# Effect of Temperature and Wetness Duration on Apple Fruit Infection and Eradicant Activity of Fungicides Against *Botryosphaeria dothidea*

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**ABSTRACT**

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A model to forecast infection of apple (*Malus domestica*) fruit by *Botryosphaeria dothidea* was developed based on results of studies with a combination of six temperatures (from 8 to 28 C) and nine wetness durations (from 2 to 48 hr). Fruit infection increased with wetness duration and temperature and could be described by the model $y = -0.1546 + 0.0123T + 0.0329W - 0.00169W^2 + 0.0000225W^3 - 0.00153(TW) + 0.000111(TW^2) - 0.00000151(TW^3)$, where $y$ = percentage of diseased apple pieces, $T$ = temperature (C), and $W$ = wetness duration (hr). Reduction of growth of *B. dothidea* on fungicide-amended agar was used to determine $EC_{50}$ values of nine fungicides: benomyl, bitertanol, fenbuconazole, flusilazole, mancozeb, myclobutanil, penconazole, tebuconazole, and triflumizole. Three of the most active fungicides (benomyl, flusilazole, and tebuconazole), triflumizole, and mancozeb were tested for protectant activity in the field and for after-infection activity in the laboratory and field. No significant difference was detected among the five fungicides in after-infection or protectant activity.

*Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not. causes fruit rot (white rot or Bot rot) and a limb canker on apples (*Malus domestica* Borkh.). Fruit symptoms are usually observed 6–8 wk before harvest. The diseases occur in warm apple-growing regions of the world and account for up to 50% fruit loss (9), extensive limb loss, and tree death.

White rot on apples is controlled by a combination of cultural practices and fungicides. Botryosphaeria cankers, colonized dead limbs and fire blight strikes, and mummies are inoculum sources and should be pruned out and either removed from the orchard or flail-chopped (8). Preventive fungicide spray programs are generally not initiated until the soluble solids level reaches 10.0%, when fruit are thought to become susceptible to infection by *B. dothidea* (2,4). However, soluble solids levels fluctuate throughout the growing season (6) and may not be a reliable indicator of fruit susceptibility. Benomyl, captan, and thiophanate-methyl are effective fungicides when applied preventively; however, the possible after-infection activity of these and other fungicides on *B. dothidea* has not been examined.

Different temperatures have been reported as optimal for germination of spores of *B. dothidea*, fruit infection, and subsequent symptom development. Eid (3) found 25–30 C to be optimal for spore germination, whereas Kohn and Hendrix (4) found the greatest incidence of infection of Top Red Delicious fruit at 30–35 C and the most rapid development of symptoms at 26–32 C. Sutton and Arauz (10) studied the combined effects of temperature and moisture on germination of ascospores and conidia of *B. dothidea*. Isolates varied in the percentage germination of conidia at different combinations of temperature and relative humidity; no germination occurred at 8 C even in free water with a 12-hr incubation. The predicted optimal temperatures for germination of ascospores and conidia were 24.6 and 26.7–29.5 C, respectively. None of these studies investigated the interaction of moisture duration and temperature with regard to fruit infection.

The objective of this study was to investigate the effect of moisture duration and incubation temperature on infection of apple fruit by *B. dothidea* in order to ascertain the most favorable conditions for infection of apple fruit. The protectant and after-infection activity of selected fungicides against *B. dothidea* was also investigated.

## MATERIALS AND METHODS

**Temperature-wetness duration study.** Six temperatures (8, 12, 16, 20, 24, and 28 C) and nine incubation durations (2, 4, 8, 12, 16, 20, 24, 36, and 48 hr) were examined (except in the first run, when the 2-, 4-, and 8-hr treatments at 8 and 12 C, the 36- and 48-hr treatments at 20 C, and the 24-, 36-, and 48-hr treatments at 24 and 28 C were omitted). Soluble solids levels were determined with a refractometer from a subsample of 10 fruit used in each test. Four wounded and four nonwounded apples (unsprayed Golden Delicious) were used for each temperature-wetness combination in 1990. Wounds 1–2 mm deep were made with five no. 1 insect pins pushed through a cork stopper. Only nonwounded fruit were used in 1991. Fruit were surface-disinfested in a 0.53% NaOCl solution for 20 sec, rinsed, placed in plastic boxes (17.5 × 12.7 × 6.4 cm), and preconditioned at the appropriate temperature for 12 hr before inoculation.

