Ecology and Epidemiology

Effect of Pod Development Stage, Temperature, and Pod Wetness Duration on the Incidence of Purple Seed Stain of Soybeans

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Contribution 1814, Department of Plant Pathology.
Accepted for publication 21 October 1991 (submitted for electronic processing).

ABSTRACT

Schuh, W. 1992. Effect of pod development stage, temperature, and pod wetness duration on the incidence of purple seed stain of soybeans. Phytopathology 82:446-451.

The incidence of soybean seed infected by *Cercospora kikuchii* was influenced by temperature, length of pod wetness period, and developmental stage of the soybean pods at the time of inoculation. The statistical significance was determined using logistic regression. Infected seeds were observed when small pods (<0.5 cm in length) and large pods (0.5–2.0 cm in length) were inoculated with a conidial suspension. No infection was observed when flowers in full bloom or postbloom (desiccated petals) stages were inoculated. The optimal temperature for infection was 25 C; no infection was observed at 15 and 35 C. In general, disease incidence increased with increasing pod wetness periods of up to 30 h. In some temperature/pod wetness combinations, disease incidence decreased when the pod wetness was further increased to 36 h. No disease was observed at pod wetness periods of less than 24 h. To describe effects of environment on purple stain incidence in pods, logistic regression models were developed separately for each pod developmental stage. The models contained linear and quadratic and interaction terms for both temperature and leaf wetness duration and satisfactorily described the data. Seed infection and seed germination were not significantly related.

The fungus *Cercospora kikuchii* (Matsumoto & Tomoyasu) M. W. Gardner (11) infects leaves, stems, and pods of soybeans (*Glycine max* (L.) Merr.) (18). It was first identified in the United States in 1924 (3) and has since been reported in most soybean-growing areas.

The disease, generally called purple seed stain, is most obvious and easily distinguishable on seeds (8). Most infected seeds exhibit discoloration ranging from pale to dark purple. The discolored area can be limited to small spots or can cover the entire surface of the seed coat. The fungus also has been isolated from symptomless seeds. Purple seed stain can cause reduced germination and/or diseased seedlings, which result in reduced plant stands in the field (5). Seed infection also can lead to economic losses because of inferior grain standard designation. The U.S. Department of Agriculture grain standards allow no more than 5% purple-stained seeds in No. 1 yellow soybean seed lots (16).

Field research on the soybean pod developmental stage susceptible to infection has reached conflicting conclusions. Flowers in full bloom have been found to be susceptible, as have both young and old pods (2,4,9,13). Roy and Abney (13) also implicated temperature and moisture conditions in the canopy as important factors in the infection process. They did not, however, determine the quantitative relationship between these parameters and infection. Similarly, conflicting results have been reported regarding the influence of seed infection on seed germination (1,10,15,19,21).

The purpose of this study was to investigate the effect of flower and pod developmental stage, temperature, and wetness duration on pod infection. Additionally, the influence of seed infection on germination was evaluated under controlled conditions. The results of this study should help determine optimal conditions for resistance screening under controlled conditions, aid in the timing and evaluating of field inoculations, and potentially lead to predictive schemes for disease management.

MATERIALS AND METHODS

Plant production and maintenance. Two seeds of soybean cultivar Amsoy 71 were planted in plastic containers holding steam-disinfested soil (1:2:2, v/v/v, sand:peat:loam) and maintained on greenhouse benches. Plants were watered with deionized water and fertilized every 2 wk with Peter's 20-20-20 (N-P-K) fertilizer. Sunlight was supplemented with 1,000-W high-pressure sodium lamps for 12 h per day. Approximately 3 wk after planting, plants were thinned to one per pot. Greenhouse temperatures, as monitored by a hygrothermograph, ranged from 20 to 25 C. Relative humidity was in the range of 40–60%. Plants were maintained in the greenhouse for 6 wk before the start of the experiment.

