Histopathology of Strawberry Infected with *Colletotrichum fragariae*

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Journal Series Paper 8160 of the North Carolina Agricultural Research Service, Raleigh 27650.
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The author thanks Marilyn Daykin for technical assistance.
Accepted for publication 12 April 1982.

ABSTRACT

Milholland, R. D. 1982. Histopathology of strawberry infected with *Colletotrichum fragariae*. Phytopathology 72:1434-1439.

Strawberry cultivars resistant and susceptible to anthracnose were inoculated with *Colletotrichum fragariae* and examined histologically. The susceptible reaction on cultivar Surecrop was characterized by intercellular and extracellular growth of the fungus in the cortex and vascular tissue resulting in cellular collapse and necrosis. Sporulation was abundant 7 days after inoculation. Ingress into the crown from infected petioles was primarily through the vascular tissue after 14 days. In resistant cultivars, Apollo and Sequoia, the fungus was confined to a few cells beneath the infection site after 21 days. A thickening of the cell walls and deposition of pectic material in the intracellular spaces of the cortex as well as the accumulation of tannins in the surrounding parenchyma cells were associated with this restriction. Sporulation was absent 21 days after inoculation.

Strawberry anthracnose, which is caused by *Colletotrichum fragariae* A. N. Brooks, is an important disease in plant production nurseries throughout the southeastern USA (1,3–5). The fungus does not overwinter in soil but survives in infected crowns of apparently healthy strawberry plants (*Fragariae* × *ananassa* L.) that can serve as a source of primary inoculum the following spring (4). A reliable inoculation technique for evaluating strawberry plants for resistance to *C. fragariae* has been developed (2). Cultivar reactions to 10 different isolates varied from small flecks on the petiole to plant death after 21 days (3). All cultivars were killed when the fungus invaded the crown.

The research reported here was undertaken to examine the histological effects of the pathogen on petiole and crown tissues of resistant and susceptible cultivars.

MATERIALS AND METHODS

Plants of strawberry cultivars Apollo and Sequoia, which are resistant, and Surecrop, which is susceptible to isolate CF-7 of *C. fragariae*, were obtained from a registered planting at the Sandhills Research Station, Jackson Springs, NC, where anthracnose has not been observed. Nine plants of each cultivar were inoculated by spraying the petioles with a suspension of 3×10^6 conidia per milliliter as previously described (3). Three plants of each cultivar were sprayed with distilled water and used as controls. The plants were placed in a moist chamber at 25–30 C for 48 hr and then removed to a greenhouse bench. Plants were evaluated after 7, 14, and 21 days, and assigned a disease index (0–5) based on petiole reaction (2,3). Inoculated and uninoculated petioles from each cultivar were selected for histological examination after 3, 7, 14, and 21 days. Petioles were cut into 10-mm sections and fixed in formalin-propionic-propanol (FPP) for 1 wk. Infected crowns of cultivar Surecrop were fixed in FPP after 14 and 21 days. Sections were dehydrated for 1 wk in an isopropyl alcohol series, then infiltrated and embedded in Paraplast (Sherwood Medical Industries, St. Louis, MO 63100) (6). The specimens were softened for 48 hr in a solution of 90 ml of 1% sodium lauryl sulfate (the household detergent, Dreft) and 10 ml of glycerol, and 12-μm-thick sections were cut with a rotary microtome. Sections were mounted on slides with Haupt's adhesive and stained with Triarch's Quadruple Stain (Triarch, Inc., Ripon, WI 54971). Germination of *C. fragariae* conidia and subsequent formation of appressoria was studied by using petioles removed from Surecrop and Apollo plants 6, 12, and 24 hr after inoculation. The petioles were cut into small sections, cleared, and stained with cotton blue in lactophenol (6). The epidermis was removed and examined microscopically.

Sections of 7- and 14-day-old lesions of Sequoia and Apollo were tested for lignin with phloroglucinol-HCl for pectin by the Ruthenium red and iron absorption methods, and for tannins with the ferric sulfate reaction (6).

