Effect of *Cercospora sojina* and *Phomopsis sojae* Alone or in Combination on Seed Quality and Yield of Soybeans

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ABSTRACT

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Two soybean (*Glycine max*) cultivars, Corsoy-79, susceptible, and Wells, moderately resistant to a *Cercospora sojina* isolate and equally susceptible to *Phomopsis sojae*, were inoculated with either fungus alone or in combination in field plots in 1981 and 1982. Frogeye leafspot, caused by *C. sojina*, which can cause latent infection in stems, was higher in *C. sojina*-inoculated plots in both years and showed a negative correlation ($r = -0.56$ and -0.50, respectively) with yield and 1,000-seed weight and occurred at a significantly higher severity in Corsoy-79 than in Wells. Recovery of *C. sojina* from pods was predictive of recovery of *C. sojina* from seeds. Recovery of either pathogen in pods, stems, and seeds was not affected by the presence of the other. Seed germination was lowest in plots inoculated with both fungi (83%), intermediate in plots inoculated with *C. sojina* or *P. sojae* alone (86.1 and 84.7%, respectively), and highest in the control (89.6%). *C. sojina* and *P. sojae* acted independently and had an additive effect.

Cercospora sojina Hara. causes frogeye leafspot of soybean (*Glycine max* (L.) Merr.) and also infects stems, pods, and seeds (9,23). *Phomopsis sojae* Leh. is part of a *Diaporthe-Phomopsis* complex of soybeans (5,6,8,15,16) that causes pod and stem blight, stem canker, and Phomopsis seed decay. Both fungi are seedborne and can reduce yields and seed quality (5,6,8,23). There are several reports concerned with the interaction between *C. kikuchii* (Matsumoto & Tomayasu) Gardner and *P. sojae* (6,9,11,13,21) but none on the interaction between *C. sojina* and *P. sojae*. We studied the separate and combined effects of these two fungi on soybean seed quality and yield. Portions of these studies were published as abstracts (1,2).

A portion of the first author's Ph.D. thesis.

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MATERIALS AND METHODS

Sources of inoculum. An isolate of *C. sojina* designated TN4 (ATCC 44087) by Yorinori (23) was maintained in test tube slants of V-8 juice agar (20,22) amended with 100 μg/ml each of streptomycin sulfate and tetracycline (V8-ST). The *P. sojae* isolate came from a soybean seed and produced colonies on acidified potato-dextrose agar (pH5) (Difco) (PDA) with yellowish mycelium and black stroma on the underside. Such cultures are called *P. sojae* by Kmetz et al (8). Stock cultures were maintained on PDA test tube slants.

Inoculum production and inoculation. A method for mass production of *C. sojina* (Cs) conidia was used (22,23). One milliliter of a spore suspension from a fresh culture was distributed over V8-ST in 9-cm culture plates and incubated at 25 C. After 7–10 days, each plate was washed with 15–20 ml of sterile deionized water and collected in a flask. The suspension was diluted with sterile deionized water to obtain about 20,000 ± 1,000 spores per milliliter. Tween 80 was added (1% by volume) before spray inoculation with a Hudson 6621 Duralite Sprayer (3.8 L) (H. D. Hudson Mfg. Co., Chicago, IL). Each plot was inoculated with 200 ml of the spore suspensions at the V5 stage (3), 1 wk later, and at the R2 stage.

P. sojae (Ps) inoculum was produced on sterilized soybean stem pieces (1–1.5 cm long) or oat seeds. Macerated mycelium with agar from two PDA culture plates incubated for 5 days at 25 ± 2 C were placed into 2-L Erlenmeyer flasks containing either stem pieces or oat seeds (three-fourths full) and incubated for 20–25 days at 27 ± 3 C. Each flask was shaken by hand during incubation to promote even mycelial growth.

Prior to hand planting of soybean seeds, 100 g of Ps-infested oat seeds (Ps-oats) were placed in each row and covered with soil. At growth stage V2 (3), 100 g of

Ps-oats and 200 g of soybean stem pieces were placed at the bases of plants in each row. At growth stage R2, a 200-ml alpha spore suspension (25,000 ± 200 spores per milliliter) was sprayed in each plot. Alpha spores were obtained from cultures grown for 15 days at 25 C on water agar sprinkled with sterilized oat pieces.