Inoculum was prepared as follows: deionized water was poured onto 2-wk-old cultures of *B. dothidea* (approximately equal proportions of isolates 1500, 1501, and 1502 grown on potato-dextrose agar [PDA]), and the surface of the mycelial mat was scraped to remove conidia. Water and conidia were blended for 15 sec and filtered through a double layer of cheesecloth. Inoculum was standardized to $1 \times 10^5$ conidia per milliliter with the aid of a hemacytometer.

The area to be inoculated on each fruit was marked with a wax pencil. A 2.5 × 2.5-cm piece of four-ply laboratory towel that had been dipped in the conidial suspension was placed on each marked area. A 6 × 6-cm piece of aluminum foil was used to hold the towel in place and prevent drying. Inoculated apples were returned to the plastic boxes (which were lined with a moist paper towel), covered with a piece of aluminum foil, and placed in incubators. Apples were removed from incubators after the appropriate incubation period and were wiped with 95% ethanol to eliminate surface inoculum. Preliminary studies indicated that this technique was effective in removing surface inoculum.

The inoculated area was sampled from each apple by removing the five wounded areas with a no. 2 cork borer (if fruit was wounded) or a 2 × 2-cm portion of the surface, which was cut into nine pieces (each approximately 6 × 6 mm, with approximately 4 mm of underlying tissue) (if nonwounded). Fruit pieces were placed in a petri dish lined with a moist paper towel. Dishes were sealed with Parafilm (American Can Co., Greenwich, CT) and left at room temperature (about 22 C) under continuous light until sporulating pycnidia were observed on the surface of the fruit pieces (usually 1 mo).

The experiment was designed as a split

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plot with temperature as the whole plot and wetting duration as the subplot. Data were recorded as the percentage of pieces with pycnidia of *B. dothidea* out of nine (or five if fruit was wounded) total pieces observed for each apple and were normalized by the arcsine-square root transformation. The experiment was conducted three times both years, about 2, 3, and 4 mo after petal fall. Data from all runs were analyzed together with linear regression (7).

**Determination of $EC_{50}$ values of selected fungicides.** Nine fungicides were tested for in vitro activity against *B. dothidea:* benomyl (Benlate 50WP), bitertanol (Baycor 50W), fenbuconazole (RH7592 2F; Rohm & Haas Co., Philadelphia, PA), flusilazole (Nustar 40W), mancozeb (Dithane 75DF), myclobutanil (Nova 40W), penconazole (Topas 10W), tebuconazole (Elite 45DF), and triflumizole (Procure 50W). All fungicides except mancozeb were solubilized in ethanol.

Molten PDA was amended with each fungicide (0.1, 1, 10, 50, and 100 g a.i./ml of mancozeb and 0.01, 0.1, 0.5, 1, and 5 $\mu$g a.i./ml of the other fungicides) before the mixture was poured into 9-cm-diam petri dishes. An equivalent amount of ethanol was added to mancozeb-amended PDA for consistency among fungicides. Three isolates—1501 (Cleveland Co., NC) and CB1 and RFC (Clayton, NC)—were evaluated; a 5-mm-diam plug of each isolate was placed in the center of fungicide-amended PDA in each culture dish. The fungus was allowed to grow for 6 days at 26 C, and two radial measurements of each colony were recorded. Radial measurements were subtracted from radial growth on unamended PDA, and the $\log_{10}$ of the fungicide concentration was regressed on the probit of the percentage reduction in radial growth to determine the $EC_{50}$ value. The concentration that resulted in 50% reduction in radial growth ($EC_{50}$) was computed for each fungicide and isolate. Each fungicide concentration-isolate combination was replicated three times, and the experiment was conducted twice. Benomyl, tebuconazole, flusilazole, triflumizole, and mancozeb were selected for use in protectant and after-infection studies.

