

## Identification and Quantitative Characterization of Rate-Reducing Resistance to *Phytophthora megasperma* f. sp. *glycinea* in Soybean Seedlings

Paul W. Tooley and Craig R. Grau

Graduate research assistant and assistant professor, respectively, Department of Plant Pathology, University of Wisconsin, Madison 53706.

The authors gratefully acknowledge the excellent statistical assistance provided by Giovanni Latorre. We also thank S. Vicen for aid in figure preparation and D. I. Rouse for reviewing this manuscript.

Research supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison as Hatch Project 2328.

Accepted for publication 21 September 1981.

### ABSTRACT

Tooley, P. W., and Grau, C. R. 1982. Identification and quantitative characterization of rate-reducing resistance to *Phytophthora megasperma* f. sp. *glycinea* in soybean seedlings. *Phytopathology* 72:727-733.

Cotyledons of 9- to 11-day-old seedlings were wound-inoculated with *Phytophthora megasperma* f. sp. *glycinea* (Pmg). A 10- $\mu$ l drop containing encysted zoospores was delivered to cotyledons by using a micropipette with a disposable tip modified to allow simultaneous wounding and delivery of spores. After incubation for 5 days, plants were scored as dead or living. Resistance was characterized by the inability of the fungus to move into the hypocotyl and kill the plant. Controlled-environment studies showed that the expression of this resistance was affected by inoculum density, temperature, photoperiod, and plant age, and was active against four

physiologic races of Pmg. Based on inoculum density studies using this method with Pmg race 7, estimated log LD<sub>50</sub> values were obtained for eight soybean cultivars that differed in susceptibility to Pmg in the field. LD<sub>50</sub> values correlated well with yield, disease severity, and (simple interest) infection rate (*R*) for each cultivar when grown in naturally infested field plots. Thus, this method may prove useful for evaluating germ plasm lines in breeding programs, in evaluating cultivar resistance in cases where field evaluations are not possible, and as a technique to study the nature of rate-reducing resistance to Pmg.

*Phytophthora* root and stem rot of soybean (*Glycine max* (L.) Merrill) is a prevalent and destructive disease throughout the soybean-growing regions of the U.S. and Canada. In previous literature, the causal organism has been named *Phytophthora megasperma* Drechs. var. *sojiae*. Since the original description (12), however, recognition of host specificity and the lack of sharp morphological distinctions between isolates within the *P. megasperma* complex has led to the use of the formae speciales concept proposed by Kuan and Erwin (18) and furthered by Pratt (26). Thus, we shall henceforth refer to the causal organism as *Phytophthora megasperma* f. sp. *glycinea*.

In the past, methods used to identify and evaluate race-specific resistance in soybean to *P. megasperma* f. sp. *glycinea* (Pmg) have involved hypocotyl inoculation with mycelial or zoospore inoculum, and plants were rated as resistant or susceptible based on plant death (15,19,28,32). The inoculation of detached cotyledons was recently proposed as an alternative to hypocotyl inoculation for this purpose (21).

Cultivars not selected for race-specific resistance to Pmg have been observed to differ in what has been termed field tolerance (10,14). This designation has been used to describe the case in which two cultivars show marked differences in yield and disease severity when grown in the presence of races of the pathogen to which they are susceptible when hypocotyls are inoculated.

Characterization of the physiological mechanism(s) responsible for the phenomenon referred to as 'field tolerance' has been inadequate to distinguish between true tolerance, in which infection is endured through a desensitization of the plant (7,23) and resistance, in which the host is able to suppress or retard the activity of a pathogen (7).

As the number of physiologic races of Pmg has steadily increased (13,16,19) methods have been devised for identifying field tolerance to root rot (10,14). However, methods are not highly quantitative and may involve time-consuming measurements of plant length and root necrosis.

Pratt et al (27) found that when intact cotyledons of alfalfa seedlings were inoculated with zoospores, differential reactions to *Phytophthora megasperma* were evident, enabling them to rate cultivars for resistance. They further found that severity of cotyledon infection was correlated with severity of root rot in eight alfalfa lines and cultivars.

The studies described herein are based on the finding that when intact cotyledons of soybean seedlings are wound-inoculated with encysted zoospores of races to which they are susceptible when hypocotyls are inoculated, differential reactions to Pmg are evident and can be quantified. The purpose of the work reported here was to characterize the disease reaction that occurs in response to cotyledon inoculation under different environmental conditions and to determine the relationship between resistance expressed in response to cotyledon inoculation and that observed in the field and referred to as 'field tolerance.'

### MATERIALS AND METHODS

**Sources of the pathogen.** Isolates of Pmg were obtained by isolation from diseased soybeans collected in Wisconsin. Race characterizations were made using a standard set of differential soybean cultivars (19).

**Preparation of zoospore inoculum.** Isolates of Pmg were grown at ~24 C for 7-10 days on V-8 juice agar. Sixteen disks (5 mm in diameter) cut from the colony margins with a No. 2 cork borer were placed in 25 ml of a sterile 1% soil extract (1 g of garden soil in 100 ml of distilled water, autoclaved 20 min) in 9-cm-diameter petri dishes and incubated at ~24 C. After 16-18 hr, zoospores (~10,000-20,000 per milliliter) were collected in the soil extract.

For inoculum density experiments in which concentrations above 20,000 spores per milliliter were desired, soil extract containing motile zoospores was first passed through a 44- $\mu$ m (325-mesh) sieve, and then placed on a magnetic stirrer for ~10 min, which caused the zoospores to encyst. The zoospore suspension was then concentrated by passing it, via a 60-cc plastic hypodermic syringe, through a 25-mm diameter, 8- $\mu$ m (pore size) Nuclepore polycarbonate membrane filter (Nuclepore Corp., Pleasanton, CA 94566) fitted inside a 25-mm-diameter Swinnex plastic disk filter holder (Millipore Corp., Bedford, MA 01730).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

Spores trapped on the filter were resuspended in 5 ml of sterile 1% soil extract in a 15-ml plastic beaker placed on a magnetic stirrer, by using forceps to submerge the filter in the actively stirring soil extract. This process was repeated until hemacytometer counts taken from the actively stirring stirred suspension revealed a spore concentration exceeding 200,000 per milliliter. Dilutions with sterile soil extract were made from the concentrated spore suspension.

