Interaction of *Phytophthora cinnamomi* and a Resistant Host, *Acacia pulchella*

Joanna Tippett and N. Malajczuk

Research student, Botany Department, Monash University, Clayton, 3168, Victoria, Australia, and research scientist, Division of Land Resources Management, CSIRO, Wembley, 6014, Western Australia.

The senior author thanks A. A. Holland and T. P. O'Brien for advice and encouragement and A. P. M. Forests Pty. Ltd. and the Victorian Forests Commission for financial assistance.

Accepted for publication 6 February 1979.

ABSTRACT

TIPPETT, J., and N. MALAJCZUK. 1979. Interaction of *Phytophthora cinnamomi* and a resistant host, *Acacia pulchella*. Phytopathology 69:764-772.

On the lateritic soils of Western Australia, the jarrah forest may be managed to encourage an understory of *Acacia pulchella* as a means of reducing the inoculum potential of *Phytophthora cinnamomi*. The cytology and ultrastructure of the interaction between *P. cinnamomi* and the legume *A. pulchella* were studied to determine whether the acacia is inherently resistant to attack by this root-infecting fungus. Penetration and attempted establishment of *P. cinnamomi* in the acacia roots suggested that the interaction was incompatible, because the host cells appeared to respond hypersensitively to infection. Darkly staining material, which was autofluorescent and presumably phenolic, accumulated in the necrotic cells, and wall appositions formed close to invading hyphae. This study reports the first resistant host reaction to infection with *P. cinnamomi*.

Phytophthora cinnamomi Rands has a very wide host range and is associated with major changes in the floristic composition of some southern Australian forest communities (20,21,33,34). Many forest *Eucalyptus* spp. and understory species are susceptible, dying rapidly or declining gradually, when infected by *P. cinnamomi*.

Earlier histological and ultrastructural studies have dealt with penetration and establishment of *P. cinnamomi* in *Eucalyptus* spp. of varying susceptibilities (17,28,29,30). No quantitative differences in these interactions were reported, and eucalypt root cells remained healthy even when adjacent to invading germ tubes. There was no evidence of a hypersensitive type of response in roots of eucalypts tolerant to *P. cinnamomi*. It was concluded that eucalypts vary in susceptibility only by degree and that events leading to establishment of *P. cinnamomi* follow a similar sequence in the roots of most species.

Both field observations and laboratory inoculations have suggested that *Acacia pulchella* R. Br. is resistant to attack by *P. cinnamomi* (22,23). It is imperative that *A. pulchella* be shown to be a highly resistant species if a fire management program is to be developed specifically to encourage an *A. pulchella* understory in the infected jarrah forests.

This report presents the results of a cytological and ultrastructural examination of the interaction between *P. cinnamomi* and roots of *A. pulchella*.

MATERIALS AND METHODS

Plant and fungal culture methods. Two isolates of *P. cinnamomi*, both of A2 compatibility type, were used as test pathogens. The first was supplied by Dr. G. Weste and was isolated from roots of *Isopogon ceratophyllus* growing among dying eucalypts in the Brisbane Ranges; the second was isolated from roots of *Eucalyptus marginata* Donn. ex. Sm. in Western Australia. Large numbers of axenic zoospores were produced in 2–3 days by the method described by Hwang et al (12).

Roots of 2-mo-old *A. pulchella* seedlings, grown in sand, were inoculated with the zoospore suspension (40 ml with an initial concentration as high as 10^3 zoospores per ml). Noninoculated control roots also were selected from root systems and left in dishes of mineral salt solution (3) or distilled water for times equivalent to those of inoculated roots.

A number of acacia roots also were inoculated with a small number of zoospores, using the method designed by Byrt and Holland (2). The roots were supported on Parafilm in a petri dish and inoculated with one drop of suspension containing approximately 20 zoospores. This refinement was attempted in case host cell responses had been masked by high inoculum.

Light and electron microscopy. The methods employed have been detailed previously (29,30). Briefly, roots were fixed 4, 7, 22, and 26 hr after inoculation in a mixture containing 2% glutaraldehyde and 4% acrolein buffered to pH 7.2 in 0.1 M cacodylate. Roots were postfixed for 1 hr in buffered 2% osmium tetroxide, dehydrated, and embedded in epoxy resin (27). Several infected roots also were embedded in 2-hydroxyethyl methacrylate (6) so that the autofluorescence of nonfixed material, under transmitted UV light excitation, could be observed against a fluorescence-free background.

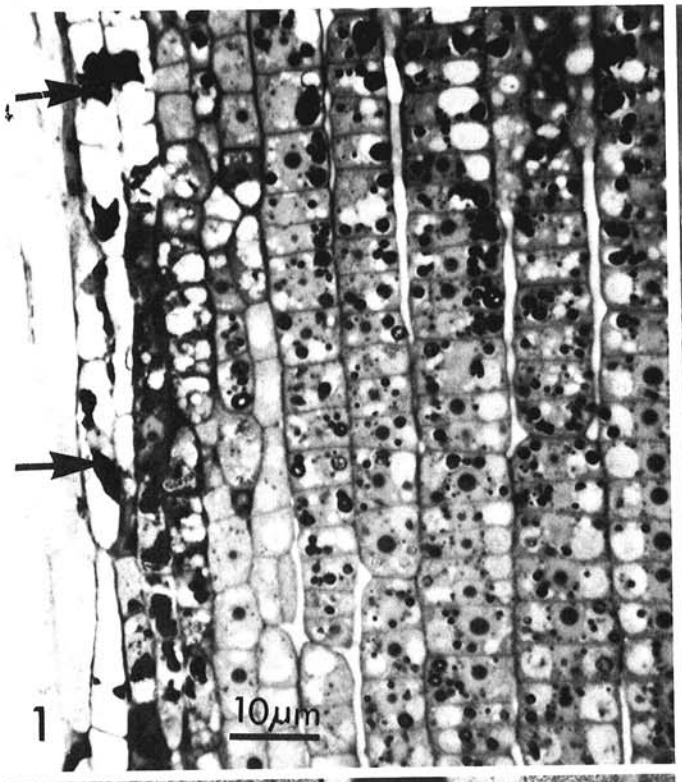
Encysted and germinating zoospores and large, colorless inclusions in the root cells were observed by Nomarski interference optics. To determine the nature of these inclusions, roots were stained in 0.1% aqueous Nile blue (Chroma-Gesellschaft Schmid & Co., Stuttgard, Untertürkheim, Germany) and 70% ethanol saturated with Sudan black B (Chroma 11595). Sudan black B could be used only on roots previously fixed in glutaraldehyde because the ethanol caused rapid collapse of the inclusions in fresh material.

