Cellular Responses of Pine Callus to Infection by *Phytophthora cinnamomi*

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ABSTRACT

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The expression of differential resistance in pine callus tissues to the fungal pathogen *Phytophthora cinnamomi* is described. The major species groups of loblolly and loblolly × shortleaf pine hybrids were more resistant to infection and invasion by the fungus than were shortleaf and Virginia pine. Within clones of each species, however, two separate reaction types were identified. These are designated as "susceptible" and "resistant." There were several major differences between these reactions at the cellular level. Fewer hyphal penetrations, a greater accumulation of electron-dense materials, and morphological changes of the host cell wall were associated with resistant reactions. No hypersensitive reaction was evident in any inoculated callus tissues. Resistance to *P. cinnamomi* appears to be regulated by physiological and biochemical mechanisms.

Additional keywords: littleleaf disease.

Phytophthora cinnamomi Rands is a major root pathogen of many species of horticultural, agricultural, and forest plants around the world. Little is known of how tolerant plants survive contact with this fungus. Response at the cellular level in the whole plant is undoubtedly complex, as each cell type apparently responds differently to infection (10). In some host-pathogen interactions, resistance expressed in intact plants is also expressed in tissue culture under proper cultural conditions. Helgeson, et al (8) demonstrated that callus tissues from resistant tobacco cultivars responded to *Phytophthora parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker in a manner similar to that of intact plants. McComb, et al (16) found that the amount of callose formed in cultured cells in response to *P. cinnamomi* correlated with field resistance. In tobacco callus-*P. p.* var. *nicotianae* interactions, deZoeton, et al (5) showed that ultrastructural relationships of colonization in tobacco roots and in callus cells were similar. Necrosis of host cells was observed in incompatible (hypersensitive) reactions. These reactions included disorganization of the cytoplasm, vesiculation, loss of tonoplast integrity, and degeneration of mitochondria (5). Necrotic reactions were also seen in tobacco callus inoculated with *Peronospora tabacina* D. B. Adam (23). The ultrastructural relationships between host cells and haustoria were very similar in callus cells and intact leaves. However, this reaction apparently did not prevent the penetration and subsequent colonization of host cells by the fungus.

Interactions of pine tissue cultures with obligate parasites have also been reported. Jacobi, et al (12) demonstrated that loblolly pine (*Pinus taeda* L.) callus was resistant to both cell penetration and intercellular growth by *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Hedgc. & N. Hunt) Burdsall & G. Snow. Tissue cultures of western white pine (*Pinus monticola* Dougl.), inoculated with *Cronartium ribicola* J. C. Fisch., showed degenerative changes in host cells several millimeters in advance of the fungus (17,18). This hypersensitive response could be assayed with cotyledons (14), with embryos cultured in vitro (6), and most importantly, with cells of sugar pine in axenic culture (7).

P. cinnamomi is an important factor in the littleleaf disease complex of pine trees in the southeastern United States. In Australia, *P. cinnamomi* has caused widespread destruction of native forests in the southeastern states and in the southwest of

Western Australia. Studies on the interactions of *Eucalyptus* spp. with this fungus indicate that the mechanism of infection is similar in both susceptible and resistant species (15,19,20,22) and have revealed that no specific anatomical or histological feature is consistently associated with resistance (3).

Our previous work strongly suggested that there is differential susceptibility among southeastern pine species and seed sources to infection by *P. cinnamomi*, and this was expressed in an in vitro inoculation system using callus tissue (13). No callus source, however, completely inhibited fungal growth on its surface. All evidence indicated that the fungus attacked all calli to some degree, but that two major reaction types were evident. The correlation, however, between host cellular change and variable growth of *P. cinnamomi* on the callus surface still is unknown. In the present investigation, this phenomenon is explored in more detail and the mechanism of penetration and invasion of *P. cinnamomi* into pine callus tissues is revealed by light and electron microscopy.

MATERIALS AND METHODS

Callus tissues of loblolly pine, shortleaf pine (*P. echinata* Mill.), loblolly × shortleaf pine hybrids, and Virginia pine *(P. virginiana* Mill.) were produced and inoculated as described previously (13). That work suggested that when callus cultures derived from seedlots of selected pine species are inoculated with *P. cinnamomi*, two distinguishable responses develop within 72 hr. Callus lines of the various pine species or seed sources had been categorized as "resistant" or "susceptible" according to relative amount of hyphal growth of *P. cinnamomi* on the callus surface.