**Method of cotyledon inoculation.** Intact cotyledons of soybean seedlings were wound-inoculated ~5 mm from the point of attachment with an encysted zoospore-soil extract suspension. Ten microliters of the encysted zoospore suspension were delivered to one cotyledon per plant by using a Gilson Pipetman micropipette. Disposable plastic tips were modified by heating the tip to seal the tip opening and form a small plastic bead. A new hole was bored just above the bead, perpendicular to the longitudinal axis, to allow delivery of inoculum. Inoculation was performed by placing a finger under the cotyledon for support, piercing the cotyledon with the micropipette containing the modified tip, and discharging inoculum into the wound as the tip was withdrawn. By using this method, we could inoculate ~200 plants per hour.

**Relationship between inoculum density, temperature, photoperiod, and plant death following cotyledon inoculation.** Soybean cultivars were selected for differing susceptibility to Pmg in the field. Cultivar Steele was chosen for being ultrasusceptible to all races except 1 and 2, to which it contains race-specific resistance. Asgrow 2656 and Wayne yield well with few plants killed when grown in fields infested with compatible races of Pmg. Asgrow 2656 has race-specific resistance to races 1 and 2, but Wayne contains no race-specific resistance. Harosoy 63 has an intermediate field reaction to multiple races of Pmg and contains race-specific resistance to races 1 and 2.

For all experiments, we used the following standard inoculum densities: 1,000, 10,000, 50,000, 100,000, and 200,000 spores per milliliter. Since only 10  $\mu$ l of inoculum was injected into one cotyledon per plant, these inoculum densities represented 10, 100, 500, 1,000, and 2,000 spores per plant. Inoculum density was expressed as the log of the spore concentration as measured by hemacytometer counts. All four cultivars were included in each experiment, and 25–30 plants were inoculated at each inoculum density for each experiment. Plants were regarded as dead if Pmg had progressed into the hypocotyl from the inoculated cotyledon resulting in its complete girdling and/or collapse within 5 days after inoculation. Plants were grown in vermiculite in 15.2-cm-diameter plastic pots placed in clay saucers and watered daily. Plants were inoculated 9–11 days after sowing; thereafter watering was performed by filling the clay saucers. All growth chambers had a light intensity of 21,520 lux and 70% relative humidity.

**Correlation between cotyledon reaction and field reaction.** Information on disease reactions and yield of eight soybean cultivars was obtained from cultivar evaluation plots in fields naturally infested with numerous races of Pmg in southeastern Wisconsin.

In 1978, the cultivars Steele, Amsoy 71, Corsoy, Wayne, Harosoy 63, and Asgrow 2656 were evaluated at one location. Single-row plots were hand planted on 23 May 1978 in 4.6-m rows 76 cm apart and 3.7 m of each row was hand harvested on 20 October 1978.

In 1979, the following cultivars were grown at multiple locations: Steele, four locations; Northrup King S1492, three locations; Asgrow 2575, two locations; Asgrow 2656, three locations. Single-row plots were hand planted on 18 May and 23 May 1979 in 4.9- to 6.1-m rows spaced 76-cm apart, and 1 m of each row was harvested on 23 October 1979.

A randomized complete block design with 4–10 replications per treatment was used at all locations. Seedling emergence counts were made ~2 wk after planting. Disease severity readings, assessed as numbers of plants killed by Pmg, were taken at ~2-wk intervals from June to August. Seed weights in grams per meter of row were used as an estimate of yield. In addition, disease severity readings taken throughout the season at each location were used to calculate the (simple interest) infection rate ( $R$ ) for each cultivar (31). Values

of  $R$  were estimated as the slope of the line by regressing  $\ln(1/1-x)$  on time (days after planting), in which  $x$  = the cumulative proportion of dead plants (31).

## RESULTS

**Symptomatology of plants subjected to cotyledon inoculation.** Symptoms were first evident on cotyledons of susceptible plants 24 hr after inoculation and appeared as sunken lesions radiating outward ~1 mm from the point of inoculation. After 48 hr, lesions had developed into larger, sunken necrotic patches encompassing up to 75% of the cotyledon, and hypocotyl invasion could be observed as a discoloration near the point of cotyledon attachment. By the third day, cotyledons of many susceptible plants were completely necrotic and shriveled, and hypocotyl invasion often resulted in light-tan, water-soaked lesions that expanded rapidly, resulting in complete collapse of the hypocotyl.

For resistant plants, cotyledon colonization initially proceeded as in susceptible reactions, resulting in the necrosis and shriveling of the inoculated cotyledon. However, the advance of the pathogen was restricted at the hypocotyl junction, and a small, discrete, reddish-brown lesion was formed on the hypocotyl at the point of cotyledon attachment.

In very few cases, plants were apparently highly resistant and no symptoms developed following inoculation. The cotyledons of highly resistant plants did not appear different from uninoculated controls that received 10  $\mu$ l of sterile soil extract.

Some plants exhibited intermediate symptoms characterized by hypocotyl invasion and the formation of large, but slowly developing, dark-brown cankers that sometimes enlarged enough to kill the plant. These plants were classified as dead only if the cankers completely girdled the hypocotyl within 5 days after inoculation.

**Relationship between inoculum density, temperature, photoperiod, and plant death via cotyledon inoculation.** Data were collected on the effect of inoculum density, temperature, and photoperiod on the proportion of plants killed for each of the four soybean cultivars (Steele, Harosoy 63, Asgrow 2656, and Wayne) known to differ widely in field reactions to Pmg. These experiments were performed with isolate 36 of Pmg (race 7) in controlled environment chambers at three different temperatures and two different photoperiods as follows: three experiments at 24 C, 12-hr photoperiod; one experiment at 24 C, 16-hr photoperiod; three experiments at 28 C, 12-hr photoperiod; three experiments at 28 C, 16-hr photoperiod; three experiments at 32 C, 12-hr photoperiod. Therefore, the total number of different experimental conditions was 4 cultivars  $\times$  5 inoculum levels  $\times$  13 experiments = 260. For each cultivar, 65 pairs of values ( $r_i, n_i$ ) were thus obtained in which  $r_i$  = the number of dead plants at the  $i$ th set of experimental conditions and  $n_i$  = the number of plants inoculated at the  $i$ th set of experimental conditions. In Fig. 1A–E the percentage of dead seedlings is plotted against the log of the doses of Pmg used for each cultivar under all experimental conditions. Although the plots show a variability in response, the cultivars fell into three distinct groups; Steele was the most susceptible, Asgrow 2656 the most resistant, and Harosoy 63 and Wayne intermediate.

