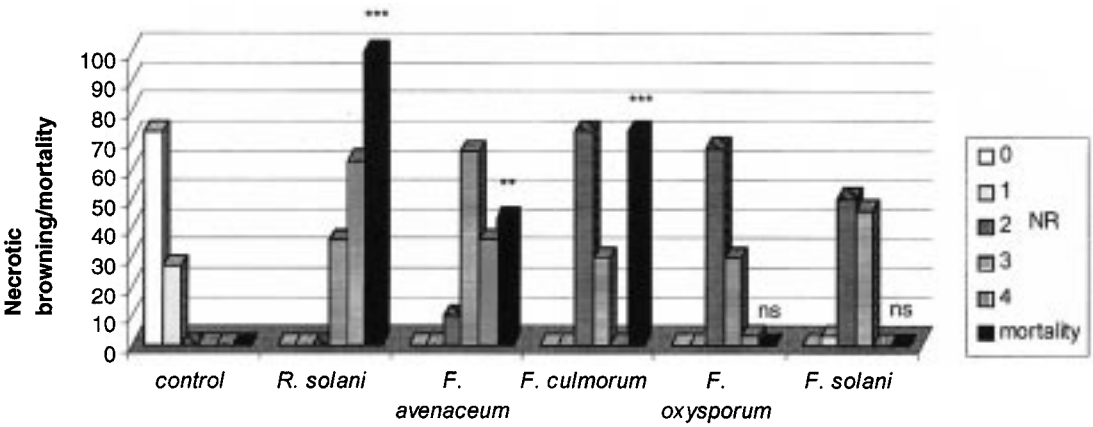
Data analyses

Results were analysed by analysis of variance (ANOVA). Data analyses with ANOVA was also complimented using the Student's *t*-test.

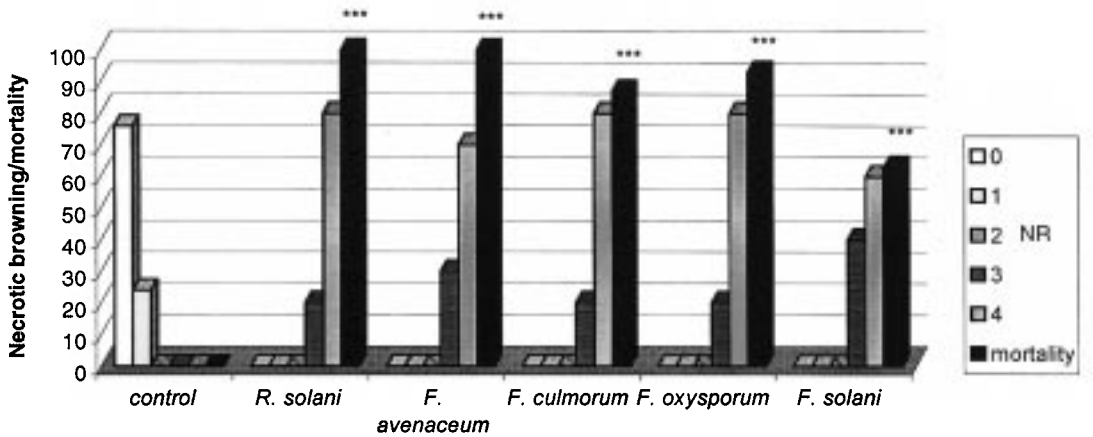
A. Larch



B. Norway spruce



C. Scots pine



Results

Host–parasite interactions; symptoms of disease on Petri dishes

The first symptoms of damping-off were observed 3–5 days p.i. on seedlings, inoculated with *R. solani*. Different degrees of host response recorded as a necrotic browning reaction were observed, with very strong necrosis noted 5–10 days p.i. At 10 days p.i., 100% mortality was recorded irrespective of the tree species infected (Figure 1). In the case of inoculations with *Fusarium* spp., differential levels of virulence were expressed (Figure 1). Results on both Petri-dish and pot experiments (Figures 1 and 2) showed that *F. avenaceum* was the most pathogenic fungus to Scots pine ($P < 0.001$), and only *F. culmorum* was significantly pathogenic to Norway spruce at $P < 0.001$, whereas in Petri-dish experiments *F. oxysporum* ($P < 0.001$) was most parasitic to European larch at 5 days p.i.; *F. solani* was observed to be the least pathogenic of the four *Fusarium* spp. studied. A considerable number of Norway spruce roots infected with *F. solani* did not show any sign of a necrotic browning response even after 15 days p.i. However, rapid induction of the necrotic browning reaction was observed when a high spore inoculum potential (10^7 spores ml^{-1}) was used compared to a much lower inoculum (10^3 spores ml^{-1}).

In terms of susceptibility, Scots pine and European larch were more susceptible than Norway spruce irrespective of the type of pathogen used. However, seedling mortality was much more rapid and usually visible after a relatively short time (ca. 5 days p.i.) with European larch than with Scots pine. Due to the high level of mortality, most experiments for larch in Petri dishes were terminated at 5 days p.i.

Symptoms of disease in pots

Symptoms of damping-off in pots were observed much later by comparison with Petri-dish experiments, irrespective of the tree species infected. The number of dead seedlings increased linearly with prolonged exposure but remained apparently unchanged after 30–40 days p.i. (Figure 2). When compared with the Petri-dish experiment, fewer seedlings with disease symptoms were observed in pots at 40 days p.i. but as was noted for Petri dishes, the initial symptoms of disease and significant mortality was observed in pots containing seedlings inoculated with *R. solani*, irrespective of the tree species.

In the case of Norway spruce, at 40 days p.i., it was noted that with the exception of *R. solani*, only seedlings infected with *F. culmorum* showed visual symptoms of infection. However, by careful removal of the inoculated seedling from the pot and ensuring that the root systems were intact, certain attributes of the resistant seedlings were noted, which were not observed amongst susceptible individuals. With resistant seedlings, the initially inoculated primary roots were rapidly killed but the secondary and tertiary roots remained alive (Figure 6a–c). In contrast, no secondary or tertiary root formation was observed amongst the diseased seedlings.

Effect of incubation temperature on infection

With Scots pine in Petri dishes, the necrotic browning reaction was observed much later in seedlings incubated at 10–15 °C, compared with seedlings incubated at 20–25 °C (Figure 3). With seedlings inoculated with *R. solani*, the first symptoms occurred about 5 days later at 10–15 °C than at 20–25 °C. After 10 days p.i. at 20–25 °C, almost all seedlings showed very strong necrotic reaction and mortality symptoms. However, little necrotic browning response or seedling mortality was recorded following inoculation with *Fusarium* isolates for 5 days at 10–15 °C, but after 20 days p.i., there was also a very strong necrotic reaction and a significant ($P < 0.01$) increase in the number of dead seedlings incubated at the lower temperature after inoculation with *F. avenaceum*, *F. culmorum*, *F. oxysporum* but not *F. solani* (data not shown).

With pot-grown seedlings, symptoms of damping-off also occurred much later at 10–15 °C than at 20–25 °C. In the case of *R. solani* inoculation, seedling mortality at 10–15 °C increased linearly over time and after 40 days p.i. 70% of the seedlings were dead. Seedling mortality was much more rapid at

Figure 1. Degree (0–4) of necrotic browning reaction and mortality of the coniferous seedlings in Petri dishes at 10 days p.i. except for larch which was at 5 days p.i. Degree of significance (mortality) as compared with control: ns = not significant, *significant at $P < 0.05$, **significant at $P < 0.01$, ***significant at $P < 0.001$ (NR = Necrotic browning reaction).

