Effects of Pod Moisture on Soybean Seed Infection by *Phomopsis* sp.

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ABSTRACT

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Increases in soybean seed infection by *Phomopsis* sp. have been associated with wet weather after physiological maturity. To determine the relationship of pod and seed moisture to the rate of seed infection by *Phomopsis* sp., pods with high incidence of carpel infection, but low seed infection, were collected after physiological maturity, maintained at 25 C at various water contents, and sampled periodically. Seed infection rates were linearly related to water contents between 35 and 19%. At water contents below 19%, no seed infection was observed. At water contents above 35%, there was a great deal of variability in seed infection rates between experiments, suggesting antagonism by other microorganisms. Growth of

Phomopsis sp. occurred at osmotic potentials as low as −186 bars and was optimal from −10 to −30 or −40 bars on potato-dextrose agar osmotically adjusted with KCl or sucrose, respectively. Growth was faster on media osmotically adjusted with sucrose than with KCl. The relationship of water content to water potential was similar for carpels and seeds and was used to express seed infection rates in terms of water potential. Below −45 bars (35% water content), seed infection rates responded to water potential in a manner similar to that of growth rate of *Phomopsis* sp. to osmotic potential on media.

Additional key words: *Diaporthe phaseolorum* var. *sojae*, *Glycine max*, *Phomopsis sojae*, *Phomopsis longicolla*, *Phomopsis phaseoli*, pod and stem blight.

Infection of soybean (*Glycine max* (L.) Merr.) seeds by *Phomopsis* sp. sensu Kmetz et al (12), the primary causal agent of pod and stem blight, reduces seed germination in the laboratory and can reduce emergence in the field (4,9,14,17,24). While *Phomopsis* sp. can infect vegetative plant parts at any time during the growing season, most seed infection occurs after physiological maturity (8,11,13,23). After physiological maturity, seed water content declines from approximately 55% to between 12 and 14% and the fungus grows from infected carpels into the seeds. Environmental conditions during this period which would be expected to delay or reverse pod drying (e.g., rain and high relative humidity) have been shown to favor seed infection (15,18,20–22). However, the quantitative relationship between seed infection and pod moisture has not been described.

The objectives of this study were to evaluate the effects of pod water content on seed infection rate in naturally-infected soybean pods and the effect of in vitro osmotic potential on growth rate of *Phomopsis* sp. and to determine the relationship between soybean seed water content and water potential. A preliminary report has been published (19).

MATERIALS AND METHODS

Seed infection. Soybean seeds of cultivar Williams were planted near Lexington, KY, in a field that had been consecutively planted with soybeans for at least 5 yr and which contained a high level of inoculum of *Phomopsis* sp. Seeds were planted at approximately 2-wk intervals so that there would be pods at different maturities and water contents from which to select at the end of the growing season. Planting dates were 15 May (planting I), 30 May (planting II), and 15 June (planting III) 1983. Cultivar Williams was used because it was the most widely grown soybean cultivar in Kentucky at the start of the study, and due to its early maturity growers in Kentucky have frequently had problems with pod and stem blight.

Pods were collected after physiological maturity (yellow pods) from the lower half of plants. To obtain pods having different water

contents, pods were either collected at different water contents in the field, or collected dry (11% water content) and rewetted to specific water contents. Pods at various water contents were collected on 22, 27, and 29 September. Pods were selected on the basis of appearance and rigidity and fell into four groups: yellow pods (physiological maturity); brown, flexible pods; brown, moderately stiff pods; brown, rigid pods (harvest maturity). After collection, these pods were stored at 5 C for 1 day before use. Dry pods were collected from plantings I and II on 22 October, placed in large plastic bags, and stored for 40 to 118 days at 5 C until used. Dry pods were initially rewetted by misting them with deionized water, placed between moist germination towels, and stored overnight at 5 C. The pods were then transferred to double plastic bags, misted with amounts of deionized water calculated to bring them to specific water contents and allowed to equilibrate at 5 C for 1 wk.