Statistical literature reporting dosage-response data provides three possible transformations suitable in representing a sigmoidal relationship between responses (proportion dead plants) and stimulus (log doses of Pmg). These are the probit, logit, and arc sine square root or angular transformations (4,8). If these transformations are indicated by "g" a more general model that considers variables in addition to logarithm of the dose has the form

$$g(p_i) = Y_i = B_0 + \sum_{j=1}^k B_j X_j + \epsilon_i \quad (1)$$

in which  $p_i$  is the proportion of dead plants at the  $i$ th set of experimental conditions (equal to  $r_i$  divided by  $n_i$ ), and  $k$  is the number of explanatory variables. This model has the general form of a regression model, so the parameters  $B_j$  can be estimated (6).

Although maximum likelihood estimation of the parameters is the preferred method, computer programs involving maximum likelihood estimation were available only for the logit transformation (5) and for the probit transformation with only one explanatory variable, typically the doses (11). Alternatively, the method of weighted least squares was used to estimate the parameters for all three transformations (5,6).

Weighted least squares analysis was performed for the probit, logit, and angular transformations and the explanatory variables  $X_1 = \log$  doses of Pmg,  $X_2 = \text{temperature}$ , and  $X_3 = \text{photoperiod}$  for each cultivar (Table 1). All  $R^2$  values except one were highly significant (29, page 453), indicating that the fitted regressions account for a large proportion of the total variation about the mean (6). Lowest  $R^2$  values were associated with the cultivar Wayne, indicating a greater amount of unexplained variation. The angular transformation was superior to logits or probits for satisfying the

model (equation 1), so this was the transformation used for further analysis.

Table 2 shows the parameter estimates of the angular transformation model

$$Y_i = \sin^{-1} \sqrt{p_i} = B_0 + B_1 X_1 + B_2 X_2 + B_3 X_3 + \epsilon_i \quad (2)$$

in which  $B_0$  represents the intercept term,  $B_1$  represents the effect of log dose of Pmg,  $B_2$  the effect of temperature, and  $B_3$  the effect of photoperiod. Significant and highly significant values of the parameter estimates indicate a significant linear relationship between the corresponding explanatory variable and plant death. Goodness-of-fit tests (6, page 26) performed to check the adequacy of this model showed that the relatively small  $R^2$  values are caused by the high initial variability of the data (pure error) rather than lack of fit of the model.

