Presymptom Histopathology of Peach Trees Inoculated with *Botryosphaeria obtusa* and *B. dothidea*

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ABSTRACT

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Peach trees were wounded mechanically in April and July and inoculated immediately with spore suspensions of the fungal gummosis pathogens, *Botryosphaeria obtusa* and *B. dothidea*. Samples for histopathological study were taken at 7, 14, 28, and 56 days after inoculation, before the onset of macroscopic symptoms. At 7 and 14 days, there were no differences in lignin and suberin deposition or periderm and callus formation between uninoculated wounds and wounds inoculated with either fungus. Fungal hyphae were visible on the inoculated wound surface of xylem and bark and were colonizing the outer layers of necrotic tissue delimited by the wound reaction. By day 28, new callus tissue was being initially colonized by fungal hyphae located on the xylem surface beneath the nonsuberized ventral callus surface. The nonsuberized ventral region of callus was primarily parenchymatous without a ligno-suberized outer layer and was the focal point for fungal pathogenesis. By day 56, the breakdown of this tissue was directly associated with fungal hyphae in close ventral proximity. Direct fungal penetration, although occasionally observed, was not required to induce gum pocket formation, cambial alteration, and tissue breakdown. The formation of gum pockets was followed by the sloughing of the parenchymatous portion of callus external to callus xylem tissue as the gum pockets expanded. Sloughed tissues usually were colonized by fungal hyphae. Both fungi appeared to incite disease in a similar manner. There were no differences in the types of cells colonized or the extent to which various tissues were colonized, although the frequency of positive isolation of inoculated fungi was higher in April and May than in July and August. Plants wounded in July had a thicker phellem layer in the new periderm compared with those wounded in April, independent of whether or not the wounds were inoculated with *Botryosphaeria*.

Peach (*Prunus persica* (L.) Batsch.) tree fungal gummosis was first observed in central Georgia in 1970 (15). Surveys in central Georgia from 1980 to 1984 showed that three species of *Botryosphaeria* were present in most gummosis cankers (5). Wound inoculations on symptomless trees with *B. obtusa* (Schw.) Shoem. and *B. rhodina* (Berk. & Curt.) Arx produced symptoms identical to those caused by *B. dothidea* (Moug. ex Fr.) Ces. & deNot. (4). The major infection court for all three species is pruning wounds (6), although *B. dothidea* also can penetrate lenticels (12,16). Penetration of lenticels by *B. obtusa* and *B. rhodina* has not been observed, but these species invade lesions caused by *B. dothidea*. Isolation of *B. obtusa* and *B. dothidea* from naturally infected tissue was more common than *B. rhodina*. *B. dothidea* was most active in the summer, whereas *B. obtusa* predominated in cankers in the winter and spring (5).

The pathological anatomy of *Botryosphaeria* infections on peach has not been extensively investigated. Preliminary observations suggested that hyphae colonizing the stem cortex were delimited repeatedly by consecutive layers of new phellogen. The flaking layers of phellem, bound together by mycelium and gum deposits, imparted to older cankers a characteristically swollen and crusty appearance. Hyphae occasionally penetrated xylem vessel elements, depending on the type of wound invaded, but systemic movement usually was accomplished by colonization of the cortex, phloem, and vascular cambium (K. O. Britton, *unpublished*). Gummosis cankers usually lacked definite margins, and fungi could be isolated up to 30 cm beyond visible symptoms (5). No information exists on the histopathology of the initial (presymptom) phase of the host/pathogen interaction.

The objective of this investigation was to elucidate the presymptom pathological anatomy and host responses of peach stems inoculated with spore suspensions of the peach tree fungal gummosis pathogens, *B. obtusa* and *B. dothidea*.

MATERIALS AND METHODS

Sixty 1-yr-old cultivar Blake peach trees on Lovell rootstock were planted at a 1.0- × 3.0-m spacing in March 1985 at the University of Georgia Horticulture Farm at Watkinsville. On 25 July 1985, a 1-cm-diameter branch from each tree was mechanically wounded by removing a portion of bark down to the xylem with a 4-mm-diameter cork borer. Wounds on two sets of 20 trees were inoculated with 10 μl of conidial suspension (10^4 conidia/ml) of either *B. obtusa* or *B. dothidea*. The third set of 20 wounds was inoculated with sterile water. Wounds were wrapped in Parafilm, which was removed at the time of sampling. The above procedure was repeated on a different branch on each of the same 60 trees on 2 April 1986.

Branches from each inoculated and uninoculated treatment were selected randomly and removed from each of five replicate trees at 7, 14, 28, and 56 days after inoculation. A 4-cm branch piece with the wound in the center was divided into four 1-cm transverse segments (the two segments in the middle each supported one half of the wound). Each segment was halved along the longitudinal axis centered through the wound. One half of each segment was surface-disinfested in 10% ethanol-0.5% sodium hypochlorite solution and placed on acidified potato-dextrose agar in 9-cm-diameter petri dishes. Segments were incubated for 2 wk under fluorescent lights at room temperature to determine the presence of *Botryosphaeria* spp. Data were collected on the number of segments colonized per treatment for the two experiments. Data on percentage of segments with *Botryosphaeria*, as determined by isolation, were transformed to the arcsin $\sqrt{\text{percentage}}$ (14) and analyzed as a split plot design. The second half of each segment was fixed in Formalin-acetic acid-50% ethanol (10:10:100, v/v/v) for histological study.

Fixed tissue was dehydrated in *t*-butyl alcohol and embedded in paraplast (1). Tissues were sectioned at 8 μm thickness with a rotary microtome and were mounted on chemically cleaned glass slides with Haupt's adhesive (10). For anatomical observation, sections were cleared of paraffin with xylene and stained with Sam's polychrome stain (13) modified for peach tissue (A. R. Biggs, *unpublished*).

Histochemical tests were conducted to determine the location of lignin and suberin in inoculated and uninoculated tissues. Phloroglucinol (saturated in 18% HCl) (9) and the Maule reaction

TABLE 1. Results of two experiments showing percentage of branch segments yielding colonies of *Botryosphaeria* on acidified potato-dextrose agar following mechanical wounding, inoculation with spores, and sampling over time[a]

Days postinoculation	Uninoculated				*B. obtusa*				*B. dothidea*			
	P2	P1	D1	D2	P2	P1	D1	D2	P2	P1	D1	D2
July/August 1985												
7	0	20	20	40	40	80	60	40	0	100	100	0
14	0	0	0	20	20	40	20	0	0	20	80	20
28	0	0	20	0	0	20	20	0	0	20	40	0
56	0	0	0	0	25	67	100	33	0	60	100	40
April/May 1986												
7	0	0	0	0	0	100	100	0	0	100	80	0
14	20	0	0	20	20	80	100	0	0	100	100	20
28	0	20	0	0	0	100	100	0	0	100	100	40
56	0	0	0	20	0	100	100	20	40	100	100	60

[a] P2, P1, D1, and D2 indicate position of isolation relative to the wound, i.e., P2 = 2 cm proximal to wound, D2 = 2 cm distal to wound, etc. Each value represents the percent isolation from five replicate trees except for the 1985 experiment, where $n = 4$ for 56-day uninoculated P2, P1, and D1, 56-day *B. obtusa* P2 and D1, and $n = 3$ for 56-day *B. obtusa* P1 and D2. Isolations from inoculated treatments were recorded as positive only if the inoculated species was recovered.

