Histological Changes in the Roots of an Avocado Cultivar, Duke 7, Infected with *Phytophthora cinnamomi*

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ABSTRACT

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Roots of the moderately resistant avocado cultivar, Duke 7, formed limited lesions when inoculated with the fungal pathogen, *Phytophthora cinnamomi*. Histological examination of tissue changes revealed two distinct anatomical responses at the lesion boundary; the formation of necrophylactic periderm in the cortex and whorls of cells walling off infected phloem bundles in the stele. Necrophylactic periderm divided necrotic, infected tissue from nonnecrotic, uninfected cortical tissue from the epidermis through to the endodermis and always coincided with the very rapid disappearance of infected cortical tissue. Phloem bundles, the site of intensive hyphal colonization in lesioned tissue, were devoid of hyphae after the formation of cell whorling and cell wall thickening at the lesion boundary. These anatomical responses appear to be an important component in determining the resistance response of this rootstock.

Avocados (*Persea americana* Mill.) have been described as highly susceptible to the soilborne pathogen, *Phytophthora cinnamomi* Rands. In this respect, it is of interest that *Persea* spp. belong to the same family of woody dicots, Lauraceae, as *Cinnamomum burmanni* Blume (camphor-tree), from which *P. cinnamomi* was first isolated (28). In recent years, the development and screening for resistance in rootstocks of avocado have resulted in the selection of two cultivars, Duke 7 and G6, exhibiting moderate field resistance to *P. cinnamomi* induced root rot (35). The level of resistance shown by various avocado rootstocks has been compared (17).

However, the basis of this host resistance has not been defined and may be physiological or anatomical or a combination of both. Tippett and Hill (33) noted that once a pathogen such as *P. cinnamomi* had progressed into *Eucalyptus* roots with secondary structure, the host had the potential for a greater variety of resistance mechanisms than was possible in primary root tissue. Meristematic tissue, such as cambium and phellogen, is continuous along such roots. In the susceptible *Eucalyptus marginata* Sm, lesion form in roots exhibiting secondary structure reflected and was controlled by the pattern of post-inoculation periderm development (33). Resistant cultivars of *Pinus strobus* L., attacked by white pine blister rust, formed a layer of cork cambium (wound periderm) between infected and healthy tissue that serves as a protective barrier. This was absent from susceptible varieties (31).

In slash pine, resistance to *Cronartium fusiforme* Hedgc. & Hunt ex Cumm. was examined and the invasion delimited by regions of atypical vertical parenchyma, which were dark staining and rich in tannins (15,16). Cell proliferation is a common response to wounding in tissues of many dicotyledonous plants and does not occur in monocotyledons, such as onions (21). Seedlings of *Persea borbonia* (L.) K. Spreng (field-resistant) and *P. indica* (L.) K. Spreng (susceptible) were reported to show no anatomical characters that would account for differences in their degrees of susceptibility (14). However, Dugger and Zentmyer (7) reported that during early stages of infection by *Phytophthora*, disease development in resistant *Persea* seedlings was slower and more restricted when compared with susceptible seedlings.

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This paper reports results of a study of the histology of tissues in lesioned zones of a clonally propagated avocado cultivar, Duke 7, resulting from inoculation with zoospores of *P. cinnamomi* onto primary roots. The results show anatomical changes that suggest that physical isolation of the infected portion of the root is a part of the resistance response.

MATERIALS AND METHODS

Plant material and growth conditions. Clonally propagated avocado rootstock (prepared as described by Frolich and Platt [11]; Whiley and Inch, *personal communication*) of the field-resistant cultivar, Duke 7, was obtained commercially from Birdwood Nursery, Nambour, Queensland. Test plants, 3–4 mo old, were transferred to and allowed to establish in boxes prepared from polyvinyl chloride drainpipe with one side sealed by a removable film of transparent plastic that allowed access to the roots (12).

The sterilized propagation medium consisted of two parts vermiculite to one part coarse sand, sieved to 2 mm grade. Plants were maintained in a controlled growth chamber with root temperatures controlled independently by inserting the boxes into small incubators that allowed the root zone to remain at a designated temperature, ± 0.5 C, while the temperature of the aerial parts of the plant was controlled independently (9). The growth chamber was maintained at 25 ± 1 C with a day/night period of 14/10 hr, respectively. Light intensity was 700 μE m^2 sec^{-1} photosynthetically active radiation. Humidity levels were not controlled and fluctuated between 60 and 90%. Root temperatures were set at a series of temperatures between 15 and 30 C, at five intervals. The plants were watered three times per week with one-half-strength nutrients solution (2).

Pathogen sources and maintenance. The isolate of *P. cinnamomi* was obtained from the roots of *Isopogon ceratophyllus* R. Br. growing in the Brisbane Ranges, Victoria, and was of A2 mating type. It was maintained on 20% V-8 agar (4). Axenic zoospore suspensions were prepared as described previously (4), and zoospore concentrations determined with a hemacytometer.

