The Influence of Temperature and Moisture on the Quantitative Production of Pseudothecia of *Venturia inaequalis*

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ABSTRACT

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The influence of temperature and moisture on the quantitative production of pseudothecia by *Venturia inaequalis* in sterilized, inoculated leaf disks and in naturally-infected leaf disks was studied under laboratory conditions. The disks were exposed in a factorial experiment to seven temperatures (0, 4, 8, 12, 16, 20, and 24 C), four moisture levels (wet, 95% RH, 88% RH and dry), and two treatment durations (3 and 7 days) at five stages of pseudothecial development (stage 2, pseudothecial initial; stage 3, ascogonium formation; stage 5, dormant period; stage 7, asci one-half mature size; and stage 9, spore formation). More pseudothecia were produced in leaf disks in the wet and 95% RH treatments than in the 88% RH and dry treatments. The optimum temperatures for pseudothecial development at stages 2, 3, 5, 7, and 9 were 4, 8, 8, 16, and 20 C, respectively. The mean number of pseudothecia produced in naturally-infected leaf disks over all treatments was lower than the mean number of pseudothecia produced in the inoculated leaf disks; however, the general response to the treatments was similar in the two types of disks. Number of pseudothecia produced in the inoculated leaf disks was significantly correlated with inoculum concentration and length of initial incubation at 20 C.

Additional key words: *Malus domestica*.

Venturia inaequalis (Cke.) Wint., the causal organism of apple scab, is one of the most important pathogens of apple (*Malus domestica* Bork.). After leaf fall, hyphae from a subcuticular stroma grow into the inner leaf tissues and if the opposite mating types are present, pseudothecial initials are formed. The pseudothecia mature in the spring and ascospores are discharged when the leaves are wet.

Moisture is essential for initiation and development of pseudothecia. Wilson (11) observed that no pseudothecia were initiated in dry leaves after leaf fall and James and Sutton (6) found that pseudothecial development ceased in air-dried leaves. In the field, development was highly correlated with measures of rainfall and high relative humidity (6).

When moisture is not limiting, temperature has a major influence on pseudothecial development. Gadoury and MacHardy (4) showed that the number of pseudothecia initiated after leaf fall was inversely proportional to temperature, but they did not investigate effects of moisture on pseudothecial initiation and effects of temperature and moisture on the number of pseudothecia that reach maturity. Jeger and Butt (8) observed that when temperature in the field was low and moisture was high, more pseudothecia were initiated than when opposite conditions prevailed, but equations based upon temperature and moisture for forecasting the number of mature pseudothecia failed because confounding factors such as time of leaf infection, extent of fungal growth in the leaves prior to leaf fall, and decomposition of overwintering leaves by saprophytes were not included. Wilson (10) pointed out that high moisture and high temperature conditions favor saprophytic organisms that decompose fallen leaves and thereby influence the development of pseudothecia.

The objective of this study was to investigate temperature and moisture as interacting variables affecting the quantitative production of pseudothecia of *V. inaequalis* in naturally infected leaves and in sterile, inoculated leaf disks. The sterilized leaf disk technique was also used to quantify the effects of inoculum concentration and amount of vegetative growth prior to pseudothecial initiation on the production of pseudothecia.

MATERIALS AND METHODS

Inoculated leaf disk technique. Four isolates of *V. inaequalis*, two of each mating type, were grown on potato-dextrose agar in 9-cm diameter petri dishes for 3 wk at 20 C under fluorescent lights. Conidia were collected by placing 8 ml of sterile, distilled water in each dish, scraping the agar surface with a sterile spatula and pouring the suspension through sterile cheesecloth. Conidial suspensions of each isolate were combined and the final concentration was adjusted to 6.0×10^4 conidia per milliliter. Five drops of Tween-20 were added to each 100 ml of conidial suspension.

McIntosh apple leaves with no scab symptoms were collected (10 July 1983 and 6 June 1984) from an orchard in Clayton, NC. Disks (1-cm diameter) punched from the leaves were placed in 1-L glass jars, moistened with distilled water, and sterilized by placing 1 ml of propylene oxide in each jar once a week for 3 consecutive weeks. Cellulose sponges ($15 \times 10 \times 25$ cm) were moistened and autoclaved for 1 hr at 137.9 kPa; molded plastic boxes ($16.5 \times 11.5 \times 6.5$ cm) with lids were sterilized with propylene oxide for 24 hr.

Under aseptic conditions, the sponges were placed in the plastic boxes and 72 leaf disks were placed on each sponge so that none of the disks were touching. The moistened sponges maintained the leaf disks in a constant pliable condition throughout the experiment. A 0.1-ml drop of inoculum was placed on each disk. The boxes were placed in plastic bags and incubated at 20 C for 2 wk and then at 8 C in the dark.

Naturally infected leaves. McIntosh apple leaves naturally infected with *V. inaequalis* were collected at leaf fall (5 October 1983) from an orchard in East Lansing, MI. Leaf disks (1-cm diameter) were punched from heavily infected leaves with a cork borer. Cellulose sponges were moistened and placed in the plastic

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boxes. Seventy-two leaf disks were placed on each sponge. The boxes were placed in plastic bags and incubated at 8 C in the dark.

