To the Graduate Council:

I am submitting herewith a thesis written by Leigh Ann Klein entitled "Scanning electron microscopy, histological, and tissue culture studies of powdery mildews infecting dogwoods." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Mark T. Windham, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

SCANNING ELECTRON MICROSCOPY, HISTOLOGICAL, AND TISSUE CULTURE STUDIES OF POWDERY MILDEWS INFECTING DOGWOODS

A Thesis
Presented for the
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Leigh Ann Klein
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DEDICATION

This thesis is dedicated to my parents

Mary Ann and James Prewitt Lisle

who give me strength.

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ABSTRACT

Powdery mildew has become a common and widespread foliar disease of dogwoods. *Microsphaera pulchra* Cooke and Peck, and *Phyllactinia guttata* (Wallr.:Fr.) Lev. are two causal agents of powdery mildew on dogwood species in eastern North America. A clear understanding of the host-pathogen relationship between these fungi and their host must be obtained to advance research in disease management and resistance breeding.

A necessary step in studying a disease is to identify the causal agent. Leaves of *C. florida* 'Cherokee Sunset' and *C. amomum* (silky dogwood) were observed under a compound microscope to determine causal agents of powdery mildew based on ascocarp morphology. Ascocarps of two different species of powdery mildew, *M. pulchra* and *P. guttata*, occurred together on 'Cherokee Brave' and *C. amomum* leaves. *Microsphaera pulchra* ascocarps occurred at a higher density on 'Cherokee Sunset', whereas *P. guttata* ascocarps were more prevalent on *C. amomum* leaves. Scanning electron microscopy (SEM), paraffin based histology, and low magnification light microscopy revealed that the ascocarps in lesser density may have landed on the leaves after dissemination and likely were not formed on the leaves. Both flowering dogwood and silky dogwood appeared to have only one powdery mildew

pathogen.

A reliable inoculation procedure is necessary to study powdery mildew disease processes. Three inoculation techniques were compared to determine the most efficient method to transfer conidia from diseased tissue to an uninfected leaf surface. The inoculation methods were as follows: (1) infected leaves were shaken over non-infected leaves, (2) conidia were deposited by wiping an infected leaf over the surface of a non-infected leaf, and (3) conidia were deposited onto non-infected leaves with a camel-hair brush. Inoculation with a brush was the least damaging method to deposit suitable numbers of viable *M. pulchra* conidia on the surface of dogwood leaves.

Microshoots and callus tissue were inoculated with conidia of *M. pulchra* in attempts to establish powdery mildew colonies *in vitro*. Sporulating colonies were formed on microshoots. Scanning electron microscopy and histology indicated that disease processes in microshoots were similar to those in intact 'Cherokee Sunset' leaves. The establishment of powdery mildew colonies *in vitro* through tissue culture techniques would provide a continuous source of inoculum, a possible means to screen for resistance, and the means to study powdery mildew disease in a controlled environment.

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CHAPTER 1

INTRODUCTION

1.1 The host, *Cornus* L. species

Dogwoods (*Cornus* L. species) are members of the Cornaceae family. The family Cornaceae consists of 18 genera, mostly small trees and shrubs, that are mainly distributed in temperate regions of North America, Europe, and Asia (Elias 1980). There are approximately 65 species of *Cornus* (Eyde 1988) distributed throughout the northern hemisphere, with centers of diversity in eastern North America, the Pacific Northwest, eastern Asia, and Central America (Ranney et al. 1994). Morphological characteristics of this genus include small tetramerous flowers that are bisexual in North American species, styles surrounded by nectariferous discs, drupes that are usually bilocular, and simple, opposite or, rarely alternate, deciduous leaves with arcuate venation (Elias 1980, Murrell 1993).

Dogwood species are separated into two groups based on fruit color and presence of bracts. The red-fruited line has basal bracts and consists of the dwarf cornels, cornelian cherries, and big-bracted dogwoods (Eyde 1988). The blue-or white-fruited line has bracts that are rudimentary or lacking (Eyde 1988). Blue-fruited species

native to North America include *C. amomum* Mill., *C. asperifolia* Michx., *C. drummondii* C.A. Meyer, *C. excelsa* H.B.K., *C. glabrata* Benth., *C. obliqua* Raf., *C. occidentalis* (Torrey and Gray) Cov., *C. racemosa* Lam., *C. rugosa* Lam., *C. stolonifera* Michx., *C. stricta* Lam., and *C. alternifolia* L. (Santamour and Dudley 1992).

Flowering dogwood, *C. florida* L., is a native under story species common to the eastern United States deciduous forests. The tree reaches a height of 15 m with mature tree diameters up to 50.8 cm (Elias 1980). *Cornus florida* produces mostly white bracts in the wild (Williams et al. 1983). Flowering dogwood ranges from Massachusetts to Florida and west to Ontario, Texas and Mexico (Dirr 1990). The tree grows in the shade of other hardwoods in soils varying from well-drained, light upland soils to deep, moist soils along lower slopes and streams (Bailey and Brown 1989, Elias 1980).

Flowering dogwood plays an important role in the forest ecosystem by providing habitat and food for wildlife (Elias 1980, Eyde 1988). Bobwhite quail, ruffed grouse, songbirds, wild turkey, squirrels and raccoons, use the fruit as a source of food, whereas the leaves and twigs are browsed by whitetail deer (Elias 1980).

Flowering dogwood has been used for many purposes

throughout history. The generic name *Cornus* was derived from "cornu", or horn, which described the strength and heaviness of close-grained dogwoods (Keeler 1912). Wood of *Cornus* species was used for hubs of small wheels, handles of tools, mallets (Keeler 1912), barrel hoops, engravers' blocks (Elias 1980) and for shuttle blocks in the textile industry (Hall 1949). "Daggerwood", or sticks once used to skewer meats, is a possible derivative of "dogwood" (Elias 1980). The name "dogwood" may have also derived from the value of this tree's astringent bark as a cure for mange of dogs (Keeler 1912). Bark of different species have yielded a substitute for quinine as a remedy for fevers (Brinkman 1974). The fruits of *C. mas* L. have been used to make jelly (Trigiano et al. 1992), whereas the fruit of *C. officinalis* Sieb. and Zucc. have been used as an astringent, in a tonic, and as a hemostatic agent in China (Yazaki and Okauda 1989).

Today, dogwood is valued primarily as an ornamental tree. Several species of dogwoods are cultivated for their showy bracts, attractive foliage, fruit and/or twig color. The following species are commonly grown for commercial use: *C. florida* (flowering dogwood), *C. kousa* Hance (Chinese dogwood), *C. nuttalli* Audubon (Pacific dogwood), *C. canadensis* L. (bunchberry), *C. mas* (cornelian cherry), and *C. stolonifera* (red-osier dogwood) (Trigiano et al. 1992).

1.2 Reported causal agents of powdery mildew disease of *Cornus* species

Powdery mildews of *Cornus* species rarely were reported before 1994, but have since become a common foliar disease of dogwoods (Hagan et al. 1995, McRitchie 1994, Ranney et al. 1994, Windham 1996). Two powdery mildew species have been reported to infect dogwoods in eastern North America, *Microsphaera pulchra* Cooke and Peck (Braun 1987, Farr et al. 1989, Ranney et al. 1994) and *Phyllactinia guttata* (Wallr.:Fr.) Lev. (Burrill 1887, Farr et al. 1989, McRitchie 1994). The asexual stage of *Microsphaera* spp., *Oidium* Link, infects flowering dogwood (Farr et al. 1989, Hagan et al. 1995), whereas *Ovulariopsis* Pat. and Har., the asexual stage of *Phyllactinia* spp., infects various *Cornus* spp. (Braun 1987). *Microsphaera pulchra* has many synonyms including *M. penicillata* (Wallr.:Fr.) Lev., *M. penicillata* var. *alni* Cooke and Peck, *M. alni* (Wallr.: Fr.) G. Wint., *M. japonica* P. Henn., *M. pulchra* var. *japonica* (P. Henn) U. Braun, *Erysiphe penicillata* (Wallr.: Fr.) Fr., and *Alphitomorpha penicillata* var. *alni* Wallr. (Braun 1987, Cooke 1952, Farr et al. 1989, Parmelee 1977, Salmon 1900). *Phyllactinia guttata* is the only species within this genus to have a *Cornus* species host (Braun 1987, Farr et al. 1989). Common

synonyms of *P. guttata* include *P. corylea* (Pers.) Karst. and *E. guttata* Fries (Cooke 1952).

1.3 Morphology and anatomy of *Microsphaera pulchra* and *Phyllactinia guttata*

Classification of powdery mildew species is based on both the sexual and asexual stages. Morphology and size of mycelia, conidiophores, conidia, ascocarps, and ascocarp appendages are used to classify powdery mildews (Yarwood 1957). Other traits examined include mycelial growth habit and number of asci and ascospores.

Powdery mildews are usually named for their perfect stage (Yarwood 1978). *Microsphaera pulchra* and *P. guttata* are obligate biotrophs that belong to the phylum Ascomycota, order Erysiphales, and the family Erysiphaceae (Alexopoulos et al. 1996). The sexual stage of powdery mildews can neither be properly called a cleistothecium nor a perithecium. Ascospores are produced in asci that are aligned in a basal hymenial layer similar to a perithecium, but the ascocarp is completely closed similar to a cleistothecium (Alexopoulos et al. 1996). As asci swell, the ascocarp wall ruptures, forming apical openings with vertical slits to release the ascospores (Braun 1987).

Phyllactinia ascospores are released by an opening around the equatorial line of the ascocarp resulting in the entire upper half becoming offset at times (Cullum and Webster 1977, Webster 1979). In the past, Yarwood and others used the term perithecia to describe the fruiting structures of the Erysiphales (Yarwood 1978), however Braun preferred "Erysiphaceous cleistothecium" in his monograph of the powdery mildews (Braun 1987). In this thesis, the sexual fruiting body of the Erysiphales will be referred to as an ascocarp.

Ascocarps of the Erysiphales are more or less spherical and somewhat flattened, with a wall, or peridium, that consists of two conspicuous layers in most genera (Braun 1987). The outer layer is thick-walled, made of mostly irregular polygonal cells, 5-25 µm in diameter, with sparse protoplasm (Braun 1987). The nuclei are not visible through these sclerotized cells. The inner layer is loosely arranged, composed of polygonal, thin-walled cells, 8-20 µm in diameter, with abundant protoplasm. These cells are binucleate or partly uninucleate. The outer layer of cells are darkly pigmented, whereas the inner layer is paler (Braun 1987).

Ascospores produced by the members of the Erysiphaceae are among the largest one-celled ascospores of fungi

(Yarwood 1957) with a size range of 10-50 x 8-30 µm (Braun 1987). *Microsphaera pulchra* ascocarps measure 80-130 µm in diameter and contain three to eight asci that are rarely sessile, with a short stalk measuring 40-75 x 30-55 µm, containing four to eight ellipsoid-ovoid ascospores with a size of 14-28 x 10-15 µm (Braun 1987). *Phyllactinia guttata* ascocarps are much larger, measuring 150-250 µm in diameter and contain six to 30 asci that are broadly clavate to slender, subcylindric, measuring 60-100 x 25-40 µm, containing two ellipsoid-ovoid ascospores with a size of 25-45 x 14-25 µm (Braun 1987).

Powdery mildew ascocarps have many appendages that serve various functions. Fruiting bodies are held to the host surface by basal hyphae, which function to obtain nutrients for the maturing ascocarp (Blumer 1967 as cited in Braun 1987). Appendages that derive from pseudoparenchymatous cells of the ascocarp are used in species identification (Braun 1987).

Diagnostic features of equatorial appendages include length and width, consistency (flexuous, stiff, geniculate), and mode of apical branching (Braun 1987). *Microsphaera pulchra* has 8-22 equatorial appendages that are rather stiff, and straight to curved (Braun 1987). The appendages are 1-2 times as long as the cleistothecial diameter,

closely and regularly branched with tips distinctly recurved (Braun 1987). *Phyllactinia guttata* has 3-15 equatorial, bulbous-based, acicular appendages that are 1-2.5 times as long as the ascocarp diameter (Braun 1987).

Phyllactinia spp. have a third appendage type that functions in the attachment of ascocarps to new surfaces after dissemination (Yarwood 1957). These short apical appendages secrete a muciloid substance and are found on the crown of the ascocarp that becomes attached to the lower epidermal layers upon landing on a new host (Cullum and Webster 1977). The appendages are penicillate cells that are simple or moderately branched, mostly about 40-60 µm long (Braun 1987).

Powdery mildew imperfect stages belong to the form class Deuteromycetes, form order Moniliales, and the form family Moniliaceae. In the past, authors have disagreed on the validity of using the asexual stage of powdery mildews in taxonomy. Since conidial size varies greatly in different environments (Yarwood 1978), factors other than shape may prove more efficient for taxonomy. Yarwood (1978) suggested that shape and presence of vacuoles, fibrosin bodies, and oil drops should be used to determine genera.

There are several diagnostic differences between the asexual stages of *Phyllactinia* and *Microsphaera* spp. (Braun

1987). The conidia of *Microsphaera* spp. are more or less ellipsoid, 23-34 x 10-15 µm, and are formed in chains on conidiophores that are usually 3-celled (Braun 1987). The conidia of *Phyllactinia* spp. are more or less clavate, 50-100 x 15-25 µm, and are formed singly on conidiophores that are 3-5 celled (Braun 1987). The appressoria of *Microsphaera* spp. are distinctive and lobed. The appressoria of *Phyllactinia* spp. are unlobed or occasionally branched to moderately lobed, hooked, elongated or nipple-shaped, and occur singly or frequently in opposite pairs (Braun 1987). Hyphae of *Phyllactinia* spp., along with *Pleochaeta* Sacc. and Speg. and *Leveillula* G. Arnaud. spp., grow hemiendophitically entering host tissues through stomata (Braun 1987, Yarwood 1957). Hyphae of *M. pulchra* are epiphytic, penetrating host cells directly (Braun 1987).

1.4 Signs and symptoms of powdery mildew disease of dogwoods

Powdery mildews are characterized by their superficial, fluffy, mycelial growth. Mycelial mats usually appear white, and at times grey or yellowish on the leaf surface, although the hyphae are transparent (Braun 1987, Yarwood 1978, Yoder 1992). Mycelia usually grow on both leaf

surfaces, but occasionally mats are predominantly on one surface (Yarwood 1957). One such case of predominant growth on a leaf surface is that of *Phyllactinia corylea* (Pers.) P. Karst., which occurs exclusively on the lower leaf surface where stomata are more abundant for hyphal penetration (Yarwood 1957). *Microsphaera alphitoides* Griff. & Maubl., which causes oak powdery mildew, produces conidiophores on both the upper and lower leaf surfaces (Hewitt 1974). Mycelial growth of *Oidium* spp. on dogwood was reported to occur on the upper surface of new leaves (Hagan et al. 1995). Mycelia can also be seen in dormant buds where the fungus can overwinter (Braun 1987).

Although hyaline conidia and mycelia of powdery mildews are produced throughout the growing season, ascocarps are produced in the cool autumn season on dogwood trees (Windham 1996). The ascocarps of *M. pulchra* and *P. guttata* usually appear on the abaxial leaf surface of dogwoods. When mature, the ascocarps appear dark brown, however immature ascocarps are yellow or reddish-brown (Yarwood 1957).

Symptoms of powdery mildew disease are seen throughout the growing season. Dogwood leaves infected with powdery mildew become strap-shaped and curled, have an increase in red pigmentation, and develop lesions that eventually become necrotic (Hagan et al. 1995, McRitchie 1994). Severe

infection may cause leaves to become brittle and fall from trees in mid-season. The disease may cause stunting of young seedlings and slow growth in older trees (Windham 1996). Nearly 100% of the foliage may be affected in seedlings and liners, resulting in possible mortality, whereas older, well-established trees generally are not killed by the disease.

Efficiency of photosynthesis and transpiration by the host plant are affected by the development of powdery mildew disease. The fungus diverts photosynthate from other regions to the site of infection, disrupting the translocation of carbohydrates within the host (Smith et al. 1968). As the fungus utilizes the products of photosynthesis, there may be a reduced growth rate in young seedlings or a general reduction in vigor of mature trees (Hewitt and Ayres 1975). Carbohydrates cease to be transported to roots, reducing the storage substances that enable the plant to overwinter (Hewitt and Ayres 1976).

Several studies have been conducted to determine the actual rate of reduction in photosynthesis. Change in photosynthetic rates of pecan leaves *(Carya illinoensis* (Wang.) K. Koch) occurred due to different percent coverages by *M. penicillata* (Gottwald 1984). Net photosynthesis was reduced 11, 38, and 42% by 25, 50, and 75% infection,

respectively. All percentages of infection, however, had no effect on dark respiration (Gottwald 1984).

Net photosynthesis rate and transpiration changed in *Quercus robur* L. when infected with *M. alphitoides* Griffon and Maublanc (Hewitt and Ayres 1975). Fungal infection caused an increase in net photosynthesis 24 h after initial infection, then caused a rapid decline. Increases in transpiration were mostly due to extra water loss from the surface of the fungus. Lower turgidity and lower leaf water potential indicated that the water loss was greater than the uptake of the leaves (Hewitt and Ayres 1975).

