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HISTOPATHOLOGY OF BOTRYOSPHAERIA RIBIS IN MELALEUCA QUINQUENERVIA: PATHOGEN INVASION AND HOST RESPONSE[1]

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The histopathology of *Botryosphaeria ribis* invasion and *Melaleuca quinquenervia* response was studied under laboratory and greenhouse conditions. Infection of excised leaves in a moist chamber occurred through wounds and stomata, and tissue discoloration followed. Development of callus tissue on wounded stems was more pronounced after spring inoculations than after fall inoculations and, likewise, more on nonstressed ramets than on stressed ramets. Callus ridges on *B. ribis*–inoculated wounds were surrounded by two-to-three-celled suberized layers, which were also present on callus ridges of noninoculated wounds and healthy bark. Cells in callus ridges of *B. ribis*–inoculated wounds had lignified cell walls and contained tanninoid substances that were invaded by hyphae. Invasion of callus tissue occurred through the interface between the callus ridges and the sapwood. Invasion and discoloration of phloem, cambium, and xylem occurred beyond the margins of callus ridges. In noncallused wounds, hyphal colonization was extensive in the cambium and phloem regions. In both callused and noncallused stems, hyphae grew inter- and intracellularly in the cortex, cambium, xylem, and pith. Callus did not limit fungal invasion of newly formed tissues or the tissues formed prior to wound inoculation. Stem girdling by the fungus appeared to be delayed by callus formation.

Introduction

Melaleuca quinquenervia (Cav.) Blake, an exotic tree species introduced into South Florida in the early 1900s (Balciunas and Center 1991), has invaded natural ecosystems. As a result, the species has been declared a federal noxious weed and a Florida prohibited aquatic weed (Bodle et al. 1994). Chemical and mechanical methods to control *M. quinquenervia* are expensive, may be environmentally inappropriate, and often are ineffective (Balciunas and Center 1991; Bodle et al. 1994). This weed may be an appropriate target for biological control using herbivorous insects (Balciunas and Center 1991). The discovery and use of fungal pathogens may supplement the biological control effectiveness of the insects. Fungi as biological control agents have been discussed by Charudattan (1988). During 1989–1990, isolates of the *Fusicoccum* anamorph of *Botryosphaeria ribis* Gross and Duggar were isolated from canker margins of declining *M. quinquenervia* trees in the Loxahatchee National Wildlife Refuge in South Florida and tested for pathogenicity (Rayachhetry 1995). The fungus caused stem cankers on *M. quinquenervia*.

The histopathology of *B. ribis* infections in stem tissues of several woody plant species has been discussed by Milholland (1972), Brown and Hendrix (1981), and Biggs and Britton (1988). This fungus colonizes wounded and unwounded plants (Luttrell 1950; Schreiber 1964; Brown and Hendrix 1981). *Botryosphaeria dothidea* (Moug ex Fr.) Ces. et de Not., often considered synonymous to *B. ribis* (Mass and Uecker 1984), does not require a wound for successful infection of blueberry (*Vaccinium corymbosum* L.) stems (Milholland 1972). Intracellular hyphae of *B. dothidea* in infected stems of peach (*Prunus persica* [L.] Batsch.) have been observed in cortical parenchyma, callus parenchyma, xylem-ray parenchyma, vessels, and tracheids, but intercellular hyphae are observed only in the phloem fibers, in necrotic cortical parenchyma outside the periderm, and in the ligno-suberized parenchyma tissues on the callus surface (Biggs and Britton 1988). In the stem blight of blueberry, caused by *B. dothidea,* xylem vessels are blocked by fungal hyphae and by tyloses and protrusions originating from adjacent parenchyma cells (Milholland 1972).

The histopathological relationship between *M. quinquenervia* and *B. ribis* has not been investigated. Our research should enhance the understanding of tissue and cellular level pathogenesis in the *B. ribis–M. quinquenervia* pathosystem. Specifically, we assessed (1) the anatomical responses of *M. quinquenervia* to infection by *B. ribis* and (2) the pattern of hyphal invasion and colonization of foliage and stem tissues.

Material and methods

Inocula acquisition and preparation

Six monoconidial isolates of *Botryosphaeria ribis* (BR-1 through BR-6) cultured from the margins of stem cankers on declining *Melaleuca quinquenervia* trees in the Loxahatchee National Wildlife Refuge in South Florida were tested for pathogenicity (Rayachhetry 1995). Isolate BR-2, shown to be more aggressive than others, was chosen for histopathological evaluation. The isolate was grown in potato-dextrose broth (Difco Laboratories, Detroit, Mich.) continuously agitated on rotary shaker at 100 rpm for 5 d at ca. 25°C. Mycelial aggregates were removed from the broth by filtering through sterile cheesecloth. Fungal residue was washed with sterile deionized water (DW), blended for 25 sec in a blender, and the hyphal suspensions adjusted to 1% dry w/v with DW. Hyphal suspensions were stored at 5°C and used for foliage and stem inoculations.

