Hyphal Morphology of *Botryosphaeria dothidea* in Vessels of Unstressed and Drought-Stressed Stems of *Betula alba*

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ABSTRACT

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Hyphae in unstressed stems of white birch seedlings were thin, contorted, highly vacuolated, and restricted to xylem vessels within 5 mm of inoculation wounds. Hyphae in drought-stressed stems were thick, branched, rectilinear, and spread extensively through vessels. SEM photographs revealed a two-layered sheath on all hyphae appressed to vessel walls in stressed stems, but the sheath was seen only rarely in unstressed stems. A significantly greater number of hyphal apices in unstressed stems were either swollen or had burst and released amorphous cytoplasm. Removal of cytoplasm with H_2O_2 + KOH revealed distinct holes in hyphal walls at bursted apices. A microfibrillar mesh was observed occasionally in vessels of inoculated unstressed stems.

Additional key word: predisposition.

Fungi that cause annual or diffuse cankers on stems of woody plants under stress are often considered nonaggressive or "secondary" parasites. Although these fungi gain entrance through wounds, they either grow saprophytically on dead tissue or remain latent until the plant is weakened by environmental stress (17). Evidence is lacking on mechanisms limiting spread of nonaggressive canker fungi and on changes that occur under predisposing stress. This paper reports results of a microscopic investigation of hyphal morphology in stressed and unstressed woody stems inoculated with a typical nonaggressive canker fungus.

Botryosphaeria dothidea (Morig.:Fr.) Ces. & deNot. is cosmopolitan. It forms cankers on a wide range of woody hosts. Crist and Schoeneweiss (3) found that stems of European white birch, *Betula alba* L., became predisposed to attack by *B. dothidea* when plant water potentials fell below −1.2 MPa (−12 bars). Wene (22) observed robust, branched hyphae of the fungus in vessels of drought-predisposed birch stems, while hyphae in unstressed stems were thin, relatively unbranched, and stained poorly. No tyloses formed in response to wounding or infection in *B. alba*. Although gum plugs occasionally appeared in vessels of stressed stems, they were ineffective barriers to hyphal spread. In wound-inoculated plants that wilted at water potential levels below the threshold predisposing level of −1.2 MPa, hyphae of *B. dothidea* grew rapidly in xylem vessels and eventually invaded adjacent cortical and phloem tissues, causing tissue collapse and canker formation. No cankers appeared and hyphae remained confined to vessels adjacent to inoculation wounds in unstressed stems (22).

Critical studies on the morphology of fungal hyphae in resistant and susceptible woody stems are few. Since one of the limitations may have been the resolution capabilities of light microscopy (LM), histological examinations with both LM and scanning electron microscopy (SEM) were included in this study.

MATERIALS AND METHODS

Fourteen 3-yr-old seedlings of *B. alba* were grown in 5.7-L containers in a medium composed of loam soil:peat:vermiculite (1:1:1, v/v) until root systems were well established. In April 1982, nine plants were subjected to predisposing drought stress in a growth chamber (24 C, 30 ± 5% RH, and 96 W/m^2 incandescent and cool-white fluorescent light for 16 hr/day), by withholding water for 7–9 days until plant water potentials were <−1.2 MPa, measured with a pressure chamber (18). The remaining five plants were watered daily. Stems were then wound-inoculated with an isolate of *B. dothidea* (ATCC 42212) as described by Crist and Schoeneweiss (3), and placed in a humidity cabinet (95 ± 5% RH) under equilibrium conditions until the plant water potential gradients collapsed, which allowed stable measurements of plant water potentials (16). Replicate measurements of two leaves per plant each day for 4 days were taken to determine the mean water potential for each seedling. All seedlings, including unstressed plants, were incubated for 7 days, then watered and transferred to the greenhouse where they were monitored for disease development for an additional 4 wk. At this point, 10 stems (five from unstressed plants and five from randomly selected, drought-stressed plants exhibiting cankers) were excised and prepared for microscopic examination.

Specimen preparation for LM. Stem segments 5 mm long were fixed in 5% formalin-acetic acid (FA) for a minimum of 24 hr, then sectioned longitudinally at 20 μm with a sliding microtome, and postfixed in FA for 30 min. The sections were mounted on glass slides and stained with 1% safranin and counterstained with warm picro-analine blue (8).