1.5 Disease processes of powdery mildews

Powdery mildew species overwinter by means of ascocarps, mycelia in dormant buds, as persistent mycelia or as chlamydospores in certain species (Yarwood 1957). Apple powdery mildew fungus, *Podosphaera leucotricha* (Ellis and Everh.) E.S. Salmon, and hawthorn powdery mildew, *P. clandestina* (Wallr.:Fr.) Lev., overwinter in buds and cause a primary infection on newly emerging leaves (Khairi and Preece 1979, Xu et al. 1995). Upon conidiophore formation, the conidia are dispersed into the air to land on other growing shoots, causing a secondary infection (Khairi and

Preece 1979, Xu et al. 1995).

The disease cycle of the asexual stage of powdery mildews, which is prevalent throughout the growing season, has been studied by many researchers. Mount and Slesinski (1971) reviewed the work of many authors and summarized the following nine developmental stages of *Erysiphe graminis* DC. on wheat or barley: (1) germination of conidia, (2) production of "club-shaped" appressorial initials, (3) maturation of appressoria, (4) penetration of the cuticle and epidermal cells, (5) formation of haustoria, (6) formation of secondary hyphal initials, (7) elongation of secondary hyphae, (8) initiation of additional infections, and (9) sporulation.

Aist and Bushnell (1991) reviewed the disease mechanism and progression of *E. graminis* in cereal hosts, providing a detailed time-line of penetration by the fungus, host cell response and development of disease. Table 1-1 summarizes Aist and Bushnell's (1991) findings and provides a general guideline for the disease process of other powdery mildew species.

Table 1-1. Time-line of the disease processes of *Erysiphe graminis* in cereal hosts.

Time After Inoculation	Event
upon contact	-conidium excretes esterases that obscure surface features of spore and dissolves host surface waxes
20 min	-conidium exudes a flocculent material that glues it to the host leaf surface
1-2 h	-primary germ tube (PGT) emerges from conidium and attaches to host cuticle
2 h	-host response to PGT is a circular area of autofluorescence in the host wall subjacent to PGT tip
2 h 2 min	-host response to PGT is a subjacent cytoplasmic aggregation (CA)
2 h 15 min	-host response to PGT is the formation of a halo in host wall
3 h	-penetration peg emerges from tip of PGT to penetrate host cuticle and cell wall
3-4 h	-appressorial germ tube (AGT) emerges from conidium and produces a terminal appressorium
4 h	-papilla is deposited by host cell below the PGT
4-5 h	-biochemical host response to the appressorium occurs with the production of 3 enzymes involved in phenolic metabolism: phenylalanine ammonia lyase, tyrosine ammonia lyase, peroxidase
6-10 h	-appressoria continues to develop producing a primary appressorial lobe

Table 1-1. (continued)

Time After Inoculation	Event
10-12 h	-host response is CA beneath the appressorial lobe and the production of a cell wall halo
11-13 h	-CA secretes a papilla below the appressorial lobe -penetration peg grows from appressorial lobe, through the host cuticle, cell wall, and into the cell lumen -penetration peg tip enlarges to form the central body of a young haustorium -CA disperses and papilla deposition discontinues
18 h	-the haustorial central body swings sideways and starts to produce fingerlike lobes, which could continue to develop up to 5 days
24 h	-a primary hypha grows from the apex of the appressorium
>24 h	-the hypha continues to grow, forming secondary and tertiary haustoria -conidia and conidiophores are produced after the tertiary haustoria generation

1.6 Effect of environment on powdery mildew disease

Powdery mildew disease occurs most readily in dry, warm areas with cool nights. Germination begins when the conidia land on the leaf during warm, dry conditions (Hewitt 1974). After nightfall, germ-tube production occurs and the infection process proceeds as humidity level rises (Hewitt 1974).

The effects of moisture on conidial germination and penetration, and powdery mildew disease in general, are controversial (Braun 1987). Rain has been simulated by daily spraying of leaves with water as a control for powdery mildew disease (Yarwood 1939). However, free moisture promoted swelling and bursting of the ascocarps to release ascospores into the environment (Schnathorst 1965). Although rain may damage fungal tissues, increased moisture levels may also increase disease due to a rise in atmospheric humidity (Butt 1978).

Research on conidial germination of various powdery mildew species in the presence of free-standing water has provided varied results (Sivapalan 1993b). *Microsphaera alphitoides* Griff. and Maubl. germinated in a similar manner on the leaf surface of *Q. robur* L. as it did in/on water in a polystyrene box. *Podosphaera clandestina* (Wallr. ex Fr.)

Lev., powdery mildew of *Crataegus monogyna* Jacz. (hawthorn), *P. leucotrich* (Ell & Everh.) Salm., powdery mildew of *Malus sylvestris* (L.) Mill. (apple), and *Uncinula bicornis* (Wallr. ex Fr.) Lev., powdery mildew of *Acer pseudoplatanus* L. (maple), all exhibited reduced germination in the presence of free water.

The results of rainfall impaction on conidia attached to leaf surfaces can vary based on the developmental stage of the disease (Sivapalan 1993a). Rain can remove conidia that have made contact with the leaf surface and not yet germinated. After conidia have formed appressoria and germ tubes, the fungus resists washing from the leaf surface. Various species of mildew withstood washing during the early stages of germ-tube growth, but most tended to withstand washing only in later development. Impaction of rain can damage mycelia and conidiophores by detaching them from the leaf surface, or can cause the fungus to sporulate abnormally (Sivapalan 1993a).

Conidia of *M. alphitoides* germinated in a wide range of relative humidities (RH) and temperatures, with an optimum at 81% RH and 20-23°C (Hewitt 1974). Germination was defined as the length of the germ-tube exceeding conidial width. Germ-tube elongation increased with relative humidity up to 96% and temperatures up to 25°C.

Temperatures studied were 0, 15, 20, 23, 25, and 30°C with relative humidities at 32, 58, 76, 81, 96, and 100% (Hewitt 1974).

Temperatures and relative humidity also affects the germination of *P. clandestina* Lev., powdery mildew of hawthorn (Khairi and Preece 1979). Germination occurred when the length of a germ-tube exceeded the breadth of the spore. Conidia germination was optimum at 18 and 20°C, and with a relative humidity of 70 and 90%. The association between relative humidity and temperature varied at different temperatures, with a nonsignificant effect of RH on percent germination at low temperatures, and a significant effect at higher temperatures.

1.7 Management of powdery mildew disease

Sulfur dust applied to grape powdery mildew involved three mechanisms of disease management (Yarwood 1957). Sulfur acted as an eradicant by killing spores that were already present, but had not caused disease. The fungicide acted as a protectant by killing spores that subsequently arrived on the plant tissues. Lastly, sulfur was therapeutic for powdery mildew disease by killing established mycelia (Yarwood 1957).

Sulfur dust has successfully managed powdery mildew for many years. In the early nineteenth century, sulfur dust was used to manage powdery mildews on fruit trees and later on grape vines (Bent 1978). Today, volatilized sulfur is used in greenhouses, whereas wettable powder and colloidal liquid formulations of sulfur are used to treat field plants (Bent 1978). Sulfur does not penetrate deep into plant tissue, but remains on the surface layers of leaves, stems, flowers, and fruits. Therefore, sulfur must be reapplied as new plant parts emerge (Bent 1978). Direct application of sulfur to the leaves, may result in toxicity especially in more sensitive cultivars (Bent 1978).

Several fungicides have proven effective to control powdery mildew on dogwoods. Windham and Windham (1997) determined fungicide effects on two-year-old white flowering dogwoods by rating disease control, new growth and foliage scorch. Myclobutanil (Systhane), dimethyl 4,4'-o-phenylenebis-(3,thiallophanate) (Clearys 3336), and propiconazole (Immunex) all provided significant control with less than 10% of foliage symptomatic for disease. However, dogwoods treated with dimethyl 4,4'-o-phenylenebis-(3,thiallophanate) and propiconazole produced more new growth than trees treated with other fungicides. Myclobutanil, dimethyl 4,4'-o-phenylenebis-(3,thiallophanate), and propiconazole caused the least

amount of leaf scorch when compared to the other treatments.

Several cultural techniques could control powdery mildew disease. Water sprays were used to "wash" powdery mildew off leaves (Yarwood 1939). However, this method usually proved less effective than chemical control and required daily labor (Yarwood 1957). Resistant cultivars offer cultural protection against powdery mildew disease. Removal of "volunteer" plants along with pruning and destroying mildewed shoots are alternative cultural techniques (Bent 1978). However, the application of chemicals, particularly the use of sulphur, is the principal defense against powdery mildews (Bent 1978).

1.8 Importance of powdery mildew disease to the nursery industry

Dogwoods are a very important component of the nursery industry. Tennessee's dogwood seedlings, root cuttings, or grafted plants make up 75% of all the dogwoods produced for sale in the United States (Southards 1995). Sixteen percent of Tennessee's woody plant sales are from flowering dogwood that brings the state a total of $30 to 40 million dollars annually. One acre of *C. florida* cultivars, depending on the production system, can generate between

$40,000 to 60,000 per harvest (Badenhop et al. 1985, Witte 1995). In addition to the nursery sales, in Knoxville, the Tennessee Dogwood Arts Festival nets an additional $10 to 13 million each year (Southards 1995). Dogwoods are celebrated in other southern states, demonstrating the trees aesthetic value throughout its range. Powdery mildew poses a threat to the nursery industry as it distorts dogwoods for sale, dampens enthusiasm for purchases, and damages trees established in urban settings.

1.9 Objectives of this research

Powdery mildew on dogwoods has only recently become a considerable threat to the nursery industry. Several researchers have studied the most immediate aspect of the disease, the tolerance of various dogwood cultivars to powdery mildew (Hagan et al. 1995, Ranney et al. 1994, Windham 1996). These studies provided the nursery industry with readily needed information to avoid losses and provided the general public with advice on purchasing dogwoods.

Research conducted for this thesis involved the next steps in the study of powdery mildew of dogwoods--understanding of the host-pathogen relationship. Disease control and resistance breeding requires an understanding of

what particular species of powdery mildew infects which hosts and how the disease progresses within that host.

To conduct these studies, ideally a large amount of inoculum would be available from a laboratory setting through tissue culture techniques. Currently, the only continuous inoculum source would be from diseased trees grown in greenhouses. Insect pests, spray treatments, humidity, and temperature are inconsistent variables in greenhouses and often affect the health of conidia. Establishment of powdery mildew colonies on callus tissue or microshoots would eliminate some variability in conidial health and provide a continuous supply of inoculum.

The objectives of this research are the following: (1) to determine the causal agent(s) of powdery mildew disease on a red-fruited species, *C. florida* 'Cherokee Brave' and a blue-fruited species, *C. amomum,* (2) to determine the best technique for the inoculation of multiple trees in a greenhouse setting, and (3) to establish and maintain powdery mildew colonies on both callus tissue and microshoots.

CHAPTER 2

DETERMINATION OF POWDERY MILDEW PATHOGENS ON TWO *CORNUS* SPECIES

2.1 Introduction

There are approximately 65 *Cornus* L. species (dogwoods) (Eyde 1988) distributed throughout the northern hemisphere, with centers of diversity in eastern North America, the Pacific Northwest, eastern Asia, and Central America (Murrell 1993). Ornamentally, several species of dogwoods are cultivated for their showy bracts, attractive foliage, fruit and twig color. The following species are commonly grown for commercial use: *C. florida* L. (flowering dogwood), *C. kousa* Hance (Chinese dogwood), *C. nuttalli* Audubon (Pacific dogwood), *C. canadensis* L. (bunchberry), *C. mas* (cornelian cherry), and *C. stolonifera* Michx. (red-osier dogwood) (Trigiano et al. 1992).

Two powdery mildew species have been reported to infect dogwoods in eastern North America, *Microsphaera pulchra* Cooke and Peck (Braun 1987, Farr et al. 1989, Ranney et al. 1994) and *Phyllactinia guttata (Wallr.:) Lev.* (Burrill and Earle 1887, Farr et al. 1989, McRitchie 1994). The anamorph

of *Microsphaera* spp. *(Oidium)* was reported on flowering dogwood (Farr et al. 1989, Hagan et al. 1995), whereas *Ovulariopsis,* the anamorph of *Phyllactinia* spp., was reported on various *Cornus* spp. (Braun 1987). *Microsphaera pulchra* has many synonyms including *M. penicillata* (Wallr.:Fr.) Lev., *M. alni* (Wallr.: Fr.) G. Wint., *M. japonica* P. Henn., *M. pulchra* var. *japonica* (P. Henn) U. Braun, and *Erysiphe penicillata* (Wallr.: Fr.) Fr. (Braun 1987, Farr et al. 1989). *Phyllactinia guttata* is the only species within this genus reported to occur on *Cornus* spp. (Braun 1987, Farr et al. 1989). Common synonyms of *P. guttata* include *P. corylea* (Pers.) Karst. and *E. guttata* Fries (Cooke 1952). Table 2-1 is a short composite of *Cornus* species infected by *M. pulchra* and *P. guttata* and their asexual stages.

Cornus florida 'Cherokee Brave' is valued for its pink bracts, red fruit and seasonal red foliage, as well as its resistance to *M. pulchra* (Hagan et al. 1995, Windham 1996). *Cornus amomum* Mill., the silky dogwood, is a common blue-fruited shrub found in swampy areas of the eastern United States (Eyde 1988). Although *C. amomum* has not been evaluated for resistance to powdery mildews, *M. pulchra* has been reported to infect this dogwood (Braun 1987). The

Table 2-1. *Cornus* species infected by sexual and asexual stages of *Microsphaera pulchra* and *Phyllactina guttata*.

HOST	PATHOGEN	SOURCE
C. alba	*M. pulchra*	Braun 1987
	M. penicillata	Parmelee 1977
C. alternifolia	*M. pulchra*	Braun 1987 Salmon 1900
	M. penicillata	Parmalee 1977 Farr et al. 1989
	P. guttata	Farr et al. 1989
C. amomum	*M. pulchra*	Braun 1987 Salmon 1900 Windham et al. 1997
	P. guttata	Salmon 1900 Windham et al. 1997
C. asperifolia	*M. pulchra*	Farr et. al 1989
C. brachypoda	*M. pulchra*	Braun 1987
C. canadensis	*M. pulchra*	Farr et al. 1989
C. candidissima	*M. pulchra*	Braun 1987
	P. guttata	Salmon 1900
C. capitata	*M. pulchra*	Braun 1987 Windham et al. 1997
C. circinata	*P. guttata*	Salmon 1900
C. controversa	*M. pulchra*	Braun 1987
	Oidium	Hagan et al. 1995

Table 2-1. (continued)

HOST	PATHOGEN	SOURCE
C. florida	*Oidium*	Hagan et al. 1995
	M. pulchra	Ranney et al.1994 Braun 1987 Farr et al. 1989 Windham et al. 1997
	P. guttata	McRitchie 1994 Farr et al. 1989 Salmon 1900 Windham et al. 1997
C. glabrata	*M. pulchra*	Farr et al. 1989
C. kousa	*Oidium*	Hagan et al. 1995
	M. pulchra	Ranney et al. 1994 Windham et al. 1997
C. macrophylla	*M. pulchra*	Braun 1987 Salmon 1900 Windham et al. 1997
C. mas	*M. pulchra*	Salmon 1900
C. nuttalli	*Oidium*	Hagan et al. 1995
	P. guttata	Farr et al. 1989 Salmon 1900
C. obliqua	*M. pulchra*	Farr et al. 1989
C. occidentalis	*M. pulchra*	Braun 1987 Farr et al. 1989
C. racemosa	*M. pulchra*	Braun 1987
	M. penicillata	Farr et al. 1989
	P. guttata	Farr et al. 1989

Table 2-1. (continued)

HOST	PATHOGEN	SOURCE
C. rugosa	*M. pulchra*	Braun 1987
	M. penicillata	Farr et al. 1989
C. sanguinea	*P. guttata*	Salmon 1900
C. stolonifera	*M. pulchra*	Braun 1987
	M. penicillata	Farr et al. 1989 Salmon 1900 Farr et al. 1989
C. florida x kousa	*Oidium*	Hagan et al. 1995
C. x californica	*M. pulchra*	Farr et al. 1989
Cornus species	*M. penicillata*	Farr et al. 1989
	M. pulchra	Farr et al. 1989

purpose of this study was to identify the powdery mildew species infecting *C. florida* 'Cherokee Brave' and *C. amomum*.

2.2 Materials and Methods

Leaf disks were collected from three 'Cherokee Brave' and one *C. amomum* tree in the fall of 1996 on The University of Tennessee Agriculture Campus. The dogwood trees were located at opposite corners (11 mm apart) of a research tree compound. Disks were cut from leaves using a 7-mm-diameter cork borer, bisected, then fixed and vacuum-aspirated in Histochoice® (Amresco, Solon, Ohio 44139). Samples were dehydrated in a graded series of 30, 50, 70, 90, 95, and 100% of isopropyl alcohol, each for 30 min. Dehydrated samples were embedded with TissuePrep (Fisher Scientific, Fair Lawn, NJ 07410) and cast into blocks. Leaf disks were sectioned at 10 μm on a rotary microtome and placed on clean glass slides. Tissues were stained with a modified Flemming's Triple Stain (Johansen 1940). Coverslips were applied with Eukitt Mounting Medium (Calibrated Instruments, Hawthorne, NY 10502) and sections viewed with an Olympus BH-2 compound microscope (Olympus America Inc., Lake Success, NY 11042-1179) at 200-1000x magnification. Photographs were taken with an Olympus C-35AD-4 camera with Techpan 2415 film

(Eastman Kodak Company, Rochester, NY 14650) and positives printed on Ilford MGIV RC (England) black and white paper.

