Influence of Temperature and Wetness Duration on Infection of Apple Leaves and Virulence of Different Isolates of *Alternaria mali*

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The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service or criticism of similar ones not mentioned.
We thank Ray Pope for technical assistance and Marie Davidian, Department of Statistics, North Carolina State University, for help in the statistical analysis.
Accepted for publication 16 July 1992.

ABSTRACT

Filajdić, N., and Sutton, T. B. 1992. Influence of temperature and wetness duration on infection of apple leaves and virulence of different isolates of *Alternaria mali*. Phytopathology 82:1279-1283.

The effect of combinations of nine different temperatures (4–36 C) and eight wetness periods (2–48 h) on the infection of Delicious apple (*Malus domestica*) seedlings by *Alternaria mali* was studied. Disease severity increased with increased wetness duration over all the temperatures tested and was greatest from 12 to 28 C. The influence of temperature and wetness duration on infection of apple seedlings by *A. mali* was described by the model: $Y_{ij} = -6.4580 + 0.1853T_i + 0.0912W_j - 0.0033T_i^2 - 0.0030W_j^2 + 0.0194T_iW_j - 0.0005T_i^2W_j$, in which $Y = \log_{10}$(percentage of leaf area covered with lesions + 0.01), T = temperature (C), W = wetness duration (h), i = individual temperature treatment, and j = individual wetness duration treatment. The predicted optimum temperature for infection was 23.5 C. At this temperature, 5.1 h of wetness was required for light infection (0.2% leaf area covered with lesions). The model derived from laboratory data was tested in the field with healthy seedlings that were surrounded by inoculated ones. Conidia of *A. mali* were trapped during 14 wetness periods; infection criteria were met during 10 of these periods. No false negatives occurred; however, six false positives were recorded. A predictive model for first occurrence of Alternaria blotch developed in South Korea was evaluated under North Carolina conditions; it predicted first occurrence of the disease 1 wk earlier in 1990 and 5 days later in 1991 than disease was observed in the field. Eight isolates of *A. mali* from western North Carolina were tested for pathogenicity and virulence in the laboratory. All were pathogenic on Delicious seedlings but varied in virulence.

Alternaria blotch of apple (*Malus domestica* Borkh.), caused by *Alternaria mali* Roberts, has recently become a problem in the southeastern United States (3) and has the potential of becoming an important disease throughout the apple-producing regions of the eastern United States. Alternaria blotch is economically the most important disease of apples in Japan, South Korea, and other Asian countries. An outbreak of the disease in Japan was noted in 1956 and appeared to correspond with increased cultivation of susceptible varieties such as Delicious and Indo (12). Delicious is the most common variety grown in the southeastern United States. Symptoms on leaves appear as circular brown spots 2–5 mm in diameter, and severe infection can result in 60–80% defoliation (3). Fruit of some very susceptible varieties such as Indo also can be affected.

The biology and epidemiology of *A. mali* have been studied in Japan and South Korea (6,12). An empirical model for forecasting the first occurrence of Alternaria blotch (based on rainfall and temperature after the phenophase tight cluster) was developed by Kim et al (6). Although data are available on the biology and epidemiology of Alternaria blotch, criteria necessary for infection during individual wetness periods have not been established.

The objectives of this study were to determine criteria for infection of apple leaves by isolates of *A. mali* from North Carolina and to use these data to develop a predictive model that could eventually be incorporated into a disease management plan for use in the southeastern United States. The forecasting model developed in South Korea was also evaluated for its applicability in North Carolina, and virulence of different isolates of *A. mali* from North Carolina was compared. A preliminary report has been published (4).

