

Pathogenicity and Histopathology of *Botryosphaeria dothidea* on Apple Stems

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ABSTRACT

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In vitro *Botryosphaeria dothidea* grew best at 29 C under fluorescent lights and produced mature pycnidia after 12 days. Apple stem and fruit isolates did not differ in growth and development from 25–35 C. Lesions developed on both wounded and unwounded apple stems inoculated with *B. dothidea*, but wounded trees became infected more readily and had larger lesions than unwounded trees. Conidia of *B. dothidea* germinated and germ tubes penetrated pruned and punctured areas on apple stems within 4 and 6 hr, respectively, after inoculation. Conidial germ tubes consistently grew toward injured tissue on stems. In stems wounded by

cutting or injured by cold or heat, the mycelium was first associated with the disrupted cortical tissue. Rapid, unobstructed vertical growth occurred once the mycelium was established in the xylem vessels. Hyperplasia of parenchyma cells initially restricted movement of *B. dothidea* into the xylem of heat-treated trees and resulted in only 56% establishment of cankers. Cankers developed on 100% of cut-wounded and cold-injured apple stems and on 12% of the unwounded, inoculated stems. Proliferation of parenchyma cells restricted infection to the cortex on unwounded stems which became infected via the lenticels.

Botryosphaeria dothidea (Moug. ex Fr.) Ces & de Not. (syn. *B. ribis* Gross. & Dug.) has been described as both a wound and nonwound parasite (8,13). Wound inoculations were used to demonstrate the pathogenicity of *B. dothidea* on a wide range of hosts (15). Neely (11) successfully inoculated tupelo (*Nyssa sylvatica*) only if inoculated trees were under stress or in unthrifty condition prior to inoculation. Stem canker of elm (*Ulmus americana*) caused by *B. dothidea* and twig blight of Arizona cypress (*Cupressus arizonica*) caused by *Botryosphaeria* sp. have been artificially induced with conidia placed on unwounded bark (7,8). Witcher and Clayton (17) were able to produce die-back and canker symptoms on blueberry (*Vaccinium corymbosum*) only by introducing *B. dothidea* into wounds, but Milholland (9,10) later showed that unwounded stems could become infected.

Drought stress and winter injury also have been associated with the increased infection and canker expansion of *B. dothidea*. Shay and Sitterly (14) concluded that drought favored the development of *Botryosphaeria* canker (Bot canker or tree canker of apple) in a severely affected apple orchard in Indiana. Conner (3) suggested that moisture stress may affect the host's ability to wall off the pathogen; the fungus advances until the stress condition is relieved and the host plant initiates a wound periderm from the cortex. In southern New York state apple trees with extensive canker development also showed severe winter injury (6). Fenner (5) isolated *B. dothidea* from apple fruit collected in middle and north Georgia in 1923. Taylor (16) reported that the incidence of apple disease caused by *B. dothidea* in Georgia was minor.

The objectives of this investigation were to determine the effects of wounding on the predisposition of apple stems to infection by *B. dothidea* and to study the histopathological effects of the pathogen on stem tissue.

MATERIALS AND METHODS

Mycelial growth and conidial germination. The *B. dothidea* isolate used in all studies was isolated from the margin of an active stem lesion on an apple tree in McDuffie County, Georgia. Apple fruit isolates were isolated from an apple fruit collected in a Jones County apple orchard. Cultures for inoculum were grown for 4 days on Difco potato-dextrose agar (PDA) at 20–22 C under fluorescent lights. Agar plugs were removed from the advancing

mycelial margins with a 5-mm-diameter cork borer, one plug was placed in the center of each petri dish of PDA, and 10 dishes were placed in each of seven incubators at 10, 15, 20, 25, 30, 35, and 40 C. Since these studies indicated that the optimum growth temperature for the canker isolate of *B. dothidea* was between 25 and 35 C, a second study was conducted to compare growth rates of apple fruit and canker isolates at 25, 27, 29, 30, 33, and 35 C. In both studies radial growth was measured after 4 days.

Conidia from 14-day-old cultures grown at 29 C under fluorescent light were suspended in sterile distilled water. A loopful of the conidial suspension was streaked on water agar in a petri dish, and conidia were observed for germination at 15-min intervals for 4 hr. Three replications were prepared at the same time to insure that 200 conidia could be observed.