Specimen preparation for SEM. In preliminary studies, use of several standard techniques for specimen fixation resulted in hyphal distortion and/or the appearance of artifacts. The most effective method of fixation without producing recognizable artifacts was longitudinal sectioning of stem segments between fixation (4% gluteraldehyde in 0.1 M cacodylate buffer) and postfixation (1% OsO_4 in 0.1 M cacodylate buffer) after Brotzman and Brown (1). In a modification of the above fixation procedure, stems were severed under water 50 mm below inoculation points, immediately transferred to the buffered gluteraldehyde, and allowed to take up the fixative through transpirational pull for 8 hr, in place of soaking sections overnight. This greatly improved in situ fixation of hyphae in sapwood vessels, in which *B. dothidea* was colonized most actively. The modification was used in the subsequent SEM studies reported herein. To remove deposits of free cytoplasm from sections for maximum resolution of hyphal

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wall morphology, the technique described by Kinden and Brown (9) was employed, except that sections were treated with 1% H_2O_2 for 1.5 min rather than 1% periodic acid for 2–3 min. All specimen segments were longitudinal sections 5 mm long and 100 μm thick, dehydrated in a graded ethanol series, critical-point dried in CO_2, sputter coated with gold, and mounted with Tube Koat (Ted Pella, Inc., Tustin, CA 92680) on Cambridge aluminum stubs. They were examined and photographed with either a JEOL JSM-U3 or ISI DS-130 scanning electron microscope operating between 10 and 20 keV accelerating voltage.

RESULTS

Plant water potentials during incubation in the humidity cabinet remained stable at $\geqslant -0.05$ MPa for unstressed controls and at $\leqslant -1.2$ MPa for drought-stressed plants. Examination of stained stem sections under the light microscope revealed that hyphal growth was extensive in drought-stressed stems, averaging 73 mm from inoculation sites. In plants with water potentials $\geqslant -0.05$ MPa, no hyphae were observed farther than 5 mm from the site of inoculation. In drought-stressed stems, hyphae were relatively thick (mean diameter 2.0 μm), rectilinear, and absorbed stain densely (Fig. 3). Hyphae primarily proliferated in the youngest xylem vessels, but in stressed stems they were also found in older xylem vessels, tracheids, vascular parenchyma, pith parenchyma, and in the cortex near inoculation wounds. In stems of unstressed plants, hyphae were thin (mean diameter 1.1 μm), contorted, and lightly absorbed stain. Many hyphal apices were capitate (Fig. 5). Vascular occlusions such as gum plugs were bypassed in both unstressed and stressed hosts. Scalariform end plates retained their integrity in unstressed stem xylem (Fig. 4), but were disrupted by robust hyphae in stressed plants (Fig. 3).

Several other features of hyphal morphology were revealed with SEM that were not detectable with LM. A sheath was observed on all hyphae appressed to vessel walls in drought-stressed stems but only infrequently in unstressed plants. Close observation at high magnification revealed two layers, one composed of a dense, mucilaginous material directly beneath the hyphae, the other composed of thin, smooth material extending 1 μm or more from beneath the hypha along the vessel wall (Fig. 2). The capitate hyphal apices observed in stained sections were revealed under the SEM to be swollen and/or burst hyphal tips (Figs. 5–7). This bursting, marked by extrusion of amorphous cytoplasm, was observed in both terminal and lateral hyphal tips. Specimen preparation using H_2O_2 + KOH to remove extruded cytoplasm exposed distinct holes in the cell walls of the bursted tips (Fig. 8). Swelling and bursting of hyphal tips appeared to be much more frequent in vessels of unstressed stems. To quantify this observation, 20 randomly chosen segments of stressed or unstressed stems were examined with the SEM. Twenty-five hyphal apices were randomly selected in each segment and judged to be either normal or aberrant (swollen or burst). The data were converted to percentage aberrant apices and analyzed by Fisher's least significant difference test. The percentage of swollen or burst hyphal apices was 28.8% in unstressed stems and 8.8% in drought-stressed stems. The increased frequency of aberrant tips in unstressed (ie, resistant) stems was highly significant ($P = 0.01$).

A microfibrillar mesh (Fig. 1) was observed in vessels of several segments from unstressed stems. The occurrence of this mesh was random and infrequent, but no similar structure could be found in any vessels of stressed stems.

DISCUSSION

Resistance to fungal pathogens in woody stems has been attributed to the formation of vascular occlusions in the case of wilt diseases, or to the formation of reaction zones which limit colonization by canker and decay fungi. In the present study no tyloses were found in wood of *B. alba*. Gum plugs and scleriform end plates were ineffectual barriers to hyphal growth in xylem vessels, and there was no evidence of reaction zone formation at the end of 1 mo following inoculation.

