

A BIOLOGICAL COMPARISON OF *DISCULA DESTRUCTIVA* ISOLATES
FROM FOUR GEOGRAPHIC AREAS

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ABSTRACT

A Biological Comparison of *Discula destructiva* Isolates From Four Geographic Areas

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A series of biological tests were conducted to ascertain whether differences among isolates of the dogwood anthracnose fungus (*Discula destructiva*) could be measured. *Discula destructiva* isolates were recovered from forty symptomatic eastern flowering dogwood trees growing at four locations in West Virginia and Pennsylvania. Before sampling, each tree was rated for percent foliage infected. Symptomatic leaf and twigs were collected in the summer of 1994 and *D. destructiva* was isolated from each leaf and twig sample. Isolates were cultured on potato dextrose agar (PDA) medium. Subcultures onto the same medium were made in order to evaluate growth and colony morphology. Two identical cultural tests were conducted and the diameters (mm) of all cultures were measured after three weeks of growth under 16/8 hr light at 24 C. No statistically significant differences in colony diameters were found among the isolates, although a small percentage of slow growing isolates were identified from each of the four sites. Only two slow growing isolates retained this feature in the second trial. Cultures typically produced slightly fluffy mycelium with concentric zonation patterns. Most of the isolates initially were pigmented light green but darkened with age. The few slower growing isolates were hard and rubbery to the touch and did not display concentric zonation. Another test was designed to establish whether a system of vegetative compatibility exists in *D. destructiva*. The isolates were paired on half strength PDA, water agar and each type of medium amended with dogwood leaves. Half of the plates were placed in the dark and half in 16/8 hour light. After 10 days of growth, the paired isolates were scored for the presence or absence of a barrage. The presence of a barrage was used as evidence of incompatibility between pairs. Barrage formation did not occur between any of the pairs, thus all paired isolates were considered to be vegetatively compatible. A double-stranded RNA (dsRNA) extraction procedure was the third assay attempted and was used to determine whether dsRNA was present in the isolates tested. All isolates contained dsRNA, as did their single conidial progeny. Many bands, however, were difficult to detect visually. The ability of isolates to invade plant tissue was measured in a fourth comparison by inoculating three different plant substrates, including apples, dogwood leaves and dormant dogwood stems. Lesion formation was observed in apples, but *D. destructiva* was rarely reisolated. A few lesions formed on the dogwood foliage but *D. destructiva* was never isolated. Lesions did not develop on inoculated dogwood stems. In all the apple, leaf and stem assays, a variety of other organisms were routinely isolated. Over all, when the inoculation procedures were employed, *D. destructiva* proved to be an unsuccessful pathogen. In all tests, *D.*

destructiva failed to display detectable variation, supporting the hypothesis that variability does not exist among the *D. destructiva* isolates collected from the widely separated geographic areas chosen for this study.

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INTRODUCTION

Eastern flowering dogwood, *Cornus florida* L. is not only an aesthetically pleasing understory forest tree, but is also widely planted as an ornamental. The showy floral bracts, which subtend the true flowers are particularly notable when they appear in the early spring before leaf expansion. Unfortunately, in the late 1970's, foliar symptoms, dieback and subsequent mortality were observed on dogwoods in the Brooklyn Botanical Gardens (Pirone, 1980). This decline was thought to be attributed to the particularly wet and cold winter of 1979 and the presence of the anthracnose fungus, *Colletotricum gloeosporioides* Penz. (Pirone, 1980). Since then, the disease has spread in a southwesterly direction, generally following the Appalachian Mountains.

During the same time that disease symptoms were observed on *C. florida*, they also were noted on western dogwood, *C. nuttallii* Audb. growing in the state of Washington. The symptoms resembled those on *C. florida* (Byther and Davidson, 1979). In 1983, Daughtery and Hibben associated a *Discula* sp. Sacc. with disease symptoms on both *C. florida* and *C. nuttallii*; with symptoms being less severe on *C. nuttallii* (Salogga and Ammirati, 1983). Hibben and Daughtery (1988) studied the differences between symptoms and fungal isolates taken from the two infected dogwood species. They showed that the same organism occurs at both locations on both *Cornus* species. Fulfillment of Koch's postulates proved that *Discula* sp. was the causal agent. Redlin (1991) described the organism as a new fungal species and established the name *Discula destructiva* Red.

Although the same organism is capable of infecting two species of *Cornus* growing in very different geographic locations; the question that remains is whether variability exists among *D. destructiva* isolates. One objective of this study was to determine if morphological differences could be detected among isolates recovered from foliar lesions and infected epicormic shoots taken from four different geographic locations. Since fungal variability may not be exhibited morphologically, a second objective was to examine whether a system of vegetative compatibility (VC) existed and if so, could reliably be used to divide *D. destructiva* into separate VC groups. A dsRNA analysis was the third part of this study and was used to ascertain whether differences exist in the presence or absence of dsRNA among *D. destructiva* isolates. A fourth component of this study involved using a variety of tissue assays in an attempt to develop a method to measure an isolate's ability to invade tissue.

LITERATURE REVIEW

There are a dozen genera and 100 species in the Cornaceae family. Sixty *Cornus* species occur in North America, Europe and Asia; but are rare in South America and Africa (Eyde, 1988). The species within *Cornus* are quite diverse in form and habit. Most are deciduous trees or shrubs and two from China are evergreen (*C. paucinervis* Hance and *C. capitata* Wallich.). Two species, *C. canadensis* L. and *C. suecica* L. are herbaceous perennials. Leaves are simple, entire and usually opposite, except in *C. alternifolia* L. and *C. controversa* Hemsl. Inflorescences occur as cymes, panicles, umbels or heads. Some species have showy bracts (*C. florida*, *C. kousa* Hance, *C. capitata*, and *C. nuttallii*) whereas others have leafy and small bracts (*C. mas* L. and *C. officinalis* Siebold & Zucc.) (Eyde, 1988). Flowers are usually bisexual, rarely unisexual, and in four parts. The fruit is a two-celled, two-seeded blue, white or red drupe, or a fleshy globose head as is the case with *C. kousa*. All dogwood leaves are distinguished by veins that curve inward toward the leaf tip (Bailey, 1976).

Cornus florida is mainly a southeastern tree, but it occurs from southern New England to central Florida, westward to eastern Texas and north to southern Michigan. The tree grows on stream banks and drier upland soils. Optimum growth occurs at a soil pH values from 6.0 to 7.0. Flowering dogwood has one of the highest foliage contents of calcium (2.0 to 3.5%) of any eastern deciduous forest tree. Its role as a soil builder is enhanced by the rapid rate at which its litter decomposes (Hepting, 1971). Dogwood fruit (drupes) are valuable for migrating birds as they contain 16% fat (Whitmore, 1992). *Cornus florida* grows to an average height of about 15-20 feet and flowers before the

leaves begin to expand. In contrast, western dogwood (*C. nuttallii*) grows natively from British Columbia to northern California and can reach heights of 75 feet. This species also produces floral bracts before leaf expansion (Orton, 1993).

There are over 100 cultivars of *C. florida*. One naturally occurring variety, *C. florida* 'rubra', possessing pink floral bracts, was discovered in Virginia by an English horticulturalist, Mark Catesby in 1712. This was subsequently propagated in Virginia, taken to England and cultivated there. The 'rubra' variety was first cultivated in this country in 1910, in Franklin County, Tennessee. In 1958, a *C. florida* cultivar named 'Cherokee Chief' became the first patented red-bracted cultivar (Nicholson, pers. comm., Commerical Nursery Co., Decherd, TN). Other cultivars having red bracts include 'Sweetwater' and 'Red Beauty'. Some white-bracted *C. florida* cultivars include 'Cherokee Princess', whose floral bracts hold up well in rain and wind; 'Rainbow', with variegated foliage; and 'Pygmy', a dwarf variety. The red-bracted cultivars have been shown to be less vigorous in growth than those possessing white-bracts (Orton, 1993).

Pests and diseases

Cornus florida is susceptible to a number of insect and disease problems. The most serious insect pest is the dogwood borer, *Synanthedon scitula* Harris. The larvae girdle the trunk or limb by tunneling beneath the bark and feeding on the phloem and cambial tissues. Other dogwood pests include dogwood twig borer, *Oberera tripunctata* Swederus; dogwood clubgall midge, *Resseliella clavulsa* Beutenmuller, which causes swelling at the branch tips; leafhopper, *Edwardsiana* sp. produces stippling on leaves;

dogwood spittle bug, *Clastoptera proteus* Fitch; and several species of scale (Grant, 1993).

Diseases that can cause symptoms on dogwood include *Botrytis cinerea* Penz., affecting floral bracts and *Elsinoe corni* Jen. & Bit., causing leaf spot (Hagen and Mullen, 1995). Symptoms of angular leaf spot produced by *Septoria floridae* Tehon & Daniels are found later in the growing season (Farr, 1991). Similar symptoms are produced by *Phyllosticta cornicola* DC., but the spots are not as angular and symptoms are detected earlier in the growing season (Sinclair et al., 1987). *Colletotrichum gloeosporioides* Penz. has been isolated as a causal agent of a leaf spot on dogwood (Toole and Filer, 1965), with *Colletotrichum acutatum* Penz. causing limb dieback (Chellemi and Knox, 1992) and infection of dogwood fruit (Smith, 1993). *Lasiodiplodea theobromae* Pat, causal agent of dogwood canker, is a serious disease of nursery grown trees and is largely influenced by stress (Mullen et al., 1991). Basal trunk canker, caused by *Phytophthora cactorum* Lebert & Cohn, is a major problem for urban dogwoods and is associated with mechanical injury and/or drainage stress (Mullen et al., 1991). Powdery mildew has been evident on dogwood and has been detected throughout the eastern U.S. A statewide survey in Indiana has found extensive powdery mildew (*Microsphaera pulchra* Wallr.:Fr or *Phyllactinia guttata* Wallr.:Fr.) infection on dogwoods since 1993 (Marshall, 1996). Twig dieback, caused by *Phomopsis corni* Sacc. has been reported on dogwood, but is considered to have a negligible impact (Redlin, 1991).

Dogwood anthracnose symptoms

The most devastating disease affecting dogwood in North America today is dogwood

anthracnose, caused by the fungus, *Discula destructiva* (sp. nov. Red.). The disease affects leaves, twigs and the main stem cambium. Three types of foliar symptoms can indicate the presence of dogwood anthracnose. These include leaf spots, blotch and blight. Spots consist of necrotic brown lesions on foliage that are surrounded by purple rimmed margins. Shot holes can result when the necrotic tissue breaks away from healthy leaf tissue. Blotches form between or along main veins and along the edge of the leaf blade. Blight results from the advancing infection throughout the leaf tissue and sometimes into the petiole. These three types of foliar symptoms may appear singly or together on infected leaves. Epicormic shoots can form on the main stem of stressed trees. Infected epicormic branches may be a source of stem cankers, as the fungus grows down the shoot and into the main stem. Multiple cankers may cause girdling of the cambial tissues, leading to tree death. Tree mortality can occur within a few years of initial infection (Hibben and Daughtery, 1988).

Discula destructiva

Discula destructiva produces one-celled conidia, within an acervular conidiomata. The conidiomata are orange-to reddish brown or black (Daughtrey, et al., 1988). This fruiting structure is found in the necrotic tissues of leaves and stems and serves as overwintering structures on twigs and unabsconded leaves (Mielke and Daughtrey, 1989). Typically *D. destructiva* develops conidiomata below the trichomes, especially on the lower leaf surface. The conidiomata-trichome association is not known in other species of this genus (Redlin, 1992). The conidia are 7-12 x 2.5-4.0 micrometers in diameter and contain one or two polar oil droplets (guttules) (Redlin, 1991). Conidia may be exuded

from cirrhi, especially under drier conditions. The fungal hyphae penetrate leaves directly; without requiring a wound or stomata (Graham et al., 1991). Histological studies have shown that when the pathogen enters a leaf, it invades inter-and intracellularly (Walkinshaw and Anderson, 1991). Necrosis in leaves without visible hyphae indicate that a toxic metabolite (Venkatasubnaiaha and Chilton, 1991) may be involved in lesion formation (Walkinshaw and Anderson, 1991).

Field studies have shown that conidia may be disseminated on the ventral body surface and mouthparts of convergent lady beetles, *Hippodamia convergens* Geurin-Meneville (Colby et al., 1993 and 1995). Long-distance spore dispersal may occur by birds, as the fungus has been isolated from the fruit and seed of severely infected trees (Britton et al., 1993b).

Disease distribution

One of the first thorough surveys to determine the impact and distribution of dogwood anthracnose was performed in 1984 at Catocin Mountain Park in Maryland (Mielke and Langdon, 1986). This survey established that 33% of the flowering dogwoods were dead and only 3% of the trees that were surveyed were disease free. Another survey, conducted in 1988, using the same plots, found that 79% of the trees were dead (Schneeberger and Jackson, 1989). In 1994, Sherald et al. (1996) examined the same survey plots and established new plots in the general area. The number of total dogwood stems/acre declined from 395 found 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.

In order to assess the amount and extent of dogwood anthracnose in the range of *C. florida*, workers from the eastern states performed an impact and distribution survey, beginning in 1986. Impact plots were established and a rating system for infected trees, devised by Mielke and Langdon (1986), was used to score trees in five different decline classes according to percent foliage affected. Presence of twig canker, mainstem canker and epicormic shoots were criteria also included in the impact survey. The distribution survey was conducted within each county of the participating states. A county was considered to be infected with dogwood anthracnose if one leaf per county was confirmed in the laboratory to possess *D. destructiva* conidia.

In 1987, a dogwood anthracnose working group was formed in the southeastern United States to review the findings of the 1986 survey and to discuss current studies performed by the members (Anderson et al., 1988). At this time, the disease had been reported in over 60 counties in nine northeastern states (CT, DE, MD, MA, NJ, NY, PA, VA and WV). By October 1988, the disease also had been found in North Carolina, South Carolina, Georgia, Kentucky and Tennessee. The disease affects all age classes and is more prevalent at elevations above 2000 feet and in cool, wet valleys (Anderson et al., 1990). In 1990, *Discula* infection was difficult to find in the western part of West Virginia, but by 1992 the disease was identified in all 55 counties of the state (Haynes, 1992).

Conditions for disease development

Several studies have been conducted to determine the conditions necessary for *Discula* infection. Of five species of dogwood tested for leaf moisture retention, *C. florida* was

When supplemental, non-overhead irrigation was applied, the increased moisture level was shown to be associated with reduced disease severity (Gould and Peterson, 1994) and dogwoods that were subjected to low light intensity and drought were more vulnerable to disease development (Erbaugh et al., 1995). Other studies indicate that dieback of epicormic shoots can occur when the trees are dormant (Dowsett et al., 1990). When overhead acid mist was applied to dogwood leaves, the percentage foliage affected by *Discula* decreased with increasing pH (Anderson et al., 1989). Trichome shrinkage at pH 5.0 and below occurred with accompanying tissue damage. Hypothetically, this may facilitate nutrient metabolic leakage and hence, a site for fungal spore germination and subsequent penetration (Thornham et al., 1992).

***Discula destructiva* growth in vitro**

Discula destructiva growth 'in vitro' has been examined. The pH for optimal growth of *D. destructiva* is 6.2. Light is necessary for spore formation and temperatures between 20-24 C are optimum for germination. Stimulation and formation of acervuli occurs on oatmeal agar (Redlin, 1991) or with the incorporation of dogwood, oak, or maple leaf to the growing medium (McElreath and Tainter, 1993). Malt sucrose agar was shown to stimulate the most rapid fungal growth (Pacumbaba and Beyl, 1992).

