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Infection and Fungal Development of *Tubakia dryina* on sweet gum (*Liquidambar styraciflua*)

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Abstract: Infection and fungal development of *Tubakia dryina* were investigated on leaves of sweet gum using a combination of microscopic techniques. Conidia of *T. dryina* adhered to the leaf surface and formed septate germ tubes. Germ tubes terminated in small appressoria that formed directly over epidermal cells. Intra- and intercellular hyphae ramified extensively throughout the leaf tissue. Host cells associated with the infection site became necrotic and collapsed, resulting in macroscopic disease symptoms.

Key Words: infection process, plant pathology, ultrastructure

INTRODUCTION

The imperfect fungus *Tubakia dryina* (Sacc.) Sutton is a leaf parasite reported to occur on a wide range of host plants, including species of maple (*Acer*), ash (*Fraxinus*), oak (*Quercus*), elm (*Ulmus*), and sweet gum (*Liquidambar styraciflua* L.) (Limber and Cash, 1945; Glawe and Crane, 1987). Although the genus *Tubakia* is worldwide in distribution, *T. dryina* is the only one of five known species reported to occur in the U.S. (Munkvold and Neely, 1991). Glawe and Crane (1987) summarized the nomenclatural history of *T. dryina*. Synonyms of the organism include *Lep-tothyrium dryinum* Sacc., *Actinopelte dryina* (Sacc.) Hoehnel, and *Actinopelte americana* Hoehnel. Recently, Belisario (1991) associated *T. dryina* with the teleomorph *Dicarpella dryina* Belisario and Barr.

The pathogenicity of *T. dryina* to its various hosts has been debated in the literature. Limber and Cash (1945) reported that leaf spots produced by this pathogen on several different genera of forest trees were 2–5 mm diam with regular margins; when associated with larger lesions, *T. dryina* was present as a secondary invader. Yokoyama and Tubaki (1971) concluded that *T. dryina* was primarily a secondary invader in Japan based on inoculation tests that did

not result in symptom development. More recent reports have established that *T. dryina* is pathogenic to *Quercus* species in the U.S. (Munkvold and Neely, 1990; Zhang and Walker, 1995), but isolates from oak failed to cause symptoms on non-oak hosts including sweet gum (Munkvold and Neely, 1990). Venkatasubbaiah and Chilton (1992) identified four nonspecific phytotoxins produced by *T. dryina* in liquid culture that caused necrotic lesions on red oak, white oak, and eight weed species.

During the summer of 1994 in the Nacogdoches County area of Texas a prominent leaf spot on sweet gum was widespread. Infected leaves had numerous necrotic lesions, each surrounded by a reddish halo. The lesions tended to merge resulting in large areas of dead tissue. In the center of each spot was a cluster of fruiting bodies described as pycnothyria (rhizothyria) of *Tubakia dryina* (Glawe and Crane, 1987), umbrella-like structures that provide protection for the numerous conidiogenous cells borne on their undersurface. *T. dryina* infection on sweet gum has been associated with the disease red leaf spot (Farr, et al., 1989).

This study was conducted to examine (1) the pathogenicity of *T. dryina* from the East Texas area to sweet gum, (2) the infection process on sweet gum and to compare it to observations made on oak (Munkvold and Neely, 1991), and (3) the relationship at the ultrastructural level that develops between host and pathogen cells following *T. dryina* infection.

MATERIALS AND METHODS

Isolation of the pathogen.—Leaves of sweet gum, *Liquidambar styraciflua*, showing the characteristics of red leaf spot and bearing pycnothyria of the fungal parasite *Tubakia dryina*, were collected from Nacogdoches County, Texas. Infected leaf sections were surfaced sterilized for 1–2 min in a 0.5% sodium hypochlorite solution, rinsed in sterile water, plated onto 2% water agar, and incubated at room temperature. After 2–4 d, fungal hyphae emerging from the leaf tissue were transferred to potato dextrose agar and cultured in the dark at room temperature. Isolates began to produce conidia within 7 d.

Inoculation of healthy leaves.—Conidia were suspended in a 0.01% solution of Tween 80 and sprayed onto

healthy sweet gum leaves. Inoculated seedlings were maintained in a dew chamber for 24–48 h (98% relative humidity, 27 C). Germ tubes and appressoria that formed on leaves of the host were visualized using an Olympus epi-fluorescence light microscope. Leaf pieces were immersed in a 0.1% solution of Calcofluor in 100 mM Tris-HCl buffer, pH 8.5 (Butt *et al.*, 1989; Kuck *et al.*, 1981). After 1 to 2 min in the dye solution, samples were briefly rinsed in water and mounted on glass slides. Specimens were examined with ultraviolet epi-illumination (excitation filter 330–385 nm, dichroic mirror 400 nm, barrier filter 420 nm) that resulted in bright blue fluorescence of the walls of fungal conidia, germ tubes and appressoria.

