Infection Process of *Discula destructiva*, the Causal Agent of Dogwood Anthracnose, and Resistance Mechanism of Flowering Dogwood

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ABSTRACT

Discula destructiva, the causal agent of dogwood anthracnose, has caused severe mortality in dogwood over the last 30 years. Although considerable research has been done with dogwood anthracnose, the infection process by *D. destructiva* is still obscure. A resistant cultivar of *Cornus florida*, 'Appalachian Spring', was discovered and released by the Tennessee Agricultural Experiment Station. However, the resistance mechanisms are unknown. The objectives of this research were 1) to determine the sequence of events in the infection process of *D. destructiva* in *C. florida* and 2) to determine how host resistance affects infection events of *D. destructiva* on flowering dogwood.

At 3 days after inoculation (DAI), majority of conidia germinated and hyphae were observed on the leaf surface. Direct penetration by *D. destructiva* hyphae was observed without appressorium formation. At 8 DAI, hyphae were aggregated between the cuticle and epidermis and grew intracellularly in epidermal cells, palisade parachyma, and spongy mesophyll cells. At 16 DAI, chloroplasts were intact but decompartmentalized and infection sites were clearly defined. Acervuli were detected at 20 DAI and were fully developed at 24 DAI on adaxial and abaxial leaf surfaces. Sporulation (ruptured acervuli) was observed at 20 DAI. This clear understanding of the infection process can be used to look for resistance mechanisms in dogwood germplasm. A resistant line would expect to slow or inhibit one or more infection events.

There was no statistical difference between the percentages of germinated conidia on susceptible and resistant cultivars of flowering dogwood one day after inoculation (DAI). However, the resistant cultivar, 'Appalachian Spring', significantly suppressed the growth of *D. destructiva* conidial germ tubes at 2 DAI, 3 DAI and 4 DAI when compared

to conidial germ tubes on leaves of the susceptible cultivar ‘Cloud 9’. Observed resistance may be due to smoother wax crystals on adaxial leaf surface and significantly thicker cuticle observed on leaves of ‘Appalachian Spring’. An unknown compound, observed highly concentrated in resistant but lower in susceptible cultivars, may be important as a resistance mechanism. These strategies reduced the inoculum potential of *D. destructiva* and play important roles in why ‘Appalachian Spring’ is resistant to dogwood anthracnose. These results provide new ways to use conidia germination test and germ tube growth measurement for detecting resistant cultivars.

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Chapter I. Literature Review

1.1 The host, *Cornus florida* L

Flowering dogwood (*Cornus florida* L) is a member of the Cornaceae family, which consists of a dozen genera and 100 species. Approximately 60 *Cornus* species occur in temperate regions of North America, Europe and Asia (34). Most are small trees and shrubs. Dogwood species are separated into two groups based on the presence of bracts and fruit color. The blue or white- fruited lines, which are native to North America, have bracts that are rudimentary or lacking (34). While the red-fruited lines have basal bracts exist. All dogwood leaves are distinguished by veins that curve toward the leaf tip (4).

Flowering dogwood is a native under-story tree whose range extends from central Florida to southern New England and westward to eastern Texas and northward to southern Michigan (31). The tree can reach 15 m in height and have trunk diameters of 0.5 m (32). Flowering dogwood grows in soil varying from well-drained, light upland soils to deep, moist soils along stream banks (5).

Flowering dogwood produces mostly white bracts in the wild (83). The tree plays a critical role in the cycling of calcium where calcium accumulates in its leaves and is released for use by other plants during decomposition of dogwood leaf litter (74). Flowering dogwood also provides habitat and food for wildlife including bobwhite quail, ruffed grouse, wild turkey, songbirds and raccoons (68). Nutritional value of the ovid scarlet drupe fruit consists of 1.1% calcium, 16.7% fat, 5.5% protein, 24.6% fiber, 0.6% phosphorus and 5.0% ash (51, 57). Furthermore, flowering dogwood is valued for its bloom in the spring and its red foliage and scarlet berries in the fall. In 1998, wholesale

dogwood sales for the United States were up to $25 million (58). In Tennessee sales of dogwood have been estimated at $30 to $40 million annually (73).

There are over 100 cultivars of *C. florida*. In 1958, a *C. florida* cultivar named ‘Cherokee Chief’ became the first patented red-bracted cultivar (18). Some white-bracted *C. florida* cultivars include: ‘Appalachian Spring’, ‘Barton’, ‘Cherokee Princess’, ‘Cloud 9’, ‘Ozark Spring’ and ‘White Cloud’. *Cornus florida* ‘Comco No. 1’, ‘Purple Glory’, ‘Red Cloud’, Red Pygmy ®, ‘rubra’, ‘Sunset’, ‘Sweetwater Red’ etc. have pink bracts (18). *Cornus florida* ‘Plena’ has up to 16 bracts with some full and broad, others twisted and barely visible. Another cultivar named ‘Fragrant Cloud’ is reputed to have a sweet fragrance when in flower.

1.2 Dogwood anthracnose

Disease Distribution

Dogwood trees have been threatened by dogwood anthracnose (*Discula destructiva* Redlin [65]) for more than thirty years. In 1976, the disease was first observed on the pacific dogwood, *C. nuttallii* Audubon, in western Washington (15). By 1983, the disease had spread to Connecticut, New Jersey and Pennsylvania and was referred to as dogwood anthracnose (28). Since the first report, the disease has spread throughout most of the range of the Pacific dogwood (28) and much of the range of flowering dogwood north of Interstate 20 (19, 42, 46, 71).

Trigiano et al. (76) suggested that *D. destructiva* was an introduced pathogen; Caetano-Anolles et al. (16, 17) revealed that fungal populations were highly homogeneous and concluded the pathogen of dogwood anthracnose did not evolve

directly from a population related to other North American *Discula* sp. Yao (88) observed that banding profiles of double stranded RNA were very different between eastern and western isolates and suggested that they might have different origins.

Mielke and Langdon (56) evaluated the health of flowering dogwood populations in Catoctin Mountain Park in Maryland and attributed the observed 33% mortality of flowering dogwood to dogwood anthracnose with only 3% of the trees were observed to be disease free. Another survey, using the same plots, conducted in 1988, found that dogwood mortality had risen to 79% (70). In 1994, Sherald et al. (72) examined the same survey plots and established new plots in the general area. The number of dogwood stems/acre declined from 395 in 1988 to 82 in 1994. Of the 82, only 56 were alive (resulting in a 94% decrease in total stems/acre since the original 1984 survey). Natural seedling regeneration in the park has not occurred due to heavy deer feeding and a shortage of other trees to serve as pollen sources.

Taxonomy

The pathogen of dogwood anthracnose was first classified as *Gloeosporium corni* Green (15). When dogwood anthracnose was first observed in the northeastern U.S., the causal agent was identified as *Colletotrichum gloeosporioides* Penzing (63). Daughtrey and Hibben (27) confirmed *Discula* sp. as the pathogen of dogwood anthracnose in flowering dogwood in 1983. Scott Redlin officially named the pathogen as *Discula destructiva* Redlin in 1991 (65).

Discula destructiva produces acervular conidiomata which contain one-celled conidia (29). The acervuli develop below the trichomes, are found in the necrotic tissues of leaves and stems and serve as overwintering structures on twigs and unabscised leaves

(55). This acervulii-trichome association has not been observed for other *Discula* species (66). The conidia are 7-12μm long and 2.5-4.0μm in diameter and contain one or two polar oil droplets (Figure 1-1) (65). Conidia may exude from cirrhi in conditions of low relative humidity. A sexual stage has not been observed. Redlin (65) postulated that if the sexual stage existed, it would likely be classified as *Apiogonomia* or *Gnomoniella* and would be in the order Diaporthales. A phylogenetic study has determined that *Discula* belongs to the order Diaporthales (90).

Signs, Symptoms and Disease Cycle

Dogwood anthracnose lesions may develop on any part of the leaf (12) and lesions often have a red to purple border with a chlorotic halo (27). Purple-rimmed lesions may enlarge throughout the spring months. Necrotic lesions may enlarge until the leaf is entirely blighted. Blighted leaves appear scorched and hang on the tree throughout summer, fall and winter seasons (13). The fungus invades and colonizes young branches through the vascular tissue and may eventually infect the trunk of the tree (80). Leaf blight and twig death lead to a loss of apical dominance and epicormic shoots may develop. Annual cankers often develop at the base of epicormic shoots. Trees may die within one to five years depending on age, size, vigor and the tree's environment (13, 25, 27, 41, 49, 69). Infected trees are at greater risk to being susceptible to dogwood borer in sunny areas (81), armillaria root rot (26), and possibly other pathogens that invade stressed trees.

Fungal hyphae penetrate leaves directly without requiring a wound or stomata (38). Walkinshaw and Anderson observed that often the pathogen enters a leaf, invading inter-and intracellularly (80). Necrosis in leaves without visible hyphae indicates that a

toxic metabolite may be involved in lesion formation (80). Venkatasubnaiaha and Chilton (79) isolated four phytotoxic phenols from culture filtrate of dogwood anthracnose and tested these metabolites on dogwood and weeds. Among these metabolites, the prenylated hydroxybenzoic acid and its acetate were the most toxic compounds.

Conidia of *D. destructiva* can be spread by splashing water over short distance. Long-distance dispersal may also be by insects (30). Convergent lady beetles were used to study relationships between insects and *D. destructiva* dissemination (22, 23). Convergent lady beetles were found to disseminate viable conidia for 16 days after exposure to conidiomata. Viable conidia could also be deposited in frass pellets of the beetles (40). In the natural environment, some arthropods can also carry viable conidia of *D. destructiva* and most of the conidia-infested arthropods were observed when sporulation and spread of dogwood anthracnose were increasing (43).

Effect of environment on dogwood anthracnose

Optimum conditions necessary for *Discula* infection included low light intensity and drought (33). However, Ament (2) conducted a water stress study to determine its effect on dogwood anthracnose severity on trees of *Cornus kousa* and *C. florida* trees and found that severe drought, moderate drought, flooding and watering daily with drainage (control) had no effect on disease development. In addition, fog at high elevations has enhanced dogwood anthracnose epidemics in the southern Appalachian region (85).

Microclimate of the canopy can influence the development of dogwood anthracnose. Evaporative potential and host population densities lead to an increased disease incidence (20, 21). Dogwood anthracnose was most severe in partially harvested stands and least prevalent in clearcuts (9). Foliage in clearcuts was drier and did not favor

fungal growth. Conversely, dense stands favored fungal growth because of high levels of shade and humidity. It is possible that fire suppression has made many stands more vulnerable to anthracnose than they might otherwise be. Furthermore, most foliage in the sun had purple-rims whereas necrotic lesions were more prevalent on foliage in the shady plots and necrotic lesions were more likely than purple-rimmed lesions to contain acervuli and have D. destructiva conidia present when the fungus was allowed to sporulate in moist chambers (61).