Mycoviruses

Many fungi have been found to contain virus or virus-like particles. Mycoviruses are found in fungal cytoplasm and some can be transmitted by anastomosis and in some cases, they have been associated with loss of virulence in plant pathogenic fungi. When

80 *D. destructiva* isolates from 11 states were screened for presence of double-stranded RNA (dsRNA), all contained dsRNAs. Twenty-eight percent contained large (8-12 kilobases) segments and most also contained medium (3-4 kb) and small (1.5-3 kb) segments. Seventy-three percent contained only medium and/or small segments (McElreath et al., 1994). The finding of dsRNA in the isolates indicates the possible presence of a cytoplasmically-borne mycovirus. Although some dsRNAs in *Cryphonectria parasitica* isolates (the chestnut blight fungus) have been correlated with decreased virulence (hypovirulence) (MacDonald and Fulbright, 1991), a relationship between presence of dsRNAs and degree of virulence in *D. destructiva* isolates has not been established (McElreath et al., 1994).

Differences between *Discula destructiva* and *Discula* sp.

Two species of *Discula* have been isolated from dogwood tissue, *D. destructiva* and an unidentified *Discula* species. Morphologically, the two species are very different in culture. *Discula destructiva* has slightly fluffy mycelium and does not readily sporulate, except on certain media, whereas the unidentified *Discula* sp. grows appressed to the agar medium and produces an abundance of spores (Trigiano et al., 1995). Both species were able to utilize cellulose, hemicellulose and pectin as well as the synthetic fats, Tween 20 and 80. When the two species were grown on a gallic acid medium, only *D. destructiva* was able to oxidize this phenolic compound, imparting a muddy brown color to the medium. The ability to oxidize phenolics may prove to be important to *D. destructiva* in its ability to overcome dogwood's defense mechanisms (Trigiano et al., 1995).

A more accurate means of differentiating between the two *Discula* species, as well as

among *D. destructiva* isolates is by the use of a DNA amplification fingerprinting (DAF) technique. This procedure involves amplifying certain genomic DNA sequences by priming with short arbitrarily defined oligonucleotide primers. These primers are used to produce polymorphic amplification profiles from DNA of the fungus. DAF has been employed to identify individuals and groups of closely related organisms; in this case to differentiate between *D. destructiva* and the unidentified species of *Discula*. When the DAF technique was used, no variation in DAF profiles of *D. destructiva* isolates was apparent. This lack of variability may indicate that the fungus was recently introduced into North America. Profiles also showed that the two *Discula* species are distinctly different (Trigiano et al., 1995).

Disease resistance

Studies to determine possible host resistance have been carried out by several researchers. In 1985, Santamour et al. (1989) collected seeds of *C. florida* from a wide geographic range, extending from Connecticut through Oklahoma. The three-year-old seedlings exposed to natural *Discula* infection showed a high percentage of mortality. The authors demonstrated that with the wide geographic range of seed used in the test, no apparent natural host resistance was found among the *C. florida* seedlings.

Four dogwood trees that survived the anthracnose epiphytotic in Catoctin Mountain Park were used in a series of resistance trials in 1992. The survivors were vegetatively propagated and the resulting clones were exposed to *D. destructiva* inoculum at Ozone, TN (natural inoculum) and the USDA Forest Service Laboratory near Asheville, NC (artificially induced inoculum using infected branches and overhead misters). Clones

from one of the four trees (tree #4) survived inoculation exposure at both test sites. This tree also was the only one of the four trees to produce fruit. The seeds from tree #4 were germinated and the three-year-old seedlings are in the process of being evaluated for resistance to *Discula* infection (Graham et al., 1995).

Several studies have been undertaken to test whether various *Cornus* species are susceptible to dogwood anthracnose and if there is a difference in disease severity among species. In a resistance screening trial in 1991, Windham et al., (1993) found that *C. controversa*, *C. sericea* and *C. kousa* 'Chinensis' were as susceptible to dogwood anthracnose as *C. florida*. A similar study in 1992 also showed that three *C. sericea* cultivars, *C. alba* and *C. kousa* 'Chinensis' were as susceptible to the disease as *C. florida* (Brown et al., 1996). A key concern of the results of this study was the susceptibility of *C. sericea*, *C. alba* and *C. kousa* 'Chinensis'. These species may inadvertently have served as a means for the introduction of *D. destructiva* inoculum into disease-free areas. Similarly, cultivars and seedlings of *C. florida* and seedlings of *C. kousa* also were evaluated for resistance to dogwood anthracnose at six different locations from North Carolina to New Jersey. *Cornus florida* cultivars 'rubra', 'Cloud Nine' and 'Barton' appeared to be more susceptible than 'Cherokee Sunset', 'Cherokee Red' and seedlings of both *C. florida* and *C. kousa* (Britton, 1993c). When Sherald et al. (1994) evaluated eight species and one cultivar of *Cornus* for resistance to dogwood anthracnose, they found all but *C. florida* remained free of the disease after four years of exposure to naturally occurring inoculum of *D. destructiva*. These results conflict with those of the previous studies and indicate the need for a better understanding of the disease in relation to the other species and cultivars of *Cornus*.

Disease management

Management of dogwood anthracnose in a forest environment is an undisputed challenge, but several methods of controlling the disease in the landscape have shown to be effective. Various cultural measures have been used in an attempt to prevent the spread of *D. destructiva* on *C. florida*. Pruning epicormic shoots during the growing season can reduce the occurrence of branch or main stem canker formation and infected branches containing cankers or infected foliage should be removed during the dormant season. In order to maintain the health and keep incidence of disease at a low level, recommendations commonly have been made to plant dogwoods in open, sunny locations. The recommendation has merit, as studies have shown decreased disease severity on open grown trees (Chellemi and Britton, 1992; Parham and Windham, 1992; and Gould and Peterson, 1994). Use of fungicides such as Daconil or Banner has been successful for annual preventative control (Windham and Windham, 1995).

Planting resistant species of dogwood, as a replacement to *C. florida* has been another alternative used as a control measure. One replacement choice is the Asian dogwood, *C. kousa*, which grows to 20 feet and produces floral bracts after the leaves have entirely expanded. *Cornus kousa* is resistant to dogwood borer and relatively resistant to *D. destructiva* (Orton, 1993). The cultivar *C. kousa* 'Chinensis', selected for its larger bracts, has been found to be less resistant than *C. kousa* (Brown, et al., 1996). The majority of *C. kousa* available from nurseries and garden centers have been grown from seed (Orton, 1993). Interspecific first generation hybrids of *C. florida* x *kousa* also have been shown to possess increased resistance to dogwood anthracnose and dogwood borer (Orton,

1993).

In observing foliar symptoms in a forest setting, some trees, regardless of site, appear to have more symptoms than other trees in the same vicinity. This curiosity could be attributed to microclimatic differences (Chellemi and Britton, 1992), level of virulence of the infecting strains, or resistance of the host plant. In particular, the isolate variability may be a factor in determining degree of infectivity and level of pathogen virulence. Therefore, this project focused on different methods that could be used to assess isolate variability.

STUDY OBJECTIVES

The objective of this study was to examine the variability that exists among isolates of *Discula destructiva* from different geographic locations. Specifically, isolates were studied to:

1. determine if cultural differences exist among isolates recovered from foliar lesions and epicormic shoots;
2. determine whether a system of vegetative compatibility can be demonstrated for *Discula destructiva*;
3. examine selected isolates for the presence or absence of dsRNA; and,
4. evaluate a variety of tissue assays to determine if a method can be developed to measure an isolates ability to invade tissues.

MATERIALS AND METHODS

Study sites

Three sites utilized in this study were located in West Virginia and one in Pennsylvania. The sites were chosen on the basis of their wide geographic separation, proximity to Morgantown and existence of dogwood anthracnose. Two plots were selected at each of the four sites. Five dogwoods, exhibiting typical symptoms of dogwood anthracnose, were chosen as study trees in each plot. The descriptions of each site and the plot names are as follows:

◆ **Krepps Park-Morgantown, WV** (samples collected on July 5, 1994)

One plot contained a relatively dense population of dogwoods in a wooded area of the park. The second plot consisted of older trees growing in an open area. The elevation in this area is approximately 1000 feet. Dogwood anthracnose was first detected in 1990 (Krepps park personnel, pers. comm.). Plot names: KROP-Krepps-open grown and KRWO-Krepps-wooded.

◆ **Oglebay Park-Wheeling, WV** (samples collected on July 13, 1994)

This is the western most site. Trees in both plots are open grown. The elevation of this area is about 1700 feet. Dogwood anthracnose was first detected in 1990 (J. Baniecki, West Virginia University extension, pers. comm.). Plot names: WOG-Oglebay Park-near Wilson Lodge and RUOG-Oglebay Park near Russell cabins.

◆ **Pocahontas County-Dilly's Mill, WV** (samples collected on July 17, 1994)

This site is about 90 miles southeast of Morgantown, adjacent to the Monongahela National Forest boundry. All trees in both plots are in a forested setting and the elevation of this area is about 2500 feet. Dogwood anthracnose was first detected at this site in

1989 (Haynes, 1990). Plot names: POTO-Pocahontas Co.-top of the hill and POBO-Pocahontas Co.-bottom of the hill.

◆ **Gettysburg National Battlefield Park-Gettysburg, PA** (samples collected on July 19, 1994). Although dogwood anthracnose has been evident in the park since the mid-1980s, an ongoing dogwood anthracnose survey established in 1990 by the USDA Forest Service indicates that the majority of trees within the Forest Service plots are still alive (Gundrum, 1997). In the current study, one plot is located in a wooded area and the other is in the open. This site is at an elevation of 1900 feet and eastern flowering dogwood is the primary understory component throughout the park (Gibson, 1989). Plot names: GBPM-Gettysburg-Peace Memorial and GBDD-Gettysburg-Devils Den.

Ratings of symptoms

Once the trees were selected, each was rated for percent foliage infected (FI) and leaf area infected (LAI). The method used to do this was modified from the Mielke-Langdon crown dieback class rating system (Mielke and Langdon, 1986). Rather than observing overall crown condition, included in the Mielke-Langdon system, the ratings were broken down to include both percent foliage infected and percent leaf area infected. Percent foliage infected involved estimating the percentage of leaves that had noticeable necrotic lesions, indicative of dogwood anthracnose symptoms. Percent leaf area infected was tabulated by observing the infected leaves within the crown and estimating the average percentage of necrosis on the individual leaves. The ratings were intended to give an overview of the percent necrosis on each tree at the time of sampling.

Collections

Infected leaf and twig samples were collected from each of the 10 trees (5/plot) at each of the four sites. In this study, leaf samples were collected from the trees by removing them with hand pruners and placing them in labeled plastic bags. Epicormic twigs were collected from only 35 of the 40 trees because Krepps Park personnel had previously removed the shoots from the five trees in the open grown plot. All *Discula destructiva* isolations were made from the collected leaf and twig samples.

Epicormic shoots were examined with a 16x hand lens at the time of collection for signs of acervular conidiomata, diagnostic of the presence of *D. destructiva*. If these fruiting bodies were found, the epicormic shoots were removed from the main stem by pruning at the base and then placing in labeled plastic bags. All twig and leaf samples from all sites were returned to Morgantown, refrigerated at 4 C, and isolations were made within three days after each collection.

***Discula destructiva* isolation, identification and culture**

After removal from the refrigerator, each epicormic twig sample, representative of each study tree, was incubated at 25 C for one-to-three days on moist filter paper in glass Petri plates. Acervular conidiomata were typically found concentrated near the apical end of the twig as brown pustules. Microscopic observation of the spores was possible by making a spore mount on a microscope slide and viewing under a Nikon phase/contrast microscope (Nippon Kogaku K.K., NY). The conidia resemble spores of many other fungi (e.g. *Phomopsis* sp. and *Colletotrichum* sp.), thereby making a definitive identification somewhat difficult (Redlin, 1991). A measurement of individual spores

was necessary to determine if the spores were those of *D. destructiva* or another fungus. The spores are typically 7-12 x 2.5-4.0 micrometers in size, hyaline and possess two polar oil droplets (guttules) (Redlin, 1991).

A straight-tip probe was used to remove the conidiomata from epicormic twig samples. The fruiting bodies were more successfully removed when they were slightly gelatinous. The hardened, dark-colored ones were difficult to remove and the very wet ones didn't stay on the probe. The conidiomata were placed on a Petri plate containing potato dextrose agar (PDA; Difco, Detroit, MI) (39 grams/liter distilled water) amended with 0.05 g chlorotetracycline HCl (ICN Biochemicals, Cleveland, OH), to retard growth of bacterial contaminants. Within about 10 days, the cultures were identified as *D. destructiva* and five subcultures were made from each of the 35 twig isolates. These subcultures served as replicates of each isolate and were used to study cultural characteristics. Each plate was dated and labeled as to site, plot, tree, isolate and subculture. All cultures were stored in Petri plates in the refrigerator at 4 C.

The conidiomata on symptomatic leaves are more commonly found on the lower (abaxial) leaf surface and are often associated with trichomes (Redlin, 1991) (Figures 1a and b). Identification of the fungus with the phase/contrast microscope was performed for symptomatic leaves in question. Correct identification of the causal agent by spore size, presence of polar oil droplets and association with trichomes was necessary to clarify possible confusion in identification of causal agent (Daughtrey et al., 1996).

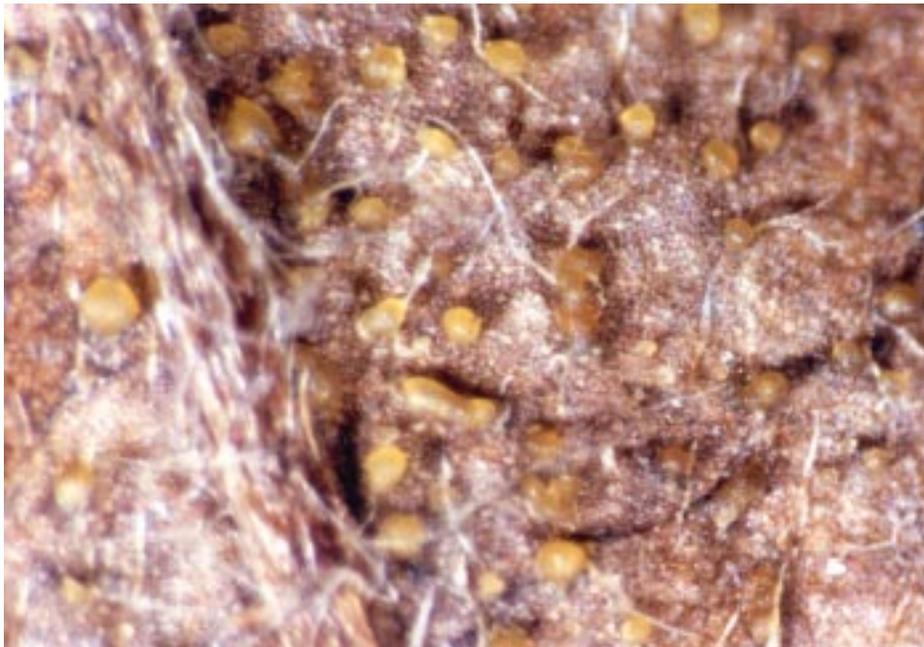


Figure 1a. Acervular conidiomata associated with trichomes on abaxial leaf surface.

