A Model for Predicting the Effects of Microclimate on Infection of Soybean by *Phomopsis longicolla*

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ABSTRACT

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A model was developed relating infection of soybean tissue by *Phomopsis longicolla* to environmental conditions. The model was based on natural infection of soybean seedlings placed in a field at weekly intervals throughout the growing season in 1981, 1982, and 1983, as well as laboratory inoculation and in vitro growth and germination studies. Parameters used in the model were rain, surface wetness, temperature, and relative humidity. Based on its average hourly temperature, each surface wetness period was assigned a rating that was the equivalent period of surface wetness at optimum temperature for pathogen growth (25–30 C). The relationship of in vitro growth rate to temperature was used to assign these ratings. An infection period was defined as beginning with rain (inoculum dispersal) and ending with surface drying. Factors were included in the model to account for exhaustion of inoculum and continuation of infection periods after interruption. When applied to the field infection results, little or no infection occurred at rating totals less than 13, but infection increased linearly with an increase in rating totals greater than 13. These results were similar to the results of the inoculation studies at 25 C. The coefficient of determination (r^2) for the model was 0.73. The model could serve as the basis of a model predicting pod infection by *P. longicolla*.

Infection of soybean (*Glycine max* (L.) Merr.) seeds by *Phomopsis longicolla* Hobbs, the primary causal agent of pod and stem blight, involves two distinct phases: infection of green pods by inoculum produced on soybean debris and infection of seeds by growth of the fungus from pod infections. Most seed infection occurs after physiological maturity when the pods senesce (4,5,8,10,11,20) and depends in part on the moisture status of the pods (17). The amount of seed infection is also dependent on the amount of pod infection, and pod infection levels at the R6 growth stage have been used to determine the need for fungicide applications to maintain seed quality (14).

Some environmental conditions have been associated with pod infection by *P. longicolla*. Rain has been strongly associated with infection of soybeans by *P. longicolla* (9,13,18) acting in part as a means of dispersal. Splash dispersal of spores of *P. longicolla* has been reported in both greenhouse and field studies (9). Temperature and relative humidity also influence pod infection. Kmetz et al (9) found that field inoculations were successful if the temperature averaged at least 26 C, relative humidity remained above 80%, and rain was 0.6 cm above normal for 7 days after inoculation. TeKrony et al used average temperature, relative humidity, and total rainfall from the R5 growth stage to physiological maturity as part of a multiple regression model predicting seed infection (19). Because no direct measurements of pod infection were made, the importance of these environmental factors to pod infection can only be inferred from this model.

Measuring pod infection, however, is difficult. Although all parts of the plant are susceptible to infection by *P. longicolla* throughout the growing season, symptoms and signs of the fungus only appear after an infected plant part dies (5,9,21). Consequently, changes in infection incidence over time must be estimated by isolation of the fungus from tissue samples.

The purpose of this research was to define and quantify how specific environmental conditions affect infection of soybean plants by *P. longicolla*. To that end, a model is presented that relates in-canopy environmental conditions directly to plant infection. Two preliminary reports have been published (15,16).

MATERIALS AND METHODS

An isolate of *P. longicolla* from an infected soybean seed was used in mycelial growth, spore germination, and seedling inoculation experiments and was identified by morphological and cultural characteristics (6). Cultures were maintained on Difco potato-dextrose agar (PDA). The soybean cultivar Williams was used in all field and greenhouse studies because, at the beginning of the study, it was the most widely grown early season cultivar in Kentucky and had higher incidences of pod and stem blight than later maturing cultivars.

Mycelial growth. The effect of temperature on mycelial growth was determined in vitro. A 0.6-cm-diameter mycelial plug was removed from the edge of an actively growing culture and was placed mycelial-side down in the center of a petri plate containing PDA. The petri plate was sealed in a plastic bag and incubated at temperatures from 3 to 41 ± 1 C. Growth was measured at various times in two directions at right angles to each other and averaged. The rate of growth was calculated from at least three measurements of four replicate plates and expressed as centimeter diameter per day. The growth rate at each temperature was based on two to six separate experiments.