Penetration by *B. dothidea*. Studies were conducted on young apple trees in the greenhouse to determine the method of penetration by *B. dothidea* and the effects of wounding on canker development. Golden Delicious apple trees grafted on MM7 rootstocks were potted in 20-cm-diameter pots containing a fumigated (methyl bromide, 454 g/m³) sand-clay-loam topsoil (1:1, v/v) soil mix. The trees were then pruned back to the first swollen bud, 15–20 cm above the graft union. Shoots were allowed to grow to about 15 cm in length before inoculation. Three shoots from each plant were selected for inoculation. Two wound treatments (pruning with a razor blade or puncturing the epidermis with a dissection needle) and a nonwounding treatment were used on each tree. Uninoculated shoots were covered with plastic bags. A suspension containing 2×10^4 conidia per milliliter was prepared from 14-day-old cultures of a canker isolate of *B. dothidea* and atomized to runoff. Five one-plant replications were placed in a humidity chamber in a randomized split-plot design. Removal times of 0, 2, 4, 6, 8, 10, 18, 24, 36, 48, and 72 hr were designated as whole plots and inoculated stems were split plots. The stems were cut into small sections, cleared, and stained with trypan blue in 50% ethanol. The epidermis was removed and examined microscopically for penetration by germ tubes. The rootstocks were then removed to the greenhouse bench and the remaining intact stems were observed for latent infections.

Histopathology of canker development. Golden Delicious apple trees on MM7 rootstocks were wounded by heat treatment, cold injury, and mechanical injury. The heat wounds were produced by exposure for 3 min to a beam projected from a 250-W infrared heat lamp equipped with a silver reflector 15 cm from the stem. The beam was directed through a 6-mm-diameter hole in a

piece of particle board placed against the stem. Cold injury was produced with a medical artery clamp modified by burning a depression in one side of the rubber nose of the clamp with a hot stirring rod. Solid CO₂ (dry ice) (5 mm³) was placed in the depression and the clamp was closed around the stem for 20 sec. Mechanical wounding was produced by making two cuts (a 25-mm longitudinal [vertical] cut and 5-mm [transverse] cut at the top of

the vertical cut) on the apple stem with a sterile scalpel, pulling the bark away from the stem like a flap, and replacing it. All injuries were made 18 cm above the soil line. Plants were inoculated by atomizing 2 × 10⁴ conidia per milliliter on stems 30 min after wounding. All inoculated and appropriate control plants were placed in a dew chamber for 24 hr. Mist was sprayed for 3 min every 30 min and the relative humidity (RH) ranged from 96 to 100% at 27 C.

Trees were moved to the greenhouse and four replications of each treatment were placed in a randomized split-plot design on the greenhouse bench. Observations were made and samples were collected at 0, 2, 4, 6, and 8 wk after removal from the humidity chamber. At each sampling time, sections (10 mm in diameter) were cut with a razor blade from the inoculated wounded and unwounded areas from each tree. Sections were cleared in a solution of 50% ethyl alcohol, glacial acetic acid, and formalin (1,000:25:65, v/v) ("Triarch" solution), for 24 hr before being transferred to 70% ethyl alcohol. Stems were sectioned (20 μm) with a sliding microtome and stained with trypan blue (2 mg/100 ml 50% ethyl alcohol) for 5 min. Sections were dehydrated with 70, 95, and 100% ethyl alcohol series for 2 min each, and then were transferred to clove oil (5 min) and xylene (4 min). Canada balsam was used to mount the sections on slides and these were placed on a slide warmer at 40 C for 48 hr.

RESULTS

Mycelial growth and conidial germination. In the first study the canker isolate of *B. dothidea* grew 1.1, 11.1, 36.1, 39.0, 65.8, 54.6, and 0.0 mm after 4 days at 10, 15, 20, 25, 30, 35, and 40 C, respectively. In the second study, the canker and fruit isolates grew

