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11. Lee, I.-M., A. Bertaccini, M. Vibio, and D.E. Gundersen. 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology 85:728–735.

12. Lin, C.P. and T.A. Chen. 1985. Monoclonal antibodies against the aster yellows agent. Science 227:1233–1235.

13. Peterson, G.W. 1984. Spread and damage of western X-disease of chokecherry in eastern Nebraska plantings. Plant Disease 68:103–104.

14. Rosenberger, D.A. and A.L. Jones. 1978. Leafhopper vectors of the peach X-disease pathogen and its seasonal transmission from chokecherry. Phytopathology 68:782–790.

15. Sinha, R.C. and L.N. Chiykowski. 1984. Purification and serological detection of mycoplasmalike organisms from plants affected by peach eastern X-disease. Can. J. Plant Pathol. 6:200–205.

An Inoculation Technique for Dogwood Anthracnose[1]

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Abstract

Researchers have been generally unable to infect dogwood foliage (*Cornus florida* L.) with dogwood anthracnose (*Discula destructiva* Redlin) in artificial environments. We tested the influence of four factors on development of *D. destructiva* lesions in intact *C. florida* leaves wounded with a pin-prick device: (1) propagule (conidia or vegetative hyphae), (2) isolate origin (Catoctin Mountain, MD or Great Smoky Mountains National Park, TN), (3) controlled environment (growth room or air conditioned, humidified chamber in a greenhouse), and (4) period of time leaves were enclosed in humidified bags following inoculation. Of the manipulated factors, time spent in moistened bags and experimental environment were most important in determining lesion size. Lesions of similar size resulted from 0, 2 and 4 days in humidified bags, but leaves enclosed for 7 days had lesions over 5 times as large. Lesions that formed on trees in the greenhouse chamber were about 15 times larger than those in the growth room. Neither fungal propagule nor isolate origin affected lesion size. Based on this information, we have successfully infected both *C. florida* and *Cornus kousa* with *D. destructiva* in subsequent studies.

Index words: dogwood anthracnose; Flowering dogwood; *Cornus florida* L.; *Discula destructiva* Redlin.

Significance to the Nursery Industry

Attempts to study dogwood anthracnose on flowering dogwood have been hampered by difficulties in reliably producing the disease under experimental conditions. This report summarizes a technique for inoculating intact dogwood leaves and consistently obtaining infection with dogwood anthracnose. We have used this information to help us test the resistance of various *Cornus* cultivars to dogwood anthracnose, and the technique should also be of interest to others investigating various aspects of the biology and control of this disease.

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Introduction

Flowering dogwoods (*Cornus florida* L.) are attractive, valuable landscape trees common in the southeastern United States that can generate gross returns of up to $60,000 per harvested acre (3). Since the late 1970s, trees of various *Cornus* species have suffered health decline and sometimes death due to a fungal disease, dogwood anthracnose (5), caused by *Discula destructiva* Redlin (13).

The nursery industry would benefit from trees which are resistant to dogwood anthracnose. Since the disease has devastated native *C. florida* trees in some areas of the eastern United States (15), resistant germplasm must be discovered in order to overcome the economic impact the disease has had on the businesses selling dogwoods in those areas. Efforts to study disease progression and resistance have been hindered by the lack of a reliable inoculation technique that consistently results in diseased foliage.

Although some researchers have succeeded in obtaining severe disease development in forested areas using natural inoculum (4), many attempts at producing the disease in laboratories and greenhouses have not been successful. Various inoculation techniques have been tried, most with only limited success (1, 12, 16). Grand et al. (11) wounded detached leaves with a pin-prick device and rubbed agar plugs of inoculum into wounds. After inoculation, leaves were enclosed

in moist chambers to provide disease-conducive conditions. Using this technique, they found that the fungus sporulated more quickly on wounded leaves than on non-wounded leaves, and that inoculation with conidia caused more severe dogwood anthracnose symptoms than did inoculation with hyphae. In this paper we describe our attempts to modify this inoculation procedure for leaves still attached to potted dogwood trees, and to compare the virulence of an isolate of *D. destructiva* that had been in culture for 18 months with an isolate recently collected from dogwood tissue.