Sample preparation for scanning electron microscopy.—Inoculated leaf pieces were fixed overnight at 4 C in 2.5% glutaraldehyde in 100mM potassium phosphate buffer, pH 6.8. The tissue was then rinsed in 50 mM buffer and post fixed in 1% OsO₄ in 100 mM buffer for 2 h at 4 C (Mims, 1981). Following thorough rinsing in distilled water, specimens were dehydrated in a graded ethanol series to 100% ethanol. Leaf pieces were critical point dried with carbon dioxide as the transition fluid, mounted on specimen stubs, and sputter coated with gold-palladium. Conidia, germ tubes, and appressoria on leaf surfaces were examined with a Hitachi S-405A scanning electron microscope operating at 15 kV.

Sample preparation for transmission electron microscopy.—Leaf pieces exhibiting symptoms of red leaf spot were fixed and osmicated as described above. Following thorough washing in distilled water, specimens were stained in 0.5% aqueous uranyl acetate overnight at 4 C. After rinsing in water, tissue was dehydrated in a graded ethanol series to 100% ethanol, then transferred to 100% acetone and infiltrated with Spurr's resin (Spurr, 1969). Specimens were embedded in Lux Contur Permanox (Miles Lab-Tech) disposable tissue culture dishes (60×15 mm) filled with a 3–4 mm layer of fresh, 100% resin (Taylor and Mims, 1991). The resin was polymerized for 24 h at 70 C. Ultrathin 80–90 nm sections were cut anticlinal to the leaf surface with a diamond knife (Diatome) on a Sorvall MT2-B microtome. Sections were collected on formvar-coated slot grids (Rowley and Moran, 1975), post-stained for 3 min each with 4% aqueous uranyl acetate and lead citrate (Reynolds, 1963) and examined with a Hitachi HS-9 transmission electron microscope operating at 60 kV.

RESULTS

Cultural features of *Tubakia dryina* agreed with previous descriptions (Glawe and Crane, 1987; Yo-

FIG. 1. Scanning electron micrograph of a germinating conidium (C) of *T. dryina*. The short germ tube terminates in an appressorium (A). The basal frill located on the conidium (arrowhead) is visible. The septum separating the appressorium from the germ tube is shown at (S). Scale bar = 5 μ m.

koyama and Tubaki, 1971). On potato dextrose agar the fungus produced a white septate mycelium and abundant conidia. Olivaceous rings of spores were formed by loosely organized masses of hyphae (sporodochia) associated with thick mucilage. The spores were single-celled and ellipsoidal in shape with a characteristic basal frill (FIG. 1) marking the point at which disjunction from the conidiogenous cell occurred (Yokoyama and Tubaki, 1971). Conidia measured 10–11 μ m $\times$ 6–7 μ m. Microconidia (Yokoyama and Tubaki, 1971; Holdenrieder and Kowalski, 1989; Munkvold and Neely, 1991) were not produced by our isolates of *T. dryina* as cultured in this study.

Following dispersal of conidia onto healthy leaves, epi-fluorescence light microscopy and scanning electron microscopy were used to monitor spore germination, germ tube growth, and penetration structure formation. As observed by Munkvold and Neely (1991) and Zhang and Walker (1995), the majority of conidia had begun to germinate by 24 h post-inoculation. Consistent with the observations of Yokoyama and Tubaki (1971), spores were able to germinate from any part of the conidial surface. Germ pores were not evident. Germ tubes appeared to emerge more often from the center of the spore than from either end (FIG. 1). The germ tubes were closely appressed to the leaf surface. These structures were septate, sometimes branched, and variable in length.

Appressoria were observed. These penetration structures were oblong in shape and separated from

FIGS. 2, 3. Transmission electron micrographs of healthy sweet gum leaf tissue. 2. Palisade parenchyma cells containing electron-dense, tannin-like deposits (T) in their vacuoles. N = plant cell nucleus. scale bar = 5 μ m. 3. Spongy parenchyma cell with granular deposits of tannin-like material (T) in the vacuole. C = host cell chloroplasts. Scale bar = 2 μ m.

the germ tube by a septum (FIG. 1). Shorter germ tubes appeared more likely to terminate in appressoria than germ tubes of considerable length.

Appressoria of *T. dryina* formed directly over the epidermal cell walls of sweet gum. Even when spores were inoculated onto the lower leaf surface, where stomata were present, appressoria were not observed to form over these natural openings. On both upper and lower leaf surfaces the fungus apparently attempted to penetrate the leaf tissue directly. Artificially inoculated plants were maintained in the greenhouse and developed symptoms of red leaf spot within 2–3 wk.

