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# Histological investigation of infection processes of Discula destructiva on Cornus florida leaves and influence of pH on growth of Discula destructiva and an undescribed Discula species

Bryan R. Cody

To the Graduate Council:

I am submitting herewith a thesis written by Bryan R. Cody entitled "Histological investigation of infection processes of Discula destructiva on Cornus florida leaves and influence of pH on growth of Discula destructiva and an undescribed Discula species." I have examined the final electronic 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:

E.T. Graham, J.W. Hilty, R.N. Trigiano

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Associate Vice Chancellor
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# HISTOLOGICAL INVESTIGATION OF INFECTION PROCESSES OF *Discula destructiva* ON *Cornus florida* LEAVES AND INFLUENCE OF pH ON GROWTH OF *Discula destructiva* AND AN UNDESCRIBED *Discula* SPECIES

A Thesis

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Master of Science

Degree

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Bryan R. Cody

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**ABSTRACT**

The infection process and colonization of *Discula* species in greenhouse grown *Cornus florida* L. leaves were studied histologically. Penetration of *Discula destructiva* Redlin isolate (TN 1) hyphae through natural openings, wounds, or directly through the leaf surface was not observed. Infection, detected by fungal colonization of leaf tissue, occurred between one and eight days post-inoculation in wounded and non-wounded leaves inoculated with TN 1 conidia. Fungal colonization was not observed in wounded or non-wounded leaves misted with water (controls). Leaves inoculated with the *D. destructiva* isolate and an undescribed *Discula* species isolate (NC 2) developed symptoms of dogwood anthracnose after two wks. Disease symptoms induced by both isolates were similar to those previously described for dogwood anthracnose caused by *Discula destructiva*. This is the first report that the undescribed *Discula* sp. is pathogenic on *C. florida*.

The influence of pH on the growth of three *D. destructiva* isolates (GA 1, TN 1 and MA 11) and an undescribed *Discula* species isolate (VA 17B) in buffered liquid culture media was studied. Citrate-phosphate buffer was used to adjust culture media to 4.0, 5.0, 6.0 or 7.0. The pH of the media remained at the initial level two wks after inoculation with the fungus. Growth of the

undescribed *Discula* sp. isolate and *D. destructiva* isolate (GA 1) decreased significantly from pH 4.0 to 7.0; whereas, *D. destructiva* isolates (MA 11 and TN 1) grew at similar rates regardless of the pH of the growth media.

## TABLE OF CONTENTS

## LIST OF TABLES

**TABLE** **PAGE**

## LIST OF FIGURES

# CHAPTER I

## HISTOLOGICAL INVESTIGATION OF INFECTION PROCESSES OF *Discula destructiva* ON *Cornu florida* LEAVES

### i. INTRODUCTION

Native flowering dogwood (*Cornus florida* L.), a small bushy tree reaching 40 feet in height, is found throughout the eastern United States (3,14). In the forest understory, *C. florida* provides nutrition for over 50 wildlife species (30). It is commonly used as an ornamental in urban landscapes and is the most important nursery-grown plant in Tennessee (3,29,30).

Decline of *C. florida* was first reported in the northeastern United States in the late 1970s and *Colletotrichum gloeosporioides* Penz. & Sacc. was suggested as the causal agent (21). In 1983, the disease was attributed to infection by a *Discula* species and other environmental phenomena (11). By 1988, *Discula* sp. was demonstrated as the cause of dogwood anthracnose by the fulfillment of Koch's postulates (17) and described as *Discula destructiva* Redlin sp. Nov. (22). The fungus naturally causes disease of *C. nuttallii*, *C. florida* and *C. kousa* in North America (22). Disease symptoms include leaf spots and blights, dieback of twigs and branches, proliferation of epicormic shoots, and stem cankers

(3,12,22).

When *Discula* spp. isolates were cultured on solidified potato dextrose medium, two distinctly different colony types were observed (10). Isolates designated as *D. destructiva* (type 1) had colony margins that were irregular; whereas, the uncharacterized *Discula* species (type 2) isolates grew in concentric circles. Trigiano *et al.* (26) demonstrated that most isolates of *D. destructiva* oxidized gallic acid; whereas, the undescribed *Discula* sp. isolates did not produce catechol oxidase. Comparison of DNA from both species of *Discula* showed very few polymorphisims between isolates of *D. destructiva* and a greater number of polymorphisims between isolates of the undescribed *Discula* sp. (26).

Interactions between *Discula* spp. and *C. florida* have been investigated by the use of light microscopy (22,28) and scanning electron microscopy (5,6,22,23,). Evidence of direct penetration of dogwood leaves or indirectly through natural openings not been reported. Wounding leaf tissue prior to inoculation with *Discula* spp. increased rate of lesion development (8,10,23,25). Therefore, objectives were the following: 1) to determine the method(s) of penetration and invasion of greenhouse grown *C. florida* leaves by *D. destructiva* and 2) to determine if mechanically wounded leaves are more susceptible to infection by *Discula* spp. than non-wounded leaves.

## ii. MATERIALS AND METHODS

**Inoculum preparation.** Isolates of *D. destructiva* (TN 1) and an undescribed *Discula* sp. (NC 2) were cultured on Difco potato dextrose V-8® juice agar (13). After sporulation was observed, (approximately one month for TN 1 and 2 wk for NC 2), conidia were collected by submerging cultures in sterile distilled water and rubbing the top of the cultures with a sterile rubber policeman. Conidial suspensions were adjusted to contain 8 x $10^4$ spores per ml and poured into Nalgene® atomizers.

