Purple Seed Stain of Soybeans

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ABSTRACT

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Field plantings of four genetically different soybean lines were inoculated with *Cercospora kikuchii.* Inoculations at different stages of host development in the same environment and inoculations at similar host stages in varying environments were compared. Incidence of purple-stained and total infected seed was not correlated with the length of the flowering period, but it was affected by weather conditions at the time of inoculation. Young pods were more susceptible to infection than old pods. Inoculated plants matured earlier, had smaller seed, and yielded less than noninoculated plants. Seed infection resulting from *C. kikuchii* inoculations in different environments at the full-bloom stage of host development ranged from 3% to 30% for P.I. 80837, the most resistant entry, and from 30% to 85% for Amsoy, the least resistant soybean entry. Soybean entries differed in the expression of purple stain in infected seed.

Additional key words: seed infection, *Glycine max*, seed-borne disease.

Purple seed stain, which is caused by *Cercospora kikuchii* (Matsumoto & Tomoyasu) Gardner, is a disease that reduces the seed quality of soybeans [*Glycine max* (L.) Merr.]. It is present in all areas of the world where the crop is grown. In certain years, highly susceptible soybean entries may have as much as 50% purple-stained seed; whereas in other years, only trace amounts occur (T. S. Abney, *unpublished*). Crane and Crittenden (4) reported a correlation between the length of the flowering period of soybean entries and the natural incidence of purple-stained seed. Plants with a flowering period of about 30 days had significantly more purple-stained seed than plants with about a 20-day flowering period (2.67 vs. 0.37%). Other field studies of purple seed stain have been based on natural infection, except for two studies (13, 18). Inoculation of soybean plants, pods, and seed with *C. kikuchii* has been confined to greenhouse and laboratories studies. Murakishi (16) reported that wounded pods of greenhouse-grown soybeans were more susceptible to invasion by *C. kikuchii* than nonwounded pods. Also, he noted that nonwounded pods became infected, but only in very high relative humidity. Data are lacking on the effect of this disease on total seed yield and seed size.

In this study, soybeans were inoculated with *C. kikuchii* at different growth stages and time of season. The incidence of both purple-stained and total infected seed was recorded. The main objectives were (i) to determine the effect of the duration of the flowering period of soybeans on the incidence of purple-stained seed, (ii) to determine if the amount of purple-stained seed is a reliable index of total infected seed, (iii) to determine the extent of pod infection, (iv) to determine the influence of environment on the incidence of purple-stained and total infected seed, and (v) determine the effect of *C. kikuchii* on total seed yield and seed size.

MATERIALS AND METHODS

The tests were conducted in 1970 and 1971 at Lafayette, Indiana, using a randomized block design with four replications. Individual plots consisted of three rows 2.9 m long and 1 m apart for the soybean entries Amsoy, Wayne, Cutler, and plant introduction P.I. 80837 representing maturity groups II, III, IV, and IV, respectively. The soybeans were planted at four different times in both years. In 1970, the planting dates were 8 May, 27 May, 17 June, and 6 July; in 1971, they were 24 May, 7 June, 23 June, and 7 July. Soybeans planted at the first planting date each year were inoculated with *C. kikuchii* at five different times during the growing season when the plants were in the following stages of growth as described by Fehr et al. (7): R2 (full bloom); R3, R5, R6 (intermediate stages of pod and seed development); and R8 (95% of pods brown, harvest maturity). Soybeans in the last three planting dates were inoculated only at the R2 stage of development which coincided with R3, R5, and R6 in the first planting date. Test rows were divided into 0.7-m sections in 1970 and referred to as sections 1, 2, 3, and 4, in order to allow for comparisons of seed samples from two different plant populations within a row (section 3 vs. 1 or 4), and wounded vs. nonwounded plants (section 2 vs. 3). Spores and mycelia of *C. kikuchii,* grown 3 days on V-8 juice agar and potato-dextrose agar (PDA) at 22 C were reduced to suspensions by adding water and fragmenting in a blender. Eight petri plate cultures per liter of water were used to inoculate each row.