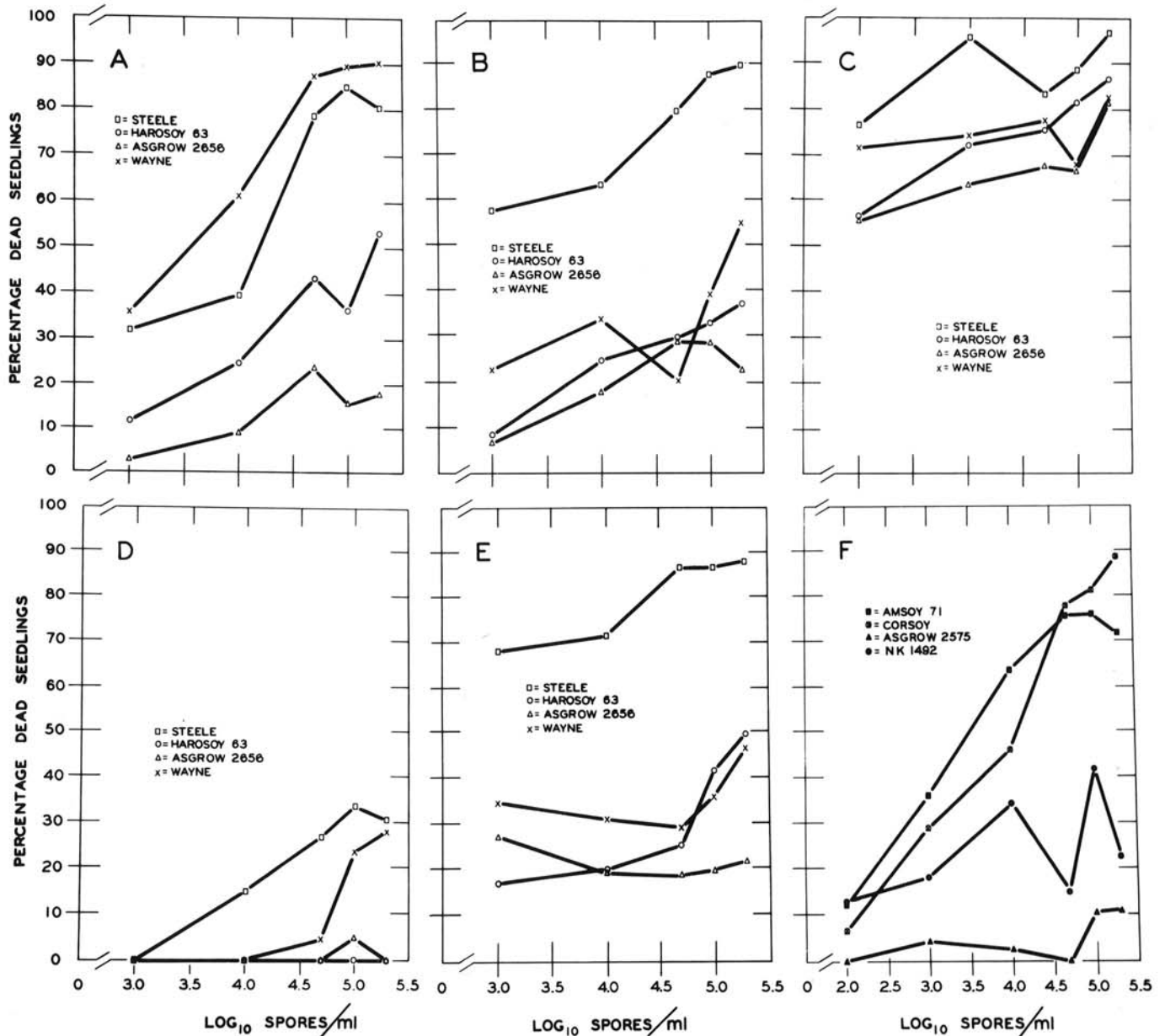


Fig. 1. A-E, Dosage-response curves for four soybean cultivars grown under five environments and subjected to cotyledon inoculation with five densities of encysted zoospores of *Phytophthora megasperma* f. sp. *glycinea* (Pmg). A, Data are means from three experiments performed at 24 C, 12-hr photoperiod. Standard errors (of the mean) for each point ranged from 2.9 to 25.4% dead. B, Data are from one experiment performed at 24 C, 16-hr photoperiod. C, Data are means from three experiments performed at 28 C, 12-hr photoperiod. Standard errors for each point ranged from 2.4 to 19.9% dead. D, Data are means from three experiments performed at 28 C, 16-hr photoperiod. Standard errors for each point ranged from 1.9 to 12.2% dead. E, Data are means from three experiments performed at 32 C, 12-hr photoperiod. Standard errors for each point ranged from 2.5 to 16.4% dead. F, Dosage-response curves for four additional soybean cultivars subjected to cotyledon inoculation with six densities of encysted zoospores of Pmg. Data are from one experiment performed at 28 C, 12-hr photoperiod.

All three variables included in the model are relevant in explaining plant death; in fact, for the four cultivars, only two of the corresponding parameters (excluding the intercept term) are not significantly different from zero (Table 2). The two cultivars that showed high levels of resistance in the field, Asgrow 2656 and Wayne, have the lowest values of  $B_1$ , indicating a lower response to increased inoculum density. Temperature was positively correlated with plant death, while photoperiod was negatively correlated with plant death.

The chosen model, which relates proportion dead plants to log doses of Pmg, temperature, and photoperiod, allows the precise estimation of log LD<sub>50</sub> values (the log of the spore concentration lethal to 50% of the plant population) for each cultivar under specified environmental conditions. In Fig. 2, the estimated log LD<sub>50</sub> values and their 95% confidence intervals are plotted against temperature for each of the four cultivars and two photoperiod

conditions. Note that the values corresponding to the conditions of 32 C and 16-hr photoperiod were obtained by extrapolation from the model since no data were collected from inoculated plants grown under this combination of conditions.

The values presented in Fig. 2 allow discrimination among the cultivars for resistance to Pmg and also provide information on the effect of temperature and photoperiod on resistance for each cultivar. Under all conditions, Steele was the most susceptible, Asgrow 2656 the most resistant, and Harosoy 63 and Wayne

TABLE 1. Values of multiple  $R^2$  obtained by regressing the transformed proportions of dead seedlings on the explanatory variables  $X_1$  = log dose of encysted zoospores per ml,  $X_2$  = temperature (C), and  $X_3$  = photoperiod (hr) for four soybean cultivars subjected to cotyledon inoculation with *Phytophthora megasperma* f. sp. *glycinea*

Transformation	Values of multiple $R^2$				
	Steele	Asgrow 2656	Harosoy 63	Wayne	Overall
Probit	0.2364 <sup>a</sup>	0.3584	0.2851	0.1125	0.1463
Logit	0.2997	0.5158	0.3564	0.2063	0.1690
Angular	0.4558	0.6165	0.6153	0.2853	0.3193

<sup>a</sup> All values of  $R^2$  are significant,  $P = 0.01$ , except for that representing Wayne with the probit transformation which was significant,  $P = 0.10$ .

TABLE 2. Multiple regression parameter estimates and their standard errors (in parentheses) for the angular transformation model<sup>a</sup> in which the transformed proportion of plants killed following cotyledon inoculation with *Phytophthora megasperma* f. sp. *glycinea* is regressed on the explanatory variables  $X_1$  = log dose of encysted zoospores per ml,  $X_2$  = temperature (C), and  $X_3$  = photoperiod (hr)

Cultivar	$B_0$	$B_1$	$B_2$	$B_3$
Steele	-1.035 <sup>ns</sup> (0.464)	0.183 <sup>**</sup> (0.039)	0.057 <sup>**</sup> (0.011)	-0.019 <sup>ns</sup> (0.017)
Asgrow 2656	-1.675 <sup>**</sup> (0.385)	0.075 <sup>**</sup> (0.033)	0.084 <sup>**</sup> (0.010)	-0.036 <sup>**</sup> (0.014)
Harosoy 63	-1.612 <sup>**</sup> (0.418)	0.169 <sup>*</sup> (0.035)	0.077 <sup>**</sup> (0.010)	-0.048 <sup>*</sup> (0.016)
Wayne	0.900 <sup>ns</sup> (0.598)	0.130 <sup>*</sup> (0.050)	0.017 <sup>ns</sup> (0.015)	-0.085 <sup>**</sup> (0.023)

<sup>a</sup>  $Y_1 = \sin^{-1} \sqrt{p_1} = B_0 + B_1 X_1 + B_2 X_2 + B_3 X_3 + \epsilon_i$ , in which  $p_1$  is the proportion of dead plants and  $B_0$  is the intercept.

<sup>ns</sup> = nonsignificant; \* = significantly different from zero,  $P = 0.05$ ; \*\* = significantly different from zero,  $P = 0.01$ .