Soybean cultivars and field plot arrangement. Soybean cultivars Corsoy-79, susceptible, and Wells, moderately resistant to TN4 isolate of Cs, and both susceptible to Ps were used. Both are maturity group II cultivars, which have a higher incidence of pod and stem blight in Illinois than cultivars in other maturity groups (15,16). The experimental design was a split plot with five replicates, with cultivars as main plots and fungal inoculations as subplots. Fungal inoculations were arranged factorially with Cs and Ps inoculated alone or in combination (Cs + Ps). Each experimental unit had six rows, 6 m long and 76 cm apart, and was separated from others by three rows of sweet corn to prevent dispersal of Cs spores from inoculated plots. The experiment was conducted in 1981 and 1982 at the Plant Pathology Research Farm at Urbana.

Disease ratings. In field studies using fungal inoculation, the presence of pathogens other than those being studied may affect results; therefore, the occurrence of the following diseases was recorded: frogeye leafspot, brown spot (*Septoria glycines* Hemmi), bacterial blight (*Pseudomonas syringae* pv. *glycinea* (Coerper) Young, Dye, & Wilkie), bacterial pustule (*Xanthomonas campestris* pv. *glycines* (Smith) Dye), and downy mildew (*Peronospora manchurica* (Naum.) Syd. ex Gaum). A disease rating scale of 0–9 was used, where 0 = no symptoms, 1 = 1–10%, 2 = 11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, and 9 = 81–100% of the leaf area showing symptoms. A rating for brown stem rot (*Phialophora gregata* (Allington & Chamberl.) W. Gams) was based on the percentage of plants in each plot showing leaf symptoms. Conditions were favorable for development of foliar symptoms caused by *P. gregata* (4).

Mycoflora of pods and stems. In 1982, 40 pods were selected at random from a bulk of pods harvested from five plants on 2 (R6) and 23 (R7) August and 3 September (R8), and four stem pieces 1–1.5 cm long were cut from five randomly selected mature plants (R8). The tissues were wrapped separately in cheesecloth, washed in a pipette washer for 2 hr in running tap water, and surface-sterilized by dipping in 95% ethanol for 10 sec, then in 0.5% NaOCl (10% Clorox) for 4 min, and washed twice for 1 min each in sterile deionized water.

Seeds were aseptically removed from the pods. Four pods and four stem pieces per 9-cm culture plate were plated separately on PDA amended with streptomycin sulfate and tetracycline (PDA-ST). After 5 days in the dark at 25 ± 2 C, the mycoflora associated with each tissue was recorded as the number of each fungus per either 100 pods or stems.

Harvest. The middle 4 m of the four center rows of each plot were hand harvested at maturity, mechanically threshed, cleaned, and air-dried. Total seed yield (kg/ha) and 1,000-seed weight (grams per 1,000 seeds) were adjusted to 13% seed moisture content.

At least 120 seeds from each plot were surface-sterilized as described previously, and 100 seeds were plated on PDA with five seeds per 9-cm culture plate. After 7 days of incubation at 25 ± 2 C, percent seed germination and occurrence of mycoflora and bacteria were recorded. Bacterial colonies similar to those described for *Bacillus subtilis* (Ehrenberg) Cohn (19) were present 90%, and the other colony type, 10% of the time. A seed was considered germinated if the radicle was 1 cm or longer. Seeds without mycoflora or bacteria were considered "clean."

Data analysis. All statistical analyses were done using Cyber 175 and IBM 360 computer systems and Statistical Analysis Systems (SAS) (14). The 1981 and 1982 data were analyzed using a split plot design and the combined-years analysis, using a split plot in time design (12,17,18). Fisher's least significant difference (FLSD) at $P = 0.05$ was used to detect differences in treatment means. Correlation coefficient (r) values were used to determine relationships between disease and soybean yield components.