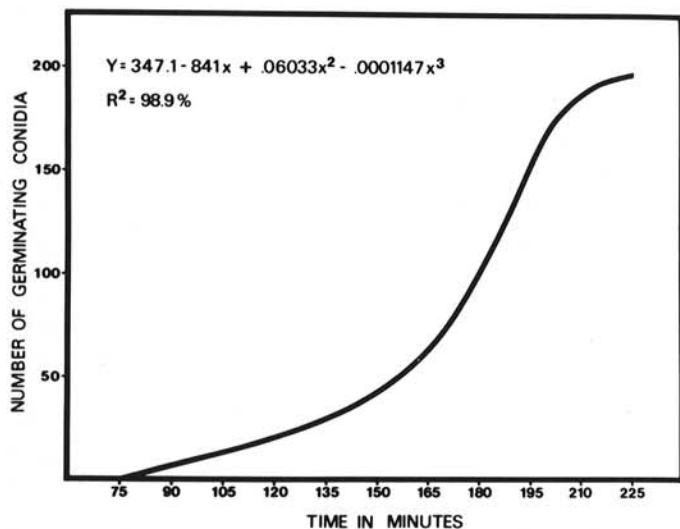


Fig. 1. Germination of *Botryosphaeria dothidea* conidia at 100% relative humidity and 21 C.

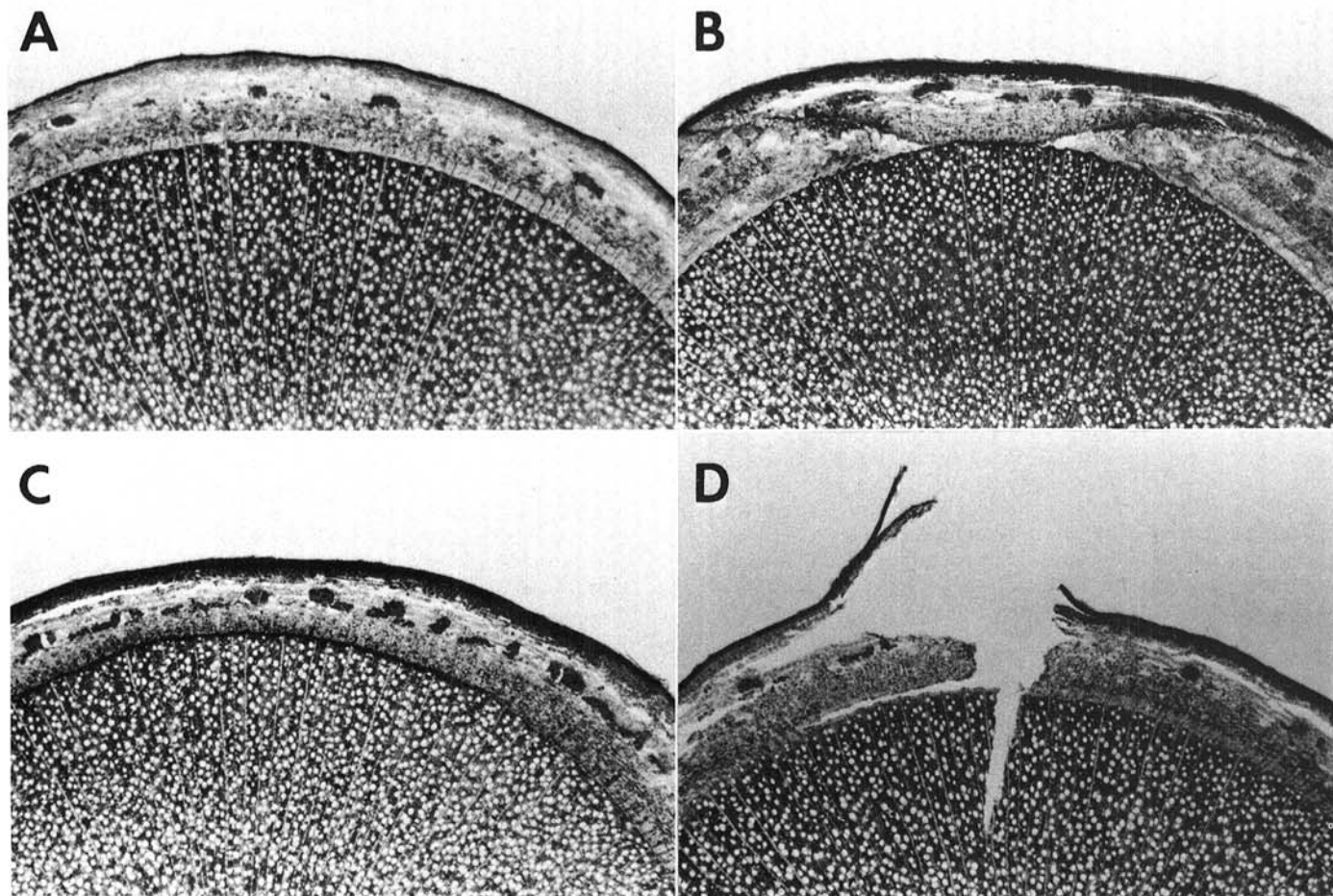


Fig. 2. Initial injury produced on apple stems by three types of wounding before inoculation with *Botryosphaeria dothidea*, ×15: A, unwounded control; B, separation of the injured from healthy cortex by the proliferation of parenchyma cells caused by heat injury; C, collapse of the discolored cortex without area separation caused by cold injury; and D, mechanical injury, but without discoloration penetrating the xylem tissue.