MATERIALS AND METHODS

Screening of apple seedlings for susceptibility to *A. mali*. Because resistance to *A. mali* is conferred by a single recessive gene (9), open-pollinated seedlings of Delicious apples used in the study were screened first for susceptibility to *A. mali*. Cultures of *A. mali* were transferred, with a sterilized loop, from test tubes onto fresh potato-dextrose agar (PDA) in petri dishes and grown under continuous light at 24 C for 7 days. We prepared a spore suspension composed of eight isolates by flooding cultures with sterile distilled water, scraping the cultures with a sterilized razor blade, and straining the mycelium and conidia through a double layer of cheesecloth into a 1-L flask. The spore concentration was adjusted with the aid of a hemacytometer to 1×10^5 conidia per milliliter. Two drops of Tween 20 (ICI Americas, Inc., Richmond, CA) were added to the suspension to ensure uniform wetness of the leaf surface.

Leaves (usually the second or third expanded leaf from the top) from individual, 1-mo-old Delicious seedlings were detached along with petioles and placed on the top of hardware cloth in plastic chambers (30.5 × 12.5 × 6 cm). Each chamber had two wet paper towels on the bottom for maintaining high relative humidity. Twenty leaves were placed in each chamber with their adaxial surfaces up (two rows of 10 leaves each). We inoculated leaves with the inoculum suspension by spraying them to the drip point with an artist's airbrush. Chambers were closed and placed in plastic bags that were sealed with masking tape. After 7 days, leaves were observed for lesions. To verify that lesions were caused by *A. mali*, we made isolations from leaves on PDA in petri dishes by cutting 2- × 2-mm pieces of lesion area along with healthy leaf tissue.

Virulence of *A. mali* isolates. *A. mali* was isolated from leaves with typical Alternaria blotch symptoms from eight orchards in Henderson, Wilkes, and Alexander counties in western North Carolina (isolates 1522, 1528, 1530, 1531, 1540, 1544, 1550, and 1556). We surface-disinfected the leaves by dipping them into 0.5% NaOCl for 15–20 s. Leaves were then dried with a laboratory towel, and portions of lesions along with healthy leaf tissue were

removed (approximately 2 × 2 mm in size) and placed on PDA in petri dishes. The fungus was grown under continuous light at 24 C for 4 days, and resulting colonies of *A. mali* were transferred to PDA in slant tubes and placed under the same conditions for an additional 6 days. Test tubes of pure cultures were then stored in a refrigerator at 4 C in the dark.

Inoculation of apple seedlings with isolates of *A. mali*. For each of the eight isolates of *A. mali*, nine susceptible Delicious seedlings were inoculated with a spore suspension of 1×10^5 conidia per milliliter. We inoculated seedlings by spraying both leaf surfaces with the conidial suspension to the drip point with an artist's airbrush. After inoculation, seedlings were placed into plastic bags, which contained one wet paper towel so that leaves remained wet, and were sealed with masking tape. Three seedlings were sprayed with distilled water (controls). A total of 25 seedlings, three seedlings inoculated with a spore suspension of each isolate, and one seedling inoculated with distilled water were placed in each of three environmental chambers at 24 C under continuous light for 7 days. Plastic bags were removed 48 h after inoculation. Seven days after inoculation, the 10 uppermost unfolded leaves from each seedling were rated for disease severity. The lower portion of the Horsfall-Barratt scale was used: 0 = no symptoms, 1 = 0–3% of the leaf area covered with lesions, 2 = 4–6%, 3 = 7–12%, 4 = 13–25%, and 5 = 26–50%. The experiment was repeated once. Variation in virulence of different isolates of *A. mali* was determined by use of the general linear model procedure from SAS (10). Means were separated by the Waller-Duncan *k*-ratio *t* test.

Temperature-wetness duration studies. Four isolates of *A. mali* (1522, 1531, 1544, and 1556 from Henderson County, North Carolina) were used for inoculation of Delicious seedlings. Isolates were transferred from test tubes to PDA in petri dishes and allowed to grow under continuous light at 24 C for 7 days. Two dishes of each isolate were then comminuted at high speed (Model 31BL92, Waring Products Division, Dynamics Corporation of America, New Hartford, CT) for 20–30 s and strained through a double layer of sterile cheesecloth into a 1-L flask. We adjusted the spore concentration with a hemacytometer to obtain 1×10^5 conidia per milliliter. Two drops of Tween 20 were added to the suspension.