**Tree conditioning.** Ten young potted *C. florida* trees (ca. 100 cm tall) were acclimatized at 23 C for 1 wk in a greenhouse incubation chamber designed to simulate forest conditions in early spring. The chamber consisted of a wooden frame (437 x 141 x 118 cm) encased in two sheets of transparent polyethylene film with 5 cm of air space between them. A shade cloth, transmitting 20% of available greenhouse light, covered the entire chamber. The chamber was equipped with an air conditioner to maintain an ambient temperature of 23 C and a humidifier to increase relative humidity within the chamber.

**Tree inoculations.** Following the acclimatization period, leaves were misted to run-off with conidial suspensions (5 trees/isolate type) or sterile water controls with the following treatment conditions: wounded leaves misted with sterile water (WW), non-wounded leaves misted

with sterile water (NWW), wounded leaves misted with TN 1 spores (WTN), non-wounded leaves misted with TN 1 spores (NWTN), wounded leaves misted with NC 2 spores (WNC), and non-wounded leaves misted with NC 2 spores (NWNC). Each treatment (ca. 8 leaves/bag) was randomly placed on a different branch (4 branches/tree).

Prior to wounding, leaves were marked with water proof india ink circles (2.5 cm in diameter) on the adaxial surface at random locations on the margin and away from the margin (6 circles/branch). Leaves were washed with a hand held spray bottle containing tap water and then allowed to dry. Wounding was accomplished by lightly pressing a leaf, within the ink circle, between a cork stopper layered with #1 insect pins and a flat surface of a larger cork stopper (Fig. 1). The insect pin-covered surface of the puncturing tool was pressed to the adaxial surface of the leaf. Following treatments, trees were returned to the incubation chamber, and temperature was maintained at 23 C and the humidifier was run constantly. Leaves inside the bags were observed for 5 wk to monitor symptoms.

**Tissue preparation.** Two discs, 11 mm in diameter, were randomly excised from a representative of each treatment at 1, 2, 4, 8 and 16 days post-inoculation. Discs were taken from both the internal and marginal areas of the leaf.

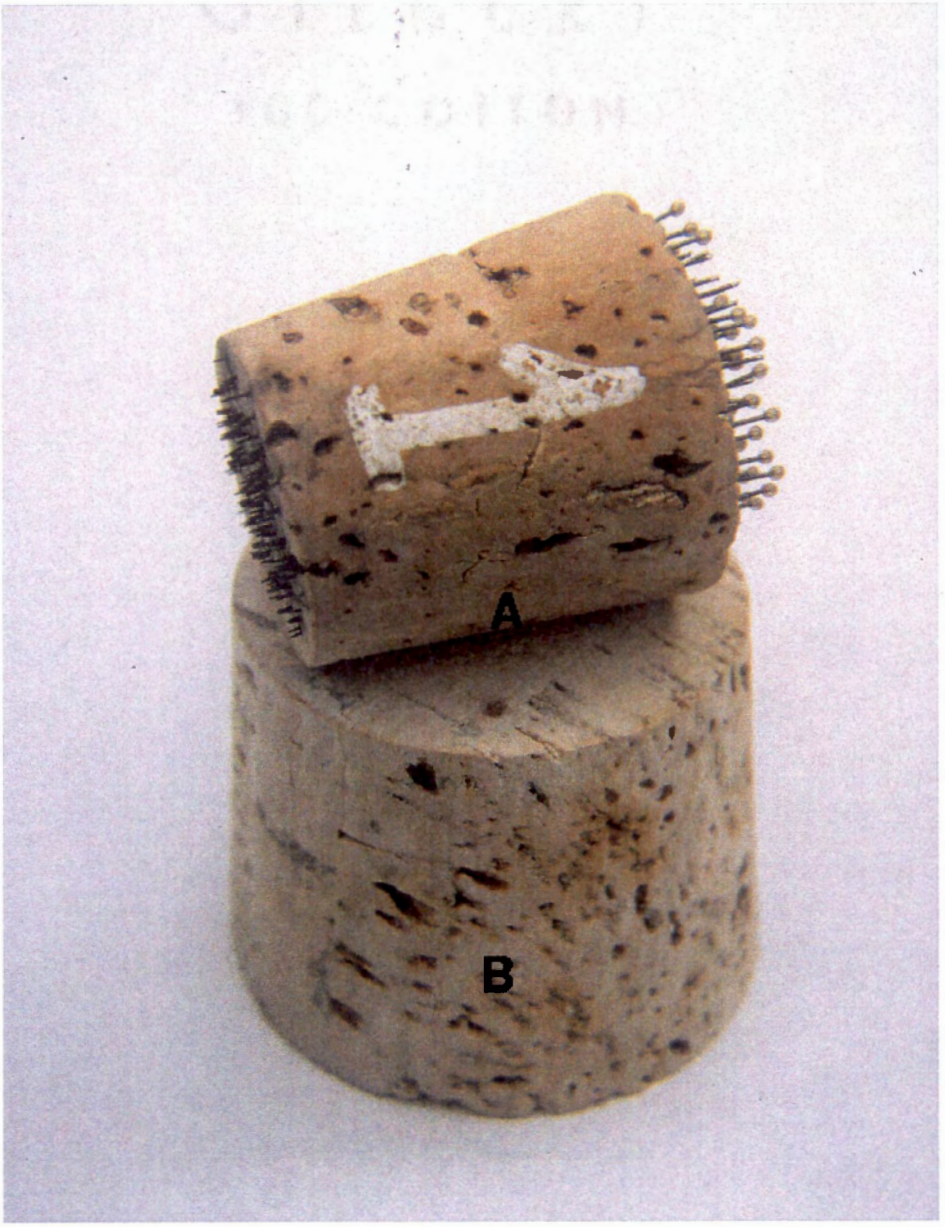



**Fig. 1.** Wounding tool: (A) Cork stopper with #1 insect pins used to wound adaxial leaf surface and (B) larger cork stopper used to support abaxial leaf surface while wounding.