At 72 hr after inoculation, callus tissues from under the outer edge of the colonies of *P. cinnamomi* were sampled. They were fixed for 2 hr at room temperature with 2% glutaraldehyde buffered to pH 7.1 in 0.1 M cacodylate buffer and rinsed three times at 10 min intervals with 50 ml cacodylate buffer (pH 7.1). Postfixation was done with 2% osmium tetroxide in cacodylate buffer at 4 C for 2 hr. Postfixed samples were then rinsed three times for 10 min each with cacodylate buffer at 4 C and dehydrated through an ethanol series followed by two changes of propylene oxide. Samples were then infiltrated and embedded in Spurr's epoxy resin. A Reichert-Jung Ultracut E ultramicrotome was used for sectioning. Semithin sections of 0.5 to 1.0 μm thickness were stained with a mixture of methylene blue and Azure A (1:1) for 1–2 min at 50 to 60 C (25). Sections were then rinsed in running distilled water, dried, and examined by light microscopy (LM). Three samples of each selected line were examined, and at least two sections were prepared from different portions of each sample of the embedded callus tissue. For transmission electron microscopy (TEM), ultrathin sections were stained with uranyl acetate and lead citrate with an LKB 2168 Ultrastainer and viewed under a JEOL JEM-100C transmission electron microscope. The entire experiment was repeated three times.

Specimens for scanning electron microscopy (SEM) were processed at the same time as preparations for LM and TEM. Infected callus tissues were fixed in 2% glutaraldehyde for 2 hr at room temperature and then rinsed with phosphate buffer 3 times for 10 min each. Tissues were then dehydrated through an ethanol series, critical point-dried with CO_2, coated with gold with a Hummer X sputter coater for 600 sec, and then examined under a JEOL JSM-IC848 scanning electron microscope at 20 kV.

RESULTS

Resistant reaction. The resistant reaction had been visually defined as having sparse hyphal growth on the callus surface with little or no visible injury to the callus cells (13). SEM confirmed that sparse hyphal growth of *P. cinnamomi* was characteristic of resistant reactions in all species (Fig. 1A). In addition, more than 50% of the hyphae observed were found only in the outer portion of the callus and were mostly restricted to intercellular spaces (Fig. 1B). The few hyphal penetrations of callus cells that did occur had a hyphal constriction at the point of penetration (Fig. 1D). Hydrolysis of the adjacent callus cell wall at the point of penetration was not evident. A thin, electron-dense layer ensheathed the penetration hypha. This amorphous, discontinuous layer, presumably of phenolic-like materials (or tannin), was intimately associated with the tonoplast as described by Robb, et al (18).

Partial collapse of surface callus cells was associated with the few hyphal penetrations in shortleaf pine. External cell morphology in resistant lines of shortleaf pine as observed by SEM was unaffected, presumably because of fewer hyphal penetrations of callus cells or tolerance of these cells to limited penetration. The plasmallema of callus cells remained attached to the cell wall when accumulations of electron-dense materials were present (Fig. 1B and C). Visible browning of the callus, although associated with the accumulation of electron-dense materials and formation of vacuolar inclusions, was not consistently associated with the resistant reaction. Granular, vesicular, and globular electron-dense materials accumulated mostly inside host vacuoles and were associated with over 50% of all the infected calli of loblolly and loblolly × shortleaf pine hybrids and with the most resistant shortleaf and Virginia pine calli.

Callus cytoplasm formed vacuolar inclusions that were concentrated in cells or those portions of cells in close proximity to the pathogen. Electron-dense granular material, comprised either of phenolic-like materials or of degenerated cytoplasm, was found within both the cytoplasm and the vacuole (Fig. 1E and F). Within these inclusions, callus mitochondria, organelles, and granular material were unusually electron-dense, suggestive of cell senescence. Conversely, presence of mitochondria, numerous vacuoles, and ribosomes in penetration hyphae reflected an active metabolism (Fig. 1D).

Susceptible reaction. Extensive hyphal growth of *P. cinnamomi* was characteristic of the susceptible reaction (Fig. 2A and B). Collapse of infected surface cells was evident in susceptible callus lines of shortleaf, Virginia, and loblolly × shortleaf pine hybrids. In all cases, callus cell deformation and collapse were associated with extensive hyphal colonization and penetration. Electron-dense materials were sparse in cells or intercellular spaces adjacent to penetration sites (Fig. 2F). Shrunken protoplasts were observed in callus cells during initial penetration. The resulting space between callus plasmallema and cell wall seemed to be preferred for initial hyphal intracellular colonization. Callus mitochondria were enveloped in a membrane to form large inclusions.