Spore germination. Spores of *P. longicolla* were collected from cultures grown in petri plates on PDA at room temperature for 16–36 days. Spores were removed by flooding each plate with 10 ml of deionized water and rubbing the surface with a stainless steel spatula. The spore suspension was filtered through four layers of cheesecloth and diluted with deionized water to give a final concentration of $5 \times 10^6 - 3 \times 10^7$ α-conidia per milliliter. A 1-ml portion of the spore suspension was spread over the surface of 2% water agar in a 9-cm-diameter petri plate with a stainless steel spatula. Each treatment was sealed in a plastic bag and incubated at a specific temperature from 3 to 41 ± 1 C. After various periods of time, 1-cm^2 blocks of agar were removed from each plate, placed on a glass slide, and stained with 0.5% lactophenol cotton blue. Percent germination was determined by examining at least 200 spores per replication at 400× in randomly selected viewing fields. A spore was considered to have germinated when the germ tube was at least as long as the widest part of the spore. Germination was determined periodically until at least 50% of the spores had germinated. Seven experiments were performed, each with four replicate plates for each of three to seven temperatures.

Seedling stem inoculations. Soybean seedlings were grown for 2 wk in a greenhouse at 20–27 C in steamed sandy loam soil. Just before inoculation, the seedling stems were cut into 8-cm lengths. A spore suspension, prepared as described above, was sprayed onto the stem sections with an atomizer until runoff. The inoculated stem sections were placed in petri plates containing a piece of moist Whatman No. 1 filter paper in the lid. Plates were placed in plastic bags. All treatments were incubated at 25 ± 1 C in the dark. The infection process was greatly slowed down after various periods of time, 0–48 hr, by placing the petri dishes at 4 C until the end of the experiment. At the end of the experiment, stem sections, excluding stem ends, were cut into 1-cm-long pieces and surface sterilized by dipping the stem sections in 95% ethanol, soaking for 5 min in 0.5% NaOCl, rinsing in sterile deionized water, and soaking for 1 min in a 1:40 paraquat (1,1-dimethyl-4,4 bipyridinium dichloride) to water solution (1). The stem sections were then placed on PDA amended with 0.1 μg/ml of streptomycin sulfate, 0.05 μg/ml of chlortetracycline, and 18 drops per liter of a nonionic surfactant (polyglycol ether, Tergitol NP-10, Sigma, St. Louis, MO), which restricted radial growth of the fungus. The stem sections were incubated at room temperature (20–25 C) for 7–14 days, and the proportion of stem sections from which *P. longicolla* grew was determined. The experiment was repeated five times with four replications per treatment. Inoculum concentrations used ranged from 2.3×10^6 to 5.9×10^7 conidia per milliliter.

Field infection. Two-week-old greenhouse grown-seedlings of the cultivar Williams were placed in the field for short periods to determine the effects of the environment on infection of soybean tissue by *P. longicolla*. The seed lots used in these experiments had less than 2% seed infection by *P. longicolla*. The seedlings were grown in steamed soil, one seedling per 15-cm-diameter pot. At weekly intervals, five potted seedlings were placed in a soybean field between rows of Williams soybeans. The pots were placed in holes so that the soil level in the pot was even with that of the field. The field, located at Spindletop Farm, Lexington, KY, had been continuously cropped to soybeans since 1975 and had high pod and stem blight disease pressure. Seedlings were left in the field for 3 days in 1981 and 7 days in 1982 and 1983. The seedlings were not watered during their field exposure. After field exposure, plants were returned to the greenhouse and incubated for 48 hr; the stems and petioles were then cut into 1-cm pieces. The plant sections were surface sterilized and assayed for infection by *P. longicolla* as described above. Between 92 and 260 plant sections were assayed each week. Seedlings were placed in the field each week from 6 July to 26 October 1981, from 8 June to 17 October 1982, and from 7 June to 18 October 1983. Another set of plants, serving as controls, was left on the greenhouse bench during the field exposure but otherwise treated similarly.