Inoculation. Plants were inoculated 2–4 mm behind the root tip with a droplet containing 80–100 zoospores or an equivalent amount of sterile distilled water for controls, using one root per plant (5). The droplet remained in place 45–60 min.

Pathogen detection. Root development and lesion extension were recorded directly onto transparent, gridded polyacetate sheet

overlays (27). Lesions were defined as heavily necrotic, brown stained tissues. The lesion front was defined as the boundary of the heavily necrotic tissue and white root.

Histology. Roots were washed, surface sterilized with 70% ethanol, and sectioned (3 mm) sequentially before plating onto P10 VP agar (34) to determine the distribution of the pathogen within the roots. Alternate sequential sections were examined histologically using both freshly cut hand sections and thin microtome sections of fixed tissue embedded in JB4 resin.

Tissue was examined on each side of the lesion front in infected tissue and at the inoculation site in sham-inoculated (control) roots (Fig. 1).

Hand sections. Hand sections were prepared from material taken immediately on completion of the trial. Plants had been maintained at either 20 or 30 C in root box incubators. Tissue was cut using degreased, double-edged razor blades (26), stained with 0.05% toluidine blue O, C.I.52040 (29), and mounted in distilled water, or counterstained with 0.05% aniline blue, C.I.42755, to reduce nonspecific fluorescence (30).

Fluorescence microscopy was carried out according to Hinch et al (13). Light microscopy was conducted using an Olympus microscope with Nomarski Optics and a M 35 camera system or a Zeiss photometer Mk III. Photomicrographs were taken using Kodak Technical Pan F print and Ektachrome 200 ASA slide film.

Thin sections. Root material was cut under water to prevent airlocks forming in the tissue, then fixed immediately on completion of the trial using 3% glutaraldehyde in 250 mM sodium phosphate buffer, pH 6.8, at 4 C for 24 hr, washed in phosphate buffer overnight (4 C), dehydrated in an ethanol series, infiltrated and embedded in JB4 resin (Polysciences, Inc., Warrington, PA). Plants had been maintained at either 15 or 25 C in root box incubators. Sections (2 μm) were cut on a Reichert Porter-Blum microtome and stained with either 1) 0.5% toluidine blue O, 2) counterstained with periodic acid/schiffs reaction, basic fuschin C.I.42510 (26), and/or 3) 0.05% aniline blue (in 67 mM phosphate buffer, pH 8.5) for fluorescence microscopy and observed unstained in distilled water for detection of autofluorescence.

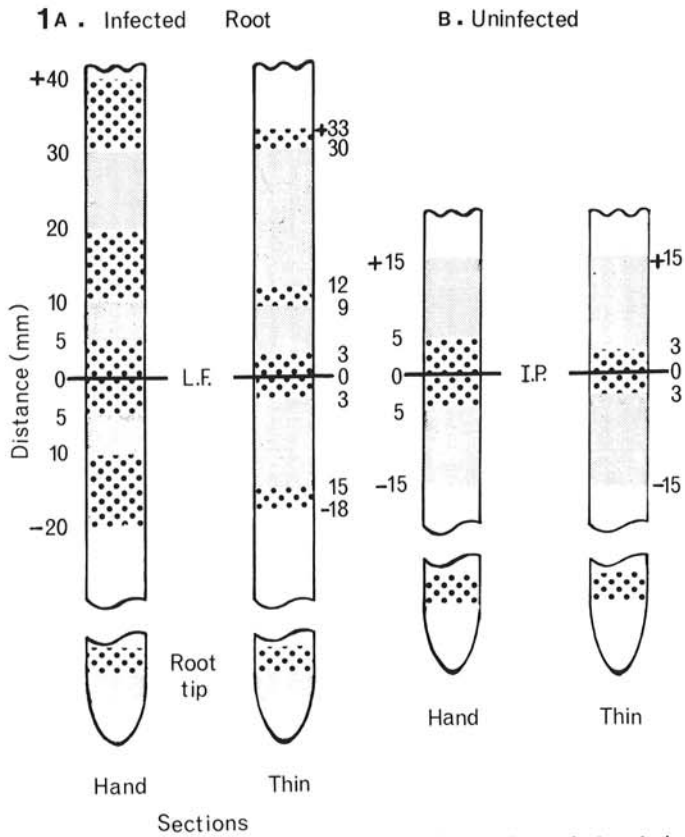


Fig. 1. Avocado root tissue sectioned sequentially for histological analysis and *P. cinnamomi* reisolation on selective agar medium, P10 VP. Large stipple shows material taken for histological analyses. Fine stipple shows material taken for *P. cinnamomi* reisolation. **A,** Roots inoculated with *P. cinnamomi*. **B,** Control roots sham-inoculated with sterile distilled water. L.F. = lesion front; I.P. = inoculation point.