Inoculum maintenance and production. An isolate of *C. kikuchii,* obtained from infected soybean seed collected at the Russell E. Larson Agricultural Research Center at Rock Springs of the Pennsylvania State University, was maintained on clarified V8 juice agar at a pH of 6.0 (20) and 25 C. Five days before inoculation, 10-day-old fungal colonies and attached agar substrate were macerated in a Waring blender. The resulting slurry was spread on petri plates containing clarified V8 agar. The plates then were incubated at 25 C under 24-h light (two 20-W cool-white fluorescent lights set 15 cm above the plate) for 4 days. This resulted in the production of numerous conidiophores, but conidial production was absent. After this treatment, plates were exposed to 12 h of darkness followed by 12 h of light for 1 day, which induced the production of mature conidia of uniform age and size. This procedure was repeated for each inoculation. After four consecutive transfers, new fungal material from the original culture was obtained from liquid nitrogen storage. This prevented loss of pathogenicity due to prolonged culture on artificial media.

Inoculation. Petri dishes containing mature conidia, produced as described above, were flooded with 15 ml of distilled water containing 0.5% Tween 20 (Sigma Chemical, St. Louis, MO). The conidia were dislodged by gentle agitation with a camel hair brush. The water/spore mixture then was strained through a single layer of cheesecloth to remove mycelial fragments and was adjusted to a density of 30,000 conidia per milliliter of water. Spore concentration was determined with a hemacytometer.

Inoculations were made at four stages of pod development: full bloom, post bloom, small pod with a length of less than 0.5 cm, and large pod with a length of 0.5–2 cm. Two flowers or pods representing each single developmental stage were tagged on each plant. Tagged flowers or pods were inoculated with conidia of *C. kikuchii* using an airbrush (Badger Air-Brush, Franklin Park, IL) with 103.5 kPa of pressure. The plant parts were sprayed for 15 s from all sides at a distance of 15 cm. Plants were left on the lab bench for 0.5 h, the time necessary for drying.

Treatments. The experiment was conducted using four Percival (Boone, IA) dew chambers. The treatments consisted of five temperatures (15, 20, 25, 30, and 35 C) and four dew periods (18, 24, 30, and 36 h) in all possible combinations. Ten plants, each with two units of each pod development stage, were inoculated per temperature/wetness duration combination. The sequence of treatments and the assignment of dew chambers were random. The entire experiment was repeated four times.

Disease assessment. After exposure to the selected temperature/dew period treatment, plants were placed on greenhouse benches and pods were allowed to mature. Leaf axils with tagged flowers or pods were monitored regularly after inoculation, and newly forming flowers were removed. At maturity, tagged pods were harvested by hand and returned to the lab. Pods were opened under a laminar flow hood. Presence or absence of disease symptoms on the seeds was recorded. Seeds from each pod were separately transferred to a petri dish containing V8 juice agar. Seeds were not surface-sterilized to aid in the detection of nonsymptomatic infection. Tweezers were sterilized between seeds. As a check, uninoculated soybean plants of similar age were positioned in the greenhouse among the inoculated plants. They were harvested with the test material to determine levels of natural infection by inoculum present in the greenhouse.

Data analysis. The effect of pod developmental stage, temperature, and wetness duration on the infection of soybean seeds was analyzed using logistic regression procedures (SAS Proc Logist [14]). Logistic regression (12) was chosen because the dependent variable is binary, i.e., the dependent variable represents observations that take only two values, 0 = no infected seeds per pod and 1 = at least one infected seed per pod. Logistic regression is based on the equation

$$P(z) = 1/[1 + \exp - (\beta_0 + \beta_1 x_1 + \ldots + \beta_n x_n)], \quad (1)$$

where $P(z)$ is the probability of event z, and the betas are the partial regression coefficients for the independent variables. Proc Logist estimates the partial regression coefficients using the linearized form of equation 1. In this case, the linear set of variables was of the form

$$P[z/(1 - z)] = f(PDS, T, WD), \quad (2)$$

in which $P[z/(1 - z)]$ is the probability of event z (probability of infection) in logit form and $f(PDS, T, WD)$ is a linear function of pod developmental stage (PDS), temperature (T), and wetness duration (WD). The terms T, T^2, WD, WD^2, PDS, $T \times W$, $T \times PDS$, and $W \times PDS$ were tested using a stepwise approach. The final model was chosen on the basis of the following criteria: 1) statistical significance of the regression coefficients, 2) chi-square values of the model, and 3) comparison of observed and predicted values. Proc Logist uses the maximum likelihood procedure for parameter estimation.