Temperature and relative humidity within the crop canopy were monitored with a hygrothermograph (Bendix, Baltimore, MD) placed in a weather shelter 15 cm above the ground. Surface wetness periods were determined 30 cm above the ground within a soybean row with a deWit leaf wetness recorder (Valley Stream Farm, Orano, Ontario, Canada). Rainfall data were obtained from a nearby weather station (400 m from the field) in 1981 and from a recording rain guage (Weather Measure, Sacramento, CA) at the edge of the field in 1982 and 1983. Weather data were recorded on an hourly basis.

TABLE 1. In vitro growth rate[a] and time to 50% germination[b] of *Phomopsis longicolla* at temperatures from 3 to 41 C

Temperature (C)	Growth rate (cm diameter/day)			Time to 50% Germination (hr)		
	N[c]	Mean	SD[d]	N	Mean	SD
3	3	0.09	0.06	3	119.7	14.1
10	2	0.21	0.02	3	25.4	2.3
13	3	0.35	0.02	4	11.8	1.6
15	4	0.52	0.02	4	10.2	1.8
18	6	0.95	0.08	3	5.9	0.3
20	2	1.30	0.28	...[e]	...	...
25	3	1.73	0.24	4	4.0	0.3
28	3	1.65	0.02	...	...	...
30	3	1.65	0.18	6	3.9	0.2
35	3	0.20	0.11	3	4.4	0.4
41	3	0.00	0.00	*[f]	*	*

[a] Growth rate was determined on potato-dextrose agar by placing a 0.6-cm-diameter agar plug of mycelium in the center of each plate and periodically measuring diameter in two directions at right angles to each other. There were four replicate plates per experiment.

[b] Germination was determined on a minimum of 200 α-conidia per temperature in each experiment. Time to 50% germination was determined by interpolation between the highest count below 50% and the lowest count above 50%. Germination experiments were conducted on 2% water agar with spores from cultures 16–35 days old.

[c] Number of experiments.

[d] Standard deviation.

[e] Temperatures at which determinations were not made are indicated by (···).

[f] * = No germination.

RESULTS

Mycelial growth. The rate of mycelial growth was fastest at 25 C, followed closely by 30 C (Table 1). Mycelial growth was very slow at 35 C, and no growth occurred at 41 C after 30 days. Below 25 C, mycelial growth rate decreased with decreasing temperature.

Spore germination. Fifty percent spore germination occurred most quickly at 30 C followed by 25 and 35 C (Table 1). No germination occurred at 41 C after 360 hr. Below 25 C, the time to 50% germination increased with decreasing temperature. Final germination was between 97 and 100% at all temperatures except 41 C.

The effects of temperature on growth and germination were compared by converting the results to percentage of maximum for each experiment. The growth rate at 25 C was used as the maximum growth rate, and the inverse of the time to 50% germination at 30 C was used as the maximum germination rate. Growth and germination responded to temperature in a similar manner (Fig. 1). However, differences in the responses to temperature occurred at 35 C and, to a lesser extent, at 13 and 15 C.