Discs were trimmed into smaller rectangular pieces (ca. 7 x 3 mm) and immediately placed in bottles containing formalin, acetic acid, and 50% ethanol. Some samples were cut specifically to include the leaf margin. Leaf sections were fixed in the FAA for 6 days, dehydrated in Johansen's *t*-butyl alcohol series (18), and embedded in paraffin (Paraplast® Oxford Labware St. Louis, MO 63103). Serial cross sections, 10-$\mu$m thick, were cut using a rotary microtome. Slides were prepared by detergent washing and distilled water rinsing. Cross sections were expanded on a puddle of distilled water at 50 C, and affixed to glass slides, without adhesive, by draining off water and drying for 12 h at 40 C. Sections were deparaffinized through three changes of Microclear™ (Micron Diagnostics, Inc., Baltimore, MD) for 15, 10, and 5 min, respectively. Sections were rehydrated by moving slides through three 5 min rinses in absolute ethanol, 5 min each in 95, 85, 70, 50, and 30% ethanol, and two deionized water rinses for 5 min each. The staining series consisted of 0.01% aqueous safranin O for 24 hr, followed by two 1 min deionized water rinses, Graham's quick-mixed hematoxylin (15) for 10 min, followed by 5 and 1 min deionized water rinses, respectively, 0.1% aqueous alcian blue for 18 min, followed by 3 and 1 min deionized water rinses, and 0.01% aqueous Bismark brown for 3 min, followed by 3 and 1 min deionized water rinses. Sections were dehydrated using a graded

series of ethanol in reverse order of the rehydration series previously described. Following the dehydration series, sections were rinsed in Microclear™ for 10 min and transferred to fresh Microclear™ for 24 hr. Coverslips were attached to slides with Eukitt® (Calibrated Instruments, Inc. Hawthorne, New York) and slides were dried for 24 hr at 40 C. Sections were examined with an Olympus BHS light microscope (4 Nevada Drive, Lake Success, NY 11042-1179, USA) and photomicrographs were taken with a Olympus PM-10AD camera system containing Ektachrome 64 T professional color reversal film.

## iii. RESULTS

The quadruple stain described above, resulted in contrast between fungal and host tissues. Hematoxylin stained conidia and hyphae light to dark purple. Chloroplasts were stained light to bright red by safranin O. Alcian blue stained cell walls blue and the cuticle was stained amber by Bismark brown.

Penetration of *D. destructiva* hyphae through natural openings, wounds, or directly through the leaf epidermis was not observed. Hyphae were seen on the adaxial leaf surface two days post-inoculation (Fig. 2A). At eight days post-inoculation, hyphae were aggregated between the cuticle and epidermis and grew intracellularly (Fig. 2B) and intercellularly (Fig. 3A) in vascular tissue, epidermal

**Fig. 2.** Sections of *Cornus florida* leaves inoculated with *Discula destructiva* conidia. (A) Hypha (h) on adaxial surface at two days post-inoculation and compartmentalized chloroplasts (arrows) in non-infected palisade parenchyma cells (1000x), (B) Hyphae (Arrows) eight days post-inoculation growing intracellularly in vascular tissue (v), epidermal cells (e), palisade parenchyma (pp), and spongy mesophyll cells (sp) (400x): Note aggregated (ag) hyphae between cuticle (c) and epidermal cells.