Temperature and moisture treatments. Subsamples of the naturally infected and inoculated leaf disks were subjected to a factorial design of seven temperatures, four moisture levels, and two treatment durations at five 2- to 3-wk intervals. The five intervals were chosen when pseudothecia were in the following stages of development (6): stage 2, pseudothecial initials showing coiling of the hyphae; stage 3, formation of the ascogonium from the initial; stage 5, lumen of the pseudothecium filled with pseudoparaphyses; stage 7, asci one-half mature size; and stage 9, asci with spores in the process of formation, but not yet septate. These stages occurred 14, 50, 83, 97, and 111 days after the naturally infected leaf disks were placed at 8 C and 8, 34, 66, 86, and 102 days after the inoculated leaf disks were placed at 8 C.

Temperature treatments were 0, 4, 8, 12, 16, 20, and 24 C, moisture treatments were wet, 95% and 88% relative humidities (RH), and dry, and treatment durations were 3 and 7 days. Each treatment was replicated twice and there were six disks per replicate. In wet treatments, the disks were placed in 9-cm-diameter petri dishes containing a 7-cm-diameter piece of filter paper and 8 ml of sterile distilled water. In dry treatments, leaf disks were placed in petri dishes with no filter paper or water added. The 95 and 88% RH treatments were maintained with aqueous saturated potassium nitrate and potassium chloride solutions, respectively, in 1-L glass jars (11). Leaf disks were suspended 4 cm above the salt solutions in Saran cloth (Chicopee Mfg. Co., Cornella, GA) baskets. At the end of the incubation period, the disks were placed on sponges in the plastic boxes and placed at 8 C in the dark. Separate subsamples of disks were treated at each of the five intervals. The experiment with the inoculated leaf disks was repeated once and the data were combined for analysis.

At maturation of pseudothecia (128 days in the naturally infected leaf disks, 114 days in the inoculated leaf disks), all disks were fixed in a mixture of 2-propanol, water, propionic acid, and formaldehyde (45:45:5:5, v/v)(FPP). Pseudothecia were removed from the disks, crushed, and observed with a compound microscope. The number of pseudothecia containing mature ascospores was recorded for each disk.

Leaf moisture content. Leaf moisture content was determined by placing five leaf disks at each of the four moisture treatments described above and incubating them at 4, 12, and 20 C for 3 or 7 days. Water saturation deficit (WSD) was used as a measure of leaf water content (1). WSD is the ratio of the amount of actual water in tissue to the potential amount of water the tissue can absorb and is determined by the equation:

$$\text{WSD} = \frac{[(\text{fully turgid weight}) - (\text{fresh weight})]}{[(\text{fully turgid weight}) - (\text{oven-dry weight})]} \times 100$$

in which fully turgid weight = the weight of leaf disks after being soaked in distilled water for 4 hr; fresh weight = weight of leaf disks when they were removed from the moisture treatments; and oven-dry weight = weight of leaf disks after drying for 24 hr at 85 C.

Inoculum concentration experiment. Inoculum was collected as described and was adjusted to give suspensions containing 3.0, 6.0, or 12.0×10^4 conidia per milliliter. Sterile apple leaf disks in two boxes (30 disks per box) were inoculated with 0.1 ml of one of the three inoculum suspensions. Disks were incubated at 20 C in the dark for 2 wk and then at 8 C in the dark for 16 wk. The number of mature pseudothecia in each disk was recorded. The experiment was repeated once and the data were combined for analysis.

Length of initial incubation period at 20 C. Sterile apple leaf disks were inoculated with 0.1 ml of an aqueous conidial suspension (6.0×10^4). Two boxes of 30 disks each were incubated at 20 C for either 1, 2, or 3 wk, and then all boxes were incubated at 8 C in the dark for 16 wk. The number of mature pseudothecia in each disk was recorded. The experiment was repeated once and the data were combined for analysis.

Statistical analysis. The experimental design for the inoculated and naturally infected leaf disk experiments was a split-split plot design and data were analyzed by analysis of variance. The experiments on inoculum concentration and length of initial incubation at 20 C were analyzed using linear correlation analysis.