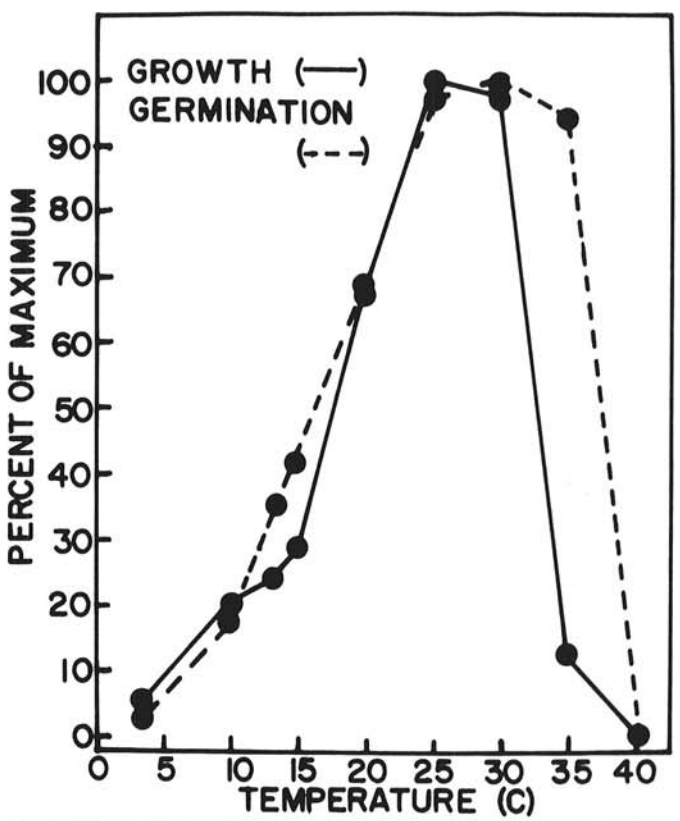

Fig. 1. Effects of temperature on growth (—) and germination (•—•) rates of *Phomopsis longicolla* expressed as percentage of maximum.

Proportional growth rates at these temperatures were lower than proportional germination rates.

Seedling stem inoculations. Results of the inoculation experiment were highly variable, but certain trends were evident (Fig. 2). Generally, a high amount of recovery was measured immediately after inoculation. The level of recovery decreased to a minimum at between 8 and 12 hr after inoculation and then increased rapidly. The rate of increase slowed after 24 hr, and infection was highest 48 hr after inoculation.

Field infection. The proportion of seedling stem pieces infected was significantly correlated with a number of environmental variables; however, no single factor was significant in all 3 yr of the study (Table 2). Stepwise multiple regression (MAXR technique [18]) was used to develop a multiple regression model for the combined infection data from 1982 and 1983. The 1981 infection data were not used because the plants were exposed in the field for 3 days rather than 7 days as in 1982 and 1983. The best three-variable multiple regression model was

$$X = 0.0673 - 0.1177*(W) + (S)\,[0.005*(T) - 0.0050],$$

in which X is the proportion of infected stem pieces, W is the number of weeks after planting at which seedlings were exposed in the field, S is the number of hours of surface wetness accompanied by rain, and T is the average hourly temperature for the exposure period. The coefficient of determination (r^2) was 0.58 ($P < 0.0001$). The incorporation of additional variables did not significantly increase the fit of the model.

An alternate model was developed to define and quantify the effect of the field environment on infection of soybean seedlings. Model development was based on 14 observations from 1981, 15 from 1982, and 12 from 1983.

There were three major steps in developing this model. In the first step, seedling infection was compared to the total duration to surface wetness periods associated with rain during the exposure period (Fig. 3A). Little infection occurred before 20 hr of surface wetness. Infection increased with increasing duration of surface wetness > 20 hr but not in a consistent manner. In the second step, temperature was incorporated into the model by expressing the duration of each surface wetness period as the equivalent duration of surface wetness at optimum temperature for infection. To do this, the response of percentage of maximum proportional mycelial growth rate to temperature was used (Fig. 1). The growth curve was partitioned into four straight lines that related the proportion of maximum growth to temperature. The lines connected the following points: zero at 0 C, 0.29 at 15 C, 1.0 at 25 C, 1.0 at 30 C, and 0.11 at 35 C. A proportion was determined for each surface wetness period based on the average hourly temperature of the period. This proportion was multiplied by the duration of the surface wetness period to give the equivalent duration of surface wetness at optimum temperature for infection, referred to as a "rating." Replacing hours with ratings for each surface wetness period associated with rain resulted in a closer grouping of the infection data (Fig. 3B).