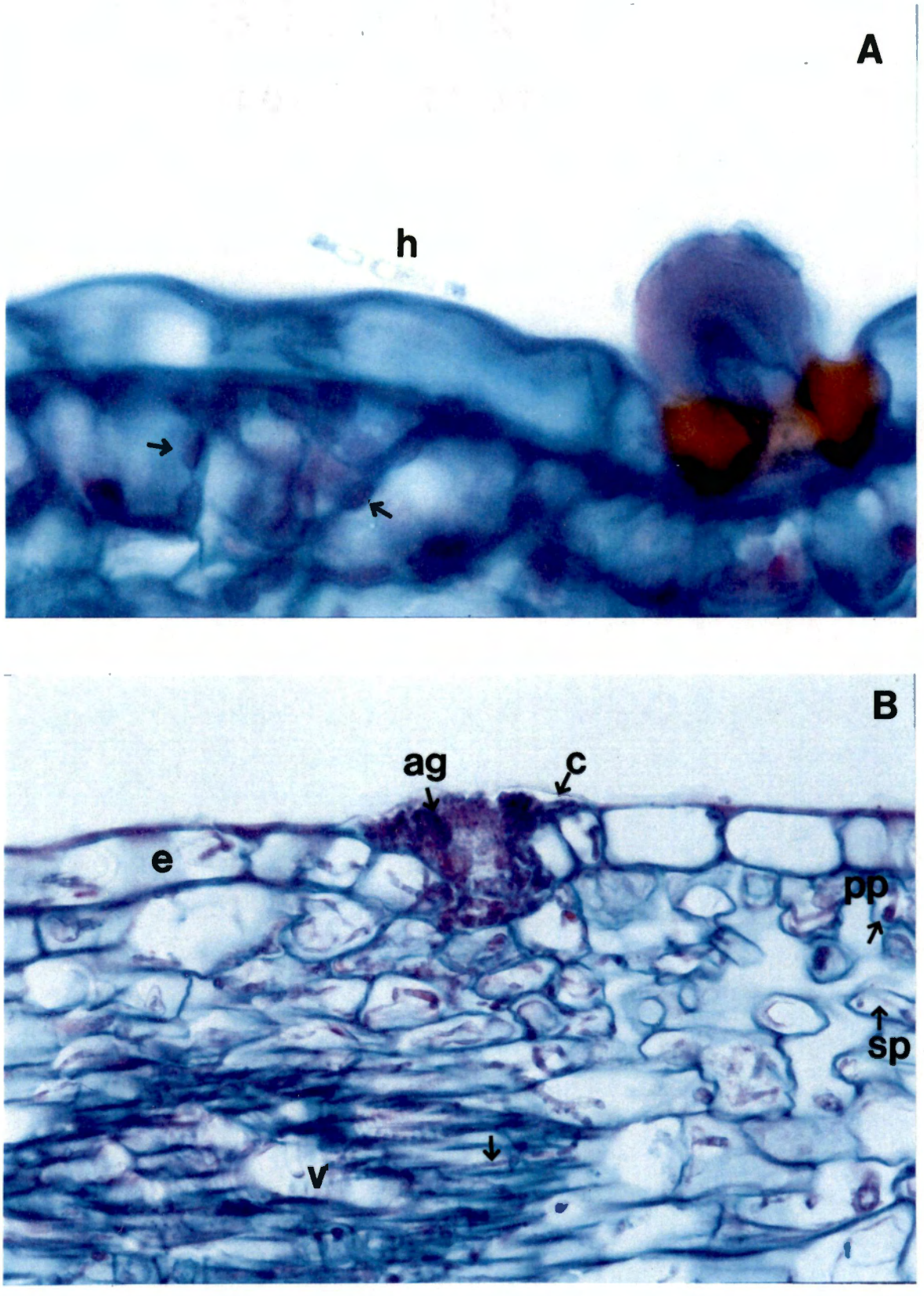
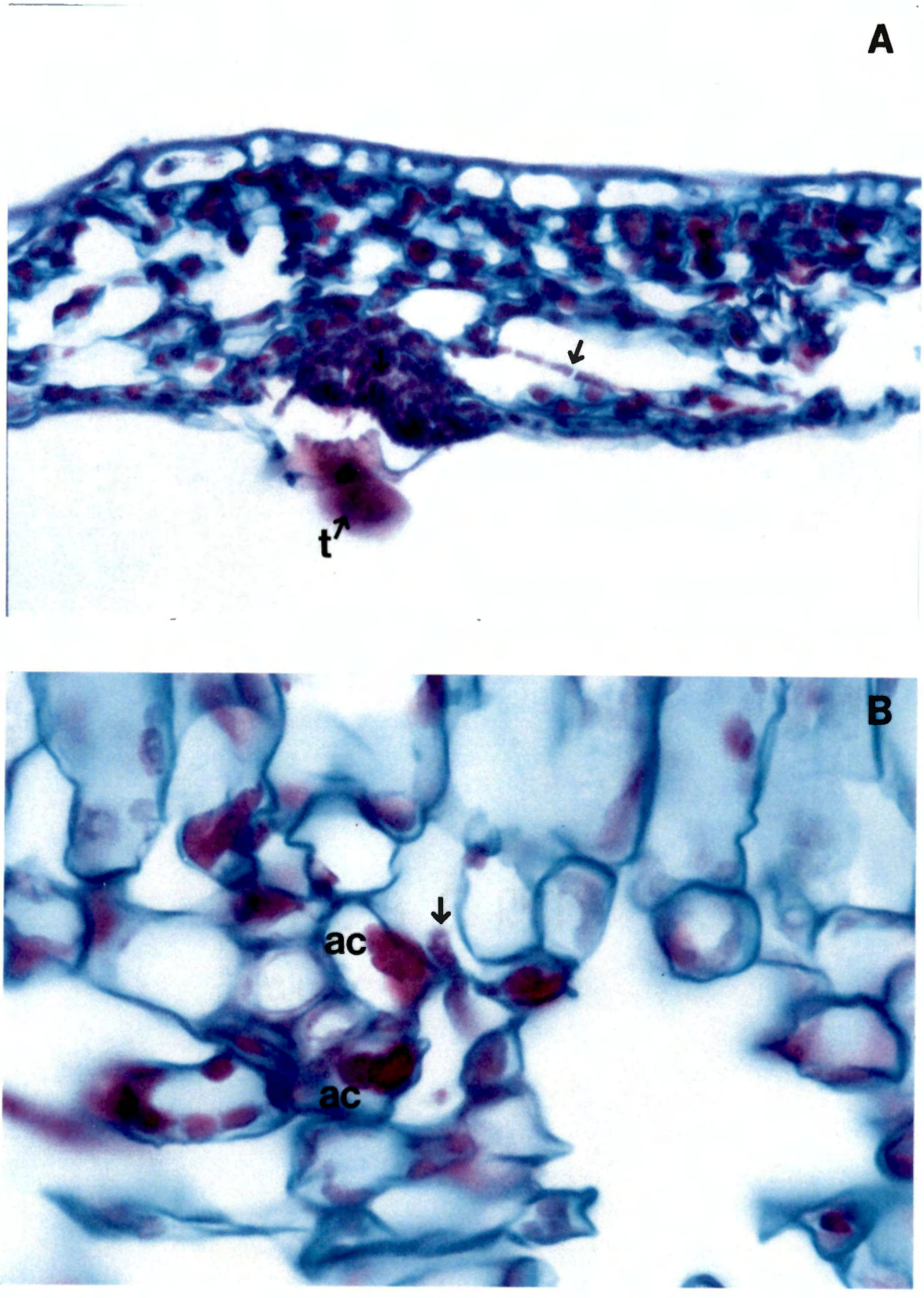

A
h
B
ag
c
e
pp
sp
v

**Fig. 3.** Sections of two wks post-inoculation *Discula destructiva* infected *Cornus florida* leaves. (A) Intracellular hyphae (arrow) and hyphae aggregating under trichome (t) (400x), (B) Hypha growing through two adjacent spongy mesophyll cells (1000x): Note decompartmentalized chloroplasts (ac) near hypha.