Additional leaves collected from the same *C. amomum* and 'Cherokee Brave' trees were dried in a plant press. A separate species of powdery mildew was observed on the leaves of each dogwood species using an Olympus SZH10 research stereo dissecting scope at 40-70x magnification. *Phyllactinia guttata* was identified on *C. amomum* using number of asci/fruiting body, ascocarp diameter, and form of appendages (Braun 1987). The powdery mildew species observed on *C. florida*, *Microsphaera* sp., was identified using asci numbers/ascocarp, form and length of appendages, and ascocarp size. Eight *Microsphaera* sp. ascocarps, from a total of three dried leaf specimens of *C. florida*, were mounted in lactophenol and examined with an Olympus BH-2 compound microscope at 100-400x magnification. The diameters of these eight ascocarps and the length of four to five appendages of each ascocarp were measured. The final ratio of appendage length to ascocarp diameter determined the identity of the *Microsphaera* sp. (Braun 1987).

Disks were cut using a 12-mm-diameter cork borer from dried leaves of each dogwood species that had both *M. pulchra* and *P. guttata* ascocarps occurring together, and were prepared for scanning electron microscopy without

fixation or dehydration. Specimens were mounted on aluminum stubs with double-sided sticky tape, sputter-coated with gold-palladium and then view with an ETEC Autoscan (Hayward, CA 94545) at 60-1200x magnification with an operation at 10 kV. Photographs were taken on panchromatic polaroid 4x5 instant sheet film (Polaroid Resource Center, Cambridge, MA 02139).

2.3 Results

Dichotomously branched appendages and the occurrence of many asci in each ascocarp identified one of the powdery mildew genera as *Microsphaera* (Plate 2-1A,B and 2-2B). Species determination was based on the ratio of ascocarp diameters to appendage length. The ratio of ascocarp diameter (92-128 μm) and appendage length (117-160 μm) was 1:1.3$\pm$0.14 (Table 2-2), which identified the species as *M. pulchra* (Braun 1987). Based on host, number of asci/ascocarp, and ascocarps with bulbous-based appendages the second powdery mildew species was identified as *P. guttata* (Plate 2-1C, 2-2A) (Braun 1987).

Ascocarps of both *P. guttata* and *M. pulchra* were observed on the same dogwood leaves (Plate 2-1D). *Microsphaera pulchra* ascocarps were randomly distributed on

Plate 2-1. Scanning electron micrographs of *Microsphaera pulchra* and *Phyllactinia guttata* ascocarps occurring together and separately on *Cornus florida* 'Cherokee Brave' and *C. amomum* abaxial leaf surface.

A. *Microsphaera pulchra* on the abaxial leaf surface *of C. florida* 'Cherokee Brave' (600x).

B. Dichotomously branched appendages of a *M. pulchra* ascocarp (1200x).

C. *Phyllactinia guttata* on the abaxial leaf surface of *C. amomum* (120x).

D. *Microsphaera pulchra* (Mp) and *P. guttata* (Pg) ascocarps on *C. amomum* (225x). Large arrow indicates appendages of *P. guttata,* small arrow indicates appendages of *M. pulchra.*

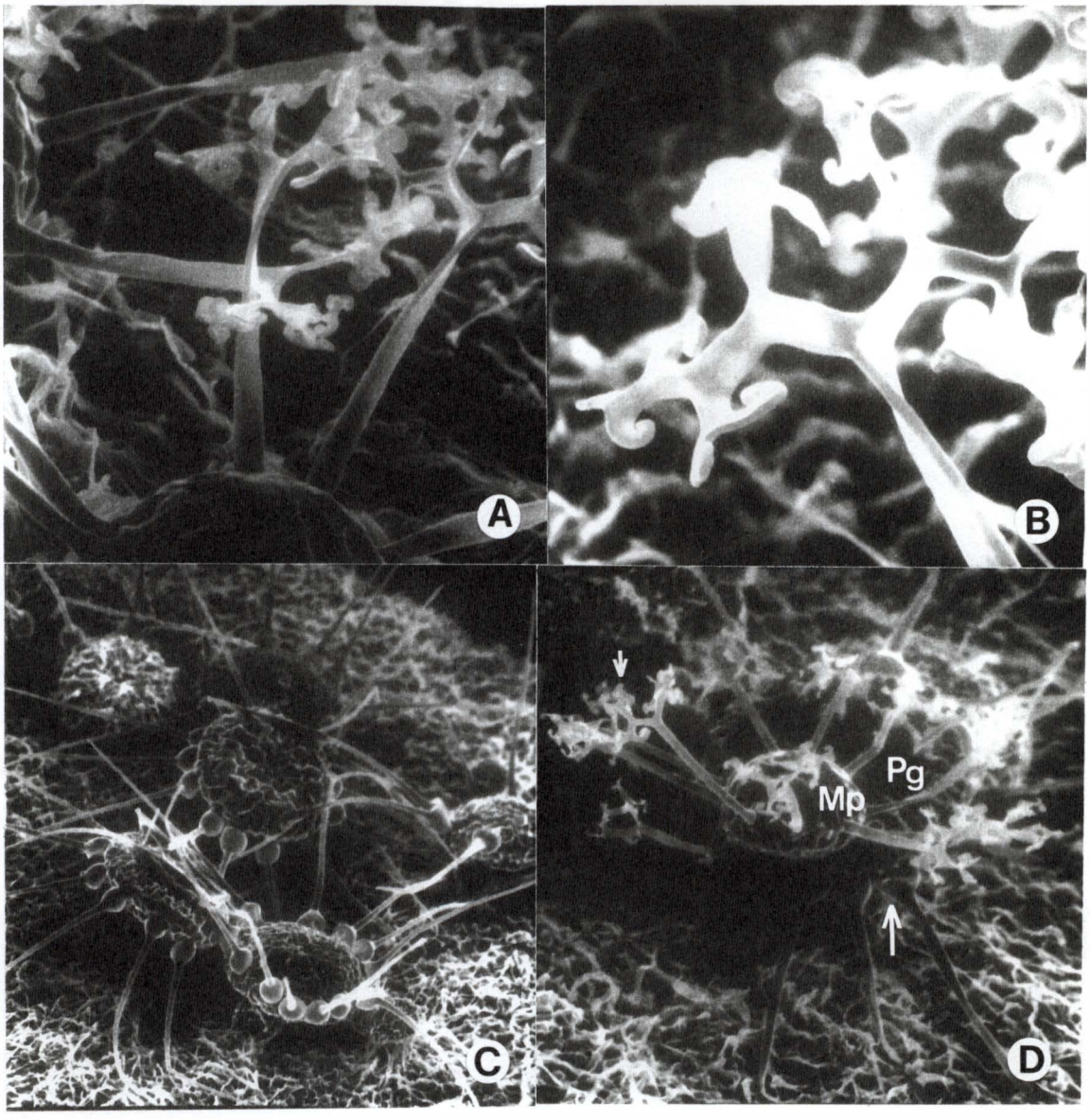

A
B
C
Pg
Mp
D

Plate 2-2. Stained sections of *Microsphaera pulchra* infecting *Cornus florida* 'Cherokee Brave' and *Phyllactinia guttata* infecting *Cornus amomum* leaves.

A. *Phyllactinia guttata* ascocarp on the abaxial leaf surface of *C. amomum* (200x). Small arrow indicates excretory appendages.

B. *Microsphaera pulchra* ascocarp on the abaxial leaf surface of *C. florida* 'Cherokee Brave' (400x).

C. *Phyllactinia guttata* hyphae entering an abaxial leaf surface stomata of *C. amomum*, ramifying through the mesophyll layer to form a haustorium (400x). G=guard cells of stomata, solid arrows indicate hyphae, hollow arrows indicate a haustorium.

D. Haustorium of *M. pulchra* formed in the adaxial leaf surface epidermal cell of 'Cherokee Brave' (1000x). Large arrow indicates a haustorium.

E. *P. guttata* entering stomata on the abaxial leaf surface of *C. amomum* (1000x).

F. Haustorium of *M. pulchra* formed in the abaxial epidermal cell of 'Cherokee Brave' (1000x). Large arrow indicates a haustorium.

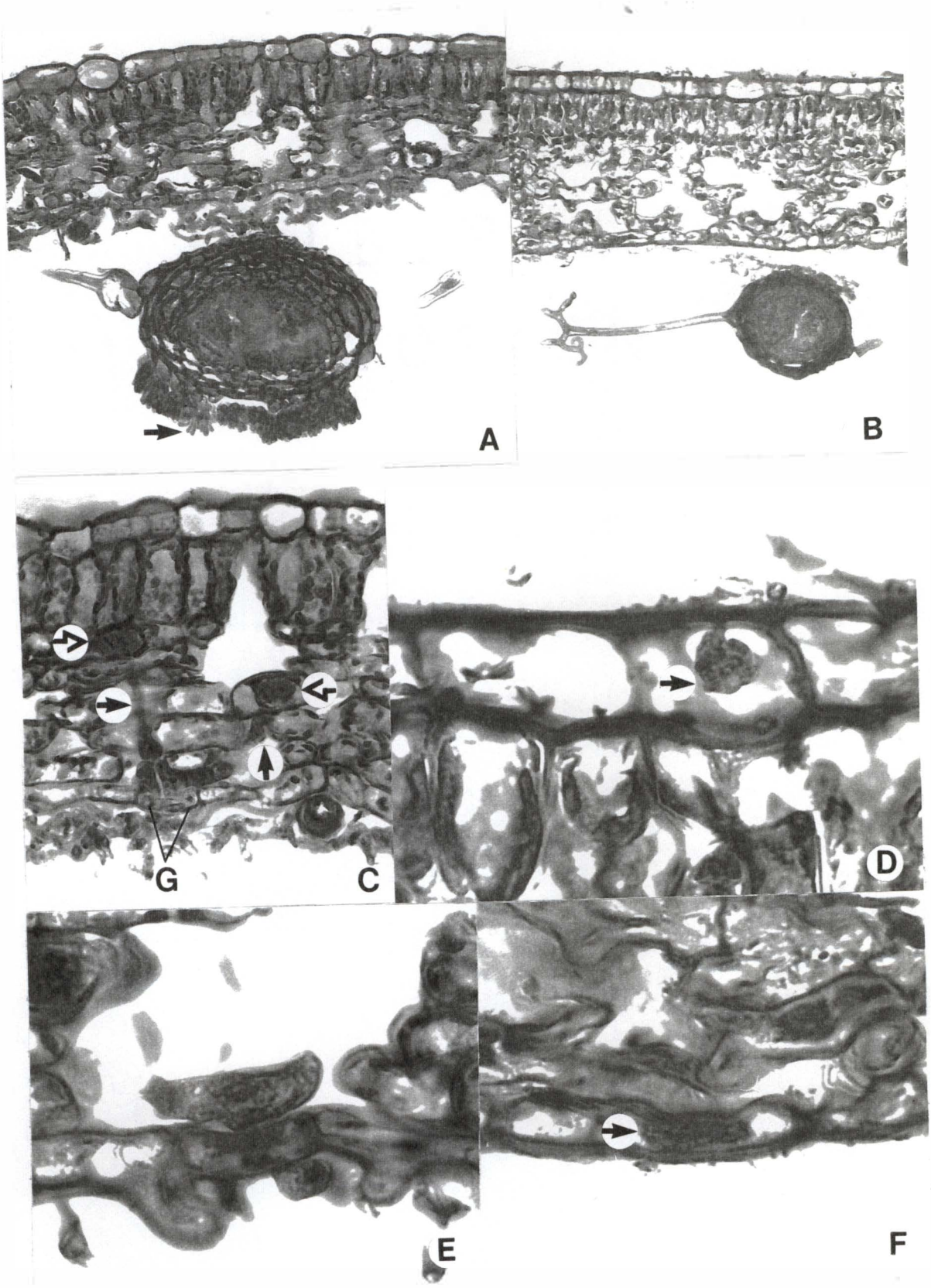

A
B
C
G
D
E
F

Table 2-2. The ratio of ascocarp diameter to appendage length to determine *Microsphaera* species.

Ascocarp diameter (μm)	Appendage length (μm)[z]	Ratio
110	160	1.5
97	133	1.4
97	119	1.2
93	117	1.3
97	120	1.2
114	140	1.2
119	136	1.1
128	145	1.1
107 $\pm$ 13	134 $\pm$ 15	1.3 $\pm$ 0.1

[z] mean of 4 or 5 appendages per ascocarp

the abaxial surface of 'Cherokee Brave' leaves and occurred at higher densities than ascocarps of *P. guttata*. *Phyllactinia guttata* ascocarps were mixed with and in close association with *M. pulchra* ascocarps on these same leaves. In contrast, ascocarps of *P. guttata* were commonly found on *C. amomum* leaves in close association with the less frequent *M. pulchra* ascocarps. Ascocarps of *M. pulchra* on *C. amomum* were at times directly on the leaf surface and at other times appeared attached to the *P. guttata* ascocarps (Plate 2-3B). However, some *P. guttata* ascocarps were situated on the 'Cherokee Brave' leaf surface with appendages pointing away from the epidermis (Plate 2-3A).

Histological studies revealed that *P. guttata* hyphae entered the abaxial leaf surface of *C. amomum* through stomatal openings (Plate 2-2E). The hyphae ramified throughout the leaf tissue of the host and produced haustoria only in mesophyll cells (Plate 2-2C). *Microsphaera pulchra* hyphae were restricted to the surface of 'Cherokee Brave' leaves and penetrated directly, forming haustoria in the adaxial (Plate 2-2D) and abaxial (Plate 2-2F) epidermal cells. Since penetration through stomata was not observed on 'Cherokee Brave' leaves, it was assumed that *P. guttata* was not a pathogen of these leaves. Likewise, since direct penetration was not observed on *C. amomum*

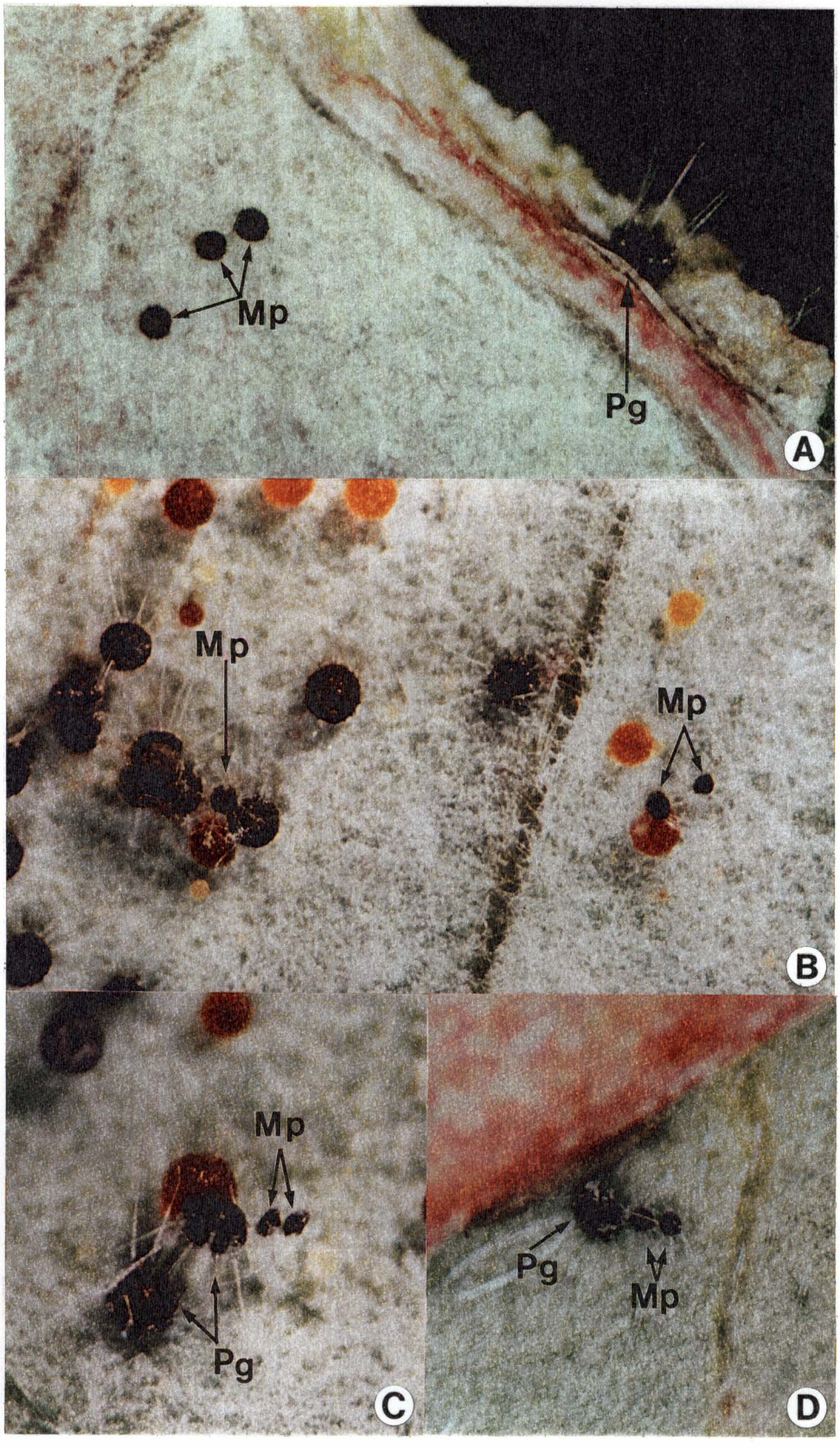
Plate 2-3. Photographs of *Microsphaera pulchra* and *Phyllactinia guttata* ascocarps occurring together on the abaxial leaf surface of *Cornus florida* 'Cherokee Brave' and *C. amomum*.