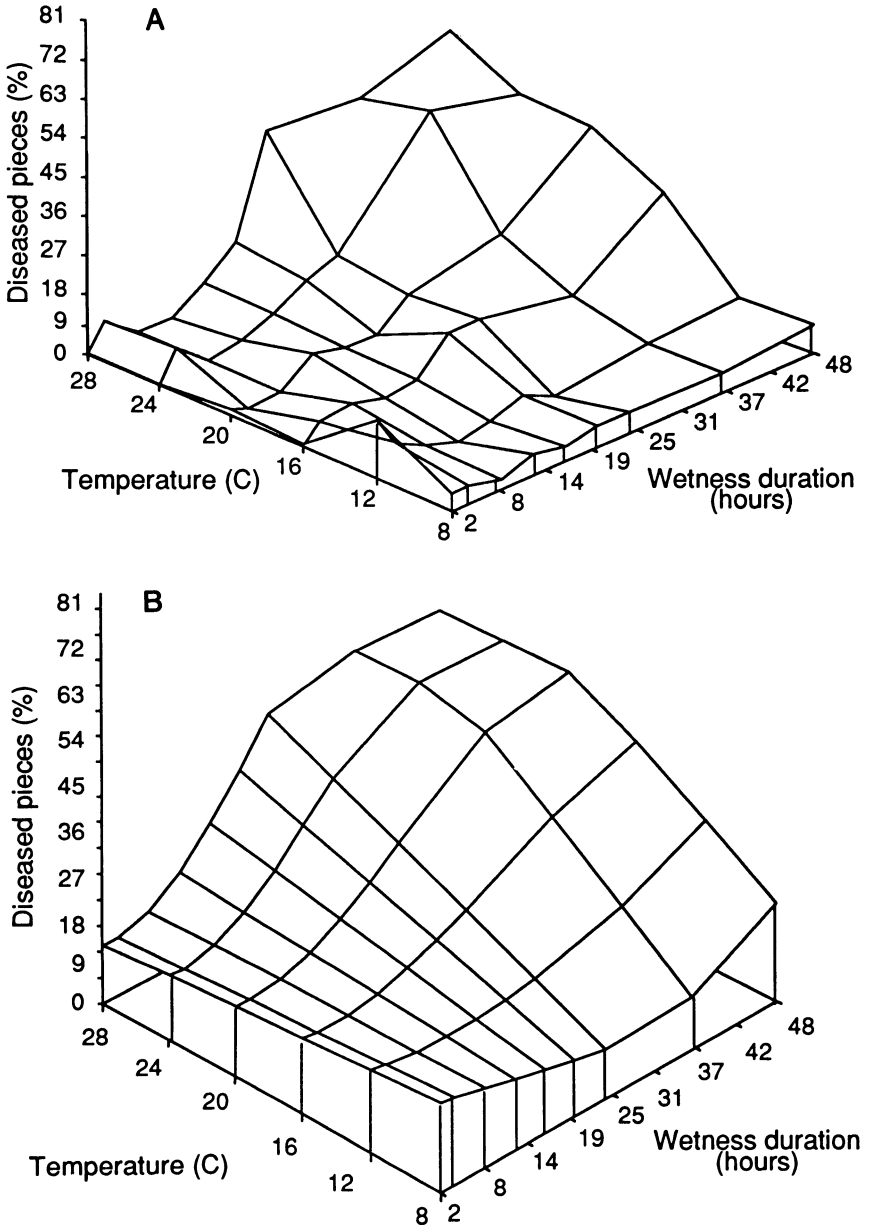


**Fig. 1.** Percentage of diseased apple pieces observed over various temperatures (8–28 C) and wetness durations (2–48 hr) **(A)** and the response surface developed through regression of wetness duration and temperature on percentage of diseased apple pieces **(B).** The relationship between wetness duration, temperature, and disease can be described with the model $y = 0.256 - 0.003T - 0.000088W - 0.00083W^2 + 0.000015W^3 - 0.00033(TW) + 0.000083(TW^2) - 0.0000013(TW^3)$ ($R^2_{adjusted} = 0.85$), where $y$ = the percentage of diseased fruit pieces, $T$ = temperature (C), and $W$ = wetness duration (hr).