RESULTS

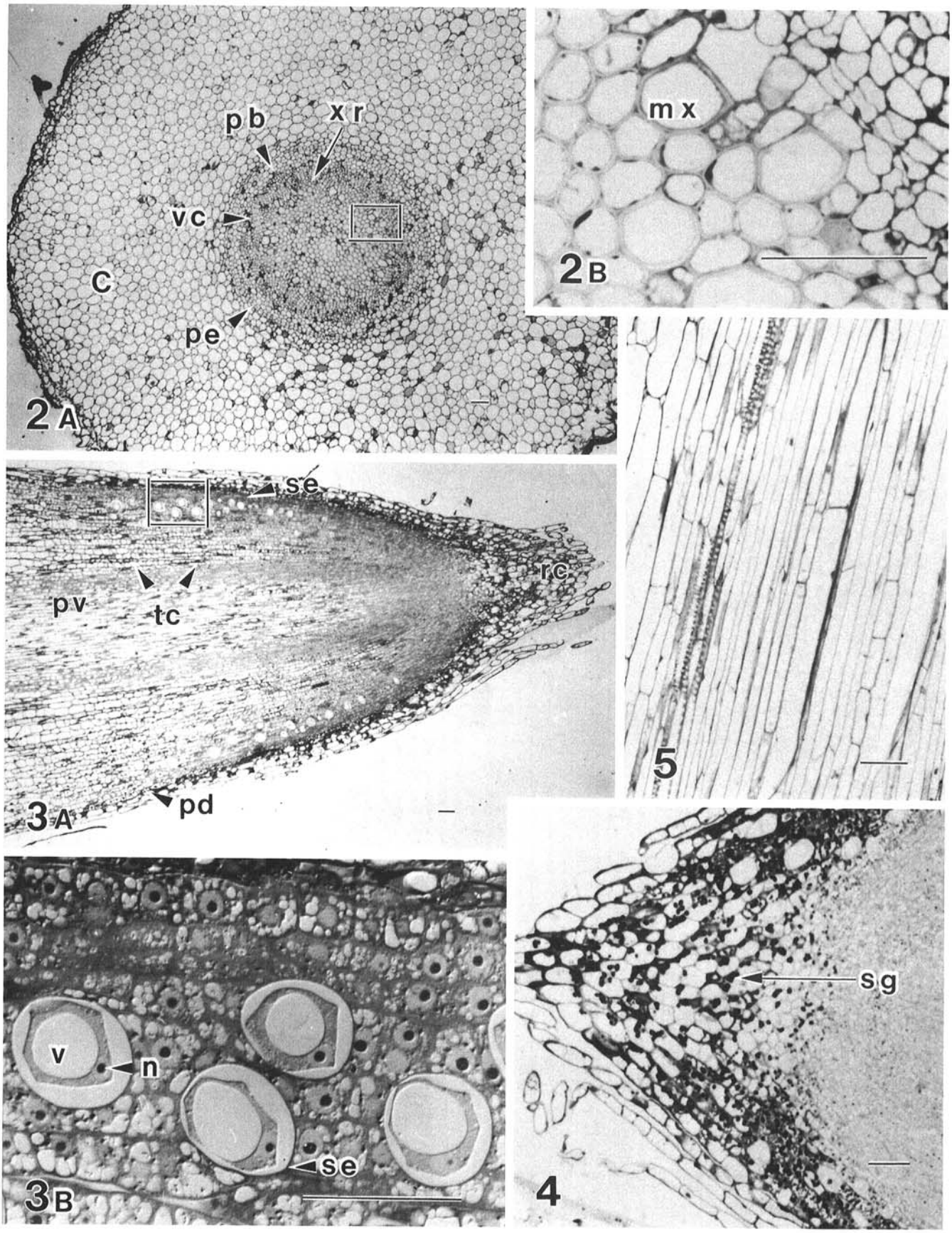
Inoculation of Duke 7 avocado roots with *P. cinnamomi* zoospores resulted in the appearance of a lesion within 24–48 hr above 25 C or within 96 hr at 15 C. Sham-inoculated roots grew normally with no lesions. Lesions that progressed into well-differentiated host tissue showing secondary thickening and lignification were eventually arrested. The time taken for lesion arrest, and hence total lesion length, varied inversely with temperature up to 25 C. Lesion extension had ceased for at least 6 days at the time roots were removed for examination. However, *P. cinnamomi* could always be isolated for up to 6 mm ahead of the lesion end point, in what was visually healthy tissue. The histology of the lesioned root revealed three zones designated as follows: Zone 1, heavily necrotic, infected tissue; Zone 2, lesion end point; and Zone 3, healthy, uninfected tissue. Each zone was characterized by distinct differences in host anatomy and in the extent of fungal development. However, the zones were not sharply defined.

Uninoculated (control) roots. Control roots were sectioned as shown in Figure 1. The root tip had usually grown 5 cm or more from the original inoculation point when finally removed for examination. A vascular cambium was present at this distance and appeared initially about 4 cm back from the root tip. The tissue exhibited well-differentiated vascular development showing secondary wall thickening and lignification, as indicated by toluidine blue O staining. Cortical and stelar tissues were healthy, cells had not yet lysed, and cell walls remained intact (Fig. 2). Root tips exhibited well-ordered, sequential development and differentiation of tissue distal to the root cap (Fig. 3A). Large vacuolate cells that may have a secretory function were numerous in the region of elongation and root cap (Fig. 3A and B). These cells become increasingly vacuolated away from the provascular tissue. The vacuoles did not stain with toluidine blue. Starch grains were concentrated in the root cap (Fig. 4). Tyloses were rarely observed in xylem bundles (Fig. 5).