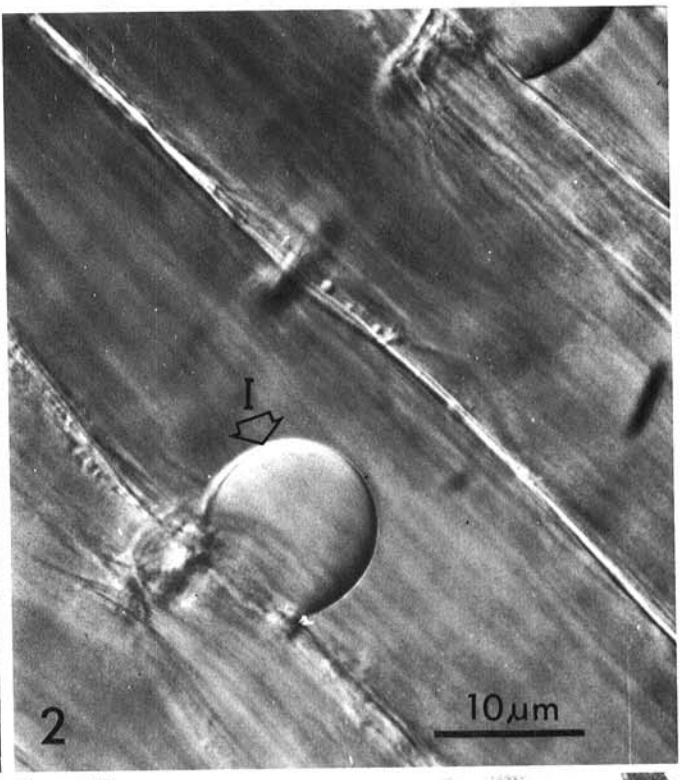
RESULTS

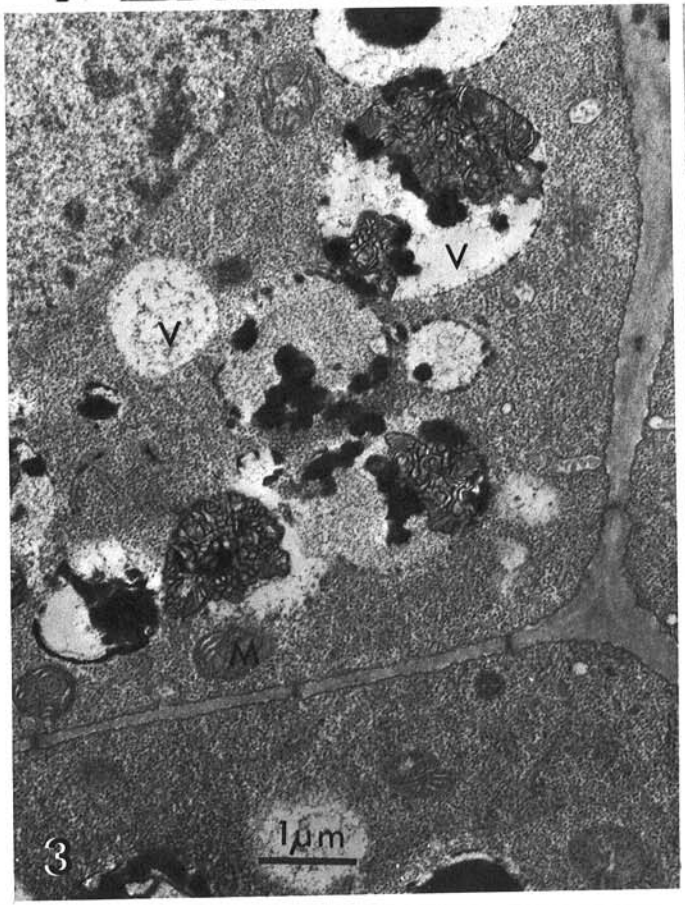
Preliminary observations. Zoospores were attracted to the zone of elongation and germinated rapidly on acacia roots, as previously observed for eucalypt roots (29). Under interference contrast microscopy, unusual, large (20 μm diameter) inclusions were observed in the epidermal cells of such whole mounts (Fig. 2). Initially, these inclusions were thought to be linked with the resistance of the acacia to *P. cinnamomi*, but this idea was abandoned. The inclusions were colorless and refractile (Fig. 2) and stained with Nile blue and Sudan black B, suggesting the presence of lipids.

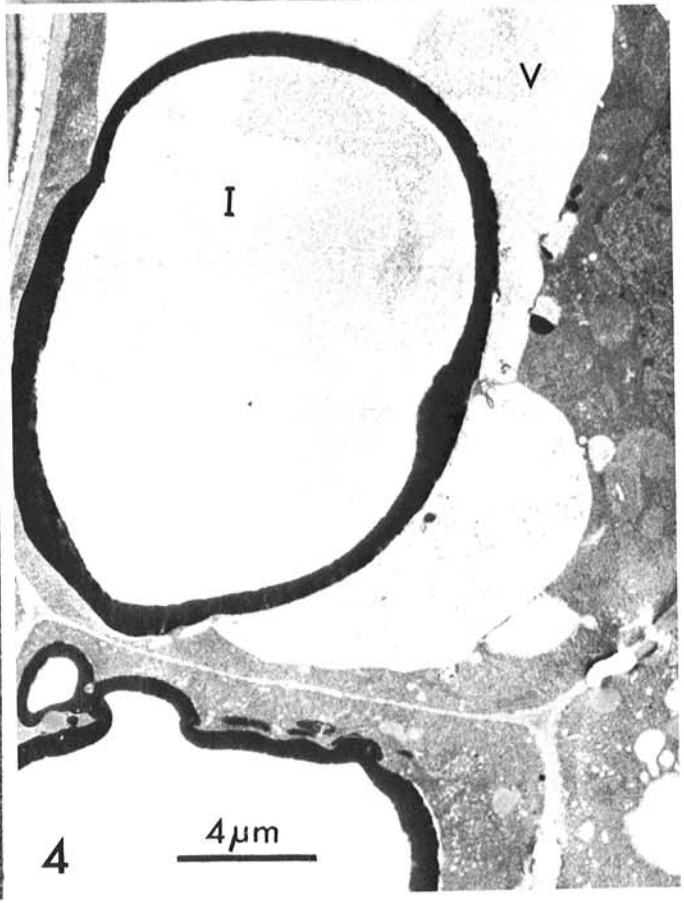
Transverse and longitudinal sections of the *A. pulchella* roots revealed a typical dicotyledonous root structure, the primary roots having a tetrarch xylem arrangement. The longitudinal root