Pods at each water content were divided into six samples of three replications each with 30–35 pods per replication. The number of seeds per replication ranged from 60 to 100. Ten additional pods were included in each replication of the first sample for water content determination. The pods in each sample were placed in a plastic bag and were incubated in the dark at 25 C. Incubation times varied according to the water content of the treatment. Maximum incubation times ranged from 6 days for the wettest treatments to 35 days for the driest. Water contents were determined on a wet weight basis at the first sampling (day 0) for each of ten randomly selected pods from each replicate. Dry weights were determined after drying the pods at 105 C for 24 hr. Seed infection in each water content treatment was assayed after six incubation times. Seeds were removed from the pods, wrapped in cheesecloth, dipped in 95% ethanol, soaked for 4 min in 0.5% sodium hypochlorite for surface-sterilization, and rinsed in sterile deionized water (3). The seeds were then placed on Difco potato-dextrose agar (PDA) amended with streptomycin sulfate at 0.1 g/ml, chlortetracycline at 0.05 g/ml, and a nonionic polyglycol ether surfacant (Tergitol NP10; Sigma Chemical Co., St. Louis, MO) at 18 drops per liter in plastic petri dishes. After incubation at room temperature for 7–14 days, the proportion of seeds with *Phomopsis* sp. emerging from them was determined. *Phomopsis* sp. was identified on the basis of colony morphology (12). The proportion of infected seeds was transformed by the multiple infection transformation to give the estimated number of infections per seed (7).

Fungal growth rate. An isolate of *Phomopsis* sp. was obtained from a soybean seed collected from the field in which the pods were grown. This isolate was used to determined the rate of mycelial growth on PDA osmotically adjusted with sucrose or KCl (16). The water potential of some treatments was confirmed with a dew-point psychrometer (Model CR-52; Wescor, Inc., Logan, UT). A 0.6-cm-diameter disk was taken from the edge of an actively growing PDA culture and placed upper side down on the agar. Each plate was sealed with Parafilm and incubated at 25 C in the dark. Periodically, average colony diameter was determined from two measurements made at right angles to each other. Growth rate (centimeters per day) was determined by linear regression, based on colony diameter measurements made on at least 3 days. There were three to five replicate plates per treatment.

Seed and carpel water potential. The Shardokov method (2) was used to determine the relationship of water content to water potential for seeds and carpels which had been adjusted to a range of water contents (2). This method detects changes in the density of a sucrose solution which has come into equilibrium with the plant material being tested. By using a series of sucrose solutions representing a range of osmotic potentials, the water potential of the plant material can be determined within a narrow range. The experiment was performed twice with three replications per pod water content.

RESULTS

Seed infection. The effect of pod water content on the rate of seed infection was determined in seven experiments (Table 1). In the first three experiments, pods in four water content groups were collected from the field on the basis of appearance and rigidity. The water content groups were 54 to 55%, 43 to 45%, 29 to 30%, and 11 to 15%, which corresponded to yellow pods, brown flexible pods, brown moderately stiff pods, and brown rigid pods, respectively. In the last four experiments, pods were collected at 11% water content and were rewetted to water contents ranging from 17 to 43%. Standard deviations ranged from 1 to 6%. Pod infection at harvest maturity was >90% for all three plantings (*unpublished*). Seed infection at time of collection was <10% for all plantings; however, some increase in seed infection occurred during the rewetting period for pods from planting I.

With either method of obtaining pods at different water contents, there was a steady increase in seed infection with incubation time until a plateau was reached, and then the level of seed infection varied along the plateau. Rates of seed infection were determined from points preceding the plateau and were based on three to six points (Table 1). Coefficients of determination (R^2) for treatment groups in which seed infection increased ranged from 0.565 to 0.999, with most values above 0.90.

TABLE 1. Background information on pods used in determination of the effect of pod water content on the rate of infection of soybean seeds by *Phomopsis* sp. [a]

