Cytopathological Response in Roots of *Picea abies* Seedlings Infected with *Pythium dimorphum*

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We thank M. Jensen, E. Reed, and T. Gulliksen for providing excellent technical assistance during this work.
Accepted for publication 30 December 1994.

ABSTRACT

Børja, I., Sharma, P., Krekling, T., and Lönneborg, A. 1995. Cytopathological response in roots of *Picea abies* seedlings infected with *Pythium dimorphum*. Phytopathology 85:495-501.

Roots of *Picea abies* seedlings were inoculated with pathogenic *Pythium dimorphum*. The ongoing lignification process, the accumulation of lignin, and the distribution of flavanols and condensed tannins (CT), were related to the concurrent visual disease symptoms, hyphal colonization, and cellular changes. The hyphae ramified in both the cortex and the stele within 24 h. Three days after inoculation the concentration of lignin had increased to a level twice that in noninfected tissues. With histochemical staining, the accumulation of lignin and the ongoing lignification were co-located in the inner cortex layer 4–6 days after infection. Comparison of temporal onset of lignification with the timing of the intruding hyphae shows that this defense response occurs too late to effectively prevent the pathogen from spreading. Flavanols and CT were also detected in both infected and noninfected roots. Increased staining of these compounds in infected roots was often detected in the inner cortex. Electron microscopic studies revealed that at least some of these phenolic compounds were localized either as spherules or as a layer appressed to the tonoplast of the central vacuole.

Additional keyword: pathogenesis-related (PR) proteins.

Plants respond to pathogen infection with an array of defense mechanisms. One such mechanism is the synthesis of pathogenesis-related (PR) proteins (26,46), which include hydrolytic enzymes such as chitinases and β-1,3-glucanases (18,19,39). These enzymes can act synergistically, both inhibiting the growth and lysing the cell walls of fungi penetrating the host tissues (4,30).

Another complementary defense mechanism is the activation of the phenylpropanoid pathway that results in the production of phenolic compounds such as phytoalexins (nonspecific fungitoxic substances), polyphenols, and lignins (33,47). The latter, together with hydroxyproline-rich glycoproteins (31) and callose (in papillae), change the architecture of the host cell wall, reinforce it, and provide a mechanical barrier against pathogen ingress. Other phenolic compounds such as flavanols and their derivatives (condensed tannins, CT) accumulate within the host cells and are involved in biochemical defense strategies (29,33).

Several studies support the concept that the ability of plants to resist a pathogen is related not only to activation of defense genes but is also largely dependent on the speed and coordination with which the different defense strategies become expressed (3,12). The efficacy of active defense is dependent on both the timing and localization of defense products during the course of infection.

We used infection of roots of *Picea abies* (L.) H. Karst. seedlings by *Pythium dimorphum* J. W. Hendrix & W. A. Campbell as a model system to study host-pathogen interactions. *Pythium* spp. belong to a group of soilborne pathogens causing disease in juvenile and succulent tissues (13,14,27). This pathogen is common in nurseries and has been isolated repeatedly from roots of coniferous seedlings with root dieback symptoms in Nordic forest nurseries (5,25,49). In our previous work (40) we have shown that more than 30 different PR-proteins accumulated in *P. abies* roots after infection with *Pythium* sp. (later identified as *P. dimorphum*). Among these, chitinases, chitosanases, and β-1,3-glucanases have been detected.

The objective of our study was to further survey the host-pathogen interaction by studying the infection pattern and assessing some of the concomitant cellular changes occurring in the host root tissue. We relate the occurrence and kinetics of phenolic compounds such as lignins, flavanols, and CT in the infected roots to parallel development of visual disease symptoms.

MATERIALS AND METHODS

Biological material. Seeds of *P. abies,* Norway spruce, were provided by the State Forest Seed Extractory and Testing Station, Hamar, Norway. They were collected in 1987 from the seed orchards in Nome, Bø, and Kviteseid, Norway. The fungal culture of *Pythium* (isolate 83-100/Øa), which was referred to in the previous work (40) as *Pythium* sp., has recently been determined to be *P. dimorphum*. It is a pathogenic strain, isolated from roots of a *P. abies* seedling with root dieback symptoms in Buskerud Forest Nursery, Norway. The strain has been maintained in the culture collection of the Norwegian Forest Research Institute.

Plant-culturing and inoculation methods. Seeds were disinfested in sodium hypochlorite (4.5%, v/v) and germinated on malt agar (1.25% malt, w/v, Difco Laboratories, Detroit, MI, in 2% agar w/v, Norsk Medisinaldepot) medium. Mycelia of *P. dimorphum* were grown for 10 days in liquid malt (1.25%, w/v) medium and subsequently homogenized with an Ultra-Turrax T25 homogenizer as described by Sharma et al (40). Mycelial fragments were adjusted to a concentration of 0.015 g dry weight per milliliter of homogenate, which equals approximately 3×10^3 cfu. Sterile, black microfuge tubes were filled with homogenate and the lid perforated. A 10-day-old seedling was inserted through the opening in the lid of each tube, submerging the whole root into the mycelial homogenate. Control seedlings were inserted in tubes containing malt medium only (1.25%, w/v). Roots were examined in the stereomicroscope and light microscope continuously until 8 h after inoculation in order to follow infection development. Infected and control seedlings were sampled after 1, 2, 3, 4, and 6 days.

Histochemical staining tests. Roots were dried gently on tissue paper. Root segments from the root hair zone were hand sectioned,

mounted directly in the staining reagents for 5–10 min, and examined in the light microscope using polarized light. Lignin was localized by a test in which phloroglucinol in acid solution reacts with aldehyde groups in the lignin molecules to give a purple-red color in the light microscope. When using polarized light, which gave a better visual resolution, the stained areas appeared as yellow-brown. Flavanols and CT were detected using vanillin-HCl. In polarized light the staining was violet-red. Staining reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and prepared according to Johansen (17). Localization of ongoing lignification was carried out by detection of H_2O_2, since it is involved in the final step of lignin polymerization. Histochemical assay for H_2O_2 was performed according to Olson and Varner (34), by treating the sections with starch-KI, in which H_2O_2 oxidizes iodide ions to iodine and iodine is complexed by the starch to form a blue-purple color, which in polarized light appears as yellow-brown.