In the third step of model development, criteria were incorporated that dealt with relationships between different periods of surface wetness and rain. The criteria were based on three possible characteristics of periods of surface wetness accompanied by rain during which infection takes place: 1) An infection period can continue after interruption of surface wetness if relative humidity is high during the interruption; 2) after inoculum is dispersed, there is a refractory period before inoculum is again available for dispersal; and 3) a minimum amount of pathogen growth is necessary before any infection can occur. The procedure used to calculate a rating for each exposure period involved assigning a temperature-based rating to each surface wetness period (as in step 2), and then combining and censoring ratings for surface wetness periods according to three criteria: 1) If the rating total for an infection period (a surface wetness period accompanied by rain) was less than 14, the rating for any subsequent surface wetness period was added to that of the initial infection period if the minimum relative humidity during the interruption was > 70%; 2) a rain occurring less than 20 hr after another rain was not considered in determining whether the accompanying surface wetness period was an infection period; and 3) if two separate infection periods occurred during the same field exposure, the shorter period was not considered.

All observations with rating totals greater than 13 had infection, and infection increased rapidly with increasing rating totals (Fig. 3C). Linear regression for the sample points with ratings greater than 13 had a r^2 of 0.63, a slope of 0.028 proportion infected stem sections per rating, and an intercept of −0.375 proportion infected

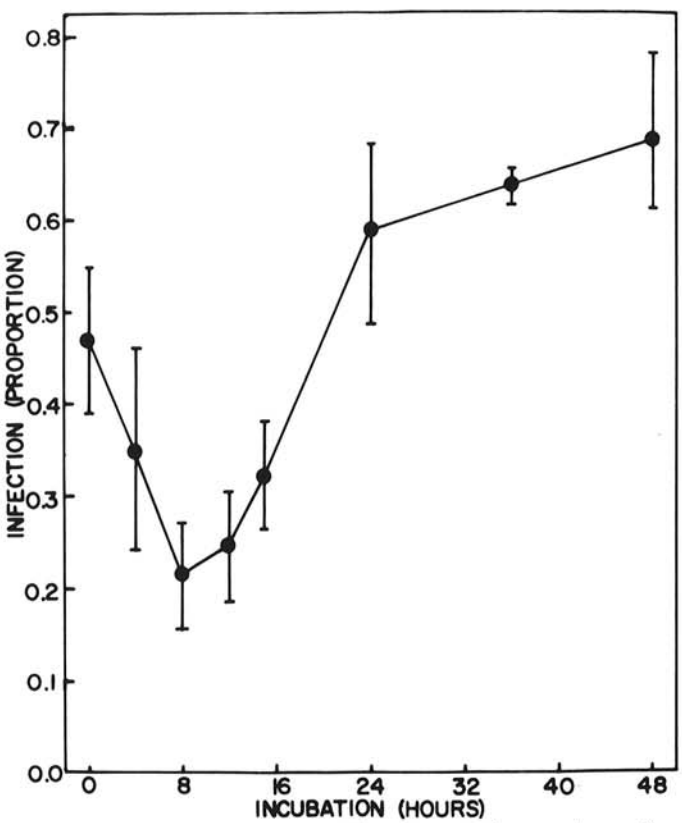

Fig. 2. Proportion of infected seedling stem sections at times after inoculation with spores of *Phomopsis longicolla*. Incubation temperature was 25 C. Vertical lines represent the standard error for results of five experiments.

