Development of an Inoculation Technique and the Evaluation of Soybean Genotypes for Resistance to *Coniothyrium glycines*

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Abstract

Red leaf blotch, caused by *Coniothyrium glycines*, is a serious disease affecting soybean in several African countries but has not yet appeared in the United States. The fungal pathogen is listed as a Select Agent by the Federal Select Agent Program because of potential consequence to U.S. agriculture if introduced. Sources of resistance to red leaf blotch have not yet been identified in spite of extensive field testing in Zambia and Zimbabwe. A seedling inoculation protocol was used to investigate the effects of inoculum density, temperature (20 and 25°C), and dew chamber incubation period (2 to 5 days) on disease development in ‘Williams 82’ soybean. Inoculum

density and incubation period had significant effects on disease severity but no significant difference was observed at 20 and 25°C. Highest levels of disease were obtained using 1.94 cm³ of mycelial inoculum per 10 ml of water and incubating plants for 5 days at 100% relative humidity. Significant differences were not observed between isolates from Zambia and Zimbabwe at 25°C. In total, 23 soybean genotypes that represent nearly 90% of the genes present in U.S. soybean were evaluated and found to be susceptible, which is consistent with previous field evaluations. This method could be useful in identifying new sources of resistance to red leaf blotch.

Red leaf blotch of soybean is a serious disease that causes blotching and defoliation of soybean (*Glycine max* (L.) Merr.) and a wild legume, *Neonotonia wightii*. The cause of the disease was first reported as *Pyrenochaeta glycines* (Stewart 1957) in Ethiopia. Since then, it has been reported in several Eastern, Central, and Southern African countries (Akem et al. 1992; Hartman et al. 1987; Levy et al. 1990). In 2002, the pathogen was renamed *Phoma glycinicola* (de Gruyter and Boerema 2002) because conidiogenesis and hyaline conidia were phoma-like. However, the most current reexamination of the species (de Gruyter et al. 2013) determined that classification in the genus *Coniothyrium* was justified, because it was noted by Stewart (1957) that the conidia were greenish-yellow in mass, resembling coniothyrium-like conidia.

Sinclair (1989) observed that, even though the disease had been restricted to several African countries, he believed it had the potential to become a major foliar soybean disease if the pathogen were to spread to other parts of the world. Red leaf blotch has been deemed a potentially devastating disease for soybean production (Hartman et al. 2011). For these reasons, *Coniothyrium glycines* is currently listed as one of the United States Department of Agriculture (USDA) Plant Protection and Quarantine Select Agents and Toxins (Federal Select Agent Program 2014; Hartman et al. 2009).

Red leaf blotch is known to cause serious soybean losses in the field (Datnoff et al. 1987; Hartman et al. 1987; Levy 1987), and it was observed (Sinclair 1989) that all commercial U.S. cultivars tested in Zambia and Zimbabwe between 1982 and 1984 were susceptible. No resistance was noted among local lines or about 5,500 exotic soybean lines tested (Levy et al. 1990). All cultivars and breeding lines tested thus far have been tested in the field in regions where the disease is endemic, requiring resources including land and the presence of natural inoculum sources. A suitable seedling inoculation method for red leaf blotch of soybean would be highly

desirable for defining conditions that allow optimal infection and, ultimately, for testing additional germplasm for susceptibility. Such a method would require less space and could be performed far more efficiently than field studies that rely on the occurrence of natural inoculum.

The objective of this study was to develop an inoculation protocol for red leaf blotch based on assessments of temperature (20 versus 25°C), inoculum density, and incubation period at 100% relative humidity using ‘Williams 82’ soybean. The two temperatures were compared because both have been used by other workers (Hartman and Sinclair 1992; Levy 1987). The optimal method was further used to evaluate 23 ancestral U.S. cultivars and breeding lines for resistance to *C. glycines*.