RESULTS

Frogeye leafspot was significantly more severe on Corsoy-79 than on Wells and was significantly higher in 1981 than in 1982 (Table 1). Frogeye leafspot occurred at a significantly higher rate on plants inoculated with Cs alone or in combination with Ps than the control or Ps-inoculated plants. The combined analysis over years showed that the occurrence of brown spot, bacterial blight and pustule, and downy mildew were unaffected by inoculation with Cs or Ps alone or in combination. Incidence of brown stem rot was significantly higher in 1982 than in 1981 and was significantly higher in the control than in plots inoculated with Cs and Ps alone or in combination.

Mycoflora associated with pods and stems. There was a significant increase over harvest dates in the recovery of Cs and total fungi from pods; the numbers of fungi recovered from 100 pods were 0.1, 14.6, and 46.1 for Cs and 220, 268.7, and 735.3 total on 2 and 23 August and 3 September, respectively. Recovery of Ps on 23 August (35.9) was not significantly higher than on 2 August (30.3), but recovery on 3 September (65.4) was significantly higher than on both of the dates. Recovery of *C. sojina* colonies from Cs-inoculated plots alone or with Ps (34.1 and 34.9, respectively) was significantly higher than from plots without Cs (control, 5.9, and Ps, 6.1). Similarly, recovery of Ps colonies from Ps-inoculated plots, alone or in combination with Cs, was significantly higher than from those without Ps (Table 2). Presence of one fungus did not influence the recovery of the other. A significantly higher recovery of total fungi from pods was obtained from plots with Ps (434.0) and Cs + Ps (418.2) than from the control (382.8) but not from plots with Cs (396.7).

More Cs and Ps were isolated from stem pieces in 1981 (32.8 and 65.8, respectively) than in 1982 (8.3 and 60.6, respectively), with the difference between

Table 1. Mean of disease ratings of foliar symptoms on soybean at full pod (R6) stage and yield and weight of seeds (1981 and 1982 combined for analysis)

Treatment	Bacterial blight	Bacterial pustule	Brown spot	Brown stem rot	Downy mildew	Frogeye leafspot	Yield (kg/ha)	1,000-Seed weight (g)[w]
Years								
1981	0.9[x] a[y]	0.9 a	4.5 a	0.02 b	0.6 a	1.8 a	2,230.1 a	175.2 a
1982	0.1 b	0.7 a	2.6 b	1.80 a	0.8 a	0.9 b	1,980.2 b	147.5 b
Cultivars								
Corsoy-79	0.5 a	1.2 a	3.6 a	1.00 a	0.3 b	2.5 a	2,055.7 a	153.6 b
Wells	0.5 a	0.4 b	3.6 a	0.80 a	1.1 a	0.1 b	2,154.5 a	169.4 a
Inoculations[z]								
Control	0.4 a	0.8 a	3.6 a	1.4 a	0.7 a	0.1 b	2,225.0 a	165.8 a
Cs	0.5 a	0.6 a	3.4 a	0.7 b	0.6 a	2.7 a	2,004.9 a	159.2 b
Ps	0.6 a	0.8 a	3.5 a	0.9 b	0.7 a	0.1 b	2,172.2 a	161.4 b
Cs + Ps	0.4 a	1.0 a	3.8 a	0.7 b	0.8 a	2.4 a	2,018.4 a	159.4 b

[w] Seed weight is adjusted to 13% moisture content.

[x] Means based on five replications for each treatment. Disease rating based on percentage of leaf area covered with leaf spots; brown stem rot based on percentage of plants in each plot showing leaf symptoms. Rating scale of 0–9, where 0=no disease, 1=1–10%, 2 = 11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, and 9 = 81–100%.

[y] Values followed by different letters are significantly different from one another, based on FLSD at $P = 0.05$.

[z] Inoculations: control = no fungus, Cs = *Cercospora sojina* spore suspension, Ps = *Phomopsis sojae*-infested oat seeds and stem pieces of soybean, and Cs + Ps = combination of Cs and Ps.

Cs recoveries being significant. However, there was a significantly higher recovery of total fungi in 1982 (271.8) than in 1981 (222.3). From stem pieces of Cs- and Ps-inoculated (alone or in combination) plots, recovery of Cs (38.9 and 34.9, respectively) and Ps (85.5 and 80.3, respectively) was significantly greater than in uninoculated plots (2.5 and 39.5, respectively). Recovery of total fungi was significantly higher from Cs- (260.8), Ps- (248.4), and Cs + Ps-inoculated (270.1) plots than from the uninoculated control (209).