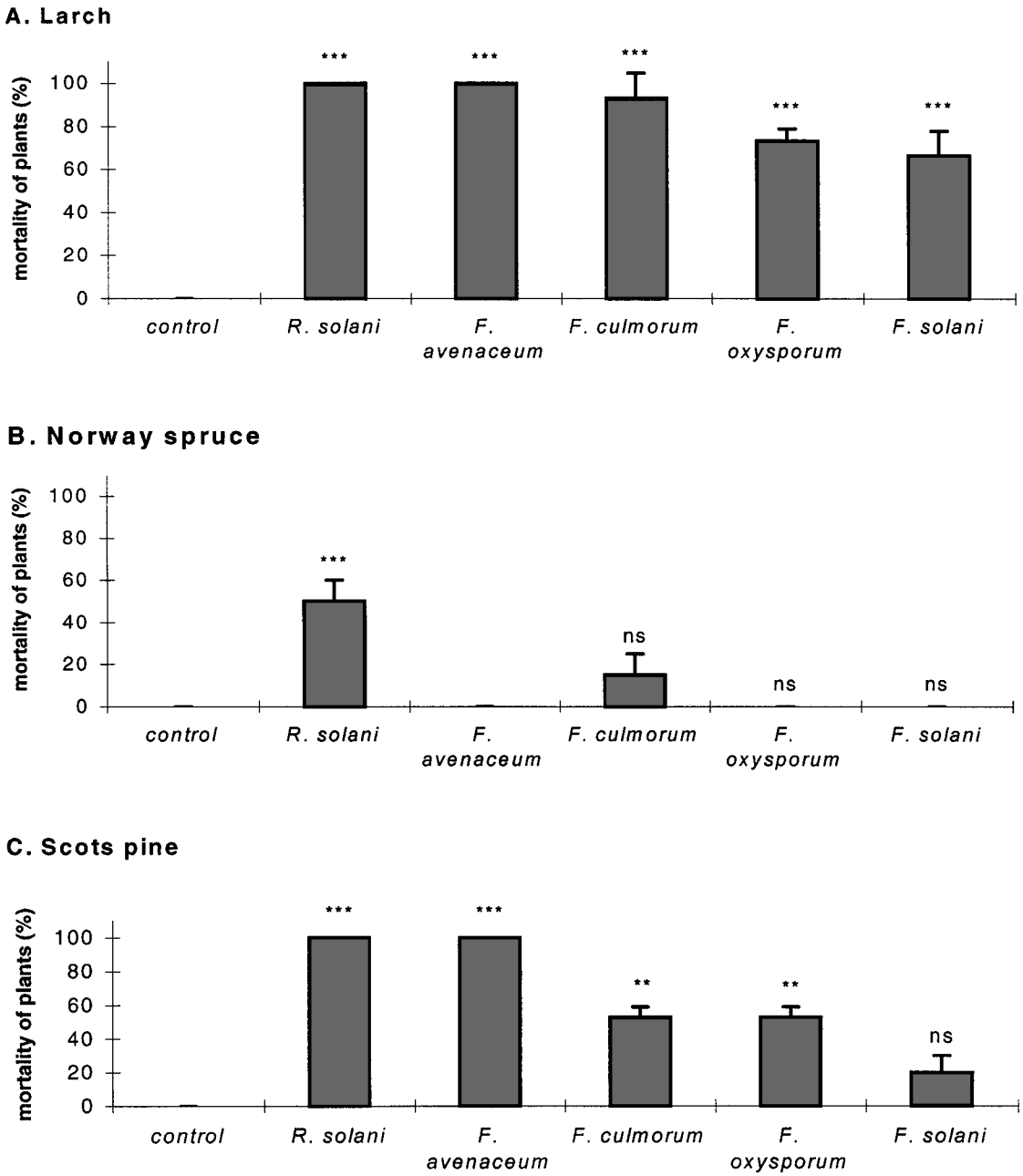


Figure 2. Mortality of the coniferous seedlings in pots at 40 days p.i. Degree of significance (mortality) as compared with control: ns = not significant, *significant at $P < 0.05$, **significant at $P < 0.01$, ***significant at $P < 0.001$.

20–25 °C suggesting that temperature had a significant ($P < 0.01$) effect on the outcome of infection. Similarly, mortality of seedlings infected with *Fusarium* spp. at 20–25 °C increased and reached a maximum at 30 days p.i. whereas, percentage mortality of seedlings, incubated at 10–15 °C increased over time and reached comparable levels only after 40 days p.i.

Effect of soil pH on the infection

No significant differences in disease severity were recorded at different soil pH levels (Figure 4). In the case of seedlings inoculated with *R. solani*, first symptoms of disease occurred much earlier at pH 4.3 than at pH 7.5, and by 20 days most of the seedlings were dead. Similar observations were made with seedlings infected with *Fusarium* spp. and grown on sand at pH 4.3. However, percentage mortality of seedlings infected with the respective fungi and incubated on sand at pH 5.8 or 7.5 increased linearly over time and at 40 days p.i. reached levels comparable to those observed at pH 4.3. Significant differences ($P < 0.05$) in terms of seedling mortality were observed between *F. avenaceum* and the other *Fusarium* spp. at all pH levels (Figure 4).

Induction of cell death in coniferous roots by pathogenic fungi

At 3–10 days p.i., a considerable reduction in the number of living cells within roots (first 10 mm) was recorded compared with control roots, irrespective of the tree species studied (Figure 5 and Figure 6d,e). After inoculation with *R. solani*, significantly fewer living cells were recorded at 3–5 days compared with uninfected controls ($P < 0.01$ or $P < 0.001$). By 5 days p.i. most roots with very strong browning (degree 4 type of necrosis) were practically devoid of living nuclei and were covered by an intense network of hyphae. However, with *Fusarium* spp. infections, considerable differences were observed in host response amongst the individual tree species with the exception of Norway spruce. Root cells of larch were very rapidly killed by each of the *Fusarium* spp. while Norway spruce was more resistant.

Results with Scots pine following infection with the *Fusarium* isolates indicated that within 5–10 days p.i. a significant ($P < 0.05$ or $P < 0.01$) decrease in the number of living cells compared with uninfected controls was visible (Figure 5). Usually, a dense network of hyphae was observed on the surface of the roots. With *F. culmorum*, very strong sporulation was observed and the formation of sclerotia was observed in some of the roots, suggesting that the fungi were no longer actively growing but had entered a dormant stage. No significant difference was recorded between individual *Fusarium* spp. in terms of the number of living cells within infected roots. In contrast to pine and larch, infected roots of Norway spruce had more living cells except in the case of infection with *R. solani* as noted.

Changes in root pH due to infection

In both Norway spruce and Scots pine, a slight increase in root pH towards alkalinity was observed at 10 days p.i. In both host trees, infection with *F. solani* affected only 0.2–0.5 change in pH units as compared to controls, whereas *F. avenaceum* caused 0.9–1.2 change in pH units above control values (Table 1).