RESULTS

Inoculated leaf disk experiments. The number of mature pseudothecia per disk ranged from 0 to 16 with an overall mean of

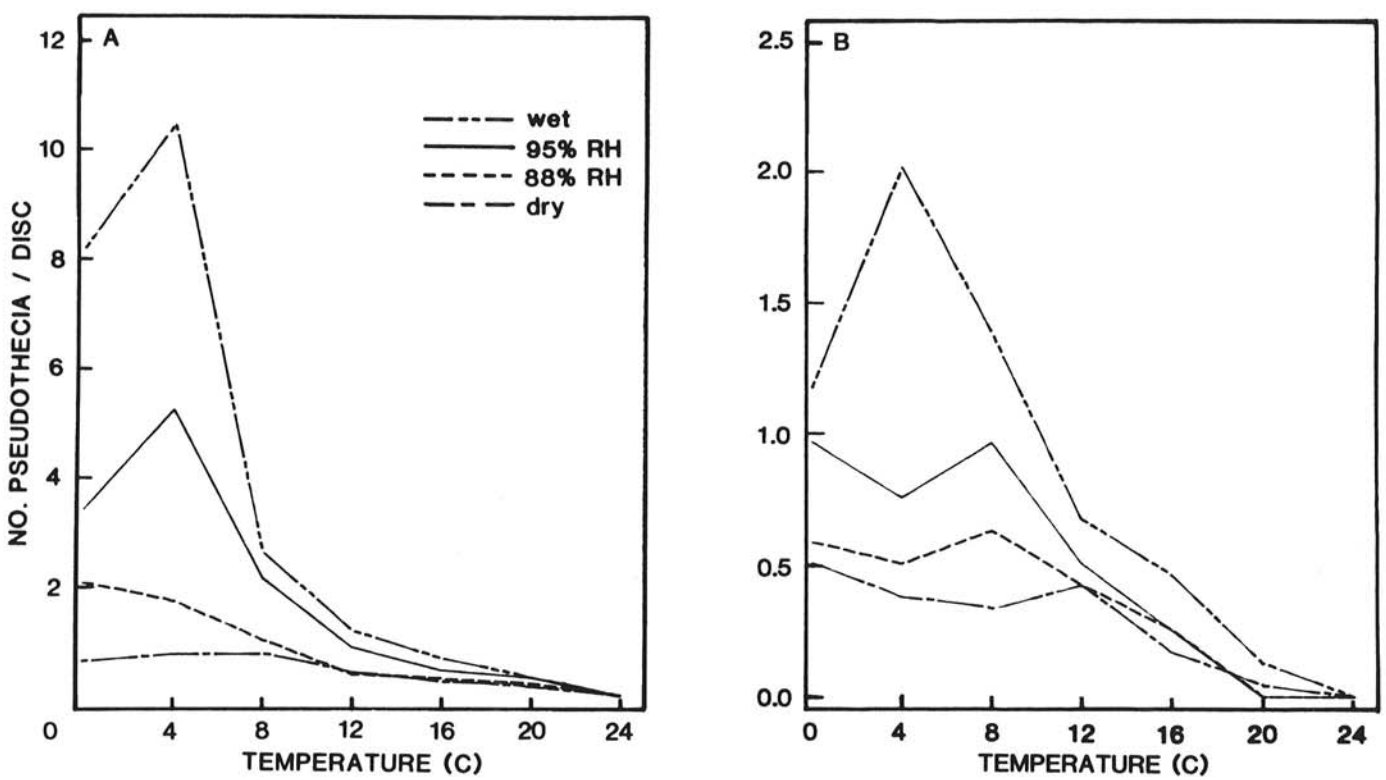

Fig. 1. Effect of temperature and moisture on the number of mature pseudothecia of *Venturia inaequalis* produced in **A,** inoculated and **B,** naturally infected apple leaf disks after a 7-day incubation period during stage 2 (pseudothecial initiation).

2.5. Numerous small pseudothecia with undifferentiated contents were also observed in the leaf disks. The number of pseudothecia produced in inoculated leaf disks was greatly influenced by temperature throughout their development. Data were similar for disks incubated for either 3 or 7 days at the temperature and moisture combinations; therefore, only data from the 7-day treatment are shown in Figs. 1A–5A. The optimum temperature for pseudothecial initiation (stage 2) was 4 C (Fig. 1A). Few pseudothecia were initiated at temperatures greater than 8 C and none were initiated at 24 C. The optimum temperature for