00031-949X/79/000137$03.00/0

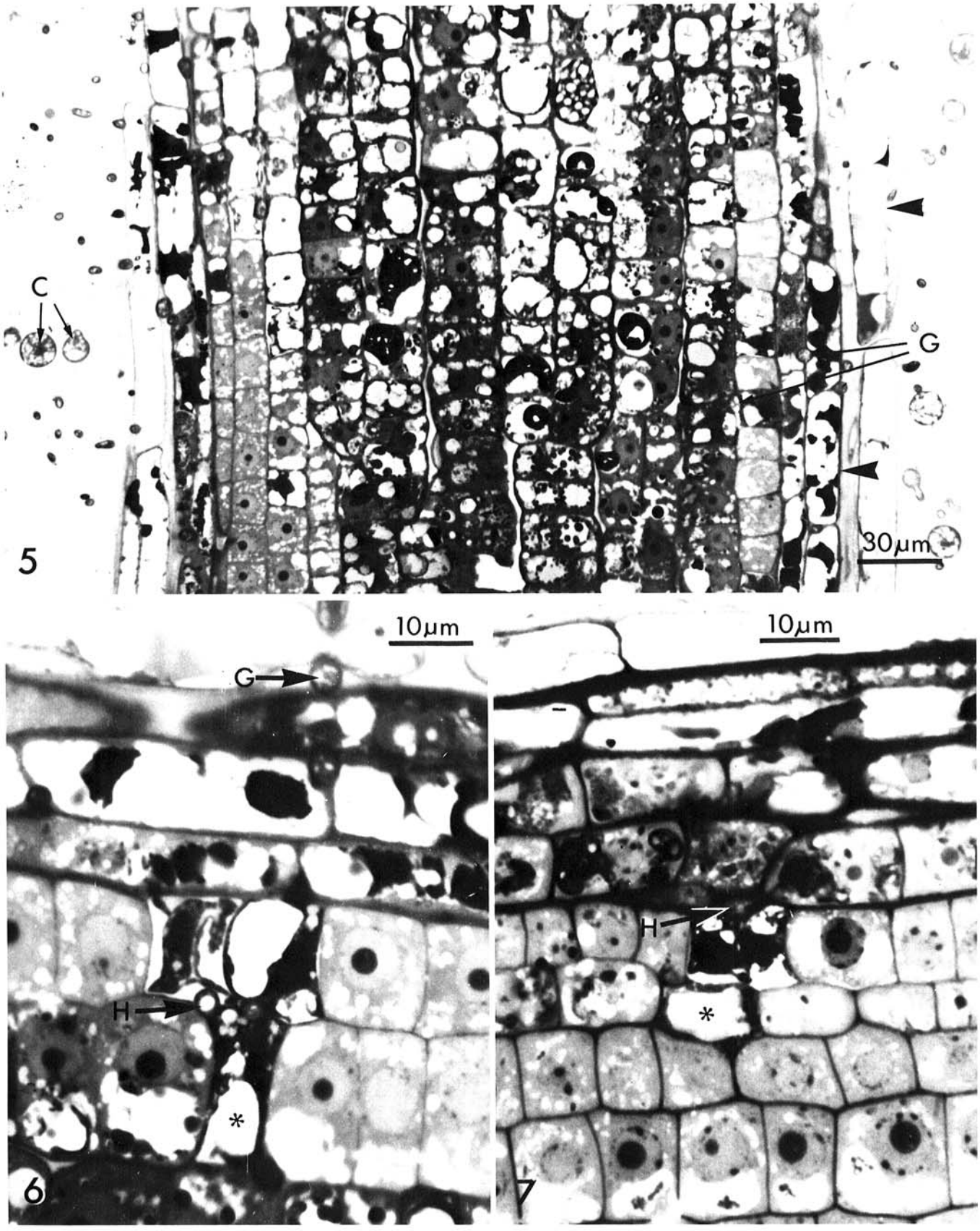








Figs. 1–4. Features of noninoculated roots of *Acacia pulchella*. **1,** Light micrograph of a longitudinal section of the cortex of a healthy root. Osmiophilic inclusions are present in most cells. Dead but persistent root cap cells (arrows) appear dense with tannins. **2,** Large, colorless inclusion (I) in epidermal cell of a root mounted in water (Nomarski interference contrast). **3,** Ultrastructural detail of outer cortical cells from a root zone similiar to that shown in Fig. 1. Cells are at an early stage of vacuolation, and darkly stained precipitates are present in most vacuoles (V). M = mitochondria. **4,** Highly vacuolate cells, showing large vacuolar inclusions (I). V = vacuole.



Figs. 5–7. Light micrographs showing germ tube growth of *Phytophthora cinnamomi* 7 hr after *Acacia pulchella* roots were inoculated. **5,** Germinated cysts (C) along surface of root longitudinal section. Germ tubes (G) have penetrated several layers of cortex, and localized necrosis of infected cells is evident. Arrows point to dead but persistent root cap cells. **6,** Higher magnification of an area from the root section in Fig. 5, showing the hypersensitive response (asterisk) of root cells to the Victorian isolate of the fungus hypha (H). **7,** Similar response 5 hr after inoculation in a Western Australian isolate.