RESULTS

Influence of pod developmental stage on disease incidence. Purple stain, if associated with a seed, was evident at the time of removal from the pods. Seeds harvested from control plants did not show purple discoloration, and nonsymptomatic infection was not detected in any seed.

Purple-stained seeds were not recovered from pods inoculated at the full and postbloom developmental stages. An average of 14.6 and 18.4% of the pods inoculated at the small- and large-pod stages, respectively, contained at least one infected seed. These values represent the mean incidence averaged over all temperatures and wetness periods and were not significantly different ($P = 0.05$). Disease incidence (the percentage of pods with at least one infected seed) was higher in the large-pod stage in six of nine temperature/dew period combinations compared with those of the small-pod stage. A higher percentage of pods with one infected seed was observed at the small-pod stage in seven of nine treatment combinations. For pods with two infected seeds, the large-pod stage had a higher disease incidence in six of nine treatment combinations. Pods with three infected seeds were found in one treatment combination in the small-pod stage as compared with five for the large-pod stage (Figs. 1 and 2).

Influence of temperature and dew period on disease incidence.

Infected seeds were not observed in pods inoculated and incubated at 15 and 35 C or at dew periods of 18 h at any temperature. This was the case for both small- and large-pod stages.

For plants inoculated at the small-pod stage, the highest percentage of pods with one or more infected seeds in each dew period was observed at 25 C for the 24- and 30-h dew periods, and at 30 C for the 36-h period (Fig. 1). Disease incidence increased when dew periods were increased to 30 h at all temperatures. Further increase in dew period length led to a reduced disease incidence at 20 and 25 C, but to an increased incidence at 30 C. At the large-pod stage, the highest percentage of pods with at least one infected seed in each dew period was observed at 25 C, again with the exception of the 36-h dew period. The effect of the dew on infection length was similar to the small-pod stage, i.e., disease incidence increased at all temperatures when the dew period was extended to 30 h (Fig. 2). Further extension resulted in a reduction in disease incidence at 20 and 25 C and an increase at 30 C. In general, 30 C was more favorable for infection than 20 C.

When comparing the separate categories (pods with one, two, or three infected seeds) for the small-pod stage (Fig. 1), the highest percentage of pods with one infected seed in each dew period was observed at 25 C for the 24-, 30-, and 36-h dew periods. The highest percentage of pods containing two infected seeds

