Influence of Temperature and Moisture on Germination of Ascospores and Conidia of *Botryosphaeria obtusa*

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ABSTRACT

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Maximum germination of conidia of *Botryosphaeria obtusa* occurred in free water and declined as relative humidity (RH) was reduced from 100 to 92%; no germination was observed at 88.5% RH. Germination in free water reached 80% in 4 hr at 16–32 C and in 12 hr at 12 C but was only 23 and 0% at 8 and 4 C, respectively, after 12 hr. Higher temperatures were required for germination at 95 and 92% RH (16 and 28 C, respectively) than at 98, 99, and 100% RH (12 C). Conidial germination at 92% RH was observed only after 12 hr at 28 C. The two isolates tested differed in temperature and relative humidity requirements for germination. Requirements for ascospore germination were similar to those for conidia. Germ tube lengths of ascospores and conidia were maximum in free water and decreased with decreasing relative humidity. After 12 hr in free water, ascospore germ tubes reached a maximum mean length of 0.78 mm at 28 C, whereas maximum conidial germ tube length was 0.82 mm at 24 C for isolate 087 and 0.99 mm at 28 C for isolate 049. Germ tube length of both types of spores declined at 32 C.

The fungus *Botryosphaeria obtusa* (Schw.) Shoemaker (synonym *Physalospora obtusa* (Schw.) Cooke) is found throughout most tropical and warm temperate regions in the world as a pathogen of many crops (4,5). The greatest economic losses due to this fungus occur on apple (*Malus* × *domestica* Bork.), on which it causes fruit black rot, frogeye leafspot, and a limb canker (10). Fruit losses as high as 60% have been reported (15).

Conidia are the main propagules of *B. obtusa* in apple orchards throughout the growing season in North Carolina although ascospores can be important early in the season (14). Conidia have been implicated as the main spore type involved in primary dispersal of *B. obtusa* in Georgia (3).

Infection of apple by *B. obtusa* is favored by free water or high relative humidity (RH) (6). The precise influence of relative humidity on spore germination of *B. obtusa* has not been determined, although, in general, relative humidity plays a major role in germination of most fungal spores (13).

Foster (6), in a study on the influence of temperature on conidial germination of *B. obtusa*, found that approximately 90% of the conidia germinated at temperatures between 12 and 32 C. After a 24-hr incubation in free water, optimal temperature for germ tube elongation was 24 C, although the average germ tube length was similar from 20 to 32 C. He did not study the effect of relative humidity on germination of conidia nor did he work with ascospores.

Knowledge of the factors that influence the germination of conidia and ascospores will result in a better understanding of

factors favoring infection and may lead to the development of more efficient management programs for the diseases caused by *B. obtusa*. The objective of this study, therefore, was to determine the influence of temperature, relative humidity, and their interaction on the germination of conidia and ascospores of *B. obtusa*.

MATERIALS AND METHODS

Conidia. Two isolates of *B. obtusa*, obtained from apple fruit collected in western (isolate 049) and central (isolate 087) North Carolina, were grown under continuous light on cellulose films (Flexel Sales, Inc., Covington, IN) placed on top of oatmeal-agar medium (Difco Laboratories, Detroit, MI). Conidia were obtained from cultures 12–14 days old by scraping off the nonsporulating mycelium from the cellulose films and blending the remaining mycelium, pycnidia, and cellulose films for 30 sec in sterile distilled water in a commercial blender. The resulting suspension was strained through three layers of cheesecloth to separate the conidia. The spore suspension was standardized to 120,000 conidia/ml to provide a minimum of 100 conidia/2-μl drop of suspension.

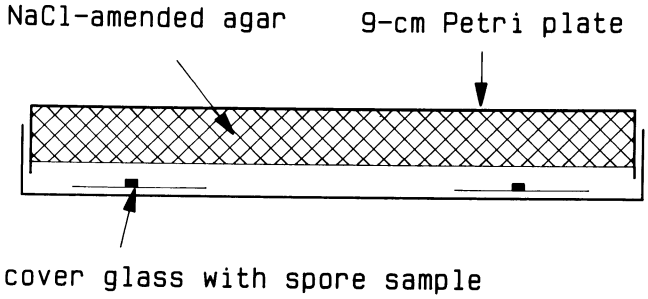

Fig. 1. Relative humidity chamber showing position of cover glass with spore sample.

The agar dish isopiestic equilibration technique, described by Harris et al (8) and modified by Alderman and Beute (1), was used to control the relative humidity. One drop of the conidial suspension of either isolate 049 or 087 was placed on each of four microscope cover glasses by means of a micropipetter (Pipetman P-20, Rainin Instrument Co., Inc., Emeryville, CA) and air dried. The four cover glasses, corresponding to four assessment times, were placed in a petri dish containing 44 ml of water agar amended with sodium chloride as shown in Figure 1. Each dish was sealed with Parafilm (American Can Company, Greenwich, CT). The relative humidity in this sealed atmosphere is related to the NaCl molality according to the values given by Lang (11). The relative humidities tested were 100, 99, 98,