Materials and Methods

Isolates and plant material. Isolates of *C. glycines* used included IMI 294986 (= *P. glycinicola* Gruyter & Boerema) (de Gruyter and Boerema 2002) (isolated from *G. max* at Mt. Makulu, Zambia in 1985), Pg-1 (isolated from *G. max* collected in Harare, Zimbabwe in 2001), Pg-21 (isolated from *G. max* collected in Harare, Zimbabwe in 2005), Pg-31 (isolated from *G. max* collected at Chisamba, Zambia in 2005), and Pg-41 (isolated from *G. max* collected at Mpongwe, Zambia in 2005). Isolations were made onto 2% water agar from field material obtained from colleagues in Zambia and Zimbabwe. *C. glycines* cultures were maintained on 20% V8-juice agar at 20°C in darkness. Ancestral soybean lines were obtained from Randy Nelson, USDA soybean germplasm collection, Urbana, IL. The selected lines account, by pedigree, for nearly 90% of the genes present in modern public soybean cultivars.

Inoculum production and plant inoculations. Soybean plants were grown in 15.2-cm-diameter plastic pots filled with Sunshine LC1 mix (Sun Grow Horticultural Products, Bellevue, WA) and fertilized as needed with 14-14-14 Osmocote (Scotts-Sierra Horticultural Products Co., Marysville, OH). To produce inoculum, a square (1.94 cm³, except for inoculum density experiments) cut from a 10-day-old 20% V8-juice agar culture of *C. glycines* containing mycelium as well as numerous sclerotia was ground in a Tenbroeck-type glass tissue grinder (Kimble/Kontes, Vineland, NJ) with 10 ml of sterile water until all lumps were removed. The ground mycelial suspension (1 ml/plate) was spread onto plates of 20% V8-juice agar containing 15 ml of medium per 9-cm-diameter disposable plastic Petri dish. The 15-ml volume produced a thin (3 mm deep) agar surface in the Petri dishes, and the inoculated dishes were incubated at 20°C for 5 days in darkness. Then, various sized squares or rectangles of agar containing mycelium and small, numerous young sclerotia were cut

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out from the thin plates and ground in a glass tissue grinder with 10 ml of sterile water per the indicated volume of inoculum, until no lumps remained. Soybean plants (approximately 2 weeks old) were sprayed to runoff with the homogenized inoculum using a 480-ml-capacity industrial spray bottle (Lab Safety Supply, Janesville, WI) with the tube extended into a 50-ml disposable conical centrifuge tube containing the inoculum suspension. Williams 82 was used as a susceptible control.

Dew chamber time of incubation. Three replicates of an experiment were performed to determine the number of days of postinoculation dew chamber incubation for Williams 82 soybean to exhibit the most pronounced degree of symptom development following inoculation with *C. glycines*. In each replicate experiment, single trifoliate (consisting of three leaflets) on one plant per 15.2-cm-diameter plastic pot were inoculated as described above using *C. glycines* isolate IMI 294986 at a density of 1.94 cm³ of agar inoculum per 10 ml of water. Inoculated plants were placed in a dew chamber at 25°C in a completely random design and three plants (containing a total of nine inoculated leaflets) were removed after periods of 2, 3, 4, or 5 days. Following more than 5 days of incubation, leaf yellowing and lesion coalescence began to occur, making results more difficult to assess. Due to space constraints, control plants were inoculated using agar inoculum minus the pathogen and were placed on a 25°C greenhouse bench at time zero and not subjected to dew chamber treatment. Plants removed from the dew chamber were allowed to dry, and the infected leaves were then scanned with a flatbed scanner. Leaf and lesion areas were determined using the ASSESS software program (Lamari 2002). Percent leaf area infected was calculated as (lesion area/leaf area) × 100.

Inoculum density-temperature. To produce varying inoculum density, the volume removed from the thin agar plates of *C. glycines* isolate IMI 294986 mycelium and sclerotia described above was altered to produce quantities higher and lower than that used for the days in the dew chamber experiment. The volume of agar inoculum used in the days in dew chamber experiment (1.94 cm³ of agar inoculum per 10 ml of water) was again used in these experiments (again, three replicate experiments were performed) but was also doubled to 3.87 cm³ per 10 ml of water and, in addition, was cut in half and in half again, yielding, 0.97 and 0.48 cm³ per 10 ml of water, respectively. Controls received only water. Soybean plants were grown three to a 15.2-cm-diameter plastic pot for approximately 2 weeks and, in each replicate factorial experiment, a single trifoliate on the three plants in each of four pots was inoculated with each inoculum density. Thus, nine leaflets were inoculated in each pot for each treatment, and two replicate pots were included in each of three replicate experiments. Two pots of plants inoculated with each inoculum density were placed in a 20°C dew chamber and the other two placed in a 25°C dew chamber for 5 days, after which time disease was assessed. All plants were assessed at the same number of days after inoculation. This yielded 18 leaflets/treatment/experiment and 54 leaflets/treatment total for the three replications. After 5 days of incubation in the dew chambers, infected leaves were removed and scanned on a flatbed scanner, and leaf and lesion areas were quantified using the ASSESS software package (Lamari 2002). Percent leaf area infected was calculated as (lesion area/leaf area) × 100.