Experiment[b]	Planting[c]	Pod developmental state[d]	Water content (%)[e] Mean	SD[f]	Initial seed infection[g] (%)	N[h]	Infection rate[i] (Inf/Day)	Coefficient of determination (R^2)
1	II	Y	55	4	0	4	0.453	0.968
	II	F	43	6	2	5	0.261	0.960
	II	M	29	6	1	3	0.226	0.992
	I	R	15	4	9	5	0.000	0.002
	I	R	11	3	5	5	0.000	0.187
2	II	Y	54	5	0	3	0.687	0.980
	II	F	45	6	1	3	0.572	0.998
	II	M	30	6	4	3	0.211	0.951
	I	R	15	1	2	5	0.000	0.119
3	III	Y	55	3	2	4	0.271	0.988
	III	F	45	5	2	3	0.290	0.999
	III	M	30	4	1	3	0.206	0.953
	II	R	12	1	1	5	0.000	0.034
4	I	R	43	6	50	3	0.230	0.690
	I	R	35	6	43	3	0.373	0.838
	I	R	30	4	42	3	0.221	0.789
	I	R	26	3	28	5	0.225	0.905
5	I	R	24	2	17	5	0.107	0.996
	I	R	20	2	7	5	0.009	0.565
	I	R	17	2	8	5	0.000	0.259
6	II	R	34	5	1	5	0.311	0.929
	II	R	29	2	1	5	0.190	0.994
	II	R	24	1	1	5	0.070	0.937
7	II	R	24	1	5	5	0.145	0.884
	I	R	22	2	12	6	0.151	0.935
	II	R	22	1	2	4	0.060	0.810
	II	R	19	1	2	5	0.001	0.960
	II	R	17	1	1	6	0.000	0.030

[a] Data for mean water content and infection rate are plotted in Fig. 1.
[b] In experiments 1, 2, and 3, pods at a range of water contents were collected from the field. In experiments 4 through 7, pods were collected dry (11% moisture) and rewetted at 5 C to a range of water contents.
[c] Pods were collected from soybeans planted on 15 May (I), 30 May (II), and 15 June (III).
[d] Pod developmental state at time of collection: Y = yellow (physiological maturity); F = brown, flexible; M = brown, moderately flexible; and R = rigid.
[e] Water content (wet weight basis) of pods (with seeds) at the first sampling.
[f] SD = standard deviation.
[g] Seed infection at the start of incubation at 25 C.
[h] N = number of incubation times out of six used to determine the infection rate.
[i] Infection rate was calculated by linear regression of the multiple infection transformation of infection incidence against incubation time at 25 C for data points preceding a plateau.

The rate of seed infection at water contents from 43 to 55% varied greatly between experiments (Fig. 1). Infection rates ranged from 0.271 to 0.687 infections per seed per day at 43 to 45% water content. However, from 19 to 35% moisture there was close agreement in seed infection rates between experiments. For example, at water contents of 29 to 30% (Table 1), seed infection rates ranged from 0.190 to 0.226 infections per seed per day irrespective of whether the pods were collected in the field or rewetted in the laboratory to these water contents and irrespective of the planting from which the pods were collected.

Regression of seed infection rate against water contents of 19 to 35% resulted in the equation

$$y = -0.381 + 0.021\,x \tag{1}$$

in which y is the seed infection rate (infections per seed per day) and x is the percent water content. The R^2 was 0.887. According to this equation, seed infection rate was 0 at water contents of 18.3% and below.

Fungal growth rate. The rate of fungal growth was measured at osmotic potentials of -3 to -186 bars (Fig. 2A). Growth was faster on sucrose-amended PDA than on KCl-amended PDA. There was an increase in the growth rate when the osmotic potential was lowered from -3 to -10 bars with either osmoticum. Maximum growth rates occurred between -10 and -30 bars with KCl and between -10 and -40 bars with sucrose. The rate of fungal growth decreased steadily with osmotic potentials lower than -40 bars. Some growth occurred at -186 bars on sucrose-amended agar. The equation,

$$r = ae^{-bp} + c/(p - d), \tag{2}$$

was fit to the fungal growth rate data; in which r is the fungal growth rate (cm/day), p is the osmotic potential (bars), and a, b, c, and d are constants. Estimates of the constants a, b, c, and d for growth on sucrose-amended PDA were 6.323, 0.014, 101.66, and 18.35, respectively, and for growth on KCl-amended PDA were 4.333, 0.023, 26.73, and 6.65, respectively.

