Cytology and Histology

Histopathology of Blueberry Twig Blight Caused by *Phomopsis vaccinii*

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ABSTRACT

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Emerging flower buds or fully opened flower clusters of highbush blueberry plants were inoculated with a conidial suspension of *Phomopsis vaccinii*. Samples were taken 3 days and 1, 2, 3, 4, 6, 8, and 10 wk after inoculation for histopathological study. *P. vaccinii* penetrated succulent floral parts. Hypertrophy and hyperplasia occurred in response to infections on ovaries and floral axes, respectively, but did not prevent growth of the hyphae. Hyphae grew intercellularly through the cortical cells of the pedicel and into the intercellular spaces in the cortex of the floral axes by 1 wk after inoculation. Stems were colonized initially by hyphal growth through the intercellular spaces in the cortex. Numerous tyloses were formed in the vessel elements in advance of this growth. All tissues in the infected stems and floral axes subsequently were colonized by *P. vaccinii* leading to necrosis and collapse of parenchyma cells in the cortex and vascular tissue. Gums were deposited in the infected vessel elements. Stem lesions ceased expanding by 8 wk after inoculation, and a meristematic layer of suberized cells developed in the cortex between the infected and healthy portions of the stem. Pycnidia were present in necrotic stem tissue by 10 wk after inoculation.

Blueberry twig blight caused by *Phomopsis vaccinii* Shear is a serious disease of highbush blueberry (*Vaccinium corymbosum* L.) in the southern United States (2,6,8). Symptoms include a rapid blighting and dieback of the previous year's twigs in early spring. Infections usually begin on the expanding flower buds and flowers and progress downward through the twigs resulting in the loss of fruit. Blueberry fruits that escape this initial infection are susceptible to rot caused by *P. vaccinii* at harvest (7). In July, the twig lesions stop expanding and fungal colonization ceases. A distinct demarcation exists between the diseased and healthy areas of the twigs.