RESULTS AND DISCUSSION

Anthracnose caused by *C. fragariae* affects the petioles, runners, and crowns of strawberry plants, resulting in wilting and death of susceptible cultivars (1). Small flecks or lesions less than 10 mm in

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0031-949X/82/11143406/$03.00/0

length develop on the petioles of resistant cultivars and fail to enlarge further after 21 days (2). However, inoculum introduced into the crown of resistant plants results in plant death, indicating that a resistance mechanism of strawberries to infection by *C. fragariae* exists in the petiole or runner tissue, but not in the crown (2). The reactions of strawberry cultivars Surecrop, Apollo, and Sequoia to isolate CF-7 of *C. fragariae* in this study were consistent with those previously reported (3).

Uninoculated tissue. The anatomy of the strawberry petiole and "runner" are similar, differing primarily in the arrangement of the vascular tissue. The runner is an elongated stem consisting of a cortex, vascular cylinder, and a large parenchymatous pith (Fig. 1). Outside the vascular tissue is a single layer of endodermoid cells filled with tannins. The vascular tissue is comprised of a bundle sheath of thick-walled fibers, phloem parenchyma, phloem, cambium, and xylem. The vascular system in the petiole is separated into three bundles: two small bundles and a large centrally located bundle. The lignified fibers (bundle sheath) and tannin-filled endodermoid cells are absent in the early stages of petiole growth, but develop as the stem matures (Fig. 2). The cortex consists of large, loosely arranged parenchyma cells enclosed by the epidermis. The epidermis is covered with elongated unicellular hairs and short multicellular glandular hairs.

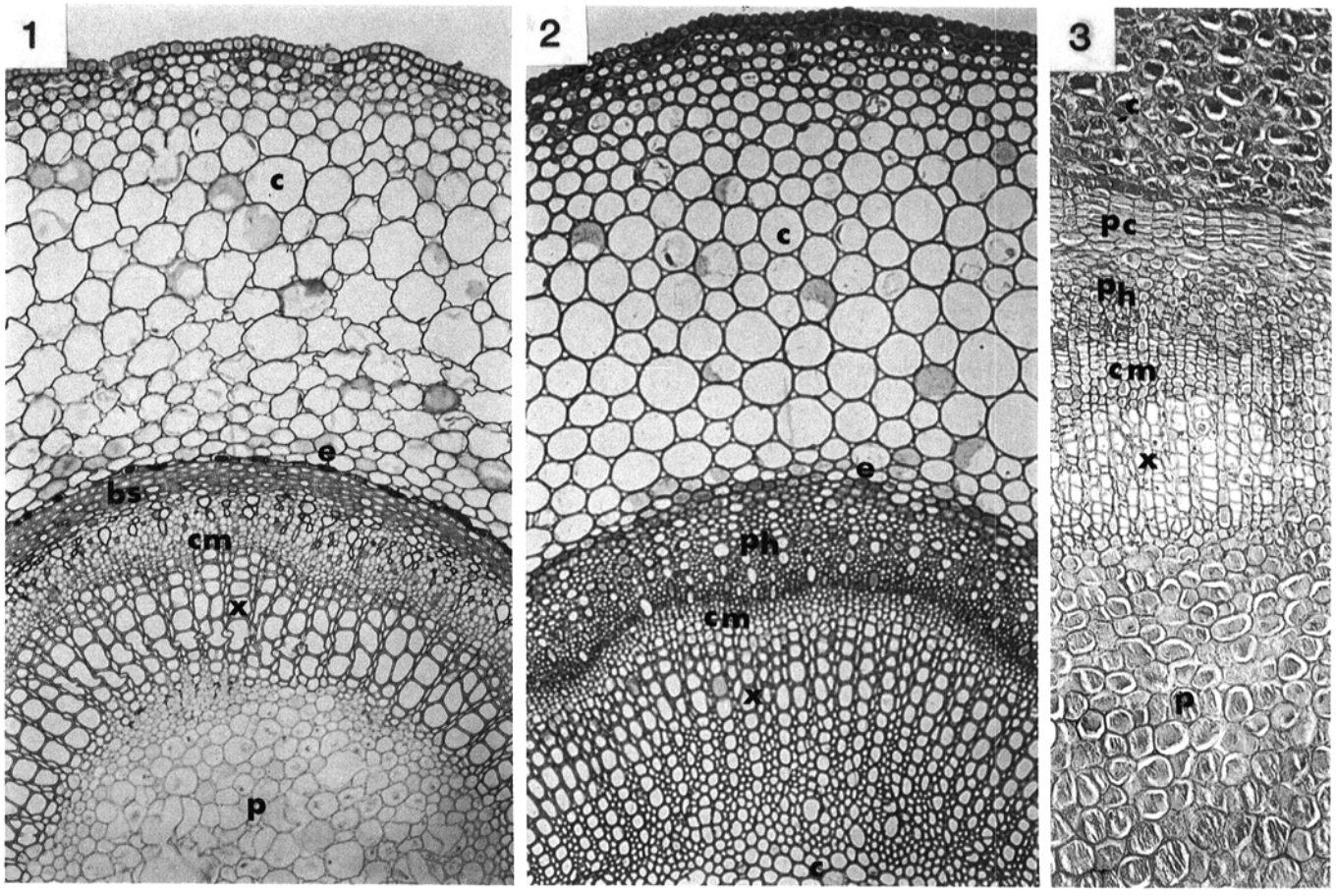
Transverse sections of crown tissue examined were similar to the findings previously reported (7). Beneath the epidermis there is a considerable amount of large, closely packed parenchyma cells separated from the vascular tissue by a single layer of endodermoid cells. The outer portion of the vascular tissue is comprised of four to five layers of thin-walled meristematic cells previously reported as the pericycle (7) (Fig. 3). Inside the vascular tissue the pith consists of large parenchyma cells often filled with a granular substance or large numbers of crystals. Cells located near the xylem vessels were compact with thick cell walls while cells near the center were more loosely arranged.

