Soybean Seed Decay: Sources of Inoculum and Nature of Infection

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ABSTRACT

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Phomopsis-type pycnidia and alpha spores (fertile spores of the imperfect stage) were abundant on overwintered soybean straw and current-season plant debris. Of alpha spores placed on acidified potato-dextrose agar only 8% produced colonies of *Diaporthe phaseolorum* var. *sojae*, whereas 92% were of an undescribed *Phomopsis* sp. In contrast, perithecia of *Diaporthe* were found infrequently and only on overwintered debris; 13% of these produced *Diaporthe phaseolorum* var. *caulivora* colonies and 87% produced *D. phaseolorum* var. *sojae* colonies. The only secondary inocula detected were alpha spores on current-season debris. Maximum production of spores occurred during the pod-filling period. Alpha spores were detected on the surfaces of immature symptomless soybeans, primarily on the lower one-third of the plants, and were recovered from plants up to 2 m from an inoculum source. *Phomopsis* sp. and *D. phaseolorum* var. *sojae* mycelia introduced into soybean plants by a toothpick method caused local, latent infection of green cotyledons, hypocotyls, stems, petioles, and pods in both field and growth chamber tests. The pathogens spread in senescing and dead plants under moist, humid conditions.

Phomopsis sp., *Diaporthe phaseolorum* (Cke. & Ell.) var. *sojae* (Lehman), and *D. phaseolorum* var. *caulivora* (Athow and Caldwell) can be isolated from symptomatic and asymptomatic immature and mature soybean plants and seed (9,10). However, neither the widespread occurrence nor nature of infection of soybean by *Phomopsis* and *Diaporthe* is understood. Major sources of *Phomopsis* sp. and *Diaporthe* spp. inoculum probably are soybean straw and debris (1,6,11).

Infection of soybean plants by *Phomopsis* sp. and *Diaporthe* spp. has been postulated to be either systemic or local and latent (2,8,9). Seed infection is associated with deterioration of the pod wall or movement of the pathogens through abrasions, cracks, or other injuries to the pod (2).

In this study we examined sources of inocula, duration of spore production, and modes of pathogen spread in soybean plant populations. Also, we examined the possibility of both systemic and local latent infection associated with the soybean seed decay.

MATERIALS AND METHODS

Monitoring sporulation in the field. Inoculum of *Phomopsis* sp. and *Diaporthe* spp. on overwintered soybean straw was monitored in 1974 and 1975 in an experimental field that had been cropped to soybean for several seasons. Sources of straw in 1974 were the cultivars Amsoy 71, Wayne, or Calland and in 1975, only Wayne. Straw was sampled weekly beginning 10 May 1974 and late February 1975 and continued through September in both years. Each sampling consisted of 25 pieces that ranged 5–20 cm long. Each section was examined with a dissecting microscope and structures resembling pycnidia or perithecia were removed and further examined with a light microscope to detect the presence of alpha spores (fertile spores of the imperfect stage) (11) or ascospores.

Secondary inoculum production was studied in fallen cotyledons and petioles in 1974 and in petioles only in 1975. In 1974, a total of 200 cotyledons and petioles from the first trifoliolate leaves (50 from each of four replications) were tagged in the field. After abscission, half of them were placed in a moist chamber and observed for sporocarp formation up to 1 mo after incubation. The other half was left in the field for 1 wk and the percentage with sporocarps was recorded. At plant maturity, the percentage of fallen petioles bearing pycnidia or perithecia was determined. Beginning in July and continuing through September, 1975, 25 abscised petioles from each of 10 fields were sampled weekly and examined for pycnidia or perithecia and mature spores.