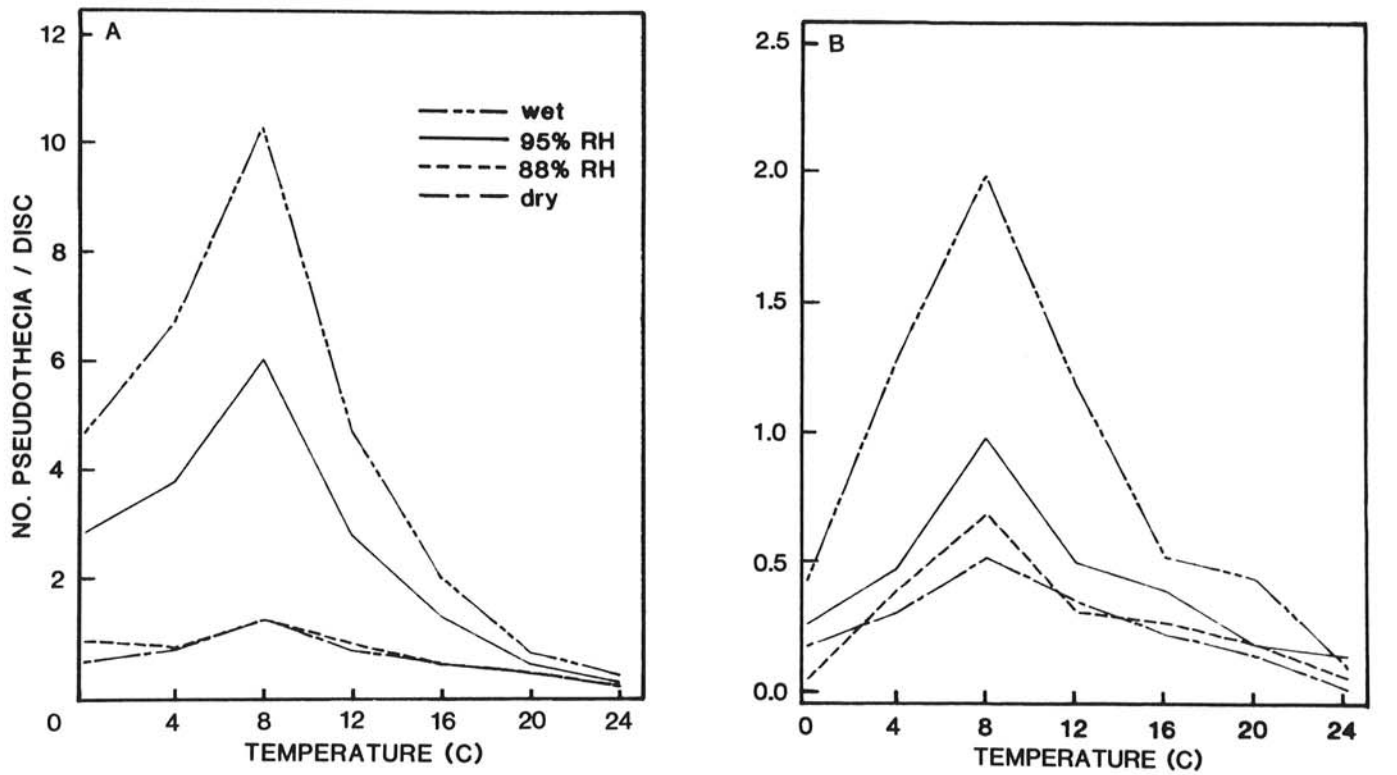

Fig. 2. Effect of temperature and moisture on the number of mature pseudothecia of *Venturia inaequalis* produced in **A,** inoculated and **B,** naturally infected apple leaf disks after a 7-day incubation period during stage 3 (ascogonial formation).