A
t
B
ac
ac

cells, palisade parenchyma, and spongy mesophyll cells (Fig. 2B and 4A). Chloroplasts, within cells that had no hyphae near them, were intact and stained from light to bright red (Fig. 2A). Whenever hyphae were associated with cells containing chloroplasts, the cytoplasm was stained bright red (Fig. 3B and 4A). Hyphae were observed near or inside most decompartmentalized cells. Acervuli were fully developed at 16 days post-inoculation (Fig. 4B), and were observed on the adaxial and abaxial leaf surface. Sporulation was first observed 16 days post-inoculation. Infection was observed in wounded and non-wounded leaves as well as on and away from leaf margins.

In the first trial, symptoms and signs of dogwood anthracnose were observed in 80% of the treatments where leaves were wounded and inoculated with spores from both TN 1 and NC 2 (Table 1) whereas, the TN 1 and NC 2 inoculated leaves that were not wounded developed signs and symptoms 60 and 40% of the time, respectively. In the second trial, *Discula* conidia were only recovered from the TN 1 inoculated leaves. The non-wounded and wounded TN 1 inoculated leaves for the second experiment were infected 40 and 20% of the time, respectively. (Table 1). Signs and symptoms of *Discula* spp. infection were found at and away from leaf margins. Control leaves did not develop symptoms of dogwood anthracnose (Table 1).

**Fig. 4.** Sections of two wks post-inoculation *Discula destructiva* infected *Cornus florida* leaves. (A) Hyphae in epidermal cells and bright red stained palisade parenchyma (pp) that are typical of diseased tissue (100x), (B) Fully developed acervulus (ac) with conidia (arrows) (400x).