Susceptible reaction. Germination of conidia and penetration of susceptible and resistant host tissue by *C. fragariae* was similar. Conidia germinated within 6 hr after inoculation, and penetration from well-defined appressoria (4–5 μm in diameter) occurred after 12–24 hr (Fig. 4). Ingress by the fungus into the host tissue occurred between epidermal cells and frequently at the basal cells of glandular hairs. The invading hyphae grew intercellularly in the cortex of the susceptible tissue, causing the walls of the parenchyma cells to collapse and a granular substance to accumulate within some of the affected cells. After 72 hr, six to eight layers of cells in the cortex had collapsed. At this stage of disease development, growth by the fungus was primarily intercellular with some hyphae inside the collapsing cells. Masses of hyphae that were the early stages of acervulus formation had accumulated beneath the epidermis and in some epidermal cells (Fig. 5).

At 7 days, necrosis of the parenchyma cells in the cortex was extensive, and the vascular bundles had been invaded (Fig. 6). The endodermoid layer, parenchyma, phloem, and cambium were the most severely affected (Fig. 7). The fungus spread both intercellularly and intracellularly throughout the cortex. As the fungus came into contact with the host cell wall, the tip of the hypha enlarged, producing a small hyphal peg that penetrated the cell wall and then enlarged as it entered the adjacent cell (Fig. 8).

The type-4 susceptible reaction was characterized by necrosis of all petiole tissue (Fig. 9). Well-developed acervuli with conidiophores, conidia, and setae were observed at this stage of disease development (Figs. 9 and 10). Colonization of the vascular bundle was almost complete. The thick-walled fibers, phloem, and cambium as well as the parenchyma cells had collapsed. The xylem vessel elements were filled with intracellular hyphae, but there was no breakdown of xylem vessel walls (Fig. 11).

