Laboratory and Greenhouse Techniques for Evaluating Selected Fungicides Against *Cristulariella moricola* on Pecan Leaves

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ABSTRACT

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Conidia of *C. moricola* were produced only on autoclaved pecan or holly leaves but not on 19 semisynthetic or plant extract media. Phialoconidia developed on media supplemented with yeast extract except on cornmeal and lima bean agar. Light was an essential factor for conidiation. Czapek-Dox + yeast extract agar supported maximal growth of sclerotia and thus served as a diagnostic medium for isolating *C. moricola* from infected leaves. Propiconazole protected mature pecan leaves in the greenhouse for 51 days. It was also effective in stopping the growth of *C. moricola* in established lesions, thus effecting a cure of zonate leaf spot of pecan.

Cristulariella moricola (Hino) Redhead (= *C. pyramidalis* Waterman & Marshall) (11,16), causal agent of zonate leaf spot of pecan (*Carya illinoinensis* (Wang.) C. Koch), can cause severe defoliation of pecan trees (2,4). This disease is most severe on pecan foliage in the southeastern United States during the July through September rainy season and absent during dry seasons. Zonate leaf spot has occurred in orchards maintained on a scab prevention schedule using dodine or triphenyltin hydroxide (4). Benomyl has been recommended for control of zonate leaf spot in Georgia (1), but the development of resistance to benomyl by many fungi (8,9) suggests that *C. moricola* might also adapt to this fungicide. Thus, effective alternative fungicides need to be found.

Fungicide evaluations for control of zonate leaf spot have been conducted in orchards with a history of the disease, but tests have not been successful because of its sporadic occurrence (3). Earlier investigations of *C. moricola* were conducted on potted pecan seedling leaves inoculated with mycelial agar disks. Leaves were wounded through the disks, and conidia produced were washed from the leaves for epidemiological studies (2,5). Procedures for producing conidia were laborious but essential, because conidia did not develop on agar media (2,3). Yokoyama and Tubaki (17) reported conidial production on malt extract agar, prompting a reevaluation of *C. moricola* growth on laboratory media. The term conidium for the propagative unit suggested by Niedbalski et al (10) is adopted in this work and refers to the large, pyramidal head 250–450 μm long and 80–120 μm in diameter, sensu Waterman and Marshall (16).

These investigations were done to determine appropriate media and techniques for growth of conidia of *C. moricola*, to define a diagnostic isolation medium that could determine the survival of *C. moricola* after fungicide treatment, and to evaluate the protective and/or curative activity of selected fungicides.

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MATERIALS AND METHODS

Culture on laboratory media. Isolates of *C. moricola* were obtained from infected, dry leaves of *C. illinoinensis* that had been stored at 4 C. Sclerotia produced on Czapek-Dox + yeast extract agar medium (CDY) were plated on water agar prepared from 20 g of agar (Difco) per liter of distilled water. A 5-mm-diameter mycelial agar disk was cut from the periphery of the colony growing on water agar and transferred to the following Difco agar media: bean pod, cornmeal, Czapek-Dox broth (+ 20 g/L agar), lima bean, malt, and potato-dextrose. BBL Czapek-Dox agar and tomato juice agar (BBL, Div. Decton Dickinson and Co., Cockeysville, MD) were included in the tests. Each Difco and BBL medium was prepared according to the manufacturer's instructions. V-8 juice agar was also included in the tests and made by combining 20 g of agar, 4 g of $CaCO_3$, and 200 ml of V-8 juice per liter. Additionally, an enriched form of each medium was prepared by amending with 4 g/L Difco yeast extract, except the BBL Czapek-Dox medium, which was amended with 4 g/L BBL yeast extract. Twelve plates of each medium were inoculated, and the petri dishes were enclosed in plastic bags, arranged in a randomized complete block design, and incubated at 21 C under fluorescent lights on a 12-hr-light/12-hr-dark cycle (41.2 $\mu E\ m^{-2}\ s^{-1}$) for 18 days. Each test was repeated four times. The development of conidia on the different agar media was determined with a stereoscopic microscope at 6, 12, and 18 days. Additionally, the sclerotia that developed on the media were counted directly. Mean sclerotium numbers per plate for each medium were compared by Duncan's multiple range test.