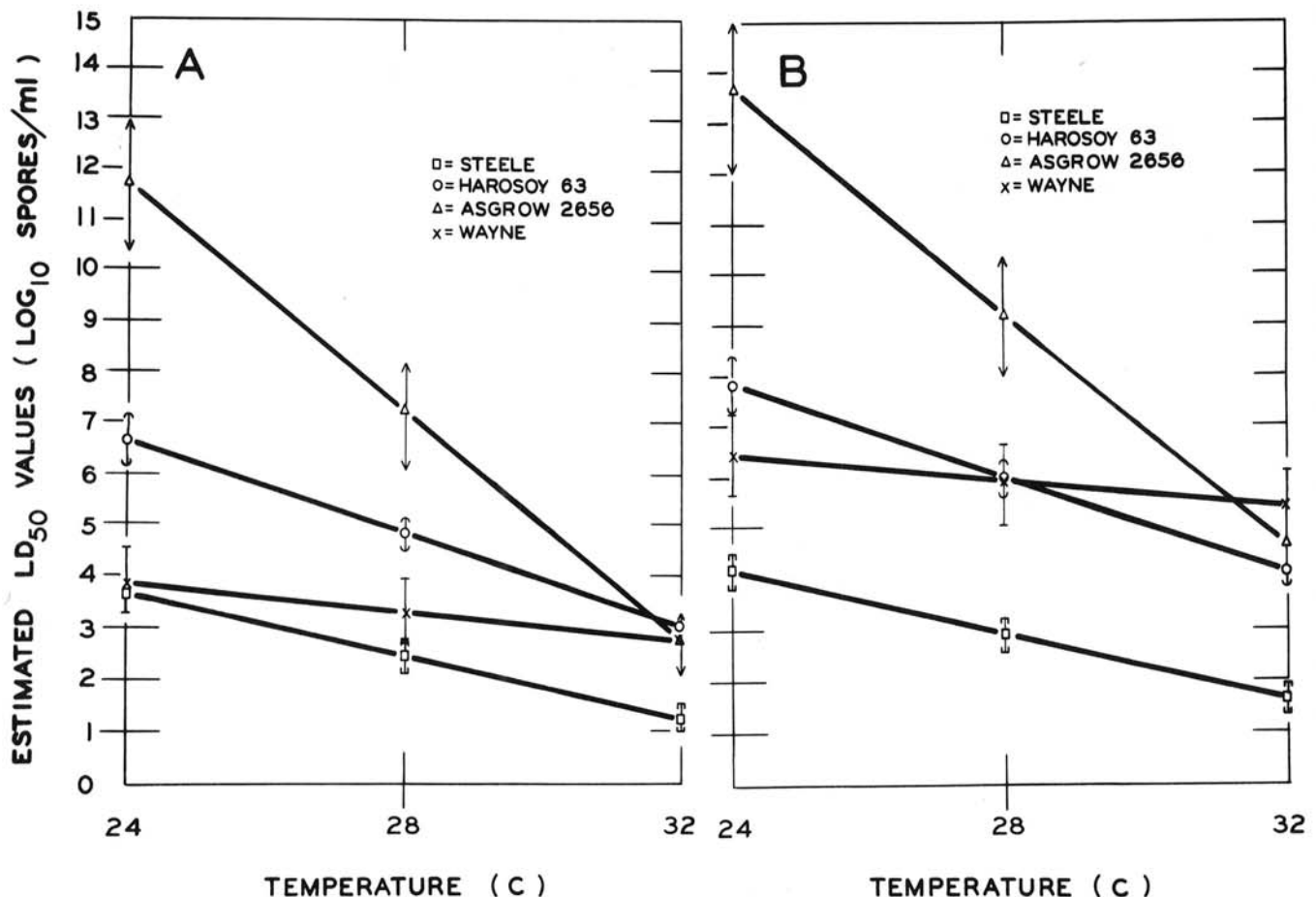


Fig. 2. Log LD<sub>50</sub> values estimated by weighted least squares regression of proportion dead plants (transformed using the angular transformation) on inoculum density, temperature, and photoperiod plotted against temperature for four soybean cultivars subjected to cotyledon inoculation with five densities of encysted zoospores of *Phytophthora megasperma* f. sp. *glycinea* under: A, 12-hr, and B, 16-hr photoperiod conditions. Values corresponding to 32 C, 16-hr photoperiod were obtained by extrapolation from the model since no data were collected under these conditions. Vertical bars represent 95% confidence intervals.

intermediate. All cultivars except Wayne showed significant responses to increased temperature, becoming gradually more susceptible as temperatures were raised from 24 to 32 C. The temperature insensitivity of Wayne was also revealed earlier by its nonsignificant  $B_2$  parameter estimate (Table 2). In addition, all cultivars except Steele showed significantly more resistance at the 16-hr photoperiod. This was most pronounced in plants of cultivar Wayne, whose rating changed from intermediate-susceptible at 12 hr to intermediate-resistant at 16 hr.

**Effect of different Pmg races on cotyledon reaction.** Cotyledon inoculation experiments involving four races of Pmg were performed in a growth chamber with 9- to 11-day-old seedlings of cultivars Steele and Asgrow 2656 incubated at 28 C. In each case, the cultivars showed highly significant differences (according to the chi-square statistic) in percentage of seedlings killed. Differences in plant death among isolates of the same race were also apparent with races 3 and 4 (Table 3).

**Effect of host plant age on cotyledon reaction.** Seedlings of cultivars Steele and Asgrow 2656 were grown in vermiculite in 19 × 22-cm plastic seed cavity trays placed inside of 24 × 30-cm aluminum pans in a 28 C growth chamber regulated to a 12-hr photoperiod. Water was added to the pans daily to a depth of 2 cm. Each pan contained 21 plants of each cultivar, which were planted at 2-day intervals to give plants of ages 6, 8, 10, 12, 14, and 16 days. Three cotyledon inoculation experiments were performed with Pmg isolate 36 (race 7) at a spore concentration of 10,000 spores per milliliter. While there was significant variability in overall plant kill between experiments, plants of cultivar Steele consistently had higher percentages of dead seedlings than those of Asgrow 2656. Mean percentages of dead seedlings for Steele were 19, 32, 47, 59, 55, and 51% at 6, 8, 10, 12, 14, and 16 days of plant age, respectively; the corresponding percentages for Asgrow 2656 were 4, 3, 5, 4, 15, and 3%, respectively. Significant or highly significant differences in plant death between the cultivars were obtained at all plant ages tested; however, in all three experiments the largest differences between cultivars were obtained when 12-day-old plants were inoculated.