Dispersal of inoculum. To study the movement of *Phomopsis* sp. inoculum from debris to adjacent plants, petioles laden with pycnidia were placed at the bases of 14-day-old Beeson soybean seedlings in Wooster soil mix (Wooster silt loam, muck, and Canadian peat, [5:5:2, v/v]) in 50 × 30 × 7-cm-deep wood boxes (12 plants per box). Boxes were placed in growth chambers at 22–24 C day (14 hr), 16–18 C night (10 hr), and 50–60% relative humidity (RH). Plants were watered as follows: (i) overhead sprinkling for 2 min, three times a day until maturity; (ii) treated as (i) for 2 wk, then soil surface was watered as needed until maturity; (iii) treated as (ii) with debris removed after 2 wk; (iv) continuous surface watering as needed; (v) as (iv) but also four rainstorms simulated by sprinkling plants from height of 12 m for 3 min, one simulated storm every 2 wk until maturity; and (vi) no debris, surface watering as needed. Plant stems were sampled every 2 wk through maturity and sections from the bottom to the top of plant were plated on acidified potato-dextrose agar (APDA) as described previously (10).

For studying movement of inoculum in a plant population, soybean straw with pycnidia and dark, blotched areas was collected in 1973 and stored at 3 C until the following season. The straw was placed on the soil surface in the field after Wayne seed had been planted in an area previously cropped to corn. The straw was spread over 1 m of the center of the middle row in each of four three-row plantings. Three plants per replication were sampled at seven selected growth stages (5) from each of the following sites: site 1, the straw site; site 2, adjacent rows opposite site 1; site 3, 1 to 2 m from site 1 in each direction in the inoculated row; site 4, same as site 3, except 4 to 5 m from site 1; site 5, in adjacent rows opposite site 4; and site 6, 20 m from nearest straw plot to serve as control. Stem sections from the lower, middle, and upper parts of the sampled plants were plated on APDA.

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Monitoring inoculum on plant surfaces. Distribution of alpha spores on plants of Amsoy 71 growing in fields previously cropped to soybeans was examined in 1974. Twelve plants were sampled 14 times during the season. Stem sections were removed from the bottom, middle, and top of each plant at each sampling time. In addition, cotyledons were sampled once and flowers and pods from the three locations were sampled separately from the stem sections. Each part was placed in 10 ml of water plus Tween-20 in 250-ml flasks at the sample site. Flasks were shaken for 15 min at 150 rpm on a rotary shaker. The suspension was filtered through 5.0 μm Nuclepore, clear, plain 47 mm diameter filter (Nuclepore, Pleasanton, CA 94566) and the filtrate was passed through 0.22 μm Millipore white, plain 25 mm diameter filter (Millipore Corporation, Bedford, MA 01730). The Millipore filter was stained with lacto-phenol cotton blue and alpha spores or ascospores were observed with a light microscope.

Spread of *Phomopsis* spp. *D. phaseolorum* var. *sojae*, and *D. phaseolorum* var. *caulivora* in soybean plants. Crall's toothpick tip method (4) was used to inoculate stems and pods with *Phomopsis* sp. and *Diaporthe* spp. mycelia in the field and cotyledons and hypocotyls in the growth chamber. In the field test, pods and stems of plants in the following growth stages (5) were separately inoculated with each of nine isolates of *Phomopsis,* six of *D. phaseolorum* var.*sojae*, and four of *P. phaseolorum* var. *caulivora*: 25 pods each at early pod (R3) (5), green bean (R5), and yellow pod (R7) stages, and 10 stems of 78- to 85-day-old plants of cultivar Wayne in fields previously cropped to corn. The three fungi were distinguished by characteristics described previously (11). Isolations for detection of these pathogens were made 1 wk after inoculation and at maturity from: (i) the inoculation point on stems and pods, (ii) 10 cm above and below the inoculation point on stems, and (iii) the base and tip of the inoculated pods. In the growth chamber test, two groups of six sets of potted 10-day- to 2-wk-old and 3-wk-old Calland soybean plants in Wooster soil mix were inoculated in the hypocotyl or cotyledon with a single isolate of *Phomopsis* sp. Half of these plants were incubated at 50% RH and the other half at 100% RH. Temperatures were 22–24 C during the day (14 hr) and 16–18 C at night (10 hr). After 1–2 wk following inoculation, tissues from the inoculation point and 10 cm above and below were plated on APDA.