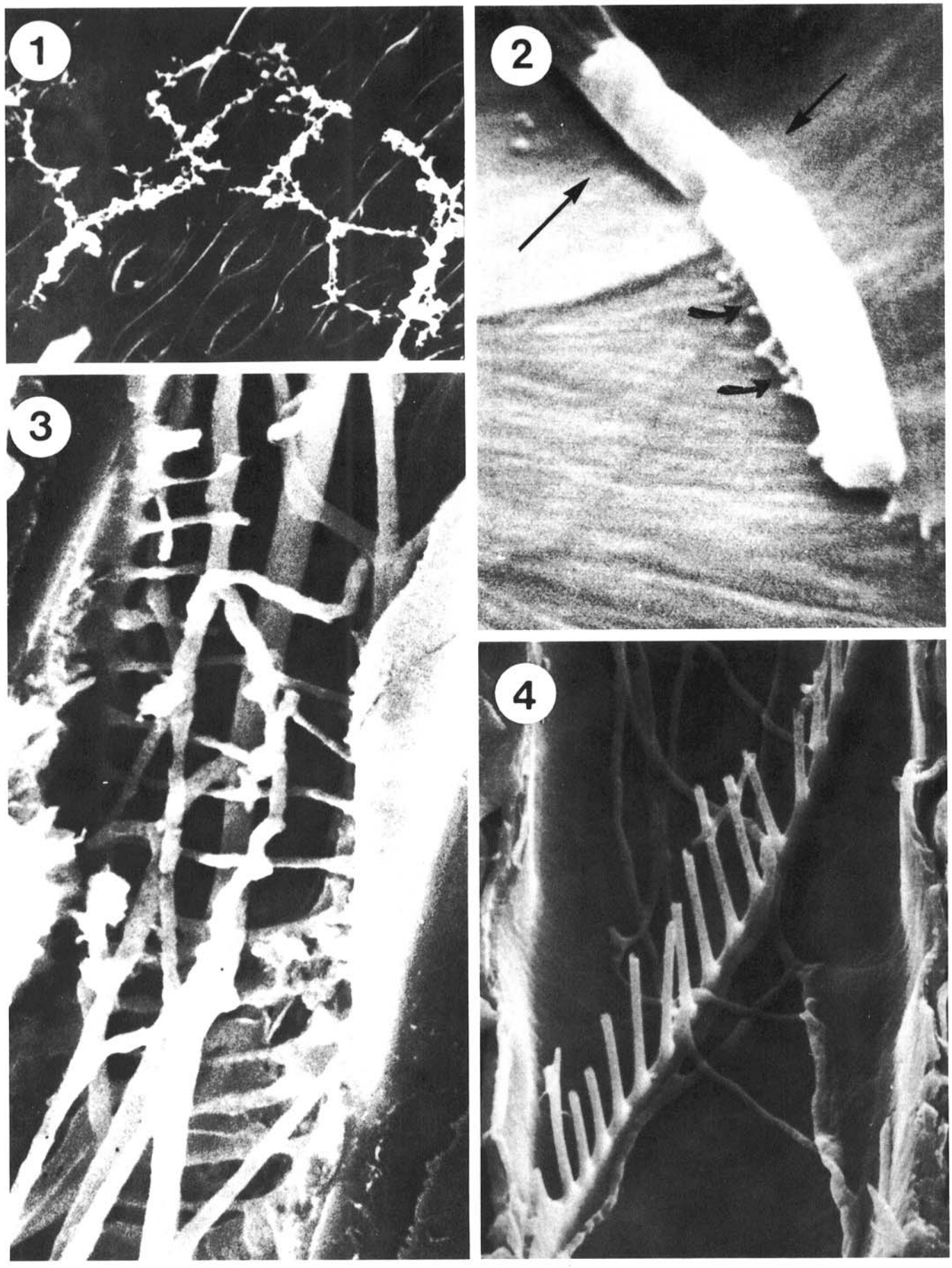
Hyphae in vessels of predisposed stems were relatively large in diameter and rectilinear in cross section, compared to thin, contorted hyphae restricted to vessels adjacent to inoculation wounds in unstressed, resistant stems. Jacobi and MacDonald (7) reported a similar phenomenon in oaks inoculated with the oak wilt fungus, *Ceratocystis fagacearum*. They observed large-diameter hyphae in susceptible red oaks and thinner hyphae in white oaks, which are more resistant to the oak wilt pathogen. They suggested that the difference may be due to a poorer nutrient base for hyphal growth in white oak xylem. Although nutrient content of sap fluid was not analyzed in the present study, prior experiments (D. F. Schoeneweiss, *unpublished*) failed to demonstrate a lack of nutrients for growth of hyphae of *B. dothidea* in vessels of unstressed woody hosts.