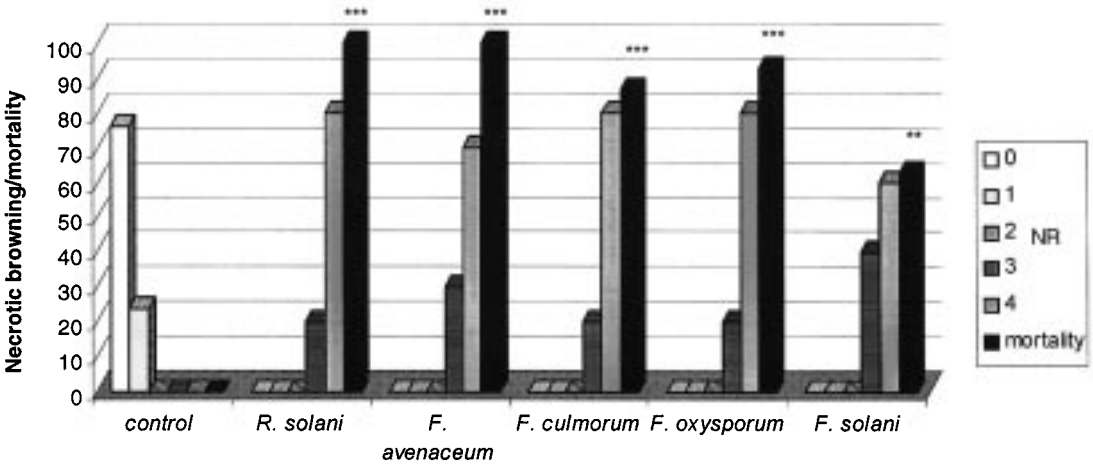
Initial events preceding fungal penetration

In the case of *R. solani*, evidence of newly emerging hyphal cells on the roots were observed by 24 h p.i., the hyphae sometimes elongating to produce dichotomous hyphal branches. It was observed that hyphal tips tightly adhered to the root surface and in some cases evidence of pre-formed hyphal penetrating structures was observed (Figure 7a). The fungus may have penetrated roots through crevices or directly through epidermal walls. Within 3 days of infection, networks of branching hyphae were recorded on the root surface. At 5 days p.i., the roots were completely covered by an intense mass of branching mycelia in contrast to control uninoculated roots, thus making it difficult to observe other modes of penetration. Chlamydospores were also frequently observed within this densely packed layer of mycelia (Figure 7b). With *F. avenaceum*, 24 h p.i., preformed hyphal structures were observed (Figure 7c) and penetration of epidermal walls usually occurred through crevices (Figure 7d). By 5 days p.i. an overgrowth of the root surface by the fungus was clearly noticeable and it had began to sporulate producing both macro- and micro-spores which also tightly adhered to the roots (Figure 7e).

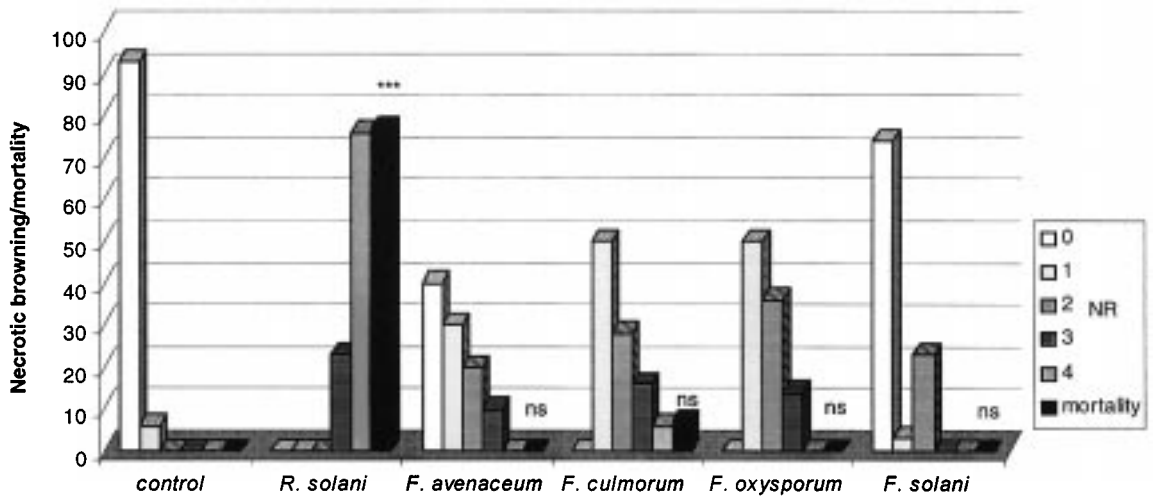
Cellular colonization of root tissues

Cellular penetration by fungi into epidermal cells began approximately within 1 day p.i. By 5–15 days p.i., presence of hyphae was recorded both inter- and intracellularly inside cortical and vascular tissues (Table 2 and Figure 6f–h). The cellular responses of the three tree species to invasion by *R. solani* were similar and the rate at which the fungus colonized the three hosts was comparable. In the case of infection by *Fusarium*

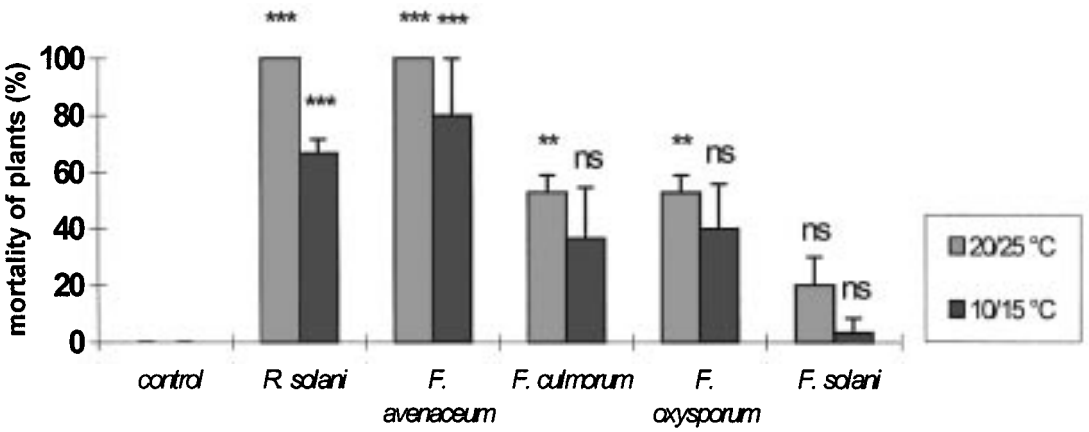
A. temperature 20/25^{0}C (Petri plates)



B. temperature 10/15^{0}C (Petri plates)



C. pots



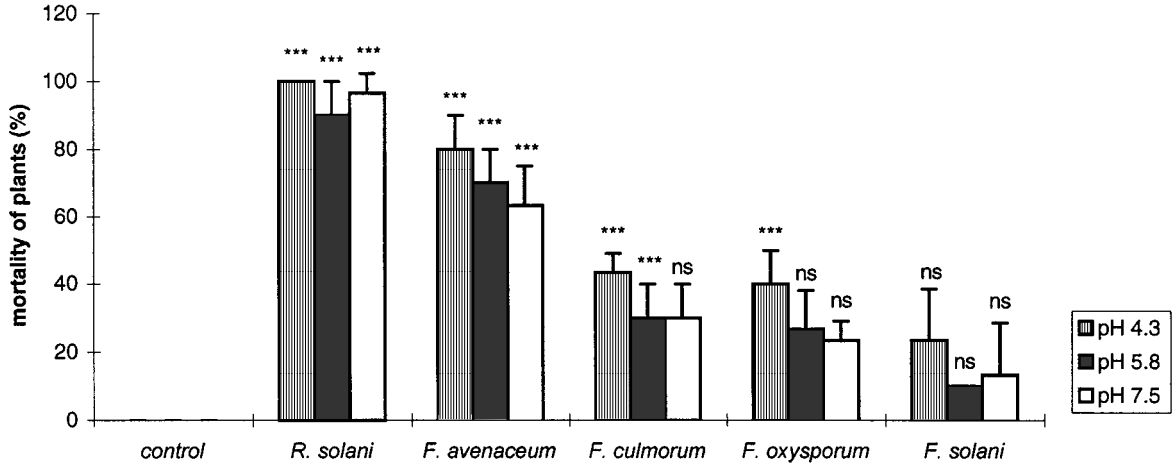


Figure 4. Mortality of Scots pine seedlings 40 days p.i. at different substrate pH values. Degree of significance (mortality) as compared with control: ns = not significant, *significant at $P < 0.05$, **significant at $P < 0.01$, ***significant at $P < 0.001$.

isolates, differences in the rates of colonization were apparent. Consequently for each host species investigated, only the most and least pathogenic isolates of *Fusarium* were investigated. With the more aggressive pathogens (i.e. *F. avenaceum*, *F. culmorum*), penetration into epidermal, cortical and vascular tissues was recorded within 15 days. However, for the weak pathogen *F. solani*, very slow growth was noted, and at 15 days p.i., the fungus was only observed within the outer cortex of Scots pine. At the TEM level (Figures 8a–f), the pathogens were observed within the middle lamellar cell corner regions. Penetration was both inter- and intracellular.