One hundred and forty-four Delicious seedlings were inoculated with a conidial suspension of *A. mali* in each run. Because of loss of the ability of some isolates to sporulate in culture, only the following isolates were used: 1531, 1540, 1544, and 1556 (run 1); 1544 and 1556 (run 2); 1522 and 1544 (run 3); and 1522, 1530, 1544, and 1556 (run 4). Seedlings were approximately 1 mo old. Two seedlings were used for each temperature-wetness duration combination in each run.

Nine temperatures (4, 8, 12, 16, 20, 24, 28, 32, and 36 C) and eight wetness durations (2, 4, 6, 12, 18, 24, 36, and 48 h) were examined. Both leaf surfaces of seedlings were inoculated with the spore suspension (1×10^5 conidia per milliliter) to the drip point by using an artist's airbrush. Nine seedlings (one for each incubation chamber) were sprayed with distilled water as a control. After inoculation, seedlings were placed into plastic bags and sealed with masking tape. One wet paper towel was placed in each bag so that leaves would remain wet for the desired period. Plastic bags with seedlings were placed into environmental chambers (Model I-35LL, Percival MFG Co., Boone, IA) and maintained in continuous darkness. Temperatures in the chambers were monitored during the course of each experiment with a maximum-minimum thermometer and/or hygrothermograph. After the desired wetness period, we removed, unbagged, and observed seedlings to confirm that all leaves had remained wet for the prescribed period. Seedlings were left at 20–25 C until leaves dried (about 20 min) and were then placed at 20–25 C for 1 wk so that symptoms would develop. The 10 uppermost unfolded leaves on each seedling were rated for disease severity with the portion of the Horsfall-Barratt scale (rating virulences of 0–5) as described previously. The experiment was repeated three times.

Statistical analysis. Disease severity data were analyzed by linear regression analysis. Disease severity levels in the first two and the fourth run of the experiment were similar, but disease severity in the third run was much higher than in the other three runs. Observations in all runs were frequently equal to 0 (no symptoms), especially at temperatures outside the range (12–28 C) favorable for disease development and at shorter wetness periods (i.e., <6 h). This resulted in disease severity data that were non-normally distributed. Therefore, data were converted to percentage of leaf area affected and transformed to normalize them as $y^{0.5}$, in which y = percentage of leaf area covered with lesions + 0.5; $\log_{10}(y + 0.01)$; and arcsin $(x)^{0.5}$, in which x = proportion of leaf area covered with lesions. The residual plot of the nontransformed data was distinctly fan-shaped; all three transformations eliminated this pattern and provided a good distribution of residuals. The transformation $Y = \log_{10}$ (percentage of leaf area covered with lesions + 0.01) was used because it provided the best distribution of residuals and because of its simplicity.

The analysis was then performed with the Mixed Procedure (11). This procedure is used to fit linear models with fixed and random effects. In our case, random effects were associated with runs. The following model was used.

$$Y_{ij} = \beta_0 + \beta_1 T_i + \beta_2 W_j + \beta_3 T^2{}_i + \beta_4 W^2{}_j + \beta_5 T_i W_j + \beta_6 T^2{}_i W_j + \delta_{ijk} + \epsilon_{ijkl} \quad (1)$$

in which: $Y = \log_{10}$ (percentage of leaf area covered with lesions + 0.01), T = temperature (C), W = wetness duration (h), i = individual temperature treatment, j = individual wetness duration treatment, δ_{ijk} = random effect of runs, and ϵ_{ijkl} = unexplained variation. All possible combinations of temperatures and wetness periods examined were evaluated for significance by using a manual procedure (10). Regression analysis was conducted with means from all four runs. The regression models obtained were used to construct curves relating wetness periods and temperature to infection. The predicted temperature and wetness duration combinations necessary for light (0.2% of leaf area covered with lesions) and severe (1% of leaf area covered with lesions) leaf infections were obtained by solving for wetness durations from the fitted model.