When overhead acid mist was applied to dogwood leaves, the percentage of foliage affected by *Discula* increased with decreasing pH (3). Britton et al. (8) concluded that nutrient available change rather than foliar damage was responsible for the increase in disease severity when foliage was treated with acid rain. Trichome shrinkage at pH 5.0 and below occurred with accompanying tissue damage, which may facilitate nutrient metabolic leakage of host dogwood tissue and may be a site for fungal spore germination and subsequent penetration (75).

Management of dogwood anthracnose

Cultural control methods have been developed for dogwoods in nursery and landscape settings, including planting resistant trees, optimum fertilization, trickle irrigation and adequate sunlight, watering during drought periods, avoiding sites near water, pruning and removing dead or diseased shoots and avoiding mechanical and chemical injury (33, 37, 86). However, in forest environments, none of these strategies are feasible.

Prescribed burning is a management technique that could alleviate pressure from dogwood anthracnose but has not been fully researched. In a long-term vegetation

monitoring study conducted in the Great Smoky Mountain National Park, Jenkins and White (46) reported an increased number of dogwood stems on three plots after a 1976 wildfire. It is reasonable to assume that this wildfire was intense due to the relatively open nature of the burned area. These three plots had lower total basal area and tree density compared with unburned plots, which resulted in increased sunlight and air movement in the understory.

Planting resistant species of dogwood has been another alternative used as a control measure. Santamour et al. (69) concluded that flowering dogwood was highly susceptible to dogwood anthracnose and resistance would be nearly impossible to find. Ranney et al. (64) and Brown et al. (11) observed resistance in mostly blue berry / bractless *Cornus* species. Native species found to be resistant to dogwood anthracnose included *C. racemosa* and *C. canadensis* (28), and *C. amomum*, *C. alternifolia*, and *C. mas* (11). In addition, a resistant cultivar of flowering dogwood 'Appalachian Spring' was discovered at Catoctin Mountain Park in Maryland and has been released as an ornamental cultivar (87).

Fungicides can also be used in controlling the disease. Protectant fungicides such as chlorothalonil (e.g. Daconil 2787®), mancozeb (Dithane M-45®, Fore) and thiophanate-methyl (Cleary's 3336®) must be applied every 7 to 14 days, when leaves begin to emerge and throughout the early growth. Systemic fungicides such as propiconazole (Banner Maxx®) and mycobutanil (Eagle, Systhane) are also effective. Additional sprays may be needed in northern climates or in unusually cool and wet summers (6, 7, 84).

1.3 Plant disease resistant mechanisms

Plants can display a remarkable ability to defend themselves from pathogens. A classic distinction is usually made between pre-existing barriers and defenses induced by the plant when pathogen interactions occur. These phenomena led to the concept of innate and induced resistance. The following paragraphs will briefly describe major defense reactions that are activated in plants by attacking pathogens.

Structural barriers

The cuticle covers all above-ground plant organs and by means of its hydrophobic nature restricts the loss of water and nutrients (53). The plant cell wall beneath the cuticle allows the generation of turgor pressure and provides the cell with its functional form. The cuticle and cell wall are the principal structures which protect the plant against injury from various kinds of biotic and abiotic stresses (67).

Composition and thickness of cuticle can vary in different species and among cultivars and are dependent on organ type, age of the plant and environmental conditions (78). Chemically, the cuticle can be described as cutin, a polyester embedded in wax (77). The cell wall consists of a thin primary wall and a thicker secondary wall. The primary walls are found exclusively in young, undifferentiated cells that may still be increasing in size. Secondary walls form after the cell has stopped growing. Cell walls are complex amalgams of carbohydrates (cellulose, hemicelluloses, and pectic polysaccharides), proteins, lignin, and encrusting substances such as cutin, suberin, and inorganic compounds (24). Structural barriers may be assumed to hinder the penetration process while host tissues develop active defense mechanisms.

Chemical barriers

Plants may have preformed substances that defend against pathogen attack. Antifungal compounds include enzyme inhibitors. These inhibitors include chemicals that can bind and inhibit ploygalacturonases (PG) of fungal origin (1), antimycotic substances such as cyanogenic glucosides which are broken down to release hydrogen cyanide (36,44,52), glucosinolates which exhibit toxicity, growth inhibition, or feeding deterrence to a wide range of potential plant enemies, including nematodes, bacteria and fungi (14, 34) and saponins which can complex with sterols in fugal membranes and cause loss of membrane integrity (47, 48, 60).

Activated defense reactions

Cell wall appositions have been studied in host resistance to pathogens. Cell wall alterations are rich in phenolic compounds and appear conspicuously at the site of infection and are often associated with resistance (54, 59). In many cases, papillae, a cell wall apposition laid down by the host at the site of attempted penetration by the fungus on the inside of the host cell wall, function as physical and chemical barriers to resist infection and are comprised of various inorganic and organic constituents, including callose and phenolic compounds (45, 82, 89).

Lignin can form a barrier to pathogen movement. Defense lignin refers to lignin deposited in response to pathogen invasion (59). Such lignin can be deposited over the entire wall of the infected cell or group of cells or only at the infection site.

Plants may also produce metabolites such as phytoalexins that can act as a defensive mechanism. More than 300 molecules have been identified as phytoalexins from approximately 900 species representing 40 plant families (39). Phytoalexins are

inhibitory to the invasion by pathogens and have variations in the rate of accumulation which causes a corresponding variation in the resistance of the plant. Many studies established a correlation between phytoalexin accumulation and resistance to disease, although correlative evidence has to be further tested (50).

Programmed cell death (PCD) occurs in multi-cellular organisms during normal physiological processes. This genetically controlled cell suicide is observed during development and in response to abiotic or biotic stresses. Hypersensitive response (HR) is a form of PCD in plants. HR is characterized by rapid collapse and death of the plant cells in and around the site of attempted infection. HR helps the plant to confine the pathogen and prevents it spreading into healthy adjacent tissues, which works only for the biotrophic pathogens.

In host-parasite interactions involving non-biotrophic parasites, the situation seems to be more complex. Induction and breakdown or tolerance of defense mechanisms seem to occur simultaneously. A delicate balance between inducing and overcoming or withstanding induced defense mechanisms favors the pathogen in cases of compatibility and favors the host in cases of incompatibility. The assessment of this kind of resistance is usually based on three aspects: infection frequency, latent period and spore production (62).

In conclusion, plants can defend against pathogen invasion through different perspectives from physical to chemical. For a particular host pathogen interaction such as flowering dogwood and dogwood anthracnose, the resistant mechanism may be just one of these mechanisms or a combination of mechanisms.

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Appendix



Fig. 1-1. Conidia of *Discula destructiva* with bi-polar oil droplets. Bar = 10 µm.

Chapter II. Histological Investigation of Infection Processes of *Discula destructiva* on Leaves of *Cornus florida*

Abstract

The infection process of *Discula destructiva* Redlin on *Cornus florida* L leaves was studied histologically. Penetration of hyphae through natural openings and wounds was not observed, while direct penetration without appressorium formation was discovered at 3 days after inoculation (DAI). Leaves inoculated with *D. destructiva* developed symptoms of dogwood anthracnose after 7 to 8 days. From histological point of view, at 8 DAI, hyphae were aggregated between the cuticle and epidermis and grew intracellularly in epidermal cells, palisade parachyma, and spongy mesophyll cells. At 16 DAI, typical necrotic chlorotic halos with red to purple border were formed on the inoculated leaves. Inside the tissue, at 16 DAI, chloroplasts were intact but decompartmentalized and infection sites were clearly defined. Acervuli were detected at 20 DAI, ruptured the plant cuticle and fully developed at 24 DAI on adaxial and abaxial leaf surfaces. Sporulation (ruptured acervuli) was observed at 20 DAI.

Introduction

Native flowering dogwood (*Cornus florida* L) is a small tree reaching 10 meters in height and is found throughout much of the eastern United States (2,7). Flowering dogwood provides nutrition for over 50 wildlife species in the forest understory (15). It is also valued as an ornamental tree (2,20).

In the late 1970s, high dogwood mortality was reported in the northeastern United States (5,9,10). In 1983, the disease was attributed to infection by a *Discula* species and other environmental phenomena (11). Redlin (13) named the pathogen

Discula destructiva Redlin in 1991. *Cornus* species susceptible to dogwood anthracnose include *C. controversa, C. florida, C. kousa* 'Chinesnsis', *C. nottallii,* and *C. sericea* (3,12,19). The most obvious symptom is leaf blight, which begins as a purple bordered lesion with tan (necrotic) centers. Infected young branches may lead to stem cankers. Multiple cankers may girdle cambial tissues, leading to tree death (2,6,13).

Interactions between *D. destructiva* and *C. florida* have been investigated by light microscopy (9,14) and scanning electron microscopy (4,13,14). The morphology of acervular conidiomata of *D. destructiva* on leaf disks of *C. florida* were described and evidence for direct penetration of dogwood leaves without requiring a wound or stomata were provided (8). Although studies suggested *D. destructiva* invaded tissues inter- and intracellularly (18), the sequence of infection events is obscure. In order to investigate the mechanisms of resistance to dogwood anthracnose in *C. florida,* it is essential to understand early stages of the pathogenesis, including conidial germination, infection structure formation, penetration and ramification. The objective of this research was to describe the sequence of events in the infection process of *D. destructiva* in *C. florida*.

Materials and Methods

Plant Material:

Fully expanded healthy leaves were collected from *Cornus florida* L (flowering dogwood) 'Cloud 9' which is susceptible to dogwood anthracnose (12), growing in a greenhouse at the University of Tennessee at Knoxville. Leaves were washed with distilled water and cut into 1 cm diameter leaf disks which were placed adaxial surface up

on two layers of moistened P8-creped filter paper (Fisher Scientific, Pittsburgh, PA) in 10 cm diameter Petri dishes (Fisher Scientific, Pittsburgh, PA).

Isolate Collection and Isolation:

Ten isolates of *D. destructiva* were obtained from leaves and twigs of diseased trees located at the University of the South (Sewanee, TN 35°12'32.22" 85°55'16.74") in March 2008. Diseased leaf and twig samples were collected from the trees, placed in labeled sterile polyethylene bags and transported to the lab.