TABLE 2. Main effect means and mean separations from analyses of variance for two inoculation experiments on percentage of branch segments yielding *Botryosphaeria* colonies

Variable	Incidence of *Botryosphaeria*[z]	
	July/August 1985	April/May 1986
Treatment		
Uninoculated	2.5 a	1.3 a
B. obtrusa	31.7 b	52.9 b
B. dothidea	31.1 b	63.9 b
Tissue location		
2-cm proximal	1.9 a	1.8 a
1-cm proximal	30.8 b	75.0 b
1-cm distal	48.5 b	71.6 b
2-cm distal	8.6 a	7.9 a
Days postinoculation		
7	39.2 b	21.7 a
14	11.3 a	35.6 ab
28	4.4 a	33.7 ab
56	28.9 b	44.6 b

[z] Means followed by the same letter are not significantly different according to Duncan's multiple range test at $P \leq 0.05$. Means are based on 80 observations for each treatment, and 60 observations for each tissue location and postinoculation time.

(10) were used to detect lignin. In addition, slides were cleared of paraffin, mounted in 90% glycerol, and examined for cell wall autofluorescence with Leitz filter block H2 (390–490 nm excitation and 515 nm barrier filters). Slides treated with phloroglucinol were examined under ultraviolet epi-illumination (Leitz filter block A, 340–380 nm excitation and 430 nm barrier filters) to detect cells with suberin lamellae (2,3).

RESULTS

Isolation of fungi. Fungal recovery from branch segments in July/August decreased with time postinoculation and increased with plants inoculated in April/May (Table 1). In July/August, the frequency of fungal isolation (both species combined) was about 40–80% lower at 14 and 28 days postinoculation relative to 7 and 56 days. Analysis of variance of isolation frequency indicated a significant first-order interaction of tissue location relative to the inoculation point and treatment due to significant differences between the control and the inoculated treatments (Table 2). Fungal isolations in both experiments were recorded with greater frequency from the tissue at the inoculated site than from sites more distant. Isolation frequencies from the inoculated sites were greater in April/May than in July/August. Within each experiment, both fungi were isolated with similar frequencies. *B. obtusa* tended to extend further proximally than *B. dothidea* when inoculated in July/August, whereas *B. dothidea* tended to be recovered more frequently than *B. obtusa* from distal tissues when inoculated in April/May. Only five of 160 isolations from uninoculated wounds yielded a *Botrysophaeria* species.

Histopathology. The response of the host to inoculation and the pathological anatomy of tissue colonization were similar for both fungi. The histopathological events were derived from observations on tissues inoculated in April because of the higher percentages of fungal isolation relative to the July/August experiment (Table 1). Results from the latter experiment will be presented only where there was considerable variation from the April/May results.

Uninoculated wounds. The appearance of a fresh wound (0 days postwounding) is shown in longitudinal orientation before inoculation (Fig. 1). Uninoculated trees in transverse (Figs. 2 and 3) and longitudinal section (Fig. 4) exhibited growth of new callus tissue approximately 200–300 μm at the wound margin 7 days after wounding. Cell division was visible in the region of the callus and in the bark tissues up to the outer cortex. Suberized boundary tissue was present in the cortex and was joined to the outer healthy bark in four of five samples. Two of five trees exhibited new periderm in contact with the original injured periderm.

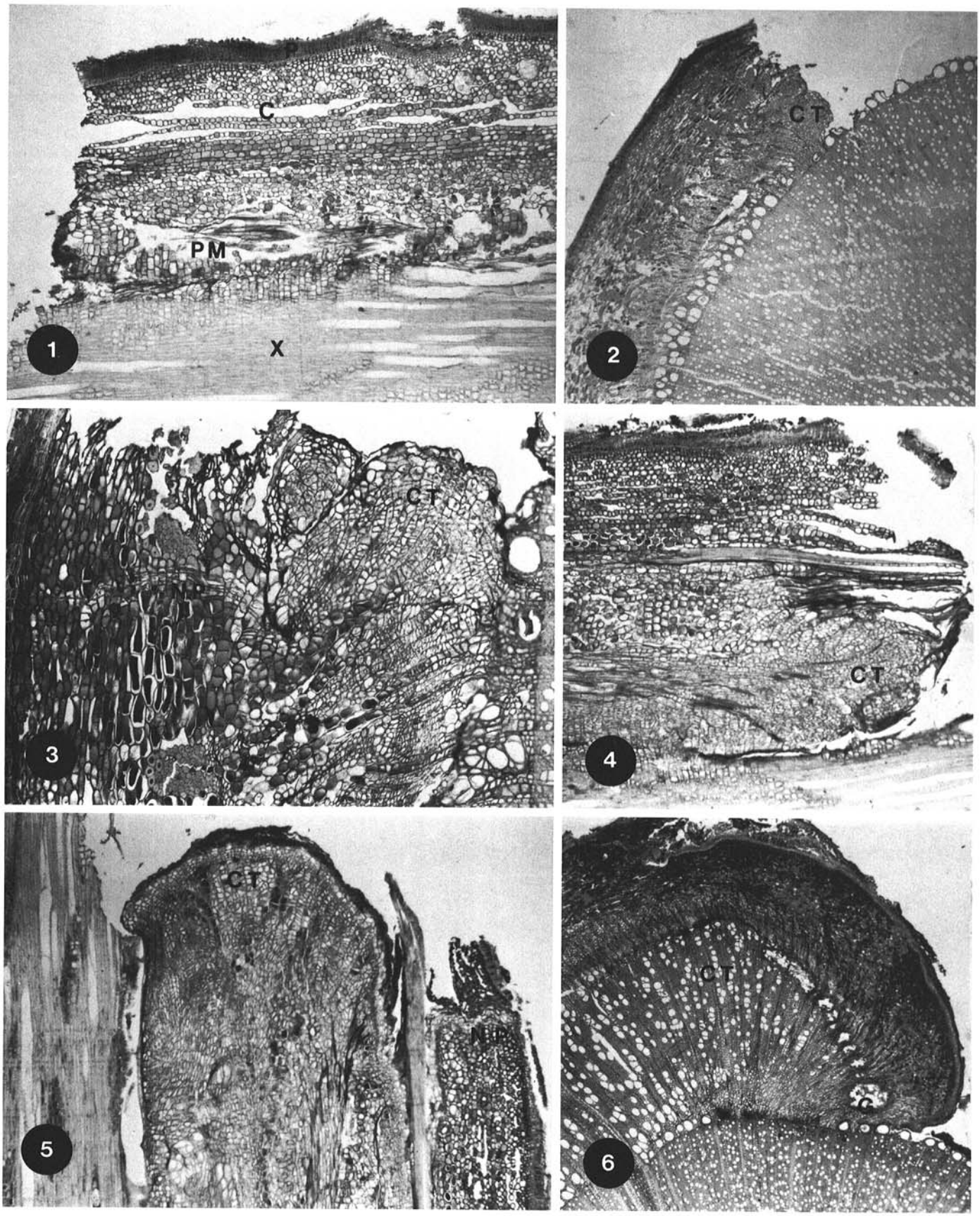
At 14 days postwounding, callus tissue had initiated growth across the wound surface (Fig. 5). The noninjured vascular cambium internal to the wound had produced abnormal cells with thickened transverse walls. The new periderm was in contact with the original periderm and possessed a layer of three to five suberized phellem cells.