Model evaluation in the field. Thirty, open-pollinated, 1-mo-old Delicious seedlings susceptible to *A. mali* were inoculated in the laboratory with a suspension of 1×10^5 conidia per milliliter by the method described previously and maintained at 24 C for 36 h under continuous light. After that time, seedlings were unbagged and placed in the greenhouse for 7 days to allow for symptom development. Thirty new seedlings were inoculated in the laboratory each month as described; they were used as a fresh inoculum source. Before each rain period, eight healthy Delicious seedlings were placed outdoors in one row under cages holding inoculated seedlings. Cages were inclined 45 degrees toward each other to completely surround healthy seedlings. Two funnel spore traps consisting of a plastic funnel 11 cm in diameter attached to a 1-L plastic bottle and containing 1 g of $CuSO_4$ dissolved in approximately 40 ml of tap water were placed among the healthy seedlings. Instruments for recording weather data (hygrothermograph and rain gauge, Belfort Instrument Co., Baltimore, MD; DeWit leaf wetness meter, Valley Streams Farm, Orano, Ontario) were placed about 2 m from the cages. After each wetness period, all seedlings were placed in a greenhouse at 20–25 C for 7 days so that symptoms would develop. After 7 days, disease severity was recorded on the 10 uppermost unfolded leaves, as previously described. The total number of conidia caught in both funnel traps was recorded after each wetness period.

Evaluation of the predictive model developed in South Korea. Cumulative degree days (CDD; referred to as cumulative degree portion (CDP) by Kim [3]) was calculated from orchards: the Central Crops Research Station in Clayton, NC, in 1990 and 1991, and the McKay orchard in Dana, NC, in 1990, beginning at the phenophase tight cluster. First occurrence of the disease was recorded in each orchard. Temperature at each location was measured with a hygrothermograph (Belfort Instruments) located in a standard instrument shelter. Rainfall was recorded with a

top-weighing gauge (Belfort Instruments). Conditions necessary for first symptom appearance according to the model are $\Sigma(T_d - 10) = 160$; with $R_f = 4$, T_d = average daily temperature and R_f = number of rainfalls after 160-cumulative degree days level was reached.

RESULTS

Screening of apple seedlings for susceptibility to *A. mali*. Over the 2-yr period, 3,270 seedlings were screened for susceptibility to *A. mali* in the greenhouse; 1,045 were susceptible (31.9%).

Virulence of isolates of *A. mali*. Overall mean disease severity was less in run 2 than in run 1. Isolates of *A. mali* have been reported to decrease in sporulation and pathogenicity after transfer and storage (6). Consequently, results from the two runs of the experiment were not combined but are reported separately. Virulence of all isolates, except 1522, diminished from run 1 to run 2. Isolates 1522, 1544, and 1556 from Henderson Co. generally resulted in the greatest amount of disease and largest lesion area on Delicious seedlings in both runs. Isolates from Henderson Co. (1522, 1530, 1544, and 1556), except 1550, generally showed a higher virulence than isolates from Alexander and Wilkes counties (1528, 1531, and 1540; Table 1).

Statistical analysis of temperature-wetness duration studies data. Disease severity on inoculated leaves increased with wetness duration over all temperatures tested. The disease was readily observed at temperatures between 12 and 28 C, whereas very little disease was observed at temperatures below and above that range. At temperatures out of the 12–28 C range, longer wetness periods were required for infection to occur.

