European Journal of Plant Pathology **104:** 225–234, 1998.

Infection of Norway spruce (*Picea abies* (L.) Karst.) seedlings with *Pythium irregulare* Buism. and *Pythium ultimum* Trow.: histological and biochemical responses

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Accepted 10 October 1997

Key words: autofluorescence, chitinase, damping-off, lignification, salicylic acid

Abstract

We have studied the reaction of *Picea abies* seedlings to infection with *Pythium.* The highly virulent species *Pythium ultimum* and the less virulent species *Pythium irregulare* germinated on the root and hypocotyl surface, formed appressoria and penetrated through the stomata as well as through the epidermis. No major differences in the growth of both fungal species were observed during the early events of colonization. The less virulent species formed about 25% more appressoria suggesting that the fungus experienced difficulties with penetration. Differences were observed in the response of the host plant to infection. Autofluorescence, possibly related to deposition of lignin or lignin-like materials increased more in cortical and endodermal tissue colonized with the highly virulent *P. ultimum* than with the less virulent *P. irregulare*. Chitinase activity was highest in the tissues most extensively colonized by the fungus. In addition, a systemic increase of chitinase activity was also detected. Interestingly, chitinase activity increased systemically in cotyledons which were never in contact with the pathogen, indicating the translocation of a systemic signal. Salicylic acid was also detected in spruce seedlings; its level increased in roots during infection with the less virulent *P. irregulare*.

Abbreviations: SA – salicylic acid.

Introduction

Damping-off of conifers causes serious annual economic losses in Northern Europe. The causal agent of this disease, *Pythium* spp., is considered a major pathogen in sylviculture as well as in agriculture (Chopra, 1976; Horst, 1990; Smith, 1988). Efforts to understand this pathogen and the disease it causes have focused on the biology of the fungus and on the epidemiology of the disease (Butin, 1989; Hendrix and Campbell, 1973; Martin, 1992). A number of studies were also directed at the defence reactions of conifers during infection by *Pythium*. Such reactions include induction of lignification and pathogenesis-related proteins (PRs) (Borja et al., 1995; Messner and Boll, 1993; Sharma et al., 1993). Defense reactions have also been studied after inoculation with the butt rot fungi *Heterobasidion annosum* (Asiegbu et al., 1994), *Fomes annosus* (Popoff et al., 1975) as well as the mycorrhizal fungus *Amanita muscaria* (Sauter and Hager, 1989). Abiotic stresses such as ozone exposure (Kärenlampi et al., 1994), frost (Polle et al., 1996) and wounding (Brignolas et al., 1995) induce biochemical changes usually associated with defence response. Tissue-cultured cells of *Picea abies* are responsive to elicitors and can be used as a model system to study induced lignification (Messner and Boll, 1994) or changes induced by an oxidative stress (Messner and Boll, 1994; Schwacke and Hager, 1992).

The objective of the present study was to compare the reaction of spruce seedlings following inoculation with *Pythium ultimum*, a highly virulent species, and with the less virulent *Pythium irregulare*. Antibodies raised against *Pythium* were used to follow the progres-

sion of the fungus in inoculated seedlings. In addition we have compared histological changes as well as biochemical responses such as the activation of chitinase and the accumulation of salicylic acid.

Materials and methods

Biological material

Seeds of Norway spruce (*Picea abies* (L.) Karst.) were obtained from the Swiss Federal Institute for Forest, Snow and Landscape Research, Birmensdorf, Switzerland. They were collected in a spruce stand in Tägerwilen, Switzerland. *Pythium* isolates were obtained from CIBA, Basle, Switzerland.

Culture conditions and pathogen inoculation

Seeds were surface-sterilized by dipping for 10 m in 30% hydrogen peroxide (v/v) then washed 4 times in sterile distilled water. Seeds were germinated on wet filter papers in darkness at room temperature for 8-10 days. Approximatively 200 seeds were then transferred into 1000 ml pots (10 × 25 cm) containing a sand-vermiculite mixture (1:1, v/v). Plants were grown in a growth chamber (22 °C day / 18 °C night temperature, 80% rel. humidity, 14 h light, 16 μmols^{-1}m^{-2}).

Pythium spp. were maintained in petri-dishes on potato carrot agar (PCA)(2%, w/v, using a 1:1 potato and carrot homogenate and 1.8% (w/v) bacteriological agar (Unipath Ltd., Hampshire, England), in darkness at room temperature. Two-week-old cultures were used for inoculation.

For infection tests, seedlings were used 10 days after emergence. Infections were carried out by pouring 10 ml of a suspension of sporangia (25 000 spores ml^{-1} of water) around the bases of each plant. We have found that this concentration was optimal to follow the development of the disease. Higher concentrations caused rapid and severe damping off, making microscopical and biochemical studies impossible.