By 28 days postwounding, new callus tissues were continuing to close over the wound (Fig. 6). Callus was about 2 mm deep and differentiated into xylem, phloem, cortex, and periderm. The new periderm had a layer of six to eight suberized phellem cells. Suberized cells were visible for the first time at the point where callus tissue joined extant xylem, although there was still an absence of ligno-suberized tissue across most of the ventral surface of the callus. Gum ducts were present in two of five samples.

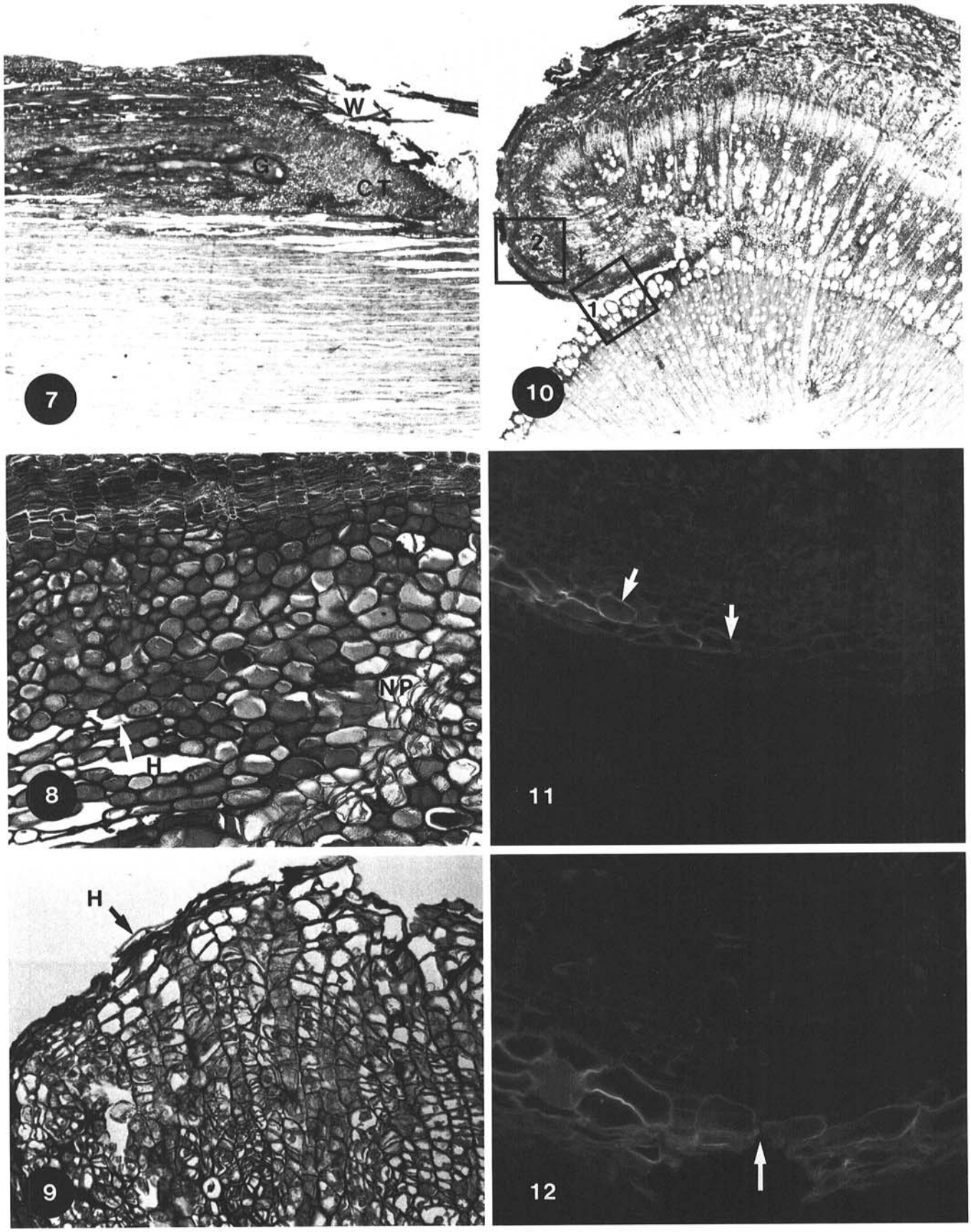
By 56 days postwounding, a considerable amount of wound xylem had been produced that appeared distinctly separated from 1985 xylem by a lignified parenchyma (sclerenchyma). One sample exhibited two gum pockets in the new callus.

B. obtusa. At 7 days postinoculation, approximately 200–300 m of callus tissue had been produced as described for uninoculated plants. Gum ducts were present only in tissues immediately proximal or distal to the wound (Fig. 7). Parenchyma cells in the primary phloem region had differentiated to form new periderm (Fig. 8). Cells in the outer cortex and on the callus surface appeared lignified but not suberized. Fungal hyphae were observed in the outer layers of wounded cortex (Fig. 8) and on the callus surface (Fig. 9).

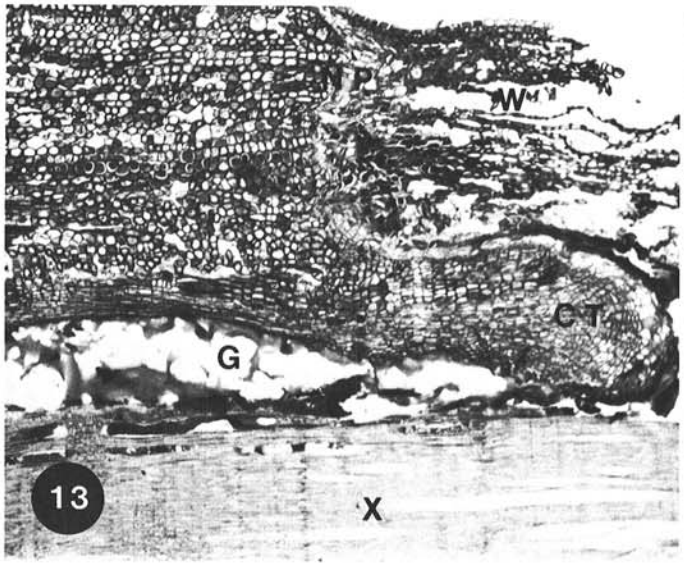
By 14 days postinoculation, the trees had produced about 400–600 μm of new callus tissue (Fig. 10). Hyphae were visible on the callus surface in 1986 xylem and in bark that had been delimited by a new periderm. The new periderm was in contact with the original periderm and possessed a layer of three to five