TABLE 2. Correlation coefficients (r) for proportion of stem segments infected by *Phomopsis longicolla* with field environmental factors[a]

Environmental Factor	Year 1981	1982	1983	1982 +1983
Week[b]	−0.21	−0.55*[c]	0.25	−0.35
Temperature[d]	−0.17	0.42	−0.01	0.17
Days with rain[e]	0.38	0.39	0.49	0.40*
Total rain	−0.40	0.29	0.69*	0.31
Hours of surface wetness	0.53*	−0.07	0.37	0.31
Hours of surface wetness with rain (S)	0.69**	0.13	0.13	0.38
Average relative humidity	0.31	−0.18	0.67*	0.14
Hours of high humidity with rain[f]	−0.19	0.66**	0.71*	0.69***
Temperature * S	0.30	0.64**	0.71*	0.54

[a] Two-week-old greenhouse-grown seedlings were placed in the field for periods of 3 days in 1981 and 7 days in 1982 and 1983. Correlations are based on 14, 15, and 12 observations for 1981, 1982, and 1983, respectively.
[b] Week after planting surrounding soybeans.
[c] *, **, and *** indicate significance at $0.05 > P >= 0.01$, $0.01 > P >= 0.001$, and $P < 0.001$, respectively.
[d] Average hourly temperature during exposure.
[e] Number of days with rain during the exposure period.
[f] Hours of relative humidity 90% or above accompanying rain.

stem sections. The line intersected the 0 infection level at a rating of < 13.4. Incorporation of a quadratic term in the regression model did not significantly increase its fit. The coefficient of determination for the overall model (i.e., including a prediction of no infection for ratings 13) was 0.73.

DISCUSSION

In this study, temperature, duration of surface wetness, and rain were the most important environmental parameters affecting infection of soybean seedlings by *P. longicolla*. This is seen in the multiple regression analysis where the number of weeks after planting, the length of surface wetness periods accompanied by rain, and the average temperature during the exposure period gave the best model describing seedling infection. Rain, as has been reported (9), is necessary for dispersal of spores of *P. longicolla*. Surface wetness is needed for germination and infection, and temperature regulates the rate of the infection process. The negative relationship of weeks after planting to infection could represent a decline in inoculum through the season or may reflect more favorable environmental conditions early in the season. At the test site, rain was more frequent and surface wetness periods longer in July than later in the season and was accompanied by higher infection levels (data not presented).