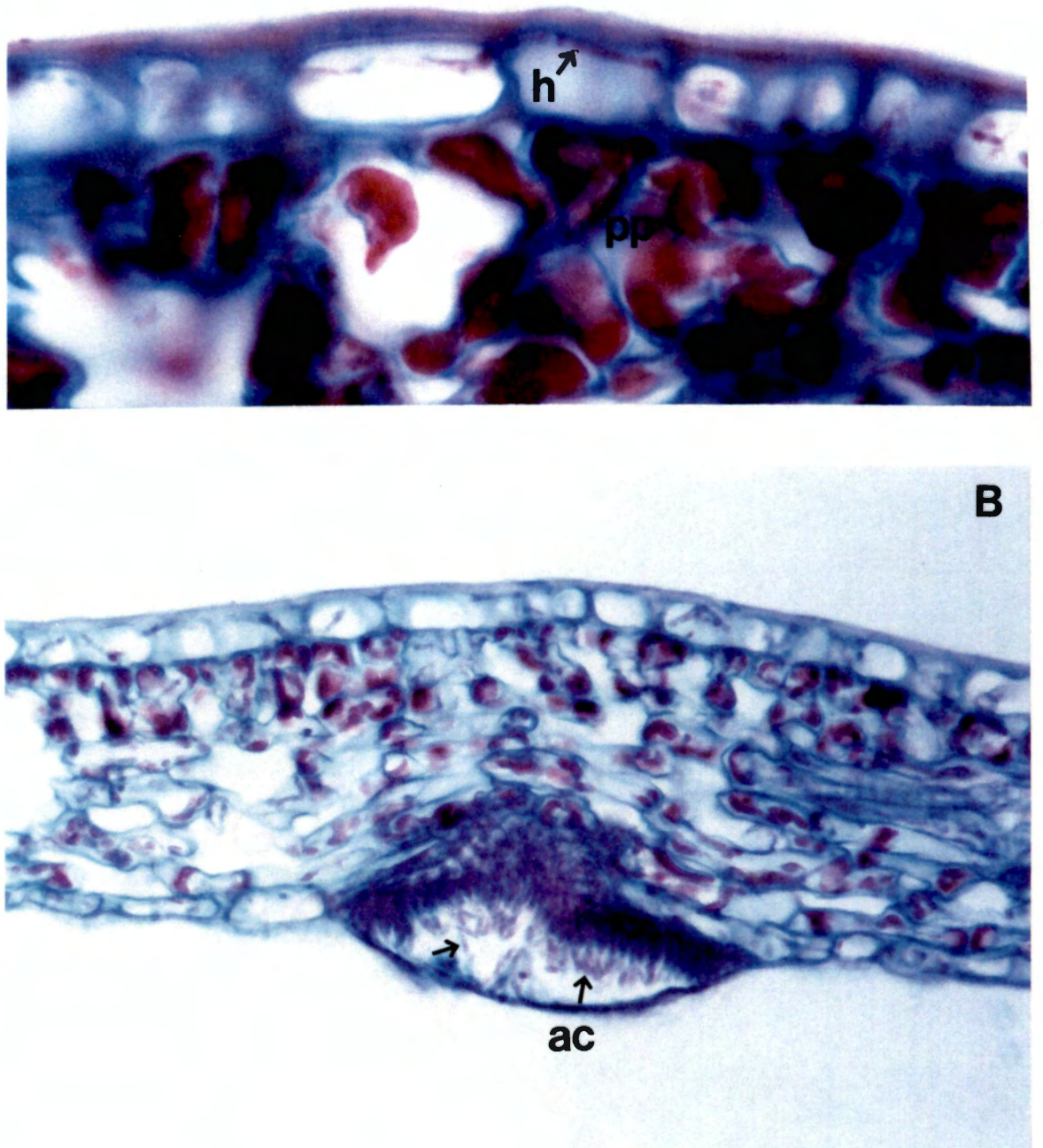

A
h
pp
B
ac

**Table 1.** Percent of two-year-old *Cornus florida* trees infected under two treatments (non-wounded and wounded) with a *Discula destructiva* isolate and an undescribed *Discula* species isolate.

| Treatments | | % Infected | |
|---|---|---|---|
| | | Experiment 1 | Experiment 2 |
| Isolate | TN 1 | | |
| | Non-wounded | 60 | 40 |
| | Wounded | 80 | 20 |
| Isolate | NC 2 | | |
| | Non-wounded | 40 | 0 |
| | Wounded | 80 | 0 |
| Control | | | |
| | Non-wounded | 0 | 0 |
| | Wounded | 0 | 0 |

## iv. DISCUSSION

Dilution of conidia necessary for germination may be too low to allow for observation of spores in cross section because of low spore frequency in 10-$\mu$m thick sections. Also, most of the conidia may wash to the distal portion of the leaf thus leaving lower concentrations in areas we observed. As hyphae became established across the surface of the leaf, chances of sectioning through them would be increased. This may explain why the first sign of the fungus being present was not until two days post-inoculation. Our results were similar to Redlin's (23), in that infection was observed within 8 days after inoculation.

Four toxins have been isolated from culture filtrates of *Discula* isolates from infected dogwoods (29). These toxins may be important in the pathogenesis of *D. destructiva* as evidenced by chloroplasts being damaged in advance of hyphal contact with cells containing chloroplasts.

Walkinshaw and Anderson (28) also observed that hyphae aggregated near epidermal cells prior to sporulation and these aggregations were usually beneath the base of trichomes. The undescribed *Discula* sp. and *D. destructiva* acervuli were mostly formed at the bases of trichomes and were observed in necrotic and living tissue. Signs and symptoms induced by the undescribed *Discula* sp. were similar

to those of *D. destructiva* as described by Redlin (22). This is the first report that the undescribed *Discula* sp. is pathogenic on *C. florida* and Koch's postulates were completed.

## CHAPTER II

## INFLUENCE OF pH ON *Discula destructiva* AND AN UNDESCRIBED *Discula* SPECIES GROWTH

### i. INTRODUCTION

Flowering dogwood (*Cornus florida* L.), a small tree reaching 40 feet in height, is native to the eastern United States (3,14). Foliage and berries of *C. florida* provide nutrition for over 50 wildlife species (30). Flowering dogwood is also a valued ornamental tree species and is often used in urban landscapes (4,30).

Flowering dogwood was reported to decline in the northeastern United States in the late 1970s and *Colletotrichum gloeosporioides* Penz. & Sacc. was suggested as the causal agent (21). In 1983, the disease was attributed to infection by a *Discula* species and environmental phenomena (11). By 1988, Koch's postulates were carried out and proved that *Discula* sp. was the causal agent of dogwood anthracnose (17). The dogwood anthracnose fungus was named *Discula destructiva* Redlin sp. Nov. and is presently known to naturally infect *C. nuttallii*, *C. florida* and *C. kousa* in North America (22). Symptoms of dogwood anthracnose include leaf spots and blights, dieback of twigs and branches, epicormic shoots, and stem cankers (3,12,22).

When *Discula* spp. isolated from diseased leaves were cultured on solidified culture medium, two distinctly different colony types have been observed (10). Isolates designated as *D. destructiva* (type 1) have colony margins that are irregular whereas undescribed *Discula* sp. (type 2) isolates grow in concentric circles.

Information on the influence of pH on *Discula* spp. growth and disease development is limited (1,2,7). In one study, where pH 2.8 and pH 7.0 water mists containing *Discula* sp. conidia were applied to *C. florida* seedlings, only seedlings subjected to the simulated acid rain developed disease symptoms (1). In another study, when *C. florida* seedlings were pretreated with simulated acid rain adjusted to pH 2.5, 3.5, 4.5 and 5.5, the severity of symptoms caused by *Discula* sp. decreased as pH increased (2). When a *D. destructiva* isolate from Georgia (GA 1) was cultured in liquid media initially adjusted with HCl to pH 2.5, 3.5, 4.5, 5.5, and 6.2, fungal dry weight increased as pH increased (7).

Common experimental inadequacies in pH studies include, not buffering growth media and not measuring the final pH of the growth media (9,16). Although effects of pH on the growth and infective capability of *Discula* spp. have been studied (1,2,7), the effects of buffered pH regimens on *D. destructiva* and the unidentified *Discula* sp. growth in culture are unknown. Therefore, the objectives of this

study were to discover if *D. destructiva* and the unidentified *Discula* sp. were affected by buffered pH of 4.0, 5.0, 6.0, or 7.0.