**Evaluation of protectant activity on detached apple fruit.** Unsprayed Golden Delicious fruit were collected, washed, surface-disinfested, and allowed to dry. Fruit were dipped into fungicide suspensions at the following concentrations ($\mu$g a.i./ml): benomyl, 112; flusilazole, 19; mancozeb, 2,000; tebuconazole, 101; triflumizole, 300; fruit were then left to dry for 24 hr. These rates were selected based on manufacturers' recommendations. Fruit were inoculated with approximately equal mixtures of conidia of isolates 1500, 1501, 1502, and 1514 and were incubated at 28 C for 24 hr. A 2 × 2-cm portion of the surface was sectioned and incubated as described previously.

The experiment was conducted as a randomized complete block, with five fruit per treatment. Control fruit were not treated with fungicides and were either inoculated or not inoculated. Data were recorded 1 mo after sectioning as the number of fruit pieces with pycnidia out of nine and were transformed by arcsin $\sqrt{y}$ before analysis of variance (7) was performed. Differences among fungicides were tested with the mean square for replication × fungicide as an error term. Duncan's multiple range test was used to compare treatment means. The experiment was repeated three times.

**Evaluation of after-infection activity on detached apple fruit.** Unsprayed Golden Delicious fruit were harvested, surface-disinfested, and inoculated with $1 \times 10^5$ conidia per milliliter as described previously; 24, 48, 72, and 96 hr after inoculation, the fruit were treated with the same five fungicides at the same rates used in the protectant test. Inoculated fruit were placed in plastic boxes and incubated at 28 C for 24 hr. After incubation, fruit were wiped dry with laboratory towels to prevent further infection after the 24-hr wetness period

and were stored at room temperature (about 22 C). Fruit in the 24-hr treatment were dipped into the fungicide suspensions immediately after the initial incubation period. Fungicide-treated fruit were allowed to dry, returned to plastic boxes, and held at room temperature. The same procedure was followed at 48, 72, and 96 hr after inoculation. Five days after inoculation, fruit were wiped with sterile water to remove fungicide residues, allowed to dry, sectioned, and placed in petri dishes as described previously. Inoculated and uninoculated fruit not treated with fungicides were included as controls.

The experimental design was a randomized complete block. The three repetitions of the experiment were blocks. Four fruit were observed for each treatment within each repetition. Data were recorded as a percentage (number of fruit pieces showing symptoms out of nine) and were normalized with the arcsine-square root transformation. Analysis of variance was performed (7) with the mean square for replication × fungicide × hour as an error to test fungicide, hour, and fungicide × hour effects.

**Evaluation of after-infection activity in the field.** Apple fruit were inoculated in the field and subsequently treated with the same five fungicides at the same rates as described in the detached fruit tests. Fruit were inoculated as in earlier experiments with a suspension of $1 \times 10^5$ conidia of *B. dothidea* per milliliter. Entire fruit were wrapped with pieces of aluminum foil, which were removed, along with the inoculum-soaked laboratory towel, after 48 hr. Fruit were allowed to dry before they were dipped in the fungicide suspension (48-hr treatment). The same procedure was followed 96 hr after inoculation.

Fruit were left on the tree for 2 wk after the last fungicide application and were then brought to the laboratory, surface-disinfested, and sectioned as described previously. Data were recorded, transformed, and analyzed as described for the after-infection test on detached fruit.

The experimental design was a randomized complete block with the two replications as blocks. Two fruit were sampled for each treatment. Poor fruit set and losses to bitter rot (caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz.) prevented the use of more fruit per treatment and more than two replications.

## RESULTS

**Temperature-wetness duration study.** Wounded fruit developed symptoms at all temperature-wetness combinations. Thus, only data from nonwounded fruit were used in the analyses. In general, disease increased as temperature and wetness duration increased (Fig. 1A). The mean percentage of diseased pieces remained below 10% at all temperatures for incubations lasting 2–16 hr, except for the 2-hr incubation at 12 C, when 16.1% of apple pieces had symptoms. Disease increased dramatically after 24 hr of incubation: fewer than 6% of the pieces from fruit incubated at 8 and 12 C developed pycnidia; as many as 45% of pieces from apples incubated at 24 C had symptoms.