Common reactions. In the reactions of either resistant or susceptible callus, cristae with attached electron-dense particles were formed near the interface of callus cell wall and hyphae (Fig. 1E). Higher magnification revealed that these particles were also formed along with callus cell wall. Robb, et al (17) suggested that this structure was a membranous elaboration of the callus tonoplast, and it may be associated with tannin synthesis. Whether the synthesized material was tannin is not known, but it is similar in appearance to the tannin inclusions described by Chafe and Duran (4) in white spruce cell suspension cultures.

A sheathlike layer of unknown origin formed on the outer surface of inoculated callus (Fig. 2C and D). With LM it appeared as a network that penetrated the intercellular spaces (Fig. 2E). As contact between the two organisms became more firmly established, the amount of this material increased. With TEM it was revealed as an electron-dense, granular substance that was concentrated at the interface of callus and fungal cell walls (Fig. 1E and G; 2F and G). There was no evidence to suggest that the sheath or the intercellular deposits were involved in resistance to hyphal growth or penetration. No sporangia or chlamydospores of *P. cinnamomi* formed on the surfaces of any callus lines of any species.

Rapid necrosis of callus cells did not occur in either resistant or susceptible reactions. The accumulation of electron-dense materials and vacuole formation in callus cells were associated with less hyphal penetration and tissue invasion, but none of these responses appeared to prevent fungal colonization. Hypersensitive reactions were not observed in resistant or susceptible calli of any pine species.