A. *Phyllactinia guttata* ascocarp (Pg) occurring with more prevalent *M. pulchra* ascocarps (Mp) on the abaxial leaf surface of *C. florida* 'Cherokee Brave'.

B. Three *Microsphaera pulchra* ascocarps (Mp) occurring with more prevalent *P. guttata* ascocarps (Pg) on the abaxial leaf surface of *C. amomum*.

C. Closer view of *M. pulchra* (Mp) and *P. guttata* (Pg) ascocarps occurring together on *C. amomum*.

D. *Microsphaera pulchra* (Mp) and *P. guttata* (Pg) ascocarps occurring together along the midvein of a *C. amomum* leaf.


Mp
Pg
A
Mp
Mp
B
Mp
Pg
C
Pg
Mp
D

leaves, it was assumed that *M. pulchra* did not infect these leaves.

2.4 Discussion

Yarwood (1957) cautions that errors in identification of host-parasite associations can be made if ascocarps become attached to a plant after dissemination. Appendage orientation of dehisced *Phyllactinia* spp. was used to determine the origin of ascocarps on specific leaf surfaces (Cullum and Webster 1977). *Phyllactinia* spp. have the following two appendage types: bulbous equatorial appendages and branched apical appendages (Plate 2-2A). The bulbous appendages point downward and function to free the mature ascocarp from the superficial mycelium on the leaf surface (Cullum and Webster 1977). The ascocarp adheres to new surfaces with the apical appendages downward and the bulbous-based appendages arranged so that the tips point away from the surface (Cullum and Webster 1977). *Phyllactinia guttata* ascocarps on the 'Cherokee Brave' leaves had bulbous-based appendages pointing away from the leaf surface, which implies the structures were not formed on these dogwoods (Plate 2-3A). *Microsphaera pulchra* appendages appear to not function to lift the ascocarp from

the host surface. Therefore, histological examination of the infected tissue for the mode of pathogen ingress and colonization of the leaf would be needed to determine if *M. pulchra* ascocarps had formed on the leaf.

Histological studies were conducted to determine the ingress and colonization by powdery mildew pathogens of each host. The mycelium of *Microsphaera* spp. is epiphytic, whereas the mycelium of *Phyllactinia* spp. is hemiendophytic, forming both mycelium on the surface and producing internal hyphae (Braun 1987, Yarwood 1957). The mycelium on leaf surfaces of both dogwood species was profuse and ascocarps were readily found in the histological sections of the leaves. Hyphae of *P. guttata* entered stomata of the *C. amomum* leaves, however there was no indication that *P. guttata* hyphae entered the stomata or ramified within mesophyllic tissue of 'Cherokee Brave' leaves. *Microsphaera pulchra* directly penetrated the abaxial and adaxial epidermal cells of 'Cherokee Brave' but did not infect *C. amomum*. This study provides documentation of both *M. pulchra* and *P. guttata* ascocarps simultaneously occurring on *C. florida* 'Cherokee Brave' and on *C. amomum,* however there was no evidence of concurrent infection by both fungi within the same dogwood leaves.

Powdery mildews can be identified using five

interrelated criteria. The most conclusive way to identify the pathogen is with ascocarp morphology, however on dogwoods, the fruiting structures are not formed until autumn. Previously documented reports of host-pathogen relationships and distribution of the pathogen are useful supplements to ascocarp morphology. Throughout the growing season, the conidial stage can be used to identify the pathogen but environmental conditions may cause variations in morphology (Braun 1987). The most accurate technique for identification is histological examination of host tissue that reveals penetration of the leaf cell, morphology and area of haustorial formation, and the area of hyphal ramification.

Observations of ascocarps of two powdery mildew species occurring on a dogwood species warrants caution. Several criteria need to be utilized to identify the powdery mildew pathogen of a particular host. Potential differences in the disease processes of *Microsphaera* and *Phyllactinia* spp. may have implications to breeding programs in the search for a general resistance mechanism for disease management. Yarwood (1957) warned that resistance to one powdery mildew species may not necessarily correlate with resistance to another, therefore correct identification of the pathogen is imperative.

CHAPTER 3

EVALUATION OF INOCULATION TECHNIQUES WITH CONIDIA OF *MICROSPHAERA PULCHRA*

3.1 Introduction

A reliable inoculation procedure is prerequisite to studies of powdery mildew disease of flowering dogwood, *Cornus florida* L.. A natural inoculation method has already been established to study resistance in the field. Non-infected healthy trees are placed under diseased dogwood trees in the field. Disease development and symptoms are monitored over time to assess powdery mildew resistance (Windham 1996, Ranney et al. 1994). Studies of the chronological progression of powdery mildew pathogenesis, however, requires a reliable inoculation technique that is applicable to greenhouse situations.

Reliable inoculation techniques for the powdery mildews use tools to transfer conidia from an infected leaf surface to a noninfected surface. Spore settling-towers are commonly used to inoculate host tissue. A spore settling-tower was used to study low humidity tolerance of *Erysiphe polygoni* DC. and other powdery mildew-causing fungi (Yarwood

1936b). Conidia were dusted onto slides, petri dishes, and leaves placed in the bottom of a large can. Settling-towers have since been used to provide an evenly-distributed, known inoculum load on leaves (Aist and Israel 1977, Carver 1987, Silvapalan 1993). A sticky coverslip was placed among the leaves at the bottom of a settling tower to monitor the density of conidia in other experiments (Carver and Adaigbe 1990, Carver and Ingerson-Morris 1989).

The "rolling method" was developed to research *E. graminis* DC. f.sp. *tritici* E´m. Marchal on wheat (Nair and Ellingboe 1962). Infected wheat leaves, with fungal sporulation, were shaken to remove old conidia. Six hours later, when new conidia were formed, young shoots were tapped gently with a glass rod and conidia were deposited onto glass slides. A stream of air was directed transversely across aggregates of conidia, leaving a single layer of viable conidia. Slides were examined under a microscope to determine uniformity of deposition and damage to the conidia, as measured by morphological abnormalities. A sterile absorbent cotton swab was gently rolled over the glass slide to pick-up the layer of healthy conidia. Conidia were transferred from the swab to the leaf by a gentle roll, once forward and once backward, over the leaf surface (Nair and Ellingboe 1962).

Advantages of the rolling method over the shaking and dusting methods are the following: (1) conidia of similar age and metabolic state are used in inoculations, (2) conidia can be examined under the microscope, prior to inoculation, to predict density, and (3) the conidia are deposited uniformly on specific portions of the host surface (Nair and Ellingboe 1962). This method provided a means to study infection on a quantitative basis (Douglas et al. 1984, Masri and Ellingboe 1966).

A third technique developed for inoculation uses a camel-hair brush. A clean, dry brush was used to collect conidia from the leaf surface and transfer them onto non-infected leaves (Edwards and Ayres 1982, Hewitt 1976, Hewitt and Ayres 1975).

An alternative technique to deposit conidia on the surface of leaves or cellophane sheets involves gently touching infected leaves onto a clean surface (Kunoh et al. 1988, Nicholson et al. 1988). Leaves infected with *E. graminis* forming conidia were gently touched to the surface of cellophane sheets. This technique ensured a precisely known time for initial contact between the pathogen and host leaf surface (Kunoh et al. 1988).

The purpose of this study was to determine the most appropriate technique for inoculation of multiple trees in

the greenhouse with powdery mildew conidia. Three techniques were evaluated in the study. The brush technique was chosen because of its simplicity, whereas the shake technique was selected to simulate inoculation with settling-towers on a larger scale. The wipe technique simulates touching infected and healthy leaves (Kunoh et al. 1988), but in this study the leaves were rubbed together.

3.2 Materials and Methods

Host plants

Cornus florida 'Cherokee Sunset' Tree A was removed from a research tree compound located on The University of Tennessee Agriculture Campus on 26 June, 1996, defoliated and placed in a greenhouse free of powdery mildew inoculum. Tree A was grown under a supplemental 15 h light/9 h dark cycle. The greenhouse was treated with volatilized sulfur at a rate of 16-23 g twice a week. Only leaves infected with powdery mildew were removed throughout the growing season. The last stripping of infected leaves was conducted on 5 September, 1996. Tree A was rinsed with tap water from a hose, washed with mild dishwashing detergent water using a sponge, then rinsed three more times prior to inoculation. The tree was washed on three consecutive days before use in

the 22 October, 1996 experiment.

The experiment was repeated using a second 'Cherokee Sunset' (Tree B) that was inoculated on 16 June, 1997. Tree B was removed from the research tree compound and transferred to a greenhouse on 4 March, 1997 to break tree dormancy. Tree B was also grown under a supplemental light cycle of approximately 15 h of light/9 h of darkness. Sulfur was not burned in the greenhouse prior to the experiment, therefore Tree B was not washed before the repeat experiment was conducted.

Powdery mildew inoculum

Inoculum was obtained from six infected *C. florida* trees grown in a separate greenhouse. The trees with powdery mildew were exposed to an approximate 15 h light/9 h dark cycle. Infected dogwood trees, in pots, were watered at the soil level to avoid wetting foliage or powdery mildew mycelial mats. Trees were shaken 24 h prior to inoculation to remove old conidia and ensure inoculum consisting of spores of the same age.

Inoculation of *Cornus florida* 'Cherokee Sunset' trees

Trees were inoculated at 9:30 a.m. on two different occasions in a closed room without light, except for approximately 15 min each day when samples were collected. Young infected leaves were carefully cut from infected trees

in the greenhouse and placed in a rectangular tray with approximately 5-cm-tall sides and transported to the room where the experiment was conducted. The trees were inoculated with three different techniques: infected leaves were shaken over non-infected 'Cherokee Sunset' leaves, infected leaves were wiped over non-infected leaves, and conidia were brushed from infected leaves onto non-infected leaves with a camel-hair brush. A permanent marker was used to place a dot on inoculated leaves. Leaves were covered with air-filled plastic bags, to create an environment of high humidity, and tied close. Bags were removed after 48 h. Trees were watered as needed throughout the experiment. The experiments continued for 7 and 8 days for trees A and B, respectively.

Sampling and assessment

Samples were collected every 24 h using a 4-mm-diameter cork borer. Disks were vacuum aspirated and fixed with cold (4°C) 3% glutaraldehyde in 0.05 M phosphate buffer, pH 6.8. Fixed samples from 48 h after inoculation were washed three times in a 0.05 M phosphate buffer (pH=6.8). Samples were then dehydrated in a 10, 30, 50, 70, 100% acetone series, each treatment for 15 min.

Four disks of each inoculation technique, from 48 h, were critically-point-dried with liquid CO_2, mounted on

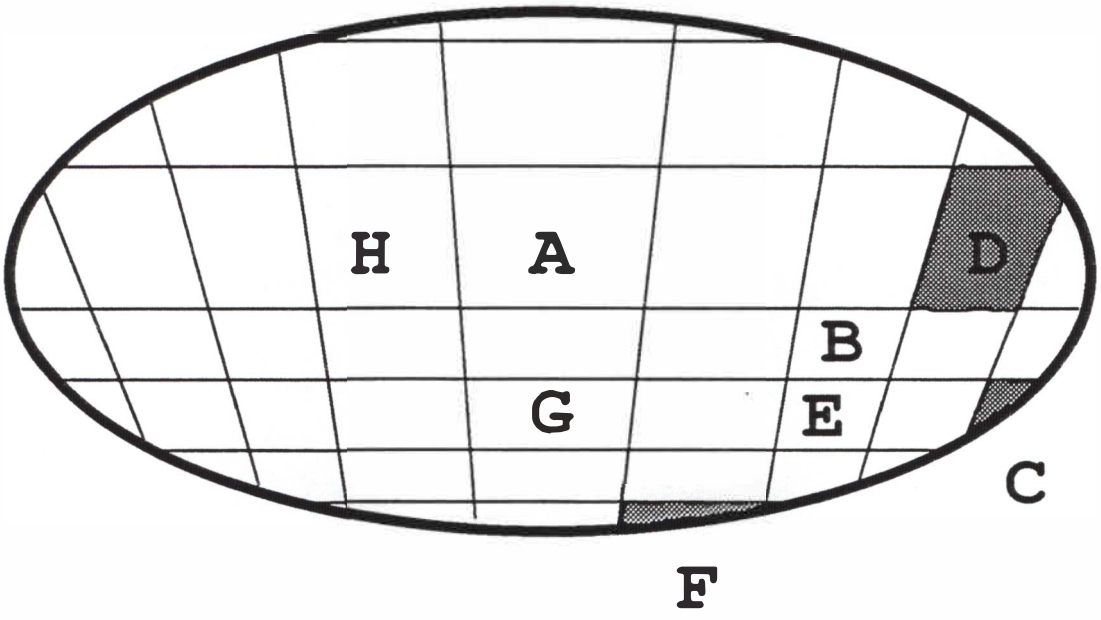
aluminum stubs with double-sided tape, sputter-coated with gold-palladium and then viewed with an ETEC Autoscan (Hayward, California 94545) at 120-600x magnification, operated at 10 kV. Photographs were taken on panchromatic polaroid 4x5 instant sheet film (Polaroid Resource Center, Cambridge, Massachusetts 02139).

Data collected included number of conidia present on the leaf surface, number of conidia germinated, and number of germinated conidia that produced appressorial swellings and penetrated the host surface. Conidia were considered germinated if any part of a germ tube was observed and penetration was inferred by the presence of appressoria.

A system was created to examine a leaf disk without overlapping view fields. A disk was examined at 60x magnification, centered in the monitor of the ETEC Autoscan, then the magnification was raised to 120x. This center screen was considered the first of 8 view fields of the disk (Figure 3-1A). The x and y axis knobs were rotated clockwise or counterclockwise to move to the next view field (Figure 3-1B, C). When the edge of the disk was reached, the view was not considered complete and therefore not counted. Conidia were counted if any part of them were visible on the view field, even if the conidia were collapsed or deformed.

Data were analyzed using ANOVA (proc glm) and when

(a)



(b)

X axis/Y axis	
center	
2cl	1cl
2cl	1cl
1cc	2cc
1cc	2cl
1cc	2cl
1cc	2cc
1cc	2cc
2cl	1cc
1cc	2cl

(c)

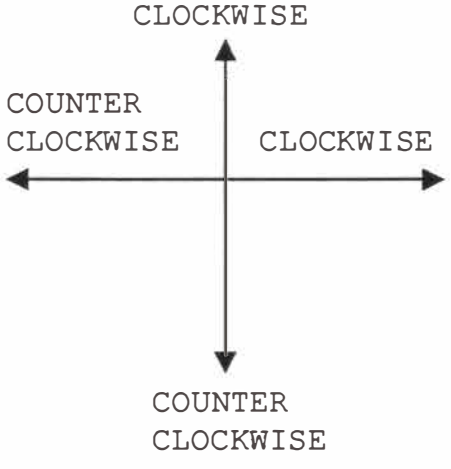


Figure 3-1. Established system to obtain views of a 4-mm-diameter leaf disk. (a) Location of views A-H on a leaf disk. (b) Rotations taken to obtain views. cl=clockwise, cc=counter clockwise (c) Relationship between each 360° turn of the x and y axis dials and movement of views on a leaf disk.

significant variation was observed, Duncan's Multiple Range Test (P=0.05) was used for mean separation. The experimental design was considered completely randomized with 4 disks collected for each treatment (wipe, brush, shake) at 48 h after inoculation. Inoculation results were noted for each of 5 complete view fields of the 4 disks resulting in 20 views for each technique.

Only the first experiment's data, Tree A, were analyzed. Two of four leaf disks from the repeat experiment, Tree B, were mounted adaxial surface down such that conidia could not be viewed. Therefore, the repeat experiment was not analyzed statistically.

3.3 Results

Significant variation was observed only for percent germination. The shake method of inoculation permitted a significantly greater percentage of germination relative to the amount of conidia on the leaf surface. Table 3-1 indicates the raw data collected for each technique and the Pr>F values obtained from the ANOVA.

Due to the high variability in the number of conidia between each view of the same technique, statistical differences in techniques were difficult to observe. The

Table 3-1. Number of conidia deposited on the leaf surface, conidia germinated, conidia penetrated, percent germination, and percent penetration for each of the three inoculation procedures.

		Treatment [z]		
Inoculation Results	**Pr > F**	**Shake**	**Wipe**	**Brush**
Conidia	0.4788	6.2	40.5	23.2
Germination	0.2246	4.6	6.4	13.3
Penetration	0.3192	3.9	5.2	10.0
% Germination	0.0032*	**72.9 a**	**35.4 b**	**49.0 b**
% Penetration	0.4541	82.3	70.7	65.7

* Significant variation (P=0.05)

[z] Different letters indicate significant difference among means within a row. Means were separated using Duncan's Multiple Range Test (P=0.05).

brush technique placed as few as 0 to 80 conidia on different views. The wipe technique resulted in more variability in the number of conidia deposited, with 0-199 conidia on different views of leaf disks. The shake method placed fewer conidia on the disks with a range of 0-19 conidia on samples.