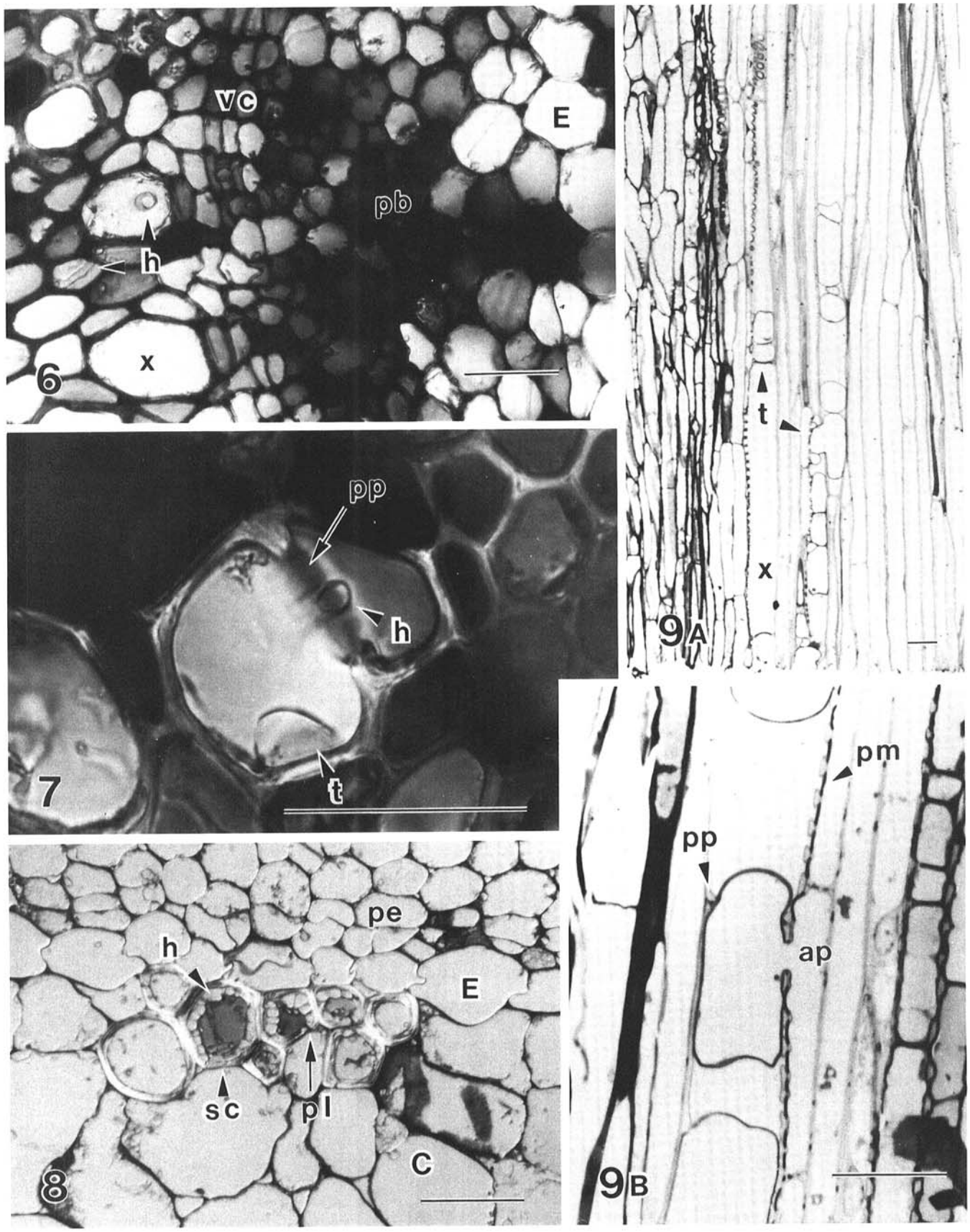
Zone 1. In infected plants, tissue necrosis always extended from the inoculation point, just behind the root cap, through primary root tissue into well-differentiated tissue with secondary wall thickenings and stelar tissue containing a vascular cambium. Zone 1 is characterized by extensive inter- and intracellular hyphal development, throughout both cortical and stelar tissue (Fig. 6). Hyphae sometimes passed along and between a xylem vessel lumen through perforation plates and bordered pits (Fig. 7). Scattered cells in the cortex and pericycle were lysed and cell walls appeared to be degraded. Cells stained strong dark blue-black with toluidine blue, denoting the accumulation of tannins and polyphenols within these cells (25) (Fig. 6). Strong autofluorescence throughout both stelar and cortical tissue confirmed the presence of high levels of phenols and tannins indicated by toluidine blue staining. Control roots did not exhibit this. Sclereid bundles with disrupted secondary wall lamellation were often seen with hyphae passing along and between adjoining cells through the cell lumen and interconnecting pores (Fig. 8). Lignification in xylem vessels and xylem parenchyma as indicated by fluorescence microscopy was reduced and confined to the largest metaxylem vessels.