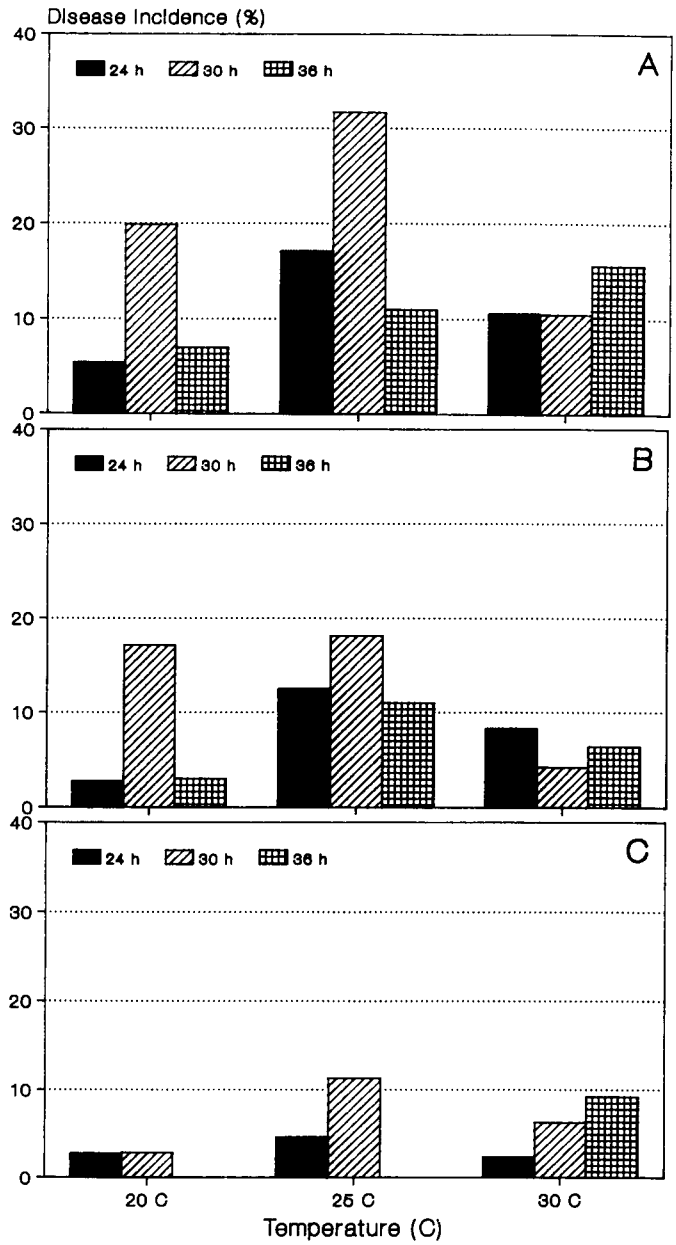

Fig. 1. Effect of temperature and dew period on disease incidence of purple seed stain of soybean cultivar Amsoy 71 inoculated at the small-pod stage (<0.5 cm in length). Percentage of pods with **A,** ≥ 1 infected seed; **B,** one infected seed; and **C,** two infected seeds.