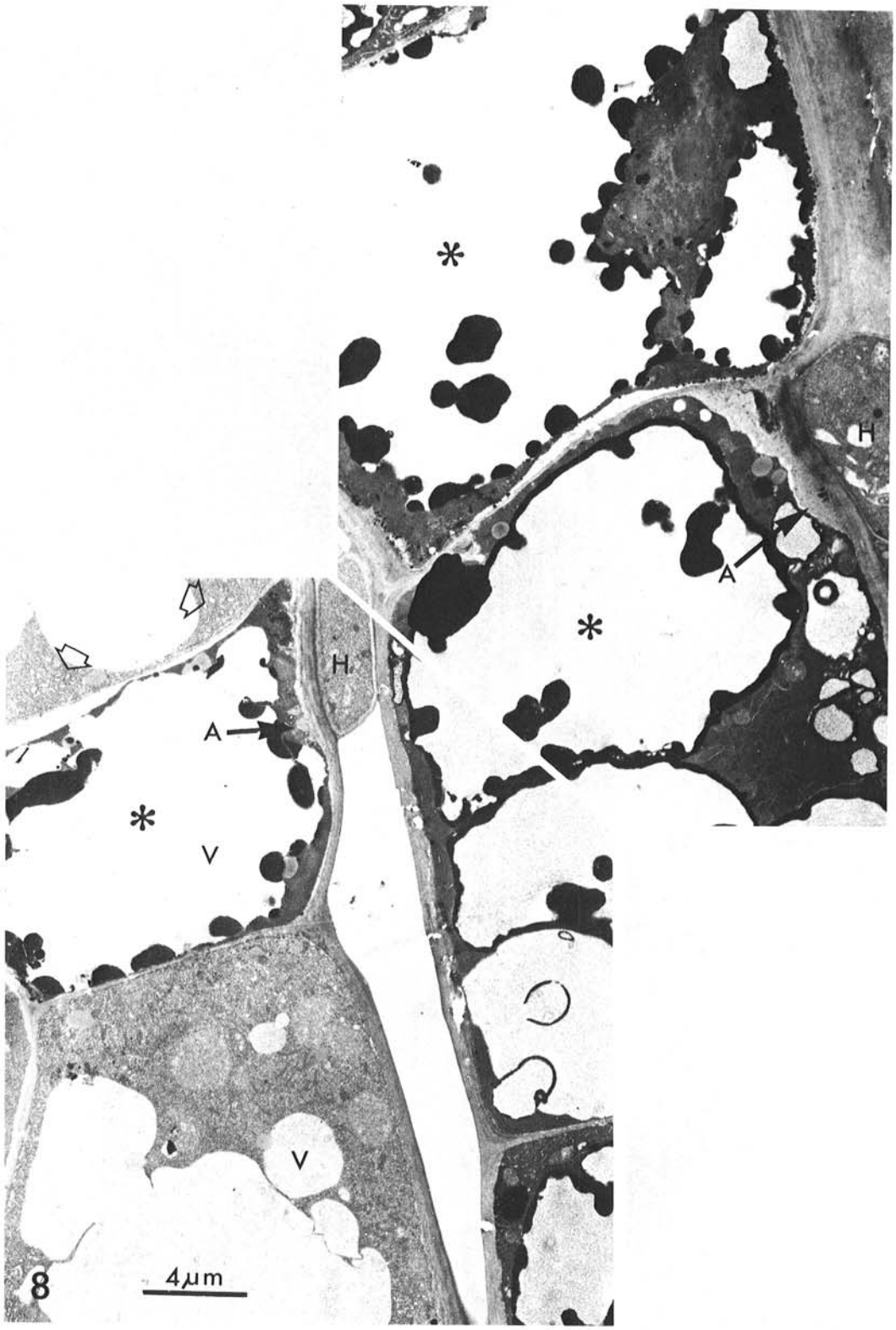
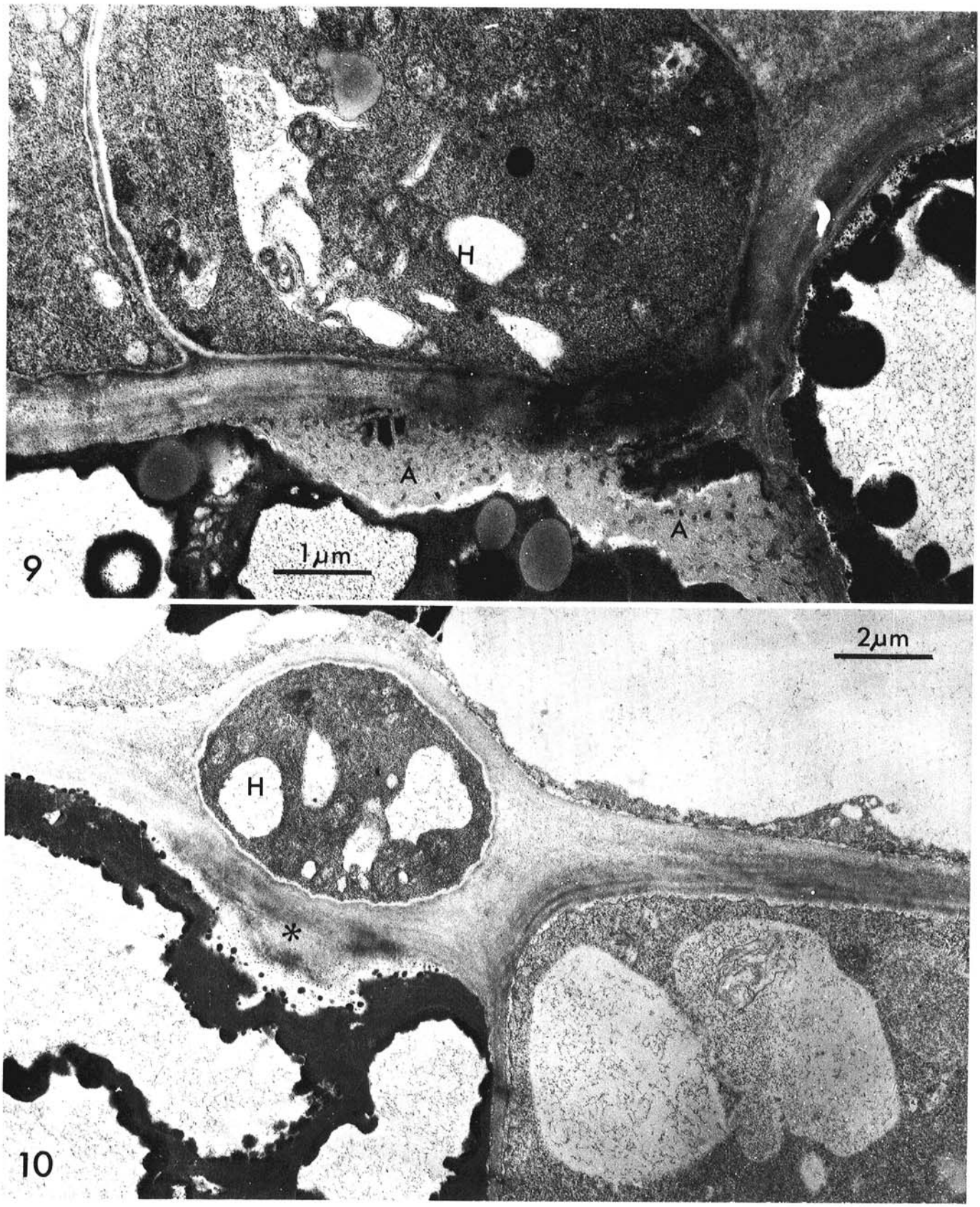
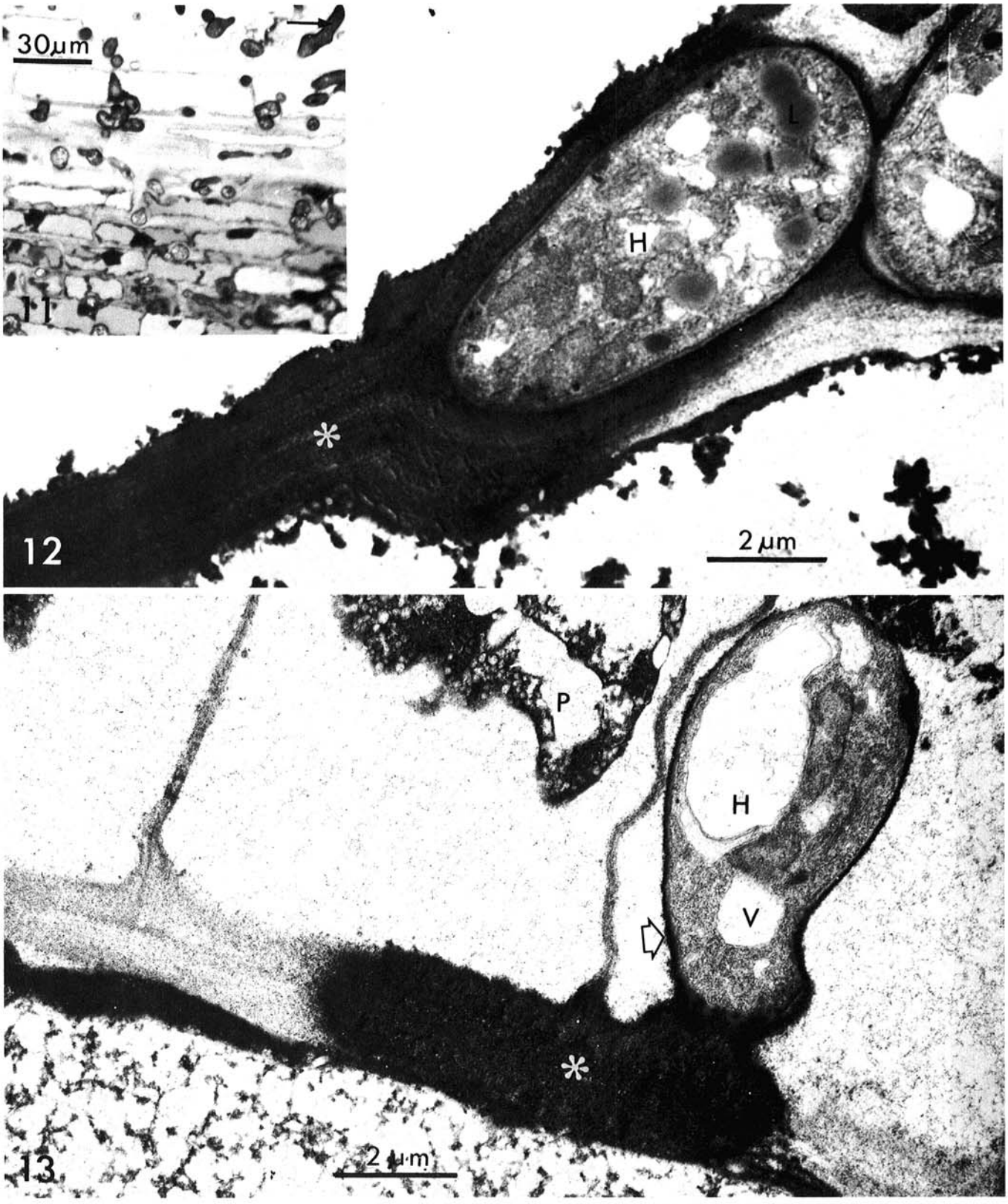