Materials and Methods

Inoculum production. To obtain a fresh isolate of *D. destructiva*, symptomatic *C. florida* leaves were collected from Great Smoky Mountains National Park (GSMNP) on July 21, 1994, and *D. destructiva* was isolated onto potato dextrose agar amended with 30 mg liter^{-1} each (0.00006 oz gal^{-1}) of chlortetracycline and streptomycin sulfate (PDA+) from leaves. An older isolate had been collected from the Presidential tree (10) on Catoctin Mountain Park, MD (CM) on June 30, 1993. It had been transferred from stock cultures on January 16, 1995.

To produce hyphal inoculum of each isolate, cultures were transferred onto PDA+. These plates were incubated in darkness at 20C (68F). To produce conidia, frozen dogwood leaves were first placed into glass petri dishes with a filter paper separating each leaf. Once full, leaves and filter papers were moistened with deionized water and the petri dish was wrapped in aluminum foil and autoclaved for 1 hr on two consecutive days and then stored in a freezer. Just before transfers of the fungus were made, the petri dish of dogwood leaves was autoclaved again, then individual autoclaved dogwood leaves were aseptically placed onto plates of PDA+. Agar plugs from stock cultures of GSMNP or CM isolates containing *D. destructiva* were then transferred to the plates with dogwood leaf for production of conidial inoculum. The petri dishes were then sealed with Parafilm and incubated at 20C (68F) in darkness. Cultures used to obtain inoculum were 57 days old when used as inoculum and were chosen for similar fungal colony characteristics: form, color and age.

Plant culture. One hundred dormant, bare root trees were potted in a pine bark mix [pine bark:sand (4:1 by vol), to each m^3 (35.3 ft^3) of which was added 4 liters (1.1 gal) of dolomitic lime, 4 liters (1.1 gal) of 17–6–10 Osmocote plus minor elements (Grace-Sierra, Malpitas, CA) and 2 liters (0.5 gal) Epsom salt (Mg_2SO_4)] in Zarn 400 pots (3 liter) (0.8 gal) on December 19, 1994, and were placed in an overwintering house. On January 10, 1995, 40 of these trees were moved to a greenhouse with supplementary lighting (400 W sodium vapor lamps) to induce budbreak. The trees were exposed to natural light in the greenhouse through the day, and the lights were used from 5 pm until 11 pm. After leaves emerged and some matured, 20 trees were moved into an enclosed greenhouse bench (GH), and the remaining twenty were placed into a walk-in environmental growth chamber (EGC) (M75, Environmental Growth Chambers, Chagrin Falls, OH).

The GH chamber was a bench enclosed by two layers of clear plastic, about 2.6 cm (1 in) apart, covered by shadecloth, and equipped with three humidifiers and an air conditioner. In both locations, average air temperature, light and relative humidity were recorded hourly with a thermocouple, PPFD sensor (Li-Cor, Lincoln, NE) and humidity probe (Li-Cor), respectively, connected to a datalogger (CR10 or 21X, Campbell Scientific, Logan, UT).

In the GH chamber, hourly average day/night air temperature ranged between 19–22C (66–72F) and 18–20C (64–68F), respectively. Hourly average light levels during the photoperiod ranged from 20 to 75 mmol m^{-2} s^{-1} (1.9 to 7.1 mmol ft^{-2}s^{-1}) on the floor of the chamber. Relative humidity ranged from 60 to 93%.

In the EGC, day/night air temperatures were set at 20/18C (64/68F), with a 14 hr photoperiod. The chamber was equipped with a 90% transmission shade cloth, bringing the irradiance from the overhead HID lamps (an equal mix of 400W high pressure sodium and metal halide lamps) to about 100 to 120 mmol m^{-2} s^{-1} (9.5 to 11.4 mmol ft^{-2} s^{-1}) at 1.4 m (4.5 ft) beneath lights. Relative humidity ranged from 68 to 85%.

Inoculation procedure. Healthy, usually terminal leaves were wounded with a 'floral frog' [several closely spaced steel needles in a steel base, (11)]. For the hyphal inoculum treatment, plugs containing hyphae were cut from the growing edge of agar cultures with a 6 mm (0.24 in) diameter cork borer and one plug was rubbed into each wounded leaf surface. For the conidial treatment, plugs were cut from dogwood leaf tissue that was laden with acervuli of *D. destructiva*, using the same cork borer. The entire agar plug was removed from the dish and rubbed into the wounded leaf surface, so the conidial treatment included some vegetative hyphae. Each inoculated leaf was enclosed in a plastic bag moistened with deionized water to maintain humidity. Bags remained on leaves for either 0, 2, 4 or 7 days. Lesions on each leaf were measured once weekly for 5 weeks, beginning 7 days after inoculation.