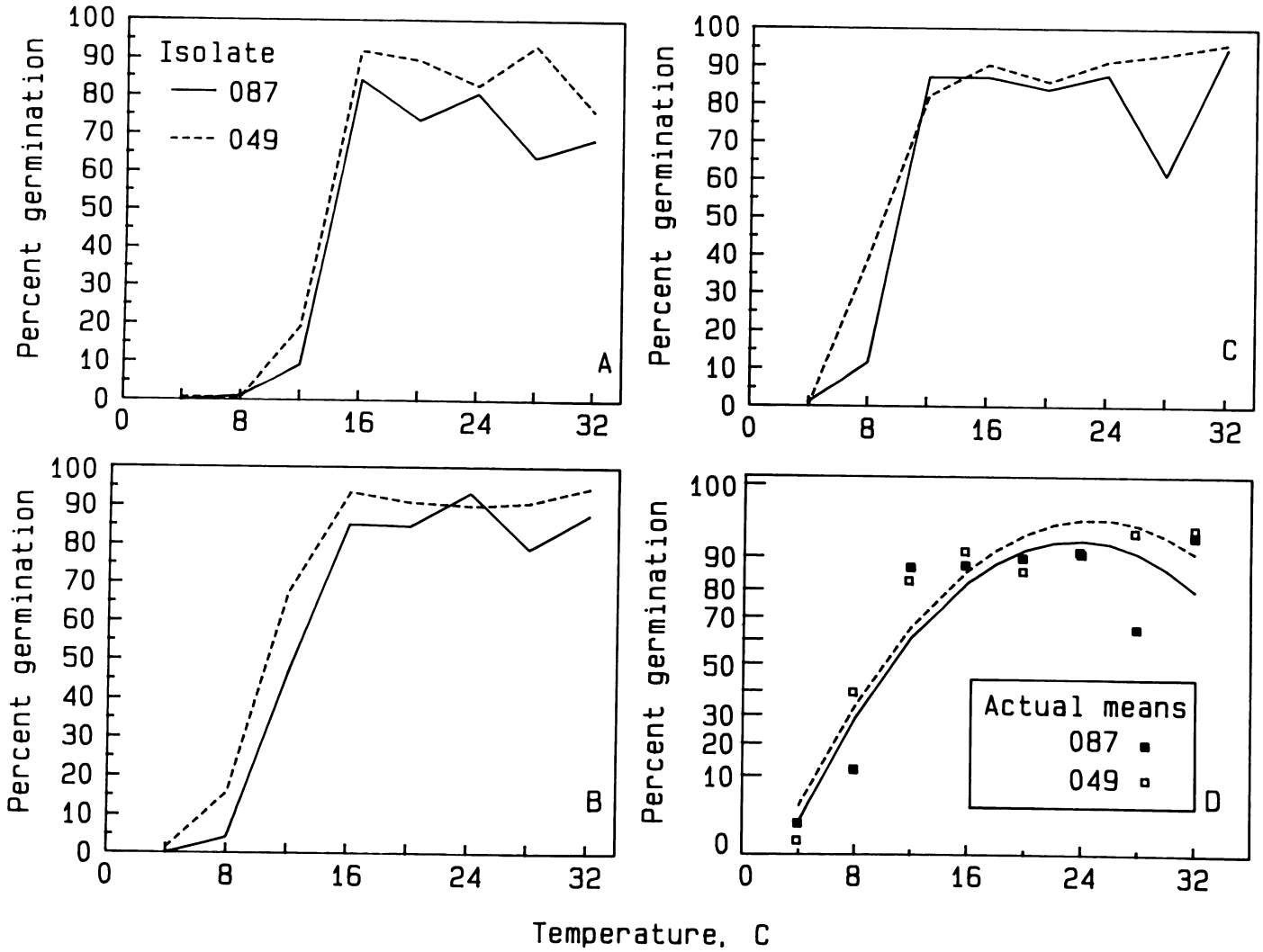

Fig. 2. Influence of temperature on conidial germination of two isolates of *Botryosphaeria obtusa* in free water; conidia were not air dried. **A,** 4 hr after spore release. **B,** 8 hr after spore release. **C,** 12 hr after spore release. **D,** Germination after 12 hr as predicted by the following regression equations: Y (isolate 049) $= -0.3110 + 0.1395T - 0.002815T^2$ (R^2adj $= 0.85$) and Y (isolate 087) $= -0.3977 + 0.1462T - 0.003094T^2$ (R^2adj $= 0.65$), where $Y =$ arcsin $\sqrt{X}$ (in radians), $X =$ proportion of spores germinated of the isolate indicated in parenthesis, and $T =$ temperature (C). The nonlinearity of the scale on the vertical axis results from transforming back to percentage the arcsin $\sqrt{X}$ scale used in the analysis of the data.

95, 92, and 88.5%, which were obtained by amending the agar with 0, 0.3, 0.6, 1.5, 2.2, and 3.1 m NaCl, respectively. Two free-water treatments were used: one in which conidia were rewetted with water after air drying, designated as "rewetted," and another free-water treatment in which the conidial suspension was not air dried, designated as "wet."

Relative humidity chambers were placed in controlled temperature incubators at 4, 8, 12, 16, 20, 24, 28, and 32 C. The calculated relative humidity varied less than 0.25% from the lowest to the highest temperature at the same molality. The relative humidity chambers were preconditioned at the desired temperatures for at least 15 hr before the spores were placed in them.

Spore germination was evaluated at 4, 8, 12, and 24 hr after the spores were placed in the relative humidity chambers. After each prescribed germination time, one cover glass from each relative humidity chamber was inverted on a drop of cotton blue in lactophenol on a glass slide to stop germination and preserve the spores and germ tubes for future observation. The relative humidity chamber then was resealed. Percent spore germination was determined by observing 50 conidia on each cover glass. A conidium was considered to have germinated if the germ tube was at least one-half the length of the spore. Germ tube length was determined at 12 hr by measuring 10 germ tubes selected randomly.

Each set of treatments was replicated three times on different days. The experiment was conducted in a split-split-plot design with temperature as the whole plot, isolates as the subplot, and relative humidity as sub-subplot. A separate analysis of variance was performed for each assessment time.

Ascospores. Naturally infected apple twigs bearing ascostromata of *B. obtusa* were used as sources of ascospores. Twigs were cut into 15-cm pieces, washed under running tap water to eliminate spores of superficial saprophytes, and immersed in distilled water for 20 min. Twigs were blotted dry with paper towels and placed in a spore tower (7). Spores were collected for 5 to 20 min on cover glasses and placed immediately after collection in controlled relative humidity chambers. Temperature and relative humidity treatments were the same as those described for conidia. Because no spore suspension was used in this part of the study, only one free-water treatment was used, similar to the rewetted treatment in the experiment with conidia.