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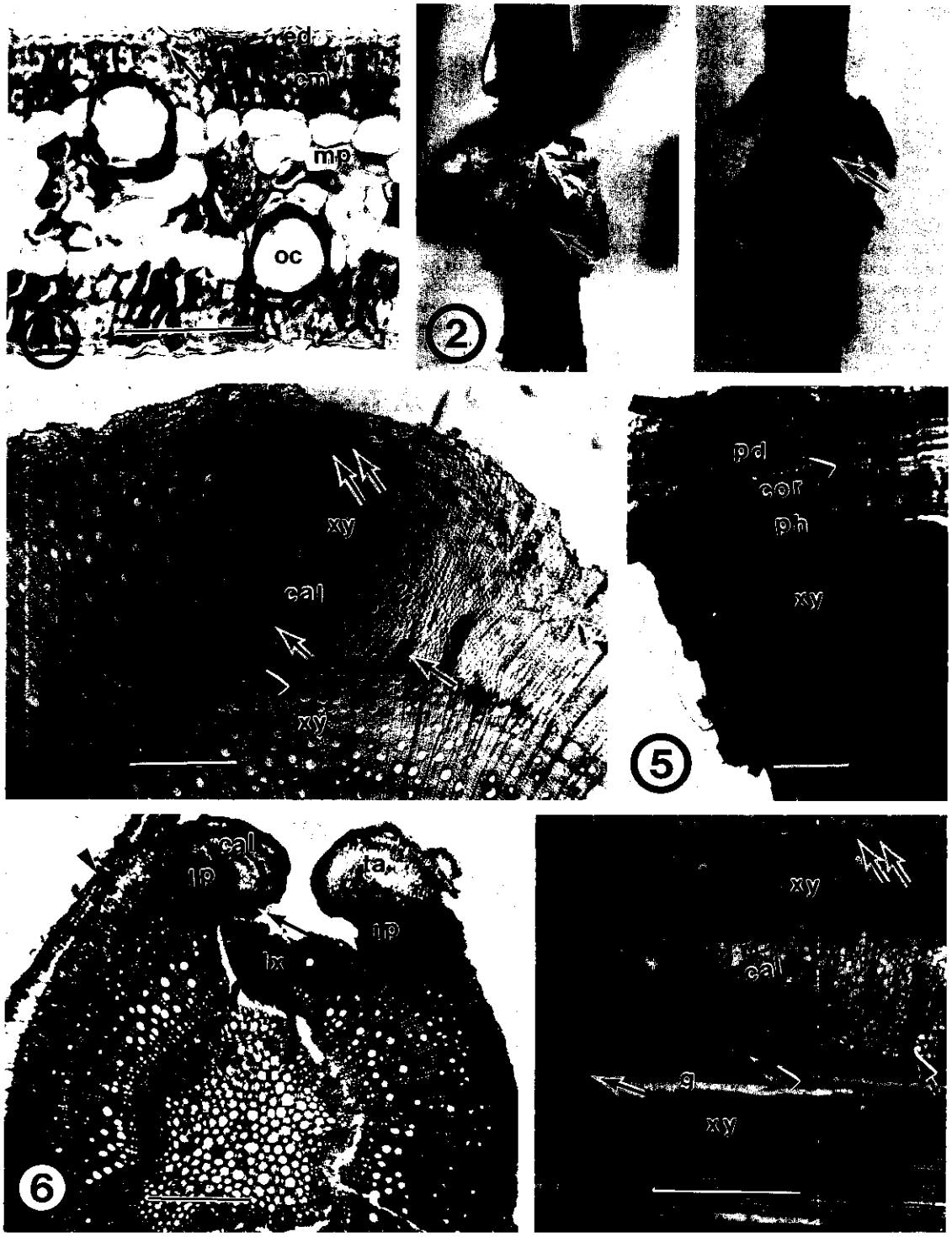


Figs. 1–7 Fig. 1, A cross section of a healthy leaf stained with Pianeze's IIIB. Note stoma (arrow), epidermis (*ed*), chlorenchyma cells (*cm*), mesophyll cells (*mp*), and oil cell (*oc*). Bar = 100 μm. Fig. 2, A wounded noninoculated stem showing callus (arrow) closing wound. Fig. 3, A stem wound-inoculated with *B. ribis*. Note that canker surrounded by callus ridges (arrow). Fig. 4, A cross section of a wounded stem stained with Pianeze's IIIB. Note callus (*cal*) filling the wound, vascular cambium (arrow head) and xylem (*xy*) at the time of wounding, and patches of ligno-suberized cells (arrows) in callus. Also note redifferentiated vascular cambium (double arrows) and newly formed xylem (*xy*) in callus. Bar = 500 μm. Fig. 5, A cross section through a nonwounded stem stained with phloroglucinol-HCl and Sudan IV. Note phelloderm (*pd*) and layers of suberized cells (arrow head), cortex (*cor*), phloem (*ph*), and xylem (*xy*). Bar = 500 μm. Fig. 6, A cross section through a portion of 4-wk-old noninoculated wound on stem stained with phloroglucinol-HCl and Sudan IV. Note suberized cell layers (arrow head) surrounding callus ridges (*cal*), extending to the ligno-suberized periderm (arrow) enclosing tanniniferous cells (*ta*) and ligno-suberized parenchyma cells (*lp*). Also note intensely lignified xylem (*lx*) beneath the wound. Bar = 500 μm. Fig. 7, A longitudinal section (stained

HOST PROPAGATION

Ramets of seven *M. quinquenervia* clones, stem-inoculated with six *B. ribis* isolates, produced morphologically similar cankers and had similar patterns of tissue discoloration in phloem, cambium, and xylem (Rayachhetry 1995). Therefore, only a representative clone was used for histopathological analysis.