Soybeans were harvested when 95% of the pods of control plants were mature. Ten plants from sections 1 and 4 of each test row in 1970 and at random from the entire row in 1971 were evaluated for purple-stained seed in top stems, top branches, bottom stems and bottom branches. Seeds from the remaining plants in sections 1 and 4 were composited and designated section 1, whereas seeds from sections 2 and 3 were kept as individual seed lots.

Total gram yield and grams per 100 seed (seed size) were determined for each seed sample except those designated top stems, top branches, bottom stems, and bottom branches. Only a visual count of the number of purple-stained seed per 50 seed was recorded for the latter samples. One hundred seeds of each field-threshed sample were viewed with an illuminated magnifier (×3) to determine the incidence of purple-stained seed and seed with imperfect seed coats. Seeds were immersed in a 95% solution of ethyl alcohol for 20 seconds, in a 1% solution of sodium hypochlorite for 1 minute, and then plated on PDA. After 8 days at room temperature, purple-stained seed, microorganisms growing from seed, and seed germination were recorded. The incidence of and interaction among fungal isolates from seed of naturally and artificially infected plants will be reported in a separate article. However, an interaction between *C. kikuchii* and *Diaporthe* spp. will be mentioned since it appeared to interfere with the determination of some *C. kikuchii* treatments effects. All field data with the exception of expression rate calculations were subjected to a computerized analysis of variance program and means were compared by the studentized range test. Percentage data was transformed to arcsine ($\sqrt{\text{percentage}}$) before analysis. Significant differences were based on $P = 0.05$. Opened flower samples collected from *C. kikuchii* inoculated and noninoculated field-grown Amsoy soybeans were used for histological studies. Specimens collected at 8, 16, 24, 48, and 72 hours after inoculation were fixed in formalin:acetic acid:alcohol (FAA). The procedures of Johansen (10) were followed in preparing samples for paraffin-embedding. Sections were cut at 10 μm with a rotary microtome, stained in safranin-fast green, and examined microscopically for the presence of *C. kikuchii*. Amsoy and Wayne soybean entries were used in greenhouse studies. When plants bloomed, the flowers of 45 plants were inoculated with mycelial plugs of *C. kikuchii* grown on PDA. The mycelial plugs were placed at the base of the sepals, next to the opening of the keel petals. Plants were placed in a moist chamber for 14-16 hours and then moved to greenhouse benches. As new flowers formed on the same plants, they were inoculated and incubated again until flowering ceased. Flowers of control plants were either inoculated with plugs of PDA or left untreated. Seeds that developed from all plants were harvested when mature, and assayed on PDA as previously described.

RESULTS

Sampling two different plant populations from different areas of a row (section 1 vs. section 3) did not result in a significant difference in the incidence of seed infected with *C. kikuchii*. As a result, in the analysis of 1970 treatment effects, seed samples harvested from section 3 were used. The incidence of infected seed harvested from wounded plants (section 2) did not differ significantly from that of nonwounded plants.

There was no relationship between the length of the flowering period of soybean entries (which decreased with each delay in planting) and the natural incidence of purple-stained and total infected seed in either year. For example, in 1970, the natural incidence of purple-stained seed in Amsoy, the most susceptible entry, was 10.2%, 18.2%, 14.0%, and 15.2%, and the natural incidence of total infected seed was 13.8%, 32.0%, 12.0%, and 14.0% for planting dates 1, 2, 3, and 4, respectively. Trends among the four soybean entries for natural incidence of both purple-stained and total infected seed were similar in both years but with higher frequencies in 1971 for the susceptible soybean entries Amsoy, Wayne, and Cutler. In 1971, Amsoy had a significantly higher level of natural seed infection than Cutler or P.I. 80837 in three of the four planting dates; Wayne, intermediate between Amsoy