Discussion

Host cell recognition and response

Results of this study show that roots of Norway spruce, Scots pine and European larch, at early stages of development, are susceptible to *Fusarium* and *Rhizoctonia* isolates, causative agents of damping-off disease. The initial host response following inoculation includes a necrotic browning reaction. Different levels of the necrotic response were expressed amongst the various tree species and even within each individual species, possibly suggesting a genetic basis for host resistance and recognition (Nelson, 1982). Although our results on necrotic browning reactions were based on visual observation which could be subjective, a more quantitative approach of estimating the number of dead cells was also considered. The high correlation between seedlings with the highest degree of necrotic browning reaction and an increased number of dead cells and mortality in such seedlings, suggests that necrotic cell death cannot be used as a measure of host resistance to necrotrophic parasites. These results contrast with similar observations with biotrophic parasites where hypersensitive cell death restricts further penetration of the pathogen (Lamb and Dixon, 1997). Consequently, the greater resistance of Norway spruce compared to Scots pine and larch to infection by *Fusarium* isolates may possibly be related to its ability to resist cell death. However, it is not clear whether host cell death is triggered by toxic microbial products or elcitors of host origin. Several studies have shown that mycotoxins secreted by fungal species are capable of inducing apoptotic cell death in plants and animal tissues (Gilchrist et al., 1995; Tolleson et al., 1996;

Figure 3. A–B. Degree of necrotic browning reaction and mortality of Scots pine seedlings infected with the pathogenic fungi in Petri dishes at different temperatures at 10 days p.i. (A = 20–25 °C; B = 10–15 °C). C. Mortality of Scots pine seedlings incubated at 20–25 °C and 10–15 °C in pots for 40 days. Degree of significance (mortality) as compared with control: ns = not significant, *significant at $P < 0.05$, **significant at $P < 0.01$, ***significant at $P < 0.001$ (NR = Necrotic browning reaction).

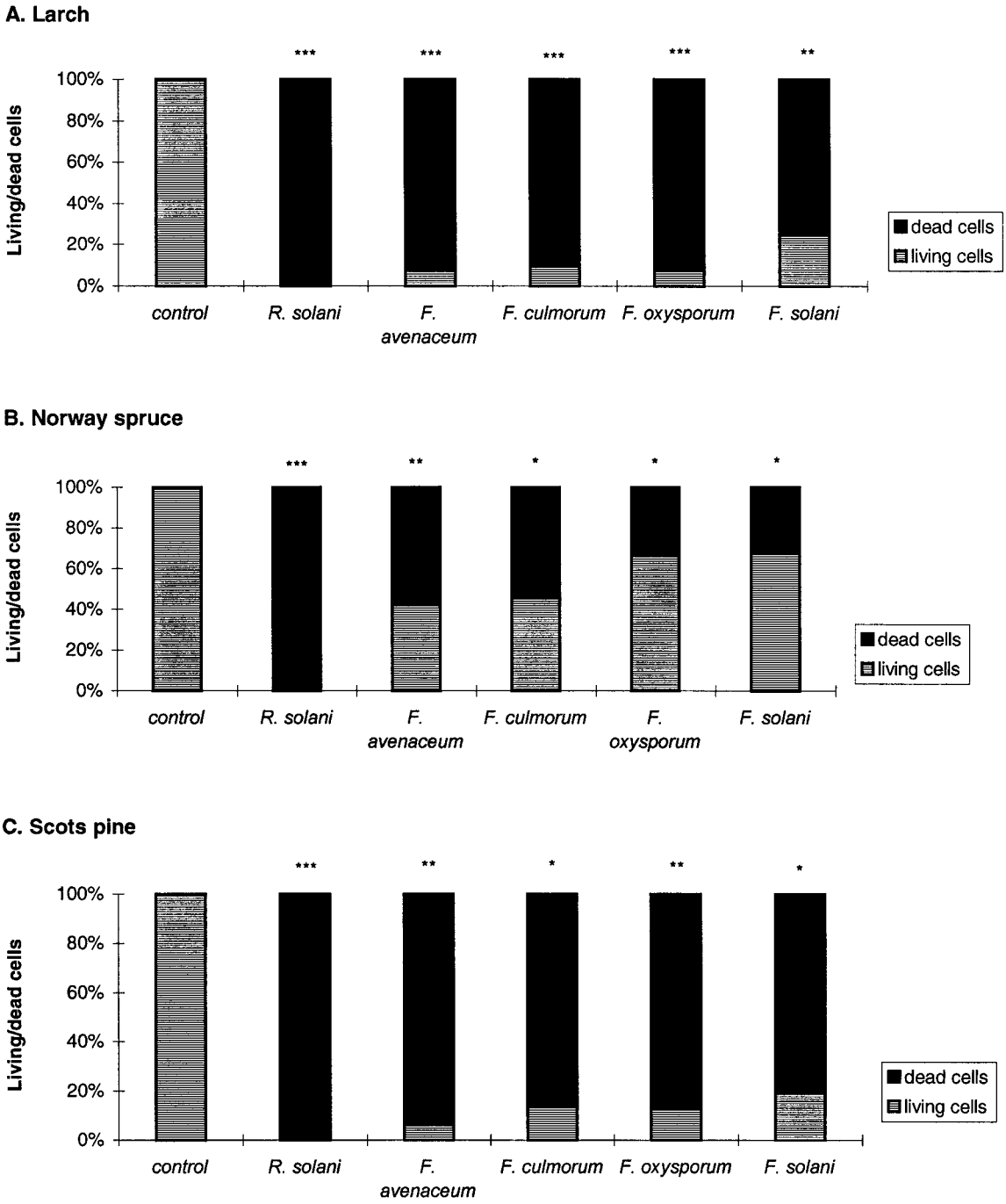


Figure 5. Percentage of living and dead cells in coniferous roots 10 days p.i. with the pathogenic fungi. Degree of significance (mortality) as compared with control: ns = not significant, *significant at $P < 0.05$, **significant at $P < 0.01$, ***significant at $P < 0.001$.