The significance of microfibrillar meshes observed with the SEM in unstressed stem vessels of *B. alba* is not known. Miller and Elgersma (11) found similar meshes in xylem vessels of cultivars of *Ulmus hollandica* resistant to *C. ulmi*. They postulated that these meshes originated from ruptured tyloses. Thus far, no tyloses have been found in *B. alba* inoculated with *B. dothidea*.

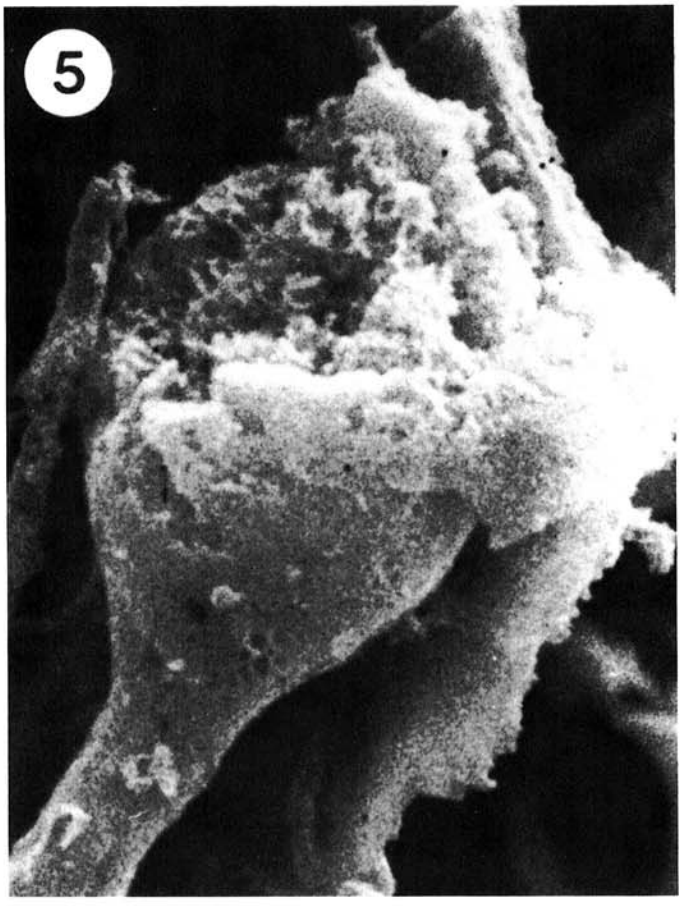
Hyphal sheaths have been reported on a number of fungal species, but their function in host-pathogen interaction is not clear. Murray and Maxwell (12) postulated that a fibrillar sheath on appressoria and germ tubes of *Helminthosporium carbonum* may facilitate conidial attachment. Exams et al (5) described a two-layered sheath on germ tubes of *Bipolaris maydis* but did not speculate on its possible function. This is the first report of a two-layered sheath on hyphae of *B. dothidea* within vessels of *B. alba*. Cooper and Wood (2) presented SEM photographs of "outgrowths" on hyphae of *Verticillium albo-atrum* appressed to vessel walls in tomato that appear very similar to the mucilaginous sheath layer on hyphae of *B. dothidea* in birch (Fig. 2). They suggested that the formation of these growths and of "blisters" on hyphae may reflect the action of enzymes on walls of vessels and pit membranes, but concluded that their function remains problematical. We believe that since these sheaths occur predominantly in predisposed stems they may have a function in pathogenicity, but clarification of their role awaits further research.