at the same rates from 20 to 30 C. Growth at 29 and 30 C was 68.4 and 65.9 mm, respectively. Both isolates grew significantly ($P = 0.05$) slower at 25 C than at 27–35 C. Mature pycnidia were produced after 12 days.

Conidia were first observed germinating at 90 min after being streaked on PDA and incubated at 21 C (Fig. 1). After 255 min, 95% of the conidia had germinated. Two or more germ tubes consistently were produced by each conidium.

Penetration by *B. dothidea*. Conidia germinated on apple stems

after 2 hr and germ tubes penetrated the pruned and punctured stems by 4 and 6 hr, respectively. Germ tubes consistently grew toward the wounded area of the stem. The average lengths of germ tubes were 16.0, 31.0, and 82.4 mm at 8, 10, and 18 hr after inoculation, respectively.

Developing cankers appeared on pruned portions of apple stems after 4 days on the greenhouse bench. Pycnidia developed and sporulation occurred at 8 and 19 days, respectively. Neither direct nor stomatal penetration was observed throughout the 72-hr

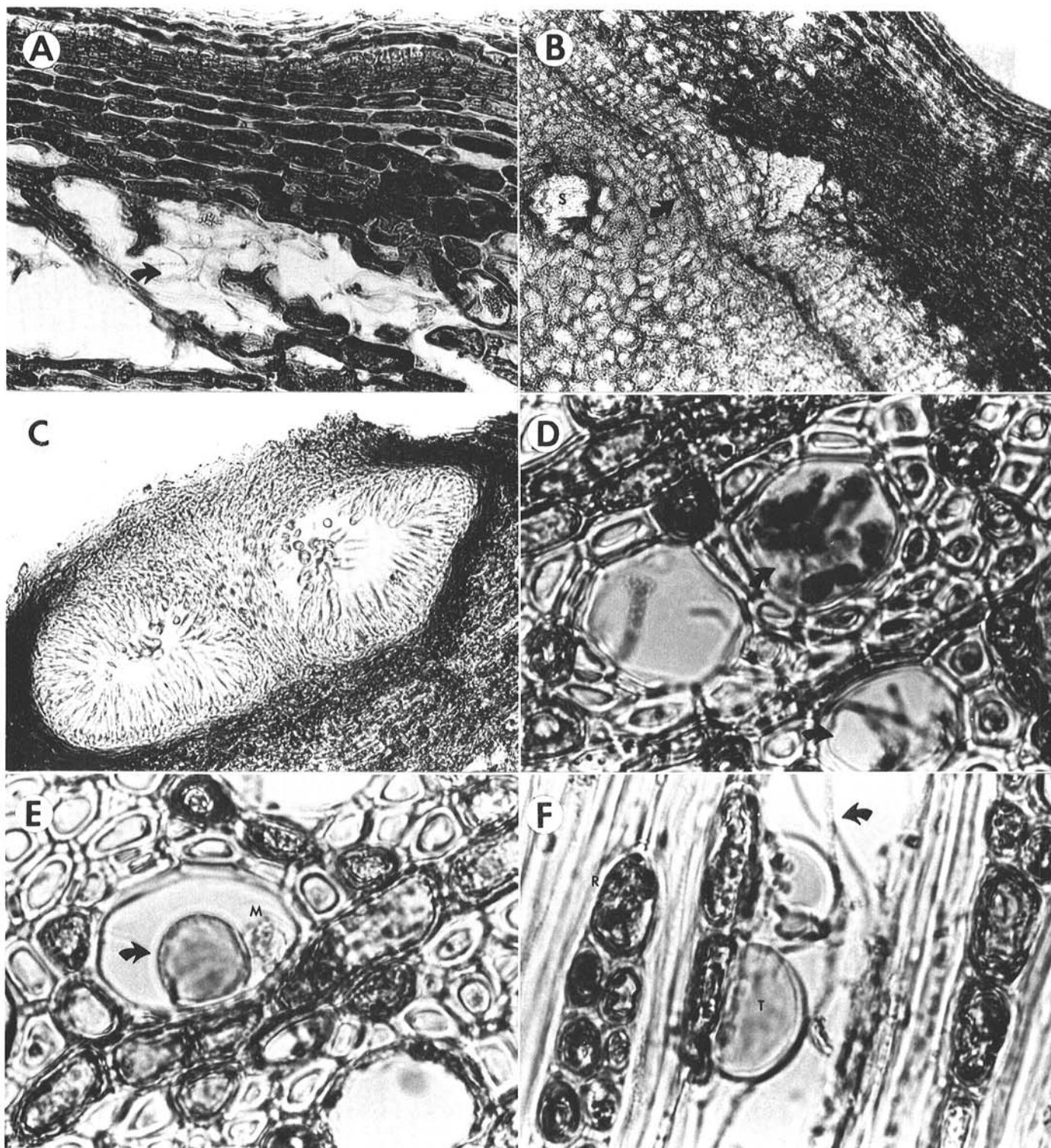


Fig. 3. A, Transverse section of a 14-day-old *Botryosphaeria dothidea* lesion on a cold-wounded apple stem showing mycelium associated with disrupted cortical tissue, $\times 200$; B, transverse section of proliferated parenchyma cells 14 days after inoculation of cut-wounded apple stems with *B. dothidea*, S = sclerenchyma cells, $\times 175$; C, well-developed pycnidium of *Dothiorella mali* in periderm of cut-wounded apple stems 14 days after inoculation with 2×10^4 conidia per milliliter, $\times 650$; D, hyphae of *B. dothidea* in the xylem of inoculated, cold-treated apple stems, $\times 1,000$; E, transverse section showing tyloses in xylem vessel, M = mycelial strand, $\times 1,000$, F, tangential section of mycelium growing unobstructed in the xylem vessels, T = tyloses, R = ray cells, $\times 1,000$.

period.

Histopathology of canker development. Different types of wounds caused marked differences in the degree of injury initially produced on the apple stems (Fig. 2). The heat treatment resulted in proliferation of parenchyma cells, which caused the separation of the healthy and injured cortex. Cold injury caused collapse and discoloration of the cortex without area segregation. No immediate discoloration was evident from injury resulting from mechanical cutting that penetrated the xylem tissue.