Because the infected apple twigs used were not uniform and numbers of spores varied from treatment to treatment, a higher coefficient of variability was anticipated in the ascospore study than in the conidia study. To compensate for this, the ascospore germination experiment was replicated four times on different days. If no ascospores of *B. obtusa* were captured on the cover glasses corresponding to any particular temperature treatment, the treatment was repeated in the next experimental run. The experimental design was a split plot with temperatures as whole plots and relative humidities as subplots.

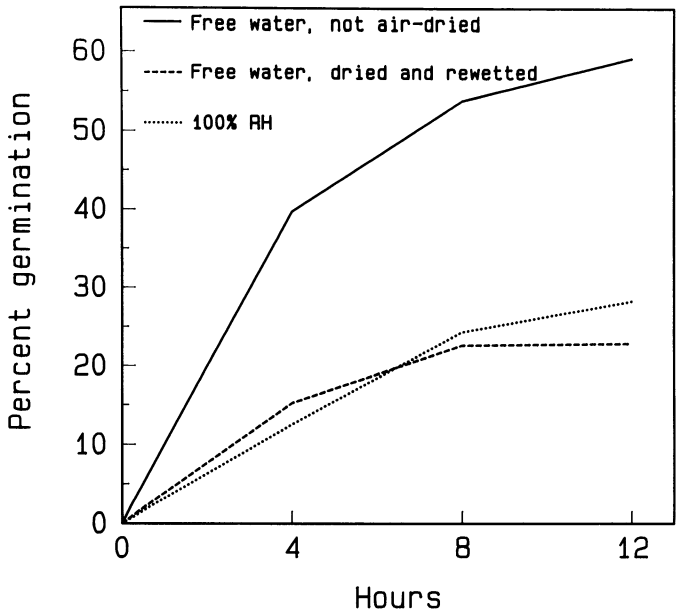

Fig. 3. Mean percent germination of conidia of *Botryosphaeria obtusa* 4, 8, and 12 hr after treatments were imposed. Average of eight temperature treatments (4, 8, 12, 16, 20, 24, 28, and 32 C).

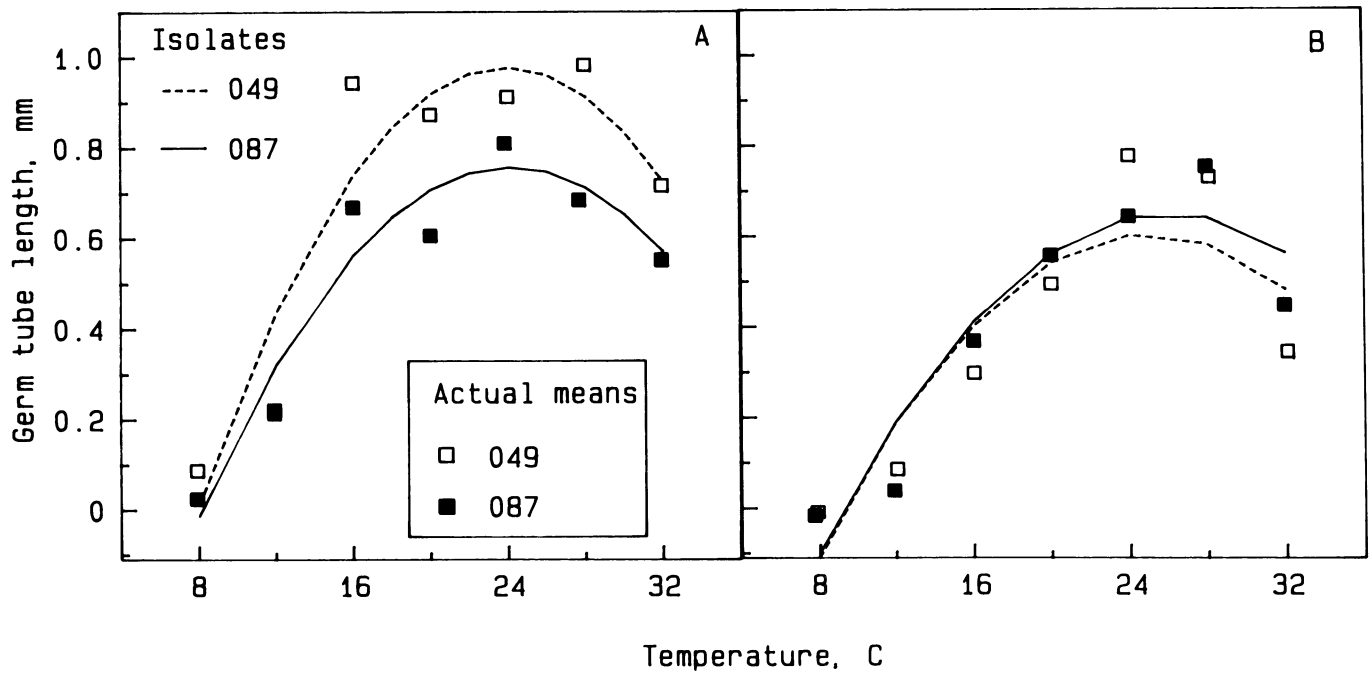

Fig. 4. Influence of temperature on conidial germ tube length of two isolates of *Botryosphaeria obtusa* in free water 12 hr after treatments were imposed. Lines are predicted values from the following regression equations. **A,** Conidia not air dried: Y (isolate 049) $= -1.1958 + 0.1823T - 0.003816T^2$ (R^2adj $= 0.64$) and Y (isolate 087) $= -0.9665 + 0.1436T - 0.002981T^2$ (R^2adj $= 0.67$); **B,** Conidia air dried and rewetted: Y (isolate 049) $= -0.9319 + 0.1224T - 0.002441T^2$ (R^2adj $= 0.65$) and Y (isolate 087) $= -0.9146 + 0.1197T - 0.002298T^2$ (R^2adj $= 0.71$), where $Y =$ germ tube length of the isolate indicated in parenthesis (mm) and $T =$ temperature (C).