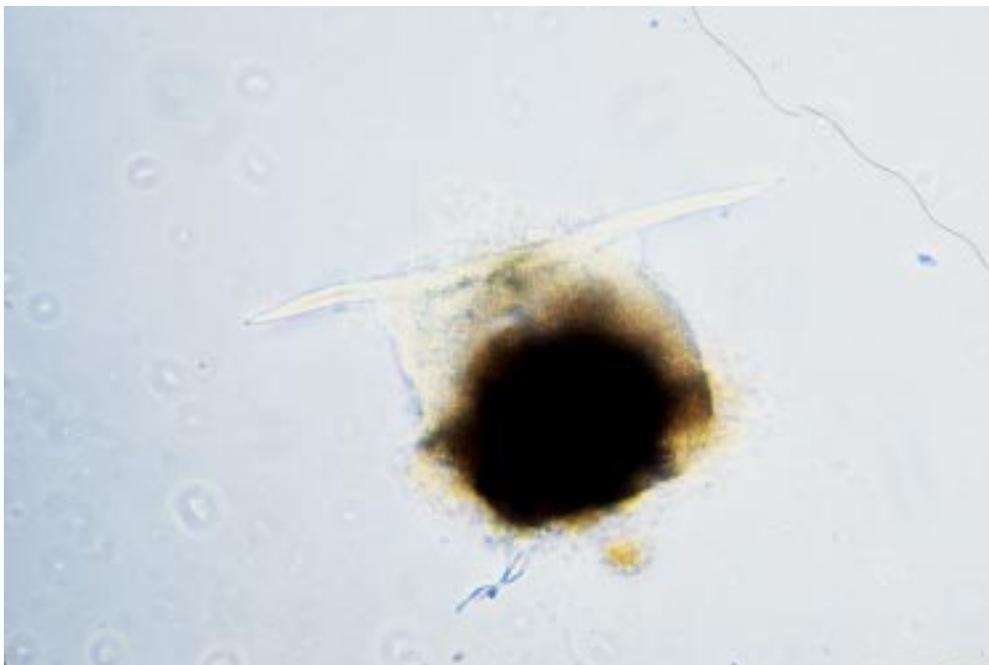


Figure 1b. Detached acervular conidiomata with associated trichome.

Other fungi, such as *Septoria floridae* (Farr, 1991), *Colletotricum gleosporoides* (Toole and Filer, 1965), or *Phyllosticta sp.* (Walkinshaw, pers. comm.) may produce foliar symptoms that are similar to those produced by *D. destructiva*.

Foliar samples from each tree were chosen from the refrigerated sample bags. One leaf was used to represent each tree. Approximately five leaf pieces measuring 0.25 cm² were cut from each leaf. The pieces were removed at the leading edge of infection (each piece contained some green as well as discolored tissue). The leaf pieces were placed in a 1.0% sodium hypochlorite solution for three minutes and immediately rinsed in sterile, distilled water. Within about five minutes, the leaf pieces were gently shaken to remove excess water and plated on PDA amended with chlorotetracycline HCl (Figures 2a and b). Within 10 days, the cultures could be identified as *D. destructiva* and five subcultures were made of each of the 40 foliar isolates. As with the twig isolates, these subcultures were used in the cultural characteristic study and each plate was labeled as to site, plot, tree, isolate and subculture. All cultures were stored in Petri plates in the refrigerator at 4 C.

For experiments that required single spores or spore suspensions, the parent isolates were subcultured on oatmeal agar (Sigma Chemical Co., St. Louis, MO, 72.5g/l). This medium induces abundant sporulation within a week. After the first week, the plates were flushed with a 1% peptone isotonic aqueous solution and swirled on a rotating plate with a bent glass rod to evenly distribute the spores. One drop of the solution was placed on a hemacytometer and the number of spores per ml was determined. In order to achieve the desired concentration of 1×10^6 /ml, generally four 10-fold serial dilutions



Figure 2a. Removal of diseased tissue at leading edge of infection. After removal, the tissue is surfaced sterilized in dilute NaOCl for 3 minutes.

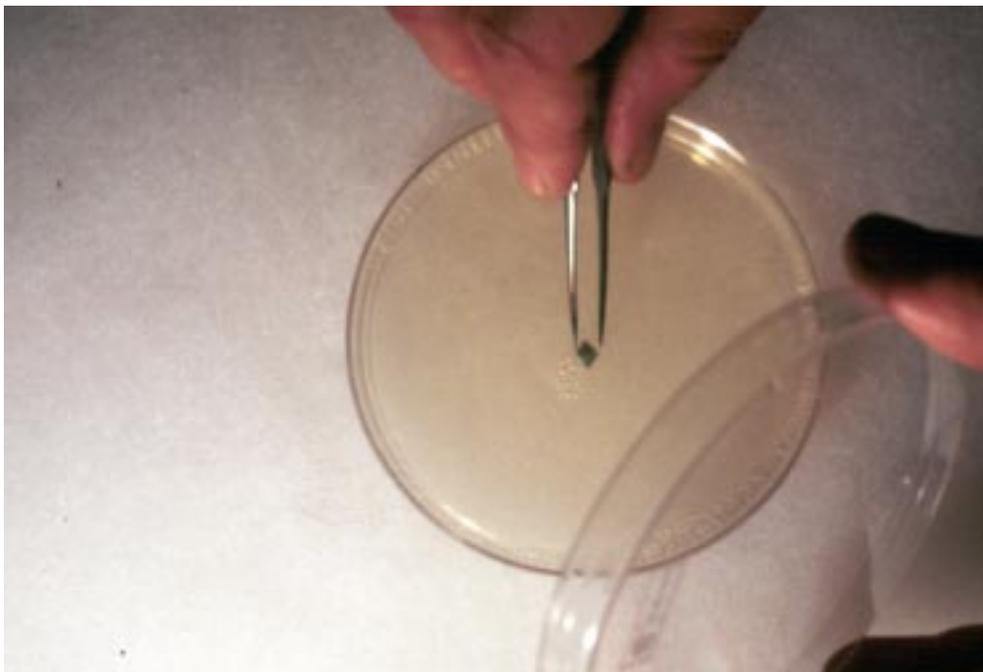


Figure 2b. Leaf piece is plated on PDA after surface sterilization. Detection of *D. destructiva* was made 10 days after isolation procedure.

were necessary. This concentration was pipetted on PDA + chlorotetracycline HCl and the single germlings were subcultured within three days.

Evaluation of cultural characteristics

In an attempt to determine if cultural differences among the isolates existed, all foliar and twig isolate subcultures were incubated on PDA + chlorotetracycline HCl at 24 C, under a 16/8 hr light regime. The number of subcultures/parent isolate ranged from one to five, depending on the success of the subculture transfer. After 21 days of growth, the diameter of each replicate isolate was measured to the nearest millimeter (mm), on the widest portion of each culture. A total of 274 subcultures were measured during the first series of tests on August 13, 1994. Differences in diameter growth were statistically analyzed by the use of a nested ANOVA procedure. Pigmentation was subjectively evaluated as was the presence of concentric zonation. When other distinctive cultural features were evident, they were recorded for each isolate. A second series of cultural testing was conducted on September 5, 1994. All isolates in the second series of tests were subcultured from the first set of subcultures and measured after three weeks of growth. Again, the number of subcultures for each isolate varied from one to five. A total of 202 subcultures were examined during the second series. The isolates were incubated for 21 days and the cultural characteristics were recorded as described above.

Vegetative compatibility

In order to test for the existence of a system of vegetative compatibility (VC), twelve

randomly chosen *D. destructiva* isolates (three from each of the four sites; with at least one twig and one foliar representative from each site) were used. A compatibility testing procedure described for *Cryphonectria parasitica* was utilized (Anagnostakis, 1977) as a guide to determine if a VC system existed for *D. destructiva*. However, a variety of different media and incubation conditions were employed. The media used in this study included:

- 1) water agar (Difco, 20g/l);
- 2) water agar amended with flaked, pre-autoclaved dogwood leaves;
- 3) half strength (19.5 g/l) PDA; and,
- 4) half strength PDA amended with flaked pre-autoclaved dogwood leaves.

Each medium was amended with 0.05 g chlorotetracycline HCl to reduce bacterial contamination.

Testing for VC required that the isolates be paired with themselves and each other. To do this, agar plugs containing the test isolates were placed approximately 0.5 centimeters apart. Cultures were incubated for 21 days at 20 C. Replicates of each pairing were made with one being placed in the dark and one in the alternating light and dark (16/8h). The cultures in the dark were placed in a wooden box and covered with a piece of black cloth.

After 21 days, each plate was viewed under an M3Z Wild (Heerbrugg, Switzerland) stereoscopic microscope for observation of the presence of a barrage, or zone of inhibition between paired isolates, indicative of an incompatibility reaction.

Detection of double-stranded RNA

Discula destructiva parent isolates and their single spore progeny were evaluated for presence or absence of dsRNA. The procedure used to extract dsRNA from *C. parasitica* was modified for this extraction (Morris and Dodds, 1979).

The extraction technique involves cellulose chromatography, which is designed to retain mostly dsRNA and eliminate the attachment of other nucleic acids. The mycelium of the isolates to be tested was first grown on cellophane, which was layered on PDA. The mycelium was scraped from the cellophane surface and frozen immediately in liquid nitrogen. The frozen mycelial tissue was ground with a mortar and pestle and was stored in a -20 C freezer in vials until ready for use.

A phenol/chloroform extraction was used to separate proteins from nucleic acids. The supernatant from each tube was pipetted into graduated cylinders and brought up to a 24.4-ml volume with ethanol and 1x STE (buffer without alcohol). This solution was passed through columns containing chromatographic cellulose powder (Whatman CF-11 cellulose) which was equilibrated with STE/16% ethanol. The charged column was washed with approximately 80 ml STE/16% ethanol and the retained dsRNA was eluted with 11 ml of 1x STE buffer. The nucleic acid solution was collected in 30-ml glass Corex tubes. Sodium acetate and ethanol were added to precipitate the nucleic acids.

After the mixture was in the -20 C freezer overnight, the precipitate was collected by centrifugation. To eliminate DNA from the preparation, DNAase was added to the mixture to digest DNA, along with magnesium chloride as a cofactor for the DNAase. After digestion at room temperature for 60 minutes, ethanol was added to stop the reaction. Sodium acetate was then added to form a salt with the nucleic acid. The tubes

were then placed in the -20 C freezer for two hours.

Approximately 1.5 ml of each sample was added to a microcentrifuge tube and spun for 5 minutes at 13,000 rpm. The mixture was decanted and the process repeated twice. The resulting pellet was resuspended with a buffer solution and bromphenol blue. Nine microliters were electrophoresed in an agarose gel with ethidium bromide for 2 hr. Bands, indicating the presence of nucleic acids (dsRNA), were detected under UV light. Polaroid film was used to record the nucleic acids present in each profile.

Virulence evaluation tests

In an effort to establish a method to compare the virulence of the isolates, three series of experiments were undertaken. An apple inoculation assay has traditionally been used to detect the presence and degree of virulence of some plant pathogenic fungi (Streets, 1982). This bioassay was previously tested for estimating the virulence of *D. destructiva* isolates (Brown et al., 1991) and was employed in this study. Excised dogwood leaves and dormant stem pieces also were used as tissues to determine if there is a difference among the isolates ability to invade host plant tissue.

Apple assay

An apple assay evaluated the amount of growth by *D. destructiva* that occurs following inoculation. Randomly selected *D. destructiva* isolates and several isolates that demonstrated reduced growth from the first subculture in the cultural characteristics assay were chosen from each study site and inoculated into “Golden Delicious” apples during eight trials from October, 1994 to July, 1996. All apples were first surfaced sterilized in a

10% aqueous solution of amphyll for 10 minutes. The isolates were then inoculated into the apple by first flaming a 7 mm cork borer, removing a piece of the apple with the borer and placing an agar plug of the fungal isolate into the hole. Sterile blank agar plugs served as controls and were used in Trials 4-8. Three-to-four inoculations were made in each apple. Following inoculation, each point was covered with labeling tape. The apples were incubated in a 30 x 9 x 17-cm plastic container with snap-down lids, to prevent desiccation. Ten days later, the lesion diameter was measured to the nearest millimeter.

Each of eight separate trials were performed, according to the aforementioned protocols. Each trial was implemented in a slightly different manner including: 1) the date of inoculation; 2) number of measurements taken; 3) number of isolates tested; 4) number of inoculation sites; and, 5) number of apples that were used (Table 1).

To confirm that the lesions were the result of colonization by *D. destructiva*, a reisolation procedure was initiated during Trial 3. After the lesion diameter measurement was taken, a small piece of apple (0.5 cm), containing both healthy and necrotic tissue, was placed in 1.0% sodium hypochlorite solution for 3 minutes and immediately rinsed in sterile distilled water. The pieces were then plated on PDA. If other fungi were retrieved, they were scored as contaminants. If it was possible, the contaminants were identified to genus and were used as inoculum in Trials 4-8. In the seventh and eighth trials, controls were paired with either the *D. destructiva* isolates or the contaminants. Pairings were made by placing the inoculum in two-7mm wide holes, which were approximately 0.5 cm apart. This procedure was used in an attempt to observe whether the establishment or growth of the *D. destructiva* isolates was influenced by sterile agar

plugs (controls) or contaminants.

Table 1. Apple inoculation dates, number of measurements per trial, number of *D. destructiva* isolates per trial, number of inoculation points per apple and number of apples used per trial.

Inoc. date	# Measurements	# Isolates	Inoc. points/apple	# Apples
10/16/94	2	21	3	16
11/23/94	3	44	3	82
5/22/95	2	12	3	40
3/27/96	1	8	4	10
4/23/96	1	4	4	6
5/24/96	1	4	4	6
8/30/96	2	4	4	10
10/2/96	1	4	4	20

Foliar inoculations

A foliar assay also was used in an attempt to measure the ability of the isolates to invade leaf tissue. All the foliage was taken from a dogwood tree near the USDA Forest Service building in Morgantown to eliminate genetic variability among hosts. The samples were removed with hand pruners, with each cutting containing four terminal leaves. The cuttings were placed in 250-ml Erlenmyer flasks containing distilled water. Fresh cuttings were taken for each of two trials occurring at the beginning and end of July, 1996 (Figure 3a).

Eight randomly chosen twig and leaf *D. destructiva* isolates from each of the four

study sites were used in the foliar inoculation study. Two of the isolates (POTO3-1 and POBO3-1) which were successfully reisolated from the infected apples in Trials 4 and 5 served as inoculum, as did two slow growing isolates from the cultural characteristics assay. All isolates used were taken from the first subculture in the cultural characteristic assay. Inoculum was generated from single spore cultures by growing the eight isolates on oatmeal agar. The same protocols for producing a solution of 1×10^6 spores/ml that were described in the isolation section were utilized in this assay.

In nature, *D. destructiva*, can enter leaves directly on unwounded tissue (Graham et al., 1991). Although natural infection occurs readily, attempts to artificially cause infection have met with limited success (Schreiber et al., 1993), and it has been suggested that wounding is a necessary prerequisite for successful infection (Redlin, 1992). In this test, pre-wounded and unwounded leaf tissues were inoculated. All four leaves on each pre-wounded cutting were pricked on the upper leaf surface with one 000 size insect pin which was seated in a 5x5 mm piece of extruded polystyrene.