Table 1. Analysis of variance for percent Williams 82 soybean leaf area infected by *Coniothyrium glycines* following incubation in a dew chamber at 100% relative humidity for 0, 2, 3, 4, and 5 days

Source	DF	P value
Experiment (Exp) ²	2	0.249
Days in dew chamber	4	0.000
Exp × days in dew chamber	8	0.322
Error	120	...
Total	134	...

² Three replicate experiments were performed in which single trifoliate (consisting of three leaflets) on one plant per pot were inoculated using *C. glycines* isolate IMI294986 at an inoculum density of 1.94 cm³ of agar inoculum per 10 ml of water. Nine leaflets were inoculated/treatment/replicate experiment.

Greenhouse inoculations. Due to space constraints in dew chambers as well as lack of lighting, which resulted in some chlorosis occurring on plants during incubation periods, it was desirable to perform inoculation studies in a greenhouse environment. Wooden lumber (35 by 19 mm) was used to construct a three-dimensional wooden framework measuring 119 cm long by 46 cm wide by 61 cm high, and this framework was covered with clear 4-mil plastic sheeting to allow light to enter. The structure was left open at the bottom so that it could be raised and lowered over potted plants on a greenhouse bench. Two layers of black plastic sheeting were placed on the greenhouse bench below the plants to allow a moisture seal when the structure was placed over the plants. At one end of the structure, a hole was made near the top to allow insertion of a 3.81-cm-diameter flexible plastic pipe. The pipe was used to conduct mist into the humidity structure generated by a Germ Guardian ultrasonic digital humidifier (model number H3010; Guardian Technologies LLC, Mentor, OH). The water reservoir on the humidifier was refilled daily to allow continuous use during experiments. Yellowing was not a problem in the greenhouse moist chamber, because plants received continual sunlight. The greenhouse assay was used to evaluate the disease reactions of Williams 82 soybean inoculated with five different isolates of *C. glycines*, and to evaluate 23 ancestral soybean lines. Each of the two types of experiments were completed three times, and were performed in a level BSL-3 containment greenhouse at 25°C on a greenhouse bench using a completely random design. For *C. glycines* isolate comparisons, inoculum of isolates IMI294986, Pg-1, Pg-21, Pg-31, and Pg-41 was produced as described above, using 1.94 cm³ of inoculum per 10 ml of water and spraying Williams 82 soybean to runoff. In each replicate experiment, three trifoliate (nine leaflets) in two pots of approximately 2-week-old Williams 82 soybean were inoculated with each isolate ($n = 18$ leaflets/isolate/experiment). To evaluate soybean lines, 10 trifoliate of each cultivar were inoculated in each of three replicate experiments ($n = 30$ leaflets/cultivar/experiment). For each cultivar, several infected leaf pieces were disinfested in 1% sodium hypochlorite for 1 to 2 min and plated onto plates of 2% water agar to confirm reisolation of *C. glycines* from the symptomatic material.

Statistical analyses. Data from the three repeated experiments were pooled and analyzed via analysis of variance using the General Linear Models procedure, as well as by linear and polynomial regression analysis in SAS (SAS Institute 2008) and Minitab (Minitab 2010).

Results

Dew chamber time of incubation. Symptoms were observed 2 days after inoculation and reached their highest level after 5 days. Mean percent leaf area infected for 0, 2, 3, 4, and 5 days in the dew chamber was 0, 0.9, 3.9, 11.6, and 17.8%, respectively. Analysis of variance (Table 1) showed that there was no significant ($P = 0.05$) experiment-to-experiment variation, nor was there a significant experiment-days in dew chamber interaction. However, highly significant differences ($P < 0.001$) were observed between days in the dew chamber. A dew chamber treatment of 5 days at 100% relative humidity resulted in the highest levels of disease (Fig. 1). The relationship between days in the dew chamber and percent leaf area infected was determined using polynomial regression analysis, and it was found that both the linear and quadratic terms were highly significant ($P < 0.01$). Thus, a quadratic equation best described the trend in the data. The best-fitting regression equation to describe the relationship was percent leaf area infected = $-0.0943 - 1.552$ (days in dew chamber) + 1.046 (days in dew chamber)², with an adjusted r^2 value of 90.2%.