Culture on sterile leaves. Preliminary evaluations showed that conidia of *C. moricola* could be produced on autoclaved leaves of pecan and holly (*Ilex cornuta* Lindl. 'Burford'). Conidial production was more abundant on the cut end of the holly leaf or along a break in the epidermis. Therefore, holly leaves were scarified with a knife or crumpled and squeezed to make numerous ruptures in the leaf tissue. Scarification or crumpling of pecan leaves was not required to obtain good conidial production.

Mature, orchard-grown pecan leaves were cut to cover the bottom of a Pyrex petri dish (100 × 15 mm). Each dish contained two 9-cm-diameter filter papers and 1 ml of distilled water. The leaf substrates were autoclaved, cooled, and inoculated with 5-mm-diameter mycelial-agar disks cut from the periphery of actively growing *C. moricola* cultures. Other autoclaved pecan leaf substrates were: leaves that had been frozen, fresh leaves grown in a greenhouse, leaves affected by sooty mold (species of Capnodiaceae), and senescent leaves collected late in October. Senescent leaves were tested 3 mo after those cited; therefore, the data were not included in the statistical analysis (Table 1). Treatments with pecan leaves included substrates incubated in the dark and substrates amended with 5 ml of water per dish. Additionally, marginal spines were cut from holly leaves, then the leaves were washed in tap water to remove insects and placed in a Pyrex petri dish containing two 9-cm filter papers and 2 ml of distilled water. These leaves were also sterilized, cooled, inoculated, and incubated. Forty-eight leaf-substrate dishes were prepared for each treatment; dishes were placed in plastic bags to prevent drying. Four dishes were placed in each bag and placed flat to allow for maximum light reception. One set of substrates was incubated in the dark; the others were incubated 36 cm under cool-white lights

model F-40T10CW/99 (Westinghouse Electric Corp., Bloomfield, NJ). Substrates were incubated with a 12-hr photoperiod in a randomized complete block design at 21 C and 20.8 μE m^{-2} s^{-1} for 8 days. The conidia produced in each leaf-substrate dish were counted with a stereoscopic microscope at 10×. Mean conidial numbers per plate were compared for differences among treatments by Tukey's test (12).

Inoculum preparation. A leaf bearing conidia of *C. moricola* was immersed in a solution containing Tween 20 (0.02 ml/100 ml of sterile tap water). The leaf surface was stroked lightly with a camel's-hair brush to suspend the conidia, the leaf was discarded, and another leaf was treated similarly until a dense suspension of conidia was obtained. The suspension of conidia was aspirated under vacuum, a 0.3-ml aliquot was pipetted into an approximately 2.5-cm-diameter circle on a glass plate, the conidia were counted with a stereoscopic microscope, and the concentration was adjusted with sterile tap water.

Evaluation of fungicides. Trees used throughout this investigation were 5-yr-old Schley pecan trees, 40–50 cm tall, grown in 12.3-L pails in the greenhouse.

The duration of protectant activity of propiconazole (Tilt 3.6E) against *C. moricola* was determined as follows. The terminal compound leaf and every other leaf to the base of the shoot were labeled with paper tags; alternate or nonlabeled leaves were covered with a plastic bag to serve as checks. Four trees were sprayed with 270 mg of propiconazole and 0.62 ml of Penetrator 3 (83% light crop oil, 15% nonionic detergent, Helena Chemical Co., Memphis, TN) per liter of water with a Handi-Sprayer (Voluntary Purchasing Groups, Inc., Bonham, TX). The leaves were sprayed with fungicide to runoff on both adaxial and abaxial leaf surfaces. Six hours later, the bags were removed from the checks and the trees were placed on greenhouse benches. Foliage of four trees was sprayed with propiconazole, then every fourth day, a new set of four trees was treated with the fungicide through 63 days (Table 2). Data presented in this paper represent days of fungicidal effectiveness in excess of 30; preliminary tests had shown control for that period.

All trees from protectant period 31 days through 63 days were inoculated after the last group of trees sprayed with propiconazole had 30 days elapsed since their treatment. A suspension of conidia of *C. moricola* (75/ml) was applied to all leaves at a pressure of 69 kPa with an artist's airbrush model 62-2 (Paasche Airbrush Co., Chicago, IL). The trees were placed in dew chambers (Percival Mfg. Co., Boone, IA) programmed to 21 C and 100% relative humidity for 48 hr with no lights. From 48 to 96 hr after inoculation, chamber lights were programmed to operate intermittently to maintain chamber humidity above 85% during the 12-hr photoperiod. After 96 hr, lesion diameters of check leaves were recorded from 12 leaves of the four trees in each protectant period evaluated. Also, necrotic flecks or lesions that occurred on fungicide-treated leaves were recorded from 24 leaves of the four trees per treatment. Subsequently, the trees were transferred to the greenhouse for 7 days, then returned to the dew chamber to test for disease recurrence.