Seed and carpel water potential. The relationships between carpel and seed water potentials and water content were similar in the two experiments which were performed (Fig. 3). A narrower range of water potentials was determined at most water contents for seeds than for carpels, but the relationship of carpel water content to water potential was similar to that of seeds. An equation relating water content to water potential for both carpels and seeds was derived by using the midpoints of each range of water
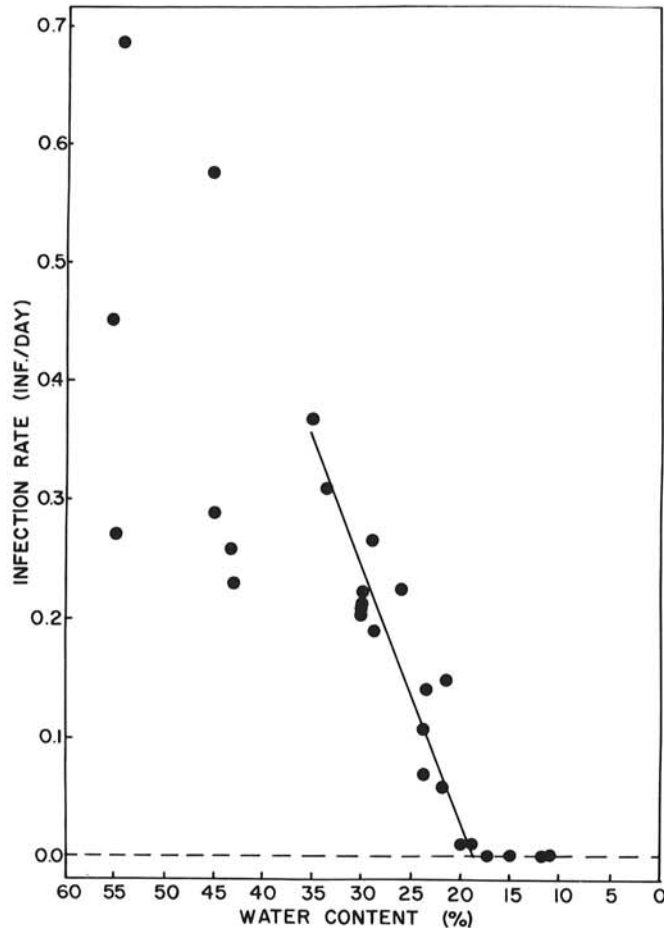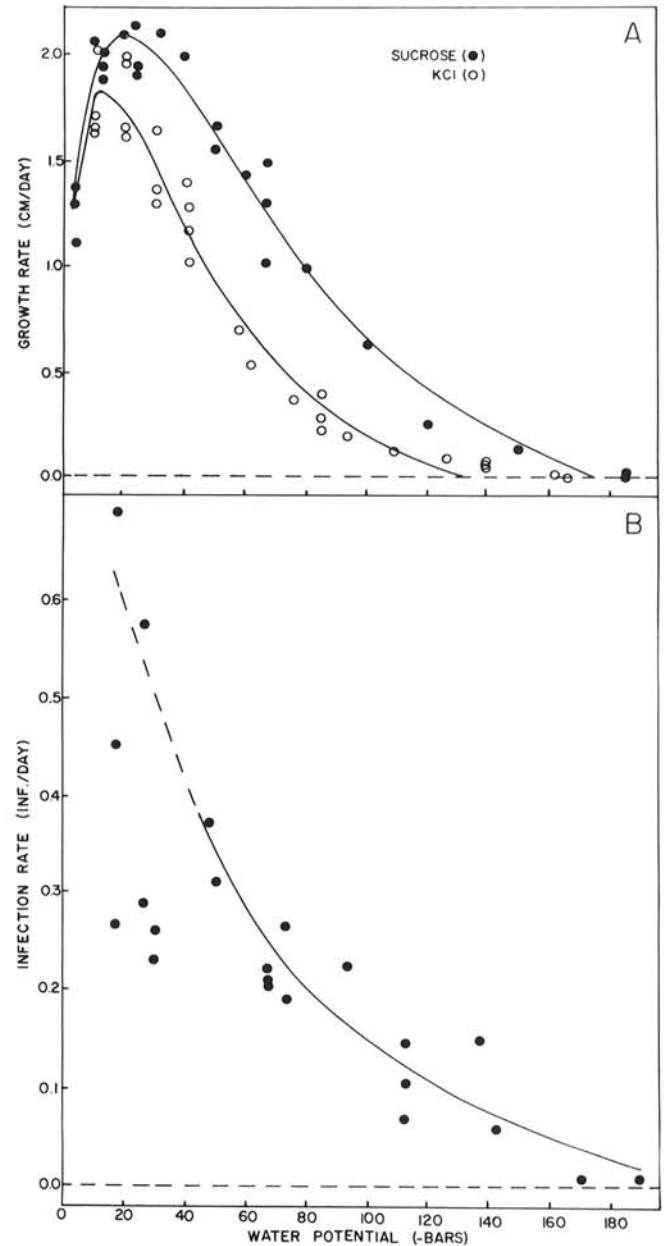