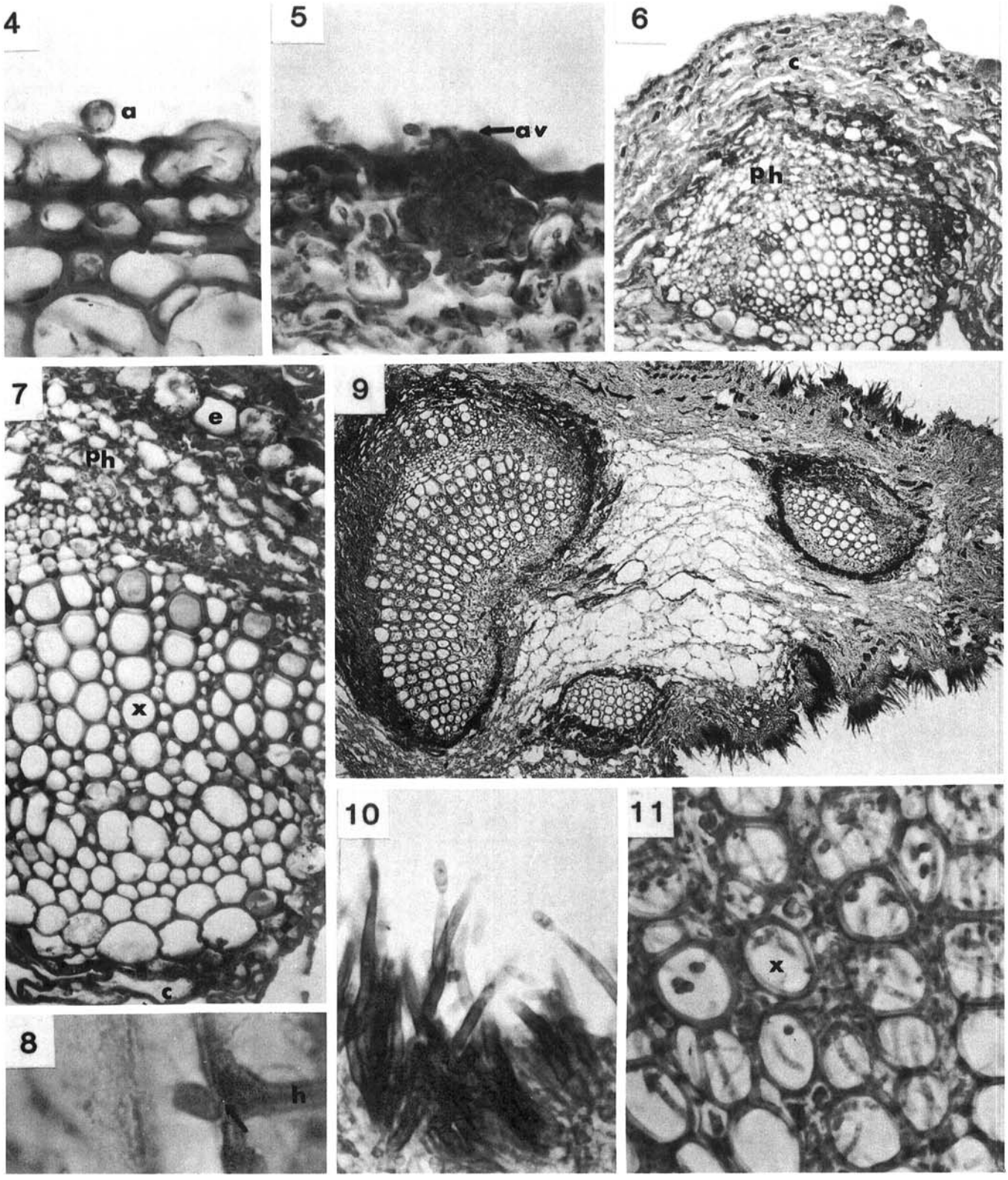
The fungus had progressed into the crown 14 days after inoculation. Transverse sections of infected crowns indicated that the vascular tissue and pith parenchyma were the most severely