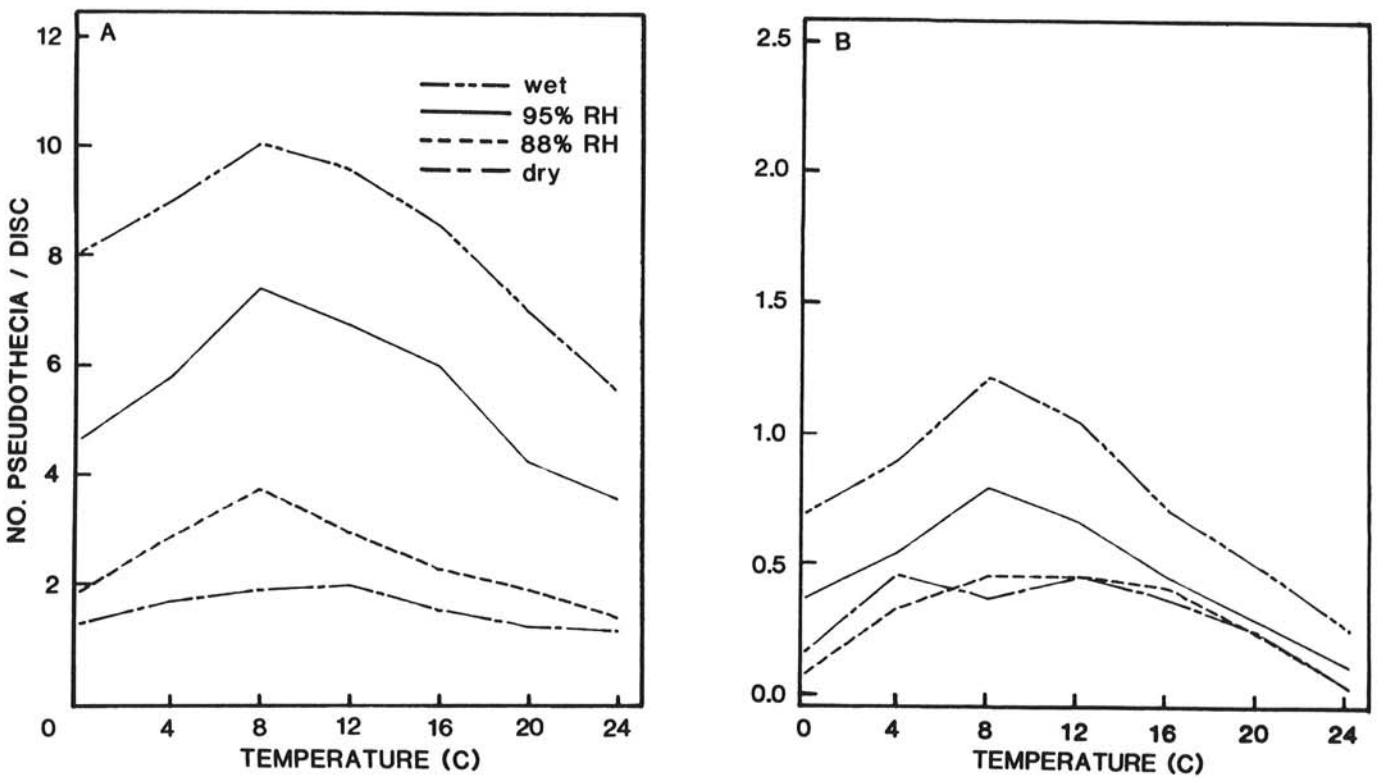

Fig. 3. Effect of temperature and moisture on the number of mature pseudothecia of *Venturia inaequalis* produced in **A,** inoculated and **B,** naturally infected apple leaf disks after a 7-day incubation period during stage 5 (dormancy period).

ascogonial formation (stage 3) was 8 C (Fig. 2A). Pseudothecia at stage 5 (pseudothecia filled with pseudoparaphyses) were not as greatly influenced by temperature as were pseudothecia at the other stages of development (Fig. 3 A). The optimum temperature for pseudothecial development shifted to 16 C when pseudothecia were in stage 7 (asci one-half mature size) (Fig. 4A), and further shifted to 20 C when pseudothecia were in stage 9 (spore formation) (Fig. 5A).

Moisture also strongly influenced pseudothecial development at all stages investigated in this experiment. The wet treatment was

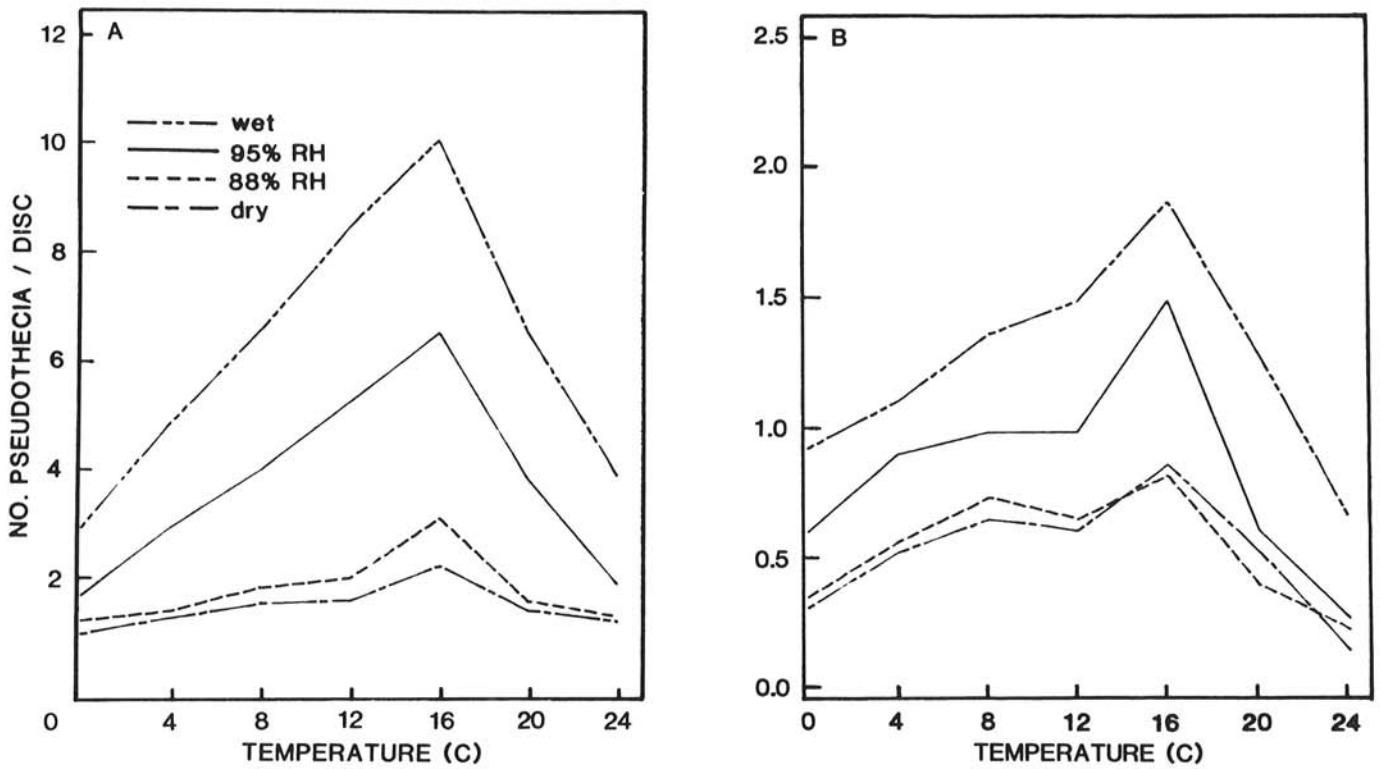

Fig. 4. Effect of temperature and moisture on the number of mature pseudothecia of *Venturia inaequalis* produced in **A,** inoculated and **B,** naturally infected apple leaf disks after a 7-day incubation period during stage 7 (asci one-half mature size).