Ten foliar cuttings were inoculated with the spore suspension with one *D. destructiva* isolate; five cuttings were wounded and five were unwounded. The spore suspension was applied by a spray bottle to the wounded and unwounded cuttings. An identical set of cuttings served as controls and were sprayed with sterile distilled water. This scheme yielded 20 cuttings, each with four leaves (10 controls; 10 treatments) per isolate used. All cuttings were incubated beneath inverted 60 x 35 x 26-cm clear plastic containers. Each plastic container could support 10 flasks with cuttings. Eight containers were used; four contained cuttings that were sprayed with isolate inoculum and four contained cuttings that were sprayed with water. The flasks were incubated at



Figure 3a. Terminal dogwood leaf cuttings. Note woody stem in flask of water



Figure 3b. Foliar incubation procedure

approximately 24 C for 10 days (Figure 3b). After 10 days, lesion diameters were measured at the widest side of each lesion. If foliar necrosis was detected within the 10-day period, isolations were made to confirm the presence of *D. destructiva*. This was done by taking a 0.25 cm² piece of leaf at the leading edge of infection and soaking it in a 1.0% sodium hypochlorite solution for 3 minutes. The sample was then transferred to sterile distilled water for rinsing and plated on PDA + chlorotetracycline HCl. Since *D. destructiva* is very slow growing, it took approximately 10 days to positively detect the presence of this fungus when cultured on PDA.

Stem inoculations

A stem inoculation procedure also was employed in an attempt to determine whether the *D. destructiva* isolates were capable of invading woody bark. Approximately 3-cm diameter stems were cut from healthy *C. florida* trees during early February 1995. The stems were cut into 30 cm lengths and the cut ends were sealed with paraffin to avoid moisture loss (Lee et al., 1992). The sealed stems were stored in a -20 C freezer until use.

Three separate stem inoculations were conducted from December, 1995 through May, 1996. The inoculations were performed by surface sterilizing the stems in a 50% aqueous solution of amphyll for 10 minutes. An upward cut was then made at the bark-cambial interface and an agar plug with a *D. destructiva* isolate was placed under the bark flap (Hibben and Daughtrey, 1988). During the first trial, six isolates were inoculated into the dogwood stems. Two slow-growing isolates from the cultural characteristics study (GBPM5-2 and KRWO2-1t) and two randomly selected isolates were used. The two Pocahontas isolates from the apple assay (POTO3-1 and POBO3-1) were used as

inocula in all three trials. A total of sixteen randomly selected isolates from the four sites were inoculated into the stems during the second and third trials. All isolates were taken from the first subculture in the cultural characteristics assay. Sterile blank agar plugs served as controls and were employed in the three trials. The bark flap, which covered inoculation points, was wrapped with labeling tape and the stems were placed in a tray with autoclaved peat, to retain moisture. The tray was covered with plastic and incubated in the dark at 24 C. In 10 days, the diameter of the lesion under the bark flap was measured to the nearest millimeter.

RESULTS

Study plots and tree ratings

Each of the five dogwood trees within the plots at the four sites were rated for percent foliage and leaf area infected. The top of the hill plot (POTO) at the Pocahontas Co. site had the highest percentage of foliage infected (27%) and the Camp Russell plot at Oglebay (RUOG) had the highest leaf area infected (22%). The wooded plot in Krepps Park (KRWO) had the lowest percentage of both foliage (9%) and leaf area infected (8%). For all sites, the trees growing in open, sunny locations had an average of 16 percent foliage and leaf area infected and the wooded plots averaged 17.5 percent foliage and 15 percent leaf area infected (Table 2). The ratings were taken with the intention of presenting a general overview of the amount of infection observed on each tree at the time of leaf and twig collections. Percent infection of each study tree was not statistically analyzed, as determining the amount of infection present on each tree was not an objective of this study.

Cultural characteristics of *D. destructiva* isolates

In order to describe the cultural characteristics of the *D. destructiva* isolates, replicate subcultures were grown on PDA for 21 days. Two separate trials were conducted with the subcultures. The colony diameters of each isolate after 21 days of growth during both subculture trials are included in Appendix Figures 1-8. After 21 days of growth, the average diameter of 274 isolates used in the first trial was 55.60 mm. Two hundred and two of these isolates were measured for the second trial. They averaged 50.31 mm in

Table 2. Percent foliage infected (FI) and leaf area infected (LAI) on dogwoods in plots within each study site.

	Krepps		Oglebay	
	Plot 1	KRWO	Plot 1	RUOG
	FI	LAI	FI	LAI
Tree #1	15%	10%	30%	25%
Tree #2	15%	15%	10%	25%
Tree #3	5%	5%	10%	20%
Tree #4	5%	5%	29%	20%
Tree #5	5%	5%	30%	20%
average	9%	8%	20%	22%
	Plot 2		Plot 2	
	Plot 2	KROP	Plot 2	WOG
	FI	LAI	FI	LAI
Tree #1	10%	20%	15%	15%
Tree #2	15%	15%	10%	10%
Tree #3	15%	10%	15%	10%
Tree #4	10%	15%	10%	10%
Tree #5	15%	10%	20%	15%
average	13%	14%	14%	12%
	Pocahontas		Gettysburg	
	Plot 1	POTO	Plot 1	GBPM
	FI	LAI	FI	LAI
Tree #1	25%	15%	10%	15%
Tree #2	35%	20%	40%	25%
Tree #3	15%	15%	20%	20%
Tree #4	20%	30%	15%	20%
Tree #5	40%	20%	20%	20%
average	27%	20%	21%	20%
	Plot 2		Plot 2	
	Plot 2	POBO	Plot 2	GBDD
	FI	LAI	FI	LAI
Tree #1	10%	10%	40%	25%
Tree #2	15%	15%	10%	10%
Tree #3	10%	5%	15%	20%
Tree #4	15%	20%	10%	10%
Tree #5	15%	10%	10%	10%
average	13%	12%	17%	15%

diameter at the end of 21 days. A nested analysis of variance procedure was used to determine whether differences in the rate of growth existed among isolates from the different study sites. When colony diameter was measured, no significant differences ($P < 0.05$) in growth were found among the isolates from the four different sites; among isolates within the same site (plots within site); among isolates taken from each tree from each site; or between leaf and twig isolates within and among sites (Table 3). However, the analysis failed to identify 13 isolates during the first trial and 20 isolates during the second trial that were appreciably smaller in diameter than the majority of those measured. Those isolates grew only 20 mm or less after 21 days (Figure 4). At

Table 3. General linear models procedure with diameter as the dependent variable

Source	DF	Type III SS	F Value	PR>F
Site	3	3830.181	4.13	0.1022
Plot (Site)	4	1237.534	1.41	0.2304
Tree (Site*Plot)	32	10445.14	1.49	0.0509
Tissue	1	2.589	0.01	0.9135

least one small isolate was recovered from each site (Appendix Figures 9 and 10).

In order to test if the reduced growth was a consistent feature, comparisons were made between the diameter of the isolates in the first and second trials. Only two isolates from the first trial remained small in the second trial. One of the isolates from Gettysburg (GBPM 5-2) was 20 mm in diameter during the first trial and 14 mm at the second trial. An isolate from Krepps Park, KRWO2-1t, was originally 19 mm in diameter; at the second trial was still only 20 mm.

The appearance of each isolate in culture was subjectively rated. Characteristically, all

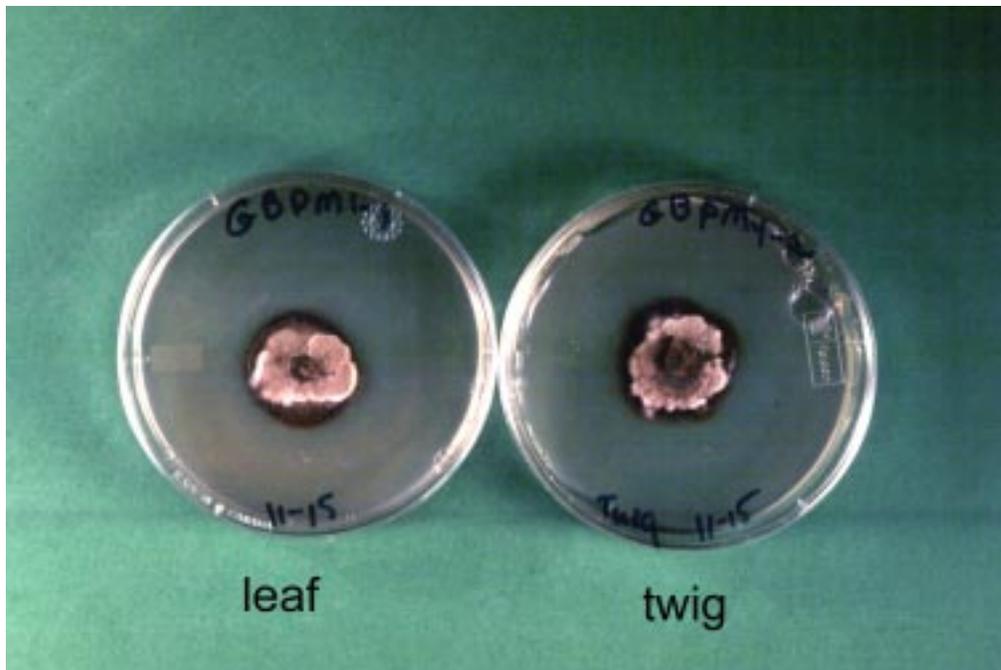


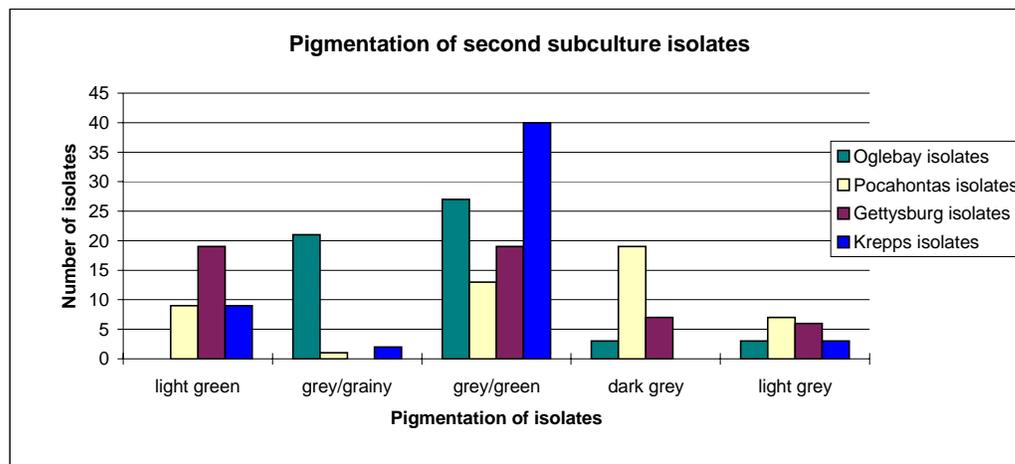
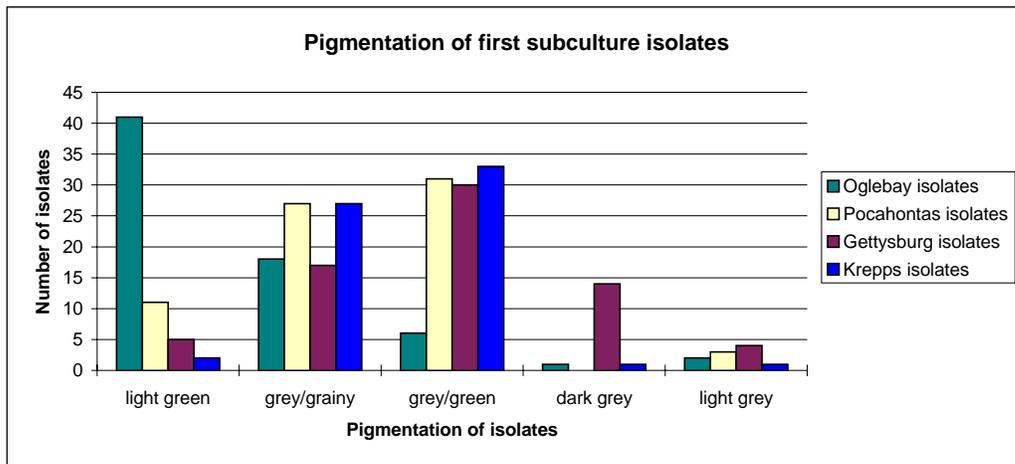
Figure 4. Slow growing *D. destructiva* isolates. Note powdery appearance and lack of concentric zonation pattern.

faster growing cultures were initially light green, darkening to greenish grey and dark grey over time (Figures 5). Cultural textures varied from smooth to grainy, with mycelial growth appearing slightly fluffy. All of the faster growing isolates had smooth or scalloped margins and a concentric zonation pattern (Figure 6). Sixteen of the 274 isolates in the first trial and 12 of 202 isolates in the second trial possessed a very distinct concentric zonation pattern (Figures 7 and 8). All isolates had watery droplets of exudate in the center of the colonies. The slow growing isolates were characterized as raised on the plate and powdery. They generally possessed a grey color and were hard and rubbery to the touch. The slow growing isolates did not display patterns of concentric zonation. In general, each site yielded isolates that were comparable in morphology and pigmentation to those from the other sites.

Vegetative compatibility

A variety of media and incubation conditions were used in a pairing test to observe whether *D. destructiva* isolates were vegetatively compatible. Twelve randomly selected isolates from the four collection sites were paired on four different media at 20 C and incubated in either a 16/8 h light:dark photoperiod or in total darkness for 21 days (Figure 9). After incubation, cultures were observed under a stereoscopic microscope for any indication of a zone of inhibition or presence of a barrage where colonies converged. A zone of inhibition was assumed to be an indication that the paired isolates were vegetatively incompatible. In no instance was a zone of inhibition or barrage evident between any of the paired isolates regardless of the substrate or incubation conditions. Sporadically dispersed fruiting bodies (acervular conidiomata) were occasionally formed

Figure 5. Frequency of five classes of pigmentation among isolates from both subcultures after 21 days of growth under 16/8 hr light.