The influence of temperature and wetness duration on infection of Delicious seedlings by *A. mali* was described by the following model.

$$Y_{ij} = -6.4580 + 0.1853T_i + 0.0912W_j - 0.0033T^2{}_i - 0.0030W^2{}_j + 0.0194T_iW_j - 0.0005T^2{}_iW_j \quad (2)$$

($R^2 = 59.2\%$; $R^2{}_a = 58.3\%$.) Parameters are as defined in Equation 1. For the model, the standard errors for the parameters are $\beta_0 = 0.6080$, $\beta_1 = 0.0676$, $\beta_2 = 0.0340$, $\beta_3 = 0.0017$, $\beta_4 = 0.0005$, $\beta_5 = 0.0028$, and $\beta_6 = 0.00007$. The relationship between temperature and wetness duration and their influence on the severity of the disease are illustrated in Figure 1A for all data and Figure 1B for the response surface predicted by the model. At 23.5 C (the predicted optimum temperature for leaf infection), only 5.1 h of wetness were required for light (0.2% leaf area affected) infection and 12.7 h for severe (1% leaf area affected) infection (Fig. 2).

Model evaluation in the field. There were 48 wetness periods between 6 April and 26 August 1990. Twenty-seven wetness periods were recorded as a result of rain, and the remaining periods were the result of dew or irrigation. Conidia of *A. mali* were not trapped during 34 wetness periods; infection criteria were met during 15 of these periods. During four wetness periods when conidia were trapped, temperature and moisture duration requirements for infection were not met. Infection of apple seedlings occurred on only four of 10 occasions when conidia were trapped and infection criteria were met (Fig. 2).

Evaluation of the predictive model developed in South Korea. Tight cluster at the Central Crops Research Station occurred on 5 April, and criteria for first symptom appearance were met on 12 May 1990. The disease was first observed on 19 May. In 1991, tight cluster occurred on 1 April, criteria for symptom appearance were met on 19 May, and the disease was first observed on 14 May. At McKay orchard (1991), tight cluster occurred on approximately 10 April, criteria for symptom appearance were met on 15 May, and the disease was first observed on 21 May.

TABLE 1. Pathogenicity of different isolates of *Alternaria mali*

Isolate	Mean disease severity[v]		Lesion area (%)	
	Run 1[w]	Run 2[x]	Run 1	Run 2
1544[y]	1.24 a[z]	0.70 a	2.52 a	1.59 ab
1556	0.81 ab	0.24 b	1.67 ab	0.43 bc
1522	0.53 bc	0.93 a	0.85 bc	1.92 a
1530	0.42 bc	0.30 b	0.80 bc	0.45 bc
1550	0.33 c	0.03 b	0.53 bc	0.04 c
1531	0.30 c	0.01 b	0.45 c	0.02 c
1528	0.29 c	0.02 b	0.55 bc	0.03 c
1540	0.20 c	0.03 b	0.30 c	0.05 c

[v] Based on a 0–5 scale in which 0 = no symptoms, 1 = 1–3% leaf area covered with lesions, 2 = 4–6%, 3 = 7–12%, 4 = 13–25%, 5 = 26–50%.
[w] First run of the experiment was conducted 3 July 1989.
[x] Second run of the experiment was conducted 19 October 1989.
[y] Isolates 1544, 1556, 1522, 1530, and 1550 are from Henderson County; 1531 and 1540 are from Alexander County; and 1528 is from Wilkes County.
[z] Means with columns followed by different letters are significantly different ($P = 0.05$), according to Waller-Duncan k-ratio t test.

DISCUSSION

Isolates of *A. mali* from the three North Carolina counties varied in virulence to Delicious seedlings as has been reported from Japan (7). Virulence in *A. mali* is correlated with AM-