Data collection and analysis. Length and width of lesions were multiplied to give a rough estimate of lesion area. Measurements over weeks were incorporated in the analysis as repeated measures. Data were analyzed using Proc Mixed (13). Least squares means for each treatment (averages adjusted for imbalances in replicate numbers) were compared using pairwise t-tests, with $P = 0.10$.

Re-isolation of the pathogen. After the fifth week's lesion measurements, some symptomatic leaves were removed from trees, brought into the laboratory, and placed into petri dishes containing a moistened Kimwipe laboratory tissue or filter paper. The dishes were sealed with Parafilm and left at room temperature for several days. Leaves were periodically checked under a microscope for production of acervuli. If acervuli were found, an inoculation needle was inserted into an acervulus, then into a petri dish of PDA+. These petri dishes were watched closely for the growth of *Discula* sp. If *Discula* was present, a pure culture was obtained on PDA+ for use as inoculum in subsequent experiments.

Experimental design. Treatments were arranged in a 2 × 2 × 2 × 4 factorial, with two isolates (CM and GSMNP), two inoculum types (conidia and hyphae), two locations (EGC and GH) and four different periods of time (0, 2, 4 or 7 d) leaves were in bags. These 32 treatment combinations (16 per location) were administered using a 4 × 4 balanced lattice design, with four inoculated leaves per tree, each leaf with a different combination of inoculum and bag times.