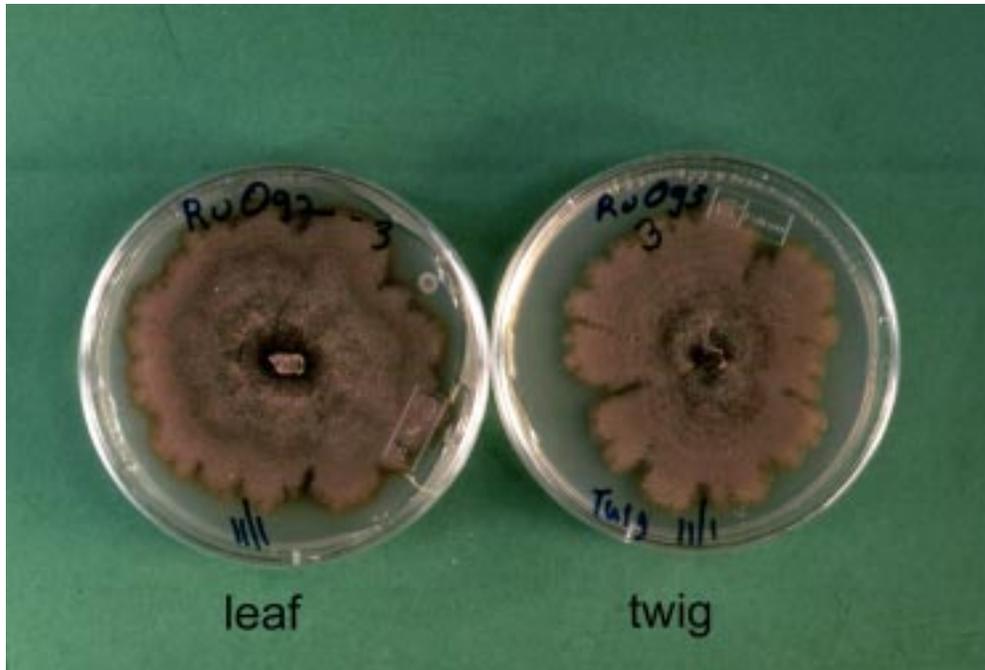
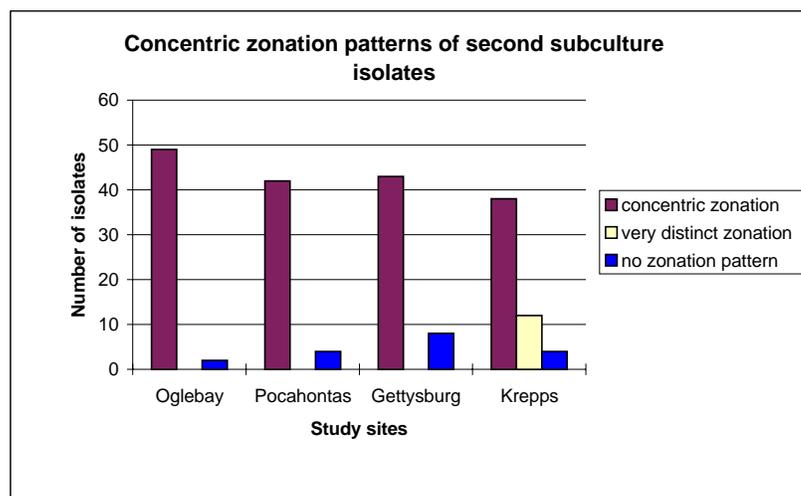
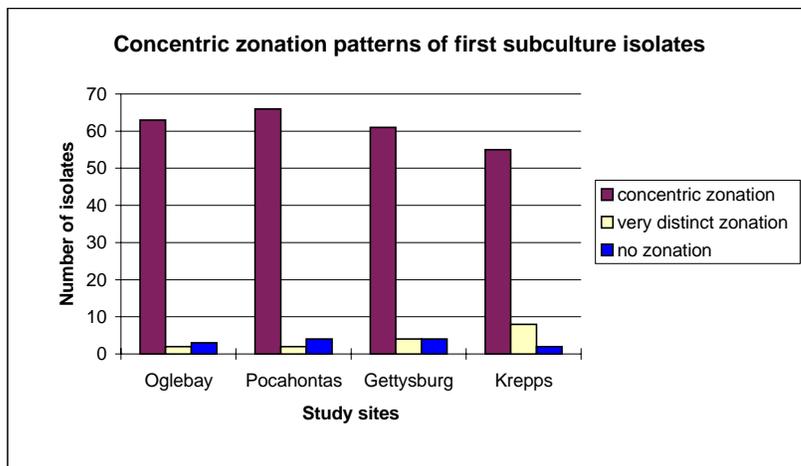


Figure 6. Growth habit of two *D. destructiva* isolates from leaf and stem tissue showing the most common colony morphology.

Figure 7. Frequency of zonation patterns of isolates from each site during both subcultures.



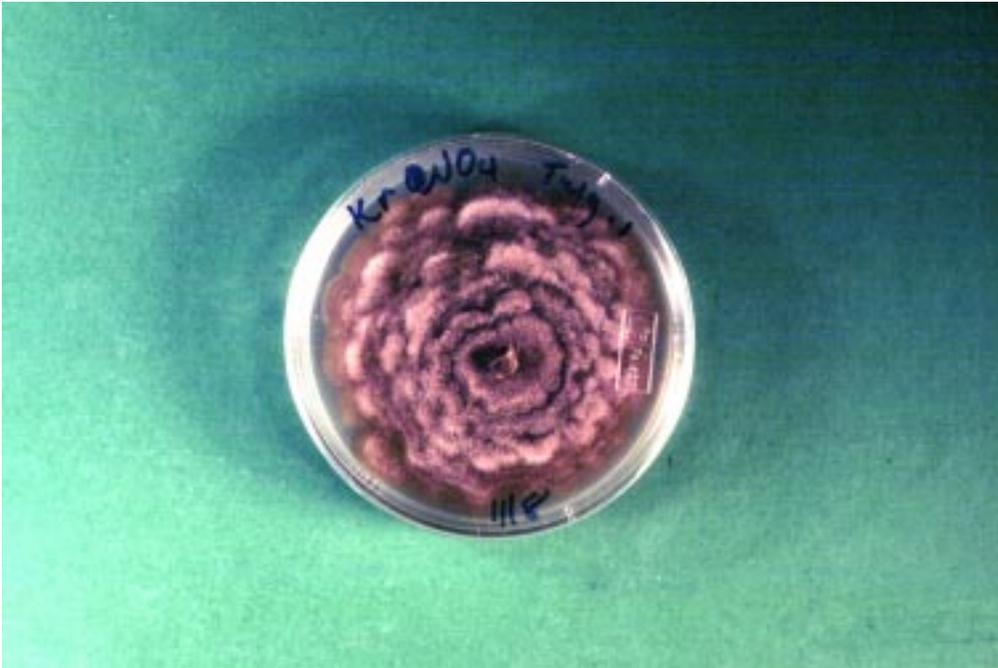


Figure 8. Distinct concentric zonation pattern evident in some isolates.



Figure 9. Paired isolates on half-strength PDA and half-strength PDA + dogwood leaves. Isolates were incubated in alternating 16/8 hr. light.

on the leaf pieces in the water agar and half-strength PDA media. The isolates grown on water agar were slow growing and almost transparent, whereas the isolates grown on PDA yielded more luxuriant growth. In no instance was hyphal anastomosis actually observed microscopically. Observing hyphal anastomosis microscopically on PDA was difficult given the abundance of hyphae in the field of view. Likewise anastomosis was difficult to view when the pairs were growing on water agar media, as hyphal growth was sparse and sporadic.

Double-stranded RNA analysis

A phenol-chloroform extraction, CF-11 chromatography and electrophoresis were used to examine *D. destructiva* isolates for the presence of dsRNA. Agarose gel electrophoresis revealed that dsRNA was present in all 56 isolates tested. The number of visible bands ranged from 1 to 9, although some of the bands could barely be seen. A longer photographic exposure (5 sec. vs. 3 sec.) provided greater clarity necessary to detect the banding patterns from isolates where the bands were difficult to detect. Patterns between twig and leaf isolates from the same site were evident, as were the banding patterns between isolates from the four different sites (Figure 10).

When the dsRNA banding patterns from 15 single spore progeny were compared to those of their parents, the patterns in the progeny were similar to those of the parent. In no instance did the process of single sporing free the *D. destructiva* colonies of dsRNA.

Virulence evaluation tests

Apple Assay

Eight apple inoculation trials were conducted over a two-year period. In each trial

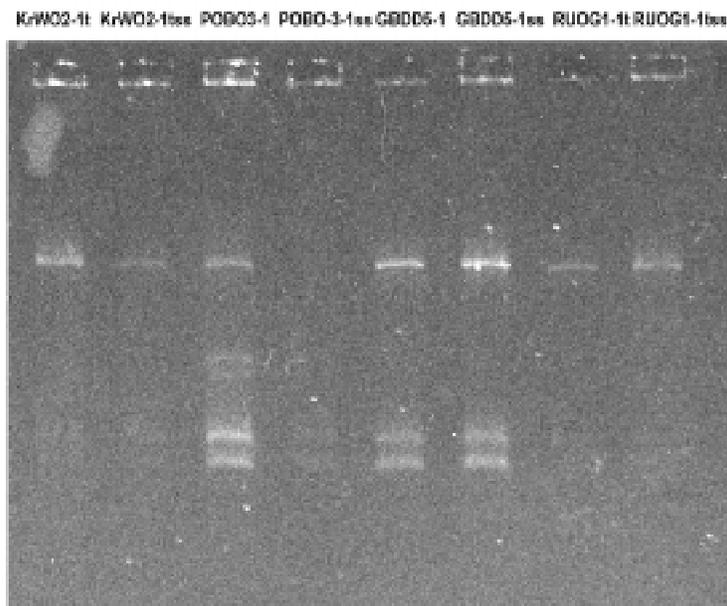


Figure 10. Double-stranded RNA banding patterns on agarose gel. One *D. destructiva* isolate and single spore progeny from each study site.

the diameter of the lesions was measured at 10-day intervals. Table 4 lists the number of lesions that resulted from each inoculation point during the eight trials. The number of apples used during each trial is indicated in the methods section. In each of the eight trials, lesion diameters were measured ten days after inoculation (Figure 11). During the first and second trials, lesions developed at almost all of the inoculation points. Reisolations were not attempted during the first two trials and the necrotic tissue that developed was assumed to be caused by invasion of *D. destructiva*. After appreciably fewer lesions developed during the third trial, attempts were made to reisolate *D. destructiva*.

Table 4. The number of lesions on apples in each trial after inoculation. Measurements were taken at 10-day intervals.

Trial	Measurement	#inoc. points	# resulting lesions
1	1	48	43
1	2	48	46
2	1	246	222
2	2	246	242
2	3	246	244
3	1	120	38
3	2	120	44
4	1	40	25
5	1	24	19
6	1	24	16
7	1	80 (paired)	22
7	2	80 (paired)	28
8	1	160 (paired)	50

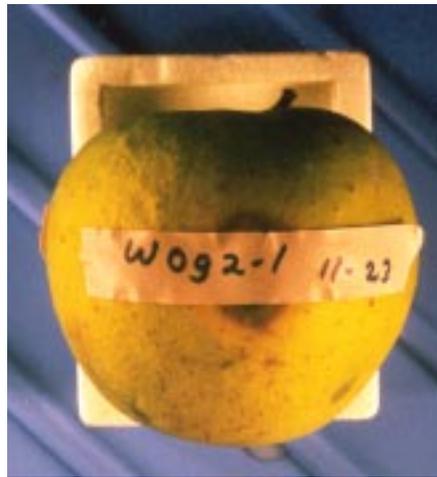


Figure 11. Lesion development on apple 10 days after inoculation with a *D. destructiva* isolate.

Reisolations were made during all remaining trials and were taken from lesions that were not obviously contaminated or degraded.

When the first reisolation was attempted during Trial 3 only species of *Penicillium*, *Chaetomium*, *Phyllosticta*, *Phomopsis* and an unidentified contaminant were retrieved. During Trial 4, one *D. destructiva* isolate, POTO3-1, was retrieved from one lesion on one apple. Three *D. destructiva* isolations were recovered during Trial 5. All were the same Pocahontas isolate, POBO3-1.

Since the two Pocahontas isolates (POTO3-1 and POBO3-1) were retrieved during Trials 4 and 5, these isolates, along with randomly selected *D. destructiva* isolates, were inoculated into apples, in the remaining trials. During Trial 6, only species of *Epicoccum*, *Trichoderma* and *Penicillium* were recovered when tissues from lesions were cultured (Table 5).

Table 5. Frequency and recovery of fungi from inoculated apple lesions in Trials 3-6.

Trial 3				
44 lesions produced				
<i>Penicillium</i>	<i>Phomopsis</i>	<i>Chaetomium</i>	<i>Phyllosticta</i>	unidentified
31	8	1	2	2
Trial 4				
25 lesions produced				
<i>Penicillium</i>	<i>Phomopsis</i>	<i>D. destructiva</i>	unidentified	
13	10	1	1	
Trial 5				
19 lesions produced				
<i>Penicillium</i>	<i>Phomopsis</i>	<i>D. destructiva</i>	unidentified	
13	3	3	0	
Trial 6				
16 lesions produced				
<i>Penicillium</i>	<i>Phomopsis</i>	<i>Epicoccum</i>	<i>Trichoderma</i>	
7	6	2	1	

Because so many contaminants were isolated, despite careful surface disinfection of the apples, the relevant question was, were lesions actually being formed by *D. destructiva*? In an attempt to answer this question, a pairing procedure was employed during Trials 7 and 8 involving *D. destructiva* isolates (including POTO3-1 and POBO3-1) and either sterile agar plugs or one of the commonly retrieved contaminants (*Phomopsis sp.* and *Penicillium sp.*). During these two trials, the isolates and/or contaminants and controls were paired. When isolations were taken from infected apple tissue near the paired sites, *D. destructiva* was never retrieved. In some cases, the introduced contaminants were recovered from paired inoculation points where either a *D. destructiva* isolate or a blank agar plug was used as inoculum. When the contaminants were co-inoculated with a *D. destructiva* isolate, either the inoculated contaminant (*Phomopsis sp.* or *Penicillium sp.*) was recovered, or no lesion was evident. A chi-square analysis ($P < 0.05$) determined that the frequency of infection was distributed differently among points on the apple that were initially inoculated with either a *D. destructiva* isolate, control or contaminant inoculum (Table 6). Pocahontas isolates, POTO3-1 and POBO3-1, successfully isolated from Trials 4 and 5, were not recovered in the remaining trials.

Table 6. Frequency of infected and non-infected inoculation points 10 days after inoculation with selected *D. destructiva* isolates, blank agar plugs (controls) and contaminant inoculum during Trials 7 and 8.

	Trial 7		
	Isolate	Control	Contaminant
Infected	11	6	11
Non-infected	13	22	7

	Trial 8		
	Isolate	Control	Contaminant
Infected	29	8	13
Non-infected	19	42	27

Foliar inoculations

A spore suspension from eight *D. destructiva* isolates was sprayed onto wounded and unwounded dogwood leaf cuttings. Each isolate was inoculated onto 20 pre-wounded and 20 unwounded leaves (four leaves/cutting; ten cuttings/isolate-five wounded, five unwounded). Lesion development was measured 10 days after inoculation. A total of 160 leaves were pre-wounded and treated with a spore suspension of eight separate *D. destructiva* isolates. Lesion development only occurred on leaves that were pre-wounded. Lesions were produced only on 11 of the wounded sites on 160 leaves. All lesions occurred on one leaf per cutting, except one cutting that had one lesion on each of two leaves. Upon isolation, nine of the 11 lesions yielded species of *Colletotrichum*, *Alternaria* and *Epicoccum*. A causal agent was not associated with the remaining two lesions. Necrosis was associated with the pin prick area and may have been attributed to wound necrosis, not *D. destructiva* symptom expression. Regardless of whether the leaves were wounded or non-wounded, lesions were not produced when water was used as a control. All of the leaves remained intact throughout the 10-day incubation period.

Since attempted retrieval of *D. destructiva* from all necrotic areas was unsuccessful on all symptomatic leaves, a correlation could not be made between lesion size and an isolate's ability to invade host tissue.

Stem inoculations

Three inoculation trials were undertaken during this study using dormant dogwood stems. A total of 26 *D. destructiva* isolates were inoculated into the stems within a 10-month period of time during 1995-1996 to observe whether infection would occur and if

differences in invasiveness could be measured.