Regression analysis. Regression analysis was conducted on the 12-hr readings. Linear, quadratic, and cubic effects of temperature and relative humidity and their interactions on spore germination and germ tube elongation were tested. Regression parameters not significantly different from zero ($P < 0.05$) were dropped from the equations unless higher degree terms of the corresponding variable were associated with significant parameters. After dropping the proper terms, regressions were conducted again. All parameters reported here are significantly different from zero.

RESULTS

Preliminary analysis of variance. Analysis of variance indicated significant ($P < 0.05$) effects of temperature, relative humidity, isolates, and all their interactions on conidial germination. No significant effects of isolates, temperature × isolates, or temperature × relative humidity × isolates on germ tube elongation were observed. In the case of ascospores, effects of temperature, relative humidity, and their interaction on percent germination and germ tube elongation also were significant ($P < 0.05$). In the following sections, all comparisons are made using the $P = 0.05$ level of significance, unless otherwise specified.

Germination of conidia in free water. Conidial germination in free water for both isolates ranged from 70 to 90% after 4 hr (Fig. 2A) and from 80 to 95% after 8 hr (Fig. 2B) and was generally > 85% after 12 hr between 16 and 32 C. No significant differences in percent germination and germ tube elongation were observed for conidia placed in this temperature range. At 12 C, 12 hr were required to reach 80% germination (Fig. 2C). Only 23.3% of the conidia placed at 8 C had germinated by this time. No germination was observed at 4 C after 12 hr.

Effect of air drying on conidial germination. Germination and germ tube elongation for both isolates in free water were higher when conidia were not air dried (wet treatment) as opposed to air drying (rewetted treatment) (Figs. 3 and 4). There was no increase in germination from 8 to 12 hr in the rewetted treatment, which indicates a loss in spore viability upon drying. Spore germination at 100% RH showed a similar trend to the rewetted treatment and was much lower than in the wet treatment. Germination increased only slightly from 8 to 12 hr (Fig. 3), indicating a loss in viability. Even at temperatures favorable for germination, conidial germination at 100% RH was less than 70% after 12 hr (Fig. 5).

Germination was lower in the rewetted treatment than at 100% RH for conidia at 12 hr (Fig. 3) and for ascospores at 4, 8, and 12 hr (Fig. 6). In general, germ tube length was similar in free water (rewetted) and at 100% RH regardless of the type of spore (Figs. 4, 7, and 8), but ascospore germ tubes were significantly longer in free water at 20 and 28 C compared with 100% RH (Fig. 8).

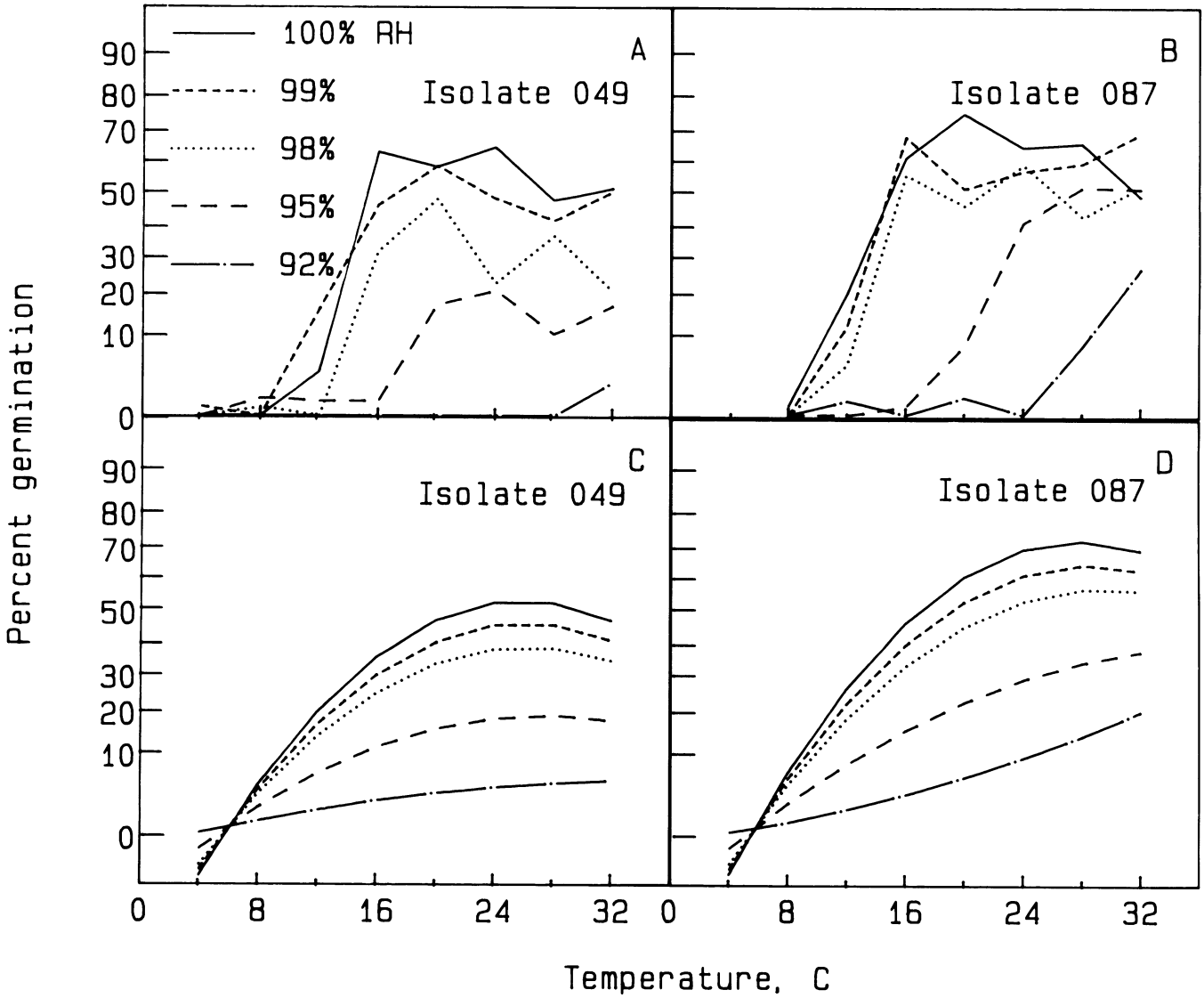

Fig. 5. Effect of temperature and relative humidity on conidial germination of two isolates of *Botryosphaeria obtusa* 12 hr after the treatments were imposed. The nonlinearity of the scales on the vertical axes results from transforming back to percentage the arcsin $\sqrt{X}$ scale used in the analysis of the data. **A, B,** Actual data means of three replications. **C, D,** Predicted values from regression analysis (equations 2 and 3 in text).