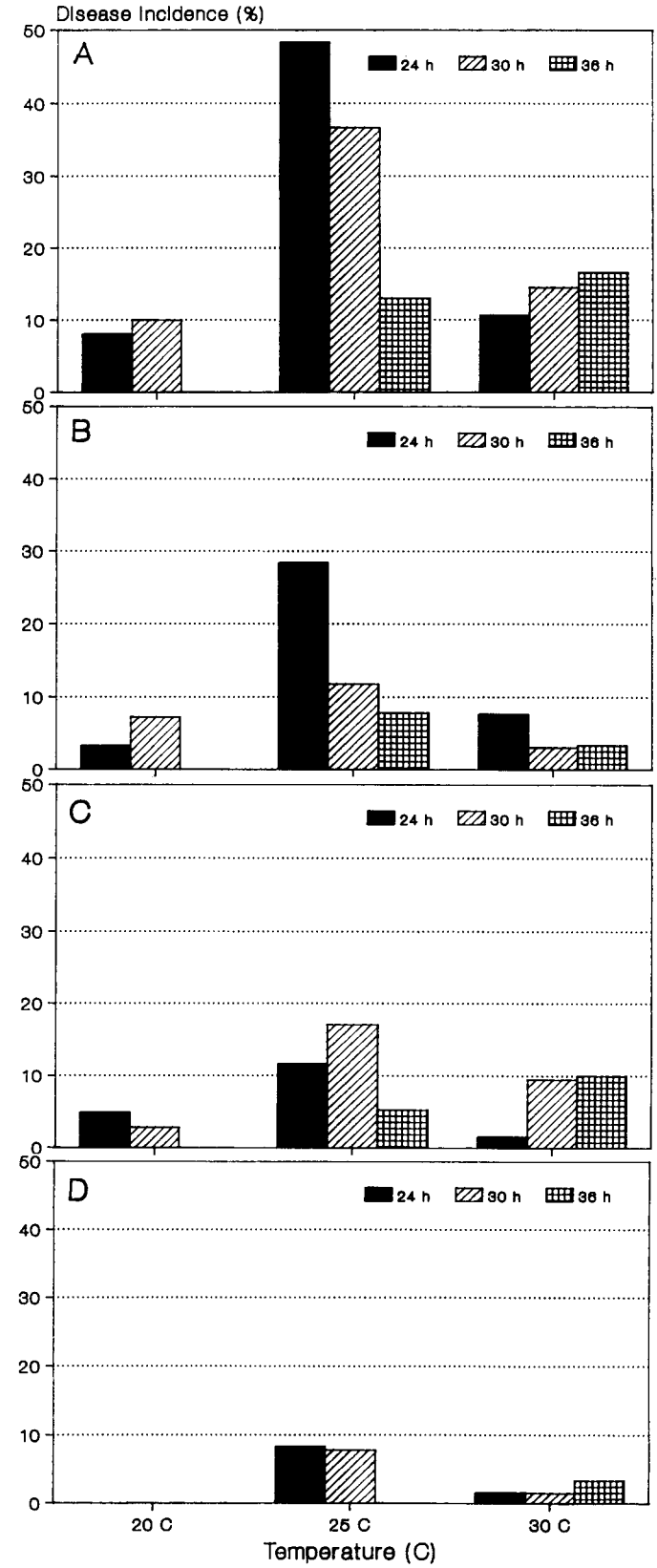

Fig. 2. Effect of temperature and dew period on disease incidence of purple seed stain of soybean cultivar Amsoy 71 inoculated at the large-pod stage. Percentage of pods with **A,** ≥ 1 infected seed; **B,** one infected seed; **C,** two infected seeds; and **D,** three infected seeds.

was observed at 25 C, again with the exception of the 36-h dew period. The effect of increasing dew periods mirrored the behavior observed for the total disease incidence. Pods with three infected seeds were observed at the 25 C/30-h treatment only (2.3%). In general, 30 C was more favorable than 20 C. When comparing the separate incidence categories for the large-pod stage (Fig. 2), the highest percentage of pods with one infected seed in each dew period was at 25 C. The percentage decreased with increasing dew periods. This can be partly attributed to the increase in the percentage of pods with two infected seeds with increasing dew periods. The highest incidence of pods containing two infected seeds was observed at 25 C with the exception of the 36-h dew period. The effect of dew period was different at each temperature. At 20 C, the incidence declined with extending dew periods, at 30 C it increased. At 25 C, the incidence of pods with two infected seeds increased up to dew periods of 30 h, then decreased. At 30 C disease incidence increased with increasing dew periods. Pods with three infected seeds were not observed at 20 C regardless of dew period. The percentage of pods in this incidence category was higher at 25 C than that at 30 C, again with the exception of the 36-h dew period. Extending the dew period beyond 30 h reduced the disease incidence at 25 C and increased it at 30 C.

Model development. A satisfactory logistic regression model that included pod developmental stage, temperature, and dew period as variables could not be developed. Models that contained temperature and dew period, however, were developed for each pod developmental stage.

Small-pod developmental stage. The best model describing the probability of obtaining at least one infected seed per pod (Y) had the form

$$\text{Prob}\,(Y) = 1/1 + \exp -(-41.64 + 1.79T - 0.03T^2 + 1.20D - 0.02D^2), \quad (3)$$

where T is the temperature, T^2 is temperature squared, D is the dew period in hours, and D^2 is the dew period squared. All parameters were significant at $P = 0.05$. The model likelihood ratio chi-square and its P value for the equation indicated a good fit. The mode likelihood ratio chi-square is twice the difference in log likelihood of the current model from the likelihood based only on intercepts. The observed percentage of pods with at least one infected seed was within the 95% confidence interval of the modeled disease incidence in eight out of nine cases (Table 1). The value outside the confidence interval was observed at the 20 C/36-h treatment combination (3.0% observed, lower 95% confidence interval = 3.1%).

A model describing the probability of obtaining at least two infected seeds per pod identified the same coefficients and parameters with a slightly different intercept (−42.82). Disease incidence estimated with this model was within the 95% confidence interval in six of nine cases. The values outside the confidence interval were observed at the 36-h dew period. The model overestimated the disease incidence twice, and underestimated it once.

A model to predict the probability of obtaining three infected seeds per pod could not be developed. The absence of this incidence class at 20 and 30 C precluded the estimation of the coefficient for the parameter temperature.