Scanning Electron Microscopy

Picea abies seedlings were cut in 0.8 cm long segments and in 2% OsO_4 and 0.1M Na-cacodylate pH 7.4 (1:1, v/v) and incubated overnight at 4 °C. The samples were then washed with 0.05M Na-cacodylate at pH 7.4 and stored at 4 °C. Before SEM observations, the fixed segments were dehydrated with acetone and critical-point dried (Polaron, Watford, UK) followed by sputtering with gold. All pictures were viewed with a 'Jeol' JSM-840 A scanning microscope and taken on Ilford FP4 Plus films.

Antibody preparation

Serum was produced against a mixture of *P. ultimum* and *P. irregulare*. A suspension of germinating spores in sterile water (5×10^5 spores ml^{-1}, 6 h germination at room temperature) was used as an antigen. This suspension was emulsified with Freund's adjuvant and injected into a rabbit. Blood was collected after 8 weeks from ear bleedings and the serum was stored in 0.5 ml aliquots at −80 °C.

Immunocytology

Longitudinal- and cross-sections of *Picea abies* were incubated with anti-*Pythium* antibody (1:1000 v/v in 5% dry milk in phosphate-buffered saline, PBS) overnight at 4 °C. They were then washed with PBS and 0.05% Nonidet P40 and incubated 2 h with alkaline phosphatase-conjugated anti-rabbit antibody (1:3000 v/v). Detection was carried out using 5-bromo-4-chloro-3-indolyl-phosphate (50 mg per ml of dimethylformaldehyde, DMF) and 4-nitroblue tetrazolium chloride (50 mg per ml 70% DMF) resuspended in incubation buffer (100 mM Tris, 100mM NaCl, 0.1% $mgCl_2$, pH 9.5). Observations were done using a light microscope (Dialux 20, Leitz) equipped with fluorescence filters (excitation wavelength set at 355-425 nm and emission wavelength set at 460 nm). The intensity of autofluorescence was quantified visually.

Quantitative evaluation of tissue colonisation

Hand-cut sections were first stained using anti-*Pythium* antibodies as described above. Quantitative evaluation of tissue colonisation was carried out by inspection at low magnification and was assessed on a five-class scale (0–20%, very little colonisation; 21–40%; 41–60%; 61–80%; 81–100%, full colonisation). Quantitative evaluation of autofluorescence was carried out by inspection at low magnification and was assessed on a five-class scale (0–20%, very little autofluorescence; 21–40%; 41–60%; 61–80%; 81–100%, strong autofluorescence). For the quantification of colonisation as well as autofluorescence, the surfaces of entire sections were considered.

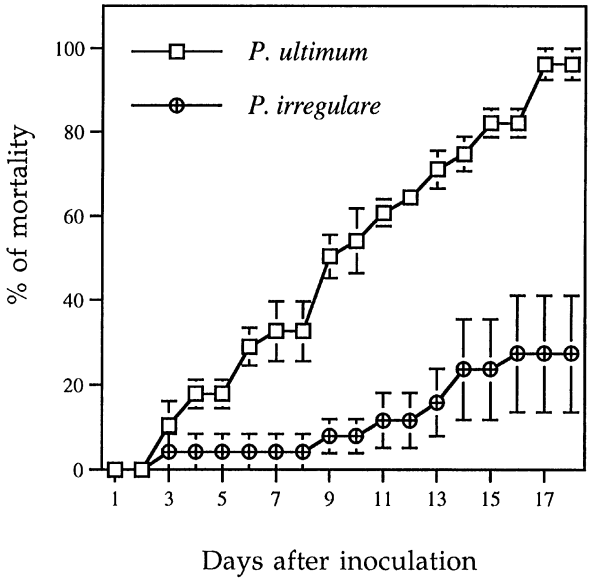

Figure 1. Mortality of *Picea abies* seedlings inoculated with *Pythium* spp. Means of 4 independent experiments (± SD), each consisting of 250 seedlings.

Chitinase activity

Picea abies seedlings were homogenized in liquid nitrogen, resuspended in phosphate buffer (50 mM) at pH 7.0, and centrifuged 10 min at 5000 x g. The supernatant was used for the chitinase assay (Métraux and Boller, 1986). One hundred μl supernatant, 50 μl of 50 mM phosphate buffer at pH 7.0, 100 μl of ^{3}H-chitin were incubated for 30 min at 37 °C. The reaction was stopped with 250 μl of 1M trichloroacetic acid. The mixture was then centrifuged at 5000 x g for 10 min and the radioactivity was measured in a 250 μl aliquot of the supernatant using a scintillation counter.

Salicylic acid analysis

Extractions and analysis using HPLC (System Gold, Beckman, Nyon, Switzerland) were performed according to Meuwly and Métraux (Meuwly and Métraux, 1993).