Twenty stem cuttings were obtained from an *M. quinquenervia* tree in the Loxahatchee National Wildlife Refuge in South Florida and rooted in a greenhouse using a growth medium containing sand:peatmoss (1:1) by volume. Excised twigs of 5–10 cm length from these rooted cuttings (ramets) were used to produce additional ramets. Additional ramets were grown for 18 mo in the same growth medium as described above in a greenhouse at ambient light and 30° ± 5°C with daily watering. Ramets of 4.5–15.0 mm diameter at 10 cm above the root collar were used for inoculations.

INOCULATIONS

LEAVES. Rayachhetry (1995) observed that excised sterile leaves of *M. quinquenervia* in a moist chamber remained green for 2–4 wk after wounding and/or inoculating with *Fusarium moniliforme* J. Sheld. var. *subglutinans* Wollenweb. & Reinking, a pathogenic fungus affecting *Pinus* sp., and *Lophotrichus* sp., a saprophytic fungus. Excised leaves similarly wounded and inoculated with *B. ribis* isolates were completely discolored within 72 h under similar conditions. Observations of discolored leaves under bright illumination revealed hyphal entry into inner tissues through stomata.

Excised leaves (fourth, fifth, and sixth from the terminal buds of the main stems) of the *M. quinquenervia* ramets were surface sterilized by dipping into 15% (5.25% stock) NaOCl for 15 min and rinsed six times each for 5 min. Sterile leaves were wounded (0.5 mm²) with a sharp needle and inoculated with 10 µL hyphal suspensions of *B. ribis*. Wounded but noninoculated leaves (control) and the wound-inoculated leaves were incubated separately in sterile moist chambers for up to 21 d.

NONSTRESSED RAMETS. Twenty stems of *M. quinquenervia* ramets were wounded by drilling holes (1.5 mm diameter and 2.0 mm deep beneath the bark). Holes were filled with the hyphal suspensions of *B. ribis* in September (fall) 1993 and March (spring) 1994. Control plants were similarly wounded, and the holes were filled with sterile deionized water. The wounded stems were immediately wrapped with Parafilm (American National Can, Greenwich, Conn.), and the ramets were maintained in a greenhouse at ca. 30°C for 4–8 wk with daily watering.

STRESSED RAMETS. Three treatments, each containing three ramets, were designed to induce physiological stresses. The treatments were (1) drought stress induced by a 7-d watering cycle of ramets, (2) cold stress induced by chilling ramets at 6°C for 3 d/wk (for the remaining 4 d the ramets were maintained on the greenhouse bench at ca. 30°C on daily watering schedule), and (3) complete manual defoliation every 7-d cycle for 4 wk and watered daily. Following one exposure to the respective treatment, the ramets were wound inoculated with *B. ribis* following the procedure for "Nonstressed ramets" (above). The respective treatment cycles were repeated for 8 wk, during which the ramets were maintained in the greenhouse at ca. 30°C (unless otherwise indicated).

TISSUE PROCESSING

LEAVES. Five necrotic leaves collected at 3, 7, and 21 d after inoculation and five control leaves collected after 21 d were cut into 10-mm × 5-mm pieces and fixed in formalin-propionic acid (FPA). Segments were dehydrated with absolute ethyl alcohol (EtOH)/tertiary butyl alcohol graded series followed by infiltration and embedding with Paraplast X-TRA (Oxford, Labware, Division of Sherwood Medical, St. Louis, Mo.; Jensen 1962). Leaf sections of 5–15 µm were obtained using a rotary microtome and affixed onto glass slides using Haupt's adhesive (Johansen 1940). Sections were dewaxed in three changes of 100% xylene then sequentially passed through xylene:EtOH (1:1), absolute EtOH, and 70% EtOH. Sections were stained with Pianeze's IIIB (Vaughan 1914) and/or lactophenol-cotton blue. Ten sections from each leaf segment were examined for fungal colonization and formation of pycnidial stroma.

STEMS. After 8 wk, one stem segment (10 mm long, 5 mm wide, and 5 mm thick) was taken from the proximal and distal margins of lesions on each of five control (callused) and 10 *B. ribis*–inoculated trees (five trees each from trees with callused and noncallused wounds). The stem segments were processed and stained as previously described for the leaf tissues.

Three freshly collected stem segments from each of the unwounded, wounded but not inoculated, and wounded and inoculated samples were infiltrated with 40% Elmer's glue, frozen, and sectioned (12 µm thick) using a freezing microtome (FRM-2, Sensortech Incorporated, Clifton, N.J.) and stained to detect lignin, suberin, and tannin formed in response to wounding and/or fungal invasion of the tissues. Lignin was detected using phloroglucinol (1% w/v in 70% EtOH) and 25% HCl (Jensen 1962). Sudan IV saturated in 95% ethanol and glycerol (1:1, v/v) was used to detect suberin (Jensen 1962; Daykin and Milholland 1990). Tannin and other phenolic substances in the tissues were detected using ferric chloride and ferric sulphate (Johansen 1940). Ten sections per segment were studied for host reaction or fungal invasion of different tissue types.