In other tests, spore suspensions were used as inocula in studying spread of *Phomopsis* sp. and *D. phaseolorum* var. *sojae* in plants. Suspensions of alpha spores of each isolate were prepared by flooding 14- to 21-day-old cultures and diluted to approximately 5,000 spores per milliliter of water. Suspensions were atomized onto flowers or pods of 25 Calland soybean plants in the field at seven reproductive stages. Pods, taken from above and below the inoculation sites, were plated after 2 wk. In growth chamber experiments, Corsoy and Beeson soybeans were grown in Wooster soil mix at the temperature, light, and RH conditions described above. Five plants (three replications) at the fourth trifoliolate and yellow pod stages were inoculated separately by spraying spores on petioles, stem internodes, and pods. On the same plants, we inoculated pods in each of three class sizes: shorter than 2.5 cm, 2.5–3.8 cm long, and longer than 3.8 cm. Corsoy plants were harvested at 130 days and Beeson plants at 123 days and numbers of pycnidia and blotches on stems and pods were recorded. Location of *Phomopsis* sp. and *D. phaseolorum* var. *sojae* in moribund tissue was determined by plating stem sections adjoining the pod node and the base and tip of inoculated pods.

RESULTS

Soybean straw and debris as sources of inoculum. On overwintered soybean straw, primary inoculum of *Phomopsis* sp. was more prevalent than that of *Diaporthe* sp. Immature pycnidia were found in straw sampled from March to May 1974 and February to May 1975. By the first week in June in both years, 80% of pycnidia examined contained alpha spores (Table 1). The percentage of pycnidia with alpha spores on overwintered straw declined during the summer. During this 2-yr period, 98% of 200 randomly sampled pycnidia formed *Phomopsis* sp. colonies on APDA, and 2% formed *D. phaseolorum* var. *sojae* colonies.

Perithecia formed in moist chambers on 80% of overwintered straw examined in the spring of 1974 and 1975. On APDA, 87% of 200 perithecia formed *D. phaseolorum* var. *sojae* and 13% *D. phaseolorum* var. *caulivora*. Perithecia were not found in the field in 1974, but in 1975 they were observed during the first and second weeks of June in 13% of the samples of which 10% contained mature ascospores.

Secondary inoculum of *Phomopsis* sp. first was observed on fallen cotyledons and subsequently was found on petiole debris approximately 1 wk after leaf abscission. Phomopsis-type pycnidia also formed on yellow petioles that had been removed from plants and placed in moist chambers. In a survey of debris at plant maturity in 1974, >90% of fallen petioles bore pycnidia. Although pycnidia were most noticeable on abscised petioles they also were found on other parts of dead soybean plants. Stem remnants of soybeans damaged by a hailstorm in 1974 showed pycnidia 3 wk after the storm. Pycnidia were not found, however, on fallen soybean leaves or old corn stubble.

Pycnidia on abscised petioles in the field produced alpha spores abundantly throughout July, August, and the first week of September but with the onset of cool weather, few of the pycnidia on fresh debris contained spores (Table 1).

Phomopsis sp. was isolated more frequently than *D. phaseolorum* var. *sojae* from pycnidia on soybean debris. Of 1,020 pycnidia, 92% formed *Phomopsis* sp. colonies on APDA and the remaining 8%, *D. phaseolorum* var. *sojae*. Perithecia were not observed on petiole debris during sampling in 1974 or 1975.