Results

Histology of the infection process

Inoculation of *Picea abies* seedlings with sporangia of *Pythium* led to disease symptoms which could be observed within 2–3 days of inoculation. The whole plant wilted and exhibited browning of the hypocotyl bases as well as the upper part of the roots and finally damping off. Inoculation with *Pythium ultimum* led to a steady increase in mortality resulting in full loss of the seedling population 17 days after inoculation (Figure 1). Inoculation with the less virulent *Pythium irregulare* led to a mortality of only about 30% of the seedlings (Figure 1). Spruce seedlings exhibited age-related resistance: seedlings older than 4 weeks (± 5 days; n = 8) after emergence remained fully resistant against either of the two *Pythium* species.

Light microscopy and scanning electron microscopy (SEM) were used to follow the time-course of *Pythium* infection. Both *Pythium* species germinated on the epidermis, formed appressoria and penetrated the spruce seedlings. Penetration occurred through the few stomata dispersed on the hypocotyl bases as well as directly through the epidermis (Figures 2 and 3). We also observed that the less virulent *P. irregulare* produced about 25% more appressoria on the surface of *Picea* seedlings (data not shown). Fungal hyphae grew on the surface and within the spruce tissue (Figures 2 and 3). The detection and visualisation of *Pythium* in freshly cut tissue sections was greatly facilitated by the use of phosphatase-labelled anti-*Pythium* antibodies (Figure 3). The vertical (up and down from the infection site, Figure 4) and horizontal progression (from the outer epidermal to the inner endodermal region, Figure 5) of *Pythium* were quantitated. The colonization of roots was much faster than that of hypocotyls (Figures 4 and 5). The less virulent *P. irregulare* grew more slowly in the roots than *P. ultimum*. Similarly, growth in the hypocotyls was faster for *P. ultimum* than for *P. irregulare* during first 48 h after inoculation (Figures 4 and 5). Roots and hypocotyls were more rapidly invaded by the virulent species than by the less virulent one. Hyphae of *P. ultimum* also reached segments closer to cotyledons than those of *P. irregulare* (Figure 4). However, hyphae of either *Pythium* species were never detected in cotyledons which are distant from the site of inoculation, even when the seedlings had damped off. Spruce cotyledons excised from the seedlings and inoculated with *Pythium* species in petri dishes containing PCA medium were never colonized (data not shown). Figure 5 reflects the horizontal penetration of *Pythium* from epidermal to endodermal region. The level of colonization by both species of the epidermal region was very high already 2 days after inoculation. The cortical region was slightly more colonized by *P. ultimum*, than by the less virulent species. The endo-

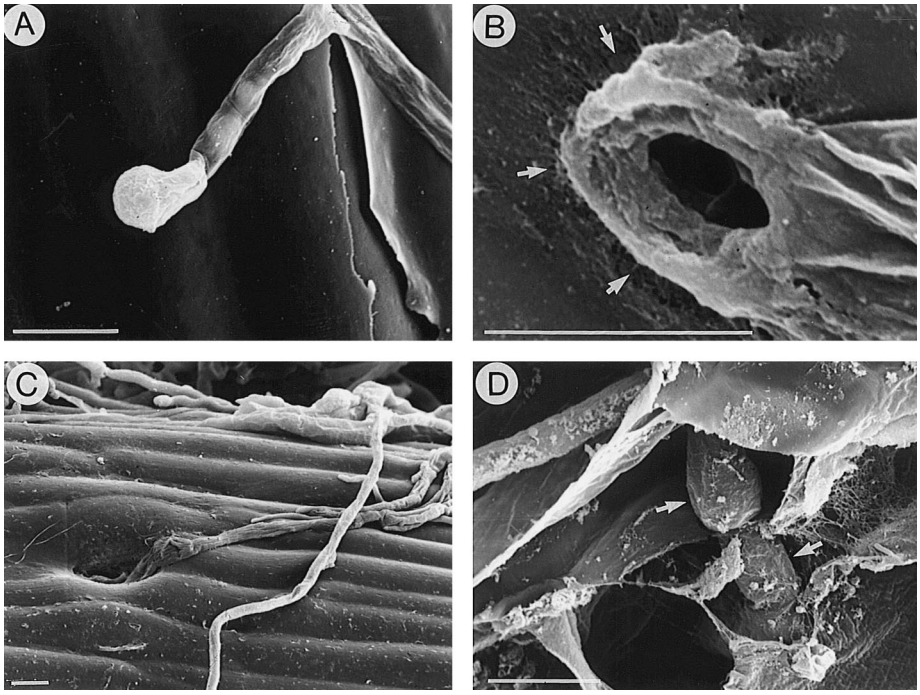

Figure 2. SEM micrographs of *Picea abies* seedling 48 h after inoculation with *Pythium* spp. A: Unstained *Pythium ultimum* hyphal tip forming an appressorium. B: Successful penetration of a *Pythium irregulare* hypha directly through the epidermal cell layer (the hypha was removed during preparation). Arrows show alteration of the cuticle surface around the penetration site. C: *Pythium ultimum* penetrating through stomatal opening on the surface of the spruce hypocotyl. D: Hypocotyl of a spruce seedling tissue with *Pythium ultimum* colonization. Arrows show hypha growing through the cell wall of neighbouring cell layers. Bar = 10 μm.

dermis was penetrated only by *P. ultimum* as early as 4 days after inoculation. *P. irregulare* was never detected in the vascular region inside the endodermis, even up to three weeks after inoculation (data not shown).