Lignin analysis. Lignin concentration was determined with thioglycolic acid (TGA, Sigma) using the assay described by Cahill and McComb (7), in which TGA reacts with the α-alkoxy function in lignin to give ligninthioglycolic acid. The relative amount of ligninthioglycolic acid was calculated as the absorbance at 280 nm ml^{-1} mg^{-1} dry weight. The assay was repeated four times and results show the average of the two last repetitions. Each sample contained 80 roots.

Electron microscopy. Scanning (SEM) and transmission (TEM) electron microscopy were used to follow the hyphal penetration and cellular changes after the infection. Flavanols and CT were detected with TEM by viewing the osmium tetroxide–fixed material. Flavanols and CT are known to be strongly osmiophilic and by osmium treatment become electron dense (35). Root segments, 3–5 mm long, were dissected from the root hair zone and fixed by immersion in 3% (v/v) glutaraldehyde in 0.1 M HEPES buffer (pH 7.3) overnight. Segments were then rinsed in buffer (three times for 15 min), postfixed (2 h) in buffered osmium tetroxide (2%, w/v), and rinsed again before dehydration with ethanol (70–100%). All steps were performed at room temperature.

For the detection of phenolic substances (flavanols and CT) root segments were fixed in 2% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 50 mM Na-cacodylate buffer (pH 7.2) and incubated overnight at 4 C. They were washed three times with the buffer and postfixed in 1% (w/v) osmium tetroxide in 50 mM Na-cacodylate buffer for 1 h at room temperature.

For SEM, dehydrated segments were critical point dried using carbon dioxide as the transition fluid, mounted with colloidal silver on aluminum stubs, and coated with platina/palladium in a sputtercoater before examination in a JEOL 850 microscope at 15 kV.

For TEM, ultrathin transversal sections (≈80 nm) were cut from segments embedded in Spurr epoxy resin (41), stained with uranyl acetate (43) and lead citrate (48), and examined in a JEOL 1200 EX microscope at 80 kV.

RESULTS

Visual symptoms. One day after fungal infection, roots became evenly light brown. Slight discoloration of the hypocotyl from fresh green to yellowish green was typical. By the second day, the upper part of the root, above the root hair zone, became darker brown with necroses of the hypocotyl. By the third day, the majority of the inoculated seedlings had withered, with symptoms such as necrotic hypocotyls and slightly swollen upper parts of the root, but the root tip remained light colored. Neither stem nor root elongation occurred after inoculation.

Hyphal colonization and cellular host-response. Within 3–4 h after inoculation the hyphae grew, adhering to the root surface (Fig. 1A,B). The whole root became encapsulated by the mycelium 2–3 h later. Infecting hyphae penetrated the root by growing between the loosely woven cells of the epidermis (Fig. 1A,B). The penetration of the cell wall by hyphae was observed in the exodermis layer (Fig. 1C), which is structurally more compact than the epidermis (Fig. 1A,B). There were visible cell wall alterations in the vicinity of penetrating hyphae (Fig. 1C). The hyphae ramified in the cortex and stele within 24 h after inoculation (Fig. 1D). At the site of penetration the hyphae formed an appressorium and infection peg (Fig. 2A). Hyphae penetrated both the cell wall and plasma membrane (Fig. 2A) or were localized between the cell wall and plasma membrane, not proliferating into the cytoplasm (Fig. 2A). Fungal ingress into the host cells was associated with severe alterations in the structure of cellular content within 24 h (Fig. 2B,C). The plasma membrane in the infected cells was convoluted. Darkly granulated, electron dense, strongly osmiophilic compounds became visible in the disrupted cells (Fig. 2B). These compounds were scattered throughout the cell interior, sometimes filling the major part of it. Control tissues remained unchanged, with a large central vacuole containing electron-lucid material (Fig. 2C). The hyphae grew by different routes in different cell layers. In the outer cortex the growth was both inter- and intracellular (Fig. 2B). Intercellular growth occurred in the middle lamella, often resulting in cell separation (Fig. 2D). Intracellular growth was predominant in inner cortex and stele.

Histochemical tests for localization of H_2O_2 and lignin. The presence of H_2O_2, indicating ongoing lignification, was detected first only in the outer cortex and vascular bundles in the control tissues (Fig. 3A). Later the staining extended evenly throughout the cortex and vascular bundles. In infected cells, the lignification process was detected first in the outer cortex, thereafter uniformly throughout the entire cortex and vascular bundles. Four days after inoculation the most intensive staining was detected in the inner layers of the cortex (Fig. 3B). The temporal development of staining lignin, as a product, with phloroglucinol-HCl in control tissue was similar to that in infected tissue, and was ultimately detected evenly throughout the whole cortex (Fig. 3C), without the intensive staining close to the endodermis. In infected tissues lignin was detected mainly in cell walls of outer cortical cells at the beginning of infection (Fig. 3D). After 4 days it was detected throughout the cortex and the sixth day after inoculation the staining was most intense in the inner cortex (Fig. 3E). There was no indicative staining detected in vascular bundles in either infected or control tissues. Histochemical staining showed that the lignification process was co-located with lignin presence in the inner layer of cortex, close to the endodermis. The lignification process, detected in vascular bundles, however, was not co-located with the lignin presence there.