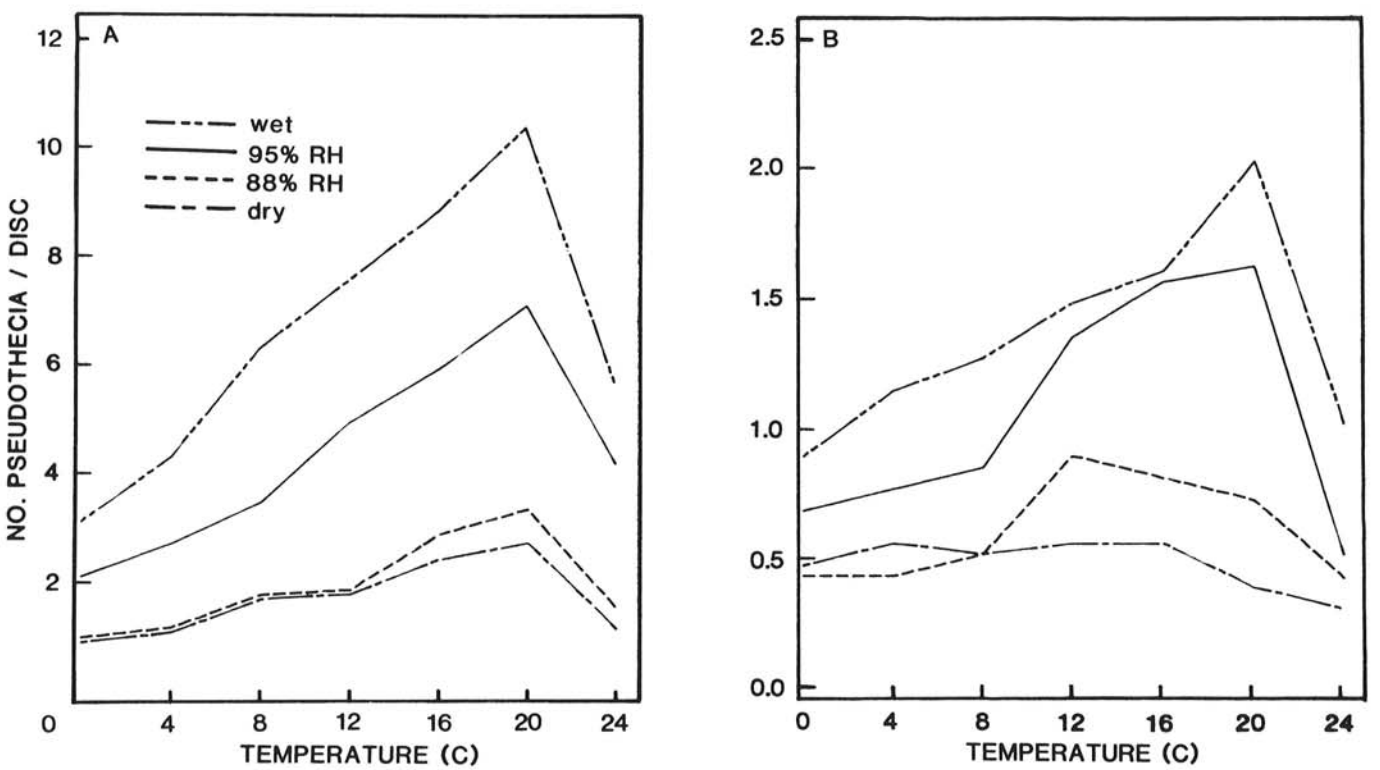

Fig. 5. Effect of temperature and moisture on the number of mature pseudothecia of *Venturia inaequalis* produced in **A,** inoculated and **B,** naturally infected apple leaf disks after a 7-day incubation period during stage 9 (spore formation).

most favorable to initiation and development of pseudothecia, followed by the 95% RH treatment (Fig. 1A–5A). Few pseudothecia were produced in disks in the 88% RH and dry treatments. Average WSD of leaf disks incubated for 3 days at the wet, 95% RH, 88% RH, and dry treatments were 34, 75, 84, and 92%, respectively. After incubation for 7 days, the average WSD of leaf disks at the wet, 95%, 88%, and dry treatments were 33, 80, 90, and 95%, respectively. No consistent differences in WSD were observed among the different temperatures.