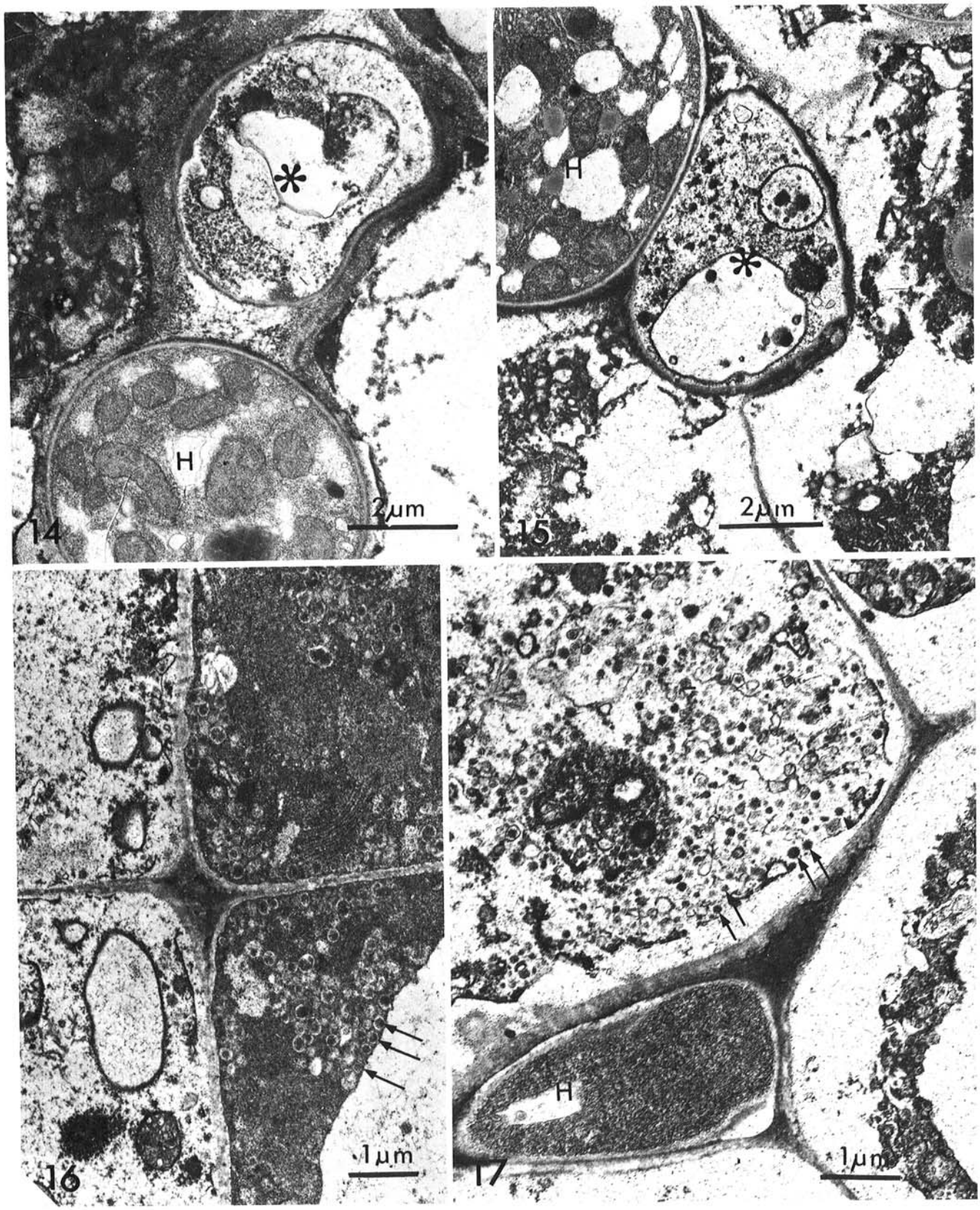
Fig. 8. Electron micrograph of outer cortical cells that have responded hypersensitively to hyphal penetration (asterisks). (Epidermal cells are to the right on the micrograph.) This micrograph was taken of a section adjacent to that in Fig. 5. Vacuoles (V) appear enlarged as if smaller vacuoles have coalesced and darkly staining material has accumulated around their periphery. The cytoplasm of these cells is darkly stained. Arrows point to smooth endoplasmic reticulum. H = hyphae; A = wall apposition.