Localization of flavanols and CT. Infected and control root tissues tested positive for flavanols and CT with vanillin-HCl, from the first day after inoculation. The staining was strong and uniform throughout the cortex and in vascular tissue (Fig. 3F). In infected roots, however, the most intense staining was detected in the inner cortex layer, adjacent to endodermis and vascular tissue. Occasionally the staining was seen in two rings: in the outer cortex layer, and in the inner cortex layer (not shown).

Examination with TEM showed that CT were found in infected and control cells. They were restricted to vacuoles, forming spherules, closely appressed to the inner layer of tonoplast (Fig. 2E).

TGA assay. The concentration of lignin was measured as a concentration of ligninthioglycolic acid and detected spectrophotometrically. The lignin concentration increased slightly until the second day in both infected and noninfected roots (Fig. 4). The enhanced increase of infection-induced lignin was detected from the second day and this rising tendency persisted until the sixth day. From the third day the concentration of lignin in the control tissue was half of that in infected tissues (Fig. 4).

DISCUSSION

Mycelial fragments were used for inoculation in our study. We tested different concentrations and 3×10 cfu per milliliter provided for an efficient infection. Kendrick and Wilbur (20) concluded that concentration of more than 500 viable propagules of *Pythium* per gram of soil is needed in order to obtain severe preemergence dieback of lima beans. Kraft et al (22) achieved a successful infection of bentgrass by *Pythium aphanidermatum* by using a concentration of $5–6 \times 10^3$ motile zoospores. It is

known that vegetative hyphae cause much heavier infection than do zoospores (28) and it may be that our concentration of cfu was unrealistically high. However, the mode of fungal penetration of spruce roots observed is in agreement with histological studies on penetration and colonization of the host tissue by *Pythium ultimum* (32), *Pythium debaryanum* (8), and other fungal pathogens (4). We could also detect visible cell wall alterations at the penetration site and cell separation during hyphal growth through the middle lamella. It is known that fungi are capable of excreting lytic enzymes, which can degrade a number of polymeric carbohydrates found in the cell walls, and may be associated with these changes (1,3).

Deposition of polymers such as callose (papillae), lignin, flavanols, and CT in infected cells has been suggested as effective in limiting

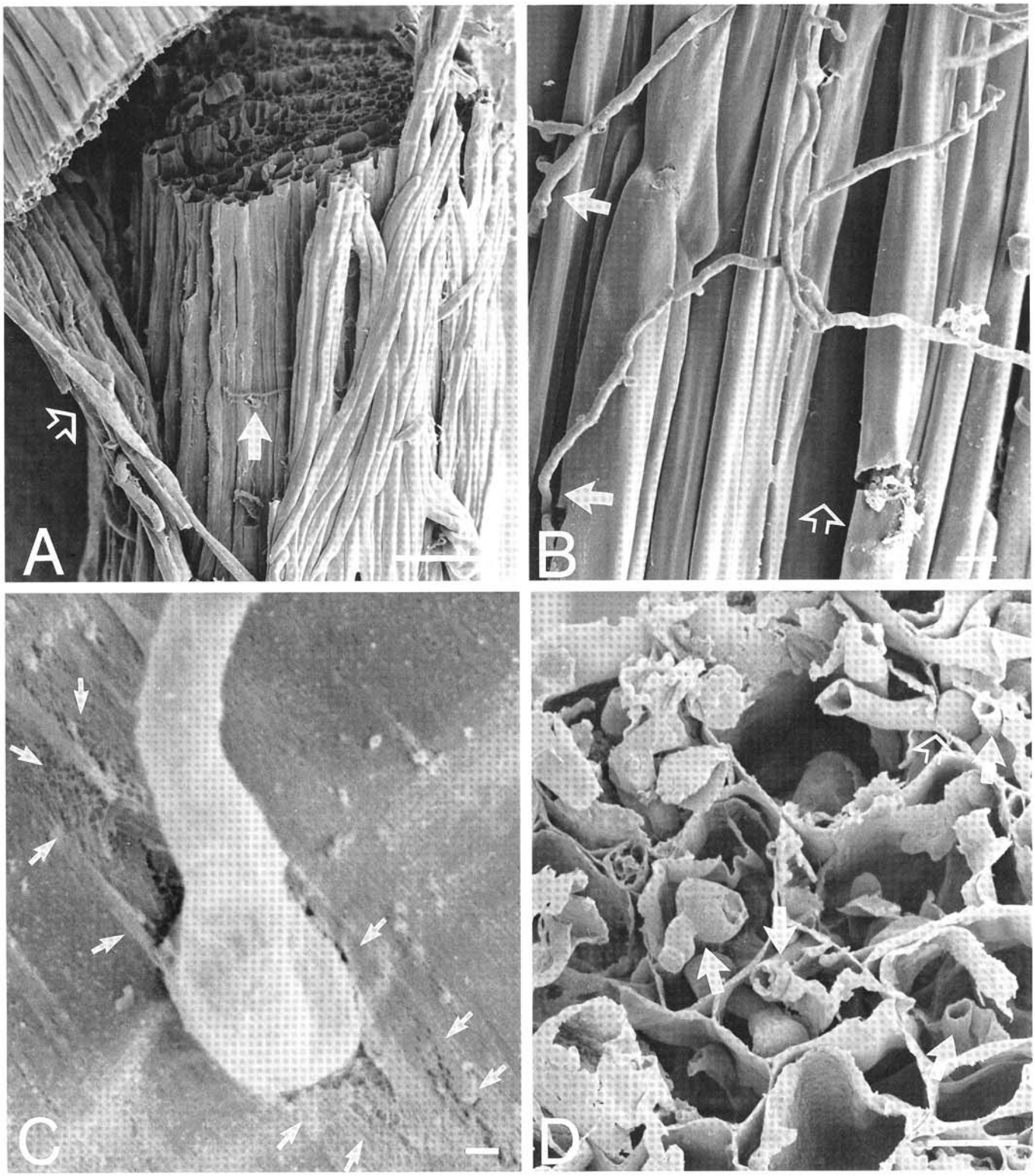

Fig. 1. Scanning electron micrographs of root segments and transversal sections of *Picea abies* roots noninfected or infected with *Pythium dimorphum*. **A,** Hyphae growing between epidermal cells (open arrow) first penetrate underlying layer of exodermis (arrow), (bar = 100 μm). **B,** Structure of root epidermis with loosely woven cells and clefts (open arrow) between them. Hyphae (arrow) do not penetrate epidermal cells but grow between them (bar = 10 μm). **C,** Hypha forming appressorium penetrates exodermis cell wall. Near appressorium a host cell wall alteration is visible (arrows), (bar = 1 μm). **D,** Section through root cortex 24 h after inoculation. Hyphae (arrows) have ramified all cortex tissue. Hyphal penetration (open arrow) from intercellular space through cell wall (bar = 10 μm).