PHOTOMICROGRAPHY

Photomicrographs of representative sections were taken using bright field illumination (Olympus/BMH, Olympus Corporation, New York, or Nikon OPTIPHOT II, Nippon Kogaku, New York).

Results

HOST RESPONSE

LEAVES. Leaves that were wounded and treated with sterile distilled water (controls) remained green up to 3 wk. Every excised leaf that was inoculated with *Bo-*

←

with Pianeze's IIIB) through the margin of callus (*cal*) on a *Botryosphaeria ribis*–inoculated stem. Note a gap (*g*) created at the interface of xylem (*xy*) and callus due to progressive necrosis of cambium (arrow) and xylem, and groups of ligno-suberized cells (arrow heads) enclosing tanniniferous parenchyma cells. Also note redifferentiated cambium (double arrows), and xylem (*xy*) in the callus. Bar = 500 µm.

tryosphaeria ribis became necrotic within 7 d and turned dark brown with discoloration of tissues rapidly expanding from the point of inoculation. Oil cells (fig. 1) in control leaves were relatively nondisrupted. As leaf discoloration progressed after inoculation, brown-colored substances leaked from leaves onto the filter paper in moist chamber. Such leakage from control leaves was not observed.

STEMS. *Melaleuca quinquenervia* stems with wound alone (fig. 2) and wound plus *B. ribis* produced callus ridges around wounds (fig. 3). In the controls of both spring and fall inoculations, wounds were completely filled with callus tissues that extended into the xylem (figs. 2, 4). As a hyperplastic response to the wounding outside the xylem, the cambium produced several layers of relatively small but thick-walled parenchyma cells, which composed the callus tissues (fig. 4). After producing several layers of parenchyma cells, a cambial layer redifferentiated in the callus tissue and produced xylem and phloem inward and outward, respectively (fig. 4). Thus, a patch of hyperplastic parenchyma cells remained surrounded by xylem tissues on both outer and inner sides.

In the wounded controls, cells in the outer layer of callus at the interface of the sapwood were filled with substances that reacted positively to ferric chloride and ferric sulphate, indicating tanninoids. Substances reacting positively to Sudan IV, presumably suberin or other fatty substances, were detected in the cell walls of a few one-to-three-celled layers at the outer phelloderm of unwounded (fig. 5) or wounded stems (fig. 6). Sudan-VI-positive substances in these layers may be constitutively produced in healthy stems but are also inducible around wound periderm. In wounded stems, the layers of suberized cells were continuous with the periderm of callus to the interface between callus and discolored sapwood (fig. 6). Also, some isolated groups of parenchyma cells in the callus gave a positive suberin reaction. Walls of all tracheids and vessels reacted positively to phloroglucinol-HCl, but those adjacent to the wound and in the callus tissues showed an intense positive reaction (fig. 6), indicating extensive lignification of the cell walls. Additionally, groups of parenchyma cells and phloem fibers in the callus showed an intense phloroglucinol-HCl reaction (fig. 6). In healthy stems, these cells stained light pink, indicating normal quantities of lignin.

Stems wound-inoculated with *B. ribis* responded in a similar manner to stems that were wounded but not inoculated, namely, by callusing and by producing suberin, lignin, and tannin. Positions of the suberized and ligno-suberized layers among inoculated stems were also similar to those in noninoculated wounds. Beneath the point of inoculation, vessels and tracheids in several tangential compartments of xylem were delimited by radial ray parenchyma cells that were extensively lignified (figs. 7, 8), as shown by the intense phloroglucinol-HCl reaction. Similarly, ray and pith parenchyma cells adjacent to necrotic xylem stained bluish-green with ferric chloride or ferric sulphate. The positive response of xylem-ray and pith parenchyma to ferric salts indicated tannin or other ergastic phenolic compounds in these cells.

Despite callus formation, sapwood discoloration among *B. ribis*–inoculated stems extended several millimeters beyond the callus ridges. The progressive disruption of cambium and inner phloem was observed at the canker margins internally to the discolored tissues (fig. 7). The periderm at the inner margin of newly formed callus adjacent to the surface of sapwood became ligno-suberized and enclosed tanniniferous cells (fig. 7).

After formation of several layers of hyperplastic parenchyma cells, a vascular cambium redifferentiated and produced phloem and xylem tissues (fig. 7) similar to those in noninoculated stems. Cells in these hyperplastic parenchyma and xylem tissues were invaded by hyphae and became necrotic. Invasion of callus tissues caused progressive necrosis; the necrotic tissues collapsed, creating an opening in the center of the callus ridge (fig. 8). Also, a ligno-suberized periderm developed within the callus wedge on both sides of the opening (fig. 8). Tyloses (fig. 9) and red-stained (Pianeze's IIIB) granular substances were abundant in the vessels located near *B. ribis*–invaded tissues.