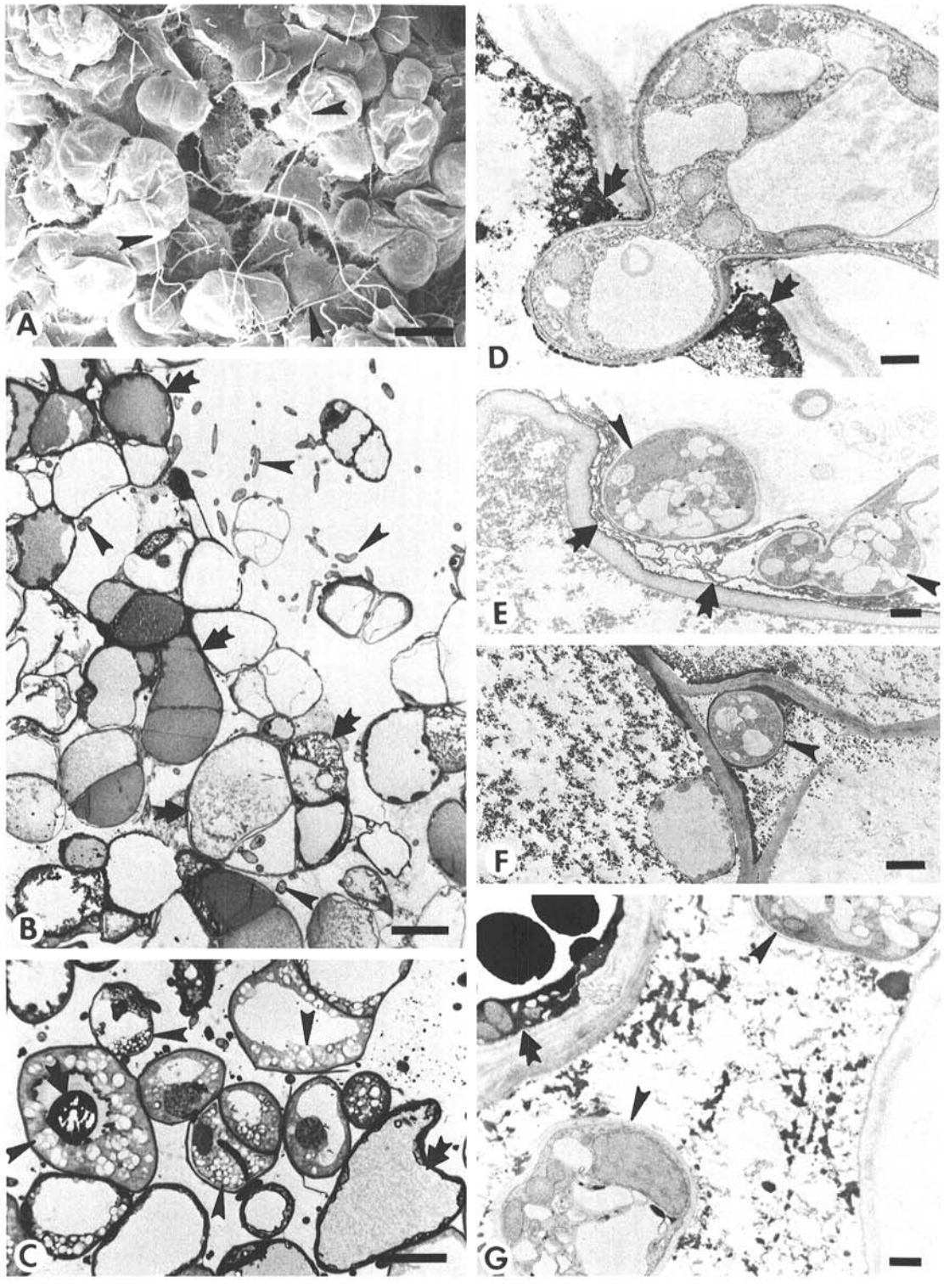

Fig. 1. Scanning electron micrograph, **A**, light micrographs, **B–C**, and transmission electron micrographs, **D–G**, of pine calli inoculated with *Phytophthora cinnamomi*, showing a resistant reaction. **A**, Loblolly × shortleaf pine hybrid, note sparse hyphal growth (arrows). Scale bar = 100 μm. **B**, Loblolly pine with sparse, intercellular hyphae (arrowheads) and accumulation of granular, electron-dense material (arrows) in callus cells. Scale bar = 50 μm. **C**, Loblolly pine with vacuolar inclusions (arrowheads) in callus cells and accumulation of globular and granular, electron-dense materials (arrows). Scale bar = 50 μm. **D**, A penetration hypha in loblolly pine, with constriction at the point of penetration and an accumulation of electron-dense materials (arrows) around the penetration hypha. Scale bar = 2 μm. **E**, Shortleaf pine with hyphae (arrows) in space between callus cell wall and cell membrane and associated with the formation of cristae and degeneration of callus cytoplasm (large arrows). An accumulation of electron-dense materials is visible in the adjacent intercellular space in the lower left. Scale bar = 2 μm. **F**, Loblolly × shortleaf hybrid, with a hypha (arrow) in an intercellular space. Granular electron-dense materials have accumulated in the adjacent callus cells, within which the host cytoplasm is either shrunken or has degenerated. Note also the dense accumulation of electron-dense materials at the interface of the host cell wall and along the adjacent host cell membrane. Scale bar = 6 μm. **G**, Shortleaf pine with two hyphae (small arrows) in an intercellular space and associated with the accumulation of granular, electron-dense materials in this space and globular, electron-dense materials in the adjacent host cell vacuole. Large arrow indicates shrunken host cytoplasm containing mitochondria. Scale bar = 2 μm.