Developing cankers on the wounded portions of the stems were visible 2 wk after inoculation. Dark red water-soaking symptoms initially developed around margins of the wounds. Mycelium was associated with disrupted cortical tissue and caused the intact periderm to sink, resulting in a cankered appearance (Fig. 3A). The collapsed cortex became filled with a watery substance, and the intact periderm expanded until it ruptured. Proliferation of the parenchyma cells was evident at 2 wk in the cut and heat-treated tissue, but not in the cold-treated tissue (Fig. 3B). Pycnidia were well developed in the periderm, and mycelium had penetrated the xylem vessels of the cut stems (Fig. 3C).

Hyphal elements were detected in the xylem and metaxylem of wounded trees 4 wk after inoculation (Fig. 3D). The mycelium was sparse in the tannin discolored cells in the center of the infected area, but was abundant in the xylem vessels on the margin of the discolored area. Tyloses and mycelium were abundant in the xylem vessels (Fig. 3E). Mycelium was sometimes detected in the xylem rays. Tangential sections of xylem in infected stems showed unobstructed movement of mycelium in the xylem vessels (Fig. 3F). Cankers continued to enlarge on infected apple stems after 6 and 8 wk.

Infection was associated with lenticels on unwounded trees. Hyperplasia was initiated beneath the site of infection, and the infected area was separated from healthy tissue (Fig. 4). Small reddish discolored areas about 2 mm in diameter were observed on the stems after 6 wk on the unwounded trees prior to canker formation. The mycelium was restricted to the periderm, and canker formation was slow. Pycnidia failed to develop in these restricted lesions.

Cut- and cold-treated apple stems exhibited 100% *dothidea* infection while the heat-treated and unwounded stems yielded 56 and 12% cankers, respectively. The mean canker length over the 8-wk period was 133.3 mm for the cut, 65 mm for the cold, 35.3 mm for the heat, and 3.5 mm for the unwounded treatments.