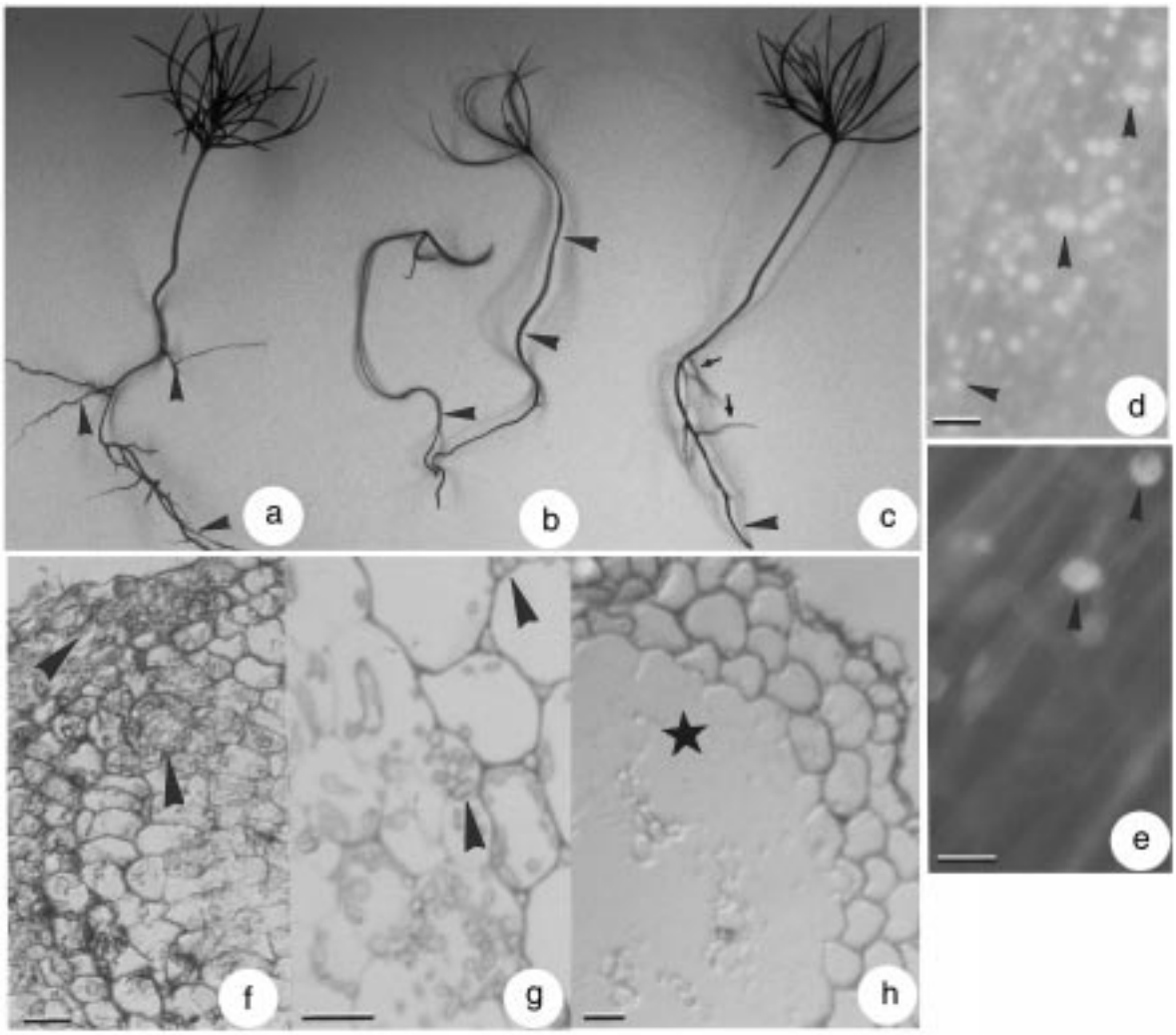


Figure 6. Secondary root formation, cell death and invasive growth of Scots pine roots following infection with *R. solani*. a. Uninfected (control) Scots pine root. Note increased number of secondary roots (arrowheads). b. Seedling roots of Scots pine infected with *R. solani*. Note flaccidity and mortality of the seedlings (arrowheads) due to the death of the primary root. c. Seedling roots of Scots pine infected with *R. solani*. Note formation of secondary roots (arrows) despite death of the primary roots (arrowhead). Note also fewer number of secondary roots as compared with the uninfected control. d. Control uninfected Scots pine root stained with acridine orange. Note abundance of fluorescent nuclei within living cells (arrowheads). e. Roots infected with *R. solani* were stained with acridine orange. Very few living nuclei was observed in host roots as compared with control uninoculaed roots. f. Disintegration of cellular architecture of Scots pine root following infection by *R. solani* at 9 days p.i. (arrowheads). g. Extensive colonization of Scots pine root by *R. solani*. Note presence of hyphae within cortical tissues at 7 days p.i. h. Disintegration of vascular tissues of Scots pine by *F. avenaceum* 15 days p.i. Bars: d, 20 μm; e–g, 10.0 μm; h, 5.0 μm.

Table 1. Changes in pH of Scots pine and Norway spruce root tissues following infection by *R. solani* and *Fusarium* species*

pH	Control	*R. solani*	*F. avenaceum*	*F. culmorum*	*F. oxysporum*	*F. solani*
Norway spruce	5.3 ± 0.1	6.3 ± 0.01	6.5 ± 0.04	6.6 ± 0.01	5.8 ± 0.01	5.8 ± 0.02
Scots pine	6.2 ± 0.02	6.4 ± 0.1	7.1 ± 0.1	7.04 ± 0.1	6.7 ± 0.04	6.4 ± 0.01

*Measured 10 days p.i.