Neither papillae nor callose pad formation was observed in this zone. Extensive tylose development was present in xylem vessels and completely occluded the vessel lumen for some distance along their lengths (Fig. 9A and B). The tyloses arose from axial parenchyma cells as is characteristic of tylose formation (8). This is in contrast to the rarity of tyloses from uninoculated, control roots.

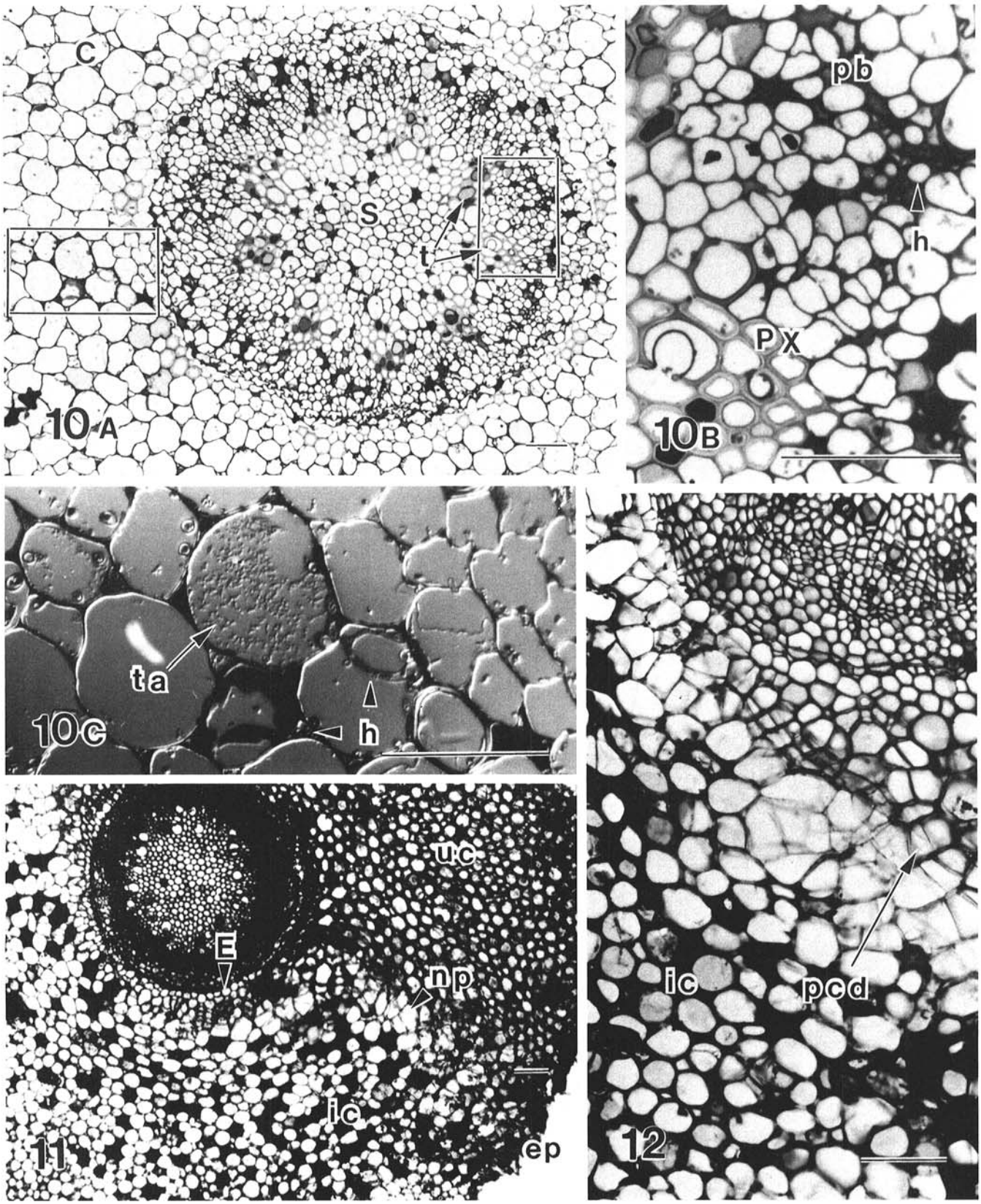
Zone 2. The lesion end point was characterized by a number of rapid changes in both root anatomy and fungal growth. Hyphal development, gross cortical cell disruption, and cell wall degradation (Fig. 10A–C) became progressively restricted to a smaller region of the cortex in this zone. The cells abutting the necrotic tissue had undergone random peri- and anticlinal cell wall