Hypocotyl tissues of Norway spruce showed autofluorescence after inoculation with *Pythium* (Figures 3 and 6). Autofluorescence was strongly associated with cell walls and might reflect lignification or suberization. Constitutive autofluorescence was observed in the epidermis. The cortex showed an increased autofluorescence only after inoculation with the highly virulent *P. ultimum*. In the endodermis region, which shows a weak constitutive autofluorescence, an increased deposition of autofluorescent materials after inoculation with both *Pythium* species could be observed. However the increase after *P. ultimum* inoculation was much stronger. The same pattern was observed already 2 and 4 days after inoculation (data not shown).

Biochemical responses to infection

We have studied two biochemical reactions typically associated with defence responses after infection with virulent or less virulent species of *Pythium*. Table 1 shows changes in chitinase activity in different parts of inoculated *P. abies* seedlings. In roots, a high constitutive chitinase activity was detected. Inoculation with either *Pythium* species did not induce marked changes (Table 1). In hypocotyls, the constitutive activity of chitinase was lower than in roots. Upon inoculation with *P. irregulare*, more chitinase activity was induced than with *P. ultimum*. Cotyledons had a lower constitutive chitinase activity than hypocotyls and roots. The induction of chitinase activity was higher upon inoculation with *P. irregulare* than with *P. ultimum.* As the cotyledon tissue was never colonized by the *Pythium* species, the increase of chitinase activity is the result of a systemic activation.

The constitutive levels of SA present in *Picea abies* are shown in Table 2. No changes could be observed in the levels of free SA after inoculation with either *Pythium* species. An increase of bound SA levels was