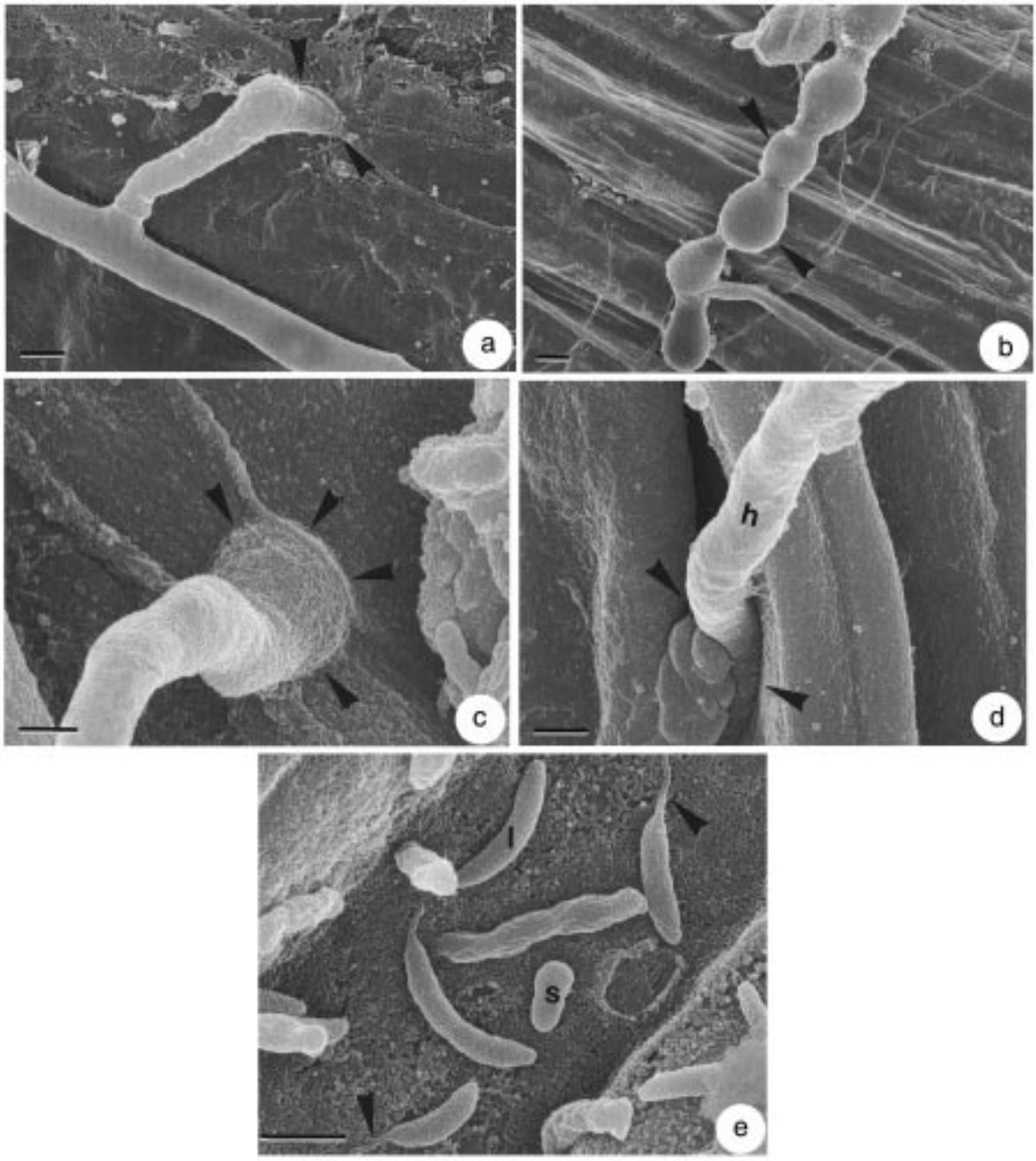


Figure 7. Scanning electron micrographs of initial developmental stages of *Rhizoctonia* and *Fusarium* progules on root surfaces prior to penetration. a. Root infected by *Rhizoctonia* sp. showing possible mode of penetration by pre-formed appressorial-like structures (arrowheads). b. Root surface of infected root. Note presence of chlamydospores (arrowheads) of *R. solani* on the root surface. c. Scots pine roots infected with *Fusarium* spp. Note presence of preformed appressorial-like structures (arrowheads) of *Fusarium*. d. Penetration of *Fusarium* hyphae through crevices on root surface (arrowheads). e. Sporulation and adhesion of (s)micro- and (l)macro-conidiospores on root surfaces. Note formation of germ-tubes from adherant spores (arrowheads). Bars: a–e, 1.0 μm.

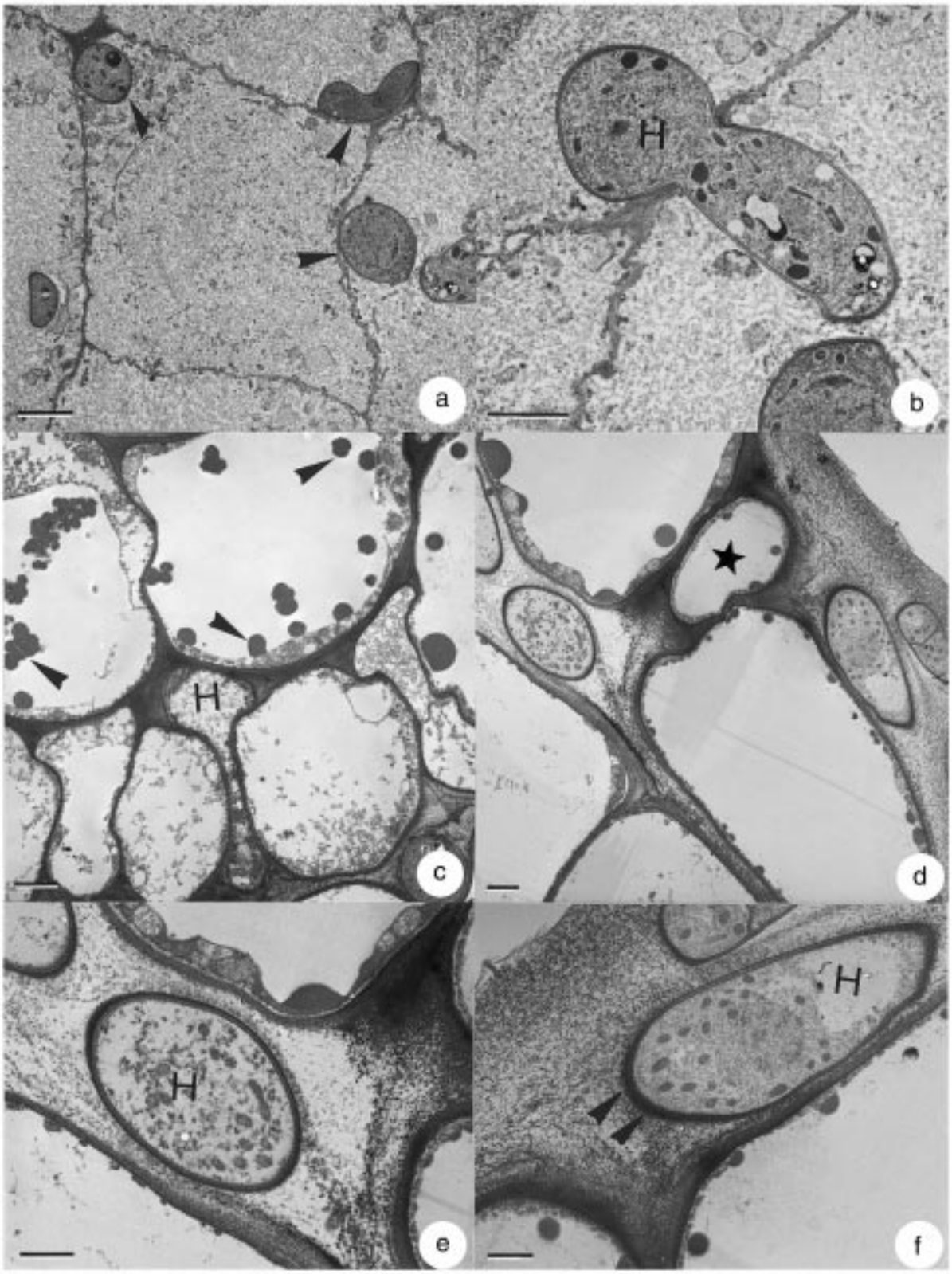
Table 2. Time-course of events in conifer seedling root tissues after challenge with selected damping-off pathogens*

Fungus	Region of root	Days post-inoculation							
		1	2	3	5	7	9	11	15
Norway spruce									
R. solani	Epidermis	P	D	D	D	D	D	D	D
	Cortex	P, L	P, L	P	P, D	D	D	D	D
	Endodermis	—	P, L	P, L	P	D	D	D	D
	Stele	—	—	—	P	D	D	D	D
F. culmorum	Epidermis	P, L	P, L	P, D	D	D	D	D	D
	Cortex	—	P, L	P, L	P, L	P, D	P, D	P, D	P, D, L
	Endodermis	—	L	L	P, L	P, L	P, L	P, L	P, L
	Stele	—	—	—	—	—	P	P, D	D
F. solani	Epidermis	P	P, L	P, L	P, L	P, L	P, L	P, L	P, L
	Cortex	—	—	L	L	L	L	L	P, L
	Endodermis	—	—	—	—	—	L	L	L
	Stele	—	—	—	—	—	—	—	—
Scots pine									
R. solani	Epidermis	P	P, D	P, D	D	D	D	D	D
	Cortex	L	P, L	P, L	D, L	D	D	D	D
	Endodermis	—	—	L	P	P, D	D	D	D
	Stele	—	—	—	P	P	P	D	D
F. avenaceum	Epidermis	P, L	P	P, D	D	D	D	D	D
	Cortex	L	L	P, L	P, L	P, L	P, L	P, L	P, L
	Endodermis	—	L	L	L	P, L	P, L	P, L	P, L
	Stele	—	—	—	—	—	P	P, D	D
F. solani	Epidermis	L	P, L	P, L	P, D	P, D	P, D	D	D
	Cortex	—	—	L	P, L	P, L	P, L	P, L	P, L
	Endodermis	—	—	—	—	L	P, L	P, L	P, L
	Stele	—	—	—	—	—	L	L	P, L
European larch									
R. solani	Epidermis	P	P	D	D	D	D	D	D
	Cortex	L	P, L	D, L	D, L	D	D	D	D
	Endodermis	—	—	P, L	D	D	D	D	D
	Stele	—	—	—	P	D	D	D	D
F. oxysporum	Epidermis	P, L	P	P, D	D	D	D	D	D
	Cortex	L	P, L	P, L	P, L	P, L	P, L	P, L	P, L
	Endodermis	—	L	L	L	P, L	P, L	P, L	D, L
	Stele	—	—	—	—	—	P	P, D	D
F. avenaceum	Epidermis	L	P, L	P, L	P, L	P, D	P, D	D	D
	Cortex	—	—	L	L	L	P, L	P, L	P, L
	Endodermis	—	—	—	—	L	L	L	P, L
	Stele	—	—	—	—	—	L	L	P, L

P – intercellular hyphal penetration. D – intracellular hyphal penetration and cell disorganization. L – lignification and/or phenolic accumulation within cellular tissues. *Semi-thin sections were stained with toluidine blue and examined under light microscope.