**Cotyledon inoculation performed in the greenhouse.** Seeds of cultivar Steele were planted in a loam, sand, peat (2:2:1, v/v) soil mixture in 19 × 22-cm plastic seed cavity trays in a 28 C greenhouse. Seed cavity trays were placed in 6 × 30 × 44-cm plastic trays in which water was maintained at a depth of 2 cm. When the seedlings were 12 days old, the cotyledons were inoculated with eight inoculum densities ranging from 500 to 60,000 spores per milliliter (19–28 plants per spore concentration). Probit analysis (8,11) of the experiment yielded a log LD<sub>50</sub> value of 2.73 (standard error = 0.29) which was in close agreement with log LD<sub>50</sub> values obtained in growth chamber studies.

Eighteen to 29 plants of the cultivars Steele and Asgrow 2656 were inoculated with 5,000 and 100,000 spores per milliliter of Pmg and were incubated in a greenhouse at 28 C. This experiment was replicated three times and results revealed differences between cultivars consistent with those obtained in a controlled environment chamber. For Steele, percentages of plants killed were 78, 94, and 85 at 5,000 spores per milliliter and 84, 95, and 100 at 100,000 spores per milliliter; for Asgrow 2656, the corresponding percentages were 28, 21, 30, and 76, 35, and 70, respectively. In all experiments significant ( $P = 0.05$ ) or highly significant ( $P = 0.01$ ) differences were obtained between the two cultivars at both inoculum densities with the exception of the higher inoculum density in the first experiment. When experiments were combined, highly significant ( $P = 0.01$ ) differences existed between cultivars at both inoculum densities.

**Additional cultivars tested.** Four additional cultivars inferred from repeated field trials to contain relatively high (Asgrow 2575 and Northrup King S1492) or low (Corsoy and Amsoy 71) levels of resistance to Pmg in the field were subjected to inoculum density experiments in a growth chamber at 28 C, 12-hr photoperiod. Two pots of each cultivar (18–29 plants per pot) were inoculated at each of the following six inoculum densities: 100, 1,000, 10,000, 50,000, 100,000, and 200,000 spores per milliliter. Results (Fig. 1F) revealed substantial differences between cultivars in response to

increased dosages of Pmg.

#### Correlation between cotyledon reaction and field reaction.

Information resistance obtained through the use of cotyledon inoculation of the eight cultivars used in these studies was compared with field data obtained for each cultivar when grown in naturally infested field plots over several locations and years. In testing for field correlation, estimated log LD<sub>50</sub> values for all eight soybean cultivars at 28 C, 12-hr photoperiod conditions were obtained by using the SAS (Statistical Analysis System) probit procedure (11), which employs maximum likelihood estimation. Although this is the preferred method of estimation, it could not be used for the data involving responses to inoculum density, temperature, and photoperiod due to the presence of more than one explanatory variable in the model. The chi-square goodness of fit statistic indicated a good fit of the data for six of eight cultivars tested. For Wayne and Northrup King S1492, the large chi-square value indicated large discrepancies between observed and expected numbers of plants killed. This could arise either because individual test subjects did not react independently, or because the calculated straight line did not adequately describe the relationship between dose and probit (8).

Table 4 shows the relative yield, percentage plants killed by Pmg, and infection rates for these eight cultivars when grown in such plots compared with the estimated log LD<sub>50</sub> values. The correlation coefficient between yield and log LD<sub>50</sub> values was statistically significant ( $P = 0.01$ ) ( $r = 0.86$ , 6 df) while those between percentage dead plants and log LD<sub>50</sub> values ( $r = -0.77$ ) as well as between infection rates and log LD<sub>50</sub> values ( $r = -0.74$ ) were statistically significant,  $P = 0.05$ .

## DISCUSSION

Our results show that resistance to Pmg in soybean seedlings can be identified and characterized through inoculation of cotyledons.

TABLE 3. Percentage of plants of two soybean cultivars killed following inoculation with eight isolates (four races) of *Phytophthora megasperma* f. sp. *glycinea* and incubation at 28 C.

Isolate	Race	Inoculum density spores/ml	Percentage dead plants		No. plants inoculated per cultivar
			Steele	Asgrow 2656	
853	3	13,600	87 <sup>a</sup>	19	23–26
909	3	20,000	52	4	27–28
904	4	20,000	100	56	26–27
912	4	27,800	73	4	26–28
842	4	23,400	89	3	26–29
36	7	20,000	100	59	27–29
839	9	20,000	100	59	27
417	9	20,000	92	52	65–67

<sup>a</sup>In each case, values for the two cultivars differed ( $P = 0.01$ ) according to the chi-square statistic, (2 × 2 contingency table and using Yates correction).

TABLE 4. Relative yields, percentage of plants killed by *Phytophthora megasperma* f. sp. *glycinea*, and infection rates ( $R$ ) averaged over years and locations compared with estimated log LD<sub>50</sub> values obtained via cotyledon inoculation for eight soybean cultivars

Cultivar	Dead plants (%)	Yield (q/ha)	Infection rate ( $R$ )	Log LD <sub>50</sub> (log <sub>10</sub> spores/ml)
Steele	49.5	13.0	0.0087 a <sup>y</sup>	2.6957 <sup>z</sup>
Amsoy 71	27.5	19.2	0.0041 b	3.7450 b
Corsoy	17.0	23.0	0.0029 bc	3.8855 b
Wayne	7.5	29.7	0.0006 cd	5.7736 c
Harosoy 63	11.0	18.5	0.0016 bcd	5.9418 c
Asgrow 2656	9.5	27.7	0.0024 bcd	6.6232 c
NK 1492	2.0	30.7	0.0002 d	8.4091 c
Asgrow 2575	12.0	34.7	0.0018 bcd	9.1190 c

<sup>y</sup>Means within a column followed by the same letter are not significantly different,  $P = 0.05$ , according to Duncan's multiple range test.