The relationship between temperature, duration of wetness, and disease could be described by the model $y = 0.256 - 0.003T - 0.000088W - 0.00083W^2 + 0.000015W^3 - 0.00033(TW) + 0.000083(TW^2) - 0.0000013(TW^3)$ ($R^2_{adjusted} = 0.85$), where $y$ = the percentage of diseased fruit pieces, $T$ = temperature (C), and $W$ = wetness duration (hr). The standard errors for the parameters are as follows: $B_0 = 0.07674$, $B_1 = 0.00384$, $B_2 = 0.01443$, $B_3 = 0.00073$, $B_4 = 0.00001$, $B_5 = 0.00075$, $B_6 = 0.00004$, and $B_7 = 0.000001$. The response surface depicted by the model is illustrated in Figure 1B.

Soluble solids were 7.7, 8.9, and 10.4% for the 3 July, 20 July, and 16 August inoculations, respectively, in 1990 and 8.5, 10.0, and 10.7% for the 11 June, 24 July, and 10 August inoculations, respectively, in 1991. No difference among runs could be attributed to the level of soluble solids.

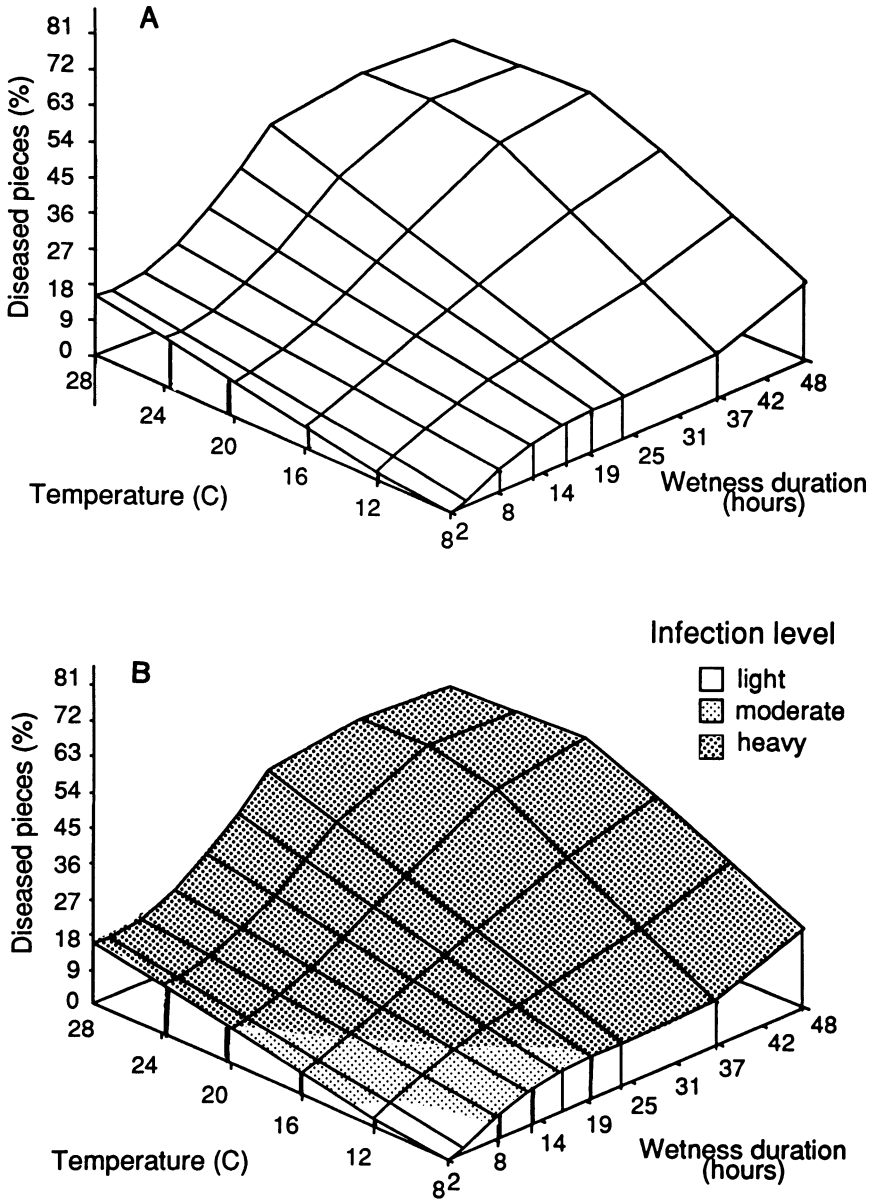


**Fig. 2. (A)** The response surface generated by regression of wetness duration and temperature on percentage of diseased apple pieces when combinations of less than 17 C and 2 hr, less than 13 C and 4 hr, and less than 9 C and up to 12 hr were set to zero. The resulting model was $y = -0.1546 + 0.0123T + 0.0329W - 0.00169W^2 + 0.0000225W^3 - 0.00153(TW) + 0.000111(TW^2) - 0.00000151(TW^3)$ ($R^2_{adjusted} = 0.84$), where $y$ = the percentage of diseased fruit pieces, $T$ = temperature (C), and $W$ = wetness duration (hr). **(B)** The same response surface with increasingly dark stippling indicating light (0–5%), moderate (6–15%), and heavy (>15%) infection.

Sutton and Arauz (10) found that ascospores and conidia of *B. dothidea* failed to germinate at temperatures below 17 C when incubated for 2 hr, below 13 C when incubated for 4 hr, and below 9 C when incubated for up to 12 hr. We assumed that infections that we observed under these conditions (Fig. 1A) were the result of natural infection. When we eliminated those specific data from the data set and reanalyzed the data (Fig. 2A), the resulting model was $y = -0.1546 + 0.0123T + 0.0329W - 0.00169W^2 + 0.0000225W^3 - 0.00153(TW) + 0.000111(TW^2) - 0.00000151(TW^3)$ ($R^2_{adjusted} = 0.84$). The standard errors for the parameters are as follows: $B_0 = 0.07236$, $B_1 = 0.00366$, $B_2 = 0.01392$, $B_3 = 0.00071$, $B_4 = 0.00001$, $B_5 = 0.00073$, $B_6 = 0.00004$, and $B_7 = 0.0000005$.