Twig and leaf isolates, including the Pocahontas isolates from the apple assay, two slow growing isolates from the cultural characteristic study and representatives from all four study sites, were used in this assay. Lesion formation did not occur on the inoculated stems and attempts to retrieve *D. destructiva* under the bark flap met with failure at each trial. The most common fungal species isolated from under the bark flap was *Trichoderma sp.* The presence of *Penicillium sp.* and *Aspergillus sp.* also was quite apparent on the outside of the stems. Although periodic washing with amphyll temporarily removed the contaminants, the growth was again evident within a few days.

DISCUSSION

The susceptibility of eastern flowering dogwood to *Discula destructiva* has been known since the mid-1970's and the pathogen continues to threaten dogwoods today. This disease is unquestionably the most serious current threat to dogwood and its impact has been acknowledged to be significant in local areas (Daughtrey et al., 1996). As a result of its impact, many studies of the disease have been conducted in heavily infected areas such as Catoctin Mountain Park, MD (Sherald et al., 1996) and Great Smokey Mountain National Park, TN (Knighten and Anderson, 1992). Factors that contribute to or enhance disease development have been attributed to various microclimatic conditions such as reduced light and evaporative potential (Erbaugh et al., 1995), proximity of trees to water (Windham et al., 1995) and north/northeast aspect (Chellemi et al., 1992). Other factors that may play a role in the incidence of the disease include the dogwood's resistance to infection and the virulence level of *D. destructiva*. In this study, attempts were made to determine if variability among the isolates of *D. destructiva* could be demonstrated. To accomplish this, diseased dogwood tissue was collected from four widely separate geographic locations and a series of tests were conducted in an effort to elucidate isolate differences.

Leaf and twig samples were taken from 40 symptomatic trees and *D. destructiva* was cultured on PDA. Isolate variability was first quantitatively assessed by measuring the diameters of the colonies the isolates produced. Although a low percentage of the isolates grew more slowly than the majority in both trials, overall differences in growth rate were not statistically significant. In this study, only two replicate isolates produced small

colonies during both cultural growth rate trials. The reason for slow growth by these two isolates is unclear. The slow growing isolates were always hard and rubbery, as opposed to those that grew at a typical rate and produced slightly fluffy mycelium with zonation. Previous workers have identified slow growing isolates and found that they will generally resume a “normal” rate of growth when subcultured (Windham and Trigiano, pers. comm.). With the exception of these two cultures, this also was the observation in my study.

When pigmentation and morphology were qualitatively assessed, all of the isolates, except the small colonies, were generally light green, progressing to grey/green and dark grey after three weeks of growth. A concentric zonation pattern was a common feature of all isolates displaying typical colony characteristics. Although the light conditions were the same for all recorded isolates, a small percentage possessed a very distinct zonation pattern. Salooga (1982) reported differences in the morphology of isolates, collected in Washington State, when grown on various nutrient media. However, when the isolates were grown on the same medium, she did not observe differences. Redlin (1991) examined 120 *D. destructiva* isolates that were collected from a wide geographic range in the eastern U.S. All isolates were found to be similar in morphology and colony characteristics. With the exception of the slow growing isolates found in the current study, the findings in my study are in agreement with those of previous studies.

Vegetative incompatibility has been a valuable phenotypic marker for studying genetic diversity and population biology in fungi (Cortesi and Milgroom, 1998). Most studies have used presence or absence of a barrage during hyphal anastomosis to determine whether paired fungal isolates are incompatible or compatible, respectively. The pairing

of fungal isolates by vegetative compatibility groups as a means to measure diversity has become common in recent years (Leslie, 1993). Under the test conditions of this study, isolates were paired and observed for barrage formation, to establish compatibility relationships between strains. Although in no case was a barrage evident between pairs under any of the test conditions employed, the process of anastomosis was not actually observed in this study. In addition, the evidence of a barrage may not be a good indicator of incompatibility between pairs, as there may not be any VC barriers within this particular species. For the purposes of this study, an assumption was made that the paired isolates were vegetatively compatible because of the absence of a barrage between pairs.

During the process of hyphal anastomosis, dsRNA containing mycoviruses may be transmitted intracellularly and have been isolated from a number of important plant pathogens (Brasier, 1998). In the chestnut blight pathogen, *Cryphonectria parasitica*, less virulent (hypovirulent) strains of the fungus can convert virulent strains to hypovirulence by the transfer of dsRNA via anastomosis (Nuss and Koltin, 1990). These hypovirulent strains are presumably responsible for effectively controlling chestnut blight in Europe (Heiniger and Rigling, 1994). In 1992, McElreath et. al. (1994) used a phenol-chloroform extraction and CF-11 chromatography procedure to examine 80 *D. destructiva* isolates, from 12 eastern states, for the presence of dsRNA. All *D. destructiva* isolates examined contained dsRNA. In the current study, dsRNA also was detected in all isolates. The number of bands ranged from one to nine, although some of the bands were barely visible. The difficulty in detecting dsRNA may have been attributed to the small amount of dsRNA in some isolates. McElreath (1994) reported that the amount of dsRNA in some of the *D. destructiva* isolates that she analyzed was

small, resulting in low-intensity bands that were difficult to detect on the electrophoretic gel. Perhaps all isolates had similar numbers of bands, but the problem with visualizing them may have reduced the number actually seen. Allowing extended exposure time during Polaroid development and enlarging the photograph during the scanning procedure provided greater clarity to the banding profiles.

The presence of dsRNA in the single-spore progeny was evidence that the single-spore process did not free dsRNA from the parent cultures. The similarity in banding patterns between parent and progeny was indicative that dsRNA was transmitted during conidiogenesis. Although dsRNA was present in the tested isolates in McElreath's and the current study, the significance of dsRNA in *D. destructiva* isolates is unknown.

In order to assess the potential ability of *D. destructiva* to invade plant tissue, three different plant substrates were utilized in separate inoculation assays. In the first assay, 'Golden Delicious' apples were inoculated with *D. destructiva* isolates. A total of eight inoculation trials were performed. Lesions developed at inoculation points on the apples during the first and second trial and initially were assumed to be caused by *D. destructiva*. When fewer lesions were produced during the third trial, reisolation of *D. destructiva* was attempted and became a standard procedure during all remaining trials. *Discula destructiva* was not successfully retrieved from any apples in Trial 3. Forty-four lesions were produced during Trials 4 and 5 and *D. destructiva* was retrieved from only four of the lesions. During Trials 6 through 8, a total of 94 lesions were produced, but *D. destructiva* was not recovered from any of the lesions. The low frequency of recovery indicated that *D. destructiva* failed to be a successful invader of apple. Brown et. al. (1991) reported that the apple bioassay may prove useful in preliminary *D. destructiva*

pathogenicity tests, but according to the findings in this study, apple was not a good substrate for the development or detection of *D. destructiva* and thus not useful as a pathoassay.

The inability of *D. destructiva* to invade apple may be attributed to the pathogen's possible lack of enzymes (pectinases) required for infection of apple fruit. The presence of competitive microflora on the apple, could be another factor responsible for the lack of successful infection. *Penicillium expansum* Link is commonly found on the exterior of apple fruit. Despite careful surface disinfection, an undetectable bruise could initiate infection at the site of the lenticel, where the fungus may be residing (Janisiewicz, 1997). The continual isolation of post harvest fungi including species of *Penicillium* and *Phomopsis*, was evidence of this possible competitive interaction.

The second pathogenicity assay involved the use of excised dogwood leaf cuttings. Foliar lesion development occurred only eleven times in 160 inoculated leaves, but *D. destructiva* was never recovered. Species of *Colletotrichum*, *Alternaria* and *Epicoccum* were isolated from nine of the lesions. A species of *Phomopsis* also was recovered from one of the stems on the cuttings. A causal agent was not associated with the remaining two lesions. *Colletotricum gleosporioides* (Toole & Filer, 1965) and *C. acutatum* (Chellemi and Knox, 1991) have both been implicated as causing leaf spot and twig dieback, respectively, on *C. florida*. *Alternaria* sp. and *Epicoccum* sp. are considered epiphytes on *C. florida* (Andrews and Kinkel, 1986). *Phomopsis corni* has been reported on the leaves and stems of *C. florida* and several other *Cornus* species (Farr et al., 1989). These epiphytic competitors may or may not be responsible for inhibiting the colonization of *D. destructiva* on the leaf surface. In my experience, the window of time that is

optimum for isolation of *D. destructiva* is early June to early August. *Discula destructiva* was not visible on PDA when foliar isolations were taken after early August, as isolated contaminants had colonized the growing medium. This may be the result of an increasing number of leaf surface microflora or a decreasing amount of inoculum produced by *D. destructiva* at this time of the year. *Discula destructiva*'s inability to compete with foliar competitors also may restrict its ability to directly infect dogwood leaves.

Several factors in the current study may have contributed in the failure to achieve foliar infection after inoculation. The use of a spore suspension and the associated mucilage, may have inhibited spore germination. *Discula destructiva* was inoculated and reisolated from pre-wounded dogwood leaves (Bruck and Grand, 1992). The authors pierced the upper epidermis with a "hole punching device" and inoculated with a mycelial plug, rather than a spore suspension. Previous lack of infection using a spore suspension was attributed to inhibition of conidial germination by the mucilaginous matrix contained within the acervulus which covers the conidia (Grand, 1995). Redlin (1992) also used mycelial plugs in a leaf-disc bioassay. He found that pre-wounding the tissue was a prerequisite for infection. Sherald et al., (1992) used a leaf-disc bioassay for evaluating resistance in various *Cornus* species and was able to achieve infection with a spore suspension. Questions in the current study include whether leaf cuttings would support symptom expression and would it be possible to delineate wound necrosis associated with infection. Overall, artificially inducing infection on dogwood has been difficult. In the current study, I used foliar cuttings placed in a flask of water accompanied by pre-wounding and application with a spore suspension. The cuttings were placed under plastic to provide humidity. Past attempts to create infections on leaves, *in vitro*, have

met with very limited success and only when certain environmental conditions were met. Hibben and Daughtrey (1988) used one-to-three year old *C. florida* seedlings in a foliar inoculation trial. The trees were grown in a greenhouse and inoculated with a *D. destructiva* spore suspension. Providing high humidity, constant leaf wetness and depriving the trees of light after inoculation, were conditions required for infection. Given these environmental requirements, foliar lesions were produced and *D. destructiva* was reisolated. Although this procedure by Hibben and Daughtrey yielded positive results, *in situ*, it has never been successfully repeated (Daughtrey, pers. comm.). In 1988, Anderson et. al. (1989) induced infection by pre-treating leaves of one-year-old *C. florida* with an acid mist, adjusting to pH 2.8 with HCl. Acid deposition on leaves has been shown to affect cuticular surface characteristics (Percy and Baker, 1987), possibly predisposing the leaves to pathogen infection.

The third assay utilized excised dogwood stems as a substrate to determine whether infection would occur when *D. destructiva* was artificially inoculated in woody tissue. Hibben and Daughtrey (1988) inoculated two-year-old living dogwood trees by applying *D. destructiva* mycelium below a bark flap. Two months later, they observed canker formation in 23% of the inoculated trees and reisolated *D. destructiva* for confirmation. In another experiment, Schreiber et. al. (1993) found no canker development in stem-inoculated dogwoods.

In the current study, *D. destructiva* infection was not achieved when the stems were inoculated with different isolates of the fungus. Although the stems were periodically washed with amphyll, a variety of fungal contaminants such as species of *Penicillium* and *Aspergillus* continually colonized the outside of the stem and *Trichoderma* species

were occasionally isolated from inoculation sites.

Inducing growth or infection on any of the tissues used in this study was unsuccessful. This further confirms what others also have found. The ability of *D. destructiva* to act as a pathogen is difficult to demonstrate. In previous studies, applying an acid mist (Anderson et al., 1989), pre-wounding leaves (Bruck and Grand, 1992) and using live trees with specific environmental criteria (high humidity, moisture and darkness) in order to induce both foliar and stem infection (Hibben and Daughtrey, 1988) all were factors necessary for successful infection, but even in these cases, infection only occurred sporadically. In nature, ingression by conidial germ tubes occurs directly, i.e. without a wound (Graham et al., 1991). Why, then, is pre-wounding a necessary procedure in order to induce infection *in vitro*?

Characteristics in culture, vegetative compatibility, presence or absence of dsRNA and the ability to invade tissues were assays used in this study to measure isolate variability. All of the tests failed to detect differences among isolates. These results are consistent with findings of others who have worked with this pathogen. Since the disease was not evident on *C. florida* before about 1976 (Redlin, 1991), it is assumed that *D. destructiva* is a recently introduced pathogen. The sudden appearance and rapid spread of the pathogen suggests that the fungus may be exotic. The lack of variation among DNA profiles taken from isolates originating from widely dispersed locations in the eastern and western U.S. is further evidence that the pathogen has not been present long enough to have expressed or developed genetic variability (Trigiano et al., 1995).

Much information has been compiled about dogwood anthracnose since the mid-1970's when the disease was first recognized. Many questions still remain about this

fungus, including the origin of the pathogen; reasons that the disease impact is more severe in some areas and not in others given similar environmental conditions; and, the importance of the interaction of this fungus with other leaf microflora in the infection process. Researchers also have been puzzled by the requirements for infection. Although the pathogen can infect without a wound, inducing infection artificially in the laboratory or field has been difficult. Prevailing environmental effects such as summer drought, severe winter conditions on shallow rooted dogwoods, and moisture in spring may be important factors contributing to disease impact.

Why is it that the current impact of the disease appears less severe in the northeastern U.S, than it was in the 1980's (Daughtrey, et al., 1996)? A reduction in pathogen inoculum may be one reason. Another may be less favorable environmental conditions for disease development or an increased competitive effect of foliar microorganisms. If climatic conditions shift in a direction favoring disease development and trends become more evident, these questions may be easier to answer in the future.