<sup>z</sup>Log LD<sub>50</sub> values followed by the same letter are not significantly different,  $P = 0.05$ , by the  $t$  test.

Inoculum density, temperature, photoperiod, and plant age all affected the expression of resistance, but the severity of reaction to cotyledon inoculation was significantly correlated with disease severity, yield, and infection rates obtained under field conditions for the eight cultivars tested.

Since the resistance identified and characterized by cotyledon inoculation acts to reduce colonization of host tissue and thus the terminal amount of disease, it may be considered a component of rate-reducing resistance (24), which would act to delay the progress of an epidemic in the field. The fact that infection rates differ among cultivars in the field indicates that such resistance is also expressed in the field and is at least partly responsible for the marked differences in disease severity and yield among 'field tolerant' cultivars.

In light of our results, which show that the host cultivars differ in ability to suppress the colonization of hypocotyls by the pathogen, we suggest that the term 'field tolerance' be used with discretion, and only in cases where differences in yield cannot be accounted for by differences in rate-reducing resistance.

Paxton and Chamberlain (25) described three distinct lesion types observed on soybean hypocotyls inoculated with mycelium of Pmg. Red lesions were formed when the tissue was totally resistant; brown to black lesions when the plants were only partially resistant; and light-tan, water-soaked lesions when the plants were completely susceptible. They further observed that in susceptible cultivars, production of phytoalexin in response to inoculation with *Phytophthora cactorum*, a nonpathogen, decreased as the plants aged, with a concomitant alteration in lesion type away from the completely susceptible type. They speculated that as plants age past 14 days, phytoalexin production becomes less important as adult plant resistance takes effect. Since these same three lesion types are readily discernible in the hypocotyl in response to cotyledon inoculation, we hypothesize that this method of inoculation allows the identification of resistance that is related to, but distinct from, hypocotyl resistance expressed in response to direct mycelial inoculation, and which very likely may involve phytoalexin production.

Although the cotyledon is not the natural infection court for this pathogen, the high correlation between reaction to cotyledon inoculation and field reaction makes it clear that either the same resistance mechanism operates in both cases, or that different mechanisms act that produce the same effect. If phytoalexin accumulation is involved in reaction to cotyledon inoculation as it is in resistance to direct hypocotyl inoculation (9,33), the former hypothesis would be favored, since phytoalexin has been detected in soybean roots subjected to inoculation with zoospores (20).

It should be pointed out that resistance to Pmg may be expressed in the hypocotyl by both root and cotyledon inoculation; other workers have assessed 'field tolerance' to Pmg in terms of the inability of the fungus to move from the taproot into the hypocotyl and kill the plant (10). Thus, results of our method should agree with those obtained via root inoculation methods (10,14). In fact, they do agree; cultivars Wayne and Harosoy 63 give intermediate ratings by the method of Jimenez and Lockwood (14). It is also apparent that in both methods, the resistance rating of cultivar Wayne is lower than its field performance would suggest. We speculate that the field performance of Wayne may be conditioned by some form of adult-plant resistance not identifiable in the seedling stage. Wayne also was one of the two cultivars that showed large discrepancies between observed and expected numbers of plants killed in response to cotyledon inoculation. The other cultivar, Northrup King S1492, has Wayne as a parent.

Cultivar rankings based on dosage-response data remained constant throughout most of the inoculum densities, temperatures, and photoperiods that were tested. However, cultivars differed dramatically in responses to these variables. The effect of increased inoculum density was less pronounced for cultivars showing resistance in the field than for cultivars with low levels of resistance.

Overall plant death increased significantly as temperature increased from 24 to 32 C. Growth studies performed with isolate 36 of Pmg on V-8 juice agar (*unpublished*) indicate that the low kill at 24 C could be due in part to slower growth of the fungus.

However, differential growth rates were not apparent between 28 and 32 C. A more likely explanation involves heat-induced susceptibility, which has been observed by other workers (1-3,22) and has been shown to involve depression of phytoalexin production in response to heat treatment.

The increased resistance with increased photoperiod may result from increased photosynthesis; a similar response has been noted in general resistance of potato to *Phytophthora infestans* (30). Other workers (17) have found that resistant soybeans grown in total darkness became susceptible to Pmg via hypocotyl inoculation. It is possible that increased light exposures result in higher levels of phytoalexin production.

In cotyledon reaction, the cultivar Asgrow 2656 was consistently more resistant than Steele to four races of Pmg to which both cultivars are susceptible when hypocotyls are inoculated. The results suggest that, while isolates may differ in aggressiveness expressed in cotyledon inoculation, the resistance identified by this method is active against numerous races of the pathogen. These findings agree with those of other workers (10,14) who have found that the levels of 'field tolerance' to Pmg expressed in root inoculations are similar for different races.

Significant increases in plant death observed in the cultivar Steele from 6 to 12 days of age at inoculation may be due to the marked softening of the cotyledons as they age, allowing the pathogen easier movement in the plant tissue. The decrease in susceptibility after 12 days may be characteristic of adult plant resistance taking effect, as suggested by Paxton and Chamberlain (25). For the more resistant Asgrow 2656, plant age was of little significance in determining its level of resistance.

In summary, the cotyledon inoculation method described in this study offers a quantitative means of identifying and characterizing rate-reducing resistance to Pmg present in soybean cultivars. Advantages of this method include its rapidity, ease of inoculum preparation and quantification, small space requirement, and ease of data collection. It may be performed in a greenhouse or growth chamber, although more consistent results are obtained with more rigorous environmental control. Also, because of variability between experiments, they should be replicated and cultivars with known reactions should be included in each inoculation. We used this method to differentiate among pure line cultivars with various levels of rate-reducing resistance to Pmg. It may also prove useful for evaluating segregating populations, and in evaluating cultivar resistance in cases where field evaluations are not possible.