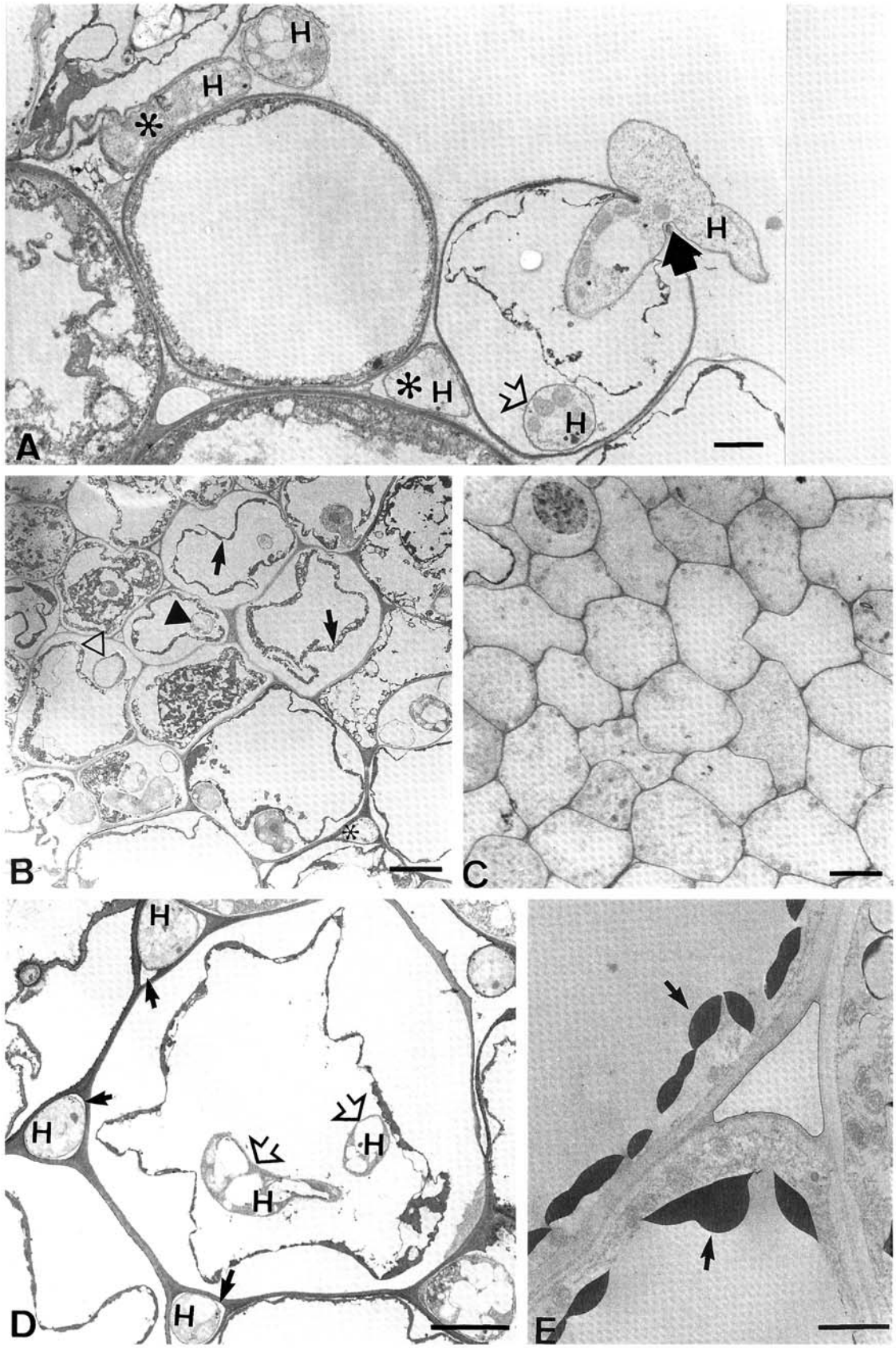

Fig. 2. Transmission electron micrographs of transverse sections of *Picea abies* roots noninfected or infected with *Pythium dimorphum*. **A,** Hyphae (H) forming appressorium and infection peg, penetrating cell wall and plasma membrane simultaneously (arrow). Hypha, localized between cell wall and plasma membrane (open arrow) or growing intercellularly (asterisk) (bar = 2 μm). **B,** Cortex of infected root, 24 h after inoculation. Infected cells show convolution of plasma membrane, cytoplasmic debris (arrows) is concentrated on inside of plasma membrane and changes from electron opaque to electron dense. Hyphal growth in outer cortex occurs frequently in middle lamella (asterisk). In inner cortex, hyphae grow inside of protoplast (triangle) and/or between cell walls and plasma membrane (open triangle) (bar = 5 μm). **C,** Cortex and stele of noninfected control root (bar = 5 μm). **D,** Hyphae (H), 24 h after infection, growing through middle lamella of cortex cells with visible cell separation (arrow). Some hyphae grow also inside protoplast (open arrow) (bar = 5 μm). **E,** Electron-dense spherules of flavanols and condensed tannins in noninfected cell. Localization (arrow) is mostly inside vacuole, closely appressed to tonoplast (bar = 2 μm).

the spread of pathogens in host tissue mechanically and biochemically (21,29). With the TEM-methods used in our study we could not detect any callose production at any stage of infection. In analogous studies, callose deposition is generally lacking in compatible host-pathogen interactions (42,44). This fits well with our system in which *P. abies* seedlings were highly susceptible to *P. dimorphum*. It has also been suggested that the plasma membrane–cell wall interface must be intact for effective callose formation (15,16). This was not the case in the infected root cells in our study and may be a possible explanation for the lack of detectable callose formation.