Spread of inoculum in the field and growth chambers. Dissemination of *Phomopsis* sp. from point sources of naturally-infected straw was monitored in a field with no overwintered straw. *Phomopsis* sp. was isolated at the first trifoliolate stage (V2) from plants emerging through Phomopsis-infested straw. By the time cotyledons dropped, the pathogen was isolated from 20% of plants directly across from, and 2 m in any direction from, the inoculum source. *Phomopsis* sp. was not recovered from control plants, 30 m from the nearest source of inoculum, or from plants more than 2 m from the straw in the experimental area. The experiment was terminated at the yellow pod stage (R7) when infections caused by inoculum from other sources was detected in control plants. The importance of rainfall for the movement of inoculum from debris to adjacent plants was investigated in the growth chamber. Rainfall was simulated by: overhead sprinkling for 2 wk followed by drip watering, continuous overhead sprinkling, or watering plants from a height of 12 m. Drip irrigation was used as the control. In the three sprinkling experiments, *Phomopsis* sp. was recovered from 42, 48, and 40% of the plants, respectively. The fungus was isolated from stem sections from the lower two-thirds of plants only and never from flowers, pods, or seeds. *Phomopsis* sp. was not recovered from the drip-water controls. Apparently, splashing of inoculum onto plants was necessary for dispersal.

A study was made on the distribution of alpha spores on soybean plants throughout the season. Alpha spores were detected on the

TABLE 1. *Phomopsis* sp. pycnidia with alpha spores on soybean straw and debris during and after 1974 soybean growing season

Sampling date	Sections with pycnidia containing alpha spores (%)	
	Straw	Debris
3 June	80[a]	...
12 June	78	...
30 June	75	...
22 July	52	89
21 August	27	61
12 September	21	81
29 September	32	21
31 October	...	0

[a]Mean of 25 straw samples (mostly stem sections from previous year) or 25 debris samples (petioles from current crop) from each of 10 fields.

surface of plants sampled under wet and dry conditions, and there appeared to be no relationship between amount of precipitation 72 hr prior to sampling and the percentage of plants with spores (Table 2). Spores were found mostly in washings from lower stem sections but also on at least one occasion in washings from flowers, pods, and petioles. In several cases, however, spores were found only in washings from the mid and top sections. In most cases, *Phomopsis* sp. was isolated in the same plant section from which alpha spores were washed, indicating that other spores had infected the stem earlier. Frequently, spores from unidentified *Septoria* and *Fusarium* spp. were observed at all sampling dates.

***Phomopsis* and *Diaporthe* spread in immature plants.** In a field test, *Phomopsis* sp. and *D. phaseolorum* var. *sojae* introduced on mycelium-covered toothpick tips caused local infection only in immature stems. Girdling stem cankers caused by one of nine isolates of *Phomopsis* sp. and one of six isolates of *D. phaseolorum* var. *sojae* killed 10% of 78- to 85-day-old Wayne soybean plants. The fungi were detected at the inoculation site in immature tissues, but the pathogens were isolated in senescent tissue away from the point of introduction as detected by plating plant sections on APDA. The fungus was not recovered from pods or petioles attached near stem inoculation sites. All three isolates of *D. phaseolorum* var. *caulivora* caused stem canker and killed an average of 70% of inoculated plants. In a growth chamber inoculation test, *Phomopsis* sp. did not spread from cotyledons into the main stem axis at 50 or 100% RH. Likewise, hypocotyl infection by *Phomopsis* sp. was local in 1- and 2-wk-old plants at 50% RH, but the fungus was isolated from the cotyledonary node in all plants placed in 100% RH and in 3-wk-old plants in 50% RH. It was concluded that *Phomopsis* sp. caused local, latent infections in immature stems.

Phomopsis sp., *D. phaseolorum* var. *sojae*, and *D. phaseolorum* var. *caulivora* infected an average of 85, 70, and 85%, respectively, of green pods inoculated with infested toothpick tips in the field. Inoculations with *Phomopsis* sp. resulted in more moldy seed at maturity than did either of the *Diaporthe* spp. In field studies involving plants inoculated with spore suspensions, *Phomopsis* sp. was isolated from 10% of the mature pods that developed from inoculated flowers and from 20% of mature pods that developed from inoculated half-size green pods. *Phomopsis* sp. was detected 2 wk after inoculation in these studies. No progressive symptoms were observed on immature pods inoculated with mycelia (toothpick tips) or spores. However, a few mature pods with pycnidia were noted from all inoculations.