**Determination of $EC_{50}$ values of selected fungicides.** Isolate RFC was more sensitive ($P = 0.05$) than isolates CB1 and 1501 to all fungicides; $EC_{50}$ values of isolate RFC were approximately 10% of those of the other two isolates for all fungicides. $EC_{50}$ values (in μg a.i./ml) for flusilazole, penconazole, myclobutanil, tebuconazole, benomyl, triflumizole, bitertanol, mancozeb, and fenbuconazole were 0.022, 0.133, 1.046, 0.024, 0.041, 0.054, 0.027, >100, and 0.262, respectively, for isolate RFC. $EC_{50}$ values for isolates CB1 and 1501 are presented in Figure 3. In run 1, isolate CB1 was more sensitive than isolate 1501 to fenbuconazole and myclobutanil; however, there was no significant difference between these two isolates in run 2.

Isolates generally responded similarly to the nine fungicides. The four most active fungicides in both runs were benomyl, bitertanol, tebuconazole, and flusilazole. Mancozeb was the least active fungicide tested. Results were consistent between runs except for myclobutanil and mancozeb, both of which demonstrated less activity in run 2 than in run 1.

**Evaluation of protectant activity on detached apple fruit.** No difference ($P = 0.05$) was detected among the five fungicides in protectant activity. The fungicides from most to least effective were benomyl, tebuconazole, mancozeb, flusilazole, and triflumizole.

**Evaluation of after-infection activity on detached apple fruit.** None of the fungicides applied after infection significantly reduced the number of diseased fruit pieces compared to the inoculated control ($P = 0.05$) in a test with the mean square for the three-way interaction as an error term (replication × fungicide × hour). The fungicides from most to least effective were mancozeb, tebuconazole, flusilazole, triflumizole, and benomyl.

In general, the number of diseased pieces increased with the length of time before fungicide treatment; however, there was no difference ($P = 0.05$) in the number of hours that elapsed after inoculation and before treatment for any of the fungicides. The fungicide × hour interaction was not significant when all three runs were analyzed together.