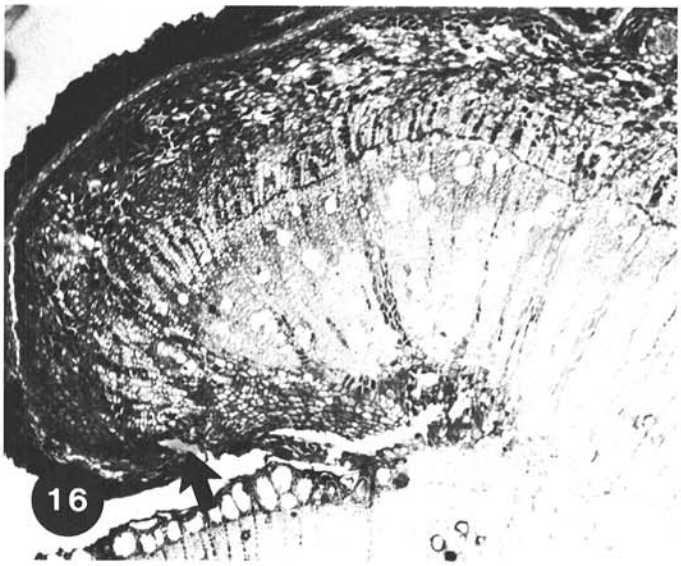


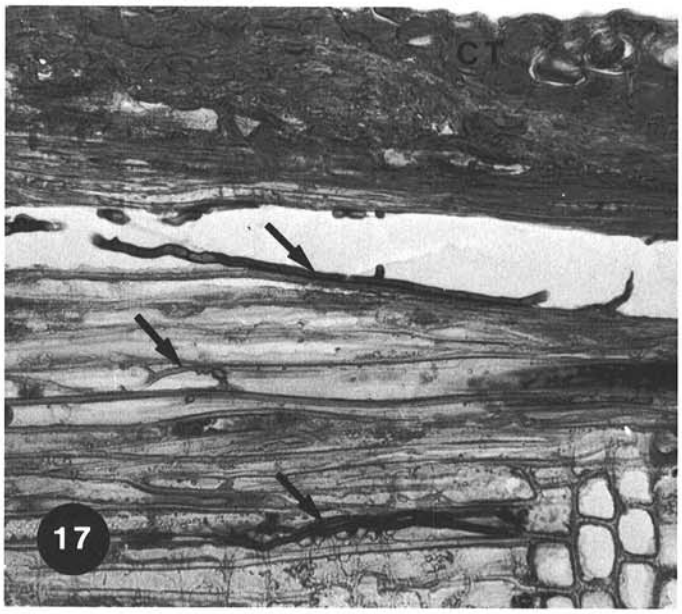
Figs. 1–6. Photomicrographs of wounded, uninoculated 1-yr-old peach shoots. **1,** Longitudinal section of a fresh wound (to the left) showing periderm (P), cortex (C), phloem (PM), and xylem (X). (160×) **2,** Transverse section of bark and xylem at 7 days postwounding showing location of newly formed callus tissue (CT) at the wound margin. (80×) **3,** Higher magnification of Fig. 2 showing callus tissue (CT) and location of new periderm in outer bark (NP). (256×) **4,** Longitudinal section of bark at 7 days postwounding showing callus tissue (CT). Note that new periderm has not been formed when compared to 3. (200×) **5,** Longitudinal section of bark and xylem at 14 days postwounding with wound at the top showing new callus (CT) and periderm (NP). (160×) **6,** Transverse section of bark and xylem at 28 days postwounding showing callus tissue (CT) with gum ducts (G). (64×)

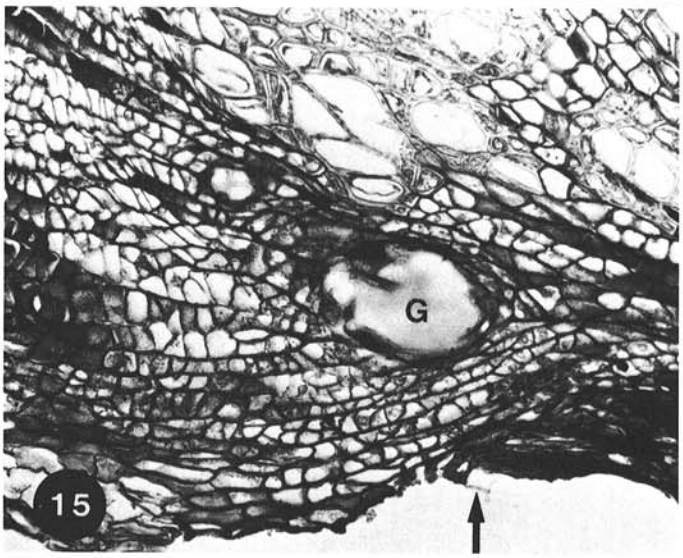


Figs. 7–12. Photomicrographs of peach tissue wounded and inoculated with spores of *Botryosphaeria obtusa*. **7,** Longitudinal section of xylem and wounded bark (W) 7 days postinoculation. Note the gum duct (G) and callus tissue (CT) produced postwounding. (160×) **8,** Transverse section from the cortex and periderm showing cell division and new periderm (NP) produced postinoculation. Note the presence of fungal hyphae (H) in cortical tissue external to the new periderm. (640×) **9,** Transverse section of a portion of callus tissue as it appeared 7 days postinoculation. Note hyphae (arrows) on the callus surface. (640×) **10,** Transverse section of callus tissue, bark, and xylem sampled 14 days postinoculation. Box 1 and box 2 correspond to Figs. 11 and 12, respectively. **11,** Box 1 from Fig. 10. Tissue was treated with phloroglucinol/HCl and examined under fluorescence to detect suberin lamellae (arrows). Note how ventral surface of the callus is nonsuberized. (800×) **12,** Box 2 from Fig. 10. Tissue was treated as described for Fig. 11. Note the gaps in the suberized layer on the callus surface (arrow) associated with cracks probably related to rapid callus proliferation. (800×)