Transmission electron microscopy was used to monitor the interaction between host and pathogen cells following infection. Healthy cells of sweet gum contained varying amounts of electron-dense material in their vacuoles (FIG. 2, 3), tentatively identified by their ultrastructural appearance as tannins (Ledbetter and Porter, 1970). These deposits varied in appearance from extremely dense (FIG. 2) to looser and more grainy (FIG. 3). Quite often the vacuole was completely filled with this material. These deposits were most extensive in the palisade and spongy parenchyma cells of the mesophyll, but also occurred

in epidermal cells and in parenchyma cells associated with the vascular tissue.

In infected leaf tissue, hyphae of the fungus ramified extensively between and within host cells (FIGS. 4–7). Epidermal and mesophyll cells were penetrated (FIG. 4), as were cells of the leaf veins (FIG. 5). The presence of tannin-like material did not affect the ability of the fungus to enter and colonize cells of the host (FIG. 6). Fungal hyphae were able to penetrate plant cell walls (FIG. 7). Several ultrastructural changes associated with host cell degradation and symptom development were observed. These abnormalities included vacuolation of the cytoplasm, collapse of cell walls, and breakdown of organelles (FIGS. 4–7).

As host cells died and a necrotic lesion developed, the fungus continued to grow on the dead plant material. Leaf material prepared at the time of sporulation revealed extensive development of mycelium within the necrotic plant cells.

DISCUSSION

Results of this investigation indicate that *T. dryina* can penetrate leaf tissue directly, thus having the ability to initiate infection on both upper and lower leaf

FIGS. 4–7. Transmission electron micrographs of sweet gum leaf tissue infected with *T. dryina*. 4. Inter- and intracellular hyphae (arrowheads) of the fungus associated with host epidermal (E) and palisade parenchyma (P) cells. Scale bar = 10 μm . 5. Infected leaf vein. Fungal hyphae (arrowheads) have colonized host cells associated with xylem elements (x). Scale bar = 5 μm . 6. Spongy parenchyma cells with tannin-like deposits (T) that have been invaded by fungal hyphae (H). Scale bar = 4 μm . 7. Penetration of the host cell wall (W) by hyphae (H) of *T. dryina*. C = disintegrating chloroplast. Scale bar = 2 μm .

surfaces. In this respect, *T. dryina* is similar to other imperfect fungi causing leaf spot diseases, including *Colletotrichum lindemuthianum* (O'Connell and Bailey, 1991) and *Guignardia bidwellii* (Luttrell, 1974).

Our results contradict those of Munkvold and Neely (1991), who examined infection structure formation on northern red oak (*Quercus rubra*). These authors identified appressorium-like structures that formed both over host stomata and directly against the cuticle. They also observed germ tubes that grew directly into stomatal openings without the formation of an appressorium. We speculate that stomatal penetration may be such a rare event that it was not observed in the ± 200 germinated spores examined, or that isolates of *T. dryina* from oak and sweet gum are distinct in terms of germ tube behavior. The latter hypothesis is supported by the fact that isolates from oak have proven to be nonpathogenic on sweet gum (Munkvold and Neely, 1990), an indication that host-specific populations of this fungus are present in nature.

Although the pathogenicity of *T. dryina* to sweet gum has been questioned (Munkvold and Neely, 1990), we contend that isolates from East Texas are pathogenic to this host. This conclusion is based on the observations that pycnothyria of *T. dryina* were the only types of fruiting structures noted on infected leaves in the field, and that the characteristic symptoms of red leaf spot were produced on inoculated plants in the greenhouse. Other fungi, in particular *Pestalotia* sp., were cultured from the larger lesions, but most likely invaded the necrotic tissue in a saprophytic manner. In other regions of the U.S., such as Illinois where Munkvold and Neely (1990) conducted their research, sweet gum populations may not be as susceptible to local populations of this fungus. Environmental stress factors may also be involved, as reports have indicated that herbicide application (Zhang and Walker, 1995) and chlorosis caused by iron deficiency (Munkvold and Neely, 1990) may increase susceptibility to *T. dryina*.

Tannins have been reported to occur in healthy tissue of a variety of plants (Ledbetter and Porter, 1970; Chafe and Durzan, 1973; Mace and Howell, 1974; Mosjidis, et al., 1990) including sweet gum (Neera, et al., 1993). They may prevent pathogen invasion by inhibiting fungal enzyme activity (Ledbetter and Porter, 1970; Mace and Howell, 1974; Mosjidis et al., 1990). Although cells of healthy sweet gum tissue appear rich in tannins, these materials apparently were not effective in preventing fungal colonization by *T. dryina*.

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