The purpose of this study was to examine histologically the

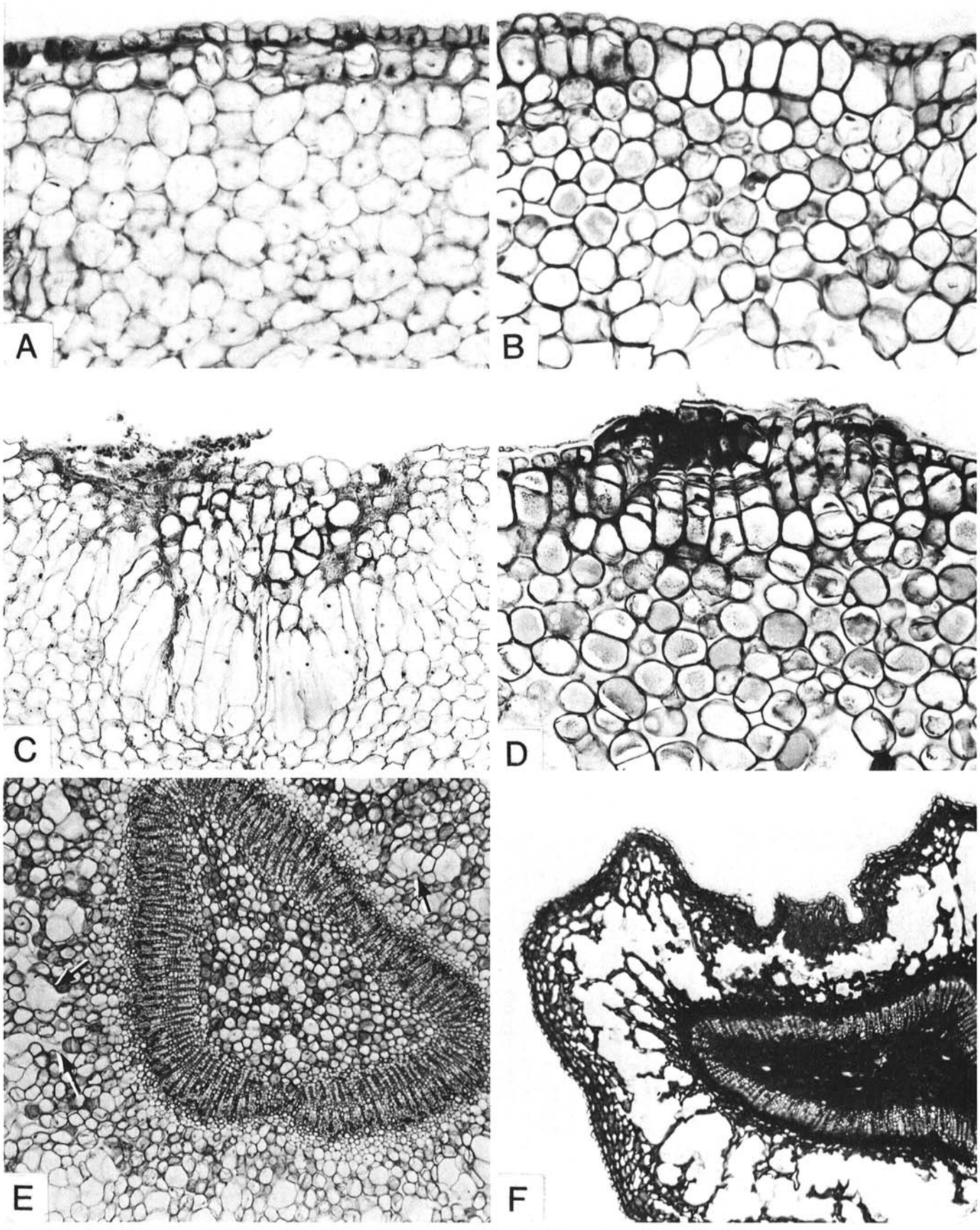

Fig. 1. Histological responses of blueberry to *Phomopsis vaccinii* at 3 days (A–D) and 1 wk (E–F) after inoculation. **A,** Transverse section of uninoculated ovary (×215). **B,** Transverse section of uninoculated floral axis (×215). **C,** Transverse section of infected ovary showing hypertrophied region of cells (×215). **D,** Transverse section of floral axis showing hyperplasia beneath an infection site (×215). **E,** Transverse section of uninoculated floral axis showing intercellular spaces (arrows) in the cortex (×87). **F,** Transverse section of floral axis colonized by *P. vaccinii* (×87). Cells in all tissues are necrotic.