From the analysis of variance (Table 1), the main-effect variables temperature and moisture, and the temperature × moisture interactions were significant at $P = 0.05$ or less at all stages of pseudothecial development. The treatment duration effect was significant ($P = 0.05$) only at stages 5 and 7. The temperature × treatment duration interaction was significant ($P = 0.05$) at stages 2, 3, and 7, and the moisture × treatment duration interaction was significant ($P = 0.05$) at stages 2, 3, and 5. The third-order interaction was significant ($P = 0.01$) only in the stage 2 treatment.

The degree of variance contributed by moisture to the total variance was greater than that due to any of the other variables or their interactions at stages 3, 5, 7, and 9 (Table 1). Temperature accounted for slightly more variance than moisture at stage 2. The degree of variance contributed by temperature was lowest at stage 5 compared to the temperature variances at the other stages of development.

Naturally infected leaf disk experiment. Fewer pseudothecia were produced in naturally infected leaf disks in all treatments than in the inoculated leaf disks with similar treatments. The number of mature pseudothecia per disk ranged from 0 to 6 with an overall mean of 0.4. Many small fruiting structures, similar to the undifferentiated pseudothecia produced in the inoculated leaf disks, were also observed.

Temperature, moisture, and treatment duration had effects on pseudothecial production in the naturally infected leaf disks similar to those found with the inoculated leaf disks (Figs. 1B–5B). More pseudothecia were produced in the wet treatments, followed by the 95% RH, 88% RH, and dry treatments. The optimum temperatures for pseudothecial production at stages 2, 3, 5, 7, and 9 were 4, 8, 8, 16, and 20 C, respectively. Analysis of variance indicated that the main-effect variables temperature, moisture, treatment duration, and their interactions accounted for considerably less of the total variability ($R^2 = 0.36$) in the data in the naturally infected leaf disk experiment than in the inoculated leaf disk experiment ($R^2 = 0.90$) (Table 2).

Length of initial incubation at 20 C and inoculum concentration studies. The number of pseudothecia produced in inoculated leaf disks was significantly correlated with the number of weeks the disks were incubated at 20 C ($r = 0.94$, $P = 0.001$) and with the inoculum concentration ($r = 0.83$, $P = 0.001$). Increasing amounts of mycelial growth were observed in the disks at the higher inoculum concentrations and after longer incubation periods at 20 C.

DISCUSSION

Temperature and moisture are important factors that influence the quantitative production of pseudothecia of *V. inaequalis*. Our results confirm the observation that low temperature and high moisture conditions are necessary for initiation of pseudothecia (6,10). Our results further indicate that once pseudothecia have been initiated, temperature and moisture continue to influence the number of pseudothecia that reach maturity. Many immature pseudothecia exposed to suboptimal temperatures and moisture conditions never matured. Some of the small, undifferentiated pseudothecia observed in the inoculated and naturally infected leaf disks may have aborted as a result of adverse conditions. Temperatures of 24 C and above combined with wet conditions are known to cause pseudothecial abortion (6).

When temperatures were favorable for the initiation and development of pseudothecia, moisture limited the number of pseudothecia produced in the inoculated and naturally infected leaf disks. Few mature pseudothecia were produced in leaf disks with a WSD greater than approximately 80% at all temperatures and at all stages of pseudothecial development. James and Sutton (6) found that moisture was the most limiting factor for development of pseudothecia of *V. inaequalis* in NC. In laboratory studies (6), the rate of pseudothecial development was retarded at WSD greater than 85% and in field studies, this rate was most highly correlated with rainfall or high relative humidities.

Temperature was less influential on the development of pseudothecia at stage 5 than at the other stages of development. James and Sutton (6) observed that the maturation process stopped when pseudothecia reached stage 5, that development did not continue until a dormancy requirement was met, and that the length of the dormancy period was not affected by temperature or moisture. Our results indicated that when pseudothecia at stage 5 were exposed to low moisture conditions, few of them matured.

Jeger and Butt (8) were unable to obtain reliable equations for predicting pseudothecial abundance in naturally infected leaves overwintered in the field by using environmental temperature and rainfall data and suggested that factors such as lesion age and disease intensity at leaf fall were not standardized. In our study, these factors were standardized with the inoculated leaf disk technique so that the effects of a range of temperatures and moisture conditions could be quantified without these confounding factors.