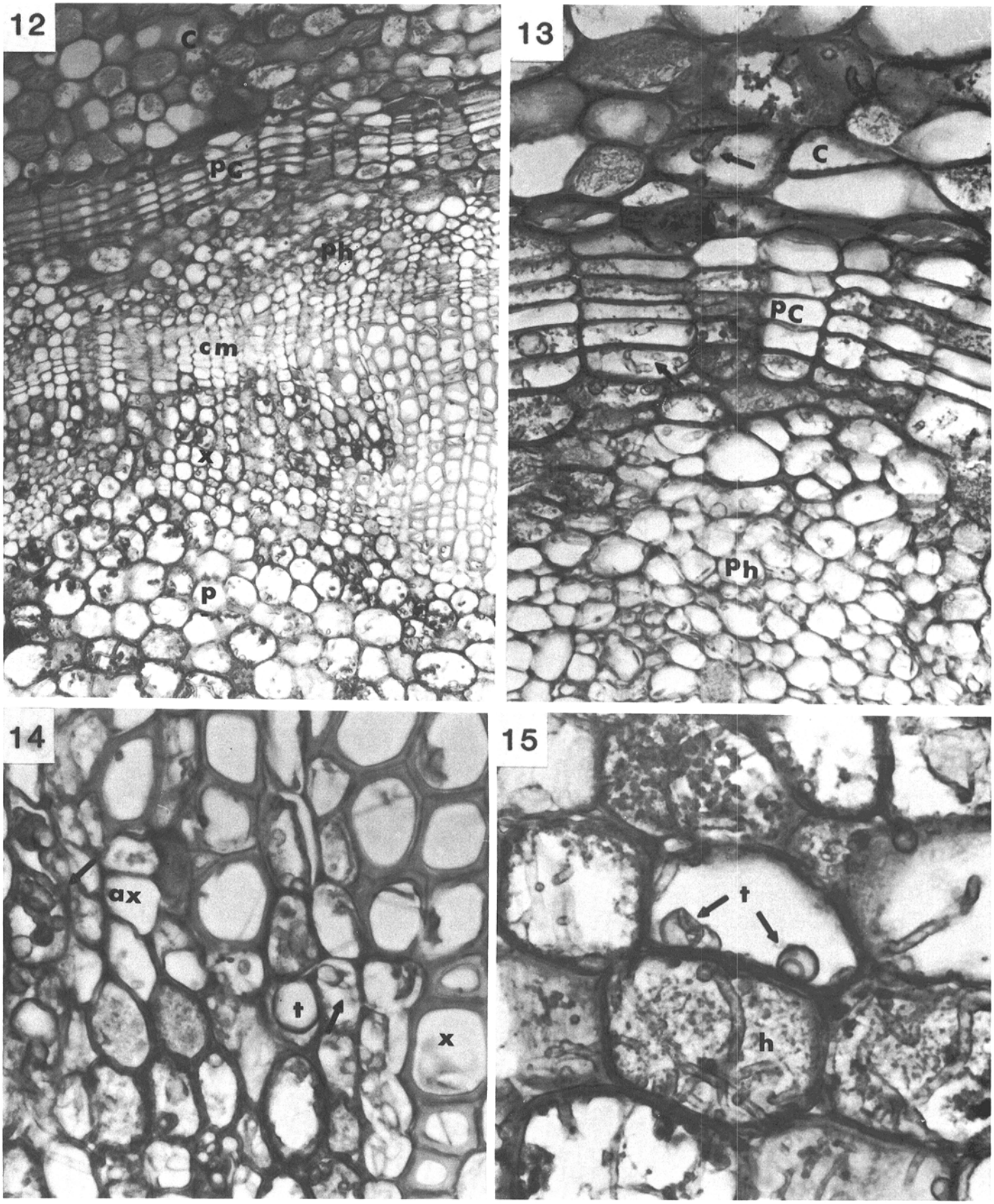
Figs. 1–3. Uninoculated stem tissues of strawberry. Transverse sections of **1,** runner (×100); **2,** petiole (×100); and **3,** crown (×100). c = cortex; e = endodermoid layer; bs = bundle sheath; cm = cambium; pc = pericycle; ph = phloem; p = pith; and x = xylem.

infected (Fig. 12). Hyphae were also observed in the cortex and pericycle (Fig. 13). The xylem was disorganized with some vessel elements developing abnormally (Fig. 14). The walls of the vessels were thinner, resulting in abnormal-shaped cells. Some tyloses were observed in the xylem tissue. Large amounts of granular substances as well as hyphae and tyloses were found in the infected pith parenchyma (Fig. 15). At least half of the tissue in the crown was severely infected and necrotic in the type-5 reaction after 21 days.