Because the three-way interaction was significant at $P = 0.05$, we analyzed individual runs separately. These analyses indicated that fungicides differed ($P = 0.05$) in their eradicant activities in the first run: tebuconazole and mancozeb demonstrated the greatest eradicant activity and triflumizole the least. There was no significant difference among fungicides in the second and third runs; however, the rankings of the fungicides in the first and second runs were similar.

The effect of time on the activities of fungicides as eradicants was evident in the first two runs; disease increased significantly with increasing time between inoculation and treatment in the first run ($P = 0.05$) but increased only slightly in the second run ($P = 0.10$). Time was not significant in the third run.

The ranking of the fungicides' after-infection activity changed over time in the first run, as indicated by a significant fungicide × hour interaction ($P = 0.05$). For example, flusilazole showed the most after-infection activity at 24 hr but was the least effective at 48 and 72 hr.

**Evaluation of after-infection activity in the field.** There was no difference ($P = 0.05$) among the five fungicides, hours between inoculation and treatment, or the fungicide × hour interaction. None of the fungicides were statistically different from the untreated inoculated controls. The treatments from most to least effective against disease development were flusilazole, triflumizole, tebuconazole, untreated inoculated control, mancozeb, and benomyl. Fewer diseased pieces were observed when fruit were treated 48 hr rather than 96 hr after inoculation.

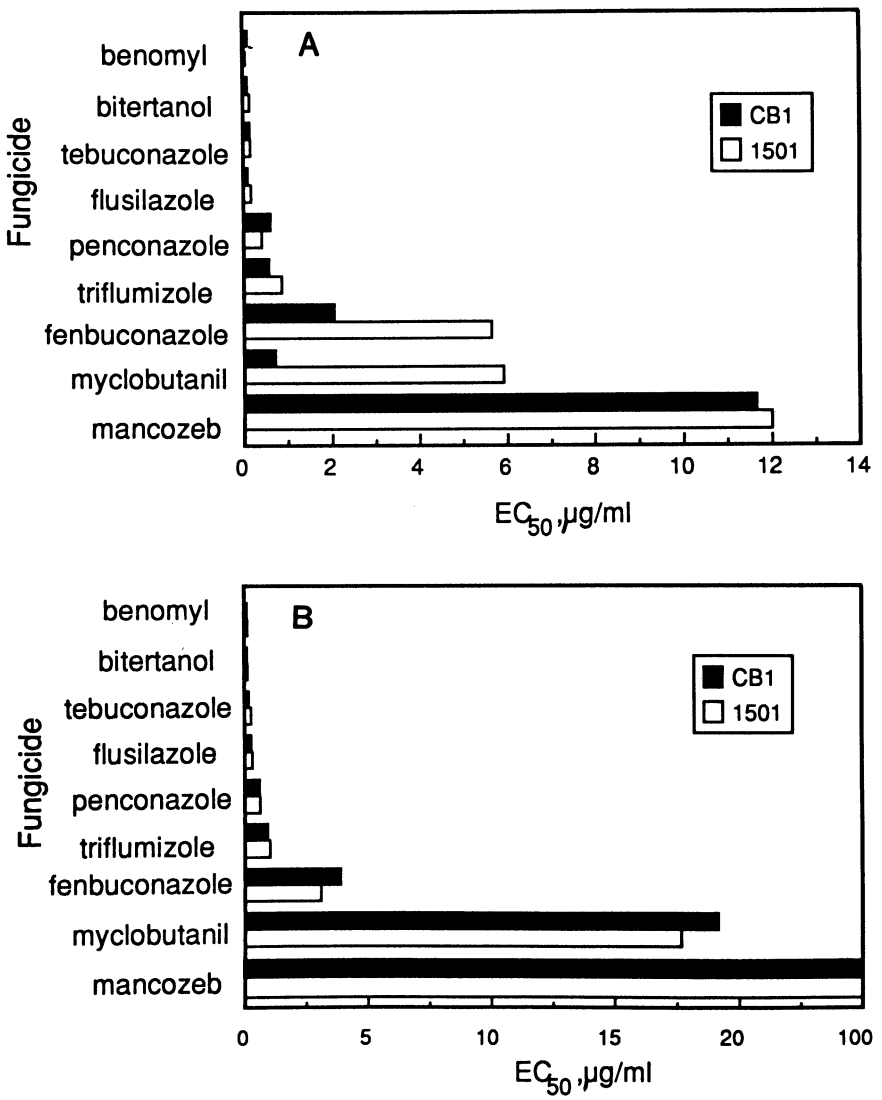


**Fig. 3.** $EC_{50}$ values for nine fungicides determined in vitro with isolates CB1 and 1501 of *Botryosphaeria dothidea* in run 1 **(A)** and run 2 **(B)**.