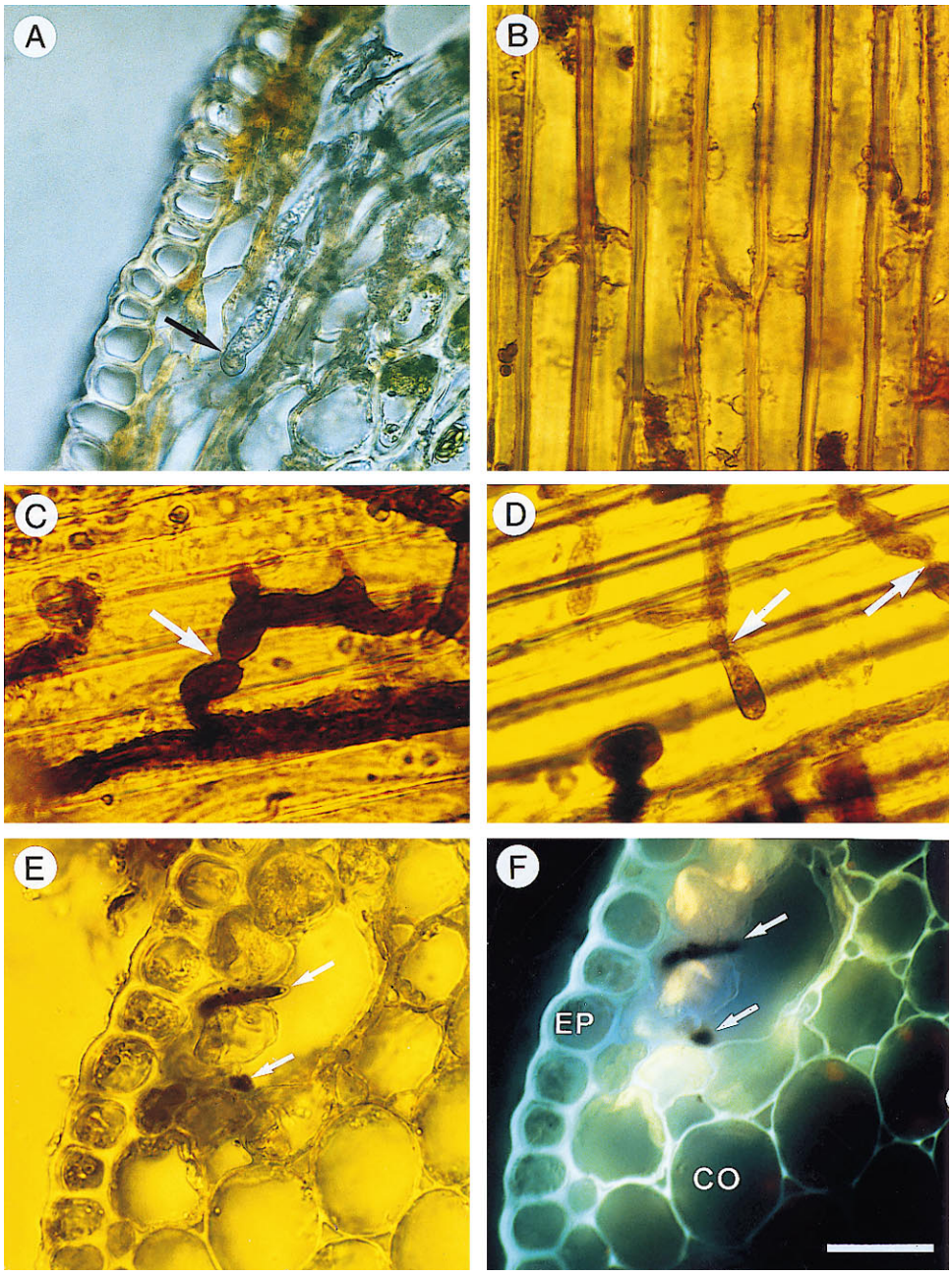

Figure 3. Light micrographs of *Picea abies* tissue 48 h after inoculation with *Pythium* spp. A: Hypocotyl cross-section with hyphal tip (arrow) of *Pythium irregulare* penetrating the cortical region. B: Longitudinal section of *Picea abies* hypocotyl incubated with anti-*Pythium* antibody (without fungal inoculation). C and D: Longitudinal sections of *Picea abies* hypocotyls incubated with anti-*Pythium* antibody and inoculated with *Pythium ultimum* (C) and *Pythium irregulare* (D). Arrows show the sites where the fungus grows directly through cell walls of neighbouring cell layers. E: Cross-section of spruce seedling hypocotyl 4 days after inoculation with *Pythium ultimum*, arrows show hyphal tips stained with anti-*Pythium* antibody. F: Autofluorescence of the same cross-section as shown in E. Abbreviations used in the figure: CO: cortical region, EP: epidermis. Bar = 10 μm.

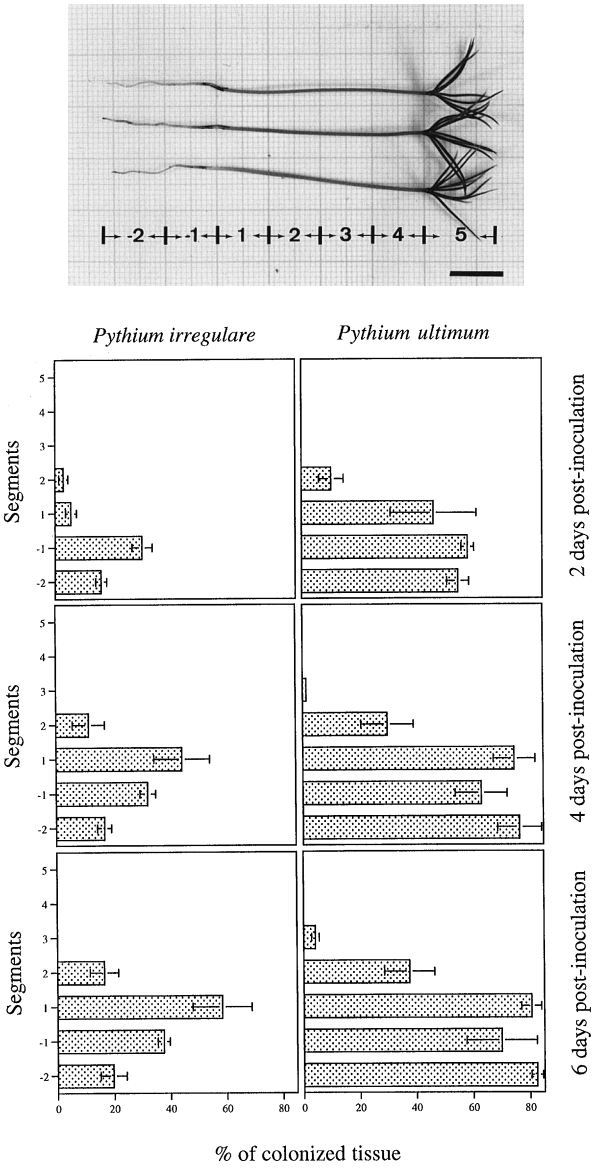

Figure 4. Vertical penetration of *Pythium* spp. hyphae into Norway spruce seedlings 2, 4 and 6 days post-inoculation. Means of 3 independent experiments (± SD), each consisting of 30 longitudinal sections taken from different seedlings. The photograph illustrates two-week-old seedlings and how they were segmented. Segments -2 and -1 are parts of the root. Segments 1, 2, 3 and 4 represent hypocotyl tissue. Segment 5 represents exclusively cotyledons. The percentage of colonized cells was assessed in an entire longitudinal section (8–10 mm long) excised from one of the segments above. Inoculation with *Pythium* sporangia suspension took place at the basis of the hypocotyl, between segment 1 and -1. Bar = 1cm.