Moisture chambers were prepared using Petri dishes containing filter paper that was moistened with distilled water. About 40 stems (1 cm long) and 20 leaf pieces were placed for up to 2 weeks at 20°C in darkness until cirri appeared on acervular conidiomata. Spore morphology was used to identify fungi as *D. destructiva* (14). *Discula destructiva* isolates were transferred using sterilized insect needles and colonized onto potato dextrose agar (PDA; Difco Laboratories, Detroit,MI) amended with 30mg/L each of chlortetracycline and streptomycin sulfate (ICN Biochemicals, Cleveland, OH). Isolates of *D. destructiva* were confirmed using the gallic acid test as described by Trigiano et al. (16), where *D. destructiva* was differentiated from other *Discula* species by turning the gallic acid medium brown. Cultures were incubated at 20°C with a diurnal 12 hour light/ dark cycle until sufficient growth occurred for cultures.

Inoculum and Inoculation:

A single agar plug from one randomly selected *D. destructiva* isolate was placed on a piece of sterilized dogwood leaf situated on PDA with its adaxial surface up in a Petri dish. Approximately 50 Petri dishes were prepared. Three weeks later, conidiomata containing conidia-masses were observed underneath trichomes. Conidia were collected

by submerging these leaves in sterile distilled water and gently rubbing the leaf surface with a sterile '7' shaped glass stick. Conidial suspensions were adjusted to 8×10^4 conidia per ml. Conidial suspensions were applied to leaf surfaces of fresh dogwood leaf disks in Petri dishes with a mist fingertip sprayer after leaf disks were wounded using a home-made wound tool that consisted of a cork stopper layered with #1 insect pins (Figure 2-1). After inoculation, the Petri dishes were sealed with parafilm (Pechiney Plastic Packaging, Menasha, WI) and incubated at 20°C with a diurnal 12 hour light/ dark cycle.

In order to examine the vigor and germination percentage of the conidia, 100 random conidia on 3 randomly chose leaf disks were counted to check whether the conidium germinated or not at 1 DAI, 2 DAI and 3DAI, respectively. As the control, conidial suspensions were also spread on PDA media and 100 random conidia on 3 randomly chose PDA Petri dishes were also examined at 1 DAI, 2 DAI and 3DAI, respectively.

Tissue Preparation for Histology

Ten random leaf disks were chosen per sampling period at 2, 4, 8, 16, 20 and 24 DAI. Disks were trimmed into smaller rectangular pieces (ca. 7×3 mm), then fixed in 50% FAA solution (Fisher Scientific, Pittsburgh, PA) in glass tubes for 2 days, dehydrated in a series of 50, 75, 85, 95% and absolute alcohol (Decon Labs, Inc., King of Prussia, PA), and embedded in paraffin (Paraplast® Oxford Labware St. Louis, MO). Serial 10 μm thick cross sections were cut using a rotary microtome. Slides were prepared by washing with detergent (Clorox, Clorox Professional Products Company, Oakland, CA) and rinsed with distilled water. Cross sections were expanded on a puddle

of distilled water at 42°C, and affixed to glass slides, without adhesive, by draining off water and drying for 12 h at 42°C. Sections were deparafinized through three changes of Microclear™ (Micron Diagnostics, Inc., Baltimore, MD) for 5 min each and rehydrated by moving slides 5 min each through a series of absolute ethanol, 95, 85, 70, 50, and 30% ethanol, and two deionized water rinses for 5 min each. The staining series consisted of 0.01% aqueous safranin O stain (Sciencelab.com, Inc., Houston, TX) for 24 hr, followed by two 1 min deionized water rinses, 1% crystal violet (Sciencelab.com, Inc., Houston, TX) for 1 min, followed by 5 min deionized water rinses. Sections were dehydrated using a graded series of ethanol in reverse order of the rehydration series previously described and then stained with fast green (Sciencelab.com, Inc., Houston, TX) for 1 min, followed by 1 and 5 min absolute ethanol rinses. Sections were then rinsed three times in Microclear™ with10 min submersion per rinse. Coverslips were attached to slides with permount (Fisher Scientific, Pittsburgh, PA) and slides were dried for 24 hr at 42°C. Sections were examined with a compound microscope (Nikon Instruments, Melville, NY). Approximately 10 slides, each with 20 leaf sections upon were viewed at each sampling period.

Tissue Preparation for Scanning Electron Microscope

Early stages of pathogenesis of the disease were examined using a scanning electron microscope (SEM). The tissue preparation protocol was used as described by Zachariah and Pasternak (21), wherein random leaf disks were chosen at 1, 2, and 3 DAI and fixed with chilled (4°C) 3% gluteraldehyde (Sigma-Aldrich, St. Louis, MO) in 0.05 M phosphate buffer (Fisher Scientific, Pittsburgh, PA) pH 6.8 and Histochoice® (Amresco, Solon, OH) for 1 hr at 20°C, followed by three washes each for 10 min, in

0.05 M phosphate buffer pH 6.8. Secondary fixation was in 2% osmium tetroxide (Sigma-Aldrich, St. Louis, MO) dissolved in 0.05 M phosphate buffer, pH 6.8, for 1 hr at 20°C. Fixed samples were dehydrated in a graded ethanol series (25, 50, 70, 95, 100% ethanol and 100% dry ethanol, 30 min for each). Samples were air dried, mounted on aluminum stubs and sputter-coated with gold palladium. Samples were examined by a FEI Quanta 200 scanning electron microscope (SEM) operating at an accelerating voltage of 10 keV. Five samples for each sampling time were examined using SEM.

Some paraffin embedded samples (3 samples for each sampling period) were also examined using SEM. These serial 10-µm-thick cross sections were attached to the special 1 cm diameter thin slides, deparafinized through three changes of Microclear^{TM} for 10 min each, air dried and sputter-coated with gold palladium. These samples were then examined under SEM.

Results and Discussion

Conidia were considered germinated when the germ tubes reached at least half length of the spore. One day after inoculation (DAI), germ tube primordial was observed with some conidia (Fig. 2-2A) but a majority (78%, Fig.2-3) had not reached the half length of conidia. At 2 DAI, most conidia (91%, Fig.2-3) had germinated (Fig. 2-2B). At 3 DAI, hyphae were seen on the leaf surface (Fig. 2-2B) and direct penetration of *D. destructiva* hyphae was observed (Fig. 2-4) without appressorium formation. Hyphae accumulated near epidermal cells prior to sporulation and these hyphal masses were usually located beneath the base of trichomes, confirming precious descriptions of Walkinshaw and Anderson (18). At 8 DAI, hyphae had accumulated between the cuticle

and epidermis and had grown intracellularly in epidermal cells, palisade parachyma, and spongy mesophyll cells (Fig. 2-5A, B). At 16 DAI, chloroplast dye color had shifted from light to bright red, which suggests that the chloroplasts were decompartmentalized and the infection sites were easily delimited. By contrast, in healthy tissues, dyed chloroplasts appeared light blue (Fig. 2-6A). Hyphae were observed near or inside most decompartmentalized cells (Fig. 2-5B). Acervulus primordia had developed by 20 DAI (Fig. 2-7) and acervuli were fully developed at 24 DAI (Fig. 2-8). Acervuli were observed on both adaxial and abaxial leaf surfaces. Acervuli were mostly formed at the bases of trichomes and were observed in both necrotic and living tissue. Sporulation through ruptured acervuli was observed at 20 DAI.

Four toxins, 4-hydroxy-3-(3'-methyl-2'-butenyl)benzoic acid, 4-hydroxybenzoic acid, (+)-6-hydroxymellein and (-)-isosclerone, have been isolated from culture filtrates of *Discula* isolates recovered from infected dogwoods (17). These toxins may be important in the pathogenesis of *D. destructiva* as evidenced by chloroplasts being damaged by hyphal contact with cells containing chloroplasts (Fig. 2-6). At the end of pathogenesis, plant tissues were disintegrated (Fig. 2-8). Toxins produced by necrotrophic pathogens are capable of breaking down host plant tissues, therefore releasing nutrients and these may explain above observations (1).

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Appendix

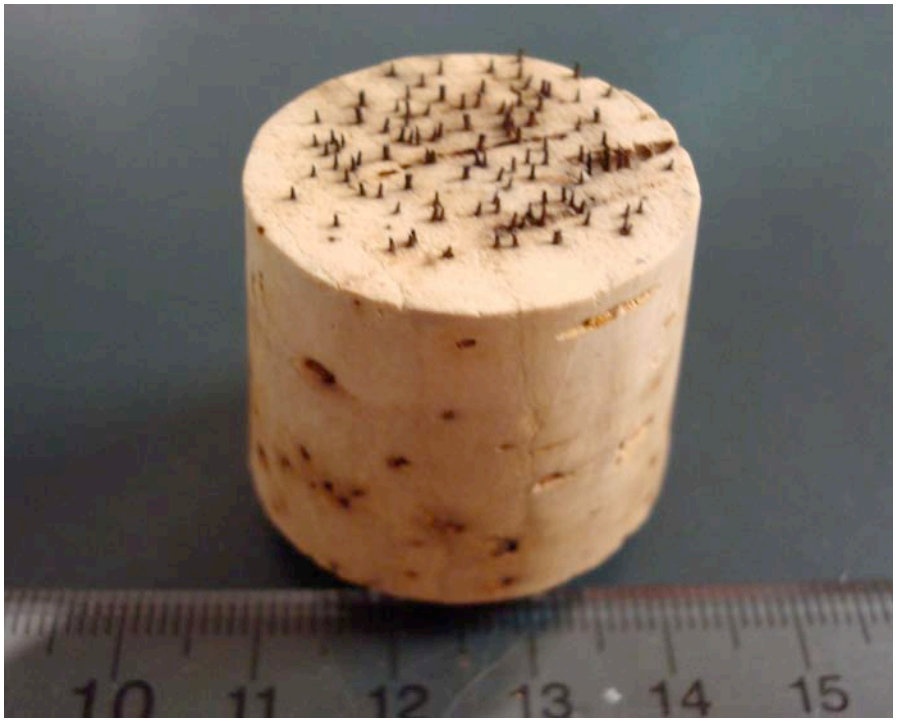


Fig. 2-1. Experimental wounding tool is a cork stopper embedded with #1 insect pins (16 pins/cm^2) used to abrade adaxial leaf surfaces.