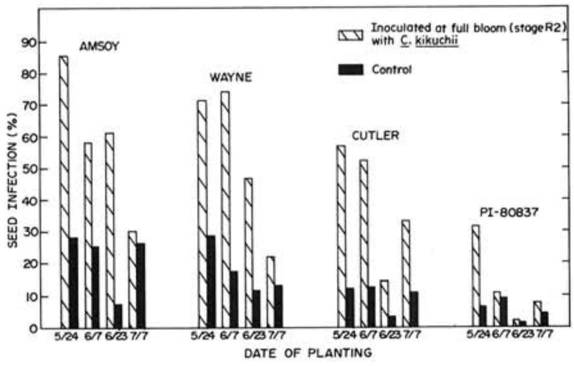

Fig. 1. Effect of planting date and time of inoculation on incidence of *Cercospora kikuchii* seed infection in soybean cultivars Amsoy, Wayne, Cutler, and P.I. 80837. Inoculation dates within corresponding planting dates were: Amsoy (9 July, 23 July, 8 August, and 23 August); Wayne (15 July, 1 August, 12 August, and 30 August); Cutler (19 July, 3 August, 15 August, and 30 August); and P.I. 80837 (1 August, 10 August, 18 August, and 30 August).

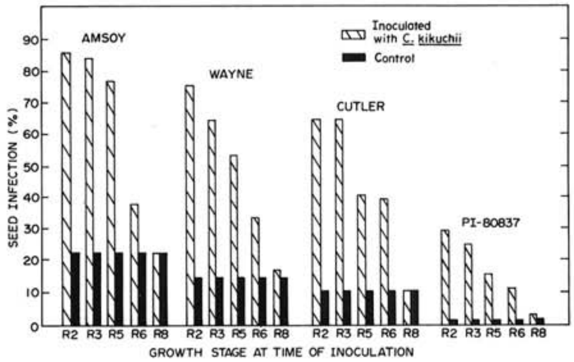

Fig. 2. Effect of plant growth stage on establishment of *Cercospora kikuchii* seed infection in soybean cultivars Amsoy, Wayne, Cutler, and P.I. 80837. Growth stages: R2 (full bloom); R3, R5, R6 (intermediate stages of pod and seed development); and R8 (95% of pods brown, harvest maturity).

and Cutler in level of natural seed infection, did not differ significantly from the other soybean entries except in the first planting date where it had a higher level of infection than either Cutler or P.I. 80837; differences in seed infection in the third planting date were not significant for any of the soybean entries (Fig. 1).

In both test years, the time of inoculation with *C. kikuchii* during the growing season influenced the incidence of both purple-stained and total infected seed. Plants inoculated in growth stage R2 in the four different planting dates in 1970 and 1971 averaged 40% seed infection compared to 12% natural seed infection. Performance of the two samples differed for the four soybean entries in the dates of planting, but the trend in purple-stained and total infected seed was similar in both years for the soybean entries and planting dates. Increases in seed infection associated with R2 inoculations for the four planting dates ranged from 10 percentage points for P.I. 80837 to 45 percentage points for Amsoy. The averages for purple-stained and total infected seed due to the R2 inoculations for both years were 50%, 22%, 14%, and 4% (purple-stained) and 65%, 50%, 33%, and 13% (total infected seed), for Amsoy, Wayne, Cutler, and P.I. 80837, respectively. Generally, the last two inoculation dates were less favorable for infection of seed (Fig. 1). Several times, inoculation periods favorable for seed infection did not favor expression of purple stain in seed, and vice versa. For example, infection of Cutler seed was significantly higher on date 4 than on date 3 (Fig. 1), but the percentages of expression of purple stain in seed did not differ. In contrast, similar amounts of Amsoy seed were infected on dates 2 and 3, but the pigment expression in seed was significantly higher on date 2 than on date 3. When plants in the first planting date were inoculated at different growth stages (R2 to R8), seed infection was favored most by inoculating at growth stages R2 and R3. The data for 1971 are shown in Fig. 2. In general, seed infection decreased as plants were inoculated in later growth stages. Inoculation of mature plants (growth stage R8) resulted in least infection. However, in comparing results of R3, R5, and R6 inoculations in the first planting date (Fig. 2) with the R2 inoculations in planting dates 2, 3, and 4 (Fig. 1), differences in seed infection due to *C. kikuchii* inoculations from R2 to R6 in the same environment were not significant (i.e., R6 planting date 1 vs. R2 planting date 4). The rate of expression of purple stain in seed by different soybean entries is presented in Table 1. All differed in rate of pigment expression. Amsoy had the highest expression rate in both years. The less susceptible soybean entries, Wayne and Cutler, had similar expression rates, but they were slightly lower in value than the rate expressed by Amsoy. The least susceptible entry, P.I. 80837, had the lowest expression rate.