The occurrence of conidia of *C. moricola* was recorded for the fungicide-sprayed and the check leaves. Subsequently, 5-mm-diameter disks were cut aseptically from each lesion in the leaf to include the lesion margin as a disk diameter. Lesions were surface-sterilized in 0.525% NaOCl for 5 min, rinsed six times with sterile water, and inserted so that 50–75% of the disk, including part of the lesion, was embedded in the CDY medium. Disks were incubated in this medium at 21 C for 14 days, at which time the presence of sclerotia growing in *C. moricola* colonies facilitated pathogen identification.

The curative activity of selected fungicides was evaluated on leaves of potted pecan trees. Fungicides and rates tested were those found effective for control of pecan scab (caused by *Cladosporium caryigenum* (Ell. & Lang.) Gottwald) under orchard conditions, i.e., bitertanol (Baycor 50W) at 0.60 mg/L, flusilazol (Nustar 40E) at 4.05 g/L, and propiconazole 3.6E at 67.5 and 135 mg/L. Penetrator 3 (0.62 ml/L of water) was included with each fungicide. A suspension of *C. moricola* conidia (55/ml) was applied to all leaves, and the plants were incubated in dew chambers as described previously. Lesion diameters were recorded 4 days after inoculation. Alternate leaves were covered with plastic bags to serve as checks. Subsequently, the uncovered leaves were sprayed with fungicide to runoff on both adaxial and abaxial leaf surfaces, using seven trees for each treatment. Plastic

Table 1. Mean number of conidia of *Cristulariella moricola* produced on autoclaved pecan and holly leaves after 8 days at 21 C

Leaf substrate	Leaf source	Light[v]	Water (ml)	Conidia (mean no. per plate)
Pecan[w]	Greenhouse	+	1	362.6 a[x]
Pecan	Orchard	+	1	69.1 bc
Pecan	Orchard, then frozen 14 days	+	1	46.3 c
Pecan	Orchard	+	5	20.7 c
Pecan	Orchard, with sooty mold	+	1	1.5[y]
Pecan	Orchard	−	1	0.0[y,z]
Holly	Ornamental bush	+	2	159.7 b

[v] Light at 20.8 μE m^{-2} s^{-1} at location of leaf-substrate dishes.
[w] Mature, disease-free leaves cut to fit into Pyrex petri dishes (100 × 15 mm).
[x] Means followed by the same letter do not differ significantly ($P = 0.05$) according to Tukey test.
[y] Data not analyzed with other treatments.
[z] Subsequent incubation of cultures under light yielded abundant conidia.

Table 2. Number of days propiconazole[a] protected Schley pecan leaves from infection by *Cristulariella moricola*

Protectant period[b]	Leaf spot diam. (mm) 4-days postinoculation		Conidiation per tree (%)[c]	Fungus reisolation CDY media (%)[d]	
	No fungicide	Fungicide	Fungicide	No fungicide	Fungicide
31	4.7[e]	0.5–1.5[f]	0.0	46.3	0.0
35	5.3	0.5–1.5	0.0	62.8	3.0
39	4.5	0.5–1.5	0.0	69.6	0.0
43	5.0	0.5–1.5	0.0	92.3	0.0
47	6.0	0.5–1.5	0.0	95.8	0.0
51	6.5	0.5–3.0	0.0	80.0	0.0
55	7.1	0.5–6.0	2.3	84.8	2.0
59	6.2	0.5–5.0	4.2	73.8	1.6
63	7.1	0.5–8.5	16.5	61.5	5.0

[a] Propiconazole applied at 270 mg/L to runoff on leaves of 4-yr-old, greenhouse-grown Schley pecan trees.
[b] Number of days that elapsed between only fungicide application and inoculation with *C. moricola*.
[c] Percentage of treated leaves per tree producing conidia of *C. moricola* after repeat incubation in dew chambers. Column for no fungicide omitted because lesions were ragged and too extensive to evaluate.
[d] Percentage of leaf disks per treatment that produced *C. moricola* sclerotia on Czapek-Dox + yeast extract agar.
[e] Mean lesion diameter from 12 leaves on four trees.
[f] Average diameter of "flecks" from 24 leaves on four trees per treatment.

bags were removed from check leaves 6 hr after the fungicides were applied, and the trees placed on greenhouse benches for 7 days. Eleven days after inoculation, the trees were sprayed with sterile water to runoff and returned to the dew chamber for 3 days to promote lesion and conidial development. The recurrence of *C. moricola* was recorded per compound leaf for treated and check leaves. Finally, necrotic and healthy leaf tissues were plated on CDY agar media to test fungal survival as described previously.