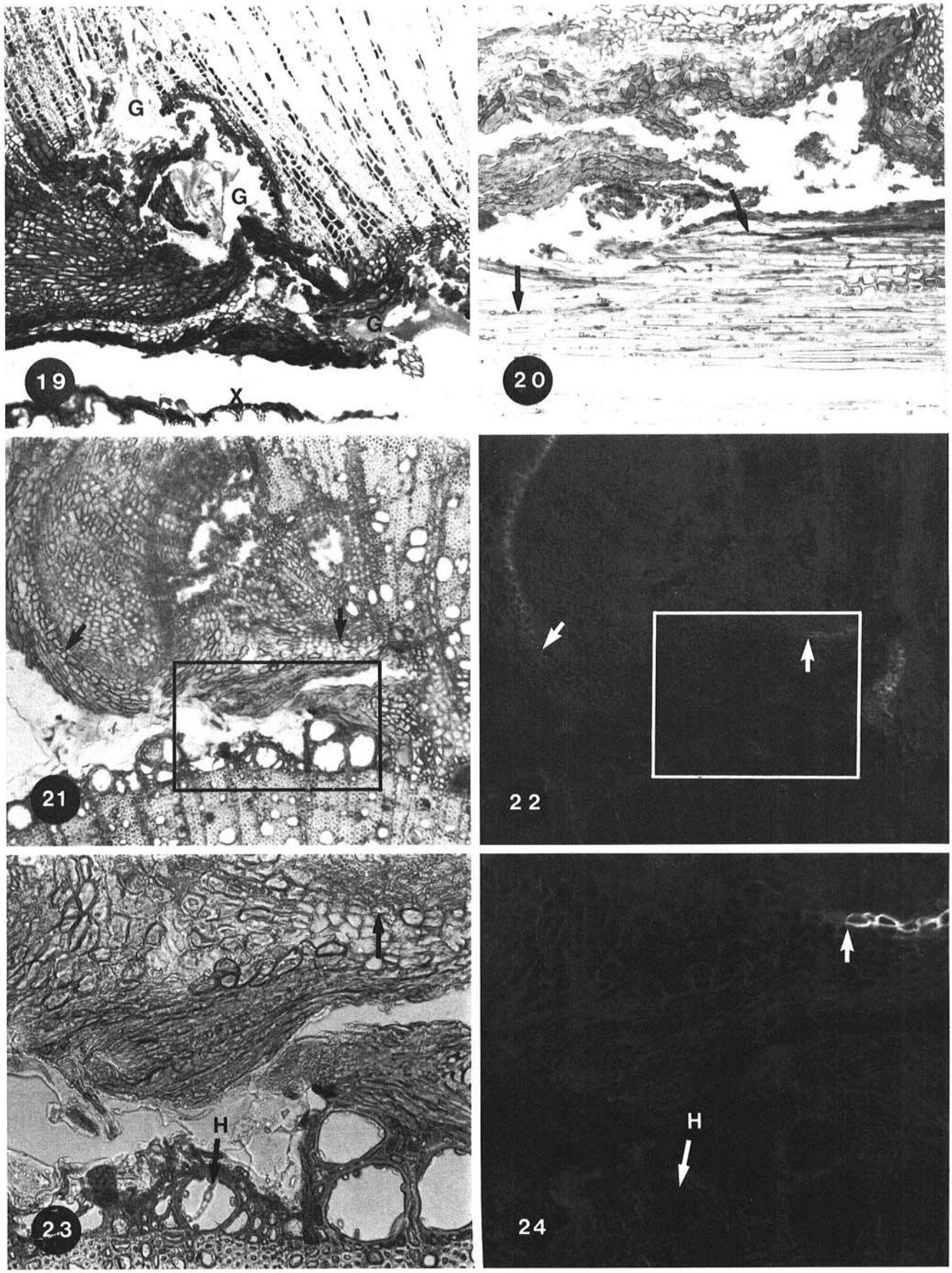




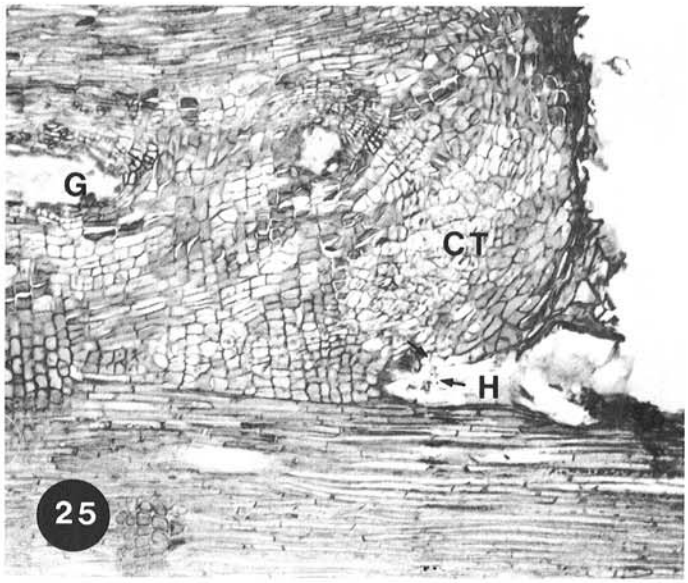


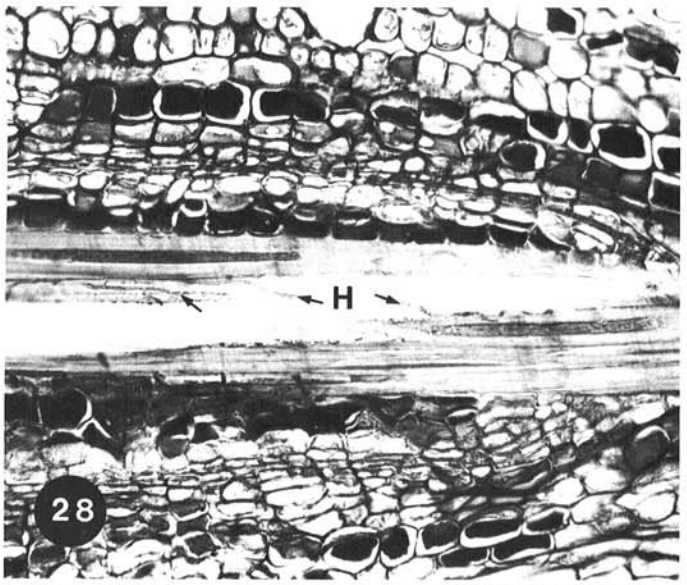


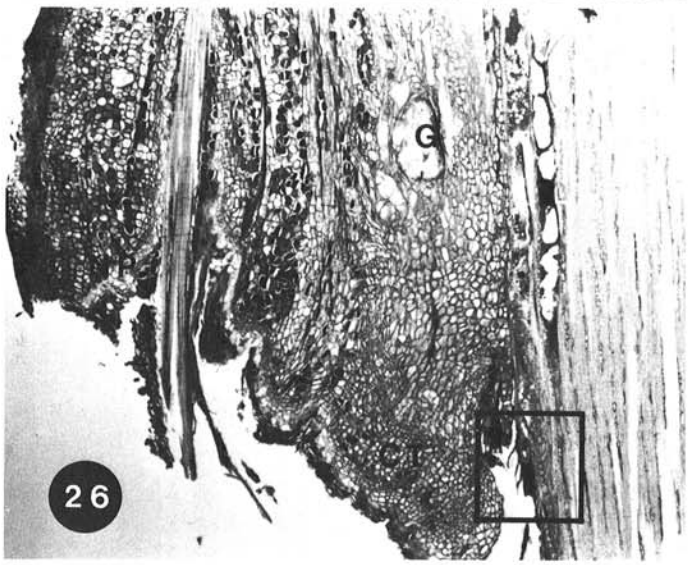
Figs. 13–18. Photomicrographs of peach tissue wounded and inoculated with spores of *Botryosphaeria obtusa*. **13,** Longitudinal section of xylem and wounded bark (W) 14 days postinoculation showing callus tissue (CT), gum duct (G), and new periderm (NP). (160×) **14,** Transverse section of the surface of callus tissue at 28 days postinoculation showing hyphal invasion (arrows) of the suberized outer layers. (640×) **15,** Transverse section of the ventral callus region showing the location of gum pockets (G) in parenchymatous tissue and association of these pockets with subjacent hyphae (arrows). (640×) **16,** Transverse section of inoculated bark showing xylem surface and callus tissue at 28 days postinoculation. Note the gum-filled crack on the ventral callus surface (arrow). (160×) **17,** Longitudinal section of xylem located immediately beneath callus tissue (CT). Note hyphae in xylem vessels and tracheids (arrows). (640×) **18,** Longitudinal section of wounded bark 56 days postinoculation showing pycnidium of *B. obtusa*. (256×)