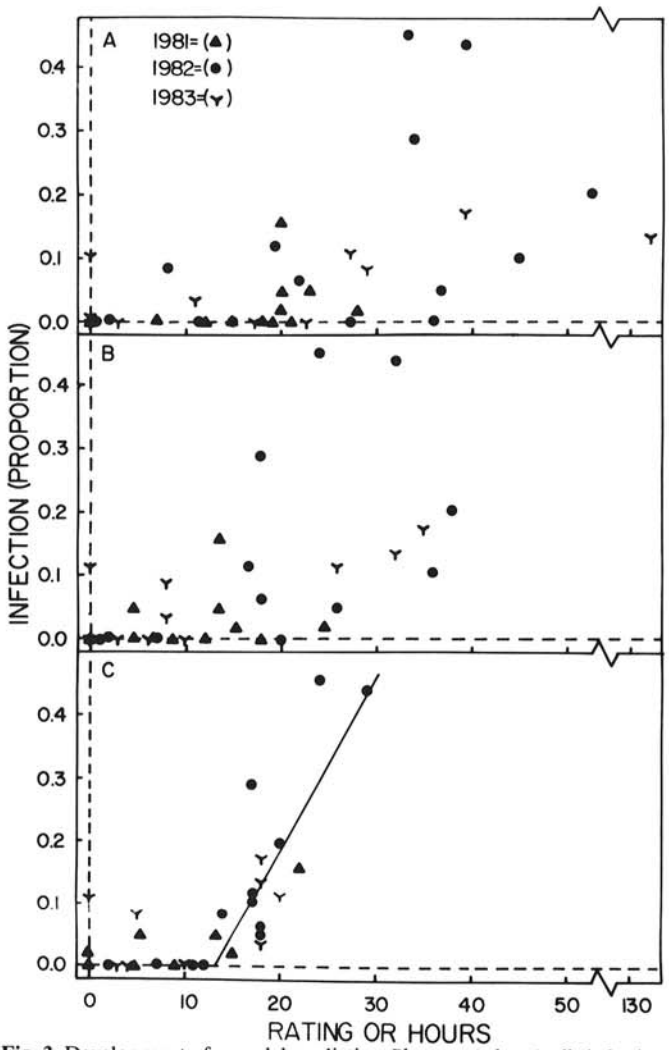

Fig. 3. Development of a model predicting *Phomopsis longicolla* infection of greenhouse-grown Williams soybean seedlings exposed in the field for short periods of time in 1981 (▲), 1982 (•), and 1983 (Y), at Spindletop Farm, Lexington, KY. Observed infection is plotted against: **A,** Model using total hours of leaf wetness associated with rain; **B,** Ratings equal to the total hours of leaf wetness associated with rain adjusted for temperature; **C,** Model ratings. The solid line in C fits the data with ratings of 13 or more with a coefficient of determination (r^2) of 0.63. The coefficient of determination for the entire model in C (ratings 0 or greater) is 0.73.

The elements of the multiple regression analysis were used to develop an alternate model. The goal of the alternate model was to describe more accurately how environmental conditions affect infection by *P. longicolla*.

Six assumptions formed the basis of the alternate model: 1) Rain is necessary for dispersal; 2) surface wetness is necessary for infection; 3) temperature affects the rate of pathogen growth and, thus, the rate of infection during periods of surface wetness; 4) the infection process can continue after surface wetness is interrupted by a period of high relative humidity without surface wetness; 5) after inoculum is dispersed, there is a refractory period during which inoculum is not available for dispersal; and 6) there is a minimum amount of pathogen growth that is necessary before any infection can take place. Assumptions 2, 4, and 6 were based on the observed behavior of the disease in inoculation experiments, whereas the other assumptions were based on the reported behavior of *P. longicolla* or similar fungi (2,7,9). In the inoculation experiments, recovery of the fungus was high initially, declined to a minimum at 8 hr after inoculation, and then increased to a plateau (Fig. 2). At 25 C, germination of spores of *P. longicolla* is nearly complete by 8 hr (data not shown). Thus, the high recovery before 8 hr may have been due to ungerminated spores being relatively resistant to the surface sterilization. This initial high recovery of the fungus was also seen in greenhouse inoculation studies where the plants were allowed to dry after exposure in a moist chamber (data not shown). If the apparently artifactual recoveries before 12 hr are omitted from the inoculation results, the overall pattern of increase in infection with time is similar to that observed with the alternate model (Fig. 3C). Thus, the field data can be considered to represent a population of single infection processes rather than the effect of multiple infection processes during a single exposure period.

A number of factors that could be important in infection of soybean by *P. longicolla* were not included in the alternate model. The availability of inoculum for dispersal is probably influenced by the physical environment, in addition to cropping history (3), and the age of colonized debris (9,12). However, inoculum production was addressed in the model only through the inclusion of a refractory period. Also, infection of soybean pods by *P. longicolla* has been observed to decrease with the height of pods above the ground (8), but such an effect was not included in the model. Additional work is needed to quantify and clarify these aspects of the disease. Although the two-stage linear model fits the data reasonably well, some form of sigmoidal model might be more appropriate. Additional inoculation experiments under controlled conditions could help clarify this; however, the problems that we encountered with apparent recovery of inoculum from the plant surface immediately after inoculation would need to be overcome.

The alternate model that we developed deals with a single episode of infection of soybean stems and petioles. Although such stem and petiole infections eventually become sources of inoculum, the phases of the disease that are of direct economic importance involve infection of pods over the entire growing season, followed by growth of the fungus into seeds after physiological maturity. To estimate pod infection at physiological maturity, the infection-rating equations that we derived (i.e., infection 0 if rating ≤ 13 and infection $= -0.375 + 0.028*$ rating if rating > 13) could be used to predict infection for each infection period during a growing season. The sum of these predictions might be related to pod infection at physiological maturity; however, it is likely that such a model would need further development to account for relationships between different infection periods and differences between pods and stems.

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