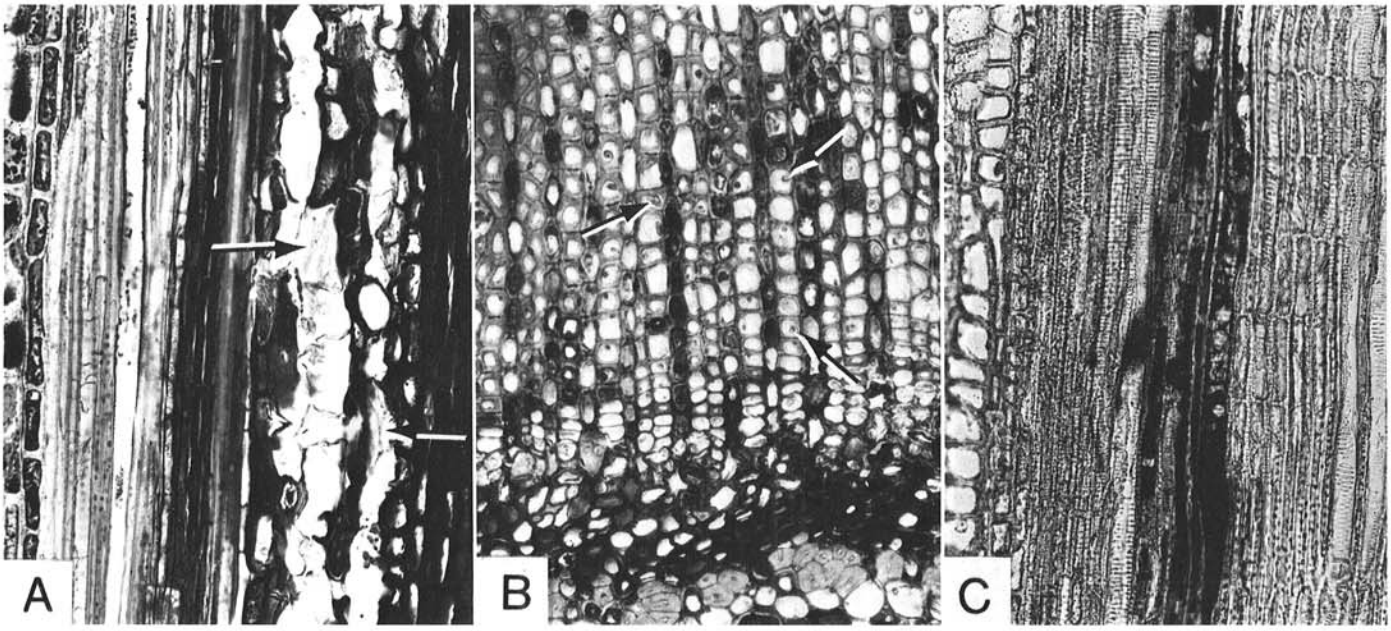

Fig. 2. Histological responses in blueberry stems to *Phomopsis vaccinii* at 1 wk (A and B) and 3 wk (C) after inoculation. **A,** Longitudinal section of stem from the margin of a lesion showing hyphae (arrows) restricted to the intercellular spaces of the cortex (×185). **B,** Transverse section of stem showing intracellular hyphae (arrows) in vessel elements (×343). **C,** Longitudinal section of stem stained with phloroglucinol (×185). Dark staining gums block the xylem vessel elements.

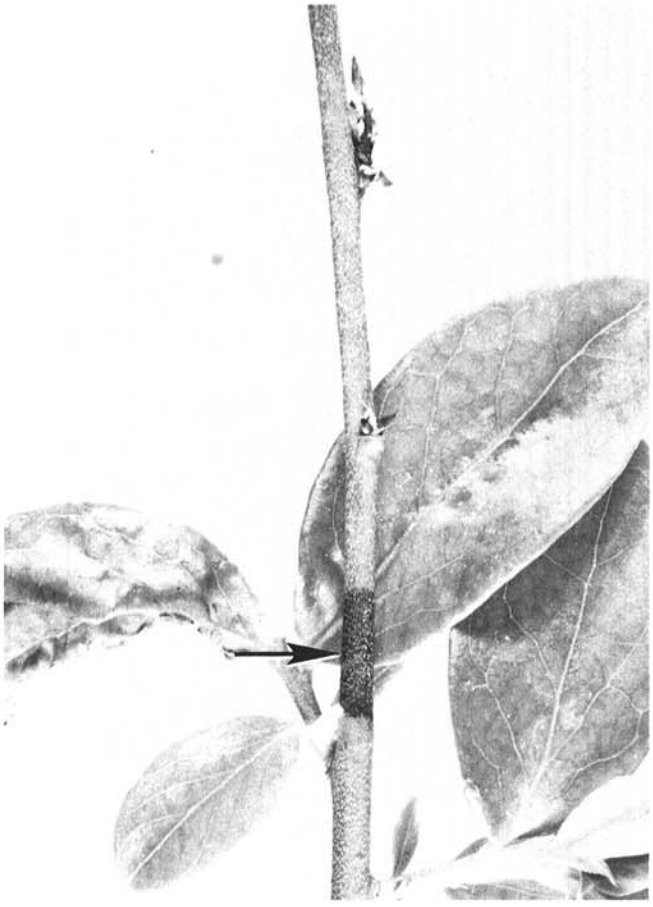

Fig. 3. Twig blight lesion 4 wk after inoculation. Dark brown region of tissue (arrow) developed between the light brown necrotic tissue at top and healthy green tissue at base.

mode of entry of *P. vaccinii* into the blueberry twig, follow its colonization through the twig, and determine if any anatomical barriers may prevent the continued growth of the fungus.