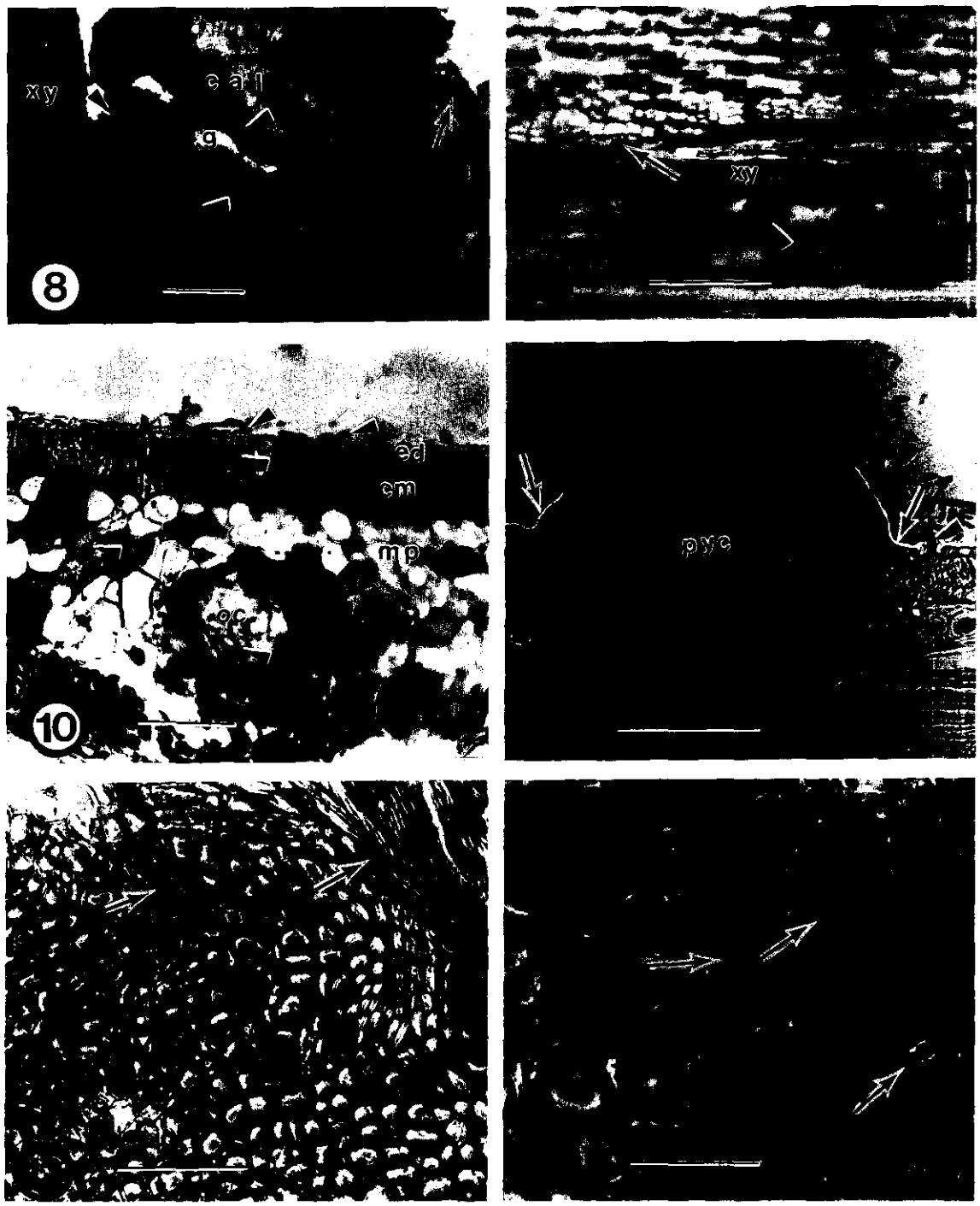
When callus was absent around the wound, the sapwood became discolored relatively quickly and the phloem and cambium collapsed in vertical and tangential directions from the point of inoculation, creating a depression on the stem surface. Phloem, cambium, and xylem tissues were blackened at the advanced stages of necrosis and the canker margins were progressively discolored, imparting brownish coloration. Several one-to-three-celled layers of suberized cells were observed in the phelloderm region of the stems similar to those detected in callused stems. The pith of stems that were inoculated in cambial region turned brown after ca. 8 wk while the pith remained pale green in the wounded noninoculated stems.

The groups of *M. quinquenervia* ramets exposed to low moisture, low temperature, and defoliation produced no visible or only a slight callus response around wounds inoculated with *B. ribis*. All of the stressed stems that produced no or minimal amounts of callus showed reactions similar to those for noncallused wounds.