Obvious differences existed between inoculation techniques when the raw data were examined (Table 3-1). The wipe technique placed the greatest mean number of conidia (40.5) on the leaf surface but only a mean of 6.4 conidia germinated. The conidia were usually flattened and often clumped around trichomes (Plate 3-1B). However, of the mean of 6.4 conidia that germinated, 5.2 formed appressoria.

The brush technique placed the second greatest mean number of conidia on the leaf (23.2) and a mean of 13.3 of these germinated with a mean of 10.0 forming appressoria. Brushing placed the greatest number of viable conidia on the host surface that succeeded in penetrating dogwood leaves. There were some damaged conidia, however the healthy conidia germinated and appeared evenly distributed on the leaf surface (Plate 3-1C, D).

The shake technique resulted in a mean of only 6.2 conidia on the host surface, however a mean of 4.6 of these germinated with a mean of 3.9 forming appressoria. The shake technique caused minimal damage to the conidia,

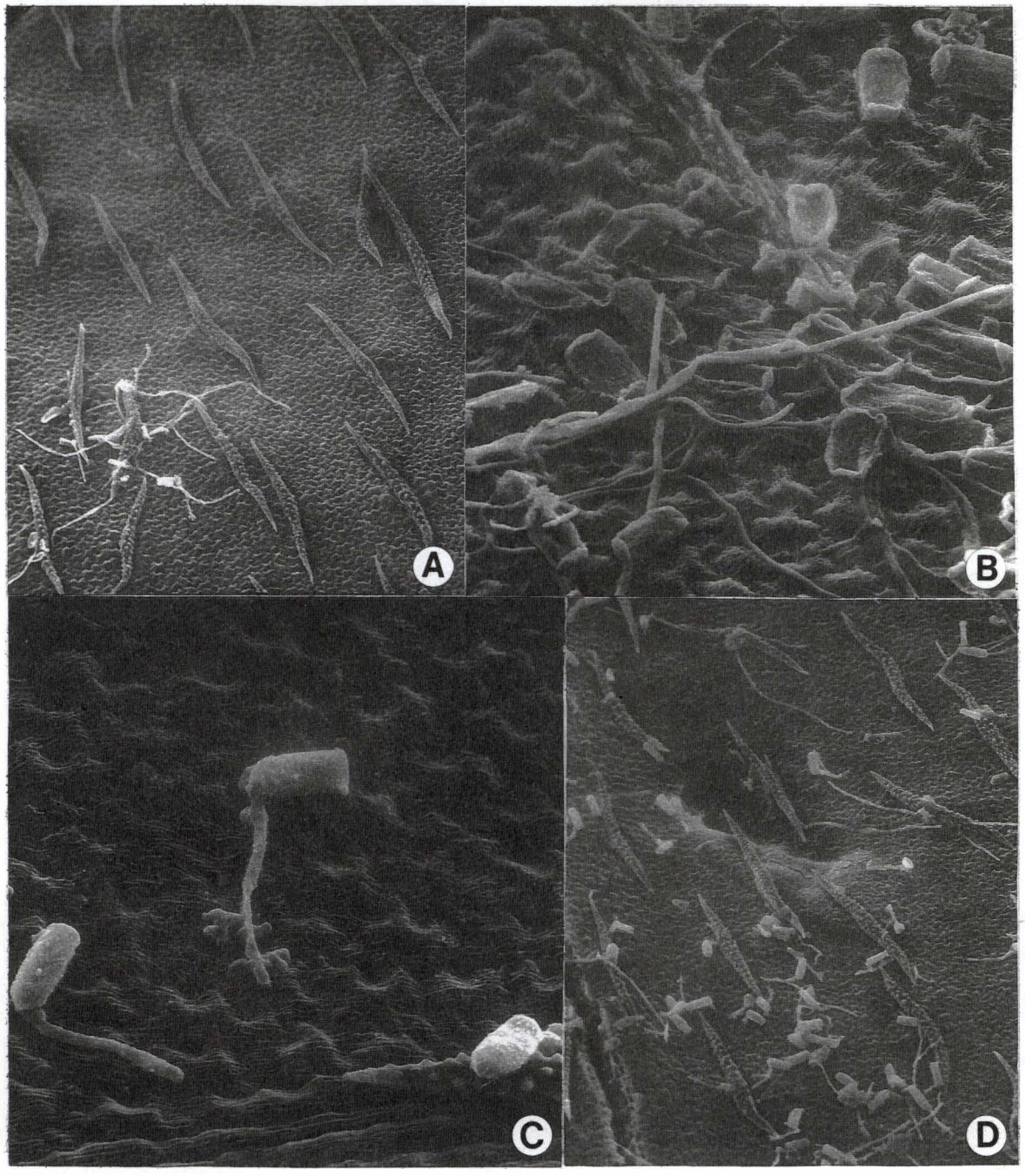
Plate 3-1. Scanning electron micrographs of conidial deposition, germination, and penetration resulting from brush, shake, and wipe inoculation techniques.

A. Conidia deposited on the adaxial leaf surface using the shake technique (120x).

B. Conidia deposited on the adaxial leaf surface using the wipe technique (480x).

C. Conidia deposited on the adaxial leaf surface using the brush technique that have germinated and formed appressoria (600x).

D. Conidia deposited on the adaxial leaf surface using a camel-hair brush (120x).


A
B
C
D

however there was a low density of deposited conidia (Plate 3-1A).

3.4 Discussion

Although there was no significant variation in the amount of conidia delivered using each technique, there were advantages of certain methods over others. The wipe technique placed the largest amount of conidia on the leaf surface, but the conidia were often damaged. Conidia of the Erysiphaceae are different than most air-dispersed fungi in that their spores do not absorb water and swell during germination, instead the water volume decreases (Braun 1987). Certainly, some of the withered conidia observed with the wipe technique (Plate 3-1B), as with shake and brush, were the effects of germination. However, the frequency of flattened conidia appeared much higher for the wipe technique. The shake technique appeared the least damaging to conidia with a significantly greater percent germination, but also placed the least amount of conidia on the leaf surface. Of the techniques studied, the camel-hair brush appears to be the best tool for transferring conidia to leaf surfaces. There was a sufficient amount of conidia delivered to the leaf without an excessive amount of damage

to spores when compared to the wipe technique, resulting in subsequent powdery mildew disease. Mycelial mats were distinguishable to the unaided eye by 162 h after inoculation indicating a normal host-pathogen relationship.

Dogwood leaves were washed prior to the experiment to remove any residual sulfur crystals. Sulfur, used to prevent powdery mildew infection prior to the experiment, was not only an eraticant, but also a protectant that killed spores that fell on the leaf surface (Yarwood 1957). When viewed with a scanning electron microscope, the adaxial leaf surface appeared free of sulfur crystals. Therefore, sulfur did not appear to alter germination and penetration of the conidia deposited by each inoculation technique.

Washing leaves prior to inoculation may have affected conidial germination, germ tube or appressoria formation, although the leaf surface appeared undamaged. Washing hawthorn (*Crataegus monogyna* Jacq.) leaves for 1 h prior to inoculation caused *Podosphaera clandestina* Lev. germination to vary depending on leaf maturity (Khairi and Preece 1979). Germination of conidia on young leaves was not significantly affected by a pre-inoculation washing with water. There was an increase in percent germination of conidia on intermediate-aged leaves whereas there was no significant difference in germination of conidia on older leaves when

compared to unwashed leaves. However, washing increased germ-tube length of conidia infecting young, intermediate aged leaves, and one of three of the older leaves (Khairi and Preece 1979). An increase in germ-tube length on prewashed leaves was assumed to be the result of removal of inhibitory materials.

Varying results were observed with *E. cruciferarum* Opiz ex Junell (Purnell and Preece 1971). Colony formation on prewashed swede (*Brassica napus* L.) leaves was reduced even though germination was not affected by preinoculation washings. Leaves of different ages were washed for 1 h prior to inoculation. Germination of conidia and subsequent appressoria formation were not affected by a prewash with distilled water. On unwashed leaves there was little variation in primary and secondary hyphae formation. Hyphae production was significantly reduced on prewashed mature, and older leaves (Purnell and Preece 1971).

If trees were allowed to stand for a few days after washing, but prior to inoculation, an increased number of conidia would produce primary and secondary hyphae (Purnell and Preece 1971). During summer months, the greatest amount of hyphae reduction was observed when trees were inoculated 0-3 days after washing. At 5 days, there was an increase in the number of primary and secondary hyphae on the leaves. A

possible explanation for this outcome is that washing swede leaves caused a depletion of nutrients on the leaf surface that adversely affected hyphal growth. Young leaves had larger amounts of wax on the surface, therefore 1 h of washing may not remove enough nutrients to stop hyphal growth (Purnell and Preece 1971).

Light could have also played an important role in the germination process. The average length of hyphae differed in various light exposures, 48 h after inoculation at 24°C (Yarwood 1936a). In complete darkness the hyphal length was 699 μm, whereas the length increased to 1070 μm for a 4 h day, 1620 μm for an 8 h day, and 1880 μm for a 12 h day. With 16 h of light, the hyphal length decreased to 1678 μm, and in continuous light to 1500 μm (Yarwood 1936).

The number of appressoria likewise reflected a difference in light periodicity verses darkness. There were a mean of 3.3 appressoria formed per colony in continuous light, 10.4 formed in a 12 h light day, and only 2.1 formed in continuous darkness (Yarwood 1936).

In this experiment, several factors could have altered the infection process when compared to normal field conditions. Leaves were washed up to 24 h prior to the inoculation, therefore wax and cuticle layers may have been damaged. Under ideal conditions, this experiment should have been conducted in early spring before trees in the

greenhouse were treated with sulfur. Although some of the leaves in experiment one (Tree A) were probably not older than a month or two, the tree was probably stressed from continuous defoliation. In the spring, the tree potentially would not be stressed, and most of the leaves would have been young and more susceptible to infection, which could result in less variation between views. In October, when the experiment was conducted, most of the leaves inoculated were probably mature or older. The leaf age, in combination with leaf washings and lack of a diurnal light cycle, could have greatly influenced the infection process. All leaves sampled in the experiment were under the same conditions, however, so the inoculation techniques could be compared to one another.

Due to the results of this experiment, it appears conidia are best transferred with a camel-hair brush. In future studies an alternative brush techniques may prove to be less damaging to the conidia. Edwards (1979) brushed the surface of *Quercus robur* L. diseased leaves with a camel-hair brush, then gently tapped the shaft of the brush to knock spores onto uninfected leaves. A variation of this technique would be tapping the infected leaves above a healthy host. These techniques could eliminate contact

damage caused by actually brushing the spores onto the leaf surface.

CHAPTER 4

INOCULATION OF DOGWOOD CALLUS AND MICROSHOOTS WITH *MICROSPHAERA PULCHRA*

4.1 Introduction

Powdery mildews are biotrophic fungi and require a living plant host to complete their life cycle. To work with powdery mildews in experimental systems, methods must be derived to produce inoculum. Typically, *Microsphaera pulchra* Cooke and Peck inoculum is obtained from infected dogwood trees grown in either the greenhouse or field. Powdery mildew disease research could be advanced if a continuous inoculum source was maintained *in vitro* under controlled laboratory conditions.

There has been limited success in the establishment of powdery mildew colonies on tissue cultures. Suspension cultures of *Rosa* L. sp. (rose) were inoculated with *Sphaerotheca pannosa* (Wallr.:Fr.) Lev., *Acer pseudoplatanus* L. (sycamore maple) with *Uncinula aceris* (DC.) Sacc., *Triticum aestivum* L. (wheat) with *Erysiphe graminis* DC. f.sp. *tritici* Em. Marchal, and *Pisum sativum* L. (pea) with *E. pisi* (Webb and Gay 1980). Wax was extracted from pea

leaves and reconstructed on wheat and pea callus prior to inoculation with powdery mildew. Another technique was removal of the lower epidermis from pea leaves and placing it, cuticle side uppermost, on pea callus prior to inoculation. Neither wax nor epidermal tissue were placed on rose and sycamore callus prior to inoculation. Conidia were transferred onto each callus culture using a dry, sterile mounting needle or transferred in a drop of filter-sterilized fluorinated hydrocarbon 43. Inoculated callus was maintained at various temperatures, photoperiod exposures, humidities, and in different tissue culture media. Callus was also inoculated at different times during growth cycles of the callus. Conidial germination was defined by authors as "abnormal" and appressoria were not formed (Webb and Gay 1980). Powdery mildew disease was not established in callus cultures of rose, sycamore, wheat or pea.

Sunflower tumor tissue, induced by *Agrobacterium tumefaciens* (Smith and Townsend) Conn., was inoculated with conidia of *Erysiphe cichoracearum* DC. in an attempt to establish powdery mildew disease (Heim and Gries 1953). A dry, sterile needle was used to transfer the conidia, or a strip of infected epidermal tissue was placed over sunflower tissue. Cultures were sealed and stored in an illuminated

room at 22-25°C. Aerial hyphae and/or conidial chains were produced on one culture, but fungal colonies were not maintained due to fluctuations in room temperature. Aerial hyphae crinkled as the temperature rose from the 22-25°C to 27°C for 24 h. Although room temperatures were lowered immediately after the fluctuation, hyphae did not reappear. In a subsequent study, powdery mildew was established again on a tissue culture of sunflower, but aerial hyphae were ephemeral. Colony establishment was influenced by temperature, moisture on individual tissue surfaces, and vigor of the callus culture (Heim and Gries 1953).

There has been success in the establishment of other plant biotrophic fungi in culture. Sporangia of *Peronospora tabacina* Adam. (*P. hyoscyami* de Bary), the causal organism of blue mold of tobacco, were used to inoculate tobacco callus (Trigiano et al. 1984). Diseased 4-cm^2-leaf segments were attached to the inner surface of petri dish lids, sporangiophores downward, using 1% water agar. Lids were attached to petri dish bottoms containing callus grown in Murashige and Skoog (1962) medium supplemented with indoleacetic acid and kinetin. Lids with inoculum remained in place for approximately 18 h. Cultures were incubated at 25°C in darkness then examined at 48 and 96 h after inoculation. Trigiano et al. (1984) observed germ tubes,

bulbous appressoria-like structures, haustoria, intracellular hyphae, intercellular hyphae, and scanty surface mycelium. Sporangiophores and sporangia were later observed (Trigiano, personal communication).

Peronospora tabacina grows internally within its host, with sporangiophore emergence only in high humidity. In the previous study, *Nicotiana tabacum* L. leaves were surface disinfected with 70% ethanol before being attached to petri dish lids (Trigiano et al. 1984). This technique resulted in 18 of the 20 callus cultures remaining free of contaminants throughout the experimental period. Since *M. pulchra* hyphae grows superficially on the leaf, disinfection with alcohol would damage and possibly kill the fungus.

Establishment of powdery mildew on dogwood culture tissues or cells would accomplish the following: (1) provide large quantities of inoculum free of other contaminants; (2) enable screening for resistance in dogwood tissue cultures from different cultivars and species; (3) and establish collections of isolates. The purpose of this study was to establish powdery mildew colonies on dogwood callus and micropropagated shoots obtained from axillary bud proliferation.

4.2 Materials and Methods

Callus tissue

Young terminal twigs were collected from *Cornus florida* L. (flowering dogwood) 'Cherokee Sunset', 'Cherokee Brave', 'Cherokee Chief', and 'Cherokee Princess' to start callus cultures. Leaves were removed and discarded. Stems were washed in distilled water, a small drop of Liqui-Nox (Fisher, Norcross, GA 30091), and Triton X-100 (Fisher, Norcross, GA 30091). Trichomes were burned from stems by dipping them in 95% ethanol, quickly passing the tissue through an alcohol flame, then immediately dropping the twigs into disinfectant. Shoots were surface-disinfected in a 50% Clorox® (2.62% NaClO)(The Clorox Company, Oakland, CA 94612) solution for 8 min, then rinsed three times in sterile distilled water. Approximately 4-mm-internodal transverse sections were cut from the shoots and placed in 60-mm-diameter petri dishes containing modified McCown's Woody Plant Medium (WPM) (Lloyd and McCown 1980). Each liter of medium was modified to contain 2 mg (9 µM) 2,4-dichlorophenoxyacetic acid, 1 mg thiamin, and 30 g sucrose. Medium pH was adjusted to 5.8 with KOH and 8 g phytoagar were added before autoclaving. Two stems were placed in each petri dish and cultures were sealed with Parafilm™

(American National Can, Neenah, WI 54958). Cultures were maintained in an incubator at 24°C with 25 µmol m^{-2} sec^{-1} light provided by cool fluorescent bulbs on a 15 h light/9 h dark cycle. Every 4 wk the callus was divided in half as necessary and maintained on fresh medium.