Table 3. Production of sclerotia of *Cristulariella moricola* on selected media at 21 C

Agar medium	No. sclerotia[x]
Bean pod	0.95 ab[y]
Bean pod + YE[z]	0.00 a
Cornmeal	0.95 ab
Cornmeal + YE	0.03 a
Czapek-Dox (BBL)	0.00 a
Czapek-Dox + YE (BBL)	14.40 d
Czapek-Dox (Difco)	0.00 a
Czapek-Dox + YE (Difco)	21.08 e
Lima bean	2.20 abc
Lima bean + YE	1.68 abc
Malt	3.60 abc
Malt + YE	2.83 abc
Potato dextrose	0.00 a
Potato dextrose + YE	4.15 abc
Tomato juice	8.73 cd
Tomato juice + YE	8.43 bcd
V-8 juice	4.38 abc
V-8 juice + YE	7.00 abc
Yeast extract	0.00 a

[x] Mean number of sclerotia per plate after 12 days of incubation.
[y] Means followed by the same letter are not significantly different ($P = 0.01$) according to Duncan's multiple range test.
[z] YE = yeast extract (4 g/L).

Fig. 1. Conidia of *Cristulariella moricola* produced on sterilized pecan leaves in petri dish after 7 days of incubation at 21 C. **(A)** Surface view of conidia in petri dish. **(B)** Conidia viewed through microscope. Scale bar = 400 μm.

RESULTS

Culture on laboratory media. Conidia were not produced on any of the proprietary agar media, V-8 juice agar, or chopped pecan leaves in water agar. Phialoconidia were abundant on laboratory media supplemented with yeast extract, except on cornmeal and lima bean agars, and were 2–4 μm in diameter. Phialoconidia transferred to CDY or other media did not germinate.

The number of sclerotia that developed on the various media was recorded after 12 days of incubation (Table 3); no appreciable increase in numbers occurred after 18 days of incubation. The addition of yeast extract to Czapek-Dox and potato-dextrose agar resulted in enhanced sclerotial development; however, sclerotial numbers were slightly depressed by this amendment in the other media, except V-8 juice agar (Table 3). Significantly more sclerotia were produced on the CDY medium than on the other media. *C. moricola* did not develop a mean sclerotial number of at least one on the following media: bean pod, bean pod + yeast extract, cornmeal, cornmeal + yeast extract, Czapek-Dox, potato-dextrose, or yeast extract agars.

Culture on sterile leaves. Autoclaved, greenhouse-grown pecan leaves incubated under lights supported conidial production with 362 conidia per dish (Table 1, Fig. 1). Good conidial production was also achieved on autoclaved holly leaves. When 5 ml of water was used per dish, sclerotial production occurred but was accompanied by less conidial production. No conidiation occurred in some trials with leaves that had been frozen. Conidia did not develop on pecan leaves incubated in the dark. After 8 days of incubation in the dark, inoculated leaves were incubated an additional 8 days under the 12-hr fluorescent light and 12-hr dark program. At the end of this period, an average of 204 conidia per dish was recorded. Poor conidial production occurred on leaves colonized by sooty molds; only four of the plates produced conidia (average of 1.5). No conidiation occurred on senescent leaves collected late in October a few weeks before leaf fall.

Pathogen survival. White sclerotial initials of *C. moricola* were observed on colonies growing on CDY media within 3 days after leaf sections from check leaves were inserted into the substrate. The sclerotial initials became dark brown-black mature sclerotia within 5 days of