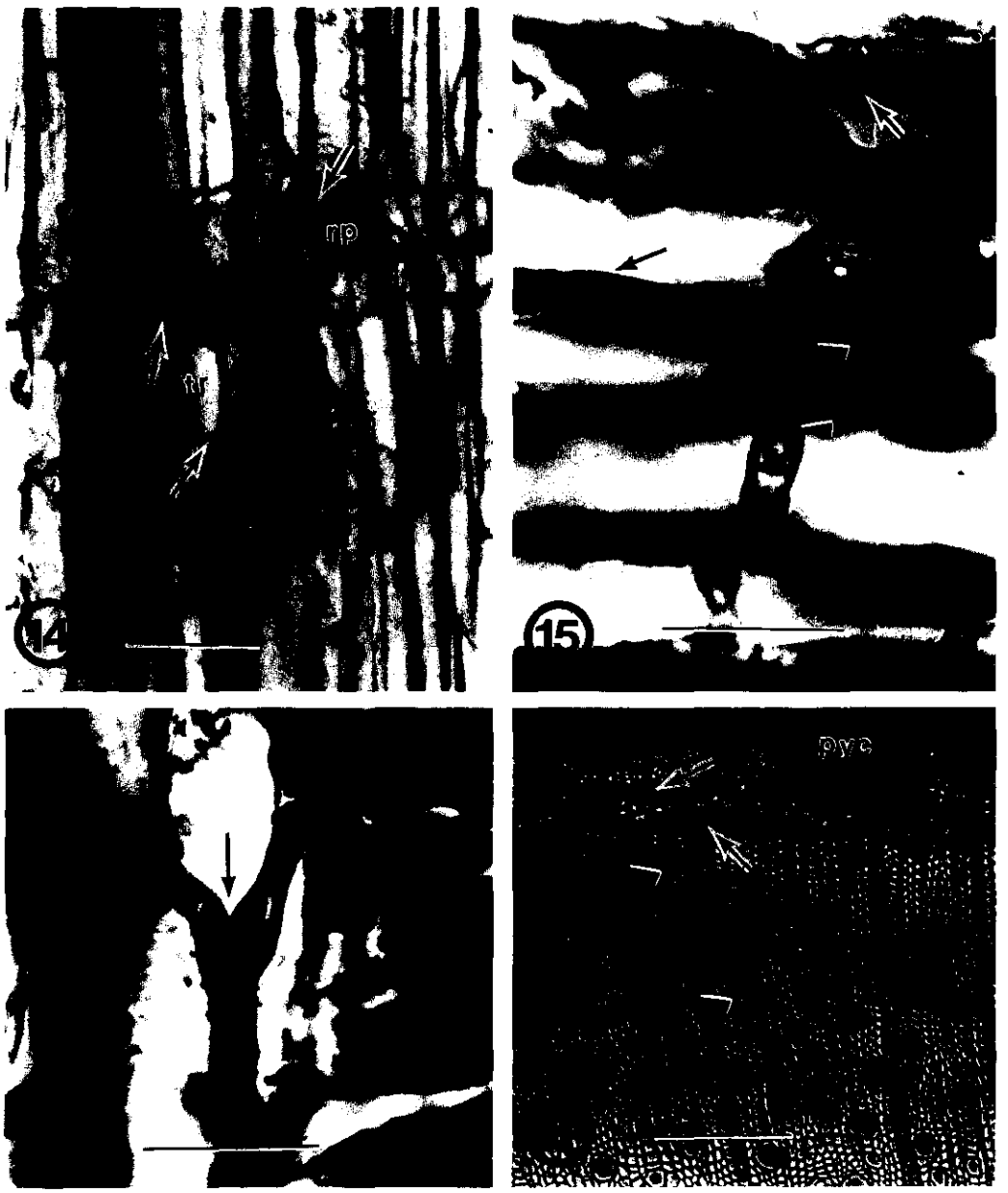
FUNGAL INVASION AND COLONIZATION

LEAVES. The fungus invaded the leaf mesophyll after gaining entry through the stomata (fig. 10). Hyphae advanced through the inter- and intracellular spaces of mesophyll and vascular tissue of the midrib and colonized the oil cells (fig. 10) where intraleaf pycnidial stroma were initiated. Those stroma grew in size, became uni- or multilocular, and ruptured the epidermis (fig. 11).

STEMS. Hyphae were present in the inter- and intracellular spaces of hyperplastic callus tissues (fig. 12).



Figs. 8–13 Morphology and histology of *Melaleuca quinquenervia* leaves and stems inoculated with *Botryosphaeria ribis*. Fig. 8, A cross section through an 8-wk-old *B. ribis*–inoculated wound of a freshly collected stem stained with phloroglucinol-HCl revealing xylem (*xy*) and callus ridge (*cal*). Note suberized (arrow) and ligno-suberized parenchyma cells (arrow heads) in and around the callus ridge. Also note gaps (*g*) in callus due to cell disruption caused by fungal invasion from the ventral surface (interface between xylem and callus ridge) of callus ridge. Bar = 500 μm. Fig. 9, A longitudinal section through the canker margin of a *B. ribis*–inoculated stem. Note tyloses (arrow head) plugging a vessel in the xylem (*xy*) near disrupted vascular cambium (arrow). Bar = 250 μm. Fig. 10, A cross section through a leaf (stained with Pianeze's IIIB) inoculated with *B. ribis*. Note fungal hyphae (arrow heads) penetrating epidermis (*ed*) through stomata. Also note hyphae in chlorenchyma (*cm*), mesophyll (*mp*), and oil cell (*oc*). Bar = 100 μm. Fig. 11, A cross section through a leaf (stained with Pianeze's IIIB) inoculated with *B. ribis* revealing a fungal hypha penetrating through stomata (arrow head), and a pycnidium (*pyc*) emerging from the epidermis (arrow). Bar = 100 μm. Fig. 12, A cross section through a callus ridge of a stem canker caused by *B. ribis*. Note cells surrounded by hyphae (arrows) resulting in deterioration of cellular integrity. Bar = 100 μm. Fig. 13, A cross section through a callus ridge of a stem canker caused by *B. ribis* revealing intra- and intercellular hyphae (arrows) in deteriorating areas as shown in fig. 12. Bar = 50 μm.