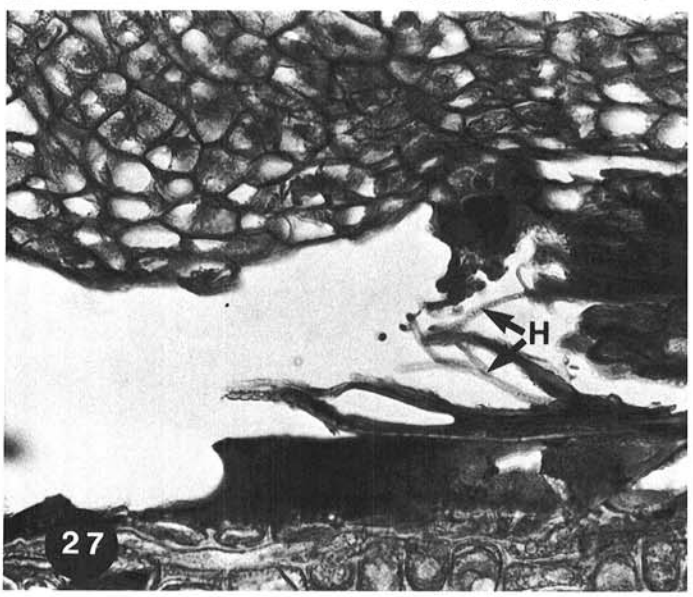


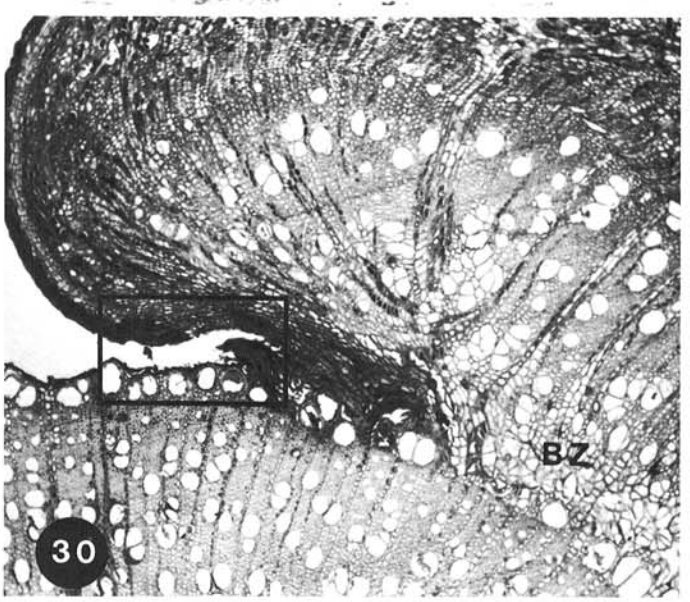
Figs. 19–24. Photomicrographs of peach tissue wounded and sampled 56 days after inoculation with spores of *Botryosphaeria obtusa*. **19,** Transverse section of the ventral callus region and surface of wounded xylem (X). Note the apparent breakdown of the parenchymatous ventral region and the occurrence of gum-filled cracks (G). (160×) **20,** Longitudinal section showing similar tissue to that in Fig. 19. Note the presence of fungal hyphae in xylem tissue (arrows). (160×) **21 and 22,** Bright field and epifluorescence micrographs, respectively, of phloroglucinol/HCl-treated callus tissue. Note the gum ducts, cracks, and limits of suberization (arrows) in the ventral region. (160×) **23 and 24,** Higher magnification of the boxed areas in Figs. 21 and 22. Note limits of suberization (arrows) and the presence of hyphae in subjacent xylem vessels (H, arrows). (640×)