Lignins are known to inhibit or retard the enzymatic digestion of host cell walls, hence hindering fungal growth through the host tissue (21,24,47). In our study the lignin increase was detected with TGA assay 2 days after infection, whereas histochemical staining with phloroglucinol-HCl was fully indicative of lignin presence 4–6 days after inoculation. Our results corroborate other observations (7,10,36) that TGA assay can detect the infection-induced lignin earlier (1–2 days after inoculation) than histochemical reaction with phloroglucinol-HCl and hence is more sensitive.

It was postulated that lignification is a part of disease resistance expression (47). However, it is unclear whether lignification is rapid enough to play a role in the first line of the plant's resistance responses (29). Cahill and McComb (7) described lignin increase in the roots of resistant *Eucalyptus calophylla* infected with *Phytophthora cinnamomi* after 24 h, compared with constant levels in roots of susceptible *Eucalyptus marginata*. In our case

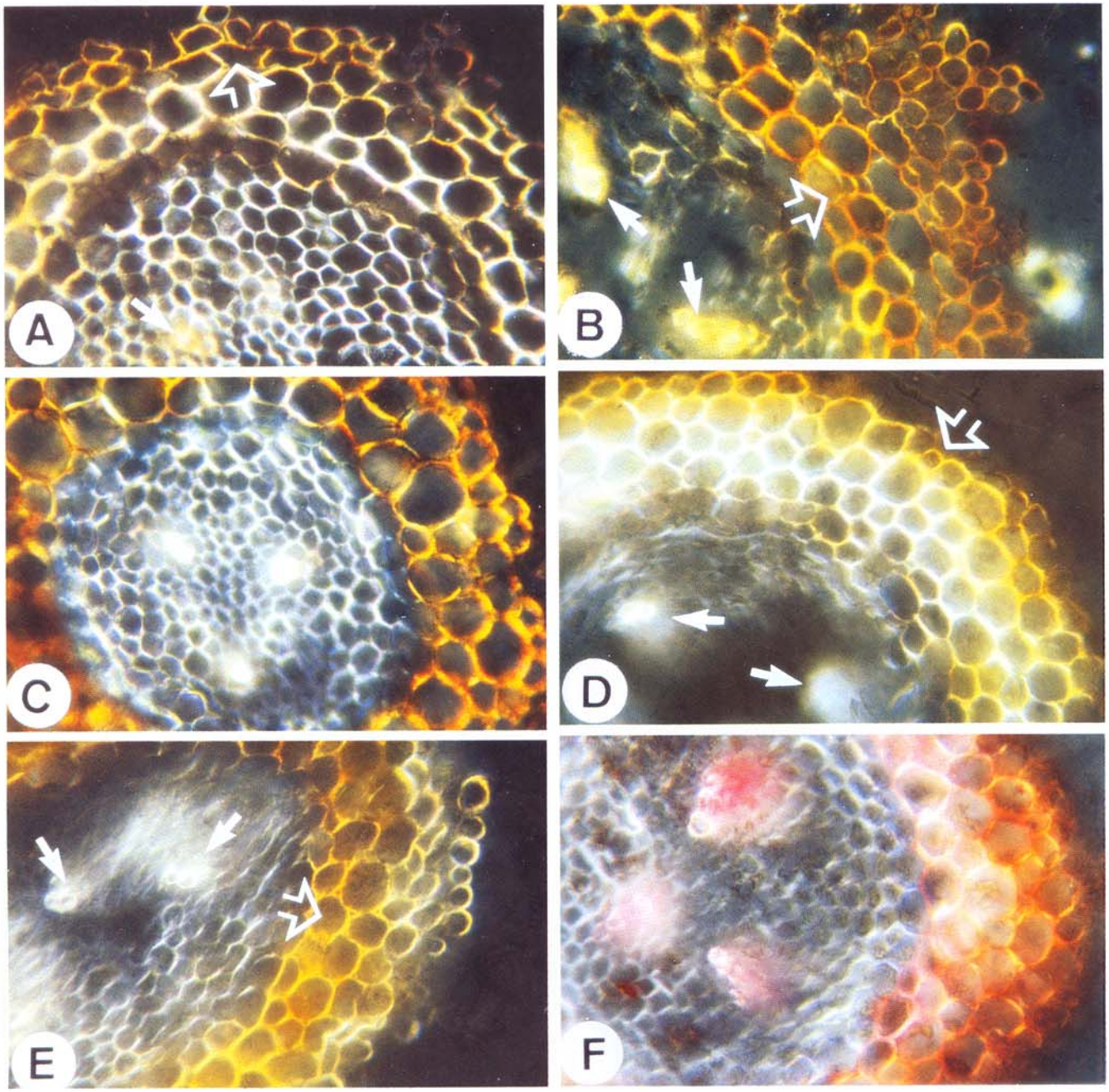

Fig. 3. Representative patterns after histochemical staining of *Picea abies* root sections infected with *Pythium dimorphum,* viewed in polarized light. **A,** Localization of H_2O_2, with KI in starch, shows ongoing process of lignification. In control tissues, presence is detected mainly in outer cortex (open arrow) and vascular bundles (arrow). **B,** In infected tissues the most intense staining for lignification process is detected in whole cortex, mainly in layer of inner cortex (open arrow), close to endodermis and vascular bundles (arrow). **C,** Lignin, detected with phloroglucinol-HCl, in control tissues 6 days after inoculation is distributed uniformly through whole cortex. No staining in vascular bundles. **D,** Lignin in root tissue 5 h after inoculation. Positive staining in outer cortex (open arrow). No staining in vascular bundles (arrows). **E,** Cell walls of whole cortex stain positively for lignin 6 days after inoculation. Most intensive staining is visible in inner cortex (open arrow). No staining in vascular bundles (arrows). **F,** Cell walls of whole cortex and vascular tissue stain positively for flavanols with vanillin-HCl in control tissue.