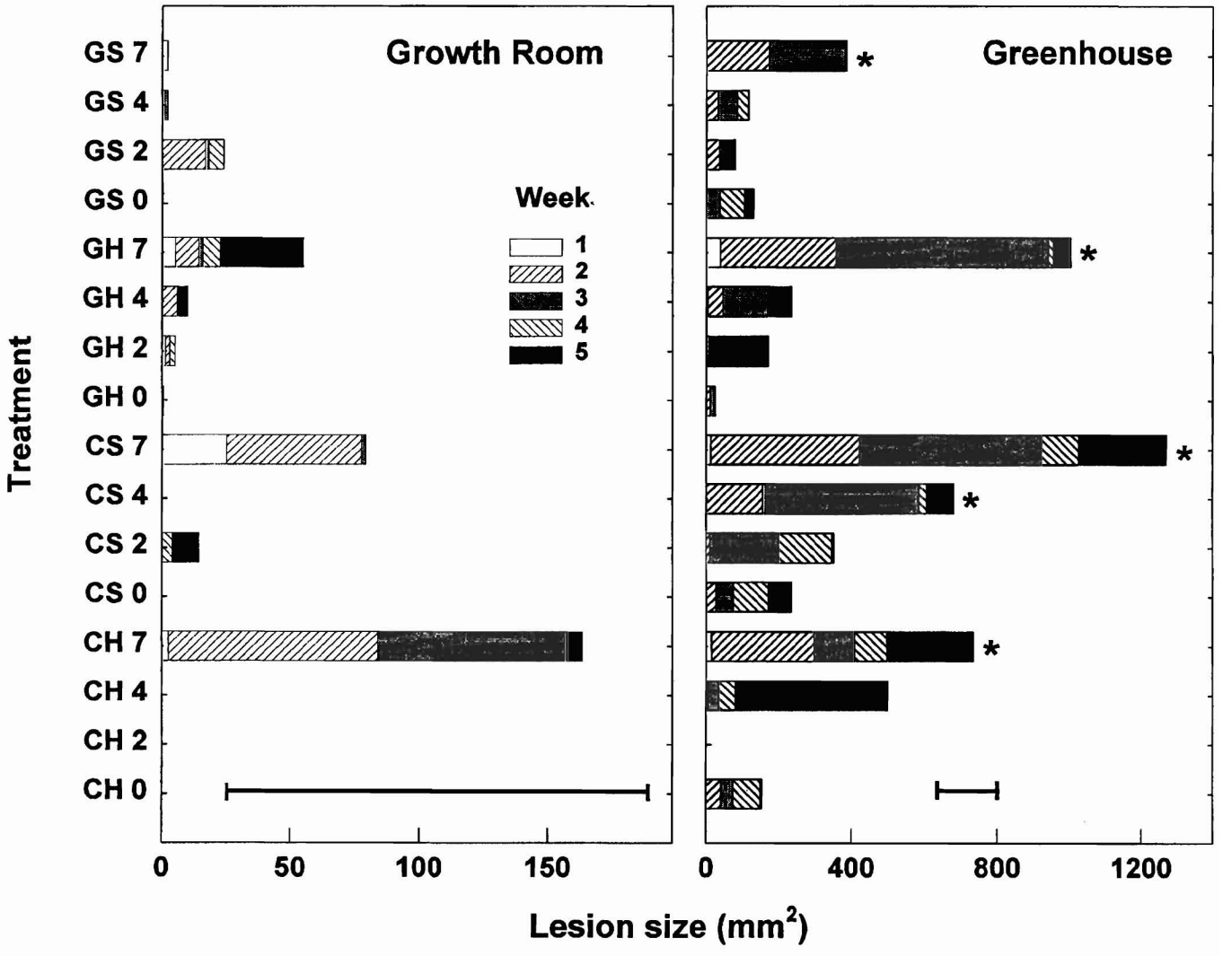

Fig. 1. **Weekly least squares means (n = 5) of lesion sizes caused by each combination of inoculation treatments. Lesion area was estimated by multiplying lesion length and width. C = Catoctin Mountain isolate; G = Great Smoky Mountain National Park isolate; S = conidia; H = hyphae; 0 = no bag applied; 2 = bag left on for 2 days; 4 = bag left on for 4 days; 7 = bag left on for 7 days. (*) denotes lesions significantly larger than zero at $P = 0.10$. Horizontal line in each plot represents standard error of all means in that plot.**

Usually five leaves were inoculated with each treatment combination and trees were randomly placed within replication blocks in either the GH or the EGC.

Results and Discussion

Lesions in the GH chamber [average of 203 ± 37 mm² (0.3 in²) across treatments] were about 15 times larger by the end of the experiment than those that formed in the EGC [average of 14 ± 37 mm² (0.02 in²) across treatments] (Fig. 1). Several researchers have found that conditions conducive to disease include high relative humidities and low light intensities (2, 6, 8, 9). Since the GH chamber was darker and more humid than the EGC, providing an environment more conducive for dogwood anthracnose, differences in lesion sizes in the two inoculation chambers were likely due to differences in environmental conditions.

Inoculations with fungal isolates from CM and GSMNP led to lesions of statistically similar size [135 ± 35 mm² (0.21 in²) and 81 ± 35 mm² (0.05 in²), respectively, across treatments]. The CM isolate had been in culture for 18 months and the GSMNP isolate had been in culture for 6 months. Propagules did not differ with respect to virulence; inoculations with conidia caused lesions that averaged 119 ± 35 mm² (0.18 in²), and inoculations with vegetative hyphae caused lesions that averaged 97 ± 35 mm² (0.15 in²), by the end of the experiment. The similarity between lesion sizes resulting from inoculation with conidia and hyphae could be due to the conidia treatment including some vegetative hyphae. Conidia and hyphae of *D. destructiva* do not appear to lose virulence in culture for up to 18 months.

Length of time that leaves were enclosed in bags after inoculation was an important factor in determining the final size of lesions. Lesions on leaves in bags for 0, 2 and 4 days were similar but significantly smaller than lesions on leaves that remained bagged for 7 days (Fig. 2A). Enclosing leaves in bags increased relative humidities around the inoculated leaves. Increased humidity has been demonstrated to increase disease severity (6).

Lesion growth was mostly linear over time (Fig. 2B). When environmental conditions are favorable for disease, symptoms are expected to become more severe over time (18).

We supplied environments conducive to disease and lesion areas grew each week, as expected.

The inoculation treatment that caused the greatest level of disease severity was inoculation with agar plugs containing primarily conidia from the CM isolate and placing leaves in bags for 7 days in the GH. By week 5, average lesion area was 1262 mm^2 (2.0 in^2). The most consistent treatment between locations, however, was CM hyphae with a bagged period of 7 days. Although lesion areas on trees in the EGC were not statistically different from zero, due to large variability among replicates, CM hyphae with a bagged period of 7 days caused the largest lesions in the EGC, and the third largest in the GH (Fig. 1).