In 1982, Cs was recovered from stem pieces only toward the end of the growing season (Fig. 1). Corsoy-79 had higher recovery of Cs than Wells on the last sampling date (3 September), but the difference was not significant. Recovery of Ps occurred throughout the growing season from both cultivars and was highest on 2 August. Recovery of total fungi increased throughout the growing season. The two cultivars did not differ significantly from each other in recovery of Cs, Ps, or total fungi on stems or over all sampling dates.

Recovery of Cs from pods was correlated (* and ** = significant at $P = 0.05$ and 0.01, respectively) with Cs from seeds ($r = 0.87$**) and stem pieces ($r = 0.89$**); recovery from pods and seeds was correlated with seed weight ($r = -0.33$* and -0.37*, respectively). Recovery of Ps from pods was correlated with recovery from seeds ($r = 0.87$**) and stems ($r = 0.85$**), with that of total fungi on pods ($r = 0.41$**), and with total fungi on stems ($r = 0.33$**) and seeds ($r = 0.51$**). Recovery of Ps from pods and seeds was correlated with seed germination ($r = -0.50$** and -0.63**, respectively). Total fungal recovery from seeds was correlated with occurrence of Cs and Ps, respectively, on seeds ($r = 0.73$** and 0.67**), on pods ($r = 0.55$** and 0.51**), and on stems ($r = 0.52$** and 0.53**). Total fungal recovery also was correlated with yield ($r = -0.41$**). Seed weight was correlated with yield ($r = 0.58$**) but not with germination.

Seed health. In the analysis of combined data, yield and 1,000-seed weight were significantly higher in 1981 than in 1982, respectively; over years, Wells had a significantly higher 1,000-seed weight than Corsoy-79 (Table 1). However, 1,000-seed weight for Cs-, Ps-, and Cs + Ps-inoculated plants was significantly lower than for the control. Inoculation with Cs and Ps, alone or in combination, reduced yields but not significantly below the control.

The analysis combined over cultivars and years showed that seed germination and percentage of clean seeds were lower in 1981 than in 1982 and that the recovery of Cs, Ps, and total fungi from seeds was significantly higher in 1981 and 1982 (Table 2). Recovery of *Alternaria* spp. and *Fusarium* spp. was lower in 1981 than in 1982, but significantly more bacteria were recovered from seeds in 1981 (17.3) than in 1982 (9.3). Seed germination for Corsoy-79 was higher than for Wells, but the two did not differ in the percentage of clean seeds. Corsoy-79 seeds had a higher recovery of Cs and total fungi, but the cultivars did not differ from each other in the recovery of Ps, *Alternaria* spp., and *Fusarium* spp. Significantly more bacteria were recovered from Wells (15.4) than from Corsoy-79 (11.1) seeds.

Inoculation with Cs or Ps alone significantly reduced germination and percentage of clean seeds below the control (Table 3). The combination of Cs + Ps reduced germination and percentage of clean seeds below that of those from the Cs or Ps inoculations. Recovery of Cs and Ps was significantly higher from plots with the respective inoculation, but their recovery was unaffected by the inoculation with the other. The interaction between Cs and Ps was not significant. Inoculation did not influence recovery of *Alternaria* spp. or *Fusarium* spp. but did result in a higher recovery of total fungi compared with the control. The recovery of total fungi was greater from plots inoculated with Cs than from those with Ps alone, and inoculation with the combination resulted in a higher recovery of total fungi than with either alone. There was no significant interaction between Cs and Ps inoculation in recovery of total fungi.

The combined analysis of both years'

Table 2. Recovery of *Cercospora sojina* and *Phomopsis* spp. from soybean pods (1982)

Treatment[w]	*C. sojina*[x]	*Phomopsis* spp.[x]
Control	5.9[y]b[z]	32.4 b
Cs	34.1 a	30.2 b
Ps	6.1 b	57.6 a
Cs + Ps	34.9 a	55.1 a

[w] Treatment: control = no fungus, Cs = *Cercospora sojina* spore suspension, Ps = *Phomopsis* spp.-infested oat seeds and soybean stem pieces, Cs + Ps = combination of Cs and Ps.