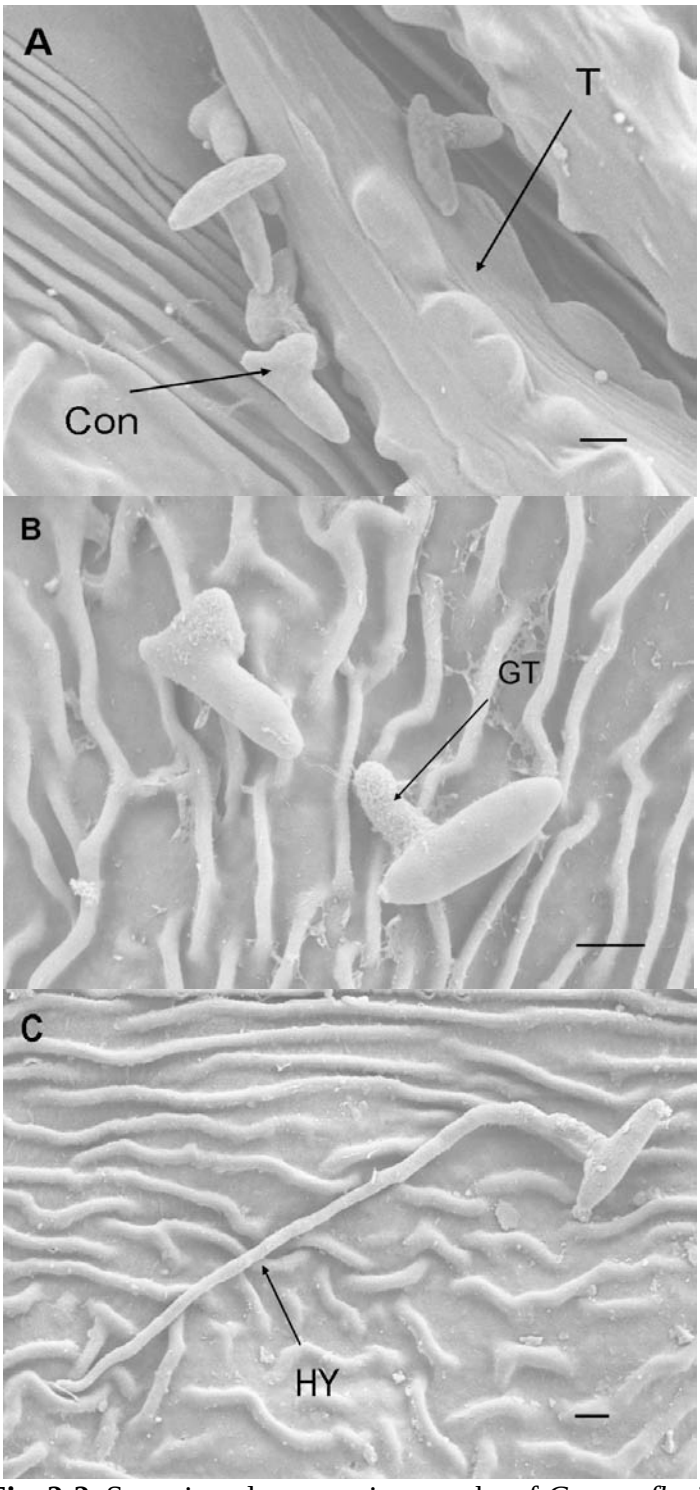


Fig. 2-2. Scanning electron micrographs of *Cornus florida* 'Cloud 9' leaves with *Discula destructiva* conidia. **A,** Conidia (Con) begin to germinate on the leaf surface underneath the trichome (T) 1 day after inoculation (DAI). **B,** Germinated conidia with germ tube (GT) on leaf surface 2 DAI. **C,** Hypha (HY) grows from conidia 3 DAI. Bar = 2 μm.

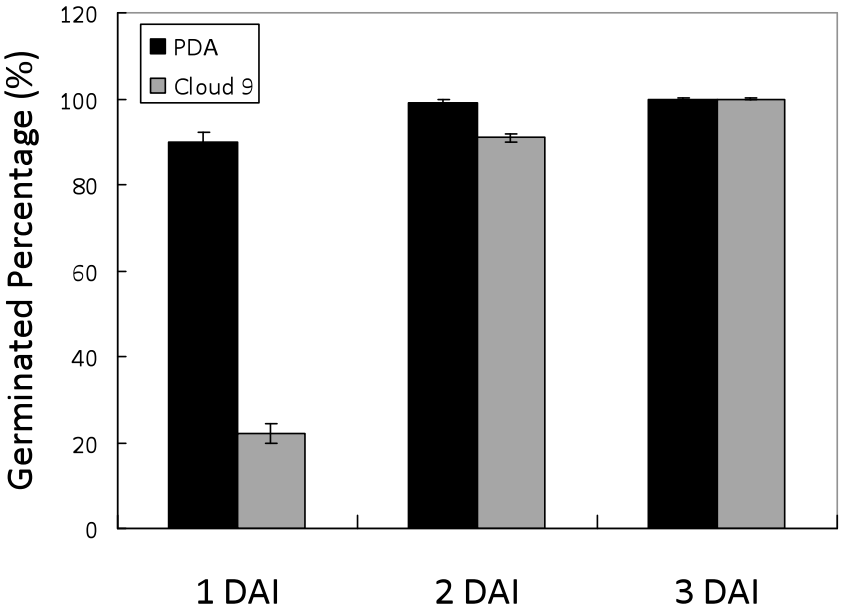


Fig. 2-3. Histogram of conidial germinated percentage at 1, 2 and 3 days after inoculation (DAI) on potato dextrose agar (PDA) and *Cornus florida* 'Cloud 9', respectively.

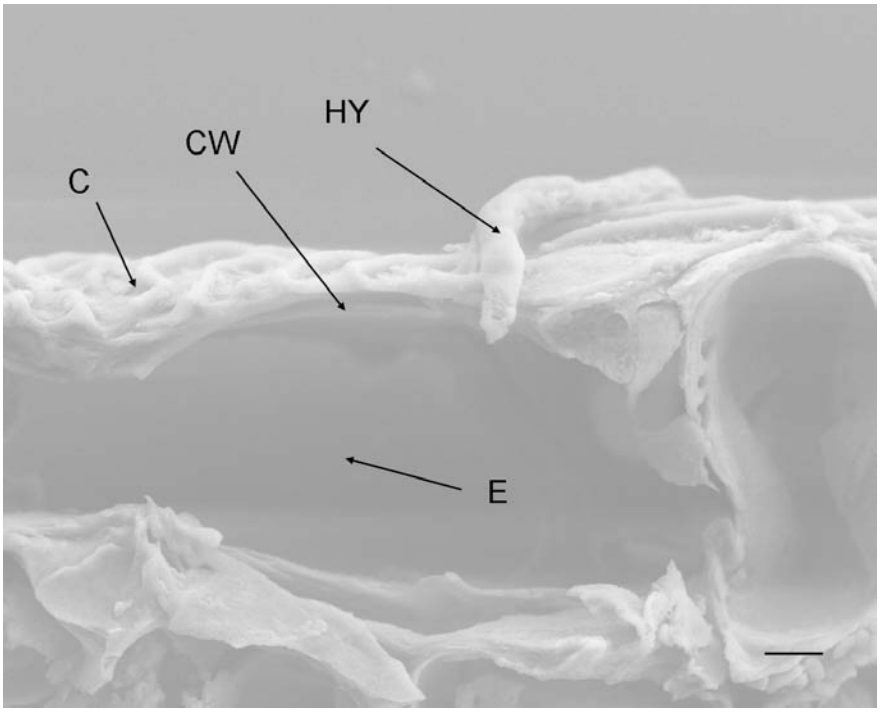


Fig. 2-4. Scanning electron micrographs of a *Discula destructiva* hypha (HY) directly penetrating through the *Cornus florida* 'Cloud 9' leaf cuticle (C) and cell wall (CW) into epidermal cells (E) at 3 DAI. Bar = 2 µm.

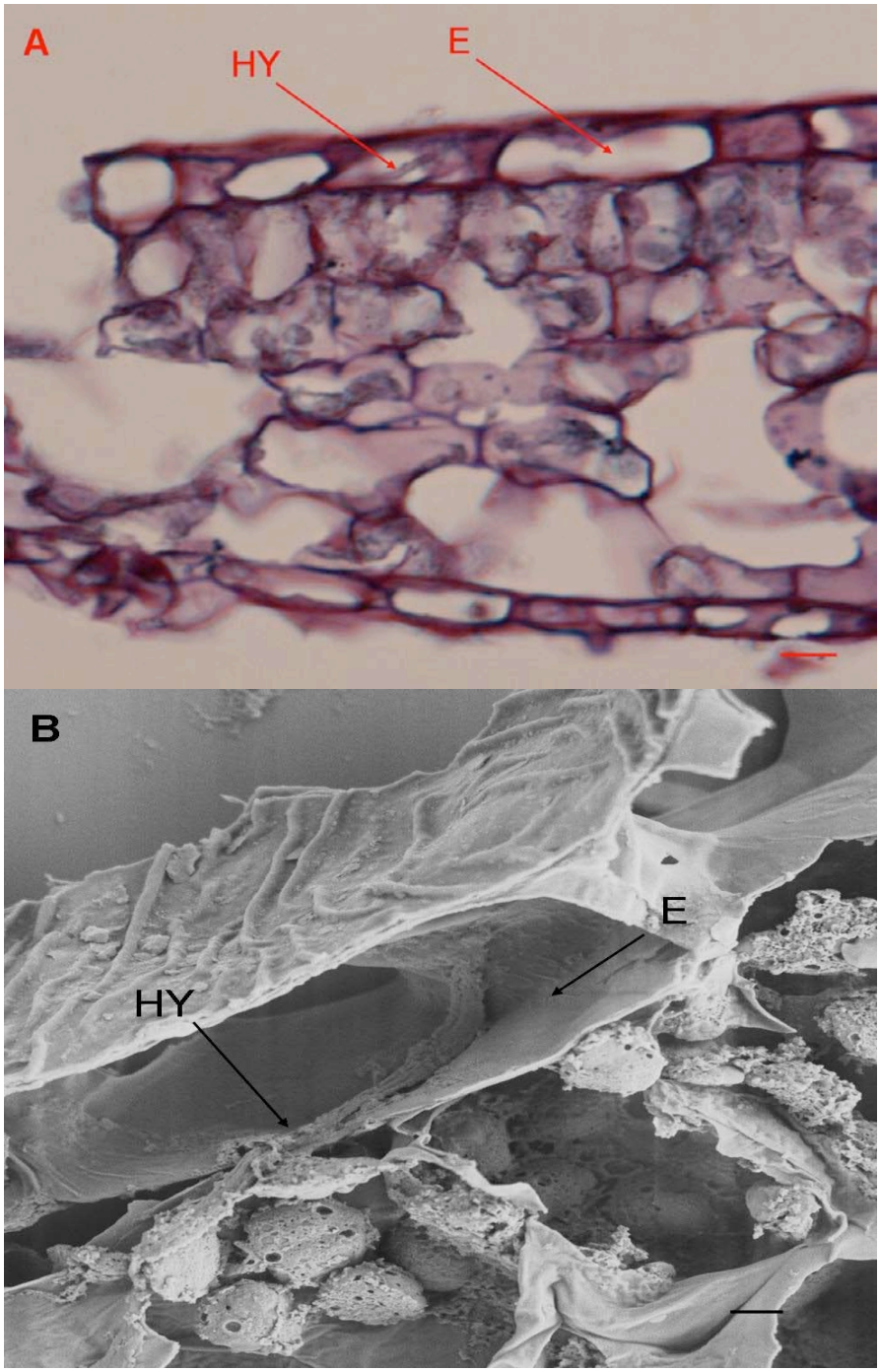


Fig. 2-5. A, B, Light micrographs and scanning electron micrographs, respectively, of cross sectioned *Cornus florida* 'Cloud 9' leaves inoculated with *Discula destructiva* conidia at 8 DAI. **A,** Hyphae (HY) are growing intracellularly within epidermal cells (E). Bar = 10 µm. **B,** Hypha (HY) is growing in epidermal cells. Bar = 2 µm.