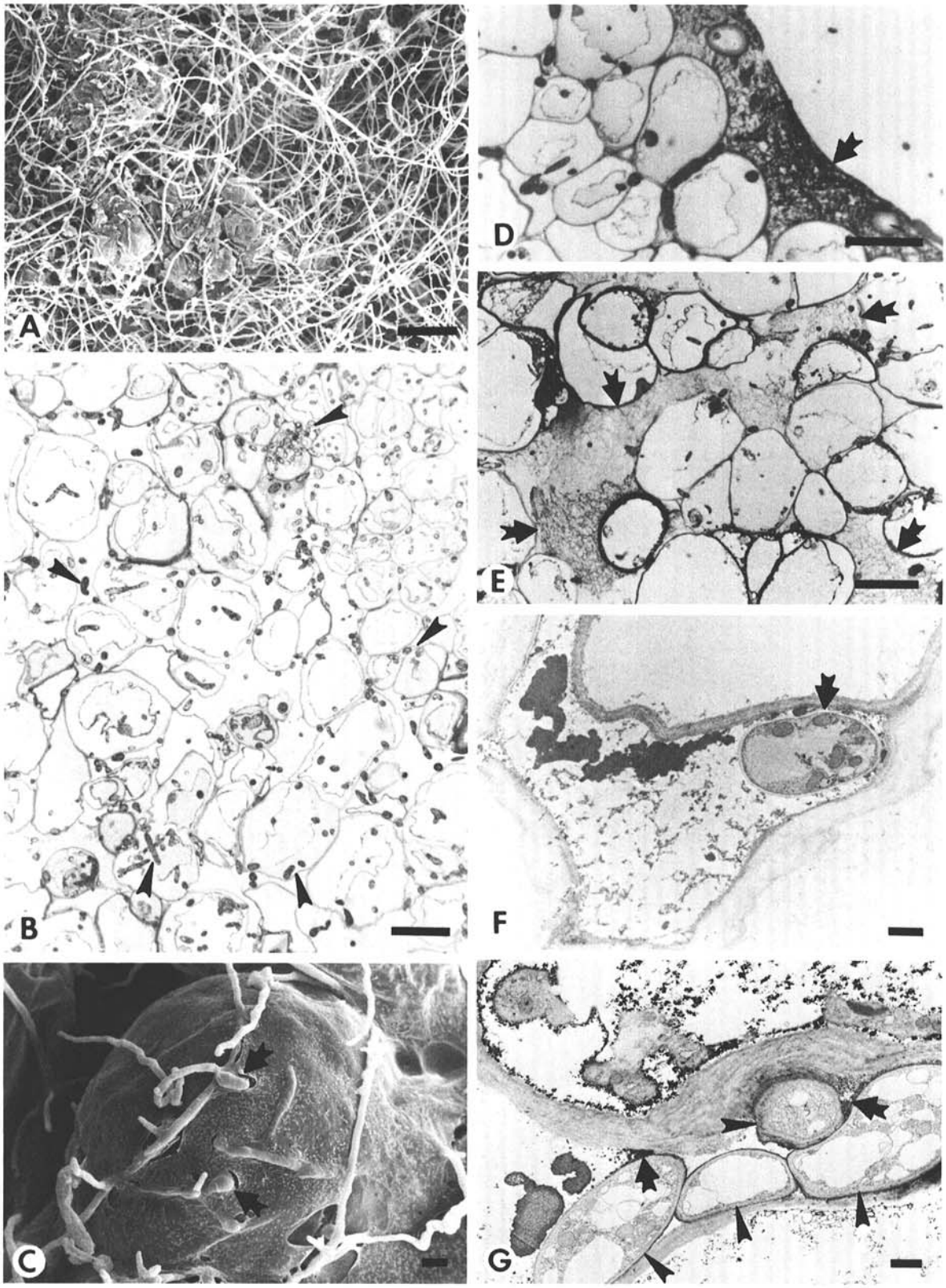

Fig. 2. Scanning electron micrographs, **A** and **C**, light micrographs, **B**, **D**, and **E**, and transmission electron micrographs, **F** and **G**, of pine calli inoculated with *Phytophthora cinnamomi*, showing a susceptible reaction. **A**, Virginia pine with dense hyphal growth. Scale bar = 100 μm. **B**, Shortleaf pine with numerous intercellular and intracellular hyphae (arrows). Note that most callus cells are plasmolyzed. Scale bar = 50 μm. **C**, Shortleaf pine with sheath covering the callus cells. Hyphae (arrows) have penetrated through this sheath into the callus cell. Scale bar = 10 μm. **D** and **E**, Shortleaf pine with the sheath (arrow) on the callus surface and the network (arrows) in intercellular spaces. Scale bar = 50 μm. **F**, Shortleaf pine, showing a hypha (arrow) in an intercellular space and accumulation of granular and amorphous electron-dense materials. Scale bar = 4 μm. **G**, Loblolly pine with several hyphae (arrowheads) in an intercellular space and associated with electron-dense materials, shrunken cytoplasm, and disruption of cell membranes. Note the electron-dense materials (arrows) that have accumulated near the interface of host and fungal cell walls. Scale bar = 2 μm.