[x] Recovery of *Cercospora sojina* and *Phomopsis* spp. was from germinated as well as ungerminated seeds.

[y] Means based on three replicates per treatment. For each replicate, 100 seeds were surface-sterilized, placed on potato-dextrose agar, and incubated in the dark for 5 days at 25 ± 2 C. Mycoflora recovered were expressed as number based on 100 seeds.

[z] Values followed by different letters are significantly different from one another, based on FLSD at $P = 0.05$.

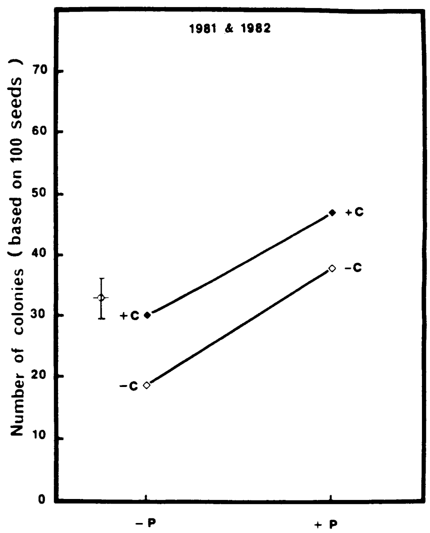

Fig. 1. Recovery of total fungal colonies from seeds of soybeans inoculated with *Cercospora sojina* (C) and *Phomopsis* spp. (P) in different combinations. Seeds were surface-sterilized and plated on potato-dextrose agar, and the number of colonies recovered was averaged for 1981 and 1982. + = Presence and − = absence of fungus in the treatment.

Table 3. Mycoflora recovered from seeds of Corsoy-79 and Wells soybeans (1981 and 1982 combined for analysis)

Treatment	Germination of seeds (%)	Clean seeds[v]	*Cercospora*[w] *sojina*	*Phomopsis*[w] *sojae*	*Alternaria*	*Fusarium*	Total Fungi
Years							
1981	82.3[x]b[y]	50.5 b	20.0 a	16.0 a	0.3 b	0.2 b	40.0 a
1982	89.4 a	67.1 a	5.5 b	10.7 b	4.3 a	1.9 a	26.6 b
Cultivars							
Corsoy-79	87.4 a	58.5 a	16.8 a	14.0 a	2.2 a	0.9 a	37.3 a
Wells	84.3 b	59.1 a	8.7 b	12.7 a	2.3 a	1.2 a	29.4 b
Inoculations[z]							
Control	89.6 a	70.8 a	3.5 b	7.9 b	2.3 a	1.1 a	18.4 d
Cs	86.1 b	57.1 b	21.3 a	9.8 b	2.2 a	1.3 a	37.8 b
Ps	84.7 bc	50.3 b	4.9 b	18.3 a	2.0 a	0.9 a	30.1 c
Cs + Ps	83.0 c	47.1 bc	21.3 a	17.4 a	2.7 a	0.9 a	47.0 a

[v] Seeds from which no mycoflora were recovered.

[w] Recovery of *C. sojina* and *P. sojae* from germinated, ungerminated, and total seeds.

[x] Means based on three replicates per treatment. For each replicate, 100 seeds were surface-sterilized, placed on potato-dextrose agar, and incubated in the dark for 5 days at 25 ± 2 C. Mycoflora recovered are expressed as number based on 100 seeds.

[y] Values followed by different letters are significantly different from one another, based on FLSD at $P = 0.05$.

[z] Inoculations: control = no fungus, Cs = *C. sojina* spore suspension, Ps = *Phomopsis* spp.-infested oat seeds and stem pieces of soybean, and Cs + Ps = combination of Cs and Ps.

data showed a correlation ($r = 0.71^{**}$) between germination and percentage of clean seeds and a negative correlation between germination and recovery of Cs ($r = -0.45^{**}$), total fungi ($r = -0.58^{**}$), and bacteria ($r = -0.61^{*}$) from seeds. Clean seed was significantly negatively correlated ($r = -0.81^{**}$) with all fungi recovered from seeds, especially Cs ($r = -0.69^{**}$).