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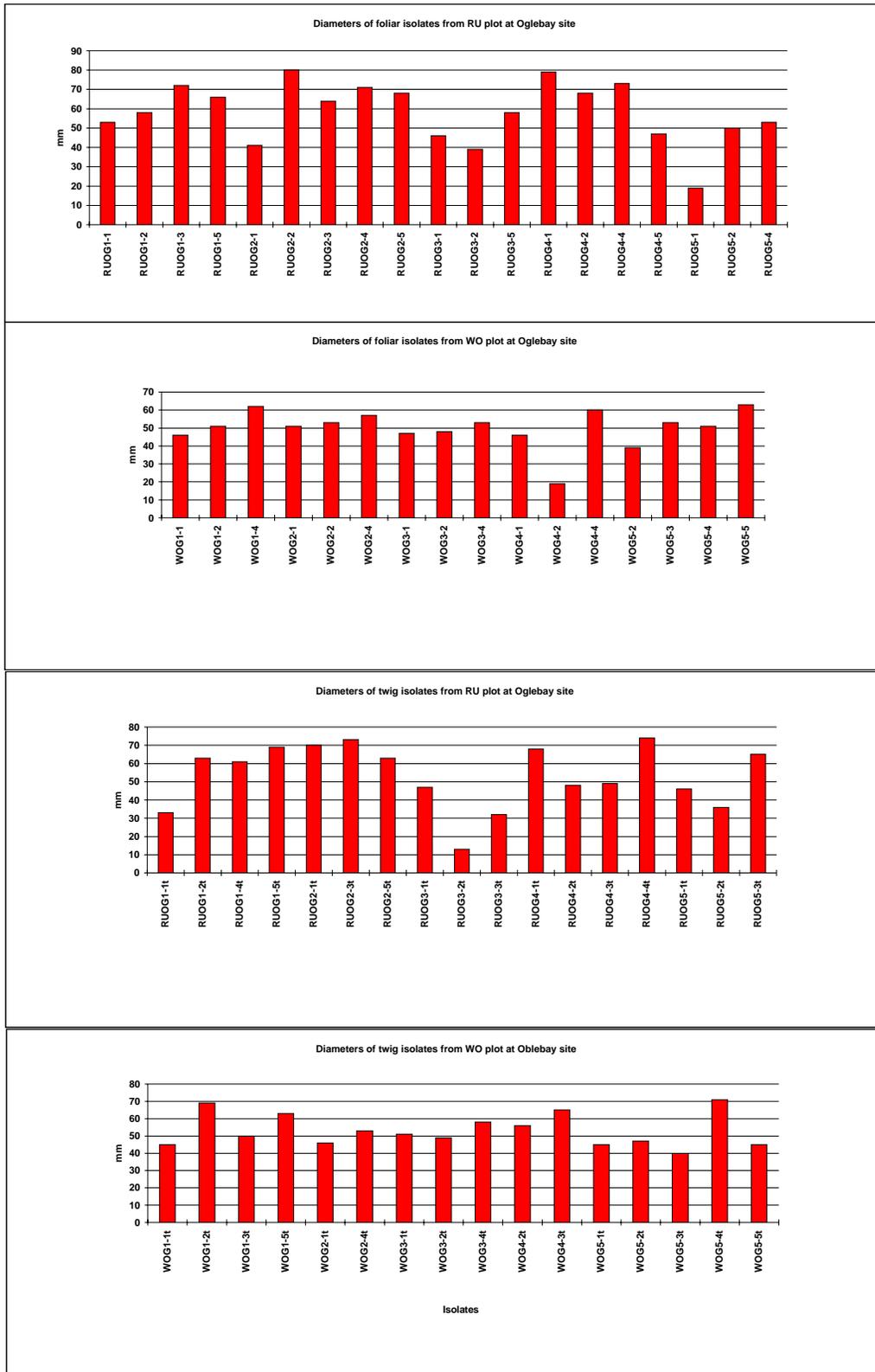
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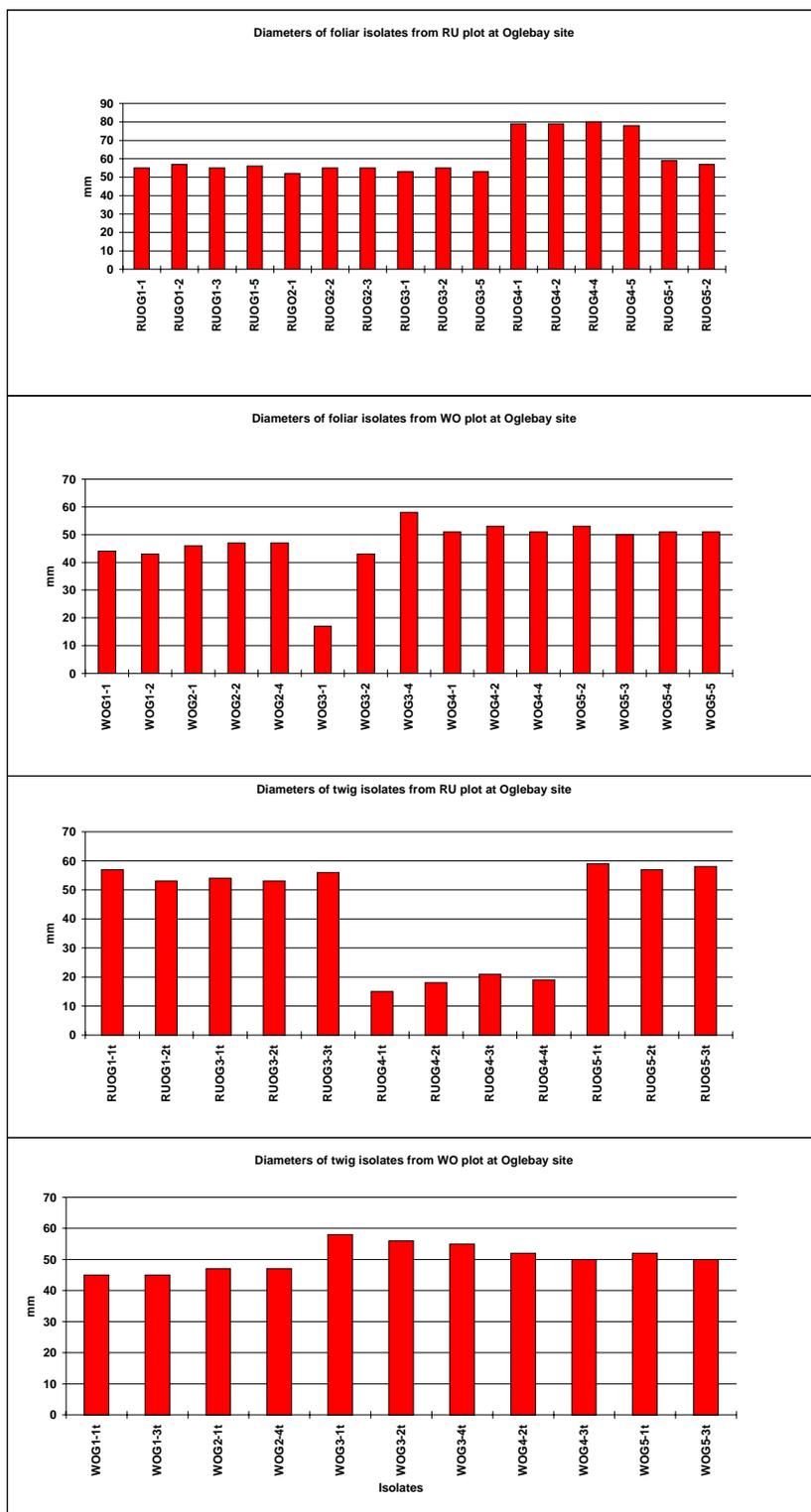
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APPENDIX

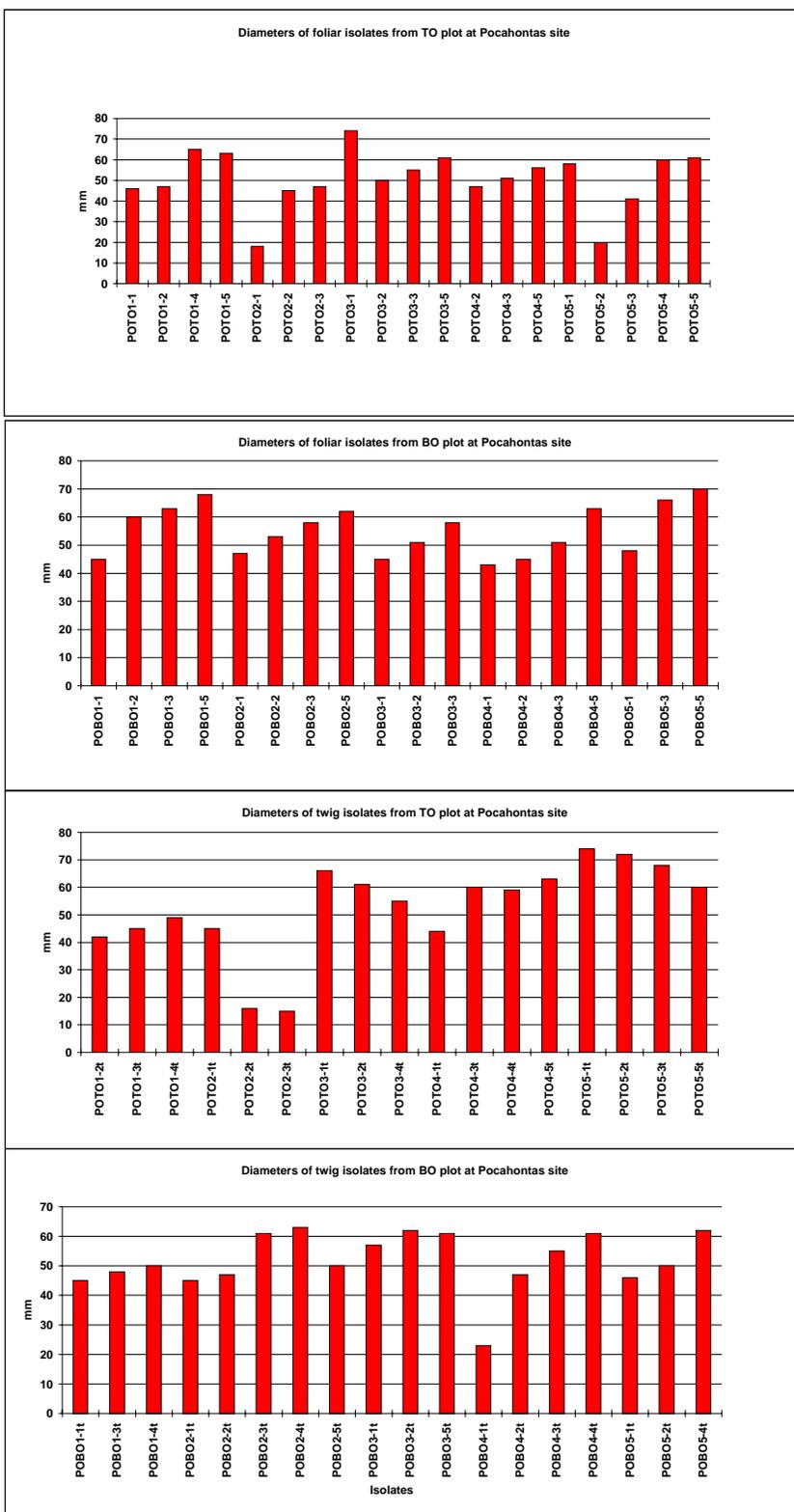
Appendix Figure 1. Diameters of first subculture foliar and twig isolates from Oglebay site after 21 days of growth under 16/8 hr light. All cultures were grown on PDA.



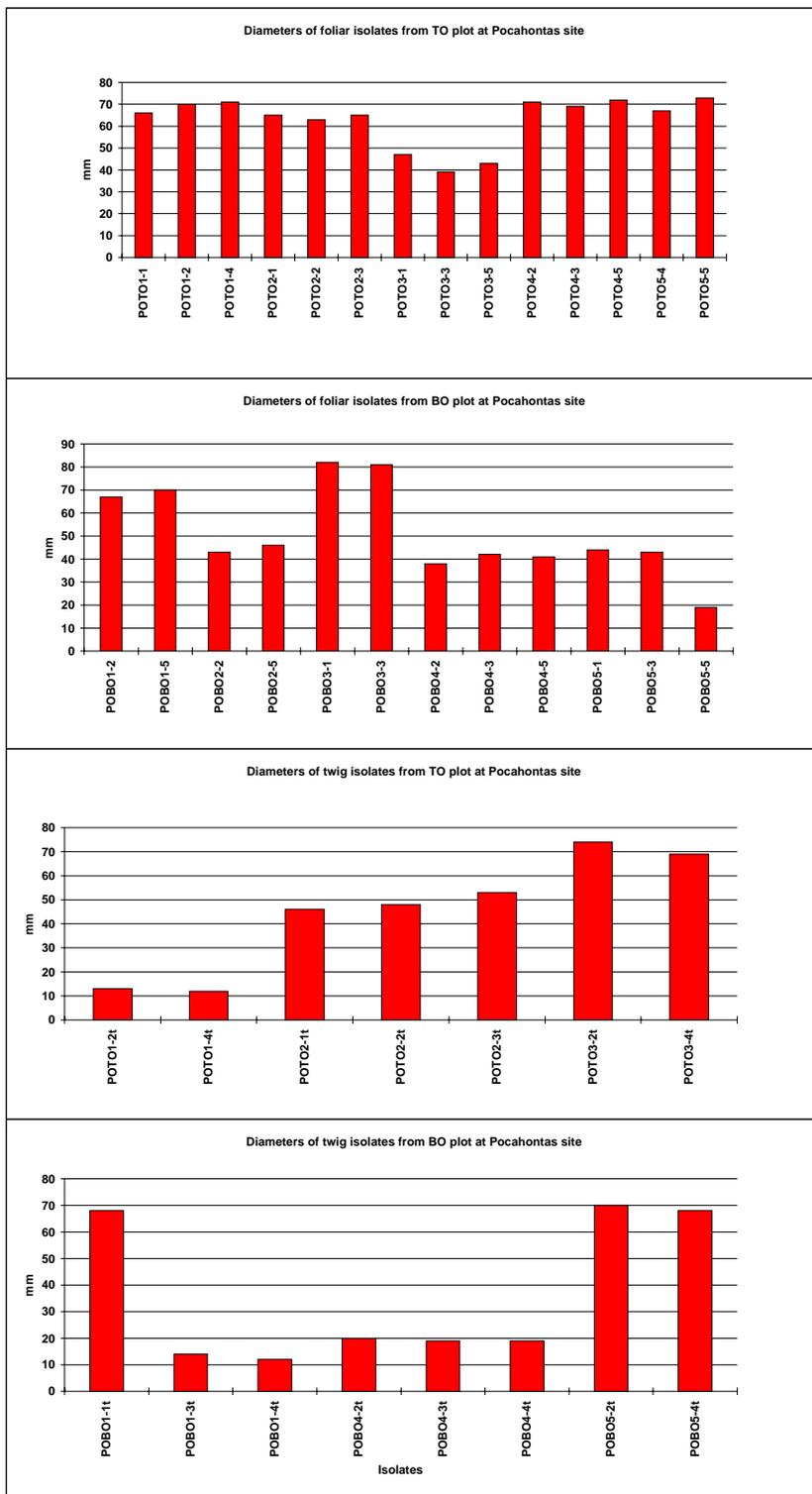
Appendix Figure 2. Diameters of second subculture foliar and twig isolates from Oglebay site after 21 days of growth under 16/8 hr light. All cultures were grown on PDA.