Large-pod developmental stage. The best model describing the probability of obtaining at least one infected seed per pod (Y) had the form

$$\text{Prob}\,(Y) = 1/1 + \exp -(-34.20 + 2.60T - 0.07T^2 - 0.01D^2 + 0.03T \times D), \quad (4)$$

where the variables are defined as in equation 2 and $T \times D$ is the temperature/dew period interaction. All parameters were significant at $P = 0.05$, and the likelihood ratio indicated a good fit. The observed values were within the 95% confidence intervals of the predicted values in eight of nine cases (Table 2). At 20 C/30-h dew period, the observed value was 10.0%, and the upper 95% confidence interval was 9.9%. A model describing the probability of obtaining at least two infected seeds per pod identified the

TABLE 1. Comparison of observed and estimated *Cercospora kikuchii* disease incidence per pod on soybean cv. Amsoy 71 inoculated at the small-pod (<0.5 cm in length) stage

Dew period (h)	Temperature (C)																							
	20								25								30							
	Y_1[a]				Y_2[b]				Y_1				Y_2				Y_1				Y_2			
	Obs[c]	Pred[d]	LCI[e]	UCI[f]	Obs	Pred	LCI	UCI	Obs	Pred	LCI	UCI	Obs	Pred	LCI	UCI	Obs	Pred	LCI	UCI	Obs	Pred	LCI	UCI
24	5.4	6.9	3.2	14.4	2.7	2.2	1.0	5.0	17.1	17.1	10.0	26.5	4.6	5.9	3.1	11.0	10.6	17.9	8.0	17.9	2.3	2.9	1.3	6.2
30	19.9	14.0	7.0	24.8	2.8	4.6	2.1	9.9	31.7	30.4	20.1	43.1	13.6	11.8	6.5	20.0	10.5	17.1	9.9	27.7	6.3	5.9	2.9	11.5
36	3.0	7.3	3.0	16.6	0.0	2.3	0.9	6.2	11.0	17.9	8.0	35.0	0.0	6.3	2.4	15.3	15.6	9.3	4.0	19.9	9.2	3.1	1.1	7.7

[a] Y_1 = Incidence of ≥ one infected seed per pod.
[b] Y_2 = Incidence of ≥ two infected seeds per pod.
[c] Observed disease incidence (mean of four runs).
[d] Estimated disease incidence from equation 3.
[e] Lower 95% confidence limit.
[f] Upper 95% confidence limit.

TABLE 2. Comparison of observed and estimated *Cercospora kikuchii* disease incidence per pod on soybean cv. Amsoy 71 inoculated at the large-pod (0.5–2 cm in length) stage

Dew period (h)	Temperature (C)																							
	20								25								30							
	Y_1[a]				Y_2[b]				Y_1				Y_2				Y_1				Y_2			
	Obs[c]	Pred[d]	LCI[e]	UCI[f]	Obs	Pred	LCI	UCI	Obs	Pred	LCI	UCI	Obs	Pred	LCI	UCI	Obs	Pred	LCI	UCI	Obs	Pred	LCI	UCI
24	8.0	12.9	7.3	21.7	4.6	6.3	3.4	11.5	48.3	41.3	32.4	50.9	20.0	24.4	17.7	32.7	10.6	10.3	5.3	18.8	3.0	5.0	2.4	9.8
30	10.0	5.1	2.7	9.9	2.8	2.4	1.2	4.7	36.7	37.3	30.0	45.2	21.0	21.4	16.0	28.1	14.5	18.8	12.5	25.6	11.0	9.2	5.9	14.1
36	0.0	0.8	0.0	2.9	0.0	0.3	0.0	1.4	13.0	16.1	10.1	24.6	5.0	8.0	4.7	13.4	16.6	14.1	8.5	22.5	13.3	7.0	3.9	12.1

[a] Y_1 = Incidence of ≥ one infected seed per pod.
[b] Y_2 = Incidence of ≥ two infected seeds per pod.
[c] Observed disease incidence (mean of four runs).
[d] Estimated disease incidence from equation 4.
[e] Lower 95% confidence limit.
[f] Upper 95% confidence limit.

same coefficients and parameters. Again, the intercept was slightly different (−35.0). The observed values were within the 95% confidence interval of the estimated values in eight of nine cases. The exception was at the 30 C/36-h treatment. As in the small-pod developmental stage, a model describing the probability of three infected seeds per pod could not be developed.