the lignin level was similar in both infected and noninfected roots until the second day after inoculation, when a larger increase was detected in the infected roots. This slow increase of lignin concentration compared with the high speed of hyphal colonization may be explained by the susceptibility of *P. abies* seedlings.

Flavanols and CT are associated with rapid response to pathogenic infection (33) but are also constitutively present within plant tissues (38). The presence of these compounds is reported to vary not only in different plant species but also in different plant organs (23,38). The vanillin-HCl test is, however, not completely specific for flavanols and CT (11,37). Anthocyanins and dihydrochalcones react with vanillin-HCL as well. When our sections were treated with HCl in ethanol alone, there was a slight coloration detected in the outer cortex, which may indicate the presence of anthocyanins and dihydrochalcones. Although the staining was very weak, the presence of these compounds in the root tissue may explain the color shift in the outer cortex layer of control tissues. The common detection pattern of ongoing lignification, lignin accumulation, and presence of flavanols and CT in a cortex layer close to the endodermis may suggest that these mechanisms are protecting the vitally important vascular tissues from pathogen. Therefore, the constitutive presence of flavanols and CT may be implicated in rapid, first-line defense. This process would ward off pathogens at the critical stage of entry, which leaves no time for de novo synthesis of enzymes or lignin in the host (29). We observed a dramatic increase in electron-dense, granular compounds in infected tissues in accordance with earlier studies (15, 16,45). The nature of these compounds is not known. Since polyphenols are also easily oxidized by osmium tetroxide, it is possible that electron-dense materials are aggregates of polyphenols and other substances such as proteins. The aggregation may also represent a necrotizing process in which cellular components are disorganized into electron-dense granular debris. In our control tissues, the flavanols and CT were restricted mainly to the vacuoles and may precipitate proteins only available there. If the protein concentration increases because of the infection (fungal extracellular proteins present in addition to cytoplasmic proteins sieving in through disrupted tonoplasts), then the increase of visible, electron-dense spherules scattered within the cells may be ascribed to higher precipitation rates of flavanols and CT (released from the disrupted vacuoles), when compared with controls. The phenomenon of shift in the cytoplasmic pH to more acidic after infection (29) may also contribute to protein precipitation.

In resistant plants PR-proteins are produced rather rapidly upon interaction with pathogens (6,19). In susceptible plants a quantitatively similar but kinetically different PR-response has been reported; the induction of PR-proteins is considerably delayed, and therefore less effective, compared with resistant plants (2,9, 18). A similar temporal pattern was described in the case of lignin deposition in susceptible and resistant plants (7). In Table 1, the temporal and quantitative changes on concurrent cellular and molecular levels in roots of *P.abies* infected with *P. dimorphum* are summarized. In our study, the whole root was colonized by the fungus within 24 h. Similar colonization speed of susceptible tissues has also been reported in other systems (3,33). Hyphal colonization of the root tissue caused parallel alteration of the cellular ultrastructure (accumulation of electron-dense, osmiophilic aggregates and spherules). It supports the view that rapid fungal colonization is accompanied by massive activation of defense mechanisms in order to halt the fungal ingress. The first detectable accumulation of PR-proteins was observed within 24 h after inoculation (40). Among these proteins some had chitinase activity. However, *Pythium* spp. lack chitin in their hyphal walls and this may explain the ineffectivness of this enzyme in hindering the rapid ingress. Both β-1,3-glucanase and chitosanase activities became detectable later, from the second day onward. Flavanols were detected in control and infected roots. Since these compounds are constitutively present in host cells, they may be relevant in the plant's rapid response immediately after fungal attack. Lignins accumulated later in the course of infection, starting with the second day. Even if lignin accumulation was almost twice as high as in the uninfected roots, at the point when the rise in its accumulation was detected, the pathogen had already ramified through the entire root. Lignification as a barrier against the fungal infection was in this case ineffective due to the late onset.

The results presented in this work support other studies showing that the resistant reaction in host plants upon infection is dependent on both temporal and spatial onset of active defense mechanisms. The failure of host cells to halt fungal ingress in our system may be explained by the susceptible, juvenile seedlings we used as well as the high inoculum potential. By the time products of defense mechanisms such as PR-proteins or lignins are detected, cortex and stele of infected roots were already colonized by hyphae. The temporal onset of these mechanisms was too late for effective pathogen inhibition. Flavanols and CT, perhaps due to their presence in host cells, have a key role in rapid defense during the infection in other systems. Their regulation, however, remains enigmatic and requires further investigation.