Figs. 14–17 Morphology and histology of *Melaleuca quinquenervia* stems inoculated with *Botryosphaeria ribis*. Fig. 14, A longitudinal section through xylem from the canker margin of a *B. ribis*–inoculated stem. Note hyphal growth (arrows) along ray parenchyma (*rp*) and tracheids (*tr*). Bar = 50 μm. Fig. 15, A longitudinal section through xylem from the canker margin of a *B. ribis*–inoculated stem. Note hyphae (arrows) growing vertically along inner wall and apparently clogging tracheids. Also note hyphae passing radially (marked by constrictions; arrow heads) from one cell to another and localized hyphal swellings beneath the arrow on the upper right-hand corner of the figure. Bar = 20 μm. Fig. 16, A longitudinal section through xylem from the canker margin of a *B. ribis*–inoculated stem. Note a hyphae branching (arrow) in a lumen of tracheid. Bar = 20 μm. Fig. 17, A cross section through a noncallused stem canker caused by *B. ribis* revealing a pycnidium (*pyc*) in phelloderm, hyphal concentration in cambium and phloem (arrows), and hyphae (arrow heads) in the lumen and intercellular spaces of vessels and tracheids. Bar = 200 μm.

At the advanced stages of hyphal invasion, parenchyma cells at the callus margin became necrotic and eventually collapsed as the fungus continued to grow within callus (fig. 13). Also, hyphal invasion of xylem elements occurred underneath the callus ridges.

Hyphae were observed in the inter- and intracellular spaces of the discolored cells of phloem and pith parenchyma. However, detection of younger (unmelanized) hyphae in these cells was difficult as these hyphae and the cellular contents of discolored cells stained similarly with Pianeze's IIIB stain. Radial and tangential growth of hyphae in the sapwood occurred through inter- and intracellular spaces of the xylem rays, tracheids, and vessels (figs. 14–16). Hyphae were observed in the lumens of parenchyma cells (fig. 13), xylem rays (fig. 14), vessels, tracheids (figs. 15, 16),

and cortical and pith parenchyma cells. Invasion of adjacent tracheids or vessels occurred through pits as indicated by constrictions at the point of emergence in lumen (fig. 15). In tracheids, hyphae grew freely or appressed to the wall (fig. 15). Hyphal swellings (fig. 15) and branching (fig. 16) often clogged the tracheids. In addition to hyphae, vessels in the infected xylem contained granular substances that stained red with Pianeze's IIIB stain.

Among noncallused stems, fungal hyphae were concentrated in the cambial region. Hyphae were also present in the inter- and intracellular spaces of the cortex but were often masked by the cellular remains of collapsed host cells in these areas. As in the case of callused stems, fungal growth in vascular tissues occurred through inter- and intracellular spaces of the cells in the xylem (fig. 17) and pith. Partially submerged pycnidial stroma emerged from collapsed tissues of the stems (fig. 17). Observations showed that fungal colonization of stem tissues exposed to low moisture, temperature, and defoliation was similar to that described for noncallused wounds of the stems.

Discussion

Histopathological relationship of *Botryosphaeria ribis* with some other woody plants has been studied (Biggs and Britton 1988). Our work is a comprehensive histopathological description of the host-pathogen relationship of *Melaleuca quinquenervia* and *B. ribis*.

Hyphae of *B. ribis* entered *M. quinquenervia* leaves through wounds and stomata. Michailides (1991) found similar stomatal penetration of the leaves and fruits of pistachio (*Pistacia vera* L.) by germ tubes of the macroconidia of *Botryosphaeria dothidea*. Previous observations (M. B. Rayachhetry, unpublished data) showed that *B. ribis* did not invade intact leaves of *M. quinquenervia* in the absence of free water on the leaf surface. Tissue discoloration of *B. ribis*–inoculated leaves of *M. quinquenervia* developed following the extrusion of brown-colored substances from the leaves. This phenomenon is suggestive of *B. ribis*–initiated changes in wall and membrane integrity of the cells in the chlorenchyma and mesophyll in the leaves.

Callus formation around wounds on trees is considered a nonspecific resistance mechanism of a host to wall off a pathogen (Biggs et al. 1983; Griffin et al. 1984). In our study, callus formation among noninoculated and inoculated wounds on stems of *M. quinquenervia* varied from completely healed wounds (no canker among noninoculated stems) to pronounced callus ridges (around cankers of most spring inoculations) to slight callus ridges (around cankers of most fall inoculations) to no visible callus (around cankers of some spring and fall inoculations). Stems that did not form callus were quickly girdled by the fungus. However, fungal growth through callus tissues and the wood formed prior to infection seemed to be slower in stems with callused wounds than in the stems lacking a callus response. A similar phenomenon was reported for pathological relationships between *Inonotus obliquus* (Fr.) Pilat and paper birch (*Betula papyrifera* Marsh; Blanchette 1982), and *Phytophthora cinnamomi* Rands and red oak (*Quercus rubra* L.; Robin et al. 1992).