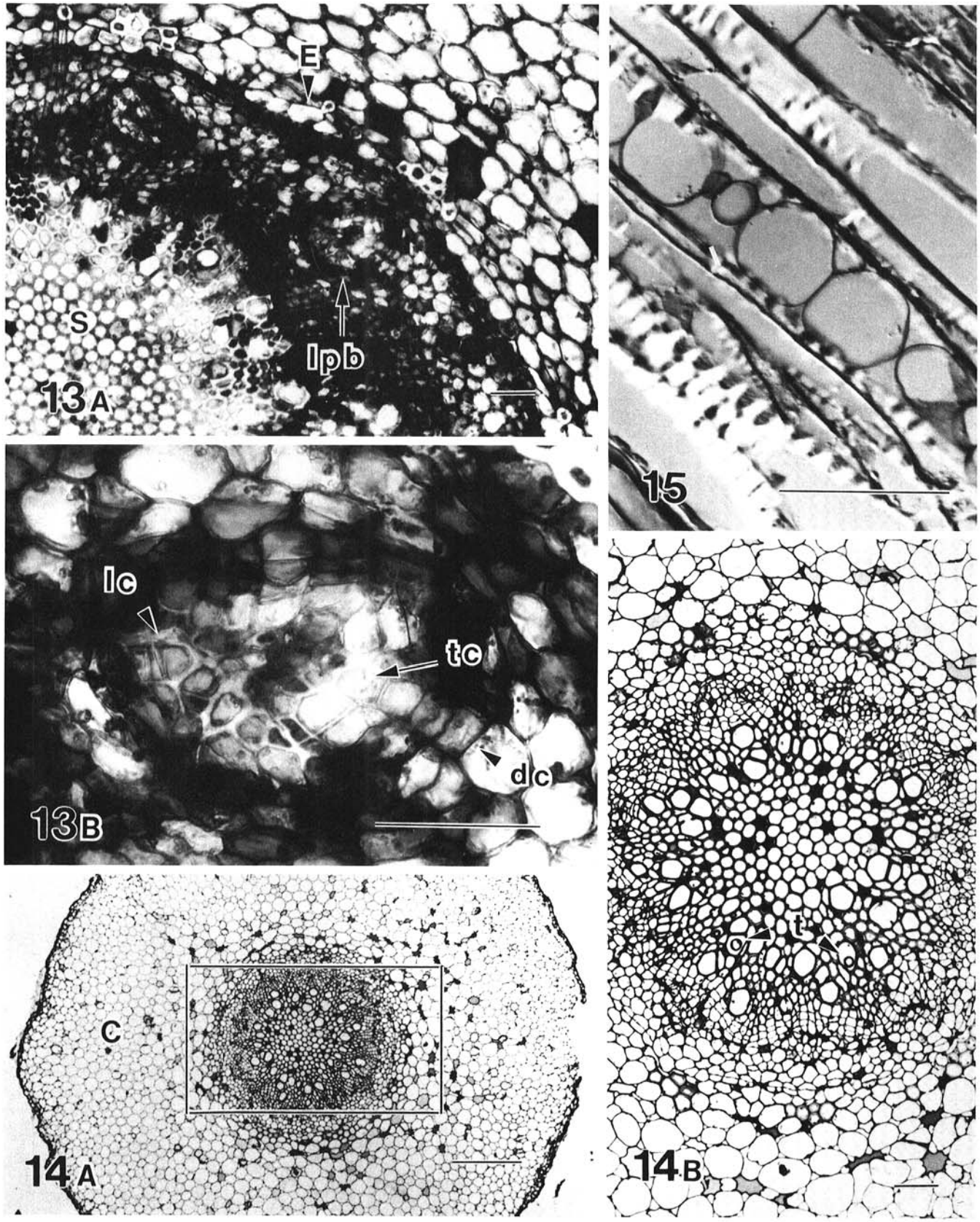
Figs. 2–5. Thin sections of control roots at the root tip or site of the original inoculation point (I.P.), stained with toluidine blue O. Plants were grown at either 15 or 25 C. Scale bar = 50 μm. **2,** Transverse section at the I.P., 8.7 cm from the root tip. Plants grown at 25 C. **A,** Note healthy vascular cambium (vc), cortex (C), xylem rays (xr), phloem bundles (pb) and pericycle (pe). **B,** Inset of **A**. Note absence of tyloses or detritus lining inside of metaxylem vessels (mx). **3A,** Longitudinal section of root tip of plant grown at 15 C. Note healthy root cap (rc), provascular tissue (pv), files of tannin rich cells (tc), and protoderm (pd) containing vacuolate secretory cells (se). **B,** Inset of **A**. Note secretory cells with nucleus (n) and vacuole (v). Vacuole is nonstaining in toluidine blue. **4,** Longitudinal section of root tip stained with *p*-aminosalicyclic acid. Note starch grains (sg) concentrated in root cap. Plant was grown at 25 C. **5,** Longitudinal section of a root at the I.P., 4.5 cm back from the root tip showing absence of tyloses in the metaxylem. Plant was grown at 25 C.