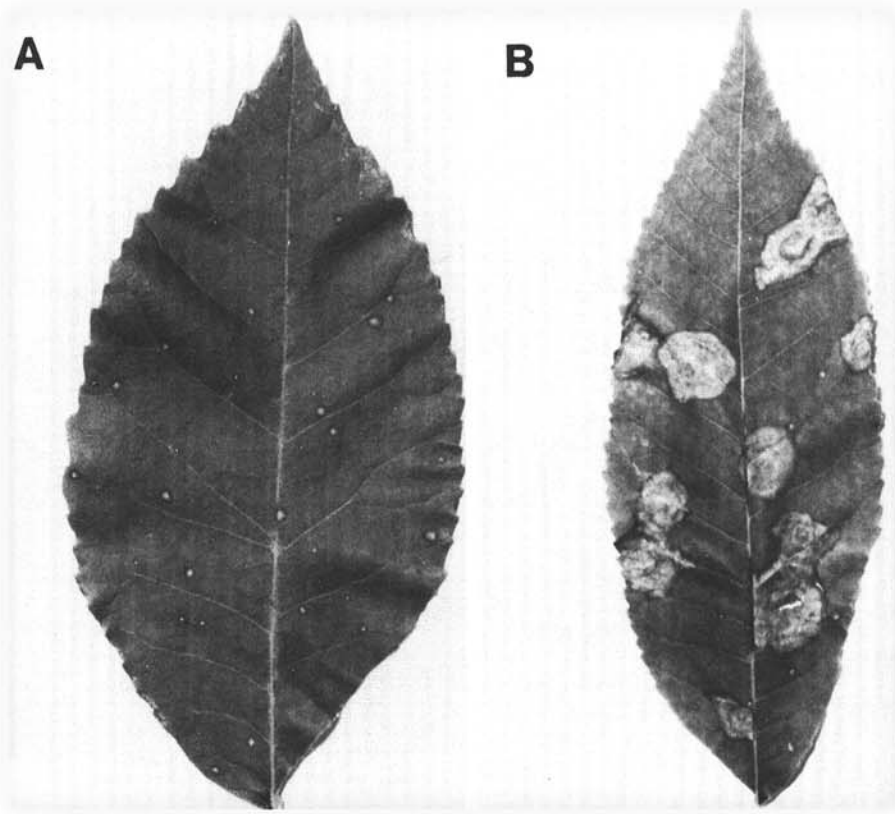

Fig. 2. Leaf spots or flecks in pecan leaflets. **(A)** Propiconazole applied as protectant fungicide, then 31 days later challenge-inoculated with *Cristulariella moricola*. **(B)** Check leaflet. Both leaflets photographed 11 days after inoculation.

incubation. Secondary organisms that occasionally grew from the lesions of fungicide-treated leaves were *Alternaria* spp., *Cladosporium* spp., and other species of fungi and bacteria. The occurrence of typical sclerotia of *C. moricola* in a grayish white fungal colony facilitated a rapid, presumptive identification of the pathogen.

Evaluation of fungicides. Evaluation of a fungicide on every other leaf of a potted tree with alternate leaves serving as checks provided an exacting method for evaluating the success of inoculations. Although continuous air circulation occurred in the dew chambers, lower humidity apparently occurred in the upper quarter of the chamber and along the inside of doors that permitted leaves to dry. Consequently, if inoculated leaves at the top of a tree were not infected, then data from the fungicide-treated leaf immediately below were omitted and it was considered similarly noninfected. Trees used in these tests provided compound leaves that were similar in size to those grown on orchard trees.

Application of propiconazole to protect leaves against *C. moricola* did not prevent infection from occurring (Table 2). Sites of infection were characterized by the presence of a circular leaf spot or fleck with a gray center ranging from 0.5 to 1.5 mm in diameter (Fig. 2A). Conidia of *C. moricola* could be seen in the centers of the spots with a stereoscopic microscope. Leaf spots on fungicide-treated leaves did not increase in diameter during the evaluation period in the greenhouse or in the dew chamber, but lesions on the check leaves incubated in the greenhouse increased in diameter (Fig. 2B). Subsequently, after 3 days of incubation in dew chambers, some check leaflets were partially deteriorated from *C. moricola* activity and only conidiation readings were practical. Three percent of the leaf spots that developed 35 days after the application of propiconazole yielded cultures of *C. moricola* on CDY media, but conidiation was not observed on these leaflets. Fifty-five days after the fungicide treatment, conidiation occurred on infected leaves tested for pathogen survival by incubating trees in the dew chamber. Cultures of *C. moricola* bearing typical sclerotia were also obtained on CDY media from these leaves. An increase in conidiation occurred with each successive 4-day test interval. Similarly, isolation of *C. moricola* was positive with each successively longer spray interval.