Wang et al., 1996). Gilchrist et al. (1995) reported that fumonisins, a toxin secreted by *Fusarium* spp. induced disruption of sphingolipid metabolism leading to cell death in plants.

The significant mortality recorded in all tree species infected with *R. solani*, suggests some differences in the pathogenicity of the fungal isolates. Other authors have also reported about 99% mortality rate of pine seedlings challenged with *R. solani* AG-5 in French nurseries (Camporota and Perrin, 1998). Differences in pathogenicity amongst the fungal isolates may also indicate that host cell death may partly reflect invasion


a
b
H
c
H
d
e
H
f
H

of the necrotroph which could be related to the characteristic nature of its secreted extracellular metabolites. *F. solani* provoked the least host response and was considered a weak pathogen. An in-depth comparative study of *F. solani* and the highly pathogenic *Fusarium* isolates may provide some insight into the mechanisms of host recognition, evolution of compatibility and pathogen virulence in conifer pathosystems. Another interesting aspect of these results is the differential levels of pathogenicity of each individual *Fusarium* isolate to different hosts (e.g. *F. avenaceum* for Scots pine/European larch, *F. solani* for larch, *F. oxysporum* for larch/Scots pine and *F. culmorum* for all three tree species) which further suggests some elements of molecular complementation between host and pathogen. A similar phenomenon of host preference has been described for the necrotrophic root rot pathogen (*Heterobasidion annosum*) on conifer trees (Korhonen, 1978).

Another marked feature of resistance in these conifer hosts which was of particular interest was the considerable reduction in mortality rate prevalent amongst seedlings which developed secondary and tertiary roots when the initially infected primary roots were killed. In most of the seedlings examined, infection did not spread to secondary and tertiary roots. It will be of interest to investigate the reduced tendency for the spread of the fungi into such newly-formed lateral roots. It is possible that factors other than morphological changes in root formation such as systemic induction of host defence compounds may have also contributed in enhancing resistance. Xue et al. (1998) noted that pre-inoculation of bean plants with non-pathogenic binucleate *Rhizoctonia* spp. prior to infection elicited a significant and systemic increase in peroxidases, glucanases and chitinases which protected the tissues from invasive growth of the pathogenic isolate.

Abiotic factors and disease occurrence

Mortality was much more rapid in seedlings incubated under stress-related conditions such as in Petri dishes compared to pots. However, the pathogenic capability and host-related responses of the tree species under both conditions were similar after prolonged incubation. Other authors have also demonstrated that stress inducing nursery practices help to pre-dispose seedling roots to infection by these facultative parasites (Beyer-Ericsson et al., 1991). Agrios (1988) noted that environmental conditions prevailing in both air and soil may greatly affect the development of damping-off disease. In this study, incubation of infected seedlings at sub-optimal temperatures (10–15 °C) significantly delayed disease progress. Other authors (Hangyalne-Balul et al., 1975) made similar observations and concluded that pine seeds should be sown at 10–13 °C before the air temperature reaches 20 °C. However, Smiley and Uddin (1993) reported that *R. solani* AG-8 in natural soil caused more severe root rot of wheat at low rather than at high temperatures. By contrast, Singh and Mehrotra (1982) noted that pre-emergence damping-off of grains caused by the tropical strain *R. bataticola* did not occur at 18 °C but the disease was maximal at 34 °C. As we noted in this study, low temperature may delay the appearance of disease symptoms but will not necessarily stop growth of the fungus.

Furthermore, in this study, pH had no adverse effect on the development of pathogens, although incidence of disease occurrence was much more rapid at lower pH. Other authors have also noted that soil pH is important for the occurrence of plant disease caused by soil-borne pathogens (Colhoun, 1973; Agrios, 1988). Scher and Baker (1980) showed that *Fusarium* wilt of flax was significantly less at pH 8.0 than at 6.0 in suppressive Metz fine sandy loam, but was more severe at both pH levels in a conducive soil. Changing the pH of soil may lead to an increased or decreased availability of the nutrients required by both host and pathogen (Simley and Cook, 1973). Simley and Cook (1973) also observed that soil-borne pathogens favoured by acid soils are favoured by ammonium ions and suppressed by nitrate ions. The converse was true for those fungal pathogens favoured by neutral or alkaline soils. Schier (1987) and Schier and Patton (1995) found that damping-off caused the death of several pine seedlings

Figure 8. TEM photographs showing cellular colonization of Norway spruce root tissues. a. Colonization of host tissues. Note presence of *F. culmorum* hyphae (arrowheads) at 9 days p.i. within cellular material. b. Colonization of host tissues by cell to cell invasive growth of *F. culmorum* hyphae (H). c. Host cellular responses during challenge with *F. culmorum* was marked by accumulation of electron dense bodies, probably phenolics (arrowheads). Note also intracellular growth of the pathogen hyphae (H). d. Presence of electron dense materials surrounding dead hyphae (star) of *F. culmorum*. e. Growth of *F. culmorum* hyphae (H) within middle lamellar cell corner regions. f. Deposition of electron dense substances (arrowheads) on hyphal tips (H). Bars: a–d, 2.0 μm; e–f, 1.0 μm.

(*Pinus rigida*, *P. echinata*, *P. taeda*, *P. strobus*) in unsterilized soil at pH 3.0 whereas seedling mortality was <3% at pH 4.0 and 5.6. Although the effects of pH on damping-off have usually been attributed to the effects of substrate pH on host vigour, these results suggests the involvement of other factors influencing the simultaneous host–parasite interaction.

Hyphal morphogenesis and cellular penetration

The germination and differentiation of spore and hyphal material on the root surface suggests the presence of stimulating factors of host origin. Initial events preceding penetration such as formation of germ-tubes and pre-formed hyphal penetrating structures on the root surface were similar to observations made in other pathosystems (Heath, 1991; Mendgen and Deising, 1993). Although the mechanisms regulating formation of appressoria-like or hyphal penetrating structures are not fully understood, other authors (Hoch et al., 1987; Read et al., 1992) considered that components of the host cytoskeleton or an ionic or electrical/topographical signal may be responsible. At the cellular level, differences in pathogenic capability were observed amongst the fungi tested. With *R. solani*, the fewer lignified cells recorded during host invasion resulted in the defence mechanism being too weak to resist, delay and arrest the significant number of mortalities recorded in the various hosts. Amongst the *Fusarium* isolates, only *F. solani* displayed weak pathogenicity effects on all host species. The high levels of lignification and phenolic accumulation observed in tissues colonized by *F. solani* suggests that this may have contributed in suppressing the invasive growth of the fungus.

These data clearly indicate differential levels of resistance amongst conifer seedlings to infection by selected damping-off pathogens. Although necrotic cell death may be a factor in determining the host's ability to defend against attack, it did not confer absolute resistance during challenge with the necrotrophic damping-off pathogens.

Acknowledgements

This work was partly supported by the Swedish Council for Forestry and Agricultural Research (SJFR), the Anna and Gunnar Vidfelts fund, and one individual VISBY programme scholarship from the Swedish Institute.