## ii. MATERIALS AND METHODS

**Culture preparation.** Three isolates of *D. destructiva* (TN 1, MA 11, and GA 1) and one isolate of an undescribed *Discula* species (VA 17B) were grown on 12.5 ml of Difco potato dextrose agar (PDA) in Petri dishes (60 x 15 mm). Cultures were incubated at 18 C in a growth chamber maintained with a 16/8 photoperiod.

**Media Preparation.** Each of 80, 125 ml erlenmeyer flasks contained 25 ml of cold filtered (Falcon 7105 0.22 micron) citrate-phosphate buffered liquid growth medium (0.5 g Difco yeast extract, 1 g of $NH_4NO_3$, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.01 g CaCl, 0.5 g KCl, 2 ml from a modified stock solution of 0.3 g $MnSO_4$, 0.439 g $ZnSO_4 \cdot 7\ H_2O$, 0.724 g $(FeNO_3)_3 \cdot 9\ H_2O$, and 5 g of glucose per liter deionized water). Stocks of citrate-phosphate buffer at 1 M and 0.2 M, respectively were used to titrate media to pH of 4.0, 5.0, 6.0, and 7.0 (13).

**Media treatment.** Agar plugs, 6.35 mm in diameter, containing actively growing hyphal tips, were removed from the margins of 10-day old fungal colonies. Plugs were quartered to increase growing points. One quartered plug was placed in each 125 ml erlenmeyer flask containing 25 ml

of the liquid medium and flasks were swirled for a couple of sec to make sure the plugs were separated into four pieces. The experiment was organized as a randomized complete block design, blocked by trial and repetition. Treatments were arranged as a factorial of pH by isolate. Each pH treatment was repeated five times and the entire experiment was performed twice.

**Incubation.** Cultures were maintained at 18 C in a growth chamber adjusted to 16/8 photoperiod for 2 wk. Flask contents were vacuum filtered onto preweighed filter paper (S/P Grade 360 Qualitative 9.0 cm in diameter). Collected material was dried at 60 C for 24 hr and weighed. Initial filter paper weights were subtracted from corresponding dry weights of fungal material plus filter paper. Initial and final pH of the media were recorded.

**Statistical analysis.** Data were analyzed by General Linear Model (24). Because the results of the analysis of variance for pH over all the isolates were not significant ($.05 < P < .0687$), orthogonal polynomial contrasts were utilized to determine if any significant differences in weight of fungi were present when pH was averaged over all isolates.

## iii. RESULTS

Final pH was never greater than .05 pH units above initial pH or less than .09 pH units below initial pH.

Growth of one isolate of *D. destructiva* (GA 1) and the undescribed *Discula* sp. (VA 17B) isolate was reduced as pH of the media increased from 4 to 7 (Fig. 5). When growth at pH 4 and 7 were compared for these two isolates, increasing pH to 7 reduced growth of GA 1 and VA 17B 35% and 49%, respectively (Table 2). Growth of the two other *D. destructiva* isolates were not affected consistently by pH treatments (Fig. 5). When comparing growth at pH 4 and 7, growth of TN 1 was reduced 16% in media adjusted to pH 7 and growth of MA 11 was increased 15% (Table 2). There were significant differences ($.05 > P > .0232$) in fungal growth when isolate dry weights were averaged over all pH. Significant differences in fungal growth ($.05 > P > .013$) at different pH levels were observed with orthogonal polynomial contrasts and differences were linear. Contrasts for individual isolates showed that an increase in pH does have a significant linear effect on the decrease in growth of VA 17B ($.05 > P > .0025$) and GA 1 ($.05 > P > .0477$). Whereas, there was no significant linear effect on a decrease in growth of either TN 1 ($.05 < P < .6017$) or MA 11 ($.05 < P < .5084$).

### iv. Discussion

Most fungi grow best in, but are not limited to, acidic environments (9,16,20). Moreover, plant pathogens usually grow best when the initial media pH is between 5.0 and 6.5

**Fig. 5.** Mean dry weights of an undescribed *Discula* species isolate (VA 17B) and three *D. destructiva* isolates (GA 1, TN 1, and MA 11) grown for 2 wk at pH 4.0, 5.0, 6.0, and 7.0. Bars represent standard errors.