In 1970, there was a greater incidence of purple-stained seed in the top half (stems and branches) than the bottom half (stems and branches) of Amsoy, Wayne, and Cutler soybean plants. However, many of these differences were not statistically significant. When they were significant, they were rather large and were most frequent in Amsoy and Cutler. In 1971, more purple-stained seed were harvested from the tops than from the bottoms of Amsoy, Wayne, and Cutler plants (Table 2). In Amsoy and Cutler inoculated plants, half of these differences were statistically significant and occurred most frequently when plants were inoculated in later growth stages (i.e., R5 and R6). In these growth stages, only pods were developing, whereas both flowers and pods were present in stages R2 and R3. In addition, the youngest pods were present in the tops of plants that were inoculated in stages R5 and R6. The differences in the incidence of purple-stained seed between stems and branches are shown in Table 3. In 1971, the majority of purple-stained seed was harvested from the stems of Amsoy, Wayne, and Cutler.

TABLE 1. Expression of purple stain in soybean seed infected with *Cercospora kikuchii*

Year and soybean entry	Expression rate[a]					
	Soybean growth stage at time of inoculation[b]					
	R2	R3	R5	R6	R8	Noninoculated
1970						
Amsoy	77	47	67	60	71	65
Wayne	48	35	55	56	61	64
Cutler	27	21	40	53	55	62
P.I. 80837	13	10	8	13	37	7
1971						
Amsoy	89	87	83	72	76	60
Wayne	41	44	40	40	49	39
Cutler	52	45	60	46	53	53
P.I. 80837	14	6	16	9	18	23

[a]Based on four replications, 100 seed per replicate; rate calculated by (number visually purple-stained seed/number of seed infected with *C. kikuchii*) × 100.

[b]Soybean plants inoculated with *C. kikuchii* at growth stages R2 (full bloom) to R8 (pods brown, harvest maturity).

TABLE 2. Influence of branch and stem positions (top vs. bottom portion of plants) on frequency of purple-stained soybean seeds in 1971

Soybean cultivar and position	Frequency of purple-stained seed (%)[a]					
	Soybean growth stage at time of inoculation[b]					
	R2	R3	R5	R6	R8	Noninoculated
Amsoy						
top	63.5	78.8	74.2*	47.8*	24.5	22.7
bottom	79.0*	83.0	62.0	29.0	18.0	20.0
Wayne						
top	29.8	25.5	25.5	14.0	7.0	5.8
bottom	33.5	23.8	22.0	8.2	6.5	3.8
Cutler						
top	35.0*	30.8	32.2*	25.0*	7.8	6.2
bottom	26.5	24.0	19.8	15.0	7.2	7.0
P.I. 80837						
top	1.2	3.0	1.2	1.5	0.2	0.2
bottom	0.5	3.0	2.5	0.5	0.2	0.8

[a]Based on four replications, 50 seed per replicate; values followed by an asterisk (*) within each top vs. bottom comparison for individual soybean entries are significantly different ($P = 0.05$).