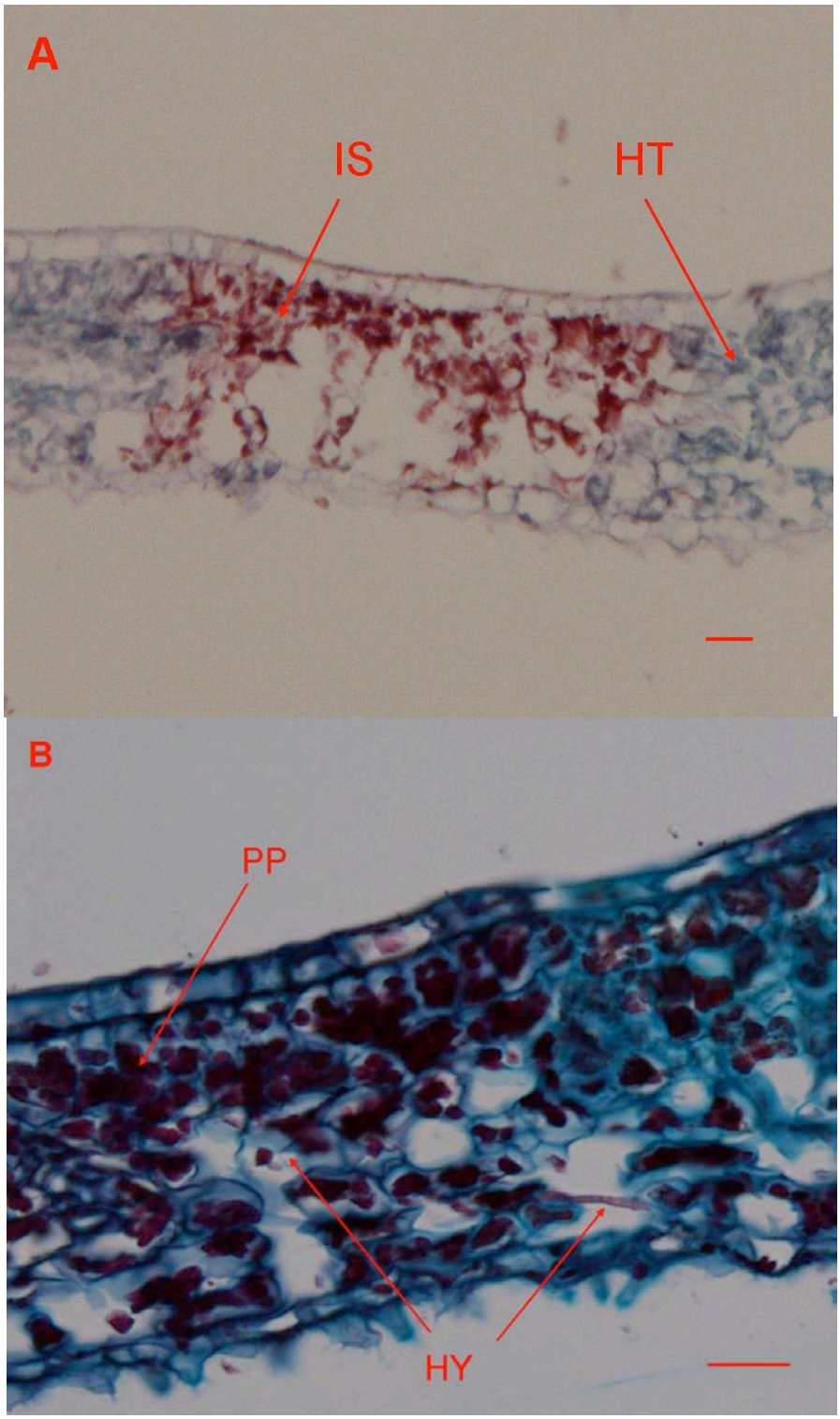


Fig. 2-6. Light micrographs of *Cornus florida* 'Cloud 9' leaves inoculated with *Discula destructiva* conidia at 16 DAI. **A**, Diseased infection sites (IS) and healthy tissue (HT). **B**, Typically diseased, palisade parenchyma (PP) were stained bright red using safranin O. Bar = 20 µm.

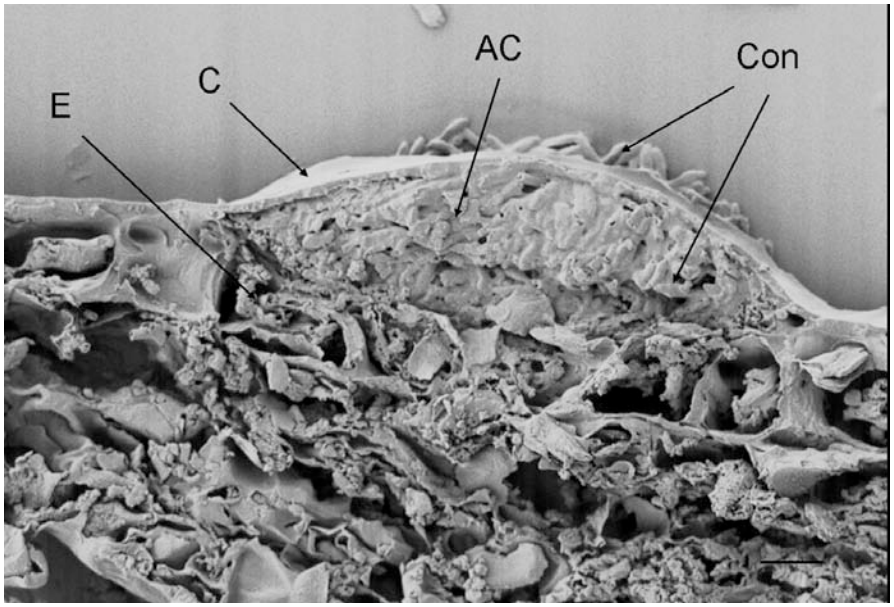


Fig. 2-7. Scanning electron micrographs of *Cornus florida* 'Cloud 9' leaves inoculated with *Discula destructiva* conidia at 20 DAI. Developing acervulus (AC) with conidia underneath the cuticle and above the epidermal cell layer (E). C, Cuticle; Con, Conidia. Bar = 10 μm.

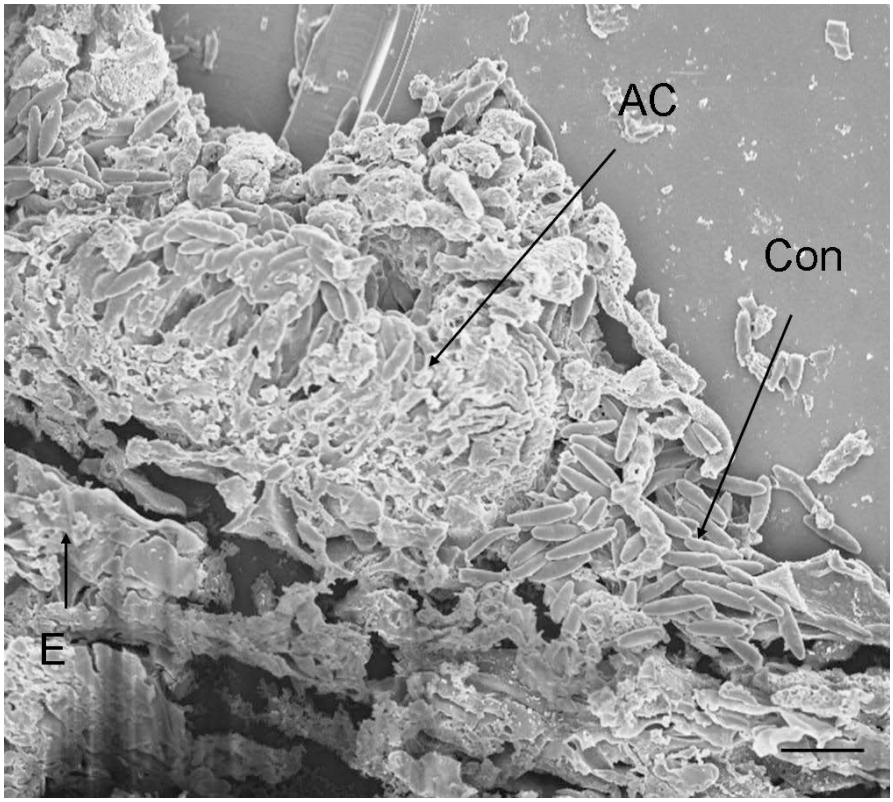


Fig. 2-8. Scanning electron micrographs of *Discula destructiva* fully developed on *Cornus florida* 'Cloud 9' leaf at 24 DAI. Note the crushed epidermal cell layer (E). AC, acervulus; Con, Conidia. Bar = 10 µm.

Chapter III. Comparison of Resistant and Susceptible Flowering Dogwoods in Early Stages of Pathogenesis of *Discula destructiva*

Abstract

Early stages of pathogenesis of *Discula destructiva*, the causal agent of dogwood anthracnose on flowering dogwood (*Cornus florida*), were studied using the susceptible cultivar ‘Cloud 9’ and the resistant cultivar ‘Appalachian Spring’. There was no statistical difference between the percentages of germinated conidia on ‘Cloud 9’ and ‘Appalachian Spring’ one day after inoculation (DAI). However, ‘Appalachian Spring’ significantly suppressed the growth of *D. destructiva* conidial germ tubes at 2 DAI, 3 DAI and 4 DAI when compared to conidial germ tubes on leaves of the susceptible cultivar ‘Cloud 9’. The smoother wax crystals of adaxial leaf surface and significantly thicker cuticle observed on leaves of ‘Appalachian Spring’ may contribute to observed resistance. In addition, resistant ‘Appalachian Spring’ also presents significantly thicker adaxial leaf cuticle than following susceptible cultivars, including ‘Appalachian Blush’, ‘Cherokee Princess’ and ‘Plena’, which suggests that a thicker cuticle may increase the resistance to the infection of *D. destructiva* since it enters the host through direct penetration. Furthermore, an unknown compound, observed at higher concentrations in ‘Appalachian Spring’ when compared to ‘Cloud 9’, may be important as a resistance mechanism. These discovered strategies reduced the infection potential of *D. destructiva* and therefore play important roles in why ‘Appalachian Spring’ is resistant to dogwood anthracnose.