By the fifth week, three treatments, (hyphae from the CM isolate, conidia from the CM isolate and hyphae from the GSMNP isolate, each enclosed in a bag for 7 days after inoculation) had produced lesions with areas of at least 10 mm^2 (0.016 in^2) in at least 40% of the leaves inoculated in both the GH and the EGC (Fig. 3). Inoculation with conidia of the CM isolate on leaves enclosed in bags for 7 days led to infection in 80% of inoculated leaves in the GH and 40% of leaves in the EGC. Eighty per cent of the leaves in the GH inoculated with hyphae from the CM isolate on leaves enclosed for 7 days had lesions, while the same treatment in the EGC caused lesions in 60% of the leaves inoculated in this manner. Hyphae from the GSMNP isolate on leaves bagged for 7 days caused lesions in 100% of the leaves inoculated with that treatment in the GH chamber, while in the EGC that treatment caused lesions in 60% of the leaves inoculated in this way.

Because there was much less disease in the EGC than the GH, we have, for succeeding experiments, adjusted the conditions in the EGC and modified the inoculation procedure at that location. We added an additional 50% light transmission shadecloth (reducing the irradiance to about 60 mmol m^{-2} s^{-1}) and left moist bags on inoculated leaves for 12 instead of 7 days. In subsequent experiments, adjustments increased the amount of dogwood anthracnose in the growth room, but the environment in the greenhouse chamber was still almost three times as conducive to dogwood anthracnose as in the growth room (Ament, unpublished).

One possible concern about the applicability of this technique to the nursery industry is that wounding leaves during inoculation might compromise some physical barrier present in resistant seedlings, which is a problem long faced by researchers interested in disease resistance. Previous research has indicated that wounds caused by insects and acidic mists are likely involved in the natural spread of the disease (7, 17). Perhaps this technique is best used for resistance screening in outdoor trials where environmental conditions are less conducive to disease than in our chambers.

In summary, inoculation success can be improved by enclosing wounded, inoculated leaves in humidified plastic bags for at least 7 days. Cool, very humid conditions with low light intensity are conducive to dogwood anthracnose and have a pronounced effect on inoculation success. Both propagules were equally successful as inoculum in our trials, possibly indicating that neither conidia nor hyphae of *D. destructiva* lose virulence after up to 18 months in culture. Isolate origin (and period of time in culture) did not affect lesion size under our conditions.

Literature Cited

1. Anderson, R.L., P. Berrang, J. Knighten, K.A. Lawton, and K.O. Britton. 1993. Pretreating dogwood seedlings with acidic precipitation increases dogwood anthracnose symptoms in greenhouse-laboratory trials. Can. J. For. Res. 23:55–58.