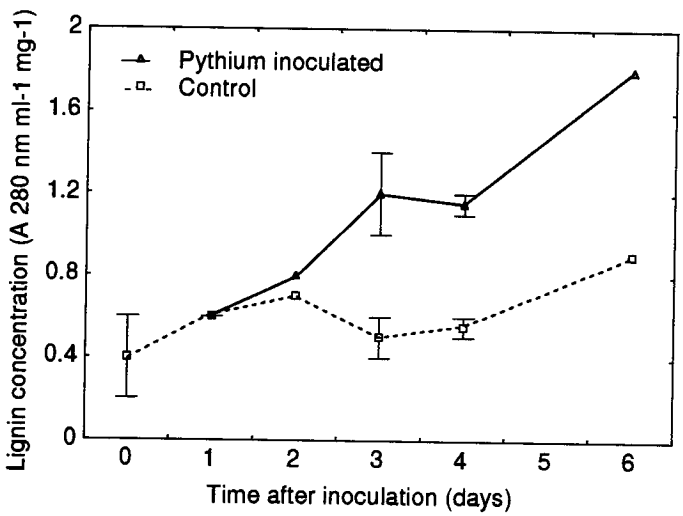

Fig. 4. Relative lignin concentration (expressed as amount of ligninthioglycolic acid) in roots of *Picea abies* seedlings noninfected (control) or infected with *Pythium dimorphum*.

TABLE 1. Temporal occurrence of chitinase, chitosanase, β-1,3-glucanase according to Sharma et al (40), flavanols, lignins, H_2O_2, ultrastructural changes, hyphal colonization, and visual necroses in *Picea abies* seedling roots infected with *Pythium dimorphum* compared with control tissues

		Days after inoculation					
		1	2	3	4	6	10
Chitinase	Infected	++[a]	++	+++	+++	+++	+++
	Control	+	+	+	+	+	+
Chitosanase	Infected	−	+	+	++	++	++
	Control	−	−	−	−	−	−
β-1,3-glucanase	Infected	−	+	++	++	+++	+++
	Control	−	−	−	−	−	−
Flavanols	Infected	+++	+++	+++	+++	+++	+++
	Control	+++	+++	+++	+++	+++	+++
Lignin (LTGA)	Infected	−	+	+++	+++	+++	+++
	Control	−	−	+	+	++	++
H_2O_2	Infected	−	++	+++	+++	+++	+++
	Control	−	+	+	+	+	+
Ultrastructural changes	Infected	++	+++	+++	+++	+++	+++
	Control	−	−	−	+	+	+
Hyphal colonization	Infected	++	+++	+++	+++	+++	+++
	Control	−	−	−	−	−	−
Visual necroses	Infected	−	+	++	+++	+++	+++
	Control	−	−	−	−	−	−

[a]Scale of − = not detectable and +, ++, +++ = minimum, moderate, and maximum amounts, respectively.