In growth chamber tests, *Phomopsis* sp. was recovered from approximately 20% of mature Beeson or Corsoy pods that had been inoculated at the half-size green, full-size green, or yellow pod stages. *Phomopsis* sp. and *D. phaseolorum* var. *sojae* were detected more often in mature pods than in pods 1 wk after inoculation in all pod maturity inoculation groups. Also, the fungi frequently were found at maturity in stems adjacent to the pod inoculation site (Table 3). No lesions developed on any of the inoculated pods. *Phomopsis* sp. was isolated from less than 5% of mature seeds when flowers, green pods (all sizes), or yellow pods were inoculated. *Diaporthe phaseolorum* var. *sojae* was isolated from 5% of mature seed from pods inoculated only at the green bean stage. Both *Phomopsis* sp. and *D. phaseolorum* var. *sojae* pod inoculations resulted in some prematurely ripened stems with blotching and linear rows of pycnidia (Table 3). Pods showed pycnidia or blotching in only a few cases.

TABLE 2. Incidence of Phomopsis-type spores on Wayne soybean stems and pods in the field[a]

Growth stage[b]	Precipitation in previous 72 hr (cm)	Stems and pods with Phomopsis-type spores disseminated from straw and debris (%)
V0	0.0	14
V1	1.1	8
V2	0.0	0
V3	0.3	0
V4	0.2	0
V5	3.8	0
R1	0.0	3
R2	0.0	11
R4	0.7	0
R5	2.3	94
R6	0.8	0
R7	2.3	5
R8	2.3	31

[a] Alpha spores were detected on Nuclepore membrane filters used to filter a water plus Tween-20 suspension containing surface material removed from plant sections by shaking.

[b] Growth stages based on key by Fehr et al (5) in which V2 = first trifoliolate, R2 = full bloom, R5 = green bean, and R8 = maturity.

DISCUSSION

Phomopsis sp. and *D. phaseolorum* var. *sojae* both cause local, latent infections of immature soybean tissue and can colonize mature and senescent plants. Consistent occurrence of local infections was shown in inoculation studies in which the fungi were reisolated from inoculation sites on cotyledons, hypocotyls, stems, and pods of immature plants 1 wk after inoculation. In most cases, *Phomopsis* sp. and *D. phaseolorum* var. *sojae* did not spread from the inoculation point in immature plants or produce visible disease symptoms. Because the method for surface disinfection used during isolation should have eliminated all surface organisms, we concluded that the fungi were established beneath the epidermis in the cortex as local, latent pathogens in stems and pods. In growth chamber inoculations, premature ripening and production of blotching and pycnidia on stems were evidence that these fungi were active in senescent plants. In contrast, our isolates of *D. phaseolorum* var. *caulivora* caused local cankers and occasionally killed plants when introduced into stems but failed to cause disease symptoms when introduced into other plant parts. The fact that *Phomopsis* sp. and *Diaporthe* spp. were not detected in pods attached to inoculated stems was evidence that those fungi did not move in immature plants. Previous research has shown a similar frequency of independent recovery of *Phomopsis* sp. from tip and base of pods (10). Therefore, it appears that pod infections occur independent of stem infections. The time, conditions, and manner of movement of *Phomopsis* sp. and *Diaporthe* spp. in immature, senescent, and mature pods needs to be investigated. Development of pycnidia on detached green pods suggests rapid colonization of

TABLE 3. Frequency of isolation of *Phomopsis* sp. and *Diaporthe phaseolorum* var. *sojae* at plant maturity in inoculated Beeson soybean pods, petioles and stems, and pods adjacent to inoculation sites, and percent inoculated plants with pod and stem blight symptoms

Inoculum	Plant part inoculated	Growth stage[a]	Isolations from[b] Petiole (%)	Stem[c] (%)	Pod (%)	Plant part showing symptoms[d]
Phomopsis sp.	Petiole	R7	50	8	8[e]	16
	Pod	R3	...	43	17	43
	Pod	R5	...	43	17	43
	Pod	R7	...	8	42	8
D. phaseolorum var. *sojae*	Petiole	R7	0	8	8[e]	0
	Pod	R3	...	8	0	8
	Pod	R5	...	8	25	8
	Pod	R7	...	17	0	17

[a] Based on Fehr et al (5).