2. Augé, R.M. 1995. Environmental stress and dogwood anthracnose. Tenn. Agri Sci. 175:24–29.

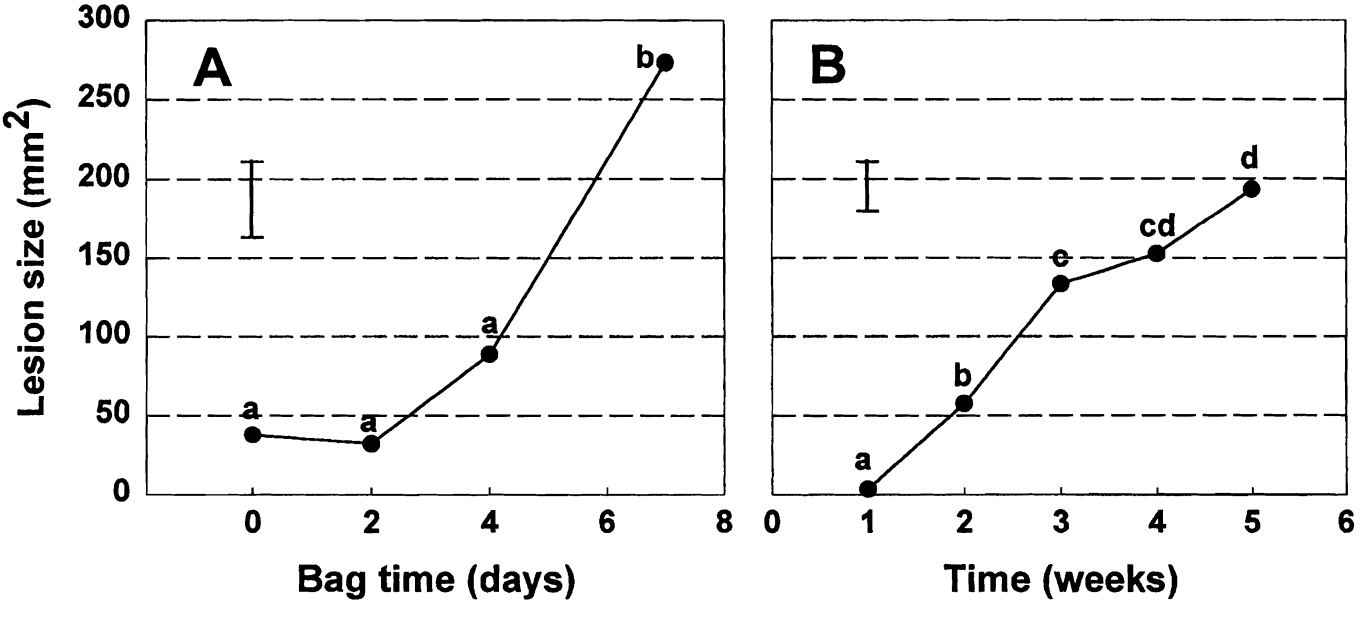

Fig. 2. **A. Least squares means of lesion sizes caused by inoculating leaves and enclosing them in moistened plastic bags for different periods (n = 40, averaged over isolate origin, location, and fungal propagule). B. Least squares means of lesion sizes each week (n = 160, averaged over bag time, isolate origin, location and fungal propagule, i.e. all leaves in the experiment). Lesion area was estimated by multiplying lesion length and width. Data points marked with different letters are different at $P = 0.10$. Vertical line in each plot represents standard error of all means in that plot.**