LITERATURE CITED

1. Albersheim, P., Jones, T. M., and English, P. D. 1969. Biochemistry of the cell wall in relation to infective processes. Annu. Rev. Phytopathol. 7:171-194.
2. Benhamou, N. 1993. Spatio-temporal regulation of defence genes: Immunocytochemistry. Pages 221-235 in: Mechanisms of Plant Defense Responses. B. Fritig and M. Legrand, eds. Kluwer Academic Publishers, Dordrecht.
3. Benhamou, N., Broglie, K., Chet, I., and Broglie, R. 1993. Cytology of infection of 35S-bean chitinase transgenic canola plants by *Rhizoctonia solani:* cytochemical aspects of chitin breakdown *in vivo.* Plant J. 4:295-305.
4. Benhamou, N., and Côté, F. 1992. Ultrastructure and cytochemistry of pectin and cellulose degradation in tobacco roots infected by *Phytophthora parasitica* var. *nicotianae.* Phytopathology 82:468-478.
5. Beyer-Ericson, L., Damm, E., and Unestam, T. 1991. An overview of root dieback and its causes in Swedish forest nurseries. Eur. J. For. Path. 21:439-443.
6. Bol, J. F., Linthorst, H. J., and Cornelissen, B. J. 1990. Plant pathogenesis-related proteins induced by virus infection. Annu. Rev. Phytopathol. 28:113-138.
7. Cahill, D. M., and McComb, J. A. 1992. A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of *Eucalyptus calophylla* (field resistant) and *E. marginata* (susceptible) when infected with *Phytophthora cinnamomi.* Physiol. Mol. Plant Pathol. 40:315-332.
8. Chi, C. C., and Hanson, E. W. 1962. Interrelated effects of environment and age of alfalfa and red clover seedlings on susceptibility to *Pythium debaryanum.* Phytopathology 52:985-989.
9. Collinge, D. B., and Slusarenko, A. J. 1987. Plant gene expression in response to pathogens. Plant Mol. Biol. 9:389-410.
10. Doster, M. A., and Bostock, R. M. 1988. Quantification of lignin formation in almond bark in response to wounding and infection by *Phytophthora* species. Phytopathology 78:473-477.
11. Gardner, R. O. 1975. Vanillin-Hydrochloric acid as a histochemical test for tannin. Stain Technol. 50:315-317.
12. Graham, T. L., and Graham, M. Y. 1991. Cellular coordination of molecular responses in plant defense. Mol. Plant-Microbe Interact. 4:415-422.
13. Hendrix, F. F., and Campbell, W. A. 1973. Pythiums as plant pathogens. Annu. Rev. Phytopathol. 11:77-98.
14. Hendrix, F. F., and Campbell, W. A. 1983. Some Pythiaceous fungi - new roles for old organisms. Pages 123-160 in: Zoosporic Plant Pathogens, a Modern Perspective. S. T. Buczacki, ed., Academic Press, New York.
15. Hinch, J. M., Wetherbee, R., Mallet, J. E., and Clarke, A. E. 1985. Response of *Zea mays* roots to infection with *Phytophthora cinnamomi.* I. The epidermal layer. Protoplasma 126:178-187.
16. Jang, J. C., and Tainter, F. H. 1990. Cellular responses of pine callus to infection by *Phytophthora cinnamomi.* Phytopathology 80:1347-1352.
17. Johansen, D. A. 1940. Plant microtechnique. McGraw-Hill Book Co., New York.
18. Joosten, M. H. A. J., and De Wit, P. J. G. M. 1989. Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1,3-β-glucanases and chitinases. Plant Physiol. 89:945-951.
19. Kauffmann, S., Legrand, M., Geoffroy, P., and Fritig, B. 1987. Biological function of pathogenesis-related proteins: Four PR-proteins of tobacco have 1,3-β-glucanase activity. EMBO J. 6:3209-3212.
20. Kendrick, J. B., Jr., and Wilbur, W. D. 1965. The relationship of population density of *Pythium irregulare* to pre-emergence death of lima bean seedlings. (Abstr.) Phytopathology 55:1064.
21. Kosuge, T. 1969. The role of phenolics in host response to infection. Annu. Rev. Phytopathol. 7:195-222.
22. Kraft, J. M., Endo, R. M., and Erwin, D. C. 1967. Infection of primary roots of bentgrass by zoospores of *Pythium aphanidermatum.* Phytopathology 57:86-90.
23. Lees, G. L., Suttill, N. H., and Gruber, M. Y. 1993. Condensed tannins in sainfoin. 1. A histological and cytological survey of plant tissues. Can. J. Bot. 71:1147-1152.
24. Lewis, N. G., and Yamamoto, E. 1990. Lignin: occurrence, biogenesis and biodegradation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41:455-496.
25. Lilja, A., Lilja, S., Poteri, M., and Ziren, L. 1992. Conifer seedling root fungi and root dieback in Finnish nurseries. Scand. J. For. Res. 7:547-556.
26. Linthorst, H. J. M. 1991. Pathogenesis-related proteins of plants. Crit. Rev. Plant Sci. 10:123-150.
27. Martin, F. N. 1992. *Pythium.* Pages 39-49 in: Methods for Research on Soilborne Phytopathogenic Fungi. L. L. Singleton, J. D. Mihail, and C. M. Rush, eds. American Phytopathological Society, St. Paul, MN.
28. Marx, D. H., and Bryan, W. C. 1970. The influence of soil bacteria on the mode of infection of pine roots by *Phytophthora cinnamomi.* Pages 171-172 in: Root Diseases and Soil-borne Pathogens. T. A. Tousson, R. V. Bega, and P. E. Nelson, eds. University of California Press, Berkeley.
29. Matern, U., and Kneusel, R. E. 1988. Phenolic compounds in plant disease resistance. Phytoparasitica 16:153-170.
30. Mauch, F., Mauch-Mani, B., and Boller, T. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. 88:936-942.
31. Mazau, D., and Esquerré-Tugayé, M. T. 1986. Hydroxyproline-rich glycoproteins accumulation in the cell walls of plants infected by various pathogens. Physiol. Mol. Plant Pathol. 29:147-157.
32. Miller, C. R., Dowler, W. M., Petersen, D. H., and Ashworth, R. P. 1966. Observations on the mode of infection of *Pythium ultimum* and *Phytophthora cactorum* on young roots of peach. Phytopathology 56:46-49.
33. Nicholson, R. L., and Hammerschmidt, R. 1992. Phenolic compounds and their role in disease resistance. Annu. Rev. Phytopathol. 30:369-389.
34. Olson, P. D., and Varner, J. E. 1993. Hydrogen peroxide and lignification. Plant J. 4:887-892.
35. Parham, R. A., and Kaustinen, H. M. 1976. Differential staining of tannin in sections of epoxy-embedded plant cells. Stain Technol. 51:237-240.
36. Ride, J. P., and Pearce, R. B. 1979. Lignification and papilla formation at sites of attempted penetration of wheat leaves by non-pathogenic fungi. Physiol. Plant Pathol. 15:79-92.
37. Sarkar, S. K., and Howarth, R. E. 1976. Specificity of the Vanillin test for flavanols. J. Agric. Food Chem. 24:317-320.
38. Sarkar, S. K., Howarth, R. E., and Goplen, B. P. 1976. Condensed tannins in herbaceous legumes. Crop Sci. 16:543-547.
39. Sela-Buurlage, M. B., Ponstein, A. S., Bres-Vloemans, S. A., Melchers, L. S., van den Elzen, P. J. M., and Cornelissen, B. J. C. 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol. 101:857-863.
40. Sharma, P., Bøjrja, D., Stougaard, P., and Lönneborg, A. 1993. PR-proteins accumulating in spruce roots infected with a pathogenic *Pythium* sp. isolate include chitinases, chitosanases and β-1,3-glucanases. Physiol. Mol. Plant Pathol. 43:57-67.
41. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
42. Stanghellini, M. E., Rasmussen, S. L., and Vandemark, G. J. 1993. Relationship of callose deposition to resistance of lettuce to *Plasmopara lactucae-radicis.* Phytopathology 83:1498-1501.
43. Stempack, J. C., and Ward, R. T. 1964. An improved staining method for microscopy. J. Cell Biol. 22:697-701.
44. Stössel, P., Lazarovits, G., and Ward, E. W. B. 1981. Electron microscope study of race-specific and age-related resistant susceptible reactions of soybeans to *Phytophthora megasperma* var. *sojae.* Phytopathology 71:617-623.
45. Tippett, J. T., O'Brien, T. P., and Holland, A. A. 1977. Ultrastructural changes in eucalypt roots caused by *Phytophthora cinnamomi.* Physiol. Plant Pathol. 11:279-286.
46. van Loon, L. C. 1985. Pathogenesis-related proteins. Plant Mol. Biol. 4:111-116.
47. Vance, C. P., Kirk, T. K., and Sherwood, R. T. 1980. Lignification as a mechanism of disease resistance. Annu. Rev. Phytopathol. 18:259-288.
48. Venable, H. H., and Coggeshall, R. 1965. A simplified lead citrate staining for use in electron microscopy. J. Cell Biol. 25:407-408.
49. Venn, K., Sandvik, M., and Langerud, B. R. 1986. Nursery routines, growth media and pathogens affect growth and root dieback in Norway spruce seedlings. Medd. Nor. Inst. Skogforsk. 39:314-328.