Effect of temperature and relative humidity on spore germination. Conidial germination decreased at the lower relative humidities (Fig. 5). Ascospore germination also decreased with decreasing relative humidities; however, germination was similar at 98, 99, and 100% RH at temperatures higher than 16 C (Fig. 6). No spore germination was observed at 88.5% RH after 24 hr. At 92% RH, no spore germination occurred after 4 hr; after 8 hr, 1.5% of the ascospores had germinated (Fig. 6). Conidial germination of isolate 087 was observed only at 32 C (16.7%) after 8 hr at 92% RH. Germination was very low after 12 hr and 92% RH, as can be seen in Figures 5 and 6.

The combined effect of temperature and relative humidity on germination of ascospores and conidia of *B. obtusa* 12 hr after spore release was described by the following equations:

$$Y\text{ (ascospores)} = 1703.8742 - 1.9213T + 0.03847T^2 - 53.9194H + 0.5693H^2 - 0.002007H^3 + 0.02156TH - 0.0004307T^2H \quad R^2\text{adj} = 0.89 \quad (1)$$

$$Y\text{ (conidia, isolate 049)} = 44.47 - 0.9974T + 0.02255T^2 - 0.000066T^3 - 0.8869H + 0.004407H^2 + 0.01038TH - 0.0002082T^2H \quad R^2\text{adj} = 0.75 \quad (2)$$

$$Y\text{ (conidia, isolate 087)} = 5.7828 + 1.1375T + 0.02455T^2 - 0.06239H + 0.01243TH - 0.0002645T^2H \quad (3)$$
$$R^2\text{adj} = 0.72$$

where Y = arcsin $\sqrt{X}$ (in radians), X = proportion of spores germinated, T = temperature (C), and H = percent relative humidity.

Predicted values associated with these regressions can be observed in Figure 5C and D and Figure 6D. The effects of temperature and relative humidity on germ tube elongation of *B. obtusa* are shown in Figures 7 and 8. The combined effect of temperature and relative humidity on germ tube length of ascospores and conidia was described by equations 4, 5, and 6, respectively:

$$Y\text{ (ascospores)} = -1994.4849 - 0.7121T + 0.01451T^2 + 62.78H - 0.6579H^2 + 0.002295H^3 + 0.007701TH - 0.0001557T^2H \quad R^2\text{adj} = 0.62 \quad (4)$$

$$Y\text{ (conidia, isolate 049)} = 8.8438 - 1.1475T + 0.02196T^2 - 0.09526H + 0.01235TH - 0.0002353T^2H \quad (5)$$
$$R^2\text{adj} = 0.79$$