References

Agrios GN (1988) Plant pathology. Academic Press: INC. Third Edition

Armstrong GM and Armstrong JK (1981) *Formae speciales* and races *of Fusarium oxysporum* causing wilt diseases. In: Nelson PE, Toussoun TA and Cook RJ (eds) *Fusarium*: Disease, Biology and Taxonomy. Pennsylvania State University Press: University Park. pp 391–399

Asiegbu FO, Daniel G and Johansson M (1993) Studies on the infection of Norway spruce roots by *Heterobasidion annosum*. Canadian Journal of Botany 71: 1552–1561

Asiegbu FO, Daniel G and Johansson M (1994) Defence related reactions of seedling roots of Norway spruce to infection by *Heterobasidion annosum*. Physiological and Molecular Plant Pathology 45: 1–19

Asiegbu FO, Kacprzak M, Daniel G, Johansson M, Stenlid J and Mañka M (1999a) Biochemical interactions of conifer seedling roots with *Fusarium* spp. Canadian Journal of Microbiology 45: 823–935

Asiegbu FO, Johansson M and Stendlid J (1999b) Reactions of *Pinus sylvestris* root tissue to the presence of mutualistic, saprotrophic and necrotrophic micro-organisms. Journal of Phytopathology 147: 257–264

Beyer-Ericsson L, Damm E and Unestam T (1991) An overview of root die back and its causes in Swedish nurseries. European Journal of Forest Pathology 21: 439–443

Bonello P, Pearce RB, Watt F and Grime GW (1991) An induced papilla response in primary roots of Scots pine challenged *in vitro* with *Cylindrocarpon destructans*. Physiological and Molecular Plant Pathology 39: 51–67

Boyce JS (1961) Forest Pathology, Mc Graw-Hill Book Company (eds). New York, Toronto, London: INC

Camporota P and Perrin R (1998) Characterization of *Rhizoctonia* species involved in tree seedling damping-off in French forest nurseries. Applied Soil Ecology 10: 65–71

Colhoun J (1973) Effects of environmental factors on plant disease. Annual Review of Phytopathology 11: 343–364

De Witt PJGM (1987) Specificity of active resistance mechanisms in plant–fungus interactions. In: Pegg GF and Ayres PG (eds) Fungal infection of plants. Symposium of the British Mycological Society (412pp). Cambridge University Press.

Farquhar ML and Peterson RL (1989) Pathogenesis in *Fusarium* root rot of primary roots of *Pinus resinosa* grown in test tubes. Canadian Journal of Plant Pathology 11: 221–228

Gilchrist DG, Wang H and Bostock RM (1995) Sphingosine related mycotoxins in plant and animal diseases. Canadian Journal of Botany 73: S459–S567

Gilman JC (1957) A manual of soil fungi. The IOWA state University Press, Ames, IOWA, USA pp 102–104

Griffin DM (1958) Influence of pH on the incidence of damping-off. Transactions of British Mycological Society 41: 483–490

Hangyalne-Balul W, Balul W and Hangyal W (1975) Pathogenicity of some fungus species causing damping-off of conifer seedlings. Erdo 24: 65–68

Hartley C (1921) Damping-off in forest nurseries. USDA Bulletin 934

Heath MC (1991) Evolution of resistance to fungal parasitism in natural ecosystems. New Phytologist 119: 331–343

Henry CM and Deacon JW (1981) Natural non-pathogenic death of the cortex of wheat and barley seminal roots as evidenced by nuclear stain with acridine orange. Plant and Soil 60: 255–274

Hoch HC, Staples RC and Boureth TM (1987) Chemically induced appressoria in *Uromyces appendiculatus* are formed aerially apart from the substrate. Mycological Research 79: 418–424

Ji C, Smith-Backer J and Keen NT (1998) Genetics of plant–pathogen interactions. Current Opinion in Biotechnology 9: 202–207

Kolattukudy PE and Gamble DL (1995) *Nectria haematococca*. In: Pathogenesis and Host Specificity in Plant Disease. Vol. II. Eukaryotes, Pergamon, Elservier Science Ltd: Oxford UK 83–102

Korhonen K (1978) Intersterility groups of *Heterobasidion annosum*. Communicationes Intitutio Forestalia Fenniae 94: 1–25

Kronland WC and Stanghellini ME (1988) Clean slide technique for the observation of anastomosis and nuclear condition of *Rhizoctonia solani*. Phytopathology 78: 820–822

Lamb C and Dixon RA (1997) The oxidative burst in plant disease resistance. Annual Rev. Plant Physiol. Plant Molecular Biology 48: 251–275

Lilja A, Lilja S and Li Z (1993) Conifer seedlings root fungi and root dieback in Finish nurseries. Scandinavian Journal of Forest Research 7: 547–556

Mañka K (1998) Fitopatologia lesna, PWRiL (eds). Warszawa

Mendgen K and Deising H (1993) Infection structures of fungal plant pathogens-cytological and physiological evaluation. New Phytologist 24: 193–213

Nelson RR (1982) On genes for disease resistance. In: Resistance to Diseases and Pests in forest trees. Edited by Heybroek HM, Stephan BR and von Weissenberg K. Centre for Agricultural publishing and Documentation, Wageningen, Netherlands pp 84–93

Nirenberg H (1981) A simplified method for identifying *Fusarium* spp. occurring on wheat. Canadian Journal of Botany 59: 1599–1609

Popoff T, Theander O and Johansson M (1975) Changes in sapwood of roots of Norway spruce attacked by *Fomes annosus*. II. Organic chemical constituents and their biological effects. Physiologia Plantarum 345: 347–356

Read ND, Kellock LJ, Knight H and Trewavas AJ (1992) Contact sensing during infection of fungal pathogens. In: Callow JA and Green JR (eds) Perspectives in Plant cell recognition. Cambridge UK: Cambridge University Press, 137–172

Scher FM and Baker R (1980) Mechanisms of biological control in a *Fusarium* suppressive soil. Phytopathology 70: 412–417

Schier GA (1987) Germination and early growth of four pine species on soil treated with simulated acid rain. Canadian Journal of Forest Research 17: 1190–1196

Schier GA and Patton RL (1995) Damping-off disease of pine seed simulated by acidic rain. Canadian Journal of Forest Research 25: 838–844

Simley RH and Cook RJ (1973) Relationship between take-all of wheat and rhizosphere pH in soils fertilized with ammonium versus nitrate–nitrogen. Phytopathology 63: 882–890

Singh PJ and Mehrotra RS (1982) Influence of soil moisture and temperature on *Rhizoctonia bataticola* infection of grain. Indian Phytopathology 35: 327–329

Smiley RW and Uddin W (1993) Influence of soil temperature on *Rhizoctonia* root rot of winter wheat. Phytopathology 83: 777–785

Sneh B, Burpee L and Ogoshi (1991) Identification of *Rhizoctonia* species. APS Press. The American Phytopathological Society, St. Paul Minnesota, USA, p 133

Stenlid J and Johannson M (1987) Infection of roots of Norway spruce (*Picea abies*) by *Heterobasidion annosum*. II. Early changes in phenolic content and toxicity. European Journal of Forest Pathology 17: 217–226

Tolleson WH, Dooley KL, Sheldon WG, Thurman JD, Bucci TJ and Howard PC (1996) The mycotoxin fumonism induces apoptosis in cultured human cells and in livers and kidneys of rats. Advances in Experimental Medical Biology 392: 237–250

Wang H, Li J, Bostock RM and Gilchrist DG (1996) Apoptosis: A functional paradigm for programmed plant cell death induced by a host selective phytotoxin and invoked during development. Plant cell 8: 375–391

Warcup JH (1950) The soil plate method for isolation of fungi from soil. Nature 166: 117–118

Xue L, Charest PM and Jabaji-Hare SH (1998) Systemic induction of peroxidases, 1,3-beta glucanases, chitinases and resistance in bean plants by binucleate *Rhizoctonia* species. Phytopathology 88: 359–365