Axillary bud proliferation

Microshoots were established from *C. florida* seedlings according to the methods of Kaveriappa et al.(1997). Depulped seeds from random trees in Knox and Blount Counties, Tennessee were stored in moist (field capacity) Farfard Canadian Growing Mix No. 2 (Conrad Farfard Inc., Agawam, MA 01001) at 4°C for a minimum of four mon. Germinated (radicle emerged) and ungerminated seeds were planted in Farfard Canadian Growing Mix No. 2 and grown in a greenhouse with a 15 h supplemental light/9 h dark cycle. Cultures were initiated from seedlings after the first set of leaves developed. Cotyledons and leaf blades were excised from seedlings, and stems with petioles washed in distilled water and a small drop of Liqui-Nox and Triton X-100. Stems were then briefly dipped in 95% ethanol, and ignited by a brief pass through a flame to remove trichomes. Explants were disinfected in 20% (1.05% NaClO) Clorox® solution for 15 min and rinsed three times in autoclaved distilled water. Petioles and apical meristems were removed

and single node explants, with 2 axillary buds, placed in petri dishes containing modified McCown's WPM. Each liter of medium was modified to contain 1 mg (4.4 µM) 6-benzyladenine, 1 mg thiamin, 30 g sucrose, and 0.1 g myo-inositol. Medium pH was adjusted to 5.8 prior to the addition of 8 g phytoagar then autoclaved. Cultures were maintained under conditions previously described for callus tissue.

Inoculum

Six diseased trees were maintained in a greenhouse with a supplemental 15 h light/9 h dark cycle. Trees were watered directly into the pots to avoid wetting foliage or conidia. Trees were shaken 24 h prior to inoculation of tissue cultures to remove old conidia and ensure inoculum of similar aged conidia.

Experiment 1

Diseased leaves, collected in the greenhouse, were used to inoculate callus tissue of *C. florida* 'Cherokee Brave', 'Cherokee Sunset', 'Cherokee Chief', and 'Cherokee Princess' on 23 February, 1997. Three dishes of tissue culture, each containing 2 calli, of each cultivar were inoculated in this experiment. Small sections of diseased leaves were attached, conidia downward, onto the inner surface of a petri dish lid using 1% water agar. Petri dish lids were

placed on petri dish bottoms containing callus in modified WPM as described previously. Callus tissue was inoculated by tapping the lids of the petri dishes 4 times to release the conidia from conidiophores. Petri dish lids were removed and replaced with sterile lids, then sealed with Parafilm™. Cultures were maintained in an incubator at 24°C with 25 µmol m^{-2} sec^{-1} light provided by cool fluorescent bulbs on a 10 h light/14 h dark cycle. Examination of callus began 7 h after inoculation. Callus was viewed with an Olympus SZH10 research stereo dissecting scope (Olympus America Inc., Lake Success, NY 11042-1179) at 40-70x magnification. Callus cells and conidia were scraped from the culture using a sterile scalpel with a number 10 blade. Cells were mounted in water on glass slides, coverslips applied, and viewed with an Olympus BH-2 compound microscope at 100-1000x magnification. Photographs were taken on the dissecting and compound scopes with an Olympus C-35AD-4 camera using Kodak Ecktachrome 160T colored slide film and images transferred to Kodak paper (Eastman Kodak Company, Rochester, NY 14650).

Experiment 2

Leaves were collected from trees grown in the greenhouse and transported to the laboratory in a rectangular pan with 5-cm-tall sides on 4 March, 1997.

Three dishes of dogwood tissue cultures, each containing 2 calli, of each of the four previously mentioned cultivars were inoculated in this experiment. Petri dish lids were removed, infected leaves were tapped above the cultures to disperse conidia onto calli, lids were replaced and dishes sealed with Parafilm™. Cultures were maintained for 3 h in the incubator under the same conditions as described in Experiment 1. Parafilm™ was removed 3 h after inoculation and dishes placed under a laminar flow hood at 23.5°C. After an additional 4 h, dishes were sealed and returned to the incubator. Examinations and photographs of the callus tissue began at 24 h after inoculation as described for Experiment 1.

Experiment 3

Callus and microshoots were inoculated in the greenhouse on 22 June, 1997 by tapping infected leaves over exposed tissue. Three plates of callus cultures, each containing 2 calli, of each of the previously mentioned cultivars and eight microshoots cultures, each containing 2 microshoots, were inoculated in this experiment. After inoculation, dish lids were replaced and sealed with Parafilm™. Cultures were maintained in an incubator as described for Experiment 1 and removed at 48 h after inoculation for examination. Observation and photographic

procedures for the callus were the same as described in Experiment 1. Microshoots were viewed with an Olympus SZH10 dissecting microscope and photographed with the same equipment and film as described in Experiment 1. Leaves were removed from the microshoots and fixed with cold (4°C) 3% gluteraldehyde in 0.05 M phosphate buffer pH 6.8 and Histochoice® (Amresco, Solon, Ohio 44139). Specimens were vacuum aspirated, then placed in fresh fixative. Histochoice® replacement fixative was Histochoice® supplemented with 20% ethanol.

Samples fixed in gluteraldehyde were washed three times in 0.05 M phosphate buffer (pH=6.8) and then dehydrated in a graded acetone series (10, 30, 50, 70, 100%), each treatment for 15 min. Leaves were critically point dried with liquid CO_2, mounted on aluminum stubs with double-sided sticky tape, sputter-coated with gold-palladium and then viewed with an ETEC Autoscan (Hayward, CA 94545) operated at 10 kV. Photographs were taken on panchromatic polaroid 4x5 instant sheet film (Polaroid Resource Center, Cambridge, MA 02139).

Samples fixed in Histochoice® were dehydrated with a series of 30, 50, 70, 90, 95, and 100% isopropyl alcohol, 30 min for each treatment. Dehydrated samples were embedded with TissuePrep® (Fisher Scientific, Fair Lawn, NJ 07410) and cast into blocks. Leaf samples were sectioned to a thickness of 10 μm with a rotary microtome and placed on

clean glass slides. Slides were stained with a modified Flemming's Triple Stain consisting of Safranin O (50% ethanol), 1% Crystal Violet, and Fast Green (Sigma Chemical Company, St. Louis, MO 63178)(Johansen 1940). Cover slips were applied with Eukitt Mounting Medium (Calibrated Instruments, Hawthorne, NY 10502) and slides viewed with an Olympus BH-2 compound microscope at 100-1000x magnification. Photographs were taken with an Olympus C-35AD-4 camera using Techpan 2415 film (Eastman Kodak Company) and developed on Ilfor MGIV RC (England) black and white paper.

Experiment 4

The same type and number of callus and microshoot cultures as described previously in Experiment 3 were inoculated in the greenhouse on 20 August, 1997. Cultures were maintained in an incubator under the same conditions as described for Experiment 1. Examinations began at 96 h after inoculation with an Olympus SZH10 dissecting scope.

Experiment 5

Twenty microshoots, 2 per petri dish, were inoculated on 29 September, 1997 in the greenhouse as described in Experiment 3. Cultures were maintained in an incubator at 24°C with 25 µmol m^{-2} sec^{-1} light provided by cool fluorescent bulbs on a 15 h light/9 h dark cycle. Microshoots were viewed every 24 h with an Olympus

dissecting scope. Microshoots were transferred to fresh petri dishes containing modified McCown's WPM at 48 h when contaminating fungi were visible with the naked eye or a microscope. Every 24 h after transfer, microshoots were viewed with an Olympus SZH10 dissecting scope, and photographed with an Olympus C-35AD-4 camera using Techpan 2415 film at approximately 12.5-140x magnification. Negatives were printed on Ilfor MGIV RC black and white paper. Specimens were fixed in both gluteraldehyde and Histochoice®, vacuum aspirated, and placed in fresh fixative. Microshoots were prepared for both histological examination and scanning electron microscopy as described for Experiment 3. Electron micrographs and photographs of stained sections were taken using appropriate film as described for Experiment 4.

4.3 Results

Experiment 1

Very few conidia were deposited on the callus when infected leaves were pasted to petri dish lids and tapped to inoculate the tissue. Both germinated and ungerminated conidia were observed, but appressoria were not formed and penetration did not occur.

Experiment 2

More conidia were deposited on the callus surface by shaking the infected leaves over the cultures than were deposited by mounting leaves over the cultures. However, much of the inoculum was inadvertently shaken off in the transfer of leaves from the greenhouse to the laboratory, the place of inoculation. Conidia were deposited as individuals and aggregates in the form of intact chains. Germinated and ungerminated conidia were observed on the cultures. Appressorial formation or penetration of cells were not observed in any of the cultures. However, there was attachment of conidia to callus cells and germ tube production (Plate 4-1A).

Experiment 3 and 4

Conidia were deposited at a higher density onto callus and microshoots when inoculation occurred in the greenhouse than when diseased leaves were transported to the laboratory for inoculation. Conidia readily formed germ tubes of variable lengths (Plate 4-1B) and the number of germ tubes produced varied from one-to-four (Plate 4-1F). Conidia formed lobed appressoria on callus cells and some subsequently formed haustoria (Plate 4-1D,E). On one occasion a conidium was observed to form a long germ tube and a nonlobed swelling that was assumed to be a haustorium

Plate 4-1. Photographs of conidial germination, appressorial formation, and haustorial formation on/in callus cells of various *Cornus* species.

A. Attachment of *M. pulchra* conidium to the surface of *a C. florida* 'Cherokee Princess' callus cell 24 h after inoculation (1000x). Arrow indicates point of attachment.

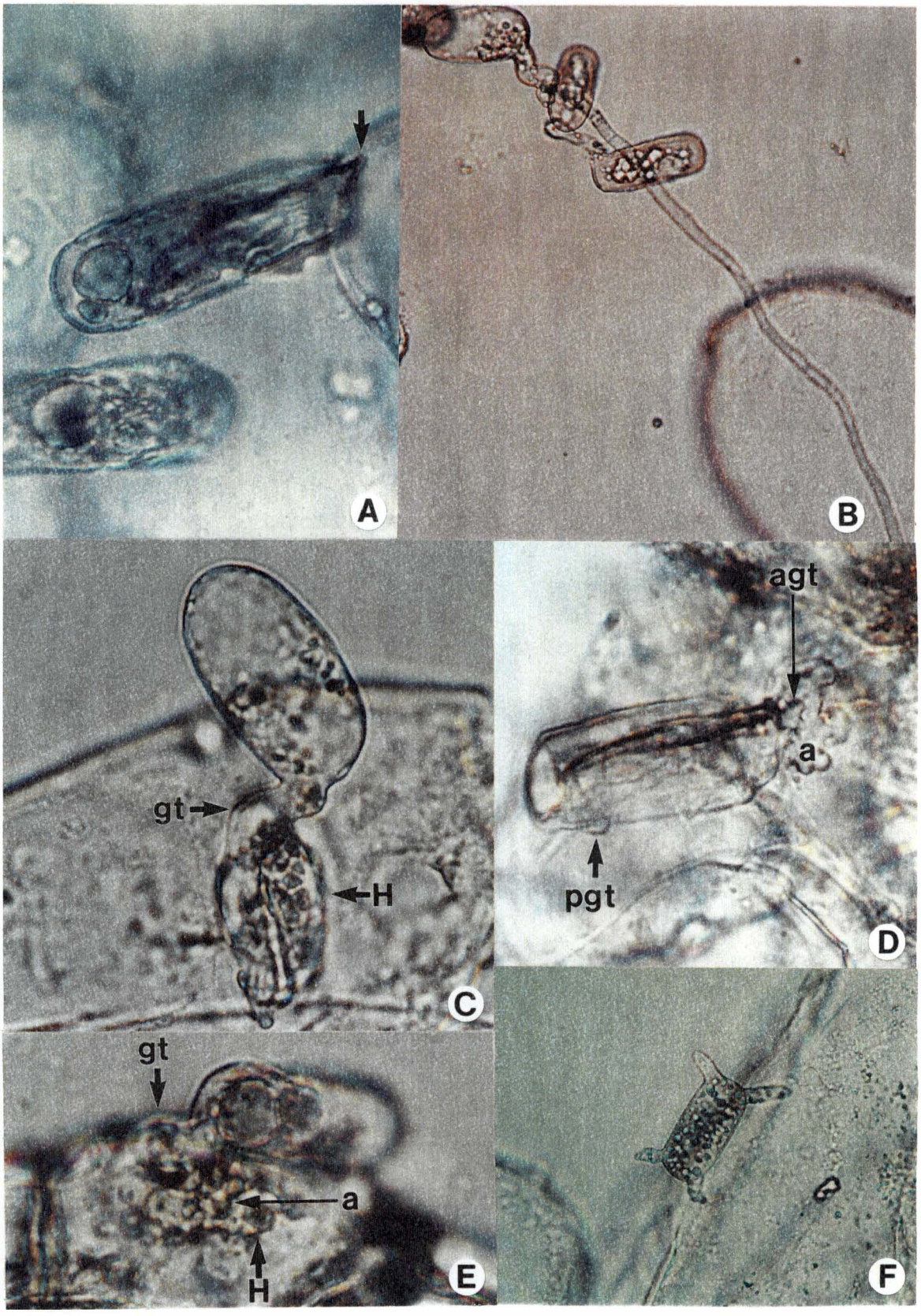
B. Extensive germ tube growth of *M. pulchra* conidium 94 h after inoculation of *C. florida* 'Cherokee Brave' callus culture (400x).

C. Germination, penetration, and possible haustorium formation of *M. pulchra* 74 h after inoculation of *C. florida* 'Cherokee Princess' callus culture (1000x). gt=germ tube, H=haustorium.

D. Primary germ tube and appressorial germ tube formation, and appressorial formation of a *M. pulchra* conidium 94 h after inoculation of *C. florida* 'Cherokee Brave' callus culture (1000x). pgt=primary germ tube, agt=appressorial germ tube, a=appressorium.

E. Germination, appressorial and haustorial formation of *M. pulchra* conidium 74 h after inoculation of *C. florida* 'Cherokee Princess' callus culture (1000x). gt=germ tube, a=appressorium, H=haustorium.

F. Multiple germ tube formation of *M. pulchra* conidium 100 h after inoculation of *C. florida* 'Cherokee Sunset' callus culture (400x).


A
B
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pgt
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gt
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H
E
F

(Plate 4-1C).

Appressorial formation and penetration also occurred on microshoots in Experiments 3 and 4. Scanning electron microscopy of microshoots from Experiment 3 verified that conidia not only germinated, but also penetrated the host and established a complete disease cycle by forming conidiophores bearing conidia (Plate 4-2A, B, C, D). In Experiment 3, there were 5 conidiophores observed between 7 microshoot leaves using SEM. Secondary hyphae on microshoots were extensive and covered large areas of the leaves in Experiments 3 and 4.

Experiment 5

Microshoots were transferred to fresh, sterile growth medium to avoid spread of contaminates. At 24 h after inoculation, germ tubes had not penetrated the leaf tissue. Therefore, the microshoots were not transferred to fresh medium until 48 h, when penetration was observed with the dissecting scope. Eighteen cultures were contaminated with fungi by 120 h, however 14 of the 20 cultures had secondary powdery mildew hyphae on leaves of the microshoots.

The two uncontaminated microshoots varied in age and surface morphology. One of these microshoots had young leaves that were folded and covered with several trichomes.

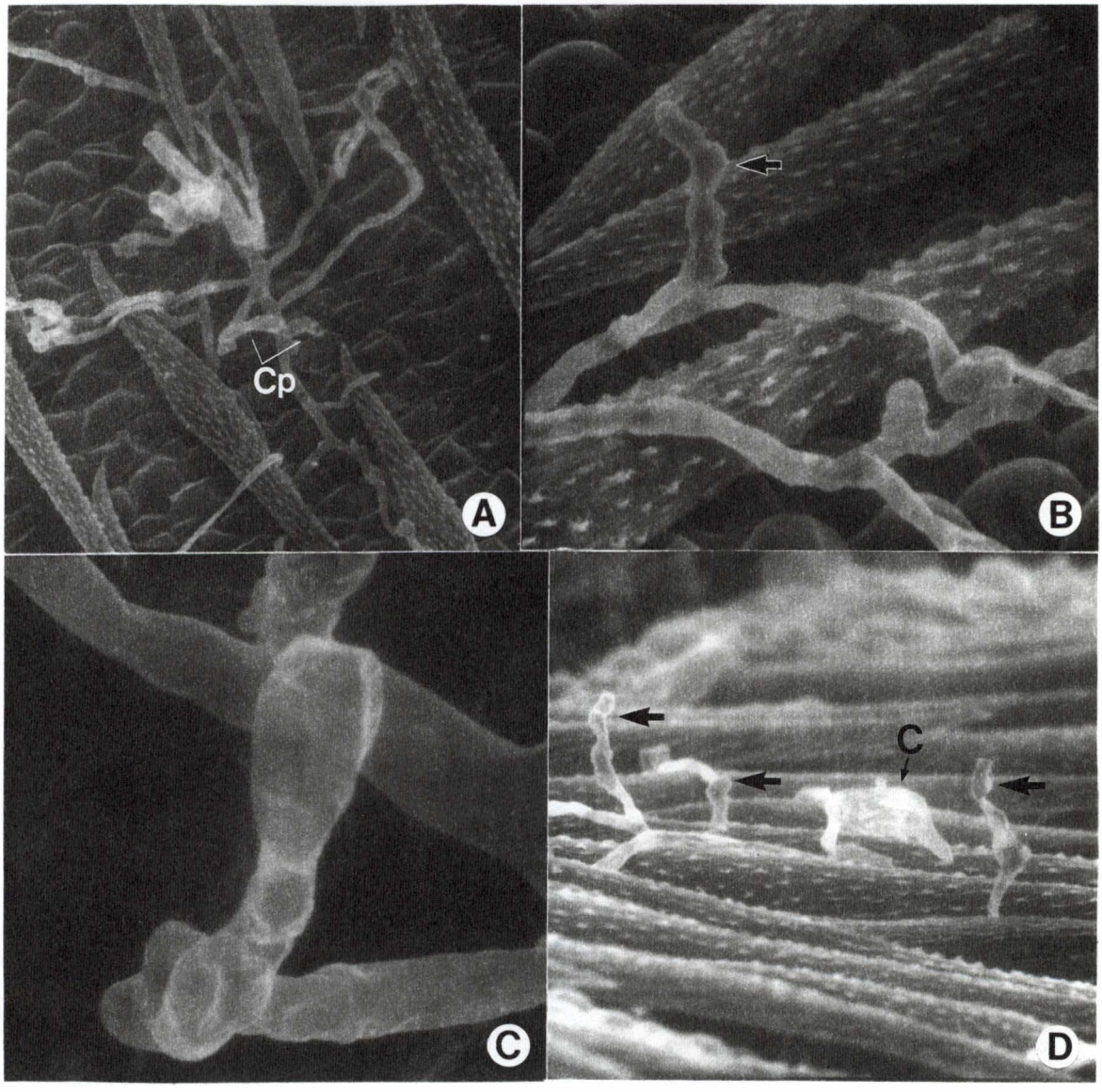
Plate 4-2. Scanning electron micrographs of conidiophore formation on the adaxial leaf surface of microshoots.