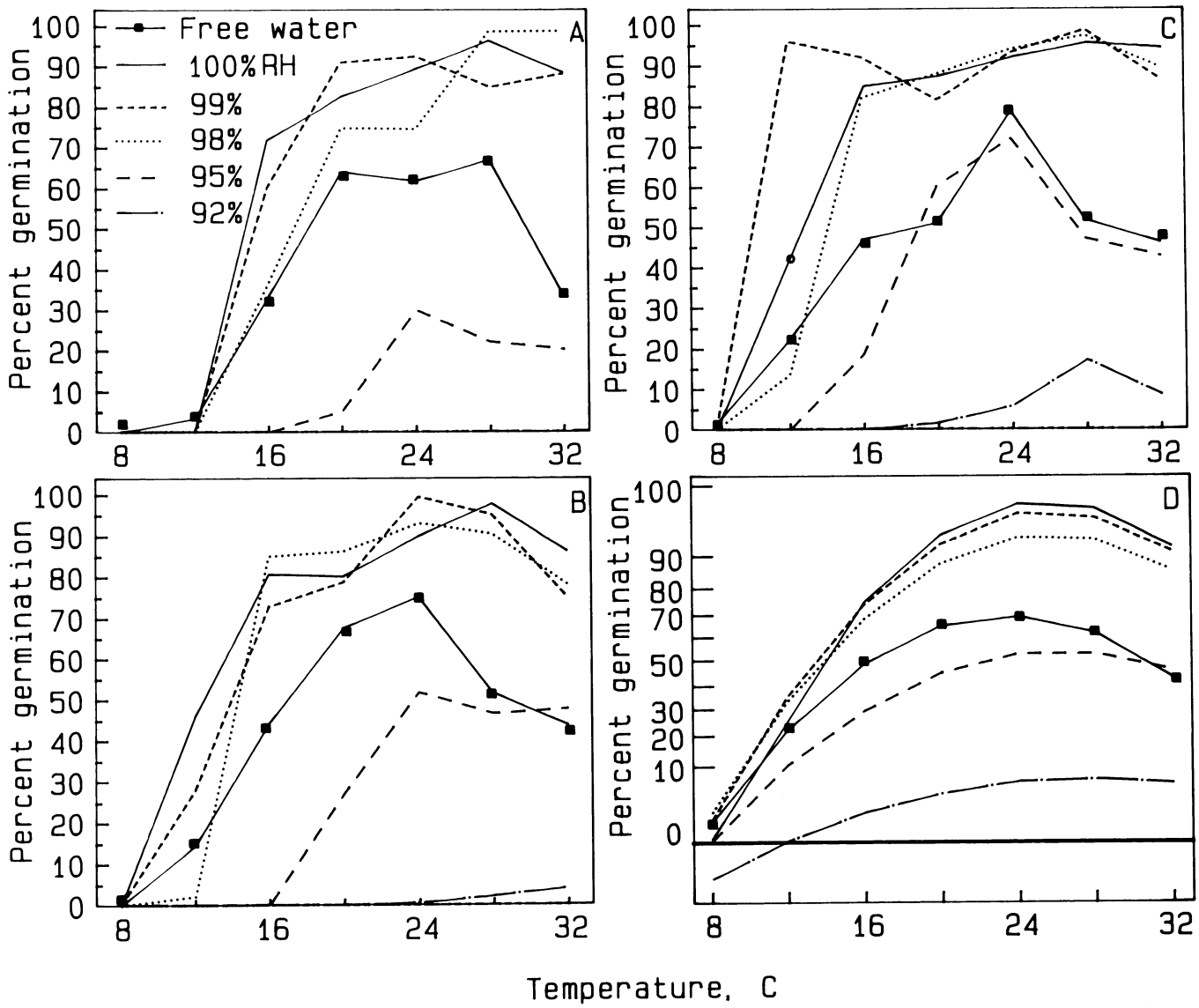

Fig. 6. Effect of temperature and relative humidity on germination of ascospores of *Botryosphaeria obtusa*. **A,** 4 hr after treatments were imposed. **B,** 8 hr after treatments were imposed. **C,** 12 hr after treatments were imposed; ascospore collection was insufficient for counts at 12 C and 100% RH. **D,** Predicted values from regression analysis. For the different relative humidity levels, see equation 1 in text. For the free-water treatment, values were predicted from the following equation: $Y = -0.9942 + 0.1712T - 0.003648T^2$ (R^2adj = 0.53), where Y = arcsin $\sqrt{X}$ (in radians), X = proportion of spores germinated, and T = temperature (C). The nonlinearity of the scale on the vertical axis results from transforming back to percentage the arcsin $\sqrt{X}$ scale used in the analysis of the data.

$$Y\text{ (conidia, isolate 087)} = 12.8232 - 1.7342T + 0.03783T^2 - 0.1378H + 0.01861TH - 0.000404T^2H \quad (6)$$
$$R^2\text{adj} = 0.67$$

where Y = germ tube length (mm), T = temperature (C), and H = percent relative humidity.

Conidial germ tube length increased as temperature increased up to 24–28 C and decreased at 32 C. Germ tubes arising from ascospores were longer as temperature increased; length was maximum for temperatures ranging from 20 to 28 C (Fig. 8). Optimum temperature for ascospore germ tube elongation was 27.1 C at 100% RH.

Increasing relative humidity also resulted in longer germ tubes for both types of spores. Germ tube length decreased linearly with decreasing relative humidity. Quadratic and cubic effects of relative humidity on conidial germ tube elongation were insignificant. Therefore, the corresponding terms were not included in the regression equations. Length was reduced at 95% RH and was close to zero at 92% RH (Figs. 7 and 8).

Germination of conidia of different isolates. The mean percent germination (over all temperatures and moisture treatments) of isolate 087 was higher (19.9%) than in isolate 049 (14.0%) after 12 hr. The isolate × temperature interaction was significant for percent germination at 8 and 12 hr but not for germ tube length after 12 hr. The calculated optimum temperature for conidial germination was similar (24 C) for both isolates in free water, but at 100% RH, optimum temperature for conidial germination of isolate 087 was estimated to be 27.8 C, and 25.5 C for isolate 049.

A highly significant ($P = 0.008$) moisture treatment × isolate interaction was detected. Germination was higher and germ tubes longer for isolate 087 than for isolate 049 at all moisture treatments except free water (wet treatment), in which germ tubes of isolate 049 were longer (Figs. 4 and 7). Percent germination was also higher in isolate 049 than in isolate 087, but this difference was not significant except at 8 C, where isolate 049 reached 39.1% germination at 12 hr compared with 11.5% germination for isolate 087 (Fig. 2E).

DISCUSSION

The results of this study on percent germination and germ tube elongation of conidia of *B. obtusa* in free water are similar to those obtained by Foster (6). He observed a high percent germination (> 80%) over a wide range of temperatures (12–32 C). In his experiments, temperatures between 20 and 32 C resulted in high values of germ tube length as well. In our experiment, temperatures favorable for both percent germination and germ