Figs. 6–9. 6 and 7, Hand transverse sections of necrotic root tissue 1 cm behind the lesion front (Zone 1) in plants set at 20 and 30 C. Toluidine blue counterstained with aniline blue. Scale bar = 30 μm. **6,** Hyphae (h) passing intercellularly through xylem parenchyma and vessels (x) with strong dark blue staining of tannins and phenols by toluidine blue in phloem bundles (pb). vc = vascular cambium; E = endodermis. **7,** Hyphae passing through a perforation plate (pp) of a metaxylem vessel. t = tylose. **8,** Thin transverse section of root 0.3 cm behind lesion front of a plant grown at 25 C. Note hyphae along inside walls of sclereid cells (sc) that have disrupted secondary wall lamellation. C = cortex; pe = pericycle; and pl = plasmodesmata. Bar = 30 μm. **9,** Thin transverse section behind the lesion front of a plant grown at 15 C stained with toluidine blue. Bar = 30 μm. **A,** Note tyloses serially blocking xylem vessels (x). **B,** A tylose arising from an axial parenchyma cell (ap) blocking a vessel and perforation plate. pm = pit membrane.



Figs 10–12. 10A–C, Thin transverse section stained with toluidine blue and counterstained with *P*-aminosalicylic acid 0.3 cm behind lesion front, 5.9 cm from the root tip of a plant grown at 15 C (Zone 2). Scale bar = 50 μm. **A,** Note widespread inter- and intracellular hyphal development, xylem blockage by tyloses (t). S = stele; C = cortex. **B,** Inset from right side of **A**. Hyphae (h) concentrated in phloem bundle (pb). PX = protoxylem. **C,** Inset from left side of **A**. Accumulated tannins (ta) and inter- and intracellular hyphae in the cortex. Nomarski optics was used. **11 and 12,** Hand transverse section stained with toluidine blue 0.2–0.4 cm ahead of lesion front, 8.7–8.9 cm from root tip of a plant grown at 20 C (Zone 2). Bar = 100 μm. **11,** Necrophylactic periderm (np) extending from the endodermis (E) to the epidermis (ep) between infected (ic) and uninfected (uc) cortical tissue. **12,** Periclinal cell wall division (pcd) acquiring the appearance of exophylactic periderm. Note the translucent nature of dividing cells in toluidine blue.



Figs. 13–15. 13A and B. Hand transverse section stained with toluidine blue, counterstained with aniline blue, 0.2–0.5 cm ahead of lesion front of a 30 C plant. Scale bar = 50 μm. **A,** Lesioned phloem bundles (lpb) surrounded by whorls of periclinally dividing cells. Metaxylem also stained yellow-brown. S = stele; E = endodermis. **B,** Inset of **A**. Central cell walls and intercellular spaces were thickened with a luminescent, nonstaining compound (lc). Note translucent inner cells (tc) and outer ring of dividing cells (dc). **14A and B and 15,** Thin longitudinal and transverse sections, stained with toluidine blue, of plants grown at 15 C, 3.0 cm ahead of lesion front (Zone 3). Bar = 50 μm. **A,** Healthy, uninfected cortical tissue (C) and stele. Bar = 200 μm. **B,** Inset of **A**. Tyloses (t) are still present in metaxylem vessels. **15,** Xylem vessels completely occluded by tyloses. Viewed by Nomarski optics.

division, appeared translucent and stained very weakly in toluidine blue. It may be cork tissue. The layers of these cells immediately adjacent to infected tissue had walls and intercellular spaces thickened with an amorphous material that was both strongly autofluorescent and fluorescent in aniline blue. This cell layer, which we identify as necrophylactic periderm (22), extended only from the epidermis through to the endodermis (Figs. 11 and 12). The healthy tissue adjacent to this was devoid of fungal material. Occasionally the necrophylactic periderm seemed to acquire the appearance of more ordered cell division of exophylactic periderm (Fig. 12).

Tissue necrosis continued further in the stele than in the cortex. Hyphae passed along the lumen of xylem vessels and were associated with a yellow-brown discoloration of the vessel bundle and surrounding xylem parenchyma cell walls. The discolored tissue did not stain in toluidine blue. Frequent tyloses and accumulated amorphous material were present along the inside of vessel walls. The discoloration became restricted to the largest metaxylem vessels as distance from the lesion end point increased. In contrast, vessel discoloration was absent from heavily infected tissue of Zone 1 and also from uninfected, control roots. Cellular disruption in the xylem parenchyma, pericycle, and vascular cambium declined markedly within the space of a few millimeters ahead of the lesion end point.

Phloem bundles, the site of dense hyphal development in Zone 1 (Fig. 10B) showed stimulated periclinal cell wall divisions resulting in a whorl of cells walling off the infected bundles from surrounding lesion. The intercellular spaces and walls of the innermost cells of these phloem bundles were filled and thickened with a translucent yellow-brown material, which did not stain in toluidine blue (Fig. 13A and B). This material is strongly autofluorescent and fluorescent under aniline blue. The cells adjacent to these central cells did not stain readily, if at all, in toluidine blue. The outer rings of dividing cells had a direct one-to-one relationship with each other, similar to that found with phellem and phellogen of exophylactic periderm (Fig. 13B).