A. Conidiophore formation on the adaxial leaf surface of a microshoot (400x). Cp=conidiophore.

B. Immature conidiophore on the adaxial leaf surface of a microshoot (1200x). Arrow indicates conidiophore.

C. Closer examination of a conidiophore (2800x).

D. Three conidiophores formed near a conidium on the adaxial leaf surface of a microshoot (600x). Arrows indicate conidiophores, C=conidium.


Cp
A
B
C
C
D

The second uncontaminated culture had leaves that were unfolded and covered with less trichomes. Conidia germinated and penetrated the host tissue of both microshoots.

Fourteen of the 20 cultures had primary or secondary hyphae and 4 of the 20 had conidiophores on leaf surfaces by 144 h after inoculation. Powdery mildew colonies were considered established when conidiophores were present that abstricted conidia. At 7 days after inoculation, 168 h, the number of colonies were counted on each of the 4 cultures that produced conidiophores. Culture 1 had 15 distinct colonies, culture 2 had 5 colonies, culture 3 had 11 colonies, and culture 4 had 9 colonies of powdery mildew. These colonies had as few as 6 to as many as 23+ conidiophores.

One of the original uncontaminated cultures, culture 4, had a small area of contamination at 7 days, 168 h, after inoculation. At 7 days, other cultures had well established contaminates on the tissue and growth medium. At 174 h the contaminant was removed from culture 4 with a scalpel. The contaminant reappeared in culture 4 by the 10th day, 240 h, after inoculation. The contaminating fungus was removed, however it was again visible on leaves at 264 h. Twelve days after inoculation the only remaining sterile culture

had conidiophores on its surface, at this point the experiment was terminated.

Disseminated conidia resulted in prolific powdery mildew disease on the microshoot culture 4 (Plate 4-3A, B) and eventually on the remaining sterile culture. Large areas of the leaves were covered with conidiophores that produced chains of conidia up to 5 spores in length (Plate 4-3 Inset).

A scanning electron micrograph of a conidium deposited onto 'Cherokee Sunset' adaxial leaf surface reveals the same features as a micrograph of a conidium on tissue culture leaves. Conidia germinated to form a primary germ tube and appressorial germ tube on opposite corners of the conidium (Plate 4-4A). Appressorial swellings formed at the base of the appressorial germ tube, penetration occurred and primary, secondary hyphae were formed (Plate 4-4A, D). Hyphae ramified across the leaf surface to form many additional appressoria, and subsequent haustoria (Plate 4-4B, C, D; 4-5B, C).

Microsphaera pulchra colonies covered large areas of the microshoot leaves (Plate 4-5A; 4-6A). Conidial fragmentation from conidiophore generative cells were observed in all stages indicating a continuous production of conidia (Plate 4-6C).

Plate 4-3. Photographs of microshoots infected with powdery mildew disease.

A. Powdery mildew colonies establishment on microshoot leaves (12.5x). Arrows are pointing to sites of infection.

B. Closer examination of a powdery mildew colony on a microshoot leaf (35x). Note conidiophores.

Inset. *M. pulchra* conidiophores with chains of conidia formed on microshoot tissue (140x).

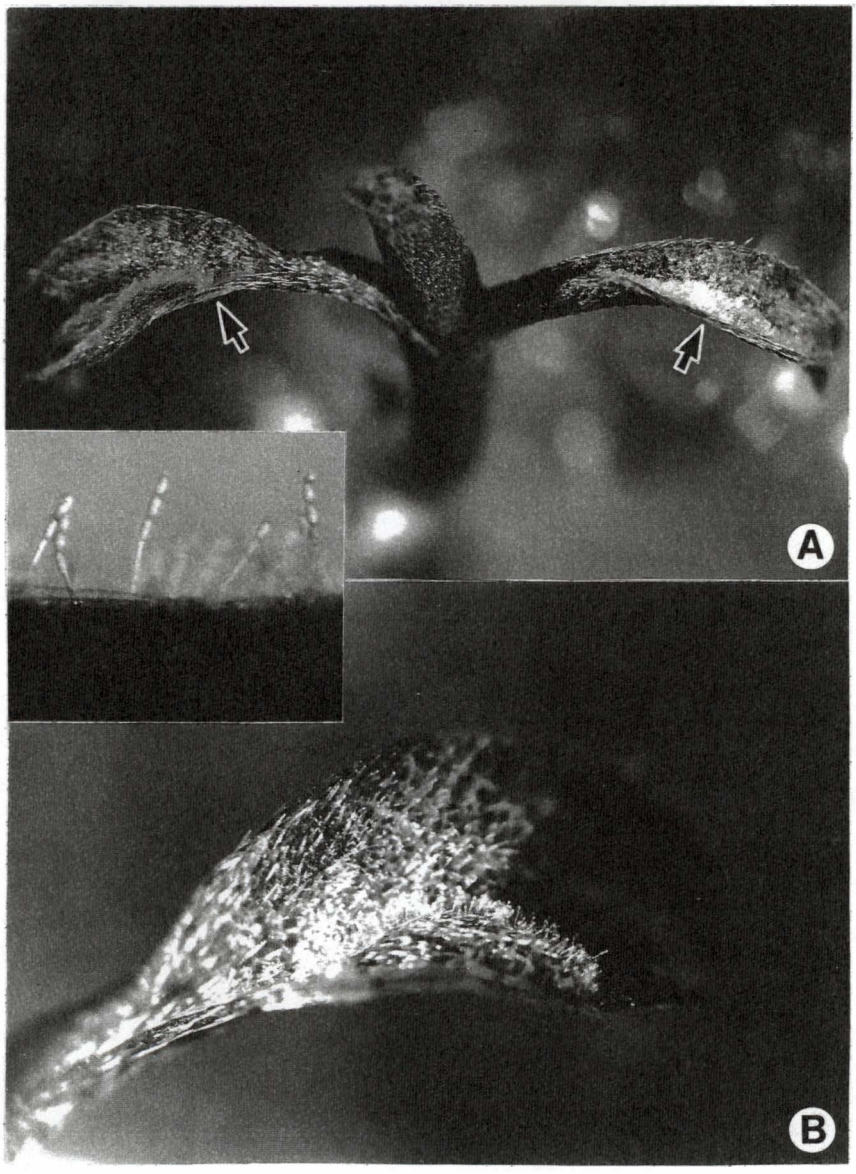

A
B

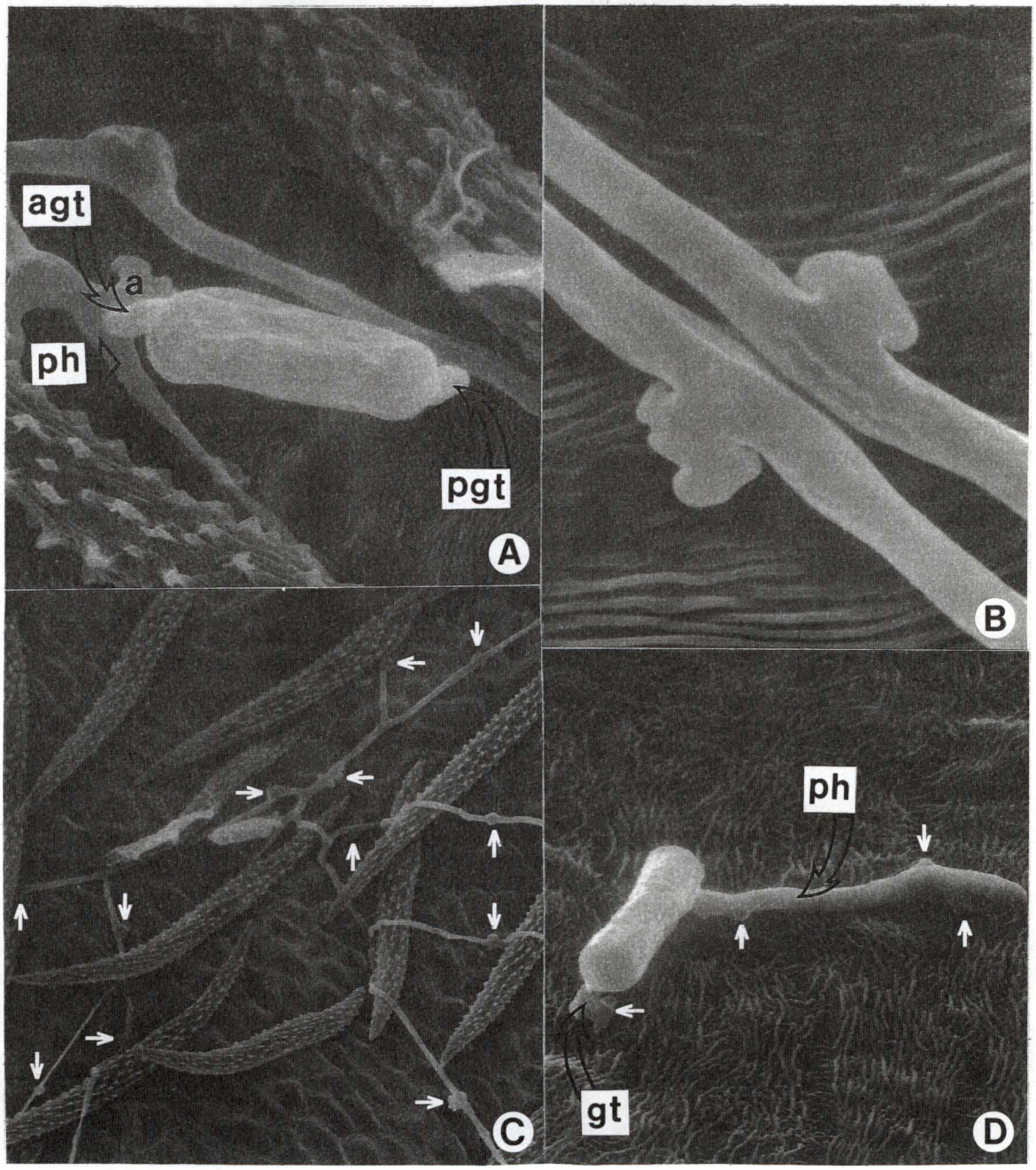
Plate 4-4. Scanning electron micrographs of conidial germination, appressorial formation, and hyphal ramification on the adaxial leaf surfaces of microshoots and *Cornus florida* 'Cherokee Sunset'.

A. Germinated *M. pulchra* conidium on the adaxial surface of a microshoot leaf (60x). pgt=primary germ tube, agt=appressorial germ tube, a=appressorium, ph=primary hyphae.

B. Appressorial formation from secondary hyphae on the microshoot leaf adaxial surface (3000x).

C. Extensive hyphal ramification on the adaxial surface of the microshoot, resulting from the germination of a *M. pulchra* conidium (300x). Arrows indicate numerous appressoria.

D. Germination, appressorial formation, primary hyphal formation, and subsequent appressorial formations of a *M. pulchra* conidium on the adaxial leaf surface of *C. florida* 'Cherokee Sunset' (960x). Arrows indicate appressoria, gt=germ tube, ph=primary hyphae.


agt
a
ph
pgt
A
B
C
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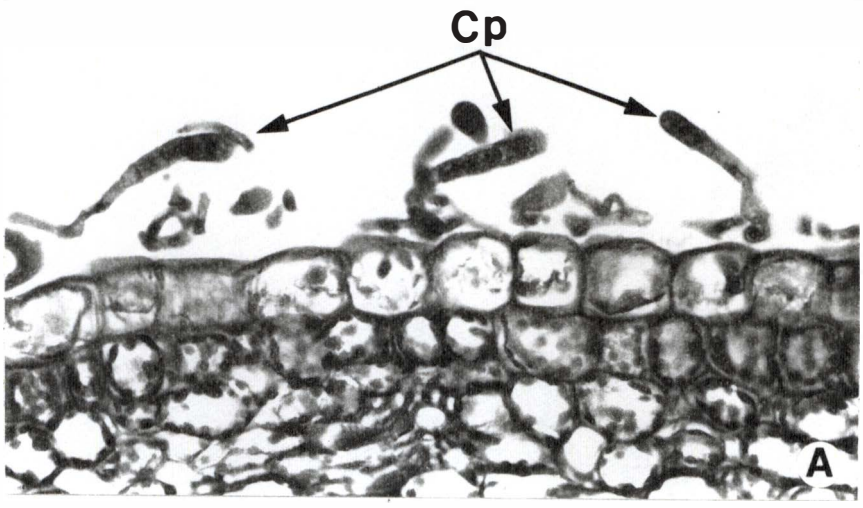
gt
D

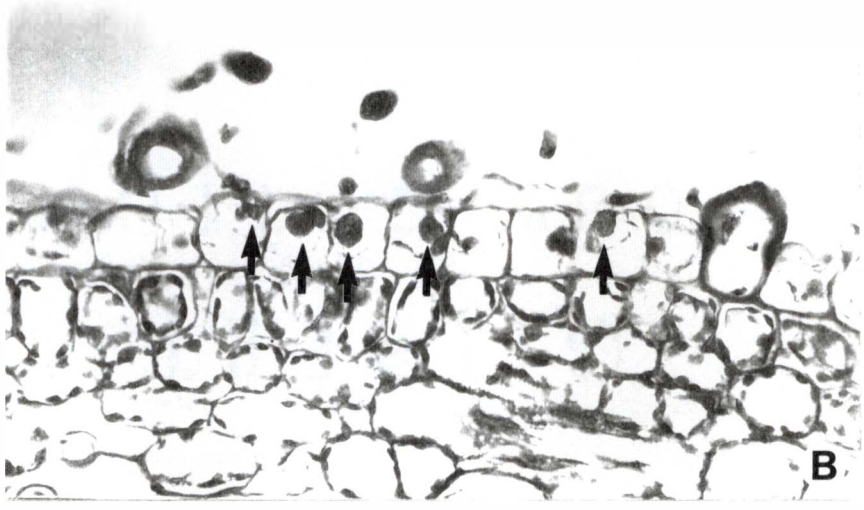
Plate 4-5. Stained sections of *Microsphaera pulchra* appressoria, haustoria, secondary hyphae, and conidiophore production on the adaxial leaf surface of microshoots.

A. Several conidiophores formed on the adaxial surface of a microshoot (400x). Cp=conidiophores.

B. Powdery mildew disease establishment due to multiple and frequent appressorial formations on the adaxial leaf surface of a microshoot (400x). Arrows indicate haustoria.