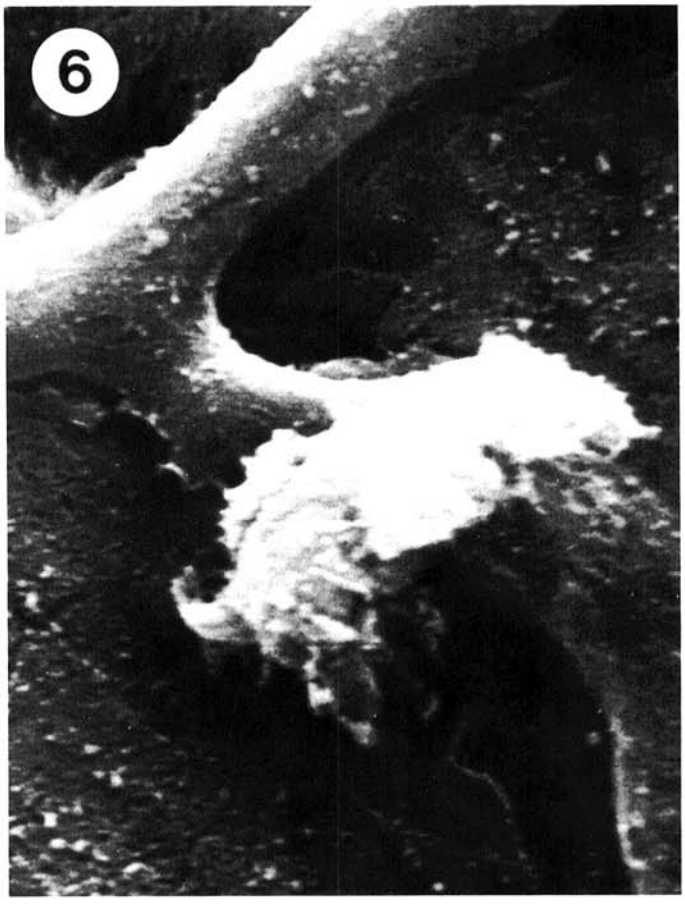
In vivo swelling and bursting of pathogen hyphal tips in host xylem is a heretofore undocumented phenomenon. The possibility of swollen tips being analogous to hyphopodia (20) was eliminated when swelling was observed on hyphal tips not appressed to vessel walls, nor exuding adhesive material (Fig. 6). Although found in other genera of the Pleosporales (19), hyphopodia have not been reported in *Botryosphaeria*. The increased frequency of swelling and bursting of hyphal tips in unstressed stems, where hyphal growth was restricted to vessels adjacent to inoculation wounds, was statistically significant ($P = 0.01$). In vitro swelling of hyphal apices of *Fusarium oxysporum* inhibited by *Laccaria laccata* (an ectomycorrhizal strain) was observed by Sylvia and Sinclair (19), whose photomicrographs also showed the inhibited hyphae to be contorted and highly vacuolated. In vivo lysis of pathogenic fungal hyphae in resistant host xylem has been reported (13–15) and has been linked to increased enzyme activity of chitinase (14) and 1,3-glucanase (13,15). Wargo (21) suggested production of these cell wall-degrading enzymes is host-mediated and acts primarily on the hyphal apex, disrupting and localizing growth of the pathogens as a resistance mechanism. Similarly, Griffiths (6) cited degeneration of the hyphal tip as the resistance mechanism in tomato and pea lignitubers, although no enzymes were isolated. Bursting may not be a result of enzymatic activity; the inability of drought-stressed *B. alba* to arrest colonization by *B. dothidea* may indicate a reduction of calcium ions in predisposed stems. Kunoh et al (10) found that host papillae were resistant to fungal penetration if formed in the presence of $Ca(H_2PO_4)_2$. They did not propose a mechanism for resistance; however, Dow and Rubery (4) demonstrated that calcium ions caused in vitro swelling and bursting of hyphal tips. X-ray microanalysis of fungal tips in stressed and unstressed hosts may eventually provide the information needed to explain the bursting phenomenon.