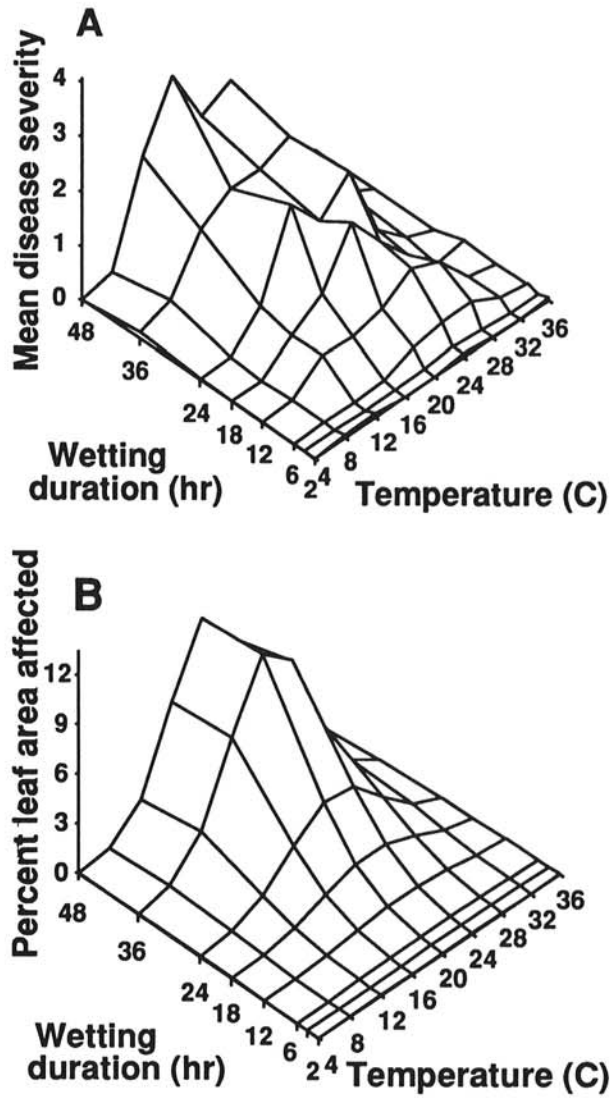

Fig. 1. A, Mean disease severity of Alternaria blotch on Delicious seedlings for different temperatures and wetness durations for all the data. **B,** Response surface fitted to the model: $Y_{ij} = -6.4580 + 0.1853T_i + 0.0912W_j - 0.0033T^2{}_i - 0.0030W^2{}_j + 0.0194T_iW_j - 0.0005T^2{}_iW_j$, in which $Y = \log_{10}$ (mean percentage of leaf area covered with lesions + 0.01), T = temperature (C), W = wetness duration (h), i = temperature treatment, and j = wetness duration treatment.

toxin production (7). In our study, all isolates (except for one) had reduced virulence from run 1 to run 2. Kohmoto et al (6) found that isolates of *A. mali* lost virulence in culture and related it to the loss of ability to produce AM-toxin.

The variability among runs in our temperature-wetness duration study is probably a reflection of isolate variation, because the condition of the plant material and environmental conditions were similar. Because of loss of pathogenicity in culture, different isolates had to be used in each run. Isolates used in the third run were more virulent on average than those used in the other three runs. Although the amount of disease recorded in the third run was substantially higher than in the first two and the fourth runs, it was uniformly distributed over all observations.

Use of different isolates in the temperature-wetness duration study reflects the natural variation that would be expected in the orchard. However, because of this isolate variability, variation in disease severity among runs was fairly large. Some variation also could be attributed to our use of seedlings. Although seedlings were screened for susceptibility, there is some variation in symptom severity (6). The use of the Mixed Procedure (11) helped us account for this variation in the disease severity data among runs. This new procedure will be useful for other biological studies that involve fixed and random effects.

The model we developed represents an attempt to characterize the relationship among temperature, wetness duration, and disease severity and to account for the variation in the data. The model presented in this paper accounted for only 59% of the variation. However, we have confidence in the minimum requirements for infection (i.e., 0.2% and 1% of leaf area affected) depicted by the curves in Figure 2. This confidence is based on perusal of the actual data for the four runs and on our validation experiments. Thresholds for light and moderate infection were chosen arbitrarily with the knowledge that disease spreads very rapidly once it begins (3). In the validation experiments when no conidia were trapped, no false negatives occurred. However, six false positives were recorded. The model differs somewhat from what we reported in the preliminary study (4), because an additional run of the experiment was conducted and is included in the analyses presented in this study.