DISCUSSION

Accumulation of electron-dense materials, granulation and disruption of cytoplasm, and shrinkage of the protoplast were seen in varying amounts in both resistant and susceptible callus lines by both LM and TEM. None of these reactions seemed to be directly related to prevention of hyphal penetration. In some aspects, intense vacuolar activity in both callus cells and the fungus resembles a similar phenomenon in the blister rust-white pine tissue culture system (17,18). We did not confirm the identity of the electron-dense substances as tannins or other polyphenolics but, from their appearance as revealed by TEM, we do not know what else they might be. An association between tannins and vacuoles may be indicative of a stage in cell deterioration (4). According to Wilson (26), the vacuolar systems of both higher plants and fungi may be of primary importance in the establishment of some pathogenic relationships. However, lomasome bodies and vesicles similar to those in hyphae of the blister rust fungus were not formed in vacuoles of *P. cinnamomi* in the present study. Lomasomes and vesicles have been postulated to be involved in the elaboration of the capsular sheath which surrounds the hyphal wall and may have adhesive or protective properties (18). The absence of a capsular sheath in our observations indicates that it is possible that penetration hyphae of *P. cinnamomi* were capable of producing enzymes or chemicals able to overcome the toxicity of phenolic compounds or tannins produced by callus cells.

Another bit of evidence in support of this hypothesis was the rapid degeneration of host cytoplasm, including mitochondria and other organelles, upon contact with penetration hyphae. That resistance to *P. cinnamomi* is regulated by physiological or biochemical mechanisms was suggested by Cahill, et al (3). However, other studies have demonstrated that anatomical and cellular changes are responsible for resistance to *P. cinnamomi* in certain species. Tippett and Malajczuk (21) examined the interaction of *P. cinnamomi* with a resistant host, *Acacia pulchella* R. Br., and found that the host cell response appeared to be a hypersensitive reaction. Hypersensitive resistance to *C. ribicola* was also displayed by calli of *Pinus lambertiana* Dougl. (7). In a tolerant eucalypt, *Eucalyptus stjohnii* R. T. Bak, a thick plug of amorphous material formed in the germ tube of *P. cinnamomi*, sealing off the hypha from the root cell (20). In subsequent reports, however, some of these same authors concluded that a hypersensitive type of reaction in tolerant eucalypts is not a resistance mechanism to *P. cinnamomi* (15,19,22). Although structures such as host cell wall appositions and sealing plugs were not seen in the present study, it should not be inferred that the pine calli examined did not possess resistance to *P. cinnamomi*.

Robb, et al (18) reported that a pine tissue culture response categorized as resistant to *C. ribicola* must include the development of callosities at points of penetration, the production of extracellular polyphenolic compounds or tannins, vacuolar fragmentation, and prepenetrational degeneration of host cell ultrastructure and ultimate cell death. This definition is apparently more complete than the ones presently described for resistance to *P. cinnamomi*. Several studies have used callose formation in cultured cells in response to the hyphal penetration of *P. cinnamomi* as a criterion to evaluate host susceptibility and have reported that use of cultured cells was correlated with resistance of assayed plants in the field (2,9,16). However, Aist (1) cautioned that correlation alone is not sufficient evidence to postulate a functional role in resistance, since the exact role of callose in plant disease is still unclear. While in the present study we did not perform a histochemical test for callose, LM and TEM did reveal other histopathological differences between resistant and susceptible callus cells, including the production of large amounts of electron-dense, phenolic-like materials and less plasma membrane disturbance and cytoplasmic damage in the resistant callus cells. It is difficult, however, to draw a clear distinction in the responses at the cellular level. Cahill, et al (3) cautioned that until interactions between *P. cinnamomi* and invaded species are adequately explained at the molecular level, this controversy will remain.

Results of SEM were more useful than those of TEM and revealed major differences in the number of penetration hyphae and changes in host cell morphology between the resistant and susceptible resistance reactions. The origin and function of the sheath or network are unclear. Presumably, the sheath was of callus origin, but it was not capable of preventing hyphal penetration. Its chemical nature was not determined. A structurally similar material was reported by Huang and VanDyke (11) and Jacobi, et al (12), the latter suggesting that it was produced as a response to stress in senescing cells. Uchiyama and Ogasawara (24) cautioned that since neither wax nor cuticle are present on the outermost layer of callus tissues, this may facilitate spore germination and penetration.

In the present study, several major differences have been found between susceptible and resistant reactions of callus at the cellular level. Less hyphal penetration, an accumulation of electron-dense, phenolic-like materials, and no morphological change of host cell wall were associated with the resistant reaction. Since no anatomically incompatible reaction was found in any combination, these results suggest that the mechanism of resistance of pine callus tissues to *P. cinnamomi* is biochemical or physiological rather than physical.

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