## DISCUSSION

Infection of apple fruit by *B. dothidea* increased with the duration of wetness at temperatures that are common in the summer in the southeastern United States (16–28 C). Some fruit infection

was observed in as short a time as 2 hr and at temperatures as low as 12 C; infection was likely during wetting periods of 8–10 hr or longer. Similarly, Kohn and Hendrix (4) observed numerous fruit infections when fruit were incubated for short durations (0–8 hr) at 30 C (the only temperature tested in their free-moisture study). In a separate study in the same paper, they found that a cubic model described the temperature-infection relationship. In our model a linear term was adequate to describe the increase in infection with increasing temperature. One reason for the difference between these models is that we tested temperatures only up to 28 C, whereas Kohn and Hendrix tested higher temperatures (up to 40 C). At the temperatures common to both studies, similar responses were observed. We did not include temperatures above 28 C, even though Kohn and Hendrix found the most infection at 30–35 C, because temperatures above 28 C are unlikely during rainy periods in the southeastern United States. Also, establishing the optimal temperature for infection was not an objective of our study.

None of the fungicides tested provided suitable protectant or eradicant activity in our tests with detached fruit. The lack of protectant activity of some fungicides was unexpected, because several fungicides demonstrated good in vitro activity and benomyl provides good control of the disease in the orchard. Arauz and Sutton (1) found that tebuconazole provided good protectant activity against *B. obtusa* on apple foliage but did not include fruit in their protectant study. Fruit treated with tebuconazole had the second lowest mean disease rating in our study.

The failure to detect fungicides with significant after-infection activity on fruit is in part the result of the large error term used to test the effect of fungicides. In addition to this statistical explanation for the similarity of after-infection activities of the fungicides tested, it is possible that many fungicides that have locally systemic activity on foliage generally do not partition into the fruit. Sutton et al (11) demonstrated that fenarimol (an ergosterol biosynthesis inhibitor fungicide) primarily remains associated with the apple cuticle. Thus, under the conditions of this test (24-hr incubation at 28 C), the fungus may have colonized fruit tissue that was not reached by the fungicides. If we had used a shorter incubation period or had incubated fruit at less favorable temperatures, some after-infection activity might have been observed. Arauz and Sutton (1) found that tebuconazole and benomyl effectively reduced disease severity on fruit when they were applied within 96 hr of inoculation with *B. obtusa,* which causes black rot of apple fruit.

Because infections could occur during most wetting events in the summer in the southeastern United States and because of the apparent lack of currently registered fungicides with useful after-infection activity, preventive fungicide applications are needed to control *B. dothidea.* Our model should provide growers with information concerning the conditions that favor infection and may aid them in modifying spray schedules.

To make our model more useful to growers, we arbitrarily identified conditions in which the potential for infection is light (0–5% infection), moderate (6–15% infection), and high (more than 15% infection) (Fig. 2B). We believe these divisions are conservative based on our observations during the course of this study. Growers with orchards with a history of white rot should be alerted to apply preventive sprays if weather forecasts indicate conditions associated with high to moderate infection risk or to shorten spray intervals if rainfall and infections are frequent. Growers with little history of disease need not be as concerned about the occurrence of light infection periods of short duration. However, lengthy wetting periods (24 hr or more) at warm temperatures (16 C or above) should concern all growers.

Our model should be tested in the field before it is recommended to growers; our definition of light, moderate, and high risk of infection was arbitrary. The development of fungicides with good after-infection activity against *B. dothidea* would improve the effectiveness of the model and perhaps enable it to be used in an after-infection program such as that currently used for apple scab (5).


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