MATERIALS AND METHODS

An isolate of *P. vaccinii* obtained in 1987 from an infected blueberry twig at the Horticultural Crops Research Station in Castle Hayne, NC, was used in all inoculations. Inoculum was grown for 7 days on oatmeal agar under fluorescent lighting (40 $\mu E\ s^{-1}\ m^{-2}$) at 25 C. The plates then were flooded with sterile distilled water, and a conidial suspension of 10^6 conidia/ml was prepared.

Thirty 2-yr-old dormant blueberry plants (cultivar Murphy) grown in a lath house in 15-cm-diameter clay pots containing a peat:sand (1:1, v/v) mixture were used for inoculation. In February, plants were moved to a greenhouse bench (20–30 C) to force budbreak.

As buds opened, an expanding but unopened flower bud was atomized with a conidial suspension of *P. vaccinii* on four stems on each of 10 plants. Four days later, a fully opened flower cluster was atomized with a conidial suspension on four stems on each of 10 additional plants. Open flower clusters were atomized with sterile water on a third group of 10 plants as a control. Plants were placed in a dark mist chamber at 20–30 C for 3 days after inoculation and then returned to the greenhouse bench.

Samples of infected and adjacent healthy tissues were collected at 3 days and 1, 2, 3, 4, 6, 8, and 10 wk after inoculation and fixed in formalin (5 cc)-propionic (5 cc) and 50% propanol (90 cc). Additional corresponding samples of blueberry flowers, pedicels, and stems were collected from the uninoculated control plants. The tissue was dehydrated in an isopropyl alcohol series, embedded in Paraplast plus (Sherwood Medical Industries, St. Louis, MO), softened for 3 days in a solution of 1% Dreft (sodium lauryl sulfate) in 10% glycerol (1), and sectioned on a rotary microtome at 12 μm. Sections were stained with Triarch's Quadruple Stain (Triarch Inc., Ripon, WI). Phloroglucinol saturated in 18% HCl was used to detect gums (10), and Sudan IV saturated in 95% ethanol and glycerol 1:1 (v/v) was used to detect suberin (4).

RESULTS

Plants with unopened flower buds and open flowers responded similarly to inoculation. Inoculated flowers showed necrosis upon removal from the mist chamber 3 days after inoculation. Necrosis also had spread to the flower pedicels in plants inoculated at

full bloom. Microscopic examination revealed inter- and intracellular hyphal growth in the mesophyll of the style, intercellular growth in the petals, and collapse of the cortical cells in the pedicels. No hyphae or tissue disruptions were observed in the bud scales. The ground tissue of uninoculated ovaries and floral axes consisted of uniform spherical parenchyma cells (Fig. 1A and B). However, on inoculated plants, conidia had germinated and penetrated into the ovaries and axes of the flower clusters. Tissue immediately below infection sites on the ovaries responded by forming a zone of hypertrophied cells (Fig. 1C), whereas tissue beneath infection sites on the floral axes underwent hyperplasia (Fig. 1D). These host responses were not successful in preventing the hyphae from growing further into the tissue because hyphae were observed growing intercellularly into the lower tissue.

The floral axes and stems of blueberry contain intercellular spaces in the cortex (Fig. 1E). By 1 wk after inoculation, floral axes and adjacent stems on inoculated plants were necrotic due to the intercellular growth of hyphae of *P. vaccinii* through the cortical cells of the pedicels and into the air spaces in the cortex of the floral axes and stems. In some samples, the ground and vascular tissues of the pedicels and floral axes were collapsed and completely colonized by *P. vaccinii* (Fig. 1F). In the stem, hyphae primarily were observed growing through the intercellular spaces of the cortex and intracellularly through the parenchyma of the outer cortex (Fig. 2A). Numerous tyloses were formed in the vessel elements in advance of the infection. Hyphae from the infected cortex grew laterally inter- and intracellularly into the xylem and phloem tissues (Fig. 2B).