The Korean model was developed to predict the time of first disease occurrence. In 1990 and 1991, disease was observed in orchards at the Central Crops Research Station and McKay within 1 wk of predicted occurrence. Because the choice of the date of the phenophase tight cluster is somewhat arbitrary, the predicted date of disease occurrence was probably within an acceptable range, and the model could be used to time the first fungicide application. On the basis of the Korean model, in 1990 and 1991, the first fungicide application would have been made at second cover. Currently, there are no fungicides registered in the United States that adequately control Alternaria blotch. In experimental plots, iprodione provided good control when applied on a 14-day schedule beginning at petal fall (5). A similar spray schedule is used in Japan where iprodione is rotated with oxine-copper and polyoxin.

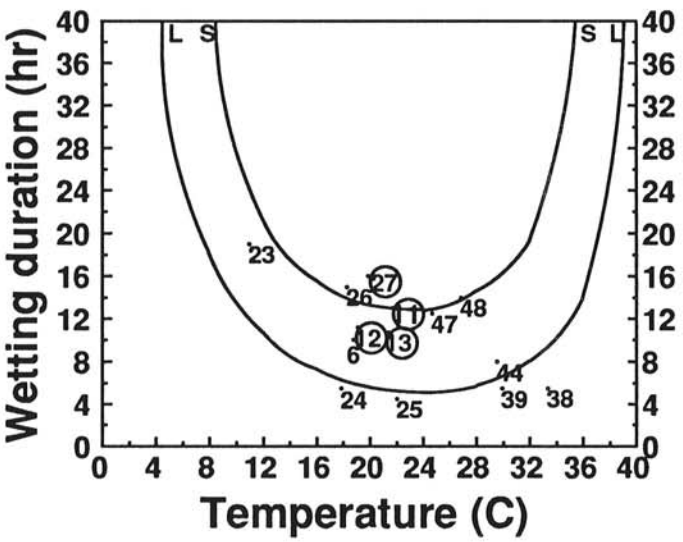

Fig. 2. Curves represent wetness duration required for light (0.2% leaf area covered with lesions) and severe (1% leaf area covered with lesions) infection of apple leaves by *Alternaria mali* at different temperatures. Numbers identify individual wetness periods when *A. mali* spores were trapped. Circled numbers represent wetness periods when infection actually occurred.

The curves that we chose for light and moderate infection are similar to those for apple scab (8), cedar apple rust (1), and black rot (2). Use of those curves is based on the availability of fungicides with "back-action" or post-infection activity. Because fungicides with post-infection activity for *A. mali* are not available and because of the rapidity of symptom development after infection (within 2 days), the curves we developed for *A. mali* will not be useful in a post-infection program.

The utility of our model, as well as other similar models (1,2,8), is limited in that the prediction of an infection period is based solely on certain environmental conditions being met regardless of inoculum availability. In our study, infection criteria were met in 15 of 34 wetness periods in which no conidia were trapped. Until factors affecting overwintering inoculum and subsequent spore production by *A. mali* in the spring are understood, the most efficient way to utilize this model and avoid unnecessary fungicide applications would be to implement it only in orchards where the disease was a problem the previous year and during periods when inoculum is most likely to be present. Consequently, we propose two uses of the model. First, predictive curves could be used in combination with the Korean model. Once criteria have been met for first symptom appearance according to the Korean model, the first spray could be delayed until the occurrence of an infection period as predicted by our curves. This should be at a stage early enough to delay the onset of the epidemic and maintain satisfactory control. Second, the model could be used to identify critical periods during the season in which a fungicide application was essential or to identify periods in which the spray interval needed to be shortened. For instance, iprodione will give approximately 70% control when applied on a 14-day schedule (5). However, if conditions for infection are met frequently, the spray interval could be shortened; conversely, if it was dry and conditions were seldom met, it could be lengthened. Before such a program is implemented, fungicides with good activity toward *A. mali* need to be registered for apples in the United States and tested in orchards with different inoculum levels of *A. mali*.

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