Fig. 1. Effect of soybean pod water content on the rate of seed infection by *Phomopsis* sp. in naturally-infected pods at 25 C. Each data point represents the mean of three replicate samples of 30 to 35 pods each. The regression line fitted to data for 19 to 35% water content represents equation 1.

Fig. 2. Effects of water potential on growth of *Phomopsis* sp. in vitro and in vivo. **A,** Rate of increase in colony diameter at 25 C on potato-dextrose agar adjusted to specific osmotic potentials with sucrose or KCl. Each data point represents the mean of three to five replicate plates. The regression lines are of the form of equation 2. **B,** Rate of seed infection in naturally-infected pods adjusted to water contents corresponding to water potentials determined by the Shardakov method. The solid and dashed regression lines represent equation 4 for the range over which it was derived and extrapolated, respectively.

potentials. Regression of ln(water potential) against ln(water content) resulted in

$$\ln(w) = 11.922 - 2.264 \ln(x) \qquad (3)$$

in which w is the negative of water potential (bars) and x is the percent water content. The R^2 was 0.964. Rearranging and combining equations 1 and 3 gave

$$y = -0.381 + 4.066 \, (w^{-0.442}) \qquad (4)$$

in which y is the seed infection rate and w is the negative of water potential. All of the seed infection rates at water potentials below −45 bars were close to the predicted values (Fig. 2b). Above −45 bars, two of the seven seed infection rates were close to the predicted values when the line was extrapolated. The response of seed infection rate to water potentials below −45 bars appeared to be similar to the response of fungal growth rate to osmotic potential (Fig. 2). The lower limit for growth on sucrose-amended PDA (approximately −180 bars) corresponded to that for seed infection.

DISCUSSION

Water potential affected growth of *Phomopsis* sp. both in vitro and as the fungus grew into soybean seeds from infected carpels (Fig. 2). The similarity of the in vitro-sucrose and in vivo data at less than approximately −45 bars may be coincidental or may be due to a similarity of the fungal environment in sucrose-adjusted PDA and in soybean pods and seeds. For other fungi, the effect of water potential on growth has been shown to depend on both whether water potential is primarily matric or osmotic and on the material used to adjust osmotic potential (1,5). The lower growth rates for

Fig. 3. Ranges of water potential determined by the Shardakov method for soybean seeds and carpels (pods) adjusted to specific water contents. Each data bar represents the range for three replicate samples. The regression line was derived from midpoints of the ranges and corresponds to equation 3.

Phomopsis sp. on PDA adjusted with KCl than on that adjusted with sucrose could have been due to toxicity of the KCl and/or nutrient effects of the sucrose. Sucrose is the primary disaccharide in soybeans (10), but its relative contribution to osmotic potential is unknown. The relative contributions of matric and osmotic potentials to total soybean pod and seed water potentials are also not known. However, the similarity between the in vitro growth rate results and the seed infection rate results at water potentials below −45 bars suggests that osmotic potential may be a major factor affecting the water potential of soybean pods and seeds.

Seed infection rates were similar between experiments at water potentials below −45 bars (35% water content) (Figs. 1 and 2b). Above −45 bars, infection rates varied considerably between experiments. This variability in the seed infection rates between experiments at high water potentials could be due to a number of factors. Host resistance, evident before physiological maturity, could still affect fungal growth shortly after physiological maturity. The intensity of pod infections could vary with planting date and the date of pod collection, although the proportion of pods infected by *Phomopsis* sp. was not different. While host resistance and the intensity of pod infections may have contributed to the variability in seed infection rates at high water potential, a more likely source of the variability is antagonism by other microorganisms. While our in vitro data indicate that *Phomopsis* sp. grows quite well at −45 bars, growth of many other microorganisms is greatly or completely restricted at this water potential (1,5). At higher water potentials, these other organisms would be expected to be more active and thus more likely to affect growth of *Phomopsis* sp.—a situation analogous to that of inhibition of the germination of *Fusarium culmorum* (W. G. Smith) Sacc. by bacteria at high water potentials (6).