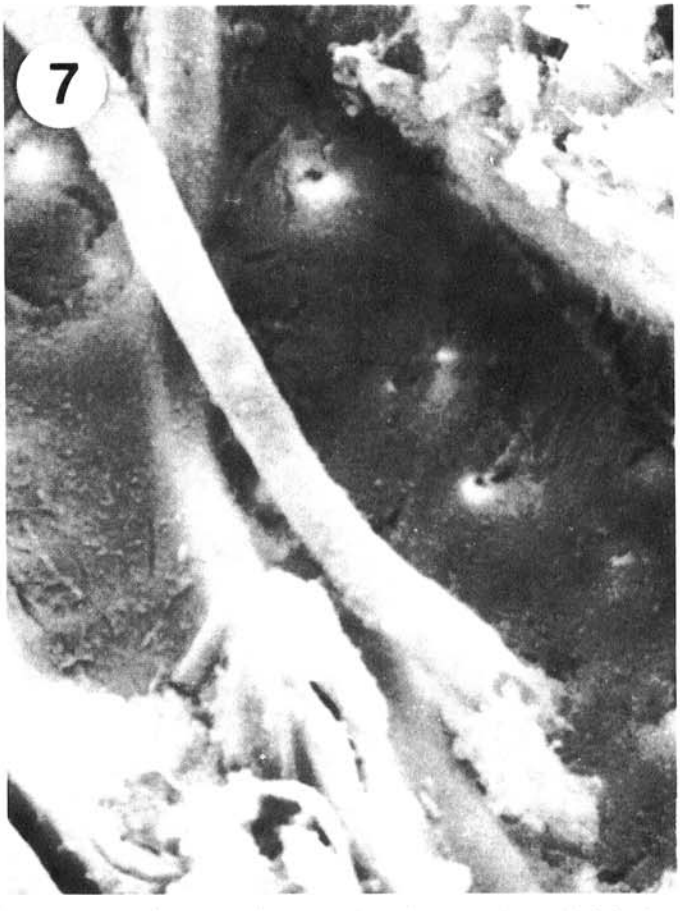
The evidence of hyphal morphology obtained in this investigation indicates that the resistance of unstressed stems of *B.*

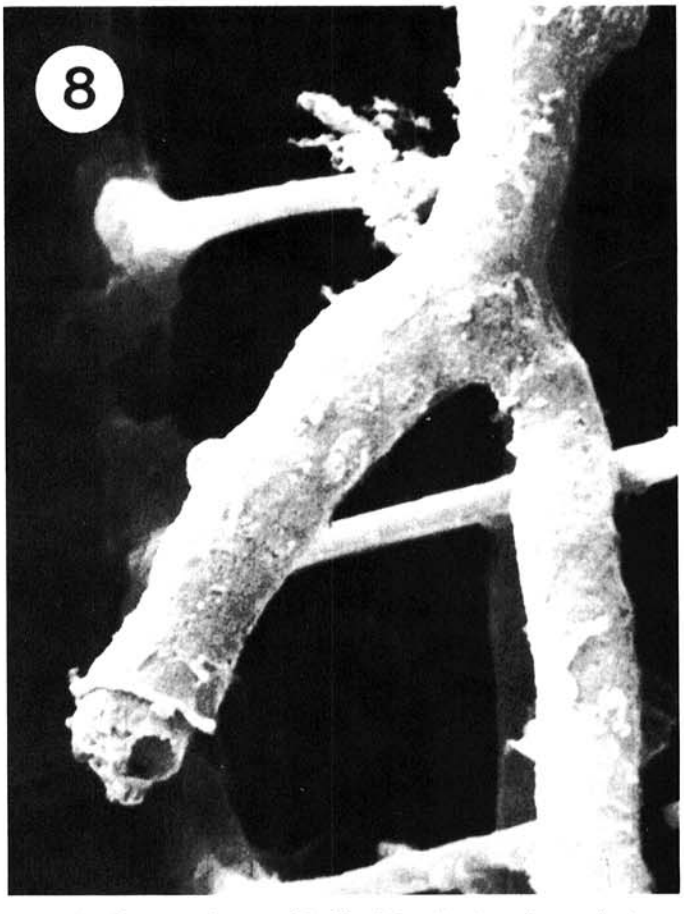


Figs. 1–4. Scanning electron microscopy of xylem vessels of *Botryosphaeria alba* in longitudinal section. **1,** Microfibrillar mesh in unstressed host (×6,890). **2,** Two-layered sheath (arrows) on hyphae of *B. dothidea* in drought-stressed host (×17,860). **3,** Robust hyphae of *B. dothidea* disrupting a scalariform vessel end plate in drought-stressed host (×1,870). **4,** Thin, appressed hyphae of *B. dothidea* penetrating scalariform vessel end plate in unstressed host (×1,880).









Figs. 5–8. SEM microscopy of hyphal apices of *Botryosphaeria dothidea* in xylem vessels of unstressed stems of *B. alba*. **5,** Swollen, bursting terminal apex (×7,870). **6,** Burst lateral apex (×10,580). **7,** Burst terminal apex with cytoplasm partially dislodged (×4,400). **8,** Burst lateral apex with cytoplasm removed by treatment with H_2O_2 + KOH, showing hole in cell wall (×9,180).

alba to attack by the nonaggressive canker fungus *B. dothidea* results from an active biochemical host defense response rather than to formation of morphological barriers, and that the host defense mechanism is not effective in drought-stressed stems.

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