Influence of seed infection on seed germination. Seed infection was associated with purple discoloration of the seed coat in all cases. Regression of percent seed germination per pod on percentage of seeds showing discoloration per pod was not significant ($P = 0.05$).

Influence of pod developmental stage on percent pod recovery. The percentage of pods recovered (number of pods harvested/number of units inoculated) was determined for each pod developmental stage. Seventy-nine, 48, 38, and 31% of the units inoculated in the large-pod, small-pod, postbloom, and full-bloom stages produced harvestable pods averaged over all temperatures and dew periods. In general, the percent recovery decreased with increasing dew periods (Fig. 3).

DISCUSSION

Previous field research aimed at determining the pod developmental stage most susceptible to infection reached different conclusions. Laviolette and Athow (9) reported from field studies that inoculation during the full flower period resulted in the highest percentage of discolored seeds. Field studies by Crane and Crittenden (2) implicated the length of the flowering period as the main factor in determining the incidence of purple-stained seeds. Roy and Abney (13) found young pods to be the developmental stage most susceptible for infection. In field studies, however, it is difficult to separate infection at the flowering stage from subsequent infection of the developing pods because of naturally occurring inoculum. Conidia of *C. kikuchii,* as determined through spore trapping, are present throughout the growing season in significant numbers (C. Orth and W. Schuh, *unpublished data*). Additionally, many small pods are already present at full bloom. Weather conditions at the time of inoculation also play an important role in the success of the infection and could lead to different conclusions regarding the pod developmental stage most conducive to infection (13). The importance of weather conditions during the time of and shortly after inoculation on the disease incidence is supported by the research of Jones (6), who suggested that once *C. kikuchii* enters the pod, influence of the environment on seed infection is limited. The approach presented in this study, namely using tagged plant parts inoculated under controlled conditions during several pod developmental stages and monitoring infection caused by naturally occurring inoculum, provides a more comprehensive method of determining key conditions for disease development.

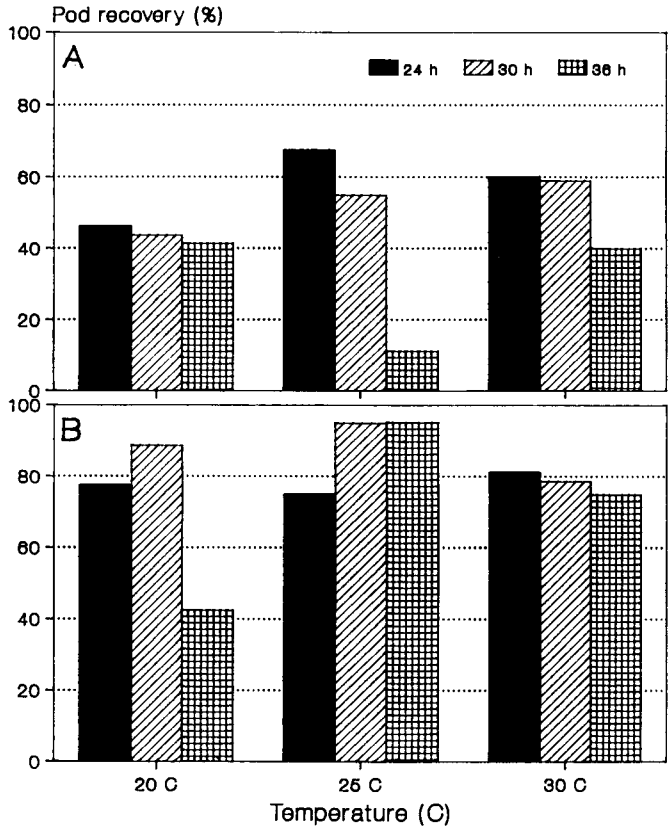

Fig. 3. Percent recovery of pods of soybean cultivar Amsoy inoculated under various temperature and dew period combinations with *Cercospora kikuchii* at the **A,** small-pod stage, and **B,** large-pod stage.