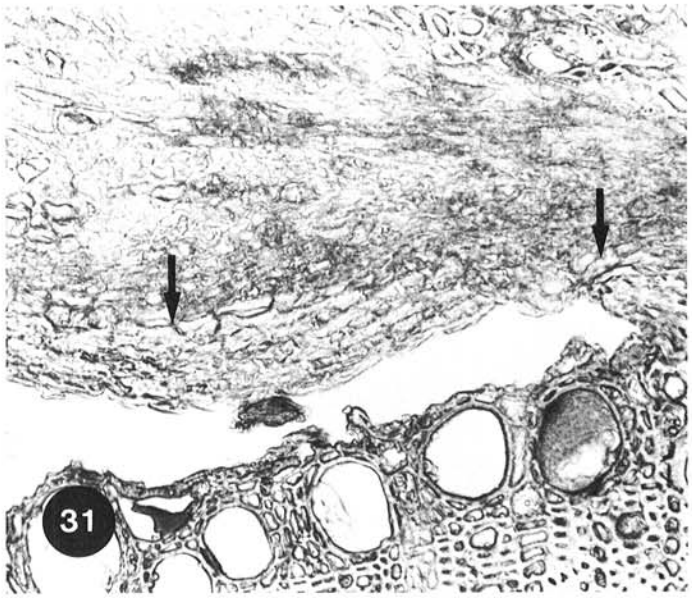


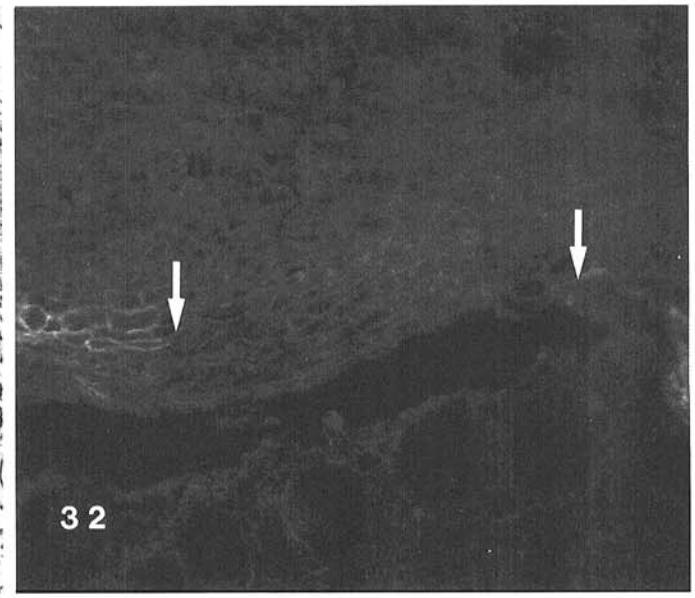


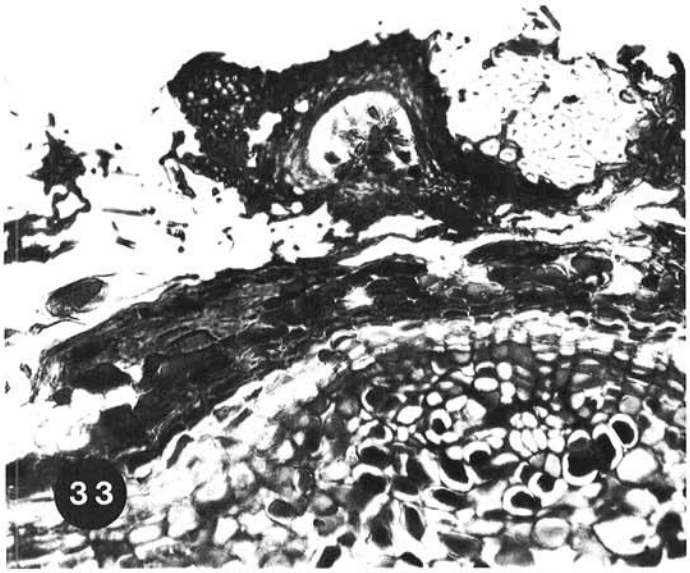


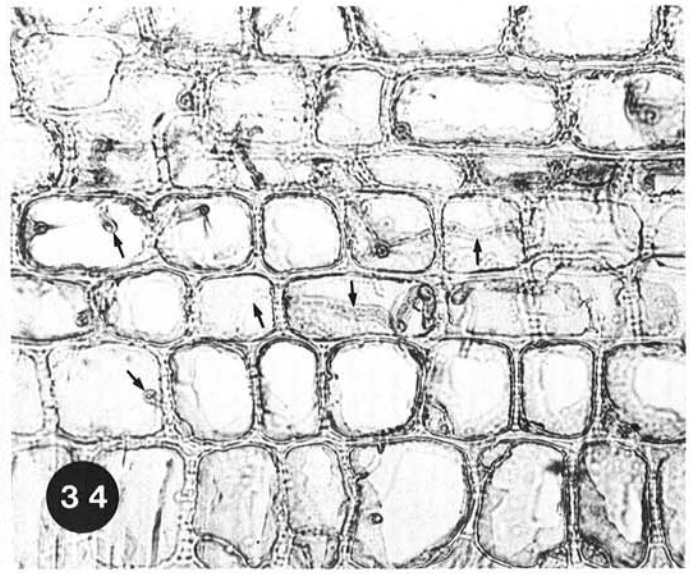


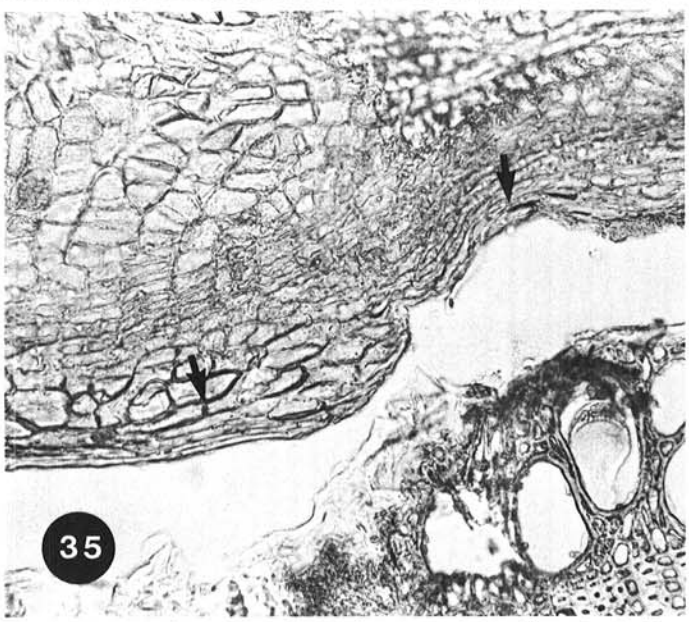
Figs. 25–30. Photomicrographs of peach tissue wounded and inoculated with spores of *Botryosphaeria dothidea*. **25,** Longitudinal section of callus tissue (CT) and xylem showing a gum duct (G) and fungal hyphae (H, arrows) located in the ventral region of new callus sampled 7 days postinoculation. (256×) **26,** Longitudinal section of an inoculated wound at 14 days showing callus tissue (CT), gum pockets (G), and new periderm (NP). (160×). **27,** Higher magnification of boxed area in Fig. 26 showing hyphae (H, arrows) colonizing the ventral region of callus and xylem surface. (1,024×) **28,** Higher magnification from Fig. 26 showing fungal hyphae colonizing the primary phloem fibers (H, arrows). (640×) **29,** Longitudinal section of callus and xylem surface 28 days postinoculation. Note the presence of gum pockets in the parenchymatous portion of callus. (256×) **30,** Transverse section of callus and xylem 28 days postinoculation. Note the barrier zone tissue (BZ) in xylem produced postinoculation. (160×)

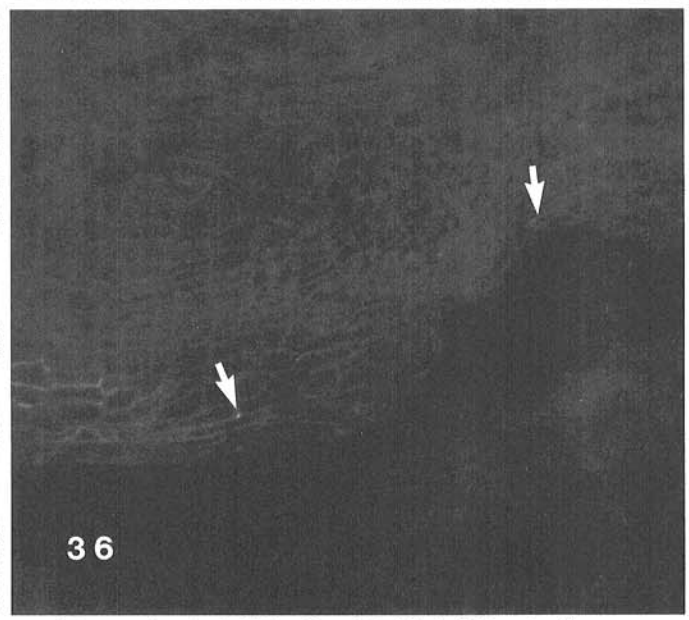












Figs. 31–36. Photomicrographs of peach tissue wounded and inoculated with spores of *Botryosphaeria dothidea*. **31 and 32,** Bright field and epifluorescence micrographs, respectively, of boxed area from Fig. 30. Note the limits of suberization on the ventral callus surface (arrows). (800×) **33,** Transverse section of inoculated bark at 28 days showing pycnidium of *B. dothidea*. (640×) **34,** Longitudinal section of xylem ray parenchyma at 56 days showing colonization by fungal hyphae (arrows). (1,000×) **35 and 36,** Transverse sections in bright field and epifluorescence showing lack of suberin (between arrows) on the ventral callus surface. (800×)