The low coefficient of determination ($R^2 = 0.36$) obtained for the effects of temperature and moisture and the low mean number of pseudothecia produced in the naturally infected disks compared to the inoculated leaf disks indicate that other factors affected the production of pseudothecia. The naturally infected disks were infested with many saprophytic organisms, and saprophytic organisms can inhibit production of pseudothecia of *V. inaequalis* through nutrient competition and antagonism (2,5). Scab incidence at leaf fall has also been shown to influence production of pseudothecia (3,10). The heterothallic nature of *V. inaequalis* requires that mycelium of both mating types be in close proximity in leaf tissue for pseudothecial initiation to occur. In a survey of orchards in New Hampshire, Gadoury (3) found that a high percentage of lesions were infertile and that the number of fertile

TABLE 1. Mean squares[w] of number of pseudothecia of *Venturia inaequalis* produced in inoculated leaf disks resulting from the interaction of seven temperatures, four moisture treatments, and two treatment durations[x] imposed at five stages of pseudothecial development

Source[z]	df	Stage of development[y]				
		2	3	5	7	9
Temperature (TEM)	6	450.49***	380.95***	179.96***	206.59**	292.09*
Moisture (MOI)	3	441.52***	752.68***	2,626.01***	1,664.72***	1,757.02***
Treatment duration (TD)	1	4.29 NS	4.19 NS	52.25*	34.71*	5.25 NS
TEM × MOI	18	97.45***	72.85***	18.67***	26.69***	26.59***
TEM × TD	6	15.62**	10.23*	2.48 NS	17.58**	9.11 NS
MOI × TD	3	8.09*	18.58*	32.03**	1.09 NS	3.12 NS
TEM × MOI × TD	18	7.24*	5.33 NS	1.78 NS	4.57 NS	1.49 NS

[w] Asterisks indicate: ***, significant at $P = 0.001$; **, significant at $P = 0.01$; and *, significant at $P = 0.05$. NS = nonsignificant.
[x] Temperatures were 0, 4, 8, 12, 16, 20, and 24 C; moisture treatments were wet, 95% RH, 88% RH, and dry; treatment durations were 3 and 7 days.
[y] Stages: 2 = pseudothecia initials, 3 = ascogonial formation, 5 = dormancy period, 7 = asci one-half mature size, and 9 = ascospore formation.
[z] Data analyzed in a split-split plot design.

TABLE 2. Selected mean squares[w] of the number of pseudothecia produced in leaf disks inoculated or naturally infected with *Venturia inaequalis*, resulting from the interaction of seven temperatures, four moisture treatments, and two treatment durations[x] imposed at five treatment times[y]

Source[z]	df	Inoculated	Naturally infected
Treatment time (TT)	4	1,163.83*	1.09*
Temperature (TEM)	6	464.29***	21.78***
Moisture (MOI)	3	6,434.59***	51.00***
Treatment duration (TD)	1	1.16 NS	0.003 NS
TEM × MOI	18	65.76***	2.09***
TEM × TD	6	5.22 NS	0.13 NS
MOI × TD	3	29.93***	1.02*
TEM × MOI × TD	18	2.41 NS	0.29 NS
R^2 (entire model)		0.90	0.36
$\bar{X}$		2.75	0.44

[w] Asterisks: ***, significant at $P = 0.001$; and *, significant at $P = 0.05$.
[x] Temperatures were 0, 4, 8, 12, 16, 20, and 24 C; moisture treatments were wet, 95% RH, 88% RH, and dry; treatment durations were 3 and 7 days.
[y] Treatment times were chosen when pseudothecia were at the following stages of development: stage 2, pseudothecial initials; stage 3, ascogonial formation; stage 5, dormancy period; stage 7, asci one-half mature size; and stage 9, ascospore formation.
[z] Data were analyzed in a split-split plot design.

lesions was proportional to lesion incidence at leaf fall. Production of increasing numbers of pseudothecia in relation to increasing inoculum concentration and incubation time at 20 C was probably due to the increased probability of mycelium of the two mating types encountering each other in the inoculated leaf disks. Although these factors may have contributed to the reduction of the number of pseudothecia produced, the temperature and moisture effects observed with the inoculated leaf disks were still evident in the naturally infected disks.

Several models for predicting maturity of ascospores of *V. inaequalis* have been proposed (7,9), but comparably little has been done to develop models for predicting the amount of ascospore inoculum. Gadoury (3) has proposed a model for predicting potential ascospore dose based on disease incidence at leaf fall, pseudothecial density per lesion, number of asci per pseudothecium, and leaf litter density. Pseudothecial density was calculated as the product of the proportion of lesions that are fertile and a constant number of pseudothecia per lesion, determined in a survey of orchards in New Hampshire. Temperature and moisture were not included as limiting factors in this model. In the United Kingdom, Jeger and Butt (8) observed that November temperatures usually occurred within the range required for pseudothecial initiation and concluded that in most years pseudothecia would develop unhindered. However, we have observed relatively low ascospore productivity in NC during some years. In the winter of 1975–1976 at the Mountain Horticultural Crops Research Station, Fletcher, NC, November temperatures exceeded 20 C for nine consecutive days, February temperatures were 20 C or above on 9 days, and rainfall was 3.6 cm above normal. Few pseudothecia were observed to mature and ascospore catch by spore traps was very low (T. B. Sutton, *unpublished*). These observations, together with results from our study, indicate the importance of including temperature and moisture as quantitative factors which influence the density of pseudothecia in the warmer apple-growing regions of the world. The influence of temperature, moisture, and saprophytes under field conditions needs to be quantified and these results could be added to the model proposed by Gadoury (3) to obtain a more widely applicable model for predicting pseudothecial abundance.

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