Figs. 9 and 10. Higher magnification of an area in Fig. 8. Gray amorphous material in wall appositions (A) adjacent to an invading hypha (H) resembles "callose." **10,** Example of cell wall modification (asterisk) adjacent to an intercellular hypha (H). Gray amorphous material has not been deposited around the swollen area of wall.



Figs. 11–13. Sections of *Acacia pulchella* roots 20 hr after inoculation with a high concentration of zoospores of *Phytophthora cinnamomi*. **11,** Light micrograph showing extensive hyphal growth and necrotic cortical tissue (longitudinal section). Some of the densely stained hyphae may be "exit" hyphae (arrow). **12 and 13,** Electron micrographs showing extensive and darkly stained wall lesions (asterisks) in contact with both intercellular and intracellular hyphae (H). Protoplasts of the host cells are totally disorganized and some appear as shrunken remnants (P). Hyphae are vacuolate (V), and some contain lipid reserves (L). The hyphal wall in Fig. 13 is surrounded by an electron-dense layer of material apparently continuous with the wall lesion (arrow).



Figs. 14—17. Electron micrographs of *Acacia pulchella* tissue 24–26 hr after inoculation with *Phytophthora cinnamomi*. **14 and 15,** Examples of senescent hyphae (asterisks). **16,** Cells at two stages of necrosis. The cells to the right, with densely stained cytoplasm, contain a number of small vesicles with electron-dense cores (arrows) that appeared to be derived from Golgi dictyosomes. **17,** Necrotic cells in proximity of intercellular hypha (H). Arrows point to persistent cores of darkly staining material, and most membrane systems appear disrupted. Limited wall hydrolysis has occurred.

sections showed progressive accumulation of osmiophilic material in vacuolar inclusions (Figs. 1 and 5). Persistent root cap cells were especially darkly stained (Figs. 1 and 5). Processing of tissues for microscopy extracted components of many inclusions seen in the sections of noninoculated control roots (Figs. 3 and 4).

Cytology and ultrastructure. Longitudinal sections of root showed germinated zoospores along the epidermis (Fig. 5). Some germ tubes had penetrated two or three cell layers of the cortex (shown in higher magnification in Fig. 6). The most noticeable feature observed by light microscopy of tissue infected for 4–7 hr was the total disruption of invaded cells or cells adjacent to germ tubes or hyphae (Figs. 6 and 7). These cells died rapidly and death was associated with accumulation of darkly staining material. The sharp demarcation between dead and apparently healthy cells (Fig. 6) suggests that the cells had undergone a hypersensitive reaction. The acacia roots responded similarly to both the Victorian and the Western Australian isolates of the fungus (Figs. 6 and 7).

The cytoplasm of healthy root-tip cells generally showed some autofluorescence, but the first cortical cells invaded by hyphae fluoresced brightly, possibly indicating accumulated phenolics.

The ultrastructural changes occurring in the invaded cells of the root's zone of differentiation were studied in some detail. Although germ tube growth appeared to be intercellular (Fig. 8), intracellular hyphae or germ tubes were observed in some dead cortical cells. Cells that had undergone rapid necrosis (Figs. 8–10) showed (i) electron-dense "droplets" lining the vacuolar membranes, (ii) "tanned" cytoplasm, and (iii) wall appositions next to "intercellular" hyphae. The cell membranes often were separated from the cell walls. The wall appositions, as defined by Bracker and Littlefield (1), were variable (Figs. 9 and 10) but most appeared to be composed primarily of lightly stained, amorphous wall material (Fig. 9). Many substances may have been deposited or incorporated in the wall appositions, but the lightly stained component resembled the "callose" described in other ultrastructural studies (9,10,16). Cells adjacent to the necrotic cells often had swollen, tubular endoplasmic reticulum (Fig. 8), which may be indicative of increased secretory activity and callose production (7,9). Aniline blue-positive material (5) was most common along epidermal or outer cortical cell walls adjacent to hyphae or germ tubes, and quite large local deposits sometimes appeared to be encasing penetrating germ tube tips. In contrast, wall lesions or appositions were absent in infected eucalypt roots (30), and some wall lesions (swellings) in the proximity of intercellular hyphae (Fig. 10) did not have associated calloselike deposits.