#### LITERATURE CITED

1. Chamberlain, D. W. 1970. Temperature ranges inducing susceptibility to *Phytophthora megasperma* var. *sojae* in resistant soybeans. *Phytopathology* 60:293-294.
2. Chamberlain, D. W. 1972. Heat-induced susceptibility to nonpathogens and cross protection against *Phytophthora megasperma* var. *sojae* in soybeans. *Phytopathology* 62:645-646.
3. Chamberlain, D. W., and Gerdemann, J. W. 1966. Heat-induced susceptibility of soybeans to *Phytophthora megasperma* var. *sojae*, *Phytophthora cactorum*, and *Helminthosporium sativum*. *Phytopathology* 56:70-73.
4. Cox, D. R. 1970. *The Analysis of Binary Data*. Methuen and Co. Ltd., London. 142 pp.
5. Dixon, W. J., and Brown, M. B. (editors). 1979. *Biomedical Computer Programs P-series*. University of California Press, Berkeley. 880 pp.
6. Draper, N. R., and Smith, H. 1966. *Applied Regression Analysis*. John Wiley & Sons, New York. 407 pp.
7. Federation of British Plant Pathologists. 1973. A guide to the use of terms in plant pathology. *Phytopathol. Pap.* 17. 55 pp.
8. Finney, D. J. 1971. *Probit Analysis*. 3rd ed. Cambridge University Press, Cambridge, England. 333 pp.
9. Frank, J. A., and Paxton, J. D. 1970. Time sequence for phytoalexin production in Harosoy and Harosoy 63 soybeans. *Phytopathology* 60:315-318.
10. Franks, R. K., and Schmitthenner, A. F. 1978. Factors affecting tolerance in soybean to *Phytophthora megasperma* var. *sojae* under greenhouse conditions. (*Abstr.*) *Phytopathol. News* 12(9):182.
11. Helwig, J. T., and Council, K. A. (editors). 1979. *SAS Users Guide*. SAS Institute Inc., Cary, NC. 494 pp.
12. Hildebrand, A. A. 1959. A root and stalk rot of soybeans caused by

- Phytophthora megasperma* Dreschler var. *sojae* var. nov. Can. J. Bot. 27:927-957.
13. Hobe, M. A., and Schmitthenner, A. F. 1980. Direct isolation of new races of *Phytophthora megasperma* var. *sojae* from NW Ohio soils. (Abstr.) Phytopathology 71:226.
  14. Jimenez, B., and Lockwood, J. L. 1980. Laboratory method for assessing field tolerance of soybean seedlings to *Phytophthora megasperma* var. *sojae*. Plant Dis. 64:775-778.
  15. Kaufmann, M. J., and Gerdemann, J. W. 1958. Root and stem rot of soybean caused by *Phytophthora sojae* n. sp. Phytopathology 48:201-208.
  16. Keeling, B. L. 1980. *Phytophthora* root and stem rot: Isolation, testing procedures, and seven new physiologic races. Pages 367-370 in: World Soybean Research Conference II: Proceedings. F. T. Corbin, ed. West View Press, Boulder, CO. 898 pp.
  17. Klarman, W. L., and Gerdemann, J. W. 1963. Induced susceptibility in soybean plants genetically resistant to *Phytophthora megasperma* var. *sojae*. Phytopathology 53:863-864.
  18. Kuan, T.-L., and Erwin, D. C. 1980. Formae speciales differentiation of *Phytophthora megasperma* isolates from soybean and alfalfa. Phytopathology 70:333-338.
  19. Laviolette, F. A., and Athrow, K. L. 1977. Three new physiologic races of *Phytophthora megasperma* var. *sojae*. Phytopathology 67:267-268.
  20. Meyer, W. A., Thapliyal, P. N., Frank, J. A., and Sinclair, J. B. 1971. Detection of phytoalexin in soybean roots. Phytopathology 61:584-585.
  21. Morrison, R. H., and Thorne, J. C. 1978. Inoculation of detached cotyledons for screening soybeans against 2 races of *Phytophthora megasperma* var. *sojae*. Crop Sci. 18:1089-1091.
  22. Murch, R. S., and Paxton, J. D. 1980. Temperature and glyceollin accumulation in *Phytophthora*-resistant soybeans. Phytopathol. Z. 97(3):282-285.
  23. Nelson, R. R. 1973. Breeding Plants for Disease Resistance. The Pennsylvania University Press, University Park. 401 pp.
  24. Parlevliet, J. E. 1979. Components of resistance that reduce the rate of epidemic development. Annu. Rev. Phytopathol. 17:203-222.
  25. Paxton, J. D., and Chamberlain, D. W. 1969. Phytoalexin production and disease resistance in soybeans as affected by age. Phytopathology 59:775-777.
  26. Pratt, R. G. 1981. Morphology, pathogenicity, and host range of *Phytophthora megasperma*, *P. erythroseptica*, and *P. parasitica* from arrowleaf clover. Phytopathology 71:276-282.
  27. Pratt, R. G., Mitchell, J. E., and Willis, D. A. 1975. Resistance and susceptibility to *Phytophthora megasperma* expressed in alfalfa cotyledons. Phytopathology 65:365-369.
  28. Schwenk, F. W., Ciaschini, C. A., Nickell, C. D., and Trombold, D. G. 1979. Inoculation of soybean plants by injection with zoospores of *Phytophthora megasperma* var. *sojae*. Phytopathology 69:1233-1234.
  29. Steel, R. G. D., and Torrie, J. H. 1960. Principles and Procedures of Statistics. McGraw-Hill, New York. 481 pp.
  30. Thurston, H. D. 1971. Relationship of general resistance: Late blight of potato. Phytopathology 61:620-626.
  31. Vanderplank, J. E. 1963. Plant Diseases: Epidemics and Control. Academic Press, New York. 349 pp.
  32. Ward, E. W. B., Lazarovits, G., Unwin, C. H., and Buzzell, R. I. 1979. Hypocotyl reactions and glyceollin in soybeans inoculated with zoospores of *Phytophthora megasperma* var. *sojae*. Phytopathology 69:951-955.
  33. Yoshikawa, M., Yamauchi, K., and Masago, H. 1978. Glyceollin: Its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. Physiol. Plant Pathol. 12:73-82.