Introduction

Flowering dogwood, *C. florida* L, is a native under-story deciduous shade tree with a geographic range extending from central Florida to southern New England, and

westward to eastern Texas and northward to southern Michigan (8). Mature trees can reach 15m high and have trunk diameters in excess of 0.5m (9). The tree naturally grows in soil varying from well-drained, light upland soils to deep, moist soils along stream banks (1).

Dogwood trees have been threatened by dogwood anthracnose, *Discula destructiva* Redlin [17], for more than 30 years. In 1976, the disease was first observed on the pacific dogwood, *C. nuttallii* Audubon in western Washington (4). By 1983, dogwood anthracnose had spread to Connecticut, New Jersey, and Pennsylvania and was referred to as dogwood anthracnose (7). Since the first report in 1970s, the disease has spread through most of the range of the Pacific dogwood and the northern central range of flowering dogwood (7). Flowering dogwood has also been severely impacted by dogwood anthracnose and significant mortality has occurred in the southeastern United States (5, 10, 12, 19).

Ranney et al. (16) and Brown et al. (2) observed resistance to dogwood anthracnose in some *Cornus* species, including some native North American species and shrub dogwoods like *C. racemosa, C. canadensis, C. amomum*, *C. alternifolia* and *C. mas* (2, 7). In addition, differences in levels of disease resistance have also been observed among flowering dogwood lines (20). A resistant cultivar of flowering dogwood ‘Appalachian Spring’ was discovered at Catoctin Mountain Park in Maryland and has been released as an ornamental cultivar (20).

Objectives of this study were to compare two flowering dogwood cultivars (‘Appalachian Spring’ and ‘Cloud 9’) with differing levels of resistance to dogwood anthracnose for spore germination and germ tube initiation of *D. destructiva* and to assess

characteristics of leaf cuticle (cuticle thickness, etc.), as a potential contributor to disease resistance mechanisms in dogwood cultivars.

Materials and Methods

Plant Material:

About 10 fresh, fully-expanded leaves (4th leaf from top) were collected from both a susceptible dogwood cultivar, 'Cloud 9', and a resistant cultivar, 'Appalachian Spring'. Leaves were taken from trees that were 2-year old, grown in containers, and held under similar environmental conditions in a greenhouse located at the University of Tennessee at Knoxville. Collected leaves were washed in distilled water and cut into 1-cm diam leaf disks, which were placed randomly with adaxial surface up upon 2 layers of moistened P8-creped filter paper (Fisher Scientific, Pittsburgh, PA).

In a separate experiment, leaves of resistant 'Appalachian Spring' flowering dogwood and the susceptible cultivars 'Appalachian Blush', 'Cherokee Princess', 'Cloud 9', and 'Plena' were collected from 2-year old, field-grown trees as previously described in late summer 2009 from the same field of a wholesale nursery located near Winchester, TN. The 10 leaves for each cultivar were measured for cuticle thickness as described, and a chemical analysis of the leaf cuticle was conducted.

Inoculum and Inoculation:

Ten isolates of *D. destructiva* were obtained from leaves and twigs of diseased trees located at the University of the South (Sewanee, TN 35^{o}12'32.22" 85^{o}55'16.74") in March 2008. Diseased leaf and twig samples were collected from the trees, and placed in labeled sterile polyethylene bags. Conidial production was encouraged by placing a

colony plug of one randomly chosen isolate on one piece of fresh sterilized dogwood leaf which had been laid with adaxial surface facing up on the surface of a Petri dish containing potato dextrose agar (PDA). Approximately 50 Petri dishes were prepared. Conidia were collected by submerging these leaves containing conidiomata in sterile distilled water and gently rubbing the leaf surface with a sterile '7' shaped glass stick. Conidial suspensions were adjusted to contain 8×10^4 conidia per ml. An atomized conidial suspension was used to inoculate the surface of leaf disks of resistant and susceptible cultivars. After inoculation, petri dishes (Fisher Scientific, Pittsburgh, PA) were sealed with parafilm (Pechiney Plastic Packaging, Menasha, WI) and incubated at 20°C with a diurnal 12 hour light/ dark cycle.

Germination and Growth of Conidia:

Percentages of germinated conidia were examined at 1 and 2 days after inoculation (DAI). Leaf disks were placed into a clearing solution (0.15% trichloroacetic acid [w/v, Sigma-Aldrich, St. Louis, MO] in ethyl alcohol [Decon Labs, Inc., King of Prussia, PA]/chloroform [Sigma-Aldrich, St. Louis, MO], 4:1 [v/v]) to stop the growth of the fungi and to remove chlorophyll. The solution was exchanged once after 24 h, during the next 48 h. Leaf segments were stained with a freshly prepared Coomassie blue solution (0.6% Coomassie brilliant blue R 250 [w/v, Sigma-Aldrich, St. Louis, MO] in methanol [Decon Labs, Inc., King of Prussia, PA] /15% trichloroacetic acid [w/v] in H_2O, 1:1 [v/v]) for 15 s, washed in water and mounted in 50% glycerol [v/v, Mallinckrodt Paker, Inc., Phillipsburg, NJ]. Percentages of germinated conidia were measured using a light microscope. A conidium was considered germinated when germ-tube length was at least

half the width of the conidium. The percentages of germinated conidia were assessed using a random sample of 100 conidia per leaf disk using 10 leaf disks for each cultivar.

Length of germ tube was measured at 2, 3, and 4 DAI using a light microscope with NIS-Elements software (Version 2.3, Nikon Instruments, Melville, NY). Each day, germ tube lengths of 20 randomly selected spores were measured on each of 10 leaf disks per each cultivar.

Physical Removal of Cuticle from Leaf Surface

In order to examine the role of cuticle, leaf surfaces were covered with a 50% aqueous solution of gum arabic (1:1, weight/volume [w/v]) using a fine hair paint brush. Once the gum arabic dried, a stable polymer film formed in which the epicuticular wax crystals were embedded. The polymer film was peeled off as described by Buschhaus *et al.* (3). This procedure was repeated to insure the epicuticular wax layer was completely removed. Estimates of conidia germination and germ tube lengths were obtained on cuticle-free leaf surface as previously described.

Experiments to determine length of germ tubes and percentage of germinated conidia were designed as a randomized complete block (RCBD). The moisture chamber, which contained one leaf disk each for the two cultivars and one leaf disk with cuticle removed for both cultivars, was the blocking factor. Ten moisture chambers were conducted for each day and each experiment. Data were sorted by day and analyzed using mixed model ANOVA (PROC MIXED) and means were compared using protected least significant difference with SAS software (Version 9.1, SAS Institute Inc., Cary, NC).

Scanning Electron Microscopy of Cuticle Wax

Cuticular wax structures of the susceptible cultivar ‘Cloud 9’ and the resistant cultivar ‘Appalachian Spring’ were examined using Scanning Electron Microscopy (SEM) as described by Zachariah and Pasternak (22). Fresh leaf disks of each cultivar were fixed with chilled (4°C) 3% gluteraldehyde (Sigma-Aldrich, St. Louis, MO) in 0.05 M phosphate buffer pH 6.8 (Fisher Scientific, Pittsburgh, PA) and Histochoice® (Amresco, Solon, OH) for 1 hr at 20°C. Samples were washed three times in 0.05 M phosphate buffer pH 6.8 for 10 min. Secondary fixation was accomplished by using 2% osmium tetroxide (Sigma-Aldrich, St. Louis, MO) dissolved in 0.05 M phosphate buffer pH 6.8 for 1 hr at 20°C. Fixed samples were dehydrated in a graded ethanol series (25, 50, 70, 95, 100% ethanol and 100% dry ethanol, 30 min for each). Samples were air dried, mounted on aluminum stubs and sputter-coated with gold palladium. Five samples for each cultivar were examined by a FEI Quanta 200 scanning electron microscope (SEM) operating at an accelerating voltage of 10 keV.

Measurement of Adaxial Cuticle Thickness

In order to measure the thickness of the adaxial leaf cuticle, Sudan black (0.5% in 70% ethanol, w/v; Sigma-Aldrich, St. Louis, MO) was used to highlight lipids in the leaf cuticle. Stained lipids turned black and were clearly distinguishable from the rest of the leaf tissue (13). Four random rectangular pieces (ca. 7×3 mm) were cut from ‘Appalachian Spring’ and ‘Cloud 9’ leaves (10 leaves for each cultivar) randomly collected from trees growing in a greenhouse. Leaf pieces were fixed in 50% FAA solution, dehydrated in an alcohol series, embedded in paraffin (Paraplast® Oxford Labware St. Louis, MO), and cast into paraffin blocks. Leaf disks were sectioned in 10 µm thick pieces using a rotary microtome, then sections were placed on glass slides.

Slides were deparafinized through 3 changes of Microclear™ (Micron Diagnostics, Inc., Baltimore, MD) for 5 min each and rehydrated by moving slides 10 min each through a series of 100, 95, 85, 70% ethanol (Decon Labs, Inc., King of Prussia, PA). After drying, the slides were immersed in a Sudan black solution for 30 min, rinsed twice in 70% ethanol for 1 min each, and then rinsed in deionized water for 5 min. Sections were dried on a hotplate and mounted with glycerin (50% in deionized water, v/v; Mallinckrodt Paker, Inc., Phillipsburg, NJ) before cover slips were applied. Sections were examined with a photonics type Olympus BH-2 microscope connected to a color camera (Nikon Instruments, Melville, NY). The images of 20 random spots (treated as sub-samples) in each rectangular leaf piece, taken by the color camera, were analyzed by Nikon NIS-elements imaging software (Version 2.3, Nikon Instruments, Melville, NY) to get the mean thickness of the adaxial cuticle (13) (Fig. 3-1). Experiments were arranged in a complete randomized design (CRD) with sub-sampling. One way ANOVA was used to analyze differences among sampled variables using SAS software (Version 9.1, SAS Institute Inc., Cary, NC). Mean separation for each variable were conducted using the least significant difference test at $P = 0.05$.

In a separate experiment, four random rectangular pieces (ca. 7×3 mm) were cut from 10 leaves taken from the five dogwood cultivars collected in the field. Estimate of adaxial cuticle thickness was obtained as described above, with measurements taken from 4 random spots per each rectangular leaf piece.

Sampling of Cuticular Waxes

Total leaf extracts from leaf surfaces of 10 pooled leaves per cultivar were collected from field-grown trees. Extracts were obtained by dipping the entire aggregate of leaves

per cultivar, excluding petioles, into 20 ml of chloroform (> 99%) for 2 min. Weights of wax were calculated by subtracting original weight of 10 leaves by the total dry leaf weights after dipping into chloroform. N-tetracosan (Sigma-Aldrich, St. Louis, MO) was added to all extracts as an internal standard (21). Five samples each for both 'Cloud 9' and 'Appalachian Spring' were prepared.