Although activated wound responses were evident in roots 6 hr after inoculation, the hyphae showed no signs of growth impedance or of senescence. The hypersensitive reaction was not immediately effective in restricting hyphal growth. Some further ultrastructural changes were observed in acacia roots sectioned 24 hr after inoculation.

The root tips exposed to a high inoculum (10^3 zoospores per ml) were invaded extensively by hyphae (Fig. 11), in contrast to limited hyphal growth in roots that had been inoculated with a few zoospores. This suggested that observations of roots inoculated with high inoculum levels should be interpreted with caution. Two observations made during later stages of the interaction included (i) the formation of darkly stained wall lesions, similar to "discs" described by Sherwood and Vance (24) or "haloes" described by Bracker and Littlefield (1), adjacent to intracellular and intercellular hyphae (Figs. 12 and 13) and (ii) the apparent production by Golgi dictyosomes of many small vesicles (less than 100 mm in diameter) with electron-dense "cores" in the root cells (Figs. 16 and 17). The electron-dense cores appeared to persist in necrotic cells after most membrane systems and organelles were totally disrupted (Fig. 17).

The intensely stained wall lesions in acacia were in marked contrast to the rapid decrease in wall staining and apparent wall hydrolysis seen in eucalypt roots (30). Wall hydrolysis was neither so extensive nor as complete as that seen in the eucalypt roots 26 hr after inoculation (Figs. 16 and 17). The wall zones that stained intensely in the acacia roots (ie, the haloes or discs) may have been biochemically altered by the action of either host or fungal enzymes. The fibrillar nature of the walls remained recognizable, but phenolics apparently accumulated or were released in the host walls.

Although some apparently healthy hyphae were present in the acacia roots exposed to high inoculum, many senescent and dead hyphae were observed 26 hr after inoculation (Figs. 14 and 15). A few mature, vacuolate hyphae containing lipid bodies also were present. The apparently healthy hyphae were cytoplasmically rich and thought to be emerging aerial hyphae (Fig. 11).

DISCUSSION

The *P. cinnamomi-A. pulchella* interaction is an example of an incompatible interaction, which infers that the host is resistant. This is the first report of a hypersensitive host response to *P. cinnamomi,* although there are many examples of phytoalexin production and hypersensitive host reactions to other *Phytophthora* spp. (11,13–15,18,25,26). The similarity with the ultrastructural changes observed in roots of a resistant tobacco cultivar infected by *P. parasitica* (8) supports the conclusion that *A. pulchella* is resistant to *P. cinnamomi.*

The abnormally rapid death of cortical cells was accompanied by the accumulation of darkly stained material, which may have been phenolic. Rate of host cell necrosis in response to hyphal penetration is generally inversely related to host susceptibility (31), and the particularly rapid necrotic reaction in *A. pulchella* roots to *P. cinnamomi* suggests a resistance mechanism.

Infected acacia roots gave no evidence of hyphal senescence within the first necrotic cells, a criterion in Müller's definition of hypersensitive reaction (19). However, in the hypersensitive reaction in potatoes to *P. infestans,* Tomiyama (32) stated that hyphal growth may not be suddenly halted in cells responding hypersensitively but that growth rate gradually decreases and dead host cells inhibit hyphal establishment.

A number of other observations made during later stages of the interaction suggested that host resistance was effective, especially when contrasted with the rapid establishment of the fungus in the eucalypt roots. First, many necrotic hyphae were observed in the acacia roots. These were not merely vacuolate, as was observed later in the eucalypt-*P. cinnamomi* interactions. Second, extensive cell death in advance of the hyphae did not occur in the acacia roots. A sharp demarcation always remained between invaded dead and healthy host cells. Third, extensive tissue maceration was not apparent in the acacia roots; darkly stained wall regions were more common than zones of almost total wall hydrolysis. Fourth, root tips of the acacia did not appear necrotic and tanned as did the eucalypt roots 1 day after inoculation.

The details and sequence of the ultrastructural changes in the nonhost pepper fruit after penetration of *P. infestans* were remarkably similar to those described in the acacia roots invaded by *P. cinnamomi* (4). The following features were noted in both interactions: (i) cell wall lesions formed, (ii) uninvaded cells, which were close to those that responded hypersensitively, had dilated, smooth endoplasmic reticulum, (iii) late in the interaction, hyphae degenerated and cell walls were often darkly stained rather than extensively hydrolyzed, and (iv) extensive death of host cells in advance of hyphae did not occur.

The formation of wall appositions in the acacia roots does not necessarily support the conclusion that the host is resistant to *P. cinnamomi.* In fact, initial growth of germ tubes was mostly intercellular and germ tube retardation was not evident. Some host cultivars that are resistant to *Phytophthora* spp. form "callosities" more readily than do susceptible cultivars (10), but there are many cases in which wall appositions form in susceptible host tissues (26).

LITERATURE CITED

1. BRACKER, C. E., and L. J. LITTLEFIELD. 1973. Structural concepts of host-pathogen interfaces. Pages 159-318 in: R. J. W. Byrde and C. V. Cutting, eds. Fungal Pathogenicity and the Plant's Response. Academic Press, New York. 449 pp.