[b] Mean percent of 10 parts in each of three replications.

[c] Stem section adjacent to pod or petiole inoculated.

[d] Mean percent of 10 plants in three replications showing at least one of the following symptoms or signs: pycnidia alone or in linear rows; dark blotching; or both pycnidia and blotching.

[e] Pod adjacent to petiole inoculated.

host tissue under certain conditions. Lehman (11) reported that *Phomopsis* sp. produced pycnidia when spores were sprayed on detached pods and incubated in a moist chamber for 9–12 days. In our field or controlled environment studies, *Phomopsis* sp. and *Diaporthe* spp. did not produce pycnidia until pods were mature. Detachment of pods undoubtedly induces senescence which would account for the rapid development of pycnidia in Lehman's tests (11).

Movement of *Phomopsis* from pod to seed and the subsequent seed decay may be enhanced by excessive rain, and high temperatures and relative humidity. During studies of soybean pod and stem blight, Lehman (11) observed that plant infection and the dissemination of *D. phaseolorum* var. *sojae* inoculum were strongly dependent on high humidity and that subsequent seed infection was markedly more abundant during rainy summers. Wilcox et al (14) suggested that high temperature and humidity in September and early October probably favor the development of *D. phaseolorum* var. *sojae* and other fungi in seed. In our field tests, seed from pods at the top of the plants sprayed with alpha spores were infected with *Phomopsis* sp. or *D. phaseolorum* var. *sojae* at maturity but control pods inoculated with water were not. Rainfall was 0.6 cm above normal during the 7 days following inoculations, average temperature was 26 C, and RH was 80%. In growth chamber tests where the relative humidity was lower (approximately 40%) similar pod inoculations did not result in seed infection at maturity. Therefore, our results indicate that optimum conditions for seed infection are high levels of rain, temperature, and relative humidity. Further studies should be performed to measure the effects of excessive moisture and high humidity and temperature on the movement of *Phomopsis* sp. from pod to seed.

The incidence of *Phomopsis* sp. in soybean seed appears to be dependent on a high inoculum level in the field. *Phomopsis* sp. is ideally adapted for proliferation and survival on soybean debris. This adaptation begins with the widespread association of the fungus with immature soybean plants indicating that it is one of the "pioneer colonists" (3) of soybeans in Ohio. Colonization of plant parts prior to death of the tissue favors pathogen survival because it minimizes competition from saprophytic fungi. In contrast, survival of *Diaporthe* spp. in soybean debris is less apparent. *Phomopsis* sp. survives overwinter as pycnidia which are much more prevalent on soybean straw than perithecia or pycnidia of *D. phaseolorum* var. *sojae*.

The increase in percent of seed infection by *Phomopsis* sp. and *Diaporthe* spp. with time (10,14) could result either from late infections or failure to detect seed surface infections occurring either at maturity or shortly thereafter. Ilyas et al (7) reported *D. phaseolorum* var. *sojae* was found most frequently in the hourglass, less so in the parenchyma, and least in the palisade layers of seed coats. The surface sterilization techniques we used may have killed mycelia in the outer layers of the seed coat, but not those in the inner seed coat or cotyledonary tissues. However, previous reports have indicated that late infection is common (2,11,14). The source of inoculum for late infection could be mycelia of latent infections in dead stems and pods colonizing seed. A second source is probably pycnidia of *Phomopsis* sp. on current crop debris or perithecia of *Diaporthe* spp. left on previous season's straw. The importance of the latter source can only be determined through studies on sporocarp maturation as has been done for *Calonectria crotalariae* (12) and *Gibberella zeae* (13).

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