[b]Soybean plants inoculated with *Cercospora kikuchii* at growth stages R2 (full bloom) to R8 (pods brown, harvest maturity).

More than half of the differences in these soybean entries were statistically significant. Soybean entries differed in the amount of purple-stained seed harvested from stems. Cutler had as much as 61% more stained seed in the stem region than branches, whereas Amsoy had only 31% more and Wayne only 45% more in the stem region than branches. Differences in the incidence of purple-stained seed among top branches, top stems, bottom branches, and bottom stems from P.I. 80837 were low (≤3%) and not significantly different.

Soybean plants inoculated with *C. kikuchii* matured earlier than noninoculated plants both years. However, Amsoy which had the highest incidence of seed infection, matured only 2 days earlier than control plants when inoculated in stage R2, whereas the other soybean entries matured 1 week earlier when inoculated in the same stage.

Soybean entries inoculated in growth stage R2, with few exceptions, produced smaller seed than control plants on all planting dates. The reduction in seed size was similar in both years for all planting dates and entries. When the susceptible entries were inoculated in growth stages later than R3, seed size did not decrease in either year. The seed size of P.I. 80837 decreased only when inoculated in growth stage R2. Total seed yield was not affected by inoculation with *C. kikuchii* as much as seed size. However, in 1971, Amsoy, the most susceptible soybean entry, yielded less than the control when inoculated in stages R2 to R8.

The germinability of seed was not reduced by *C. kikuchii*. In fact, in 1971, seed of the two soybean entries most susceptible to the fungus (Amsoy and Wayne) had the higher germination percentage (inoculated vs. controls). Seed from Amsoy plants inoculated in growth stages R2, R3, R5, and R6 had a higher germination than seed of control plants. When inoculated in growth stages R2 and R3, Wayne also produced seeds having a higher germination than controls.

Data on the incidence of imperfect seed coats in plants inoculated with *C. kikuchii* were variable and inconclusive. However, many purple-stained seed had fissured seed coats. The degree of this damage appeared to vary within and among soybean entries. Susceptible cultivars like Amsoy and Wayne had a high incidence of imperfect seed coats.

None of the seeds harvested from greenhouse-grown Amsoy and Wayne plants, in which individual flowers had been inoculated with *C. kikuchii*, had visible purple discoloration. The fungus was not isolated from any of the seeds plated on PDA. Sectioned and stained flowers of Amsoy that had been inoculated in the field with *C. kikuchii* did not contain hyphae of the fungus. That hyphae were not found in flower tissue does not rule out the possibility that some flowers were infected. Flower tissues had a great affinity for safranin stain, which made detection of hyphae difficult.

TABLE 3. Influence of soybean plant part (stems vs. branches) on frequency of purple-stained seed in 1971

Soybean cultivar and plant part	Frequency of purple-stained seed (%)[a]					
	Soybean growth stage at time of inoculation[b]					
	R2	R3	R5	R6	R8	Noninoculated
Amsoy						
branches	68.5	78.2	63.5	32.8	19.8	18.4
stems	74.0	83.5	72.8*	44.0*	23.0	24.2
Wayne						
branches	26.8	20.0	23.0	7.5	5.0	3.5
stems	36.5*	29.2*	24.5	14.8	8.5	6.0
Cutler						
branches	26.0	22.0	21.8	15.8	7.8	6.5
stems	35.5*	32.5*	30.8*	24.8*	7.2	6.8
P.I. 80837						
branches	1.0	2.5	2.2	2.0	0.2	1.0
stems	0.8	3.5	1.5	0.5	0.2	0.0

[a]Based on four replications, 50 seed per replicate; values followed by an asterisk (*) within each top vs. bottom comparison for individual soybean entries are significantly different ($P = 0.05$).

[b]Soybean plants inoculated with *Cercospora kikuchii* at growth stages R2 (full bloom) to R8 (pods brown, harvest maturity).