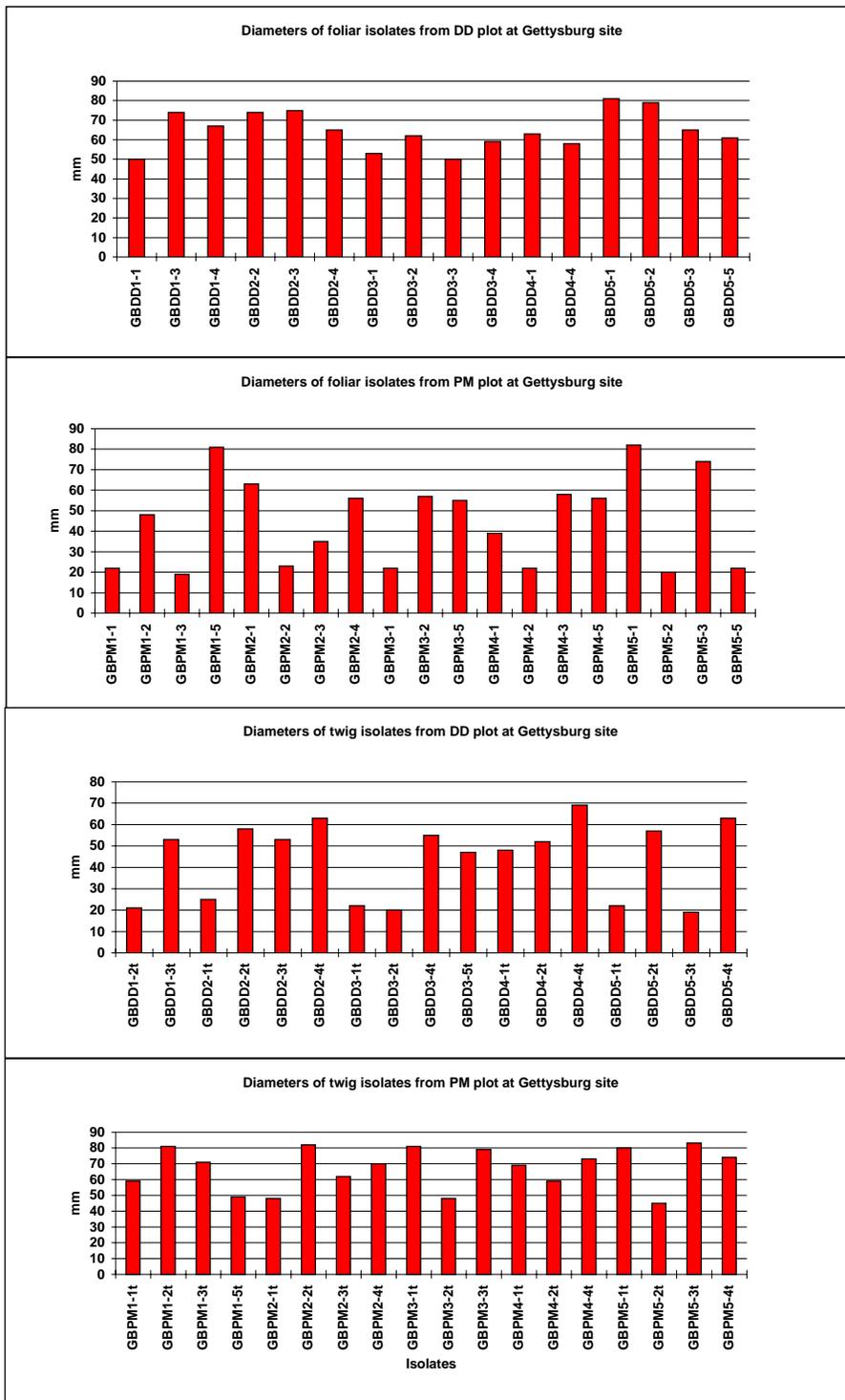
Appendix Figure 3. Diameters of first subculture foliar and twig isolates from Pocahontas site after 21 days of growth under 16/8 hr light. All cultures were grown on PDA.



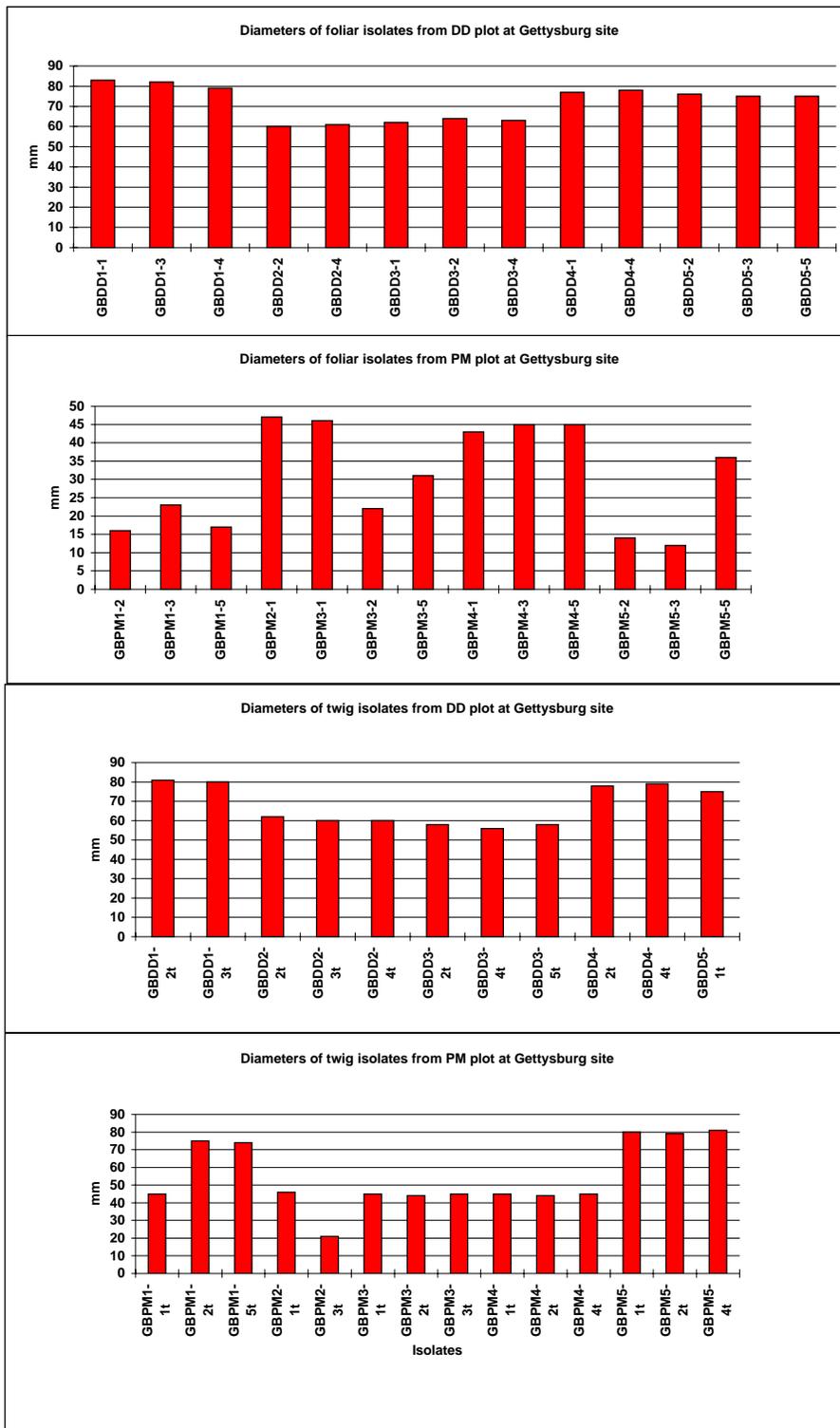
Appendix Figure 4. Diameters of second subculture foliar and twig isolates from Pocahontas site after 21 days of growth under 16/8 hr light. All cultures were grown on PDA.



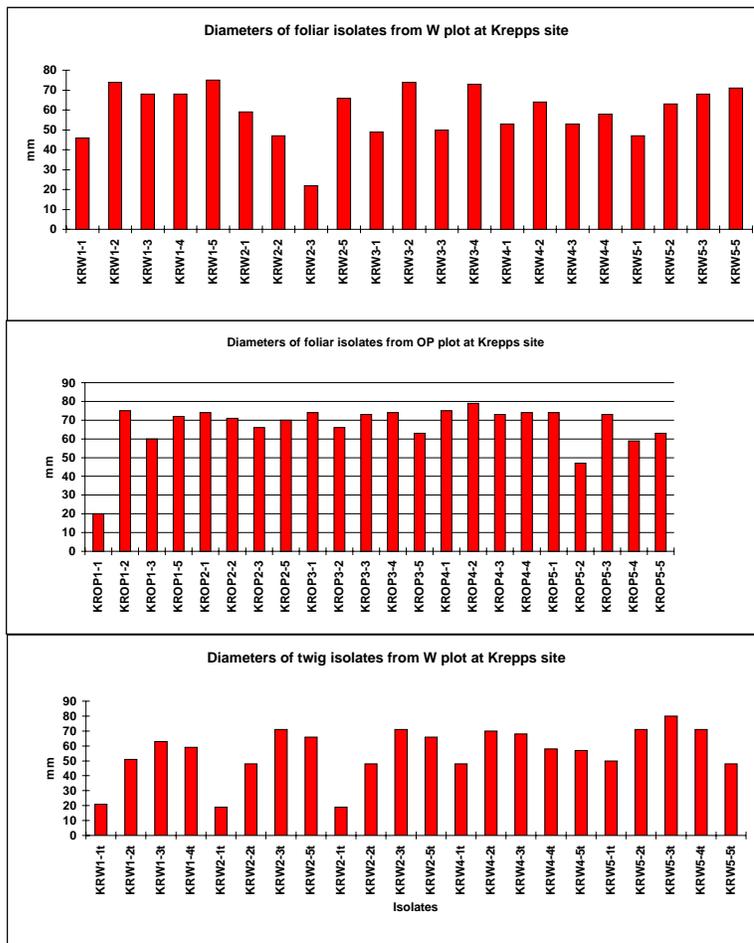
Appendix Figure 5. Diameters of first subculture foliar and twig isolates from Gettysburg site after 21 days of growth under 16/8 hr light. All cultures were grown on PDA.



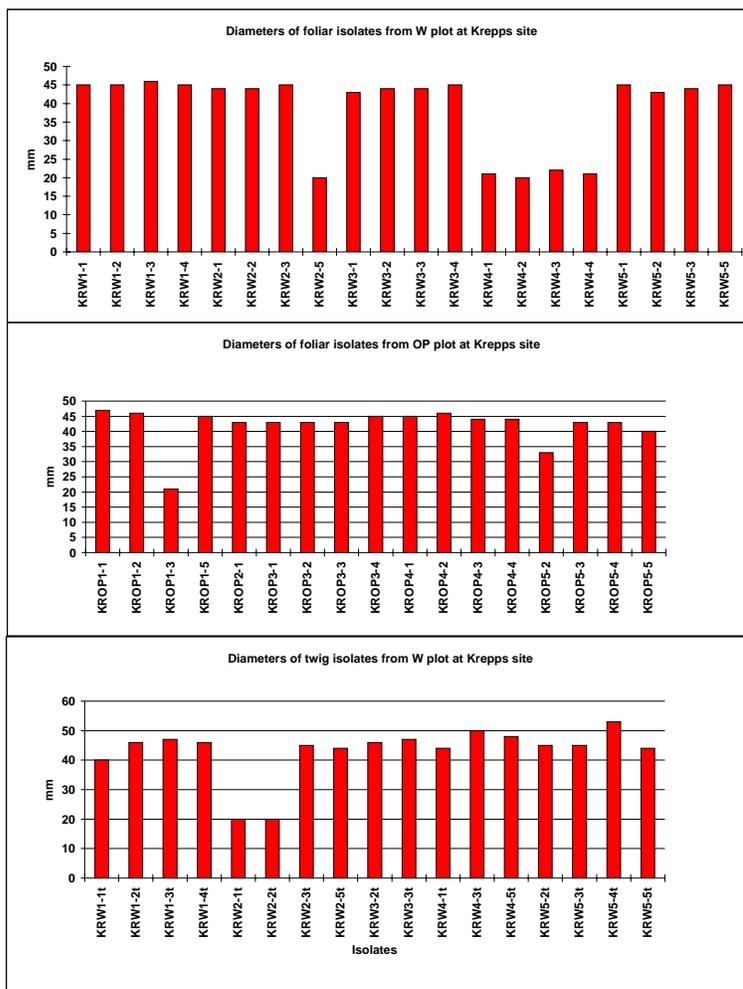
Appendix Figure 6. Diameters of second subculture foliar and twig isolates from Gettysburg site after 21 days of growth under 16/8 hr light. All cultures were grown on PDA.



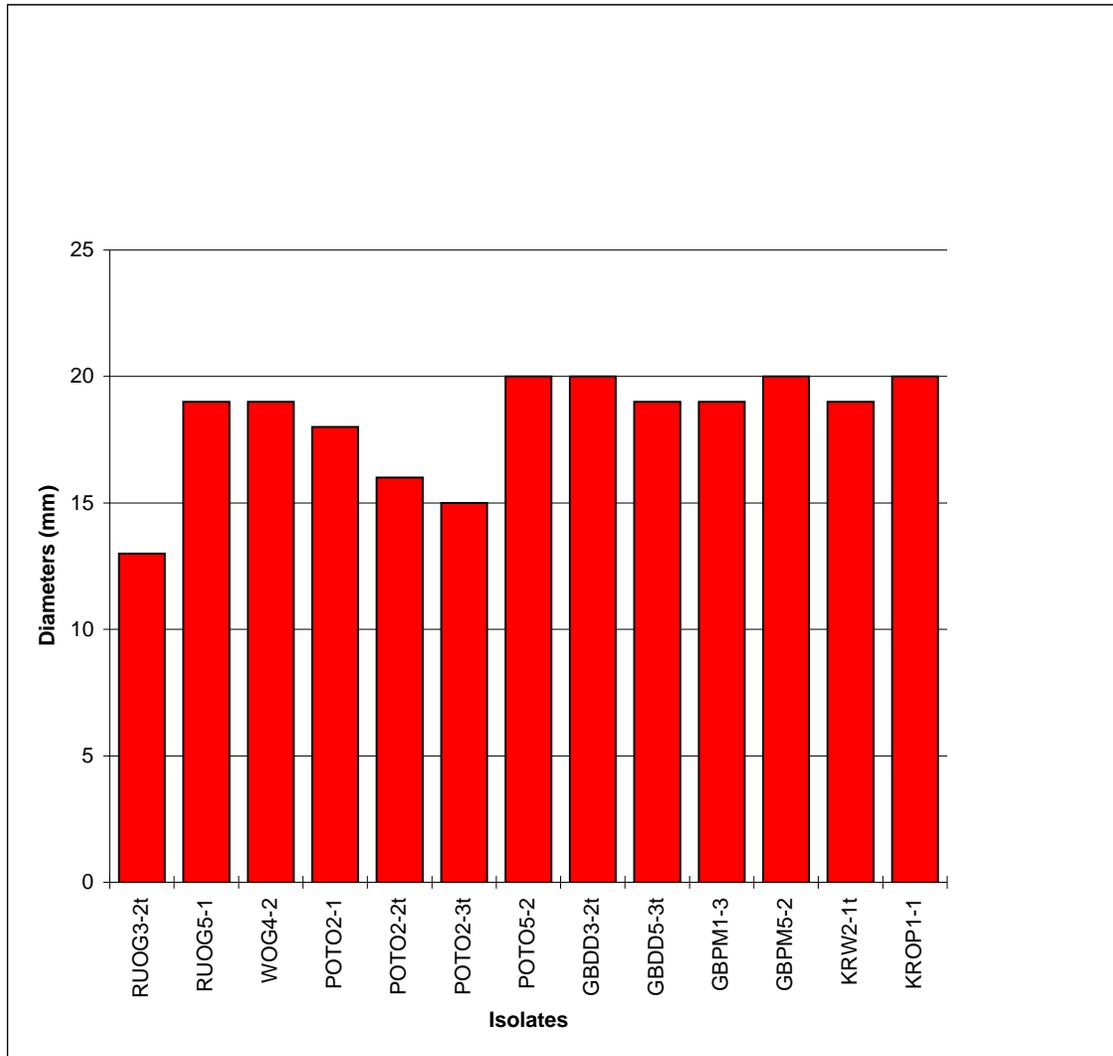
Appendix Figure 7. Diameters of first subculture foliar and twig isolates from Krepps site after 21 days of growth under 16/8 hr light. All cultures were grown on PDA.



Appendix Figure 8. Diameters of second subculture foliar and twig isolates from Krepps site after 21 days of growth under 16/8 hr light. All cultures were grown on PDA.



Appendix Figure 9. Diameters of slow growing isolates 21 days after first subculture.



Appendix Figure 10. Diameters of slow growing isolates 21 days after second subculture.