In the evaluations of curative activity, lesion diameter increase and coalescence resulting from some treatments prohibited accurate measurements of *C. moricola* growth; therefore, the percentage of compound leaves per tree showing increases in diameter of lesions was recorded (Table 4). Pecan leaves treated with bitertanol and flusilazol showed recurrence of growth of lesion diameter of 30.6 and 46.7%, respectively. Propiconazole-treated leaves had one-sixth as many leaves showing lesion diameter increase as bitertanol; flusilazol was relatively ineffective. Propiconazole demonstrated the best curative activity of the three fungicides evaluated, with only 4.8 (67 mg/L) and 4.1% (135 mg/L) of lesions continuing to increase in diameter in dew chambers. Lesions cured of *C. moricola* infection by propiconazole had a dark border that contrasted sharply with untreated lesions.

The percentages of leaf disks that produced sclerotia of *C. moricola* on CDY media were not significantly different among the fungicide treatments. Significant differences did occur, however, between the fungicide treatments and the check treatment. These results appear somewhat anomalous, because higher numbers of sclerotia did not develop from bitertanol and flusilazol treatments on CDY media.

Immature terminal leaflets on compound leaves treated with propiconazole were darker green and crinkled. Leaves that were apparently fully expanded or mature when propiconazole was applied showed no adverse effects.

DISCUSSION

That conidia of *C. moricola* can be produced on autoclaved pecan leaves in petri dish humidity chambers eliminates the need for wounding living pecan leaves through an inoculum disk to achieve infection (2,5). Also, the discovery that holly leaves support the production of conidia of *C. moricola* facilitates year-round research with this fungus wherever holly can be grown. Because the fungus has a wide host range (15), *C. moricola* can possibly be cultured on autoclaved leaves of other plant genera.

These results differ from those of Yokoyama and Tubaki (17), who reported conidial production on malt extract agar, and may indicate a physiological difference between Alabama and Japanese isolates. Fastidious characteristics of *C. moricola* were shown by abundant conidial production on healthy pecan leaves but not on senescent leaves or leaves debilitated by species of Capnodiaceae. Also, conidia developed only on leaf substrates under light.

Szkolnik (14) reported the ergosterol biosynthesis inhibitor (EBI) fungicides in general were weak to ineffective protectants against *Venturia inaequalis*, but he found that CGA-64251 (etaconazole) gave good protection compared with bitertanol, fenapanil, fenarimol, prochloraz, triadimefon, and triforine. Sutton (13) reported that application of propiconazole to foliage of wheat was highly effective against *Mycosphaerella graminicola* and other blotch diseases for at least 179 days. In my orchard evaluations for control of *C. caryigenum*, applications of propiconazole on a 3-wk preventive schedule have given superior control compared with bitertanol, etaconazole, fenarimol, and nuarimol (6,7). Apparently, propiconazole cannot be metabolized as rapidly as some EBI fungicides, thus permitting the long activity in orchard foliage and nut shucks against *C. caryigenum*.

The development of *C. moricola* sclerotia on CDY agar makes it a good indicator medium for the fungus despite the frequent presence of other fungi. Possible reasons for the low recovery of *C. moricola* from leaves treated with propiconazole to eradicate the pathogen include: 1) *C. moricola* may be a weak competitive saprophyte, 2) *C. moricola* may be very sensitive to propiconazole, or 3) a combination of 1 and 2, where the fungus may have been weakened by propiconazole and as such could not compete effectively with leaf-saprophytic fungi and bacteria.

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Table 4. Postinfection growth of *Cristulariella moricola* from pecan leaves and on agar after a curative application of a fungicide to potted trees

Fungicide treatment	Rate per liter	Lesion diameter (mm) at treatment[w]	Growth from leaves[x] (%)	Production of sclerotia from leaf disks in CDY medium[y]
Bitertanol 50W	60.00 mg	5.37	30.6 b[z]	1.7 a
Flusilazol 40E	4.05 g	4.92	46.7 bc	8.0 a
Propiconazole 3.6E	67.00 mg	4.24	4.8 a	5.4 a
Propiconazole 3.6E	135.00 mg	4.25	4.1 a	2.1 a
Check	···	4.30	55.8 c	21.9 b

[w] Lesion diameter (means) in leaves of seven trees after 4 days of incubation.
[x] Percentage of compound leaves per tree showing increases in diameter of lesions after fungicide treatment and repeat incubation in dew chambers.
[y] Percentage of leaf disks per treatment that produced sclerotia of *C. moricola* on Czapek Dox + yeast extract agar.
[z] Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

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