Vance et al. (1980) reported that lignin and suberin provide chemical barriers as a general mechanism of resistance to the infection of plants by pathogenic organisms. In our study, callus ridges with ligno-suberized periderm, tanniniferous parenchyma cells, and intensely lignified walls of tracheids and vessels beneath the wound may be viewed as nonspecific physical and chemical barriers produced by *M. quinquenervia* against fungal growth into the existing and newly formed tissues. Despite being ligno-suberized, the wound periderm of *M. quinquenervia* at the ventral side of the ridges (facing the sapwood) seemed to be an area from which fungal growth can continue into the inner portion of the callus. Biggs et al. (1983) reported similar findings for *Populus* hybrid (NE-388) infected by *Cytospora chrysosperma* (Pers) Fr. At the advanced stages of hyphal invasion, parenchyma cells at the callus margin of *M. quinquenervia* stems became necrotic and eventually collapsed as the fungus continued to grow into the newly formed cells of the callus. Similar findings have been reported for newly formed cells in the callus of *B. dothidea*–invaded peach trees (Biggs and Britton 1988).

As a morphological response to colonization of tissues by *B. ribis*, vessels in and adjacent to the infected region of the xylem frequently responded with formation of tyloses and accumulation of granular substances. Such structures are rare in uninfected stem tissues. These observations agreed with those of Milholland (1972) who found that formation of tyloses and protrusions in the vessels in blueberry stems was initiated by *B. dothidea* invasion of the tissue. Also, the formation of tyloses and gums in vessels in response to *B. dothidea* and *B. ribis* infection was reported for almond trees (*P. dulcis* [Mill.] Webb; English et al. 1975) and mango trees (*Mangifera indica* L.; Ramos et al. 1991), respectively.

Fungal hyphae were observed in the inter- and intracellular spaces of the discolored cells of phloem and pith parenchyma. However, the detection of younger (unmelanized) hyphae in these cells was difficult since these hyphae and the contents of discolored host cells stained similarly with Pianeze's IIIB stain. English et al. (1975) reported similar difficulty in detecting mycelia in the phellogen or phellem using Pianeze's IIIB stain.

In apple (*Malus* sp.), Brown and Hendrix (1981) reported that hyphae of *B. dothidea* occurred in xylem rays less frequently than in vessels. English et al. (1975) and Biggs and Britton (1988) observed that *B. dothidea* invasion occurred in all cell types and directions (tangential, radial, and vertical) from the point of infection. The growth pattern of the hyphae of *B. ribis* we observed in *M. quinquenervia* was similar to those reported by English et al. (1975) and Biggs and Britton

(1988). In apple stems, rapid downward and slow lateral development of cankers has been attributed to a higher frequency of hyphae in vessels than in xylem rays (Brown and Hendrix 1981). According to Milholland (1970), the hyphal abundance in the xylem rays was crucial to the development of cankers in blueberry stems.

Invasion of adjacent tracheids or vessels of *M. quinquenervia* by *B. ribis* occurred through pits and intercellular spaces of the tracheary elements as described by English et al. (1975) for colonization of xylem tissues of almond trees infected by *B. dothidea.* In *M. quinquenervia,* the lumens of tracheids and vessels contained one or more hyphae, with localized swellings often occluding the cellular spaces (fig. 15). Similar phenomena have been reported for almond (English et al. 1975) and apple (Brown and Hendrix 1981) stems infected by *B. dothidea.* Such occlusions restrict the flow of water through the vessels and tracheids (Milholland 1972) and possibly contribute to the decline and dieback of stems above the canker.

The cambium of noncallused *M. quinquenervia* stems was extensively colonized by hyphae of *B. ribis.* Hyphae were also present in the inter- and intracellular spaces of the cortex but were difficult to detect since they were often masked by the cellular remains of the collapsed cells in these areas. Fungal growth in vascular tissues of stems with noncallused wounds was comparable to that occurring in stems with callused wounds. However, the overall concentration of hyphae in the infected tissues of the stems with noncallused wounds was greater than that observed in the stems with callused wounds.

Adverse environmental conditions appear to inhibit callus formation and to enhance the conditions needed for growth and development of *B. ribis* hyphae in the stem tissues of *M. quinquenervia.* These observations agree with those of Doster and Bostock (1988), who found that low temperature treatments of almond trees inhibited lignin and suberin production in bark wounds.

Partially submerged pycnidial stroma developed on the bark of declining *M. quinquenervia* stems. Similar production of pycnidial stroma has been reported for *B. dothidea* on almond trees (English et al. 1975) and *B. ribis* on walnut trees (Rumbos 1987). Pycnidia produced on the bark are important sources of inocula for disease development among apple trees (Drake 1971) and pistachio vines (Michailides 1991).

Histopathological evaluation of *B. ribis* in stems of *M. quinquenervia* revealed that after successful stem infection, the fungus can perpetuate in the tissues and proliferate rapidly under conditions—such as drought, low temperature, and defoliation—that can stress the host. Callus production by the host appeared to be nonspecific response to wounding. Callus production may have been negatively affected by stress factors that favored fungal invasion of stem tissues. The biological control potential of *B. ribis* on *M. quinquenervia* may be enhanced by naturally occurring or artificially induced environmental stresses.

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