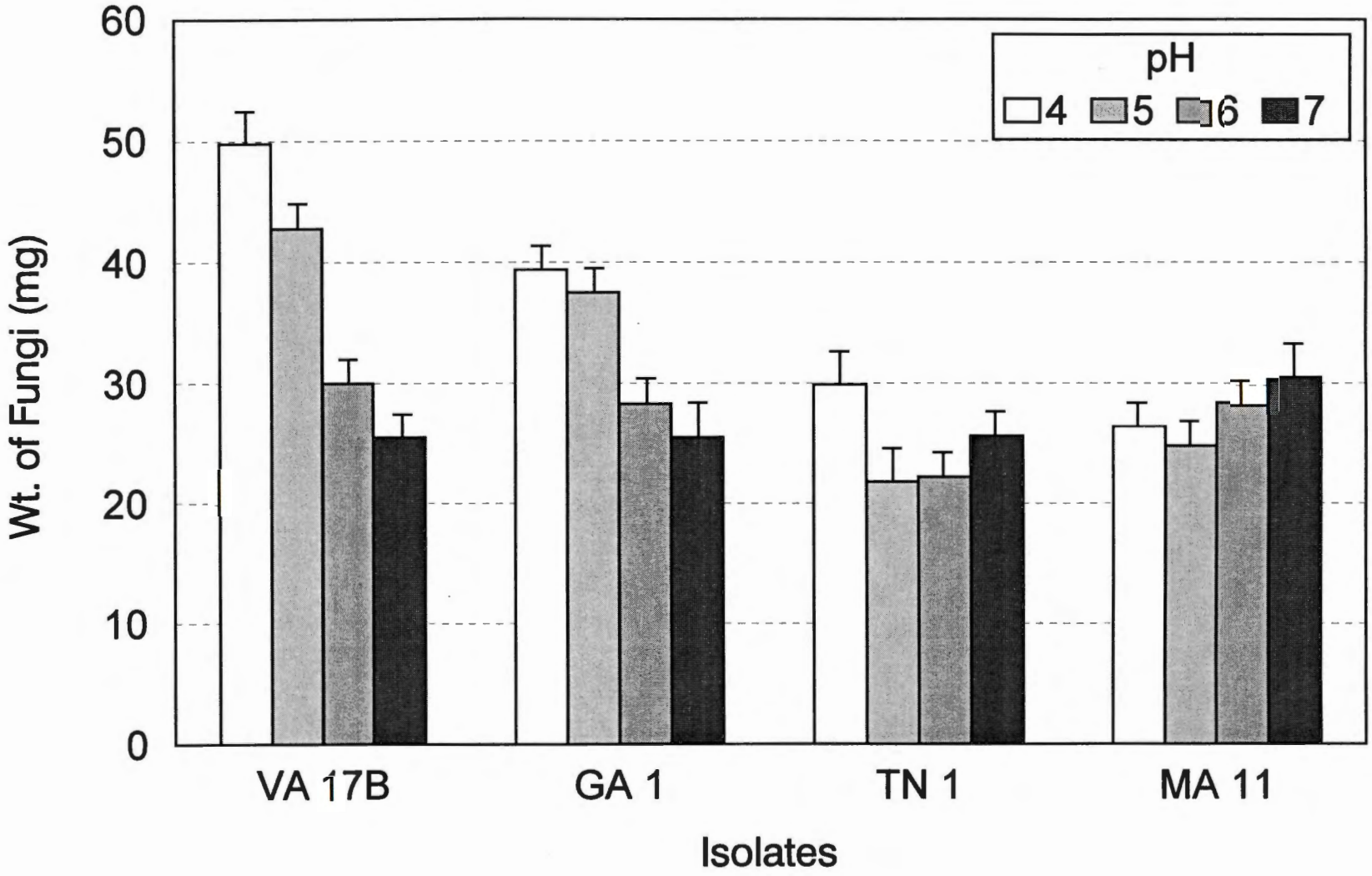

60
50
40
30
20
10
0
Wt. of Fungi (mg)
pH
4
5
6
7
VA 17B
GA 1
TN 1
MA 11
Isolates

**Table 2.** Mean percent decrease (-) or increase (+) in growth of an undescribed *Discula* species isolate (VA 17B) and three *D. destructiva* isolates (GA 1,TN 1, and MA 11) from pH 4 to 7 for trials and combined trials.

| Isolate | Trial 1 | Trial 2 | Combined |
|---|---|---|---|
| VA 17B | (-)60 | (-)35 | (-)49 |
| GA 1 | (-)46 | (-)20 | (-)35 |
| TN 1 | (+)37 | (-)40 | (-)15 |
| MA 11 | (+)32 | (+)04 | (+)16 |

(9). Previous tests have shown that fungi can produce acids as by-products of metabolism. In non-buffered media these acids can rapidly lower the pH to the point of limiting growth (9). This lowering of pH may explain why Brown *et al.* (7) found isolate GA 1 to grow well at pH 6.5 in non-buffered liquid media. Their findings were exactly opposite from our data for comparing growth of isolate GA 1 in buffered media ranging in pH from 2.5 to 6.2 (7) (Fig. 5). Furthermore, growth of all of their isolates were limited at the initial pH of 2.5 (7). Also, our results were also similar to theirs in that growth of their undescribed *Discula* sp. isolate (NY 326) and our undescribed *Discula* sp. isolate (VA 17B) decreased as pH increased (6) (Fig. 5). Other experimental differences that make comparisons to our results difficult are that all of Brown *et al.* treatments involved constant light and all but one of their treatments involved growth on agar amended media (7). The undescribed *Discula* sp. isolates typically grew faster, both in liquid and on solid media, than *D. destructiva* isolates. When comparing growth of our isolates at pH 4.0 and 5.0, the undescribed *Discula* sp.isolate grew significantly better than all *D. destructiva* isolates (Fig. 5). Because *Discula* sp. isolates outgrow *D. destructiva* isolates in a variety of media, it is likely that nutritional requirements rather than pH are responsible for this difference.

Acid rain is known to erode plant cuticles and thus provide access to pathogens (19). Brown *et al.* (5) found that when *C. florida* leaves were treated with simulated acid rain, trichome and cuticle damage increased as pH of the rain decreased from 5.5 to 2.5. Our results also suggest that if fungal propagules are in contact with acid rain on plant surfaces growth of some *Discula* spp. may be enhanced.

Further research on the influence of pH on *Discula* spp. growth should include; wider pH ranges, spore germination, and development of completely defined medium. It would also be beneficial to know dogwood leaf surface pH in a variety of geographical areas. Studies on a wider range of pH may result in finding other pH optima for some *Discula* species isolates. This research has shown that some isolates of *D. destructiva* and the undescribed *Discula* sp. are sensitive in varying degrees to pH in buffered liquid culture; whereas, other isolates of *D. destructiva* grow at similar rates regardless of the pH of the growth media.

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## LITERATURE CITED


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