DISCUSSION

Statistical analysis of two different sampling methods indicated that infection of seed by *C. kikuchii* was not influenced by location of plants within a row. Since plants at the row ends and plants within the row had similar amounts of purple-stained and total infected seed, the more vigorous plants, found on row ends (17), seem equally susceptible to infection by *C. kikuchii*.

Investigators (1, 6) have found that the flowering period of soybeans is contracted by delayed planting. The decrease in length of the flowering periods of entries, imposed by delayed planting in the present study, did not result in increases or decreases in the incidence of *C. kikuchii* seed infection (Fig. 1). Thus, the length of the flowering period of soybeans was not associated with the natural incidence of *C. kikuchii* seed infection. In fact, flowers may not be the main infection court of *C. kikuchii*. More evidence also suggests that flowers are not the main infection court: (i) Kilpatrick (12) failed to isolate *C. kikuchii* from unopened flowers, opened flowers, flowers opened 1 day, and those opened 2 days; (ii) in the present study, no seeds became infected when individual soybean flowers were inoculated with *C. kikuchii* and incubated up to 6 days in a moist chamber; and (iii) we did not observe the fungus in flowers inoculated with *C. kikuchii* in the field.

Since different times of inoculation during the growing season influenced total seed infection (Fig. 1), relative humidities and temperatures during these growing seasons were reviewed. The weather at the time of inoculation and several days afterward was considered most important. This period appears to be critical, since Jones' pod inoculation studies (11) suggest that once *C. kikuchii* enters pods, the influence of environment on infection of seed is limited. Review of the trends in weather data (7-day averages for temperature, relative humidity, and rainfall) as summarized by the Purdue University Department of Agronomy did not reveal any association between the incidence of total seed infection and relative humidities or temperatures during the growing season of either year. However, we feel that temperature at the time of inoculation was an influencing factor as it could affect survival and penetration of *C. kikuchii*.

The purple discoloration associated with *C. kikuchii*-infected seed is expressed only as plants approach maturity. The environment during this period also may be more critical for expression than earlier periods in disease development. Expression of purple stain also may be related to water loss in seed (2), which does coincide with the approximate time of expression of purple stain in infected seed.

This study confirms the earlier assumption by Murakishi (16) that *C. kikuchii* infects developing pods, but wounding was not required for infection. Results of pod inoculations also suggest that pod age, as well as environment, influences infection of seed by *C. kikuchii*. The following suggest this: (i) infection of seed decreases as plants are inoculated at successively later growth stages, (ii) seed are not infected when mature plants are inoculated, and (iii) more purple-stained seed occur in the top half of some soybean plants, where the youngest pods are located.

In 1971, the majority of soybean seed infected with *C. kikuchii* was from pods growing on the main stems. More favorable moisture conditions within the canopy of soybean plants may be a factor as reported by Mantanola (15) studying *Cercospora* infections of pepper plants.

Reduced seed germination and increased frequency of fissured or imperfect seed coats are reported as influenced by planting date (3, 6), but in our tests damage to soybean seed was primarily due to microorganisms. Most reports indicate that *C. kikuchii* does not reduce germination of soybeans appreciably, whereas *Diaporthe* spp. are associated with major reductions in seed germination (14, 19). In the present study the effects of *C. kikuchii* on seed germination was difficult to interpret because of the relationship between *C. kikuchii* and *Diaporthe* spp. Plants inoculated with *C. kikuchii* produced seed with a much lower incidence of *Diaporthe* spp. than seed from noninoculated plants. The data involving frequencies of the two suggested an antagonistic interaction. Both *C. kikuchii* (5, 8, 9) and *Diaporthe* spp. (14, 19) are associated with increased frequency of fissures in seed coats. Again, the interaction made it difficult to interpret the effects of *C. kikuchii* inoculations on seed coat damage and further identifies that the interaction between *C. kikuchii* and *Diaporthe* spp. must be considered in interpreting the effects of *C. kikuchii* on soybeans.

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