Stem lesions expanded rapidly during the second and third week after inoculation as the hyphae continued to grow through the cortex. Parenchyma cells in the infected cortex and phloem collapsed and stained darkly with safranin, one of the components used in the Triarch Quadruple Stain. A substance often observed blocking the vessel elements reacted positively to phloroglucinol (Fig. 2C). A small amount of hyphae from the colonized vascular tissue grew intracellularly into the pith. Pycnidia began to form

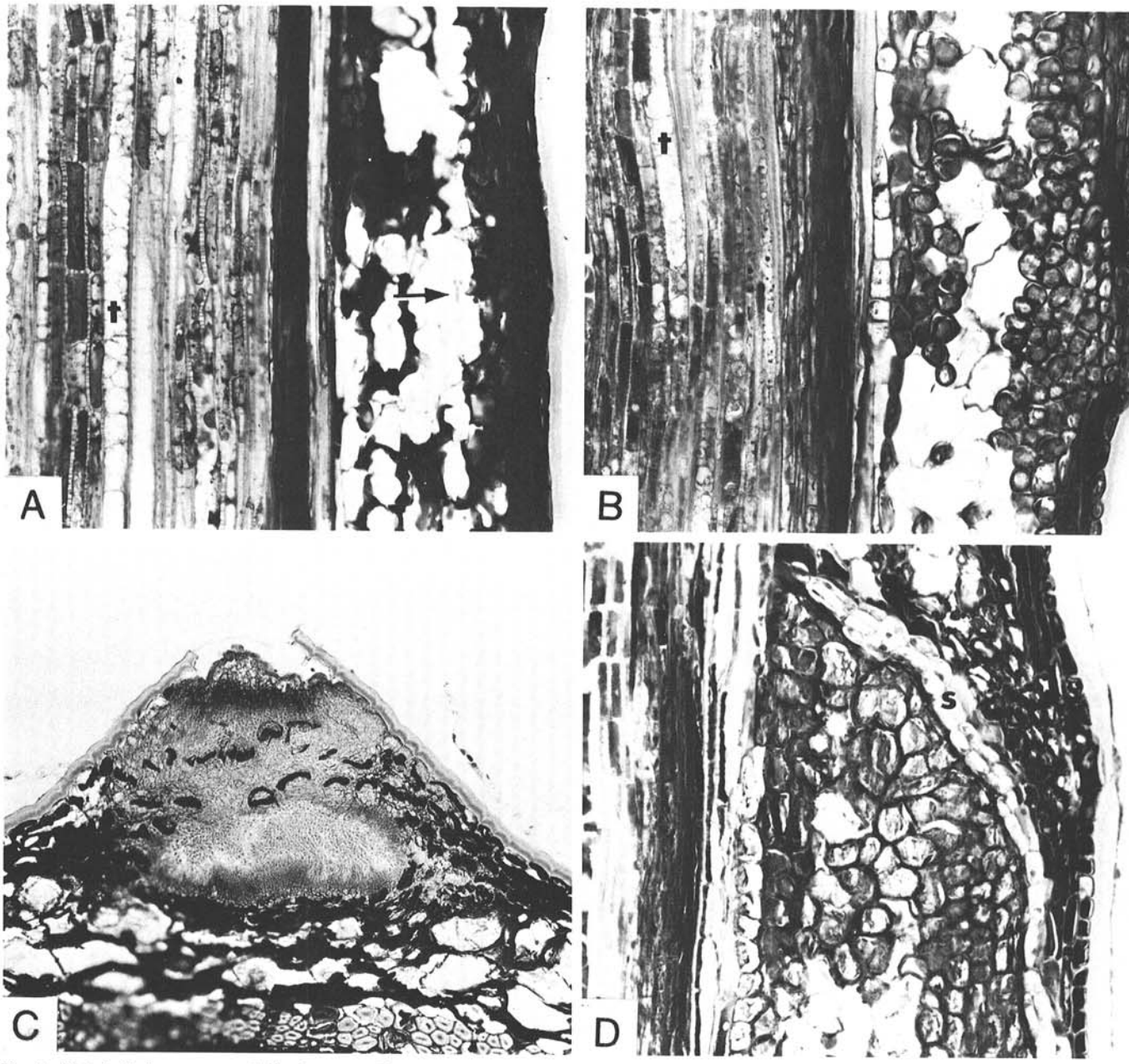

Fig. 4. Histological responses of blueberry to *Phomopsis vaccinii* at 6 wk (A and B) and 10 wk (C and D) after inoculation. **A,** Longitudinal section of stem lesion showing collapsed, necrotic cortical cells, hyphae in cortex (arrow), and tyloses (t) in xylem (×185). **B,** Longitudinal section from green stem near a twig blight lesion showing numerous tyloses (t) in the xylem but no necrosis or hyphae in the cortex (×185). **C,** Transverse section of pycnidium of *P. vaccinii* on blueberry stem (×185). **D,** Longitudinal section of blueberry stem showing meristematic layer of suberized cells (S) that developed between the necrotic cortical tissues above and the healthy cortical tissues below (×185).

between the cortex and epidermal layers at 3 wk after inoculation.

At 4 wk after inoculation, lesions on several of the stems inoculated at full bloom developed a distinct region of dark brown tissue several millimeters long between the lighter brown necrotic tissue and the bright green healthy tissue (Fig. 3). This color change occurred on all stems by 6 wk after inoculation of flowers and appeared to be correlated with a slowing down of lesion expansion. No histological differences were observed between the light and dark brown portions of the lesion. The cortical cells in the lesion were collapsed and heavily colonized by *P. vaccinii* (Fig. 4A). The vascular tissue often was colonized as well. The green stem near the lesion had numerous tyloses in the vessel elements. However, the cortical cells were intact, and hyphae of *P. vaccinii* were not observed (Fig. 4B).

Lesions stopped expanding by 8 wk after inoculation as indicated by measurements taken on five remaining lesions over the last 4 wk of the study. The average length of the twig blight lesions was 12 cm. Pycnidia were observed in the necrotic portion of the stem by 10 wk after inoculation (Fig. 4C). Also, by this time a meristematic layer of thin-walled cells staining positively with Sudan IV had developed between the healthy green tissue and the adjacent dark brown necrotic tissue (Fig. 4D). This region of dividing cells curved inward across the cortex and separated the necrotic and healthy tissue. Isolations from necrotic tissues produced typical cultures of the pathogen.