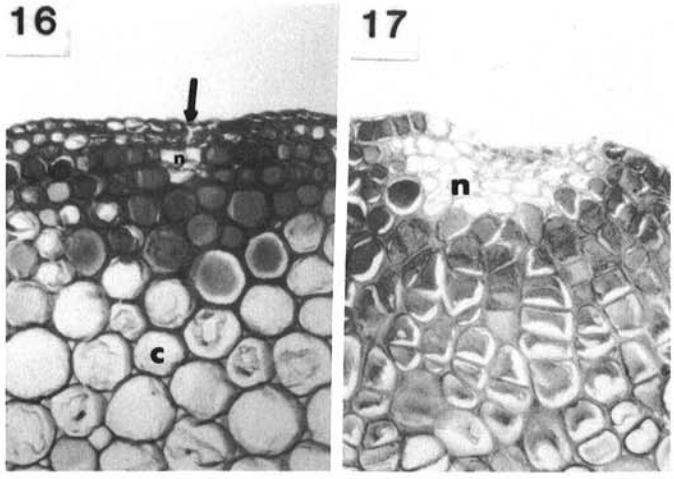
Resistant reaction. The initial stage of infection occurred in the

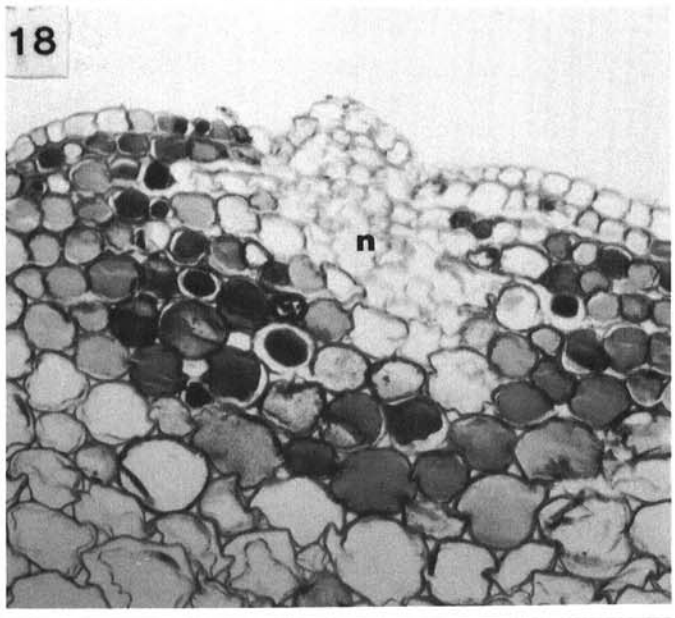


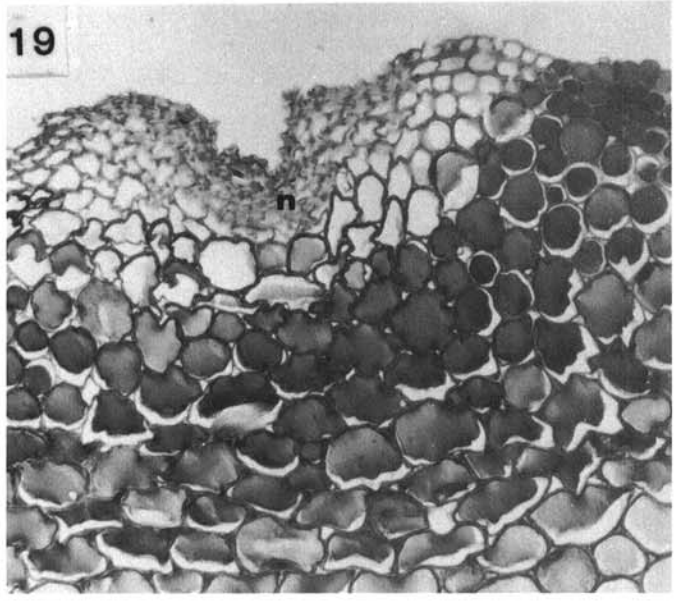
Figs. 4–11. Transverse sections through the petioles of strawberry plants (cultivar Surecrop) infected with *Colletotrichum fragariae*. **4,** Appressorium (a) and infection 24 hr after inoculation (×400). **5,** Acervulus (av) development after 72 hr (×625). **6,** Collapsing of cell walls in the cortex (c) and early stage of infection in a vascular bundle. ph = phloem (×150). **7,** Vascular bundle showing infected phloem (ph) and endodermoid cells (e). c = cortex, x = xylem (×400). **8,** Direct penetration of hypha (h) through a cell wall (arrow) (×2,000). **9,** Complete necrosis of cortex and vascular bundles 7 days after inoculation. Note numerous acervuli (×100). **10,** Acervulus with setae, conidiophore, and conidia (×1,000). **11,** Xylem (x) vessels with intracellular hyphae (×1,000).