In this study, purple-stained seeds were recovered after inoculation in the small- and large-pod stages. Chen et al (1), however, did not observe seed discoloration when soybean plants were inoculated during the R2 to R6 growth stages under controlled conditions. R2 to R6 growth stages describe pod development from flowering to pods containing fully developed green seed. The environmental conditions in their study after inoculation (3 days at 100% RH, 27 C day/21 C night) fall in the optimal range as determined in this study. Roy and Abney (13) also failed to observe infected seeds when inoculations were performed during flowering under controlled conditions. Flowers in their study, however, were inoculated with mycelial plugs as compared with conidial suspensions used as inoculum in the present study. The length of the dew period in their study (14–16 h) confounds comparison, since a minimum period of 24 h of dew was necessary even under optimal temperature conditions to cause seed infection in this study. Histological specimen of flowers collected 8, 16, 24, 48, and 72 h after inoculation in the field showed no evidence of mycelium of *C. kikuchii.* Kilpatrick (7) failed to isolate *C. kikuchii* from unopened flowers, opened flowers, and flowers opened 1 day and exposed to natural infection in the field.

The influence of temperature on disease incidence was statistically significant. This disagrees with findings by Hepperly (4), who found that temperature was not associated with the incidence of purple-stained seed. This contradiction can be explained through the narrow range of mean daily temperatures (24–30 C) observed in his experiment. In addition, little variation in either maximum or minimum temperatures was found in his study.

The influence of dew period length on the incidence of seed infection was not as clear. Disease incidence increased from 24 to 30 h, and then decreased at 36 h. This trend was more pronounced in the small-pod stage than in the large-pod stage. The different recovery rate of the pods (number of pods recovered/number of pods inoculated) may be a reason. In general, the percent recovery decreased with increasing dew periods for all pod developmental stages. That this abortion was due to infection with *C. kikuchii* cannot be determined with this experimental design. Van Schaik and Probst (17) found that abortion rates (flowers and pods combined) ranged from 43 to 81% for different cultivars in both growth chamber and field experiments. Increased abortion rates were caused by temperatures greater than 32 C, increasing photoperiods, and high number of flowers per leaf axil. In this study, greenhouse temperatures were in the range of 24–28 C, the temporal sequence of treatments was randomized, and the number of flowers was kept at a maximum of two per leaf axil. The possibility of infection as a cause of flower and pod abortion therefore cannot be dismissed with certainty.

The models developed for infection of small and large pods predicted the incidence of purple stain satisfactorily. Comparing the absolute difference between observed and predicted incidence values, the equation for the large-pod stage had smaller differences. Models could not be developed containing pod developmental stage as an independent variable. The models for small and large pods, however, identify the same significant parameters with the exception of dew (small-pod stage) and the temperature/dew period interaction for the large-pod stage. In both models, the temperature optimum is identical (25 C). Models for obtaining

two or more infected seeds were developed to investigate if different conditions during the infection process, i.e., longer dew periods or narrower temperature ranges, were needed to obtain this higher infection level. The incidence curves resulting from the models for one or more and two or more infected seeds per pod were parallel, thus indicating that the temperature and dew period requirements were identical, with different probabilities of infection associated with each incidence level.

Percentage of germination was not influenced by seed infection with *C. kikuchii,* which confirms findings of TeKrony et al (15) and Yorinori and Sinclair (22). TeKrony and Egli (15) found a correlation value of −0.27 relating germination to the incidence of infected seed. Wilcox and Abney (19), however, found a significant reduction in germination (from 82 to 72%) for infected seeds. They also found a reduction in emergence between 10 and 15% in field trials. Similar results were obtained by Yeh and Sinclair (21), who observed a reduction in germination and emergence from a sand bench. They classified the seeds according to the percentage of the seed coat showing purple stain. Seeds with 1–25% coverage were not different in germination from uninfected seeds. Divergent conclusions regarding the influence of seed infection on germination might therefore be related to different disease assessment methods, environmental conditions after infection, or length of storage period.

This study describes the environmental parameters and the pod developmental stage most conducive for seed infection by *C. kikuchii.* Inoculations for resistance screening under controlled conditions should be done using pods of 0.5–2.0 cm in length and 25 C/30 h of dew duration. These parameters also are useful in determining the infection success for inoculations under field conditions and for the development of a disease management system.

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