C. Hyphal ramification across the adaxial leaf surface of a microshoot (400x). h=hyphae, a=appressoria, H=haustorium.


Cp
A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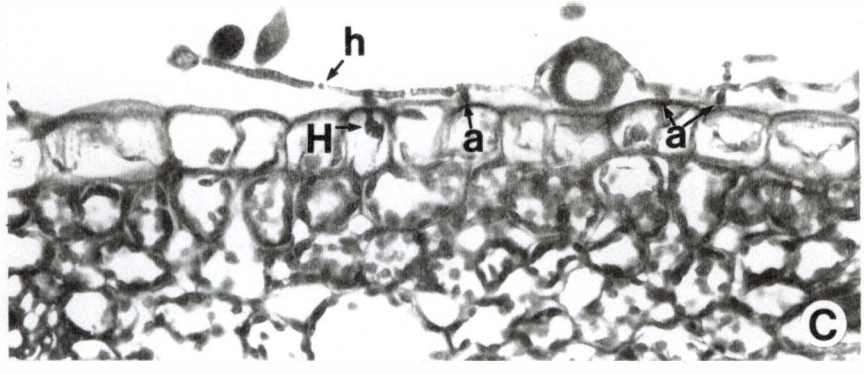
h
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C

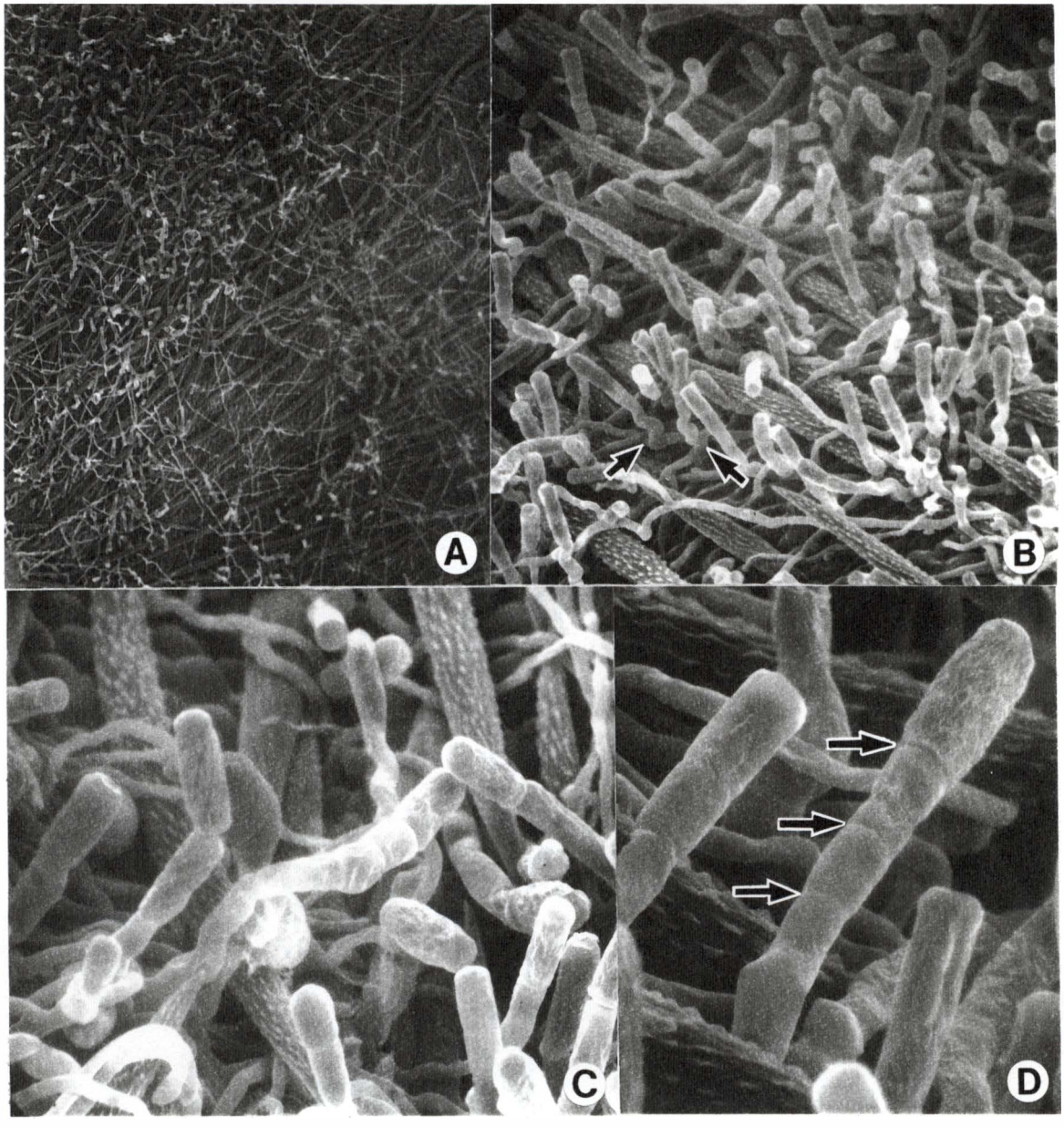
Plate 4-6. Scanning electron micrographs of powdery mildew colonies, conidiophores, conidial formation and abstriction on the adaxial leaf surface of microshoots.

A. Colony formation on the adaxial surface of a microshoot leaf (60x).

B. Conidiophores formed on the adaxial surface of a microshoot leaf (300x). Arrows indicate foot cells of conidiophores.

C. Conidiophores formed on the adaxial surface of a microshoot leaf (600x).

D. Conidiophores formed on the adaxial surface of a microshoot leaf (1200x). Arrows indicate septa separating cells of the conidiophore.


A
B
C
D

Examination of stained leaf sections with light microscopy, likewise revealed similar disease processes in microshoot cells and 'Cherokee Sunset' *in situ* cells. Both the conidia deposited on *in situ* and *in vitro* tissue germinated, formed haustoria, primary hyphae, and conidiophores (Plate 4-7A, B). Appressoria and haustoria necks were distinguishable in several of the sections of the microshoot leaves (Plate 4-7C). Multiple haustoria were formed in some cells of the microshoot leaves (Plate 4-7D). Conidial deposition, germination, haustorial formation, and hyphal ramification occurred on both leaf surfaces of the microshoots (Plate 4-7E).

4.4 Discussion

The presence of a water film on much of the callus culture probably prevented disease development. The surface of callus can vary from relatively dry to moist with a thin water film. Germ tube length is expected to be highly variable on or in a water film when compared to germination of conidia on leaf surfaces (Sivapalan 1993b). Conidial germ tubes formed in water were little more than short projections for some powdery mildew species. However, when *M. alphitoides* Griff. and Maubl., *M. berberidis* DC. ex

Plate 4-7. Stained sections of *Microsphaera pulchra* appressoria, haustoria, primary hyphae, and conidiophores on microshoots and *Cornus florida* 'Cherokee Sunset' leaves.

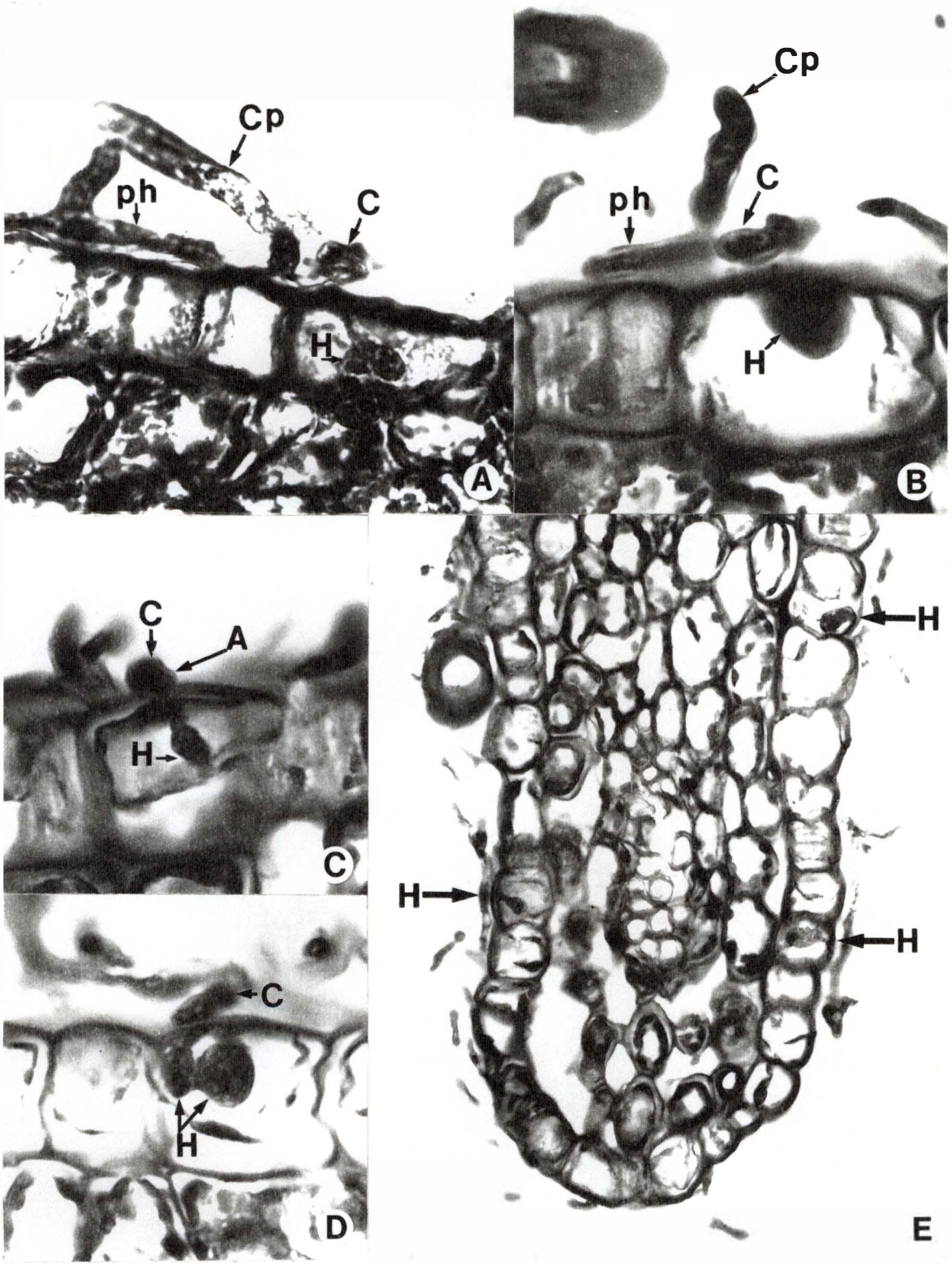
A. Germination, haustorial formation, primary hyphae and conidiophore production from a conidium of *M. pulchra* on the adaxial side of *C. florida* 'Cherokee Sunset' (1000x). C=conidia, H=haustorium, ph=primary hyphae, Cp=conidiophore.

B. Germination, haustorial formation, primary hyphae and conidiophore production from a conidium of *M. pulchra* on the adaxial surface of a microshoot leaf (1000x). C=conidium, H=haustorium, ph=primary hyphae, Cp=conidiophore.

C. Appressorial and haustorial formation of a *M. pulchra* conidium on the adaxial surface of a microshoot leaf (1000x). C=conidium, A=appressorium, H=haustorium.

D. Formation of two haustoria in the same microshoot cell on the adaxial surface of a microshoot leaf (1000x). C=conidium, H=haustoria.

E. Microshoot leaf tip with powdery mildew infection on the adaxial (right side) and abaxial (left side) surfaces (400x) magnification. H=haustoria.


Cp
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E

Merat (Lev.), *M. euonymi* (DC. ex Merat) Sacc. and *Oidium* spp. were tested for germination in the presence of water, long germ tubes were produced (Sivapalan 1993b). In this study, *M. pulchra* germ tube length varied from extensive to short projections on dogwood callus tissue. Powdery mildew conidia produce single or multiple germ tubes in water (Silvapalan 1993b), which also occurred on callus cultures in this study.

Appressorial formation may vary according to position of conidia with water films (Silvapalan 1993b). Conidia on water typically never produce appressoria, whereas conidia in water produce appressorium-like structures (Silvapalan 1993b). Appressoria formed in this study were from conidia submerged in or lying on a water film. The position of appressoria formed along the germ tube varied in this study, similar to formation in Silvapalan's (1993b) study of conidial germination in the presence of water.

Conidia may become non-viable with extended exposure to moisture. Powdery mildew conidia do not entirely lose their ability to establish a parasitic relationship with their host when they are in water for a short time (Perera and Wheeler 1975). When leaves become wet from rain or dew, conidia may infect cells more slowly after the leaves become dry again than if the conidia were never in the presence of

water. Conidia may be rendered non-viable or germinate abnormally when kept in water for extended periods of time (Perera and Wheeler 1975). The surface of callus cultures never became completely dry, therefore the conidia were continuously exposed to moisture. This could partially explain the lack of appressorial development from most conidial germ tubes.

Lack of chlorophyll in callus cells could also contribute to the absence of a normal host-pathogen relationship. Growth of germ tubes towards leaf cells may be the result of positive phototropism for green light, reflection of chloroplasts in the cell (Yarwood 1932, 1957). Since dogwood callus cells do not contain chlorophyll, negative phototropism may contribute to the explanation of why germ tubes often grew away from the cell surface. Appressorial formation is dependent somewhat upon contact stimuli (Yarwood 1932), therefore infection would not occur if the germ tubes were reaching away from the callus cells, as often occurred in these experiments.

Callus tissue does not appear to be an effective substrate for establishment of *M. pulchra* in culture. The presence of a water film on the surface and the lack of chlorophyll may deter the initial infection processes.

Leaf surface morphology did not appear to prevent *M.*

pulchra from infecting dogwood microshoots. Extensive coverage of leaf surfaces with trichomes would seem to inhibit penetration of germ tubes. A wide range of coverage was represented in Experiment 5, but tissue covered with large amounts of trichomes eventually became diseased. Penetration and hyphal ramification occurred on most cultures in Experiment 5, further illustrating the similarities of the *in vitro* and *in situ* host-pathogen relationship. However, trichome density did not appear to obstruct conidiophore development or conidia dissemination.

Altering the light cycle from 10 h of light to 15 h appeared to cause an increase in conidiophore production. In Experiment 5, many more conidiophores were produced and the mycelial mats covered a larger surface area than previous experiments. This would be expected since the inoculum was obtained from trees grown under a 15 h light/9 h dark cycle.

Tissue organization and cellular structure of microshoots were adequate enough to allow establishment of a normal disease relationship between *M. pulchra* and the host. Disease initiation and progression were compared to *in situ* 'Cherokee Sunset' leaves inoculated in the greenhouse. Conidiophores developed and appeared morphologically similar to those on *in situ* tissue (Braun

1987). Fully developed conidiophores usually contained three cells in addition to a mature conidium prior to dissemination: a foot cell, or basal cell, and a generative cell that divided into 1-2 conidium initials (Yarwood 1936, Braun 1987) (Plate 4-5B,D). Conidial germination, appressorial formation, haustorial formation, primary and secondary hyphae, and subsequent appressorial formation, all appeared normal when compared to *in situ* disease processes.

Contaminants were observed at 120 h, 5 days, after inoculation. *Alternaria* Nees, nom.cons., *Botrytis* P. Mich. ex Pers., *Candida* Berkhout, nom. cons., *Cephalosporium* Corda., *Cladosporium* Link:Fr., or *Trichothecium* Link:Fr., commonly grow within the mycelial colonies of powdery mildews (Braun 1987). *Alternaria* and *Botrytis* species were the primary contaminates of the microshoots. Contaminating fungi were often first seen at the base of the microshoot or growing on the medium, and afterwards, infesting the leaves.

Due to the high rate of contamination, 90% in Experiment 5, and the possible association of powdery mildew with other fungi, greenhouse-grown inoculum would not be a suitable sole source of conidia. However, even if pure powdery mildew colonies could be established in 10 or 20% of inoculated microshoots, these cultures could be used to inoculate other microshoot cultures. Likewise, even if a

culture became contaminated, leaves with only powdery mildew infection could be removed from the microshoot and used in inoculations. Another method of obtaining clean cultures, as in culture 4 Experiment 5, would be to remove the contaminate with a scalpel until infection is established on microshoots. At this point, the microshoot could be trimmed to the axillary buds in attempt to obtain and maintain pure isolates.

Once a method is determined to establish pure powdery mildew colonies, tissue culture could possibly be used to screen species and cultivars for resistance. Dogwood resistance to powdery mildew must first be determined as a cellular response or a morphological defense. For example, if resistance of 'Cherokee Brave' is attributed to a thicker cuticle layer, callus cultures and possibly microshoots would not reveal the resistance. If the resistance is at a cellular level, microshoots would possibly allow accurate screening. A comparison of cellular responses of tissue cultures of susceptible and resistant cultivars to powdery mildew, and *in situ* leaves would determine if microshoots are an appropriate testing material (Daub 1986).

Powdery mildew colonies grown on tissue cultured plants promises to be an effective means to obtain and maintain pure inoculum. Of the two techniques studied, microshoot

infections simulated *in situ* disease processes the most accurately. The colonies would remain viable on the microshoots for at least 2 wk and possibly longer if the culture could remain free of contaminants. Microshoot leaves, and therefore apical meristems, must be removed once a month to stimulate axillary bud development into new shoots. In Experiment 5, infection occurred on stems and buds, along with leaves. Therefore, if leaf trimming could be postponed until infection is established in shoots, infection could continue in the culture. Callus tissues would provide a substrate that is easier to maintain than microshoots. As the callus continues to grow, it could be divided along with the powdery mildew colonies. Further research should be conducted, with both callus and microshoots, in an attempt to establish pure cultures of powdery mildew in the laboratory.

The need for a continuously available, sterile inoculum to conduct research of powdery mildew disease of dogwoods has led to attempts to infect tissue cultures with the obligate parasite. This study indicates that microshoots, derived from axillary bud proliferation, would be an ideal substrate to maintain *M. pulchra* inoculum under controlled conditions. However, methods must be established to ensure

a greater frequency of sterile cultures after inoculation with conidia.

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VITA

Leigh Ann Klein was born in Winchester, Kentucky on January 15, 1973. She graduated from George Rogers Clark High School in May, 1991 and entered Eastern Kentucky University the following semester after receiving a Regents Scholarship.

While attending EKU, Leigh Ann worked in the university's herbarium and was active in the Sierra Club, Wildlife Society, and Habitat for Humanity programs. She was a member of Phi Sigma and received the Outstanding Sophomore Award and Henry LaFuze Scholarship. Leigh Ann received her B.S. degree in biology in December, 1995, graduating Summa Cum Laude.

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