Figs. 12–15. Transverse sections through the crown of strawberry plants (cultivar Surecrop) infected with *Colletotrichum fragariae*. **12,** Portion of infected crown showing cortex (c), pericycle (pc), phloem (ph), cambium (cm), xylem (x), and pith (p) (×110). **13,** Hyphae in cortex (c), pericycle (pc), and phloem (ph) (arrows) (×440). **14,** Abnormal xylem (ax), tyloses (t), and intracellular hyphae (arrows) in xylem element (×1,100). **15,** Infected pith showing tyloses (t) and hyphae (h) (×1,100).







Figs. 16–19. Transverse sections through petioles of strawberry plants (cultivar Apollo) infected with *Colletotrichum fragariae*. **16,** Resistant reaction (type-1) 7 days after inoculation. Site of infection (arrow) (×100). **17,** Type-1 reaction 14 days after inoculation. n = necrotic cells (×150). **18,** Resistant reaction (type-2) 7 days after inoculation. Note tannin-filled cells surrounding necrotic area (n) (×150). **19,** Type-2 reaction 14 days after inoculation. Note sunken area in necrotic cortex (n) (×150).

same manner as described in the susceptible tissue. However, in the type-1 reaction on Apollo, the progress of the fungus was arrested almost immediately by a host-pathogen reaction, which brought about a thickening of the cell walls in the cortex and deposition of material filling the intercellular spaces (Fig. 16). The contents of the cells surrounding the infection site developed an abnormal staining reaction. The hyphae were confined to the epidermis and one or two parenchyma cells beneath the site of infection 7 days after inoculation. There was a slight enlargement of the lesion after 14 days, and the fungus had penetrated two or three layers of cells causing a collapsing of cell walls (Fig. 17). The cells surrounding the infection site had developed a more intense staining reaction due to a heavier deposition of cellular material. Some 100 parenchyma cells measuring 100 μm in width and 50 μm deep showed the staining reaction.

The type-2 reaction was similar to the type-1 response, except the affected area was much larger, and ingress by the fungus was slightly more extensive (Fig. 18). The fungus was still confined to five or six layers of cells beneath the site of infection. Collapse of the infected cells often resulted in a small sunken area in the center of the lesion (Fig. 19). The number of dark-staining parenchyma cells surrounding the necrotic area had increased substantially, measuring 1,000 μm wide. There was little or no increase in lesion size 21 days after inoculation. No sporulation was observed on tissue showing the type-1 or type-2 reactions.

Histochemical tests to determine the nature of the material deposited in the intercellular spaces of resistant tissue were negative for lignin and positive for pectin. Since the fungus initially grows intercellularly in the cortex after penetration, it appears that cell wall thickening and deposition of pectic material in the intercellular spaces are important factors in restricting the fungus to a few cells beneath the site of infection. Many of the dark-staining cells surrounding the site of infection reacted positively for tannins. The importance of the accumulation of phenolic compounds (tannins) in the surrounding cells of type-1 and type-2 reactions was not determined. This increase in metabolic activity in resistant tissue infected with *C. fragariae* also could be an important defense mechanism; however, additional studies are needed to determine the biochemical basis for the differences between the disease reactions of resistant and susceptible plants.

The infection process, conidia germination, formation of appressoria, penetration, and establishment of *C. fragariae* occurs rapidly in strawberry petioles. In both resistant and susceptible tissues, the fungus penetrates the epidermis and invades the cortex within 12–24 hr. Although resistance to *C. fragariae* in cultivars such as Sequoia and Apollo can be overcome by many factors including high temperatures, extended wet periods, and high inoculum densities (2), under controlled inoculation and incubation conditions the fungus was restricted to a few cells in the cortex shortly after penetration and further disease development was retarded in the resistant cultivars. In susceptible tissue, the fungus is able to colonize the cortex and vascular tissue of the petioles and subsequently to invade the crown.

Resistance of the strawberry plant to the anthracnose fungus is related to fungal development after infection rather than the establishment of infection. All inoculated plants were infected. The type of lesion that develops after infection, rather than the number of infection sites, is significant in determining resistance or susceptibility.

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