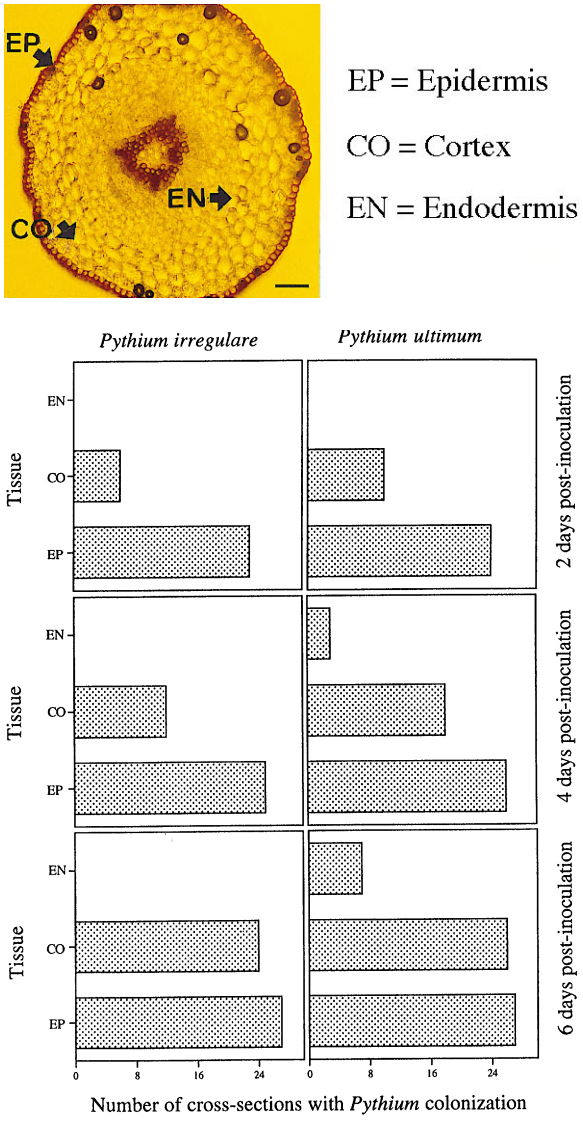

Figure 5. Horizontal penetration of *Pythium* spp. hyphae into Norway spruce tissues 2, 4 and 6 days post-inoculation at the basis of the hypocotyl, where the seedlings were inoculated with *Pythium* sporangia suspension. For each measurement, 30 cross-sections of 30 different seedlings were analysed. The micrograph illustrates a cross-section of spruce seedling 10 days after emergence. Bar = 0.1mm.

observed after inoculation in roots infected with the less virulent *P. irregulare* as compared to *P. ultimum* and mock-inoculated plants. In cotyledons, bound SA increased above the control levels only after inoculation with *P. ultimum* (Table 2).