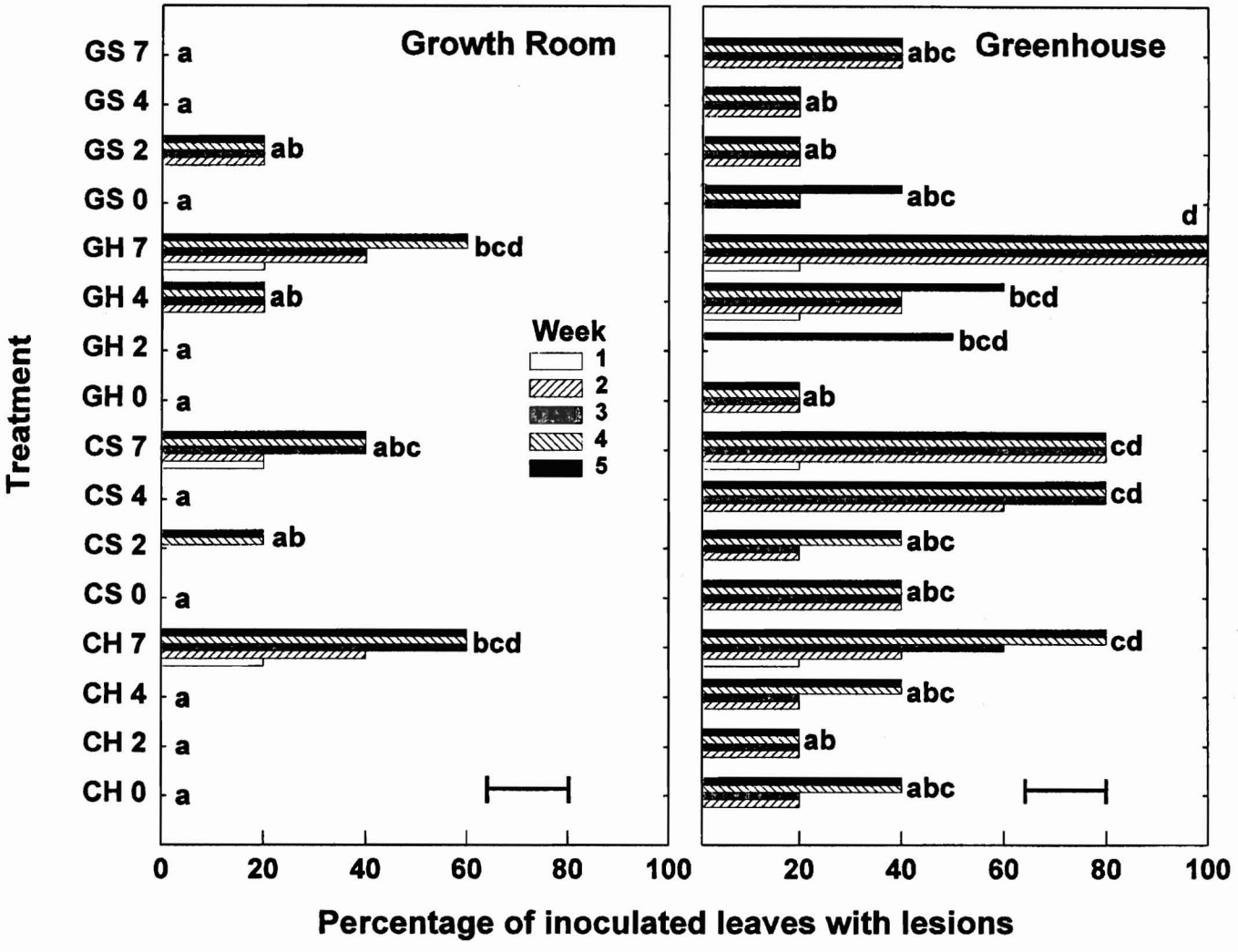

Fig. 3. Percentages of inoculated leaves having lesions at least 10 mm² (0.016 in²). Histogram bars represent averages of five leaves within each treatment combination (except for GH 2, average of four leaves). Bars marked with different letters are significantly different at $P = 0.10$. Abbreviations as in Fig. 1. Horizontal line in each plot represents standard error of all means in that plot.

3. Badenhop, B.B., W.T. Witte, and T.E. Glasgow. 1985. Production Systems and Costs for Producing Balled and Burlapped Trees of Dogwood Cultivars, Tennessee, 1984. Univ. of Tenn. Agric. Expt. Sta. Bul. 637.

4. Brown, D.A., M.T. Windham, and R.N. Trigiano. 1996. Resistance to dogwood anthracnose among *Cornus* species. J. Arboriculture 22:83–86.

5. Byther, R.S. and R.M. Davidson. 1979. Dogwood anthracnose. Ornamentals Northwest Newsletter 3:20–21.

6. Chellemi, D.O. and K.O. Britton 1992. Influence of canopy microclimate on incidence and severity of dogwood anthracnose. Can. J. Bot. 70:1093–1096.

7. Colby, D.M. 1995. Arthropod dissemination of *Discula destructiva* conidia on *Cornus florida*, MS Thesis. University of Tennessee, Knoxville.

8. Erbaugh, D.K., M.T. Windham, A.J. Stodola, and R.M. Augé. 1995. Light intensity and drought stress as predisposition factors for dogwood anthracnose. J. Environ. Hort. 13:186–189.

9. Gould, A.B. and J.L. Peterson. 1994. The effect of moisture and sunlight on the severity of dogwood anthracnose in street trees. J. Arboriculture 20:75–78.

10. Graham, E.T., R.N. Trigiano, and M.T. Windham. 1995. Learning from survivor trees: Evaluation of potential applications of Catoctin Mountain dogwoods. Tenn. Agric. Sci. 175:38–44.

11. Grand, L.F., P. Gerald, E. Gatlin, S. Lowrence, T. Burroughs, and C. Mewborn. 1994. Dogwood anthracnose research at North Carolina State University. Proc. 8th Regional Dogwood Workshop. pp. 22–23.

12. Hibben, C.R. and M.L. Daughtrey. 1988. Dogwood anthracnose in Northeastern United States. Plant Disease 72:199–203.

13. Redlin, S.C. 1991. *Discula destructiva* sp. nov., cause of dogwood anthracnose. Mycologia 83:633–642.

14. SAS Institute Incorporated. 1996. SAS/STAT software: Changes and Enhancements through Release 6.11. Cary, NC.

15. Schneeberger, N.F. and W. Jackson. 1989. Impact of dogwood anthracnose on flowering dogwood at Catoctin Mountain Park. Plant Diagnostician's Quarterly 10:30–43.

16. Schreiber, L.R., S.C. Domir, and L.S. Dochinger. 1993. Infection mechanisms in dogwood anthracnose. Proc. 7th Regional Dogwood Anthracnose Workshop. pp. 52–53.

17. Thornham, K.T., R.J. Stipes, and R.L. Grayson. 1992. Effect of acid rain deposition on trichome morphology and dogwood anthracnose biology. Proc. 6th Regional Dogwood Workshop. p. 40.

18. Windham, M.T., M. Montgomery-Dee, and J. Parham. 1995. Factors associated with dogwood anthracnose incidence and severity. Tenn. Agri Sci. 175:17–20.