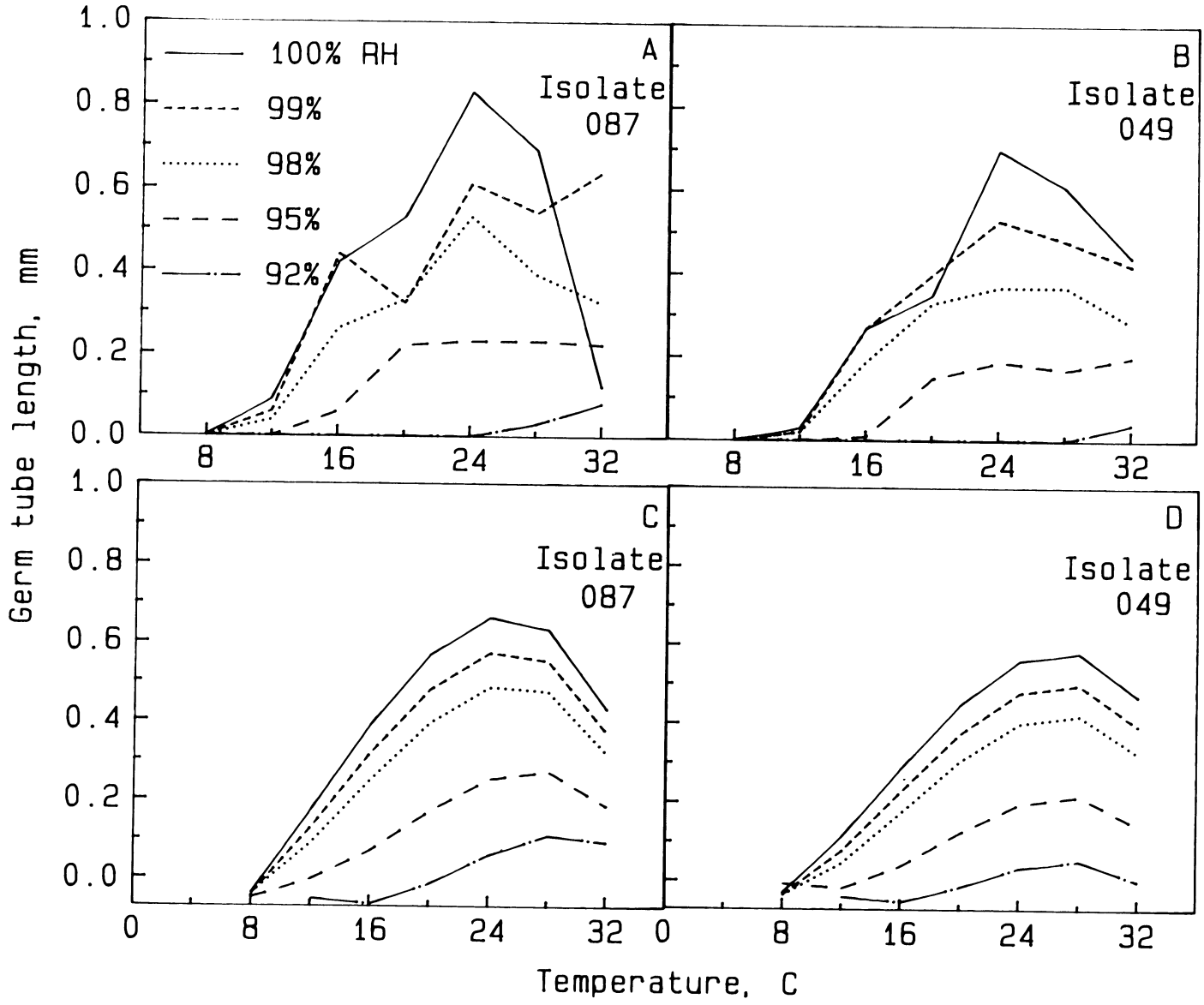

Fig. 7. Effect of temperature and relative humidity on conidial germ tube length of two isolates of *Botryosphaeria obtusa* 12 hr after treatments were imposed. **A, B,** Actual data (means of three replications). **C, D,** Values predicted from regression analysis (equations 5 and 6 in text).

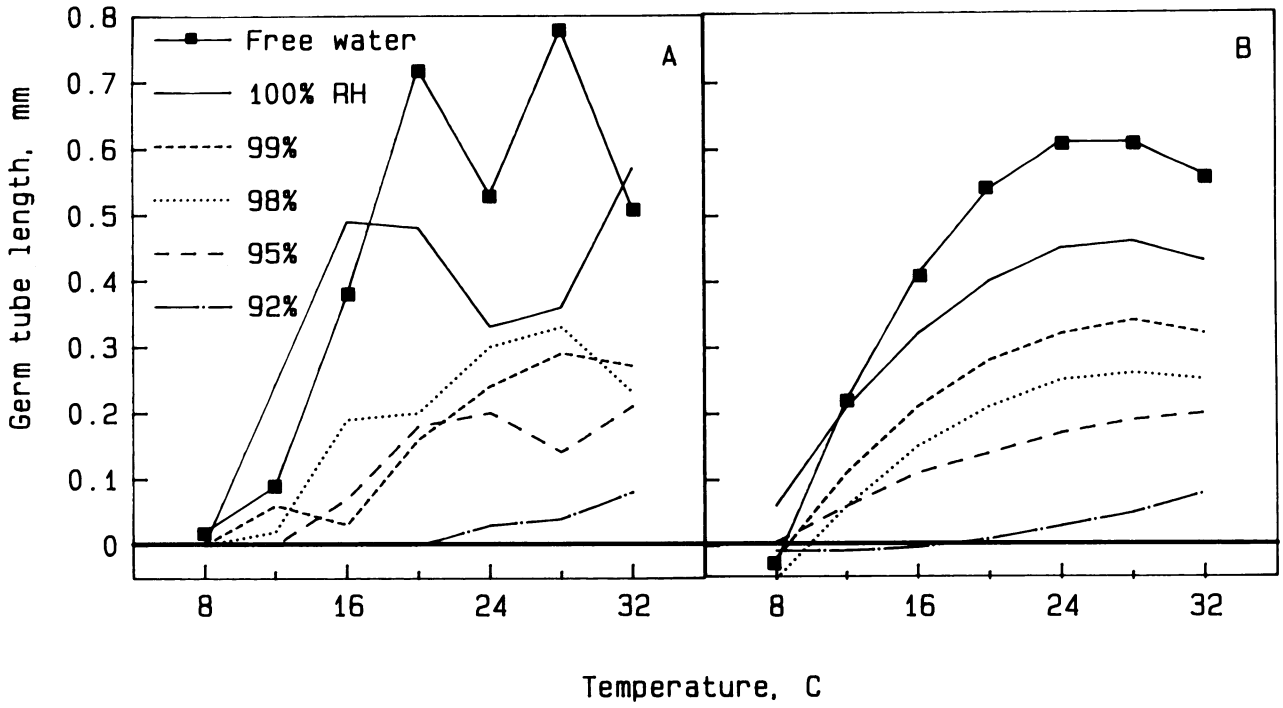

Fig. 8. Effect of temperature and relative humidity on ascospore germ tube length of *Botryosphaeria obtusa* 12 hr after treatments were imposed. **A,** Actual data (means of four replications). **B,** Predicted values from regression analysis. For the free-water treatment, values were predicted from the following equation: $Y = -0.7110 + 0.1008T - 0.001912T^2$ (R^2adj = 0.56), where Y = germ tube length (mm) and T = temperature (C). For the different relative humidity levels, see equation 4 in the text.

tube elongation ranged from 16 to 32 C.