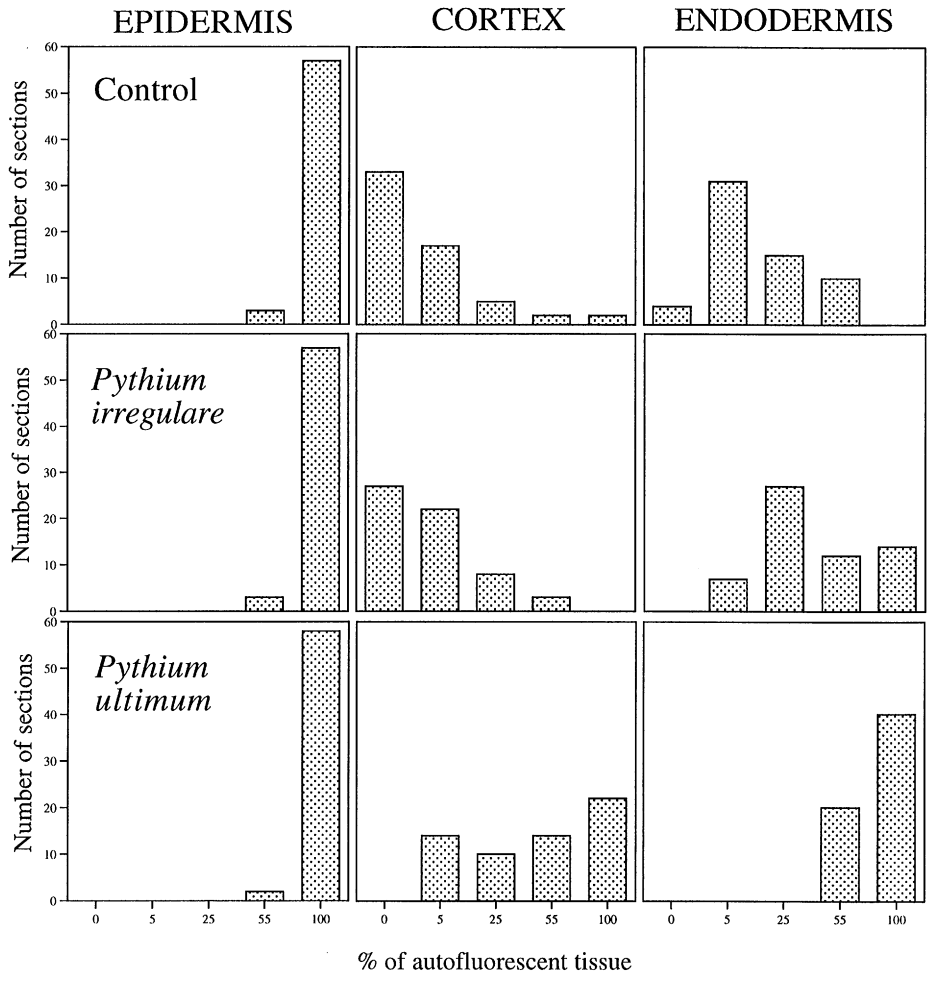

Figure 6. Induction of autofluorescence in different spruce tissues 6 days after inoculation with *Pythium* sp. For each measurement 60 cross-sections of different seedlings were investigated. Sections were taken from the basis of the hypocotyl, where the seedlings were inoculated with *Pythium* sporangia suspension.

Discussion

We have developed a test system reproducing reliably natural conditions for infection of spruce with *Pythium*. We have used *P. ultimum*, a highly virulent and *P. irregulare* a less virulent species, both closely related and belonging to the same *Pythium* group (Hendrix and Campbell, 1973; White et al., 1994). They are often isolated together from soil as well as from roots of spruce seedlings in forest nurseries (Popoff et al., 1975; Smith, 1988; White et al., 1994). Since no major differences were detected in vitro with respect to spore germination, sporangia formation and growth rate (data not shown), we conclude that the differences in virulence are due to the reaction of the plant towards the respective pathogen. In order to understand why *P. irregulare* causes less damage than *P. ultimum*, we have monitored histological as well as biochemical changes in spruce seedlings during the course of the pathogenesis.

Observations by scanning electron microscopy indicated that *P. irregulare* produced about 25% more appressoria than the virulent one (data not shown), which might reflect difficulties in penetration. This could possibly slow down the progression of the fungus. Our observations however, showed that both species penetrated through the epidermis and grew into the seedling tissue.

Inoculation with both *Pythium* species led to a severe penetration and colonization of the roots (Fig-

Table 1. Chitinase activity in different parts of *Picea abies* seedlings 6 days after inoculation with the less virulent *Pythium irregulare* and the highly virulent *Pythium ultimum* (dpm/mg protein, ± SD, 3 experiments each consisting of 150 seedlings)

Species	Roots	Hypocotyls	Cotyledons
Control	977.5 (± 477.5)	212.5 (± 72.2)	68.0 (± 8.7)
Pythium irregulare	2393.4 (± 384.4)	551.5 (± 54.6)	330.5 (± 30.5)
Pythium ultimum	2290.5 (± 330.2)	283.3 (± 73.7)	209.5 (± 9.6)

Table 2. Salicylic acid levels in different parts of *Picea abies* seedlings 6 days after inoculation with the less virulent *Pythium irregulare* and the highly virulent *Pythium ultimum* (ng/g FW, ± SD, 3 experiments each consisting of 200 seedlings)

Species	Roots	Hypocotyls	Cotyledons
A) Free salicylic acid			
Control	27.5 (± 18.6)	20.5 (± 4.4)	16.4 (± 5.5)
Pythium irregulare	50.1 (± 31.1)	15.8 (± 1.7)	23.1 (±7.0)
Pythium ultimum	25.9 (± 13.9)	19.7 (± 12.6)	20.5 (± 10.4)
B) Bound salicylic acid			
Control	44.3 (± 6.8)	37.1 (± 4.8)	72.6 (± 7.0)
Pythium irregulare	123.5 (± 9.8)	40.4 (± 14.6)	68.3 (± 2.3)
Pythium ultimum	47.4 (± 0.9)	41.0 (± 15.6)	115.4 (± 14.5)

ure 4). Two to 4 days after inoculation, mycelia appeared in the hypocotyl tissue, the time at which the first seedlings started to tip over and die (Figure 1). Death of spruce seedlings 4 days after *Pythium* inoculation was also observed by others (Borja et al., 1995; Sharma et al., 1993). The virulent *P. ultimum* grew faster horizontally and vertically from the site of inoculation. After 4 days, it caused strong inhibition of root growth and browning of the root as well as the bases of the hypocotyl. Similar symptoms have been detected after *P. irregulare* inoculation, but to a much smaller extent. Interestingly, *Pythium* hyphae were never detected in cotyledons (Figure 4). One explanation could be that the distance between roots and cotyledons is too long and seedlings die before the fungus reaches the top of the plant. Another possibility is that cotyledons produce antifungal metabolites which block the invading tissue. This is supported by our observations that cotyledon tissue was never colonized if placed close to *Pythium* mycelia in a petri dish, possibly due to the presence of antifungal compounds in this tissue (Kozlowski and Métraux, unpublished results). The strong virulence of *P. ultimum* is illustrated in Figure 5. It shows that colonization of the vascular region inside the endodermis takes 4 days. This correlates closely with the time of first damping off symptoms (Figure 1). Thus, the strong virulence of *Pythium* species is likely a result of their capacity for rapid invasion with subsequent destruction of the colonized tissue.