Chemical analysis

Before gas chromatographic (GC) analysis, bis-N,O-trimethylsilyltrifluoroacetamide (BSTFA) was added to samples from above to transform hydroxyl-containing compounds into the corresponding trimethylsilyl derivatives in pyridine (30 min at 70°C)(21). The GC analyses were conducted as described by Buschhaus *et al.* (3). The qualitative composition of the leaf extract was studied with a capillary GC (6890 N, Agilent, Avondale, PA; column 30 m HP-5MS, 0.25 mm i.d., df = 0.1 mm, Agilent), with He carrier gas inlet pressure programmed to provide a constant flow of 1.4 ml min^{-1} with a mass spectrometric detector (5973 N, Agilent). Gas chromatography identified individual wax components by comparing their mass spectra with those of authentic standards and literature-based data contained within the GC library (NIst 98.1, Agilent). The quantitative composition of the leaf extract was studied using a capillary GC with a flame ionization detector under the same GC conditions as above, but with H_2 carrier gas inlet pressure regulated for constant flow of 2 ml min^{-1}. Single compounds were quantified by comparison with the internal standard and automatically integrating peak areas. In order to find any compounds that were unique or present in higher concentrations within samples from the resistant cultivar 'Appalachian Spring', chromatographic peaks of the resistant cultivar were compared with the susceptible cultivar 'Cloud 9'. Whenever a

compound was found, the spectrum of this compound was analyzed and compared to the library (NIst 98.1, Agilent) to provide possible compound on the basis of compatible percentage of match. When the match between the two was higher than 90% and the molecular weights of the two were close, the unknown compound is likely to be the suggested compound.

Results and Discussion

Germination and Growth of Conidia:

Statistical differences between percentages of germinated conidia of susceptible and resistant cultivars were not observed at 1 DAI on leaf disks with intact cuticles and without leaf cuticles, respectively (Table 3-1). However, after cuticles were removed, germination percentages were increased significantly for both cultivars ($P < 0.001$). These results agree with observations that the cuticle presents a structural barrier that can protect the plant against leaf injury from biotic pathogens (10, 20). At 2 DAI, the germination of conidia on leaves from ‘Appalachian Spring’ was significantly lower than germination of the conidia on susceptible ‘Cloud 9’ leaves that had an intact cuticle ($P < 0.0001$). Therefore, the inoculum potential (IP) of conidia on resistant leaves is lower than the IP on susceptible leaves. After removing the cuticle at 2 DAI, percentages of germinated conidia on these two cultivars also significantly differed, with 67% germination on ‘Appalachian Spring’ and 95% on ‘Cloud 9’ leaves (Table 3-1), suggesting that besides the cuticle, other resistance mechanisms may play an important role in resistance.

Germ tube lengths were significantly shorter on leaves of ‘Appalachian Spring’ than on leaves of ‘Cloud 9’ (Table 3-2). At 2 DAI, germ tube length on leaves of

‘Appalachian Spring’ was shorter than on leaves of ‘Appalachian Spring’ from which cuticle was removed and on leaves of ‘Cloud 9’ regardless of cuticle treatment. At 4 DAI, differences in germ tube length were still apparent between leaves of ‘Appalachian Spring’, both with and without cuticle, while no differences were observed between cuticle treatments to ‘Cloud 9’ leaves. Conidial growth was suppressed by the cuticle of ‘Appalachian Spring’, thus presents additional evidence that the cuticle of ‘Appalachian Spring’ reduces the infection potential of *D. destructiva* conidia.

Wax Crystal Morphology

Qualitative differences of epicuticular wax crystal morphologies between susceptible and resistant cuticular waxes were detected (Fig.3-2). Viewed by SEM, the adaxial epicuticular wax layer on susceptible leaves was characterized by rough or folded wax crystals (Fig. 3-2a). The resistant cultivar ‘Appalachian Spring’ had a smoother wax surface than the susceptible cultivar ‘Cloud 9’ (Fig. 3-2b), which may cause rain droplets carrying conidia to drop easily from the leaf. Propagule reduction from the leaf surface would remain a decrease in inoculum density since less conidium would be left on leaf surface. Cuticle surface topography and pathogen infection interactions have been reported for other hosts and their pathogens. For example, Zabka et al. (21) observed that barley mutants with smooth cuticles were more likely to be infected with *Blumeria graminis* than wild type barley plants as smoother cuticles made finding infection courts (stomatal openings) more accessible for the pathogen. By contrast, *D. destructiva* penetrates dogwood leaves directly, thus does not require a natural opening to enter the leaf. As a consequence, adhesion to the leaf surface would be a more critical topography issue than finding an infection entry point.

Cuticle thickness

Cuticle thicknesses of initial leaf samples taken from ‘Cloud 9’ and ‘Appalachian Spring’ were assessed from plants maintained in greenhouse. Differences in cuticle thickness were observed between leaves of ‘Cloud 9’ and ‘Appalachian Spring’ with adaxial cuticle thickness averaging 1.97µm and 2.62µm (Table 3-3), respectively ($P < 0.0001$). Increased thickness of the adaxial cuticle of ‘Appalachian Spring’ (35% greater) suggested that penetration by *D. destructiva* would be more difficult.

In order to strengthen this conclusion, adaxial wax thickness of three additional susceptible dogwood cultivars (2, 16) were measured and compared with the thickness of the resistant line (Table 3-4). In this separate experiment, leaf tissue samples from all 5 cultivars were collected from field-grown trees. From these samples, resistant ‘Appalachian Spring’ leaves presented a thicker cuticle than any of the susceptible lines (Table 3-4). Since *D. destructiva* enters the host through direct penetration and without appressorium formation, a thicker adaxial leaf cuticle can definitely increase the resistance to the pathogen infection.

Similar assessments have been made regarding other plant-pathogen interactions. Nutman and Roberts (15) concluded that differences in the susceptibility of coffee varieties to berry disease (*Colletotrichum coffeanum*) were due to differences in leaf cuticle thickness which made penetration by pathogens more difficult in resistant coffee varieties. Indeed, *Colletotrichum* is a similar fungal pathogen to *Discula* and Nutman so Roberts’ conclusions were consistent with our findings. In roses, greater resistance to powdery mildew of ‘Queen Elisabeth’ roses than the cultivar ‘Cardinal’ was attributed to a thicker cuticle (6). However, cuticle thickness is not always correlated with resistance

and many plants with considerable thicker cuticles are invaded accessibly by directly penetrating pathogens. For instance, Jarosz (11) concluded that no significant correlations between cuticle thickness and resistance were observed in the interaction of powdery mildew and *Phlox* taxa cultivars. Nashaat and Moore (14) demonstrated that the leaf cuticle of wheat did not directly contribute to the resistance to powdery mildew. In another example, the infection of *Gloeosporium limetticola* was affected by the age of citrus lime leaf (18).

Chemical Components

Several compound classes were identified in the total wax extractions of both resistant and susceptible cultivars. The mixture contained larger amount of alkanes, primary alcohols, alkyl esters and triterpenoids, smaller amount of secondary alcohols and benzyl esters. When comparing the peaks in spectra of these two cultivars' epicuticular waxes, an unidentified peak appeared highly concentrated in the spectra of the resistant cultivar 'Appalachian Spring' than in the spectra for the susceptible cultivar 'Cloud 9' ($P = 0.014$, $F = 9.79$) (Table 3-5). This compound, with suggested molecular weight 154 Daltons, was observed at 5.819 min while internal standard appeared at 17.392 min (Fig.3-3). Relative abundances of this unknown compound in resistant samples ranged from 4.77% to 6.17% of the internal standard (2mg/ml) while in the susceptible cultivar 'Cloud 9' ranged from 4.02% to 5.11%.

In addition, concentrations of this unknown compound in other susceptible cultivars including 'Appalachian Blush', 'Cherokee Princess' and 'Plena', were observed in lower concentrations than in 'Appalachian Spring' as well. These results may suggest that this compound may contribute to *D. destructiva* resistance. Further studies are needed to

analyze the structure of the unknown compound from the output spectrum (Fig 3-3), synthesize an analog and apply the product in field tests to determine its affects on spore germination and germ tube growth of dogwood anthracnose on flowering dogwood.

From this study, resistant ‘Appalachian Spring’ possesses a significantly thicker cuticle, smoother wax crystals in the adaxial epicuticular wax layer, and may present compounds in epicuticular waxes that reduce germination and growth of *D. destructiva* conidia. These combined mechanisms appear to play an important role in early stages of ‘Appalachian Spring’ defense against dogwood anthracnose.

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Appendix

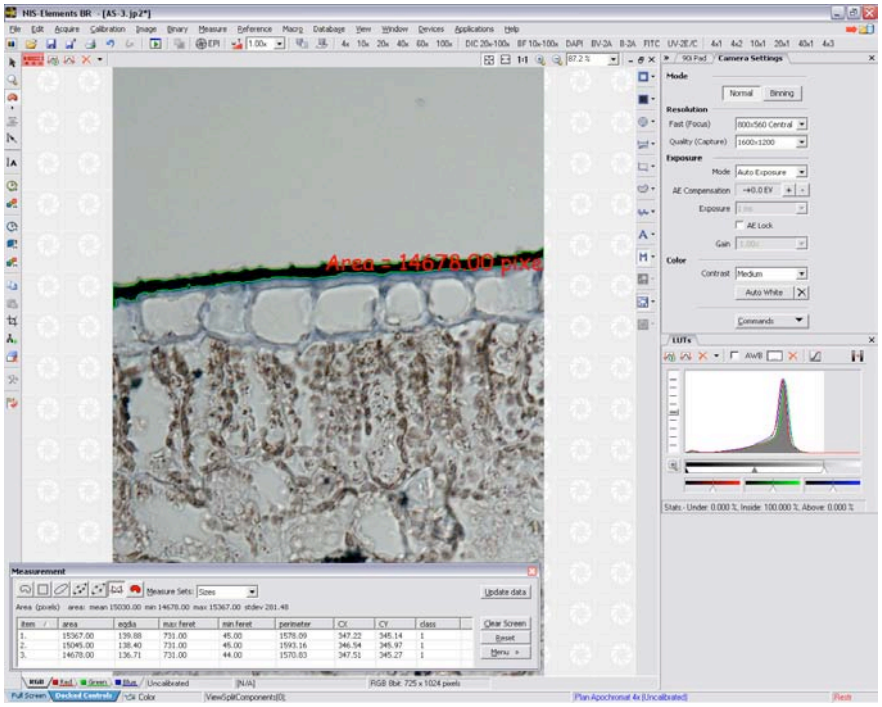


Fig. 3-1. Cuticle thickness was measured by Nikon NIS-elements imaging software. The average cuticle thickness can be determined by dividing the area by the length which can also be measured by the software. The cuticle was stained to black using Sudan b.