Conidia of *B. obtusa* proved to be very sensitive to drying. Because germination did not increase from 8 to 12 hr in the rewetted treatment and at 100% RH, it appears that drying not only delayed the germination process but also had a detrimental effect on spore viability. Because spores dried in about 20 min, a short (< 1 hr) dry period following dispersal of conidia presumably could reduce the probability of host infection by *B. obtusa*.

In many cases, 100% RH resulted in a higher percent germination than the rewetted treatment. This may be due to a low oxygen supply in the water drop used to wet the spores. Lilly and Barnett (12) suggested that germination is greater in aerated water than in nonaerated water because of the difference in oxygen supply.

Most spore germination occurred at relative humidities higher than 95%. Lower relative humidities resulted in reduced germination. The fact that some spore germination occurred at low relative humidity (92%) when temperature was high (> 20 C) may be due to an increase in the atmospheric moisture content with increasing temperature. Although relative humidity (that is, the ratio of actual humidity to saturation humidity at a given temperature) remains approximately constant for each NaCl molality, the moisture content in the atmosphere (absolute humidity) increases with temperature as the saturation humidity increases. In general, at lower relative humidities (95 and 92%), higher temperatures (16 and 20 C, respectively) were required for germination than at higher relative humidities (98, 99, and 100%, 12 C). Spores placed at low relative humidity took a longer time to germinate than those exposed to higher relative humidity. The ability of certain spores to germinate at low relative humidities has been related to high osmotic pressure of the spore (13), which would allow it to absorb water from the air. The longer germination time observed in this study at low relative humidities may be explained in terms of a longer water absorption period.

The differences observed between isolates in the present study suggest some degree of natural variability in the response of *B. obtusa* to environmental factors. Isolate 087 was obtained in a warmer and drier region and had higher temperature and lower relative humidity requirements than isolate 049, but the results obtained with only two isolates are not enough to make conclusions regarding the adaptability of this fungus to different environments. A large number of isolates collected in different regions would be required to obtain valid information in this regard. Foster (6) also observed slight variations in temperature requirements for conidial germination of five isolates in free water. He did not work with different relative humidity levels.

In this study, relative humidities higher than 95% were critical for spore germination of *B. obtusa* and, therefore, for infection to occur. The effects of duration of periods of high relative humidity in combination with specific temperatures on spore germination can be useful elements in predicting the likelihood of disease occurrence under field conditions. These parameters have been used in the construction of disease forecasting models such as the peanut leafspot warning system (9), in which the risk of disease and the need for fungicide sprays are estimated on the basis of temperature and relative humidity. It is likely that most germination of rain-splashed conidia (14) under field conditions takes place in free water. However, the results of this study suggest that high relative humidity periods following rainfall and spore dispersal may be important when the duration of the rain event is short. High relative humidity periods may be particularly important for airborne ascospores because they can be discharged for several hours after the end of a rain event (14). We have constructed a model for predicting infection by *B. obtusa*. This model incorporates the duration of wetting and temperature as infection predictors (2). The data generated in this study may be useful in refining the model.

LITERATURE CITED

1. Alderman, S. C., and Beute, M. K. 1986. Influence of temperature and moisture on germination and germ tube elongation of *Cercospora arachidicola*. Phytopathology 76:715-719.
2. Arauz, L. F., and Sutton, T. B. 1989. Temperature and wetness duration requirements for apple infection by *Botryosphaeria obtusa*. Phytopathology 79:440-444.
3. Beisel, M., Hendrix, F. F., Jr., and Starkey, T. E. 1984. Natural inoculation of apple buds by *Botryosphaeria obtusa*. Phytopathology 74:335-338.
4. Commonwealth Mycological Institute. 1973. *Botryosphaeria obtusa*.

CMI descriptions of pathogenic fungi and bacteria No. 394. Commonwealth Agricultural Bureau, Kew, Surrey, England. 2 pp.
5. Drake, C. R. 1971. Source and longevity of apple fruit rot inoculum, *Botryosphaeria ribis* and *Physalospora obtusa*, under orchard conditions. Plant Dis. Rep. 55:122-126.
6. Foster, H. H. 1937. Studies on the pathogenicity of *Physalospora obtusa*. Phytopathology 27:803-823.
7. Gilpatrick, J. D., Smith, C. A., and Blowers, D. R. 1972. A method for collecting ascospores of *Venturia inaequalis* for spore germination studies. Plant Dis. Rep. 56:39-42.
8. Harris, R. F., Gardner, W. R., Adebayo, A. A., and Sommers, L. E. 1970. Agar dish isopiestic equilibration method for controlling the water potential of solid substrates. Appl. Microbiol. 19:536-537.
9. Jensen, R. E., and Boyle, L. W. 1966. A technique for forecasting leafspot of peanuts. Plant Dis. Rep. 50:810-814.
10. Jones, A. L., and Sutton, T. B. 1984. Diseases of Tree Fruits. Cooperative Extension Service, Michigan State University, East Lansing. 59 pp.
11. Lang, A. R. G. 1967. Osmotic coefficients and water potentials of sodium chloride solutions from 0 to 40 C. Aust. J. Chem. 20:2017-2023.
12. Lilly, V. G., and Barnett, H. L. 1951. Physiology of the Fungi. McGraw-Hill, New York. 464 pp.
13. Sussman, A. S., and Halvorson, H. O. 1966. Spores. Their Dormancy and Germination. Harper & Row, New York. 354 pp.
14. Sutton, T. B. 1981. Production and dispersal of ascospores and conidia by *Physalospora obtusa* and *Botryosphaeria dothidea* in apple orchards. Phytopathology 71:584-589.
15. Taylor, J. 1952. Some north Georgia apple production problems. Phytopathology 42:288.