Conifers, as many plants, respond to infection by induction of various structural changes such as papillae formation, accumulation of lignin and suberin and by a browning response due to increased accumulation of phenolics (Asiegbu et al., 1994; Bonello et al., 1991; Borja et al., 1995; Mauch et al., 1988). In the present work, we could detect an increase in deposition of autofluorescent materials in the cell walls of the cortex and endodermis as early as 2 days after inoculation with *P. ultimum* and *P. irregulare* (data not shown). We have observed that the increase in autofluorescence of cortex end endodermal region, appears concomitantly with the colonization of the tissue by *Pythium* spp. Taking into account that at this time the *P. ultimum* is already well established in the tissue (Figures 4 and 5) it seems unlikely that the deposition of autofluorescent materials represents a serious barrier for the colonization by this species. In contrast, *P. irregulare* does not invade the vascular cylinder (Figure 5). The pattern of induction of autofluorescence in the cortex and endodermis reflects the extent of colonization of the tissue by the fungus and is strongest in *P. ultimum*-

infected tissue. Clearly, the seedlings are able to detect the invaders and react, but this response is insufficient to slow down the progression of either pathogen. Results presented in Figure 5 might suggest however, that for the less virulent *P. irregulare*, lignification and suberinization of endodermis constitutes a barrier, since this pathogen could never grow through this layer of cells. This might explain the higher tolerance of *P. abies* towards *P. irregulare*. Presumably, deposition of autofluorescent materials is a general reaction displayed against invaders but the fast-growing *Pythium ultimum* remains unaffected.

Chitinases have been shown to be antifungal (Mauch et al., 1988) and are often referred to as defence proteins (Stintzi et al., 1993). It was already reported that conifers are also producing chitinase upon attack by pathogens (Kärenlampi et al., 1994; Sauter and Hager, 1989; Sharma et al., 1993). In the present study, we have extended such observations and found a strong constitutive chitinase activity in roots and an inducible activity in the hypocotyl (Table 1). The induction of chitinase was stronger by the less virulent *P. irregulare* than by *P. ultimum*, suggesting a lesser perception of the virulent form by the plant. Similar to autofluorescence, chitinase induction might be part of a syndrome of induced barriers which may act against potential invaders, most likely non-host pathogens. The fact that both *Pythium* species can invade root tissue where chitinase activity is strongest, suggests that chitinase may not represent a serious barrier for this particular fungus. Our results also show a systemic induction of chitinase activity in the cotyledons which remained uncolonized by the pathogen (Table 1 and Figure 4). This supports the notion of a systemic signal produced at the site of pathogen attack and translocated to distant parts of the plant, a phenomenon well described in many angiosperms (Madamanchi and Kuc, 1991; Schneider et al., 1996; Sticher et al., 1997). Presumably, chitinase is part of a complex array of defence lines against pathogen or other stresses and it will now be interesting to determine against which invader and in combination of which other barriers they may act.

SA is necessary for induction of systemic acquired resistance (SAR) in several dicotyledonous species (Lee et al., 1995; Ryals et al., 1996; Schneider et al., 1996; Sticher et al., 1997). Unlike Klick and Herrman (Klick and Hermann, 1988) and Strack et al. (Strack et al., 1989), we have reported here that both free and bound forms of SA could be detected in all spruce organs. An increase in the content of free and bound SA was only detected in roots after inoculation with *P. irregulare* (Table 2). Since this is not linked to any resistance response in the root tissue and since applied SA has no effect on resistance nor associated symptoms (data not shown), we find no evidence supporting a role for SA as a signal in spruce seedlings.

Considering these results, it is clear that *Picea abies* seedling are able to react differentially to soilborne pathogens by means of structural and biochemical changes very similar to those of angiosperms.

Acknowledgements

We thank Ms. Wybrecht, for providing the *Pythium* strains; Drs. U. Heiniger and D. Rigling for providing the spruce seeds; Prof. M. Celio for generous help in the antibody preparation; Drs. U. Ryser, U. Drenhaus and D. Cao, for assistance in the microscopy work; Dr. Ph. Meuwly and W. Mölders for help with the HPLC analyses, and Drs. T. Buchala and K. Summermatter for help with the chitinase activity analyses.

Funds for this research were provided by COST 813, grant No. OFES C92.0023. Additional support was obtained from a grant from the Swiss National Science foundation (Nr. 31-37098.92).

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