The response of seed infection rate to water content did not appear to be affected by pod maturity or by initial seed infection levels. Although the seed infection rate declined with pod age after physiological maturity (experiments 1 to 3), rewetted pods (experiments 4 to 7) had seed infection rates at both 43 and 30% water content which were similar to corresponding rates in the first three experiments (Table 1). Likewise, initial seed infection levels did not affect the subsequent seed infection rates. Rewetted pods from planting I in experiments 4, 5, and 7 had initial seed infection levels as high as 50% (Table 1); still, seed infection rates were equivalent to those in other experiments at similar water contents. This increase in initial seed infection levels during rewetting did not occur in pods from planting II. Pods from planting II were exposed to the field environment for a shorter period of time after physiological maturity than were pods from planting I. This shorter exposure time in the field for pods from planting II probably resulted in less extensive pod colonization by *Phomopsis* sp. before collection, leading to less seed infection during rewetting.

Although a number of factors undoubtedly influence the rate of seed infection by *Phomopsis* sp. after physiological maturity, the results presented here demonstrate the importance of water potential in this process. The period following physiological maturity is one of pod and seed desiccation. The ability of *Phomopsis* sp. to grow and infect soybean seeds over a wide range of water potentials results in seed infection whenever environmental conditions maintain pods at water contents above 19%. This accounts for the association of increases in seed infection by *Phomopsis* sp. with wet weather (4,18,20,22,24). This ability to grow at low water potentials may also allow *Phomopsis* sp. to avoid the effects of antagonistic microorganisms active at higher water potentials. Further work to determine the interaction of water potential and temperature on the rate of seed infection may allow the prediction of seed infection by *Phomopsis* sp. in the field on the basis of environmental factors.
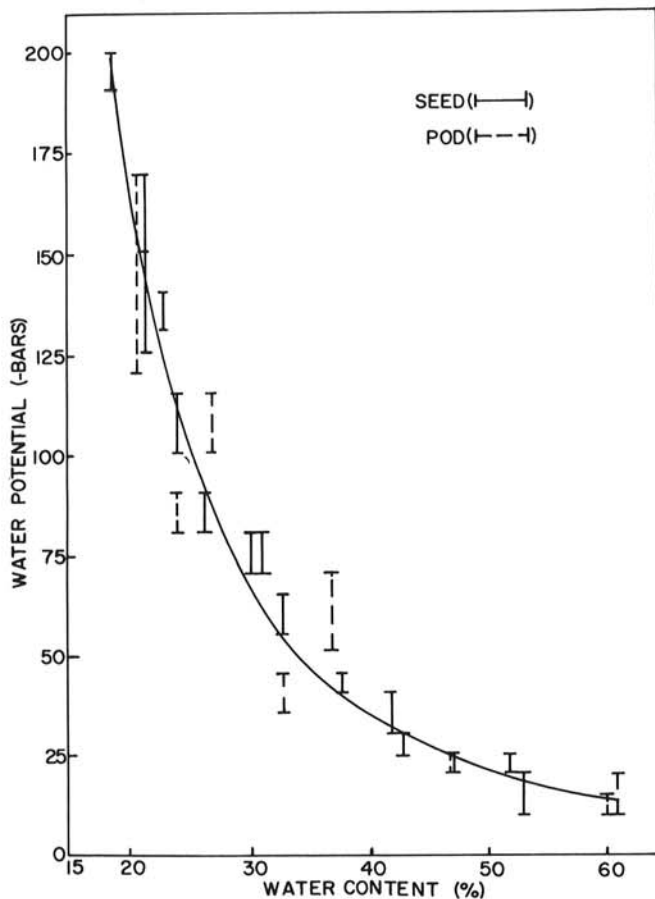
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