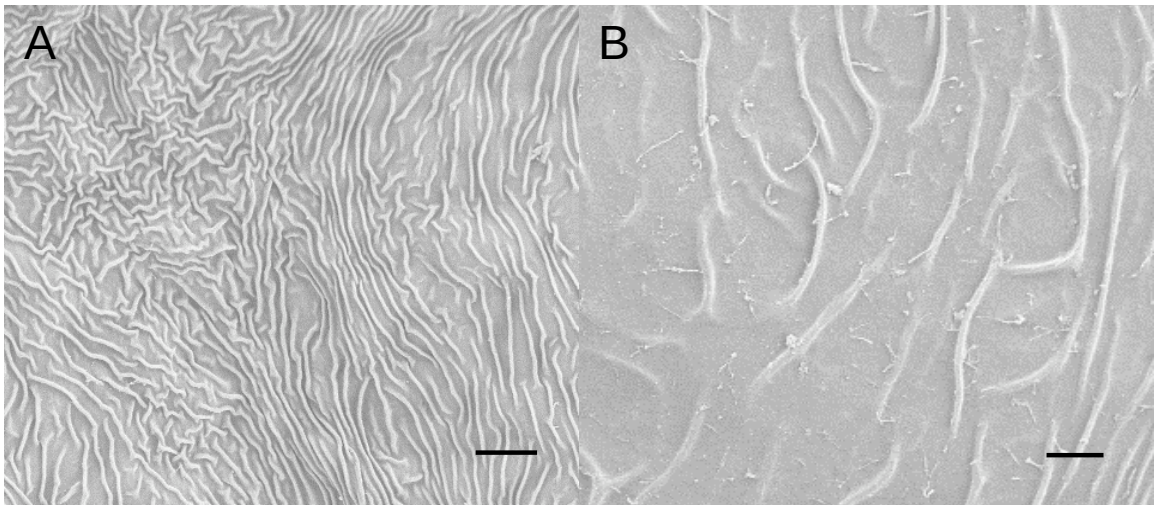


Fig. 3-2. Scanning electron microscope (SEM) images of flowing dogwood adaxial leaf surfaces. The wax crystal structure of susceptible cultivar 'Cloud 9' (A) and resistant cultivar 'Appalachian Spring' (B) are shown. Bar = 10 µm.

Table 3-1. Mean percentage of conidia germination at 1 DAI and 2 DAI with and without leaf cuticle on the resistant flowering dogwood cultivar ‘Appalachian Spring’ and susceptible cultivar ‘Cloud 9’.

Cultivar	Cuticle	1 DAI	2 DAI
‘Appalachian Spring’	Without	41% a	67% b
	With	17% b	43% c
‘Cloud 9’	Without	43% a	95% a
	With	22% b	91% a
LSD		0.074	0.054

Means followed by the same letter in a column for each variable are not significantly different from each other at the $P = 0.05$ level using the least significant difference test. In each day, for each cultivar and cuticle combination, the number of observations is 10. Total N=80.

Table 3-2. Mean germ tube length (µm) from germinated *Discula destructiva* conidia at 2 DAI, 3 DAI and 4 DAI with and without leaf cuticles in the resistant flowering dogwood cultivar ‘Appalachian Spring’ and susceptible cultivar ‘Cloud 9’.

Cultivar	Cuticle	2 DAI	3 DAI	4 DAI
‘Appalachian Spring’	Without	13.18 c	17.81 c	24.15 b
	With	11.01 d	15.81 d	20.42 c
‘Cloud 9’	Without	23.21 a	25.98 a	29.16 a
	With	21.69 b	21.80 b	30.00 a
LSD		1.27	1.22	1.16

Means followed by the same letter in a column for each variable are not significantly different from each other at the $P = 0.05$ level using the least significant difference test. In each day, for each cultivar and cuticle combination, 10 replications were conducted and in each replicate the number of observations is 20. Total N=2400.

Table 3-3. Mean cuticle thickness (µm) on leaves of the resistant flowering dogwood cultivar ‘Appalachian Spring’ and susceptible cultivar ‘Cloud 9’.

Cultivar	Resistance	Thickness(µm)	Std. Error
‘Appalachian Spring’	Resistant	2.62 a	0.032
‘Cloud 9’	Susceptible	1.97 b	0.032
LSD		0.038	

Means followed by the same letter are not significantly different from each other at the $P = 0.05$ level using the least significant difference test. Ten leaves for each cultivar, 4 pieces per leaf and 20 spots per piece give total N= 1600. $P < 0.0001$, F=200.46.

Table 3-4. Mean cuticle thickness (µm) on leaves of the flowering dogwood cultivars ‘Appalachian Blush’, ‘Appalachian Spring’, ‘Cherokee Princess’, ‘Cloud 9’ and ‘Plena’.

Cultivar	Resistance	Thickness(µm)	Std. Error
‘Appalachian Blush’	Susceptible	2.59 c	0.084
‘Appalachian Spring’	Resistant	3.93 a	0.084
‘Cherokee Princess’	Un-tested	3.05 b	0.084
‘Cloud 9’	Susceptible	2.71 c	0.084
‘Plena’	Susceptible	2.77 c	0.084
LSD		0.096	

Means followed by the same letter are not significantly different from each other at the $P = 0.05$ level using the least significant difference test. Ten leaves for each cultivar, 4 pieces per leaf and 4 spots per piece give total N= 800. $P < 0.0001$, F=41.93.

Table 3-5. Mean relative abundance (%) of the unknown compound relative to concentrations of the internal standard (N-tetracosan) of resistant cultivar ‘Appalachian Spring’ and susceptible cultivar ‘Cloud 9’.

Cultivar	Resistance	Relative Abundance (%)	Std. Error
‘Appalachian Spring’	Resistant	5.44 a	0.2156
‘Cloud 9’	Susceptible	4.486 b	0.2156
LSD		0.007	

Means followed by the same letter are not significantly different from each other at the $P = 0.05$ level using the least significant difference test. Five observations for each cultivar gives total N=10. P= 0.014, F= 9.79

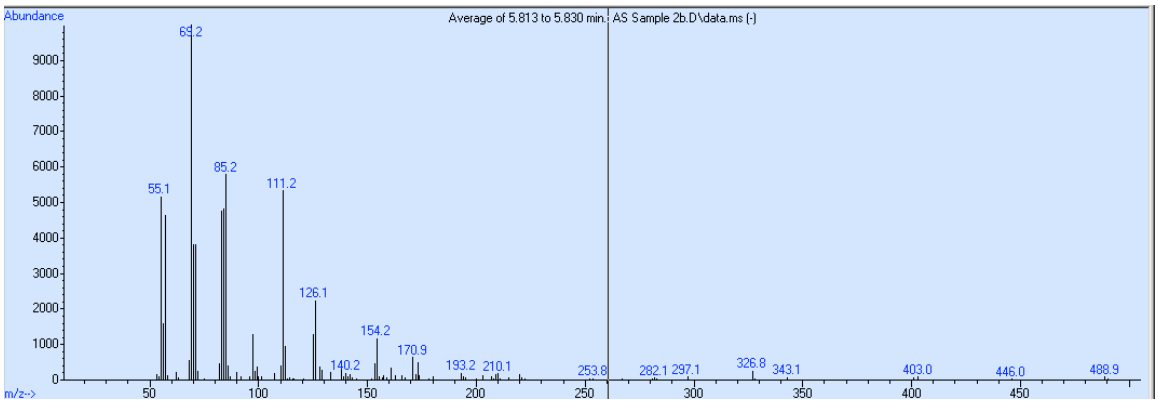


Fig. 3-3. Gas chromatography spectrum of the unknown compound observed at 5.819 min.

Chapter IV Conclusion and Future Work

The sequence of infection events for *Discula destructiva* Redlin on flowering dogwood is as follows:

Ⅰ. 2 days after inoculation (DAI)- conidia on leaf surfaces have germinated;

Ⅱ. 3 DAI- direct penetration by hyphae can occur;

Ⅲ. 7-8 DAI- visible necrotic lesions are observed;

Ⅳ. 8 DAI- hyphae have accumulated between the cuticle and epidermis and have grown intracellularly in epidermal, palisade parachyma and mesophyll cells;

Ⅴ. 16 DAI- typical anthracnose lesions (lesions with chlorotic halos and purple borders around tan (necrotic) centers) formed; chloroplasts remain intact but decompartmentalized;

Ⅵ. 24 DAI- acervuli have ruptured plant cuticle and conidia are released.

This sequence is the first documented series of infection events for *D. destructiva* on flowering dogwood. This outline can be used to look for resistance mechanisms in dogwood germplasm. A resistant line would expect to slow or inhibit one or more infection events.

Conidia germination and germ tube growth were significantly inhibited by the resistant cultivar ‘Appalachian Spring’. These results provide new ways to use conidia germination test and germ tube growth measurement for detecting resistant cultivars. By contrast with previous resistance detection methods, which include inoculating trees or

seedlings and holding them for six weeks and therefore, were much more expensive and time consuming, using in vitro tests for germination and germ tube length are much cheaper and easier.

The resistant cultivar ‘Appalachian Spring’ had a smoother adaxial wax crystal structure than ‘Cloud 9’ and a significantly thicker cuticle than susceptible cultivars ‘Appalachian Blush’, ‘Cherokee Princess’, ‘Cloud 9’ and ‘Plena’. Smoother cuticle could reduce the number of conidia (reduce inoculum density) on the leaf surface since conidia are spread by splashing rain and the thicker cuticle could make direct penetration more difficult. These combined mechanisms might contribute to some of the resistance of ‘Appalachian Spring’ to dogwood anthracnose.

Further research can be conducted to 1) look at cuticle components of abaxial and adaxial leaf surfaces for compounds that may affect conidial germination; 2) test smoother wax surface of ‘Appalachian Spring’ to see if it is more hydrophobic than rough cuticle of ‘Cloud 9’; 3) look for other mechanisms such as thick cell walls, or secondary metabolites including plant phenolics, phytoalexins and phytoanticipins etc.

Vita

Qunkang Cheng was born in Gugao, Jiangsu province, China on July 29, 1983. He attended and graduated from Gugao High school in 2000. Qunkang graduated from Nanjing Agricultural University in 2004 with a B.S. in Plant Protection. In 2007, he started his graduate studies and worked as a graduate research assistant in the Department of Entomology and Plant Pathology at the University of Tennessee at Knoxville under the direction of Dr. Mark T. Windham.