suberized phellem cells. Tissue on the ventral surface of the callus was nonsuberized (Fig. 11). Defects in the suberized layer were visible on the callus surface (Fig. 12). All samples exhibited gum ducts at the proximal and/or distal regions (Fig. 13). One sample had formed a gum duct or pocket at the lateral margin just internal to where the new callus joined the xylem present at the time of wounding.

Samples taken at 28 days postinoculation exhibited new periderm with a layer of four to six suberized phellem cells. Defects in the suberized callus surface observed on day 14 were colonized by fungal hyphae (Fig. 14). Host cells in the nonsuberized area on the ventral surface of callus exhibited increased affinity for morphological stains. Callus at the lateral margin of all samples possessed pockets of gum or gum-filled cracks in or near the parenchymatous portion of callus internal to the nonsuberized region (Figs. 15 and 16). Fungal hyphae were often located on the surface of the nonsuberized area (Fig. 15). Three of five trees showed the presence of fungus mycelium under the callus and located adjacent to the nonsuberized area. Fungal hyphae were on the wound surface and had penetrated about 200 μm in vessels, fibers, and rays (Fig. 17).

New periderm in the outer bark of inoculated trees continued to develop, exhibiting a layer of eight to 12 suberized phellem cells when examined after 56 days. Pycnidia were observed on the wound surface at this time (Fig. 18). In four of five trees, callus tissues located internal to the nonsuberized ventral region exhibited gum-filled cracks and gum pockets and/or ducts (Fig. 19) associated with fungal hyphae located subjacently (Fig. 20). The outer external callus region, and the tissue located where the xylem produced postwounding joined with extant xylem, were both heavily suberized (Figs. 21 and 22). The ventral callus surface between these regions, although partially lignified, did not become suberized (Figs. 23 and 24). Hyphae were in cracks in the nonsuberized area under the callus, in dead bark, and in vessels, fibers, and rays of extant xylem.

B. dothidea. In tissues inoculated with *B. dothidea*, callus was produced within 7 days as described for mechanically wounded uninoculated trees and trees inoculated with *B. obtusa*. The surface of this new callus was also nonsuberized. Boundary tissue and some cell division was present in the cortex, but new tissues were not in contact with the original periderm. Two of five trees exhibited bark necrosis caused by fungal colonization of the cortex. New boundary tissue and periderm were formed internal to the necrotic region. Gum ducts or pockets were observed at the proximal and distal margins of the wound (Fig. 25). Hyphae were observed on the surface of wounded xylem, in vessels of 1986 xylem present at the time of inoculation, on the surface of wounded xylem but underneath new callus, and on the outer surface of new callus (Fig. 25).

By 14 days postinoculation, the trees had produced new callus extending 400–600 μm across the wound surface (Fig. 26). New periderm had formed completely in contact with the original periderm in three of five trees and was nearly complete in the other two. Gum ducts were observed in all tissues located at the proximal and distal wound margins (Fig. 26), but none was visible at the lateral margins. The ventral callus surface was nonlignified and nonsuberized. Fungal hyphae were visible in dead tissue external to the new callus, underneath new callus subjacent to the non-ligno-suberized region (Fig. 27), and in colonized bark tissues, including the primary phloem fibers (Fig. 28).

At 28 days postinoculation, callus growth had extended 1.0–1.5 mm across the injured surface (Figs. 29 and 30). All samples exhibited a new periderm in complete contact with the original periderm. Hyphae were abundant underneath the callus, on the surface of the callus, in bark tissues external to the new periderm, and in the parenchyma, vessels, and fibers of 1986 and 1985 xylem present at the time of inoculation. Callus tissue in all five samples was lignified and nonsuberized on its centripetal surface (Figs. 31 and 32). Two of five trees exhibited gum ducts or pockets that had formed within the parenchymatous outer layer of the new callus at the lateral wound margins. Pycnidia were observed on the callus surface at this time (Fig. 33).

By 56 days postinoculation, all five samples exhibited gum ducts or pockets in the parenchymatous portion of the callus. In addition, callus periderm appeared separated from other callus tissues. Separated periderm was colonized on the external surface by fungal hyphae. The parenchymatous ventral region of callus tissue, near the confluence of the periderm and vascular cambia, in all trees appeared to be undergoing a generalized breakdown or autolysis. Fungal hyphae were not usually directly associated with this phenomenon, although they were always present on the callus surface, in dead bark, in all types of xylem cells (Fig. 34), and underneath the nonsuberized portion of the callus (Figs. 35 and 36).

DISCUSSION

The present study characterized the presymptom histopathology of *Botryosphaeria* infections of peach stems. The critical feature of this host/pathogen interaction was the anatomical character of the callus tissue produced by the host in response to wounding. The ventral surface of callus in both uninoculated and inoculated plants remained nonsuberized and, thus, provided a site susceptible to fungal colonization in inoculated plants. This site served as the focal point for formation of gum pockets and ducts which, by enlarging and coalescing, contributed to the sloughing of tissues located external to them. In infections at wounds, complete wound closure was prevented even though a new suberized periderm, with up to 14–16 phellem cells in thickness, was formed.

Intracellular hyphae of both *Botryosphaeria* spp. were observed colonizing cortical parenchyma, callus parenchyma, and xylem ray parenchyma, vessels, and tracheids. Intercellular hyphae were observed in phloem fibers, necrotic cortical parenchyma external to new periderm, and necrotic ligno-suberized parenchyma on the callus surface. In studies on apple stems, Brown and Hendrix (7) observed *B. dothidea* hyphae in xylem and metaxylem of wounded trees. Mycelium was infrequently observed in xylem rays. This histopathology was thought to account for the rapid proximal and slow lateral development of cankers on apple (7). In contrast, Milholland (11) found that xylem rays were instrumental in the development of cankers on blueberry stems. Our results, which are in agreement with English et al (8), showed that hyphae of both fungi were equally advanced in all cell types at the lateral and tangential margins of infection. At the proximal or distal infection margins, hyphae were located in the lumens of vessels and tracheids and not in parenchyma.

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