DISCUSSION

Conidia of *P. vaccinii* germinate and penetrate the succulent young floral parts of blueberry at bud break and during bloom. This infection process apparently differs from that of *P. vaccinii* on blueberry in Michigan where only wounded tissue is susceptible to infection (9). *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & de Not., the cause of stem blight of blueberry, produces similar dieback symptoms in blueberry as Phomopsis twig blight but requires a wound for infection (5).

Once *P. vaccinii* has invaded the flowers, it rapidly moves through the cortex of the floral axis into the stem cortex. Movement through the cortex takes place through the intercellular spaces and intracellularly through the parenchyma of the outer cortex. This type of spread is similar to infection by *Godronia cassandrae* Peck (11) where hyphal growth is exclusively through the air channels and differs from infections by *B. dothidea* where downward movement through the vascular tissue is most important (3,5). Although hyphae of *P. vaccinii* often were observed in the vascular tissue and pith, these regions were not invaded until the cortex had been completely colonized.

Vast numbers of tyloses were formed in advance of the infection. Abundant tyloses also were observed in stems of blueberry infected by *B. dothidea* (5). Tyloses, together with vascular plugging due to gums and hyphae, may contribute to the dieback symptoms in the distal parts of the stem.

There were no clear anatomical barriers that terminated the growth of *P. vaccinii* through the stem. The meristematic layer of suberized cells that was observed 10 wk after inoculation probably was not responsible for stopping the growth of the fungus because of the thin-walled nature of the cells and because it did not appear until 2 wk after the lesion had stopped expanding. This was rather a type of callusing over that occurred once the infection had stopped. The darker brown tissue that developed on inoculated plants 6 wk after inoculation could be the result of oxidation of phenolic compounds. Although isolations were not made from the dark brown areas to determine the viability of the fungal tissue, it is possible that the cessation of lesion expansion is due to a physiological process resulting in limiting the further growth of *P. vaccinii*.

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