2. BYRT, P., and A. A. HOLLAND. 1978. Infection of axenic Eucalyptus seedlings with Phytophthora cinnamomi zoospores. Aust. J. Bot. 26:169-176.
3. CHEN, D. W., and G. A. ZENTMYER. 1970. Production of sporangia by Phytophthora cinnamomi in axenic culture. Mycologia 62:397-402.
4. DAY, P. R. 1974. Genetics of Host-Parasite Interactions. W. H. Freeman and Co., San Francisco. 238 pp.
5. ESCHRICH, W., and H. B. CURRIER. 1964. Identification of callose by its diachrome and fluorochrome reactions. Stain Technol. 39:303-307.
6. FEDER, N., and T. P. O'BRIEN. 1968. Plant microtechnique: Some principles and new methods. Am. J. Bot. 55:123-142.
7. GUNNING, B. E. S., and M. W. STEER. 1975. Ultrastructure and the Biology of Plant Cells. Edward Arnold, London. 312 pp.
8. HANCHEY, P., and H. WHEELER. 1971. Pathological changes in ultrastructure: Tobacco roots infected with Phytophthora parasitica var. nicotianae. Phytopathology 61:33-39.
9. HEATH, M. C., and I. B. HEATH. 1971. Ultrastructure of an immune and a susceptible reaction of cowpea leaves to rust infection. Physiol. Plant Pathol. 1:277-287.
10. HOHL, H. R., and P. STÖSSEL. 1976. Host-parasite interfaces in a resistant and susceptible cultivar of Solanum tuberosum inoculated with Phytophthora infestans: Tuber tissue. Can. J. Bot. 54:900-912.
11. HOHL, H. R., and E. SUTER. 1976. Host-parasite interfaces in a resistant and a susceptible cultivar of Solanum tuberosum inoculated with Phytophthora infestans: Leaf tissue. Can. J. Bot. 54:1956-1970.
12. HWANG, S. C., W. H. KO, and M. ARAGAKI. 1975. A simplified method for sporangial production by Phytophthora cinnamomi. Mycologia 67:1233-1234.
13. JONES, D. R., C. H. UNWIN, and E. W. B. WARD. 1975. Capsidiol induction in pepper fruit during interactions with Phytophthora capsici and Monilinia fructicola. Phytopathology 65:1417-1419.
14. KEEN, M., J. SIMS, D. ERWIN, E. RICE, and J. PARTRIDGE. 1971. 6a-Hydroxyphaseollin: An antifungal chemical induced in soybean hypocotyls by Phytophthora megasperma var. sojae. Phytopathology 61:1084-1089.
15. KLISIEWICZ, J. M., and L. B. JOHNSON. 1968. Host-parasite relationship in safflower resistant and susceptible to Phytophthora root-rot. Phytopathology 58:1022-1025.
16. LAZAROVITS, G., and V. J. HIGGINS. 1976. Ultrastructure of susceptible, resistant and immune reactions of tomato to races of Cladosporium fulvum. Can. J. Bot. 54:235-249.
17. MALAJCZUK, N., A. J. McCOMB, and C. A. PARKER. 1977. Infection by Phytophthora cinnamomi Rands of roots of Eucalyptus calophylla R. Br. and Eucalyptus marginata Donn. ex. Sm. Aust. J. Bot. 25:483-500.
18. MARKS, G. C., and J. E. MITCHELL. 1971. Penetration and infection of alfalfa roots by Phytophthora megasperma and the pathological anatomy of infected roots. Can. J. Bot. 49:63-67.
19. MÜLLER, K. O. 1959. Hypersensitivity. Pages 469-519 in: J. G. Horsfall and A. E. Dimond, eds. Plant Pathology. Vol. I. Academic Press, New York. 674 pp.
20. NEWHOOK, F. J., and F. D. PODGER. 1972. The role of Phytophthora cinnamomi in Australian and New Zealand forests. Annu. Rev. Phytopathol. 10:299-326.
21. PODGER, F. D. 1972. Phytophthora cinnamomi, a cause of lethal disease in indigenous plant communities in Western Australia. Phytopathology 62:972-981.
22. SHEA, S. R. 1975. Environmental facotrs of the northern jarrah forest in relation to pathogenicity and survival of Phytophthora cinnamomi. Bull. For. Dep. West. Aust. No. 85. 83 pp.
23. SHEA, S. R., and N. MALAJCZUK. 1977. Potential for control of eucalypt dieback in Western Australia. Pages 13-19 in: Seminar Papers of Annual Conference of Australian Nurserymen's Association Ltd., Hobart.
24. SHERWOOD, R. T., and C. P. VANCE. 1976. Histochemistry of papillae formed in reed canary grass leaves in response to noninfecting fungi. Phytopathology 66:503-510.
25. SHIMONY, C., and J. FRIEND. 1975. Ultrastructure of the interaction between Phytophthora infestans and leaves of two cultivars of potato (Solanum tuberosum L.) Orion and Majestic. New Phytol. 74:59-65.
26. SHIMONY, C., and J. FRIEND. 1976. Ultrastructure of the interaction between Phytophthora infestans and tuber slices of resistant and susceptible cultivars of potato (Solanum tuberosum L.) Orion and Majestic. Isr. J. Bot. 25:174-183.
27. SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
28. TIPPETT, J. T. 1978. The response of eucalypt roots to infection by Phytophthora cinnamomi. Pages 364-368 in: M. W. Loutit and J. A. R. Miles, eds. Microbial Ecology. Springer-Verlag, Heidelberg. 452 pp.
29. TIPPETT, J. T., A. A. HOLLAND, G. C. MARKS, and T. P. O'BRIEN. 1976. Penetration of Phytophthora cinnamomi into disease tolerant and susceptible eucalypts. Arch. Microbiol. 108:231-242.
30. TIPPETT, J. T., T. P. O'BRIEN, and A. A. HOLLAND. 1977. Ultrastructural changes in eucalypt roots caused by Phytophthora cinnamomi. Physiol. Plant Pathol. 11:279-286.
31. TOMIYAMA, K. 1963. Physiology and biochemistry of disease resistance of plants. Annu. Rev. Phytopathol. 1:295-324.
32. TOMIYAMA, K. 1971. Cytological and biochemical studies of the hypersensitive reaction of potato cells to Phytophthora infestans. Pages 387-401 in: S. Akai and S. Ouchi, eds. Morphological and Biochemical Events in Plant-Parasite Interactions. Phytopathol. Soc. Jpn., Tokyo. 415 pp.
33. WESTE, G., and C. LAW. 1973. The invasion of native forest by Phytophthora cinnamomi. III. Threat to the national park, Wilson's Promontory, Victoria. Aust. J. Bot. 21:31-51.
34. WESTE, G., and P. TAYLOR. 1971. The invasion of native forest by Phytophthora cinnamomi. I. Brisbane Ranges, Victoria. Aust. J. Bot. 19:281-294.