DISCUSSION

B. dothidea can grow over a wide range of temperatures. Eid and Heuberger (4) found that optimum sporulation occurred at 25–30 C in 100% RH. Their results are confirmed by our

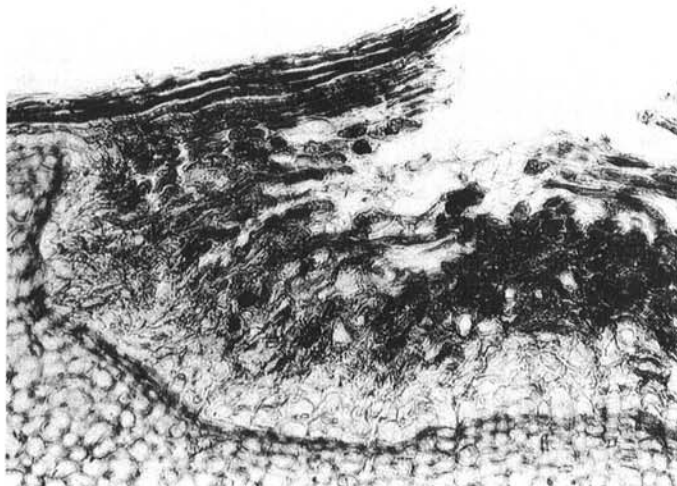


Fig. 4. Transverse section of infected apple stem lenticel 42 days after inoculation with *Botryosphaeria dothidea* (2×10^4 conidia per milliliter) showing the separation of infected tissue from healthy tissue by hyperplasia beneath the infection site, $\times 175$.

studies. No growth differences in the apple fruit and canker isolates were observed between 15 and 35 C. Further investigations confirming the similarities between canker and apple fruit isolates may help substantiate previous observations that inoculum for fruit infection originates on wood cankers.

Botryosphaeria spp. have been described as wound and nonwound pathogens. Smith (15) demonstrated the susceptibility of apple by inoculating stem wounds with three isolates of *B. dothidea*, one from walnut (*Juglans regia*), one from avocado (*Persea americana*), and one from palm (*Arecastrum romanzoffianum*). Cankers 30–80 mm long developed in about 30 days. In our studies infection was confirmed after 72 hr in wounded stems of apple. The ability of the germ tube to grow toward the wounded tissue suggests a chemotactic response. Penetration was observed after 6 hr on punctured areas of stems and after 4 hr on pruned areas.

The severity of Bot canker of apple stems depended upon the type of wound. The proliferation of the parenchyma cells in the cortex of the heat-treated apple stems resulted in the physical restriction of the *B. dothidea* mycelium to the periderm. The open unprotected wounds produced by the cut and cold treatments allowed the mycelium to freely invade the stem and move into the xylem vessels.

Mature pycnidia were produced after 14 days on wounded blueberry stems (10). Our results were similar in that mature pycnidia were formed on wounded (cut) apple stems after 14 days. Mature pycnidia developed 2 wk later on the heat- and cold-treated stems. More rapid development on cut wounds could be attributed to the greater severity of the cut wound and the inoculation of unprotected xylem vessels. Birmingham (1) and Putterill (12) reported that pycnidial formation on wounded tissue took 3–12 mo. Our findings may differ because of differences in tree age, vigor, stage of growth, environmental conditions, or virulence of the *B. dothidea* isolate.

The internal discoloration in the stem tissues was attributed to the production of tannin, suberin, and phlobaphenes by diseased or wounded cells (2). The frequent occurrence of mycelium in the xylem vessels and its infrequent occurrence in the xylem rays explain the rapid downward and slow lateral development of cankers. This is in contrast to results obtained by Milholland (9) who found that xylem rays were instrumental in the development of cankers on blueberry stems. The physical presence of the mycelium and tyloses in vessels may explain the lack of vigor of infected limbs.

Although wounded trees became infected more frequently than unwounded ones, wounding was not a prerequisite for infection. Reddish water-soaked lesions were found on both wounded and unwounded inoculated trees and are associated with the collapse of the cortical cells and invasion by *B. dothidea*. Hyperplasia initiated beneath the epidermis of the infected lenticels of the unwounded treatments, restricted the fungus to the outer periderm. Similar results were obtained on blueberry (10). Our studies confirm the increased susceptibility of wounded apple stem tissue.

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