DISCUSSION

Incidence of bacterial blight, bacterial pustule, and downy mildew was too low to be of any consequence. Although the level of brown spot severity was high, it was apparently not affected by inoculation with Cs or Ps; also, the occurrence of these diseases did not have an influence on the occurrence of Cs and Ps. The higher incidence of frogeye leafspot in Cs-inoculated plots than uninoculated plots in both years and the significantly higher recovery of Cs and Ps from pods and stems than from the control in both years confirmed the success of the inoculations. More fungi were recovered from stem pieces and pods from Cs + Ps inoculated plants than from either Cs or Ps alone, showing an additive effect of the two fungi. The correlation of Phomopsis seed infection with pod and stem infection as well as cultivar susceptibility was reported previously from this laboratory (5,6).

We show for the first time that Cs also can cause latent infection of soybean stems, but it occurs later in the season than that of Ps. Cs is primarily a leaf pathogen, and this would explain the low rate of recovery of the fungus from stem pieces early in the season. The recovery of Cs from pods was higher for Corsoy-79 than for Wells and was predictive of the recovery of Cs but not Ps from seeds. Ps can cause latent infection in soybean and can overseason in soybean residue (15), thus accounting for the recovery of Ps from stem pieces early in the season. The reduction in the recovery of Ps near the end of the season may be due to environmental factors. Shortt et al (15,16) reported a high correlation between Phomopsis seed decay and precipitation, relative humidity, dew, and temperature.

Recovery of Cs and Ps from seeds was independent of one another, and the absence of a significant interaction shows that the two fungi have an additive effect in increasing the total fungi recovered from seeds. Recovery of Cs and Ps was not correlated with isolations from stem pieces, which agrees with the results of Shortt (15) studying pod and stem blight and Phomopsis seed decay. Our results showing that recovery of Cs and Ps from stems, pods, and seeds is independent of one another contrasts with reports of antagonism between *C. kikuchii* and Ps on seeds (6,11,13,21). These data provide additional evidence of the additive effect of the two fungi.

Corsoy-79 and Wells did not differ significantly in yields, yet in 1981 when the incidence of Cs was higher, Corsoy-79 yielded less, suggesting an influence by Cs. However, when the incidence of Cs was low in 1982, there was no difference in yield. The higher seed weight of Wells over Corsoy-79 may be due to the genetic potential of the former cultivar.

The occurrence of leaf symptoms associated with brown stem rot in 1982 was highest in uninoculated plots, lowest in plots inoculated with Cs + Ps, and intermediate in plots inoculated with Cs or Ps alone. This is the first report of brown stem rot interacting with frogeye leafspot but not with the Phomopsis disease complex. Kittle and Gray (7) showed an interaction between brown stem rot and Phomopsis pod and stem blight using soil fumigation and foliar fungicides to control the two diseases. Also, our yields and 1,000-seed weights were more affected by brown stem rot in the control than in inoculated plots, resulting in the nonsignificant differences between fungal inoculations.

The recovery of Cs, Ps, total fungi, and bacteria from seeds had a high negative correlation with seed germination and percentage of clean seeds. Of the two pathogens, Cs and Ps, the latter affects seed germination much more than the former. Only seeds that were severely infected by Cs failed to germinate. The fact that high correlation exists between the recoveries of Cs and Ps from pods with those from seeds may be used to develop a strategy to control them and improve seed quality. Fungicide application at early stages of pod formation will prevent them from establishing in the pods and infecting seeds (10,15). Germination of Wells seeds was lower than that of Corsoy-79, and although the occurrence of Cs was higher, it did not affect germination as much as did the presence of bacteria. The role of bacteria (*B. subtilis*) with soybean seeds during bioassay often goes unreported. We confirm that the occurrence of bacteria affects bioassay results (19). With the absence of an interaction between Cs and Ps as shown by the use of analysis of variance, an additive effect is implied in reducing seed germination and percentage of clean seeds.

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