Zone 3. This zone of tissue extended from approximately 1/2 cm ahead of the lesion end point onward up the root. It was characterized by healthy cortical and stelar tissue, which showed no sign of cellular disruption or wall degradation. No hyphae were seen in this zone (Fig. 14A). There was no accumulation of tannins or polyphenols in cortical or stelar tissue. However, tyloses and detritus lining the inside of vessel walls were still present in some vessels as much as 3.5 cm ahead of the lesion end point (Figs. 14B and 15). These were absent in sham-inoculated (control) root tissue. The cortical periderm formation and whorling of cells around phloem bundles, described in Zone 2, were absent from this zone and from the control tissue.

DISCUSSION

The speed and magnitude with which the plant activates mechanisms for resistance determine the progressive development of the pathogen (18,19). The expression of this resistance may be anatomical or physiological or a combination of both. Kellam and Coffey (17) described cultivar Duke 7 as being moderately resistant to Phytophthora root rot. Our research has shown anatomical changes in roots of this cultivar that indicate resistance and impede pathogen invasion.

Two major anatomical responses coincided with the termination of pathogen invasion in the root. These were the production of necrophylactic periderm in the cortex and the isolation of infected phloem bundles by whorls of cells formed by periclinal cell wall division.

It is unusual for root tissue lacking in cork cambium to produce periderm tissue was a wound response (33). In secondary roots of susceptible *E. marginata*, resistance to attack by *P. cinnamomi* was exhibited in the form of periderm development in roots containing cork cambium. Necrophylactic wound periderm was formed initially in tissue directly abutting the infected tissue and always preceded the more ordered development of exophylactic periderm (33). In Duke 7, the changes observed at the lesion end point in the cortical tissue are diagnostic of necrophylactic periderm (22,23). These include: disordered cell division adjacent to necrotic, infected tissue; development of high intensity autofluorescence throughout tissue; apparent lack of vacuolar content; and thickening of cell walls and intercellular spaces, which show both autofluorescence and fluorescence with aniline blue, immediately adjacent to necrotic tissue. Mullick and Jensen (24) suggested that necrophylactic periderm was a nonspecific host response to cell death and served to protect living tissues from adverse affects associated with cell death. Necrophylactic periderm is usually obliterated with time and superseded by the more organized cell division of exophylactic periderm, which is also heavily suberized (22,33). Exophylactic periderm was not produced in the Duke 7 primary root tissue but occasionally wound periderm acquired the appearance of more ordered cell division. The capacity to produce the type of nonspecific host defense response afforded by necrophylactic periderm in woody dicots is widespread. Wound periderm was observed in resistant apple cultivars but not in susceptible ones and was considered to play a significant role in resistance to *Phytophthora cactorum* (Lebert & Cohn) Schroeter (1).

The second major anatomical response observed in infected Duke 7 avocados occurred in the phloem bundles in Zone 2. This involved a combination of cell proliferation and accumulation of an amorphous, luminescent yellow-brown material within the central cell walls and intercellular spaces, which is tentatively identified as suberin. Similar whorling of cells surrounding infected xylem parenchyma cells was observed in tomatoes infected with vascular wilt fungi (3). In addition to these major changes, the serial succession of tyloses blocking vessels may prevent the extension of hyphae along xylem vessel lumens. It may also limit water uptake, reducing tissue water potential, which has been reported to limit *P. cinnamomi* infection (32; Grant, *unpublished*). Tyloses have been observed in some susceptible *Eucalyptus* spp. when infected with *P. cinnamomi* (10,20,33). Tyloses also commonly occur in injured heartwood of most woody dicots (6). The production of mucilaginous and tanniferous cells in the root tips is a characteristic feature of the family, Lauraceae (8), to which *Persea* spp. belong and may also aid in resistance to *P. cinnamomi*, as may the large accumulation and oxidation of phenolic compounds, observed by auto- and fluorescent microscopy, in necrotic tissue.

Despite these anatomical responses, *P. cinnamomi* still remained viable in tissue up to 6 mm ahead of the lesion end point. Therefore, the resistance exhibited by Duke 7 is incomplete and does not permanently affect the pathogen's viability within the root, though it may halt its progress. Kellam and Coffey (17) report that *P. cinnamomi* remained viable in roots of Duke 7 and in fact supported a higher capacity for pathogen sporulation than infected roots of Topa Topa (susceptible) and G6 (moderately resistant). In Australian native field-resistant species such as *Gahnia radula* (R. BR.) Benth, *Lepidosperma laterale* R. Br., *Poa sieberana* Spring and *E. calophylla* R. Br., *P. cinnamomi* also remained viable up to and slightly ahead of the lesion end point although invasion of root tissue was limited in both time and space (12,27). Therefore, we conclude that in addition to the anatomical changes exhibited by Duke 7 in response to *P. cinnamomi* invasion, it is probable that there are additional factors that act in parallel at the biochemical level such as fungitoxins.

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