Inoculum density and temperature. For the lowest inoculum level used (0.48 cm³ of inoculum per 10 ml of water), a 8.6% leaf area infected was obtained on Williams 82 soybean at 20°C and 9.5% at 25°C. Disease increased up to 18.3% leaf area infected at the inoculum concentration of 1.94 cm³ of inoculum per 10 ml of water at 20°C and 18.6% at 25°C, then decreased slightly at the highest inoculum concentration of 3.87 cm³ of inoculum per 10 ml of water (Fig. 2). Thus, the relationship between inoculum density and disease was

Fig. 1. Effect of incubation time on percent leaf area infected for Williams 82 soybean inoculated with *Coniothyrium glycines* incubated in a dew chamber at 100% relative humidity at 25°C. Bars represent standard errors.

Fig. 2. Percent leaf area infected for Williams 82 soybean as a function of temperature (20 and 25°C) and inoculum density. Inoculum density values were expressed in terms of the volume of agar inoculum (0, 0.48, 0.97, 1.94, or 3.87 cm³) per 10 ml of water applied to plants by spraying to runoff. Bars represent standard errors.

curvilinear (Fig. 2), and was found to have highly significant ($P < 0.001$) linear and quadratic components. The regression equation that best fit the 20°C data (adjusted $r^2 = 82.7\%$) was percent leaf area infected = $1.142 + 4.431$ (inoculum density) – 0.2476 (inoculum density)². For the 25°C data, the corresponding equation was percent leaf area infected = $1.321 + 4.684$ (inoculum density) – 0.2700 (inoculum density)², with an adjusted r^2 value of 83.4%. Analysis of variance was performed (Table 2) and highly significant ($P < 0.001$) effects were observed for inoculum density. Significance was not observed for the factors experiment, temperature, or the interaction of inoculum density and temperature (Table 2).

Five-isolate comparison in greenhouse at 25°C. Analysis of variance showed no significant differences ($P = 0.05$) among experiments or among *C. glycines* isolates, nor was there a significant experiment-isolate interaction. Mean percent leaf area infected on Williams 82 soybean for isolates IMI 294986, Pg-1, Pg-21, Pg-31, and Pg-41 was 19.7, 17.7, 20.1, 19.9, and 18.2%, respectively.

Reactions of soybean lines. The reactions of 23 ancestral soybean lines to *C. glycines* isolate IMI 294986 at 25°C in a humidity chamber located in a level BSL-3 containment greenhouse are shown in Table 3 (Fig. 3). Soybean cultivars tested did not differ significantly from one another in percent infected leaf area using the Tukey method and a 95% joint confidence level (Table 3).

Discussion

This study describes the use of ground mycelium and sclerotia to inoculate soybean with *C. glycines*. Previous red leaf blotch workers have performed inoculations of soybean leaves or leaf disks by using agar plugs or spraying leaf surfaces with a suspension of conidia (Datnoff et al. 1986), by applying mycelial disks to leaflets (Hartman and Sinclair 1992), or by applying droplets of a suspension made from macerating pycnidia-producing, 21-day-old culture plugs to leaflets (Levy 1987). For other sclerotia-forming fungi, Faske et al. (2006) evaluated several types of inoculum in studies of *Sclerotinia minor* on peanut, including use of germinating sclerotia on peanut stems, 3-day-old dry mycelia, perlite granules coated with mycelial fragments, and mycelial agar plug inoculum and observed that all types of inoculum resulted in similar lesion development. A mixture of sclerotia and mycelium was used successfully for inoculation of lettuce with *S. minor* (Grube and Ryder 2004), while conidia were sprayed onto plants of faba bean for infection by *Botrytis fabae* (Tivoli et al. 2006). For *S. sclerotiorum* on soybean, foliar inoculations have been performed using mycelial disks (Chun et al. 1987), mycelial squares grown on sterile paper (Nelson 1985), and by spraying ascospores and placing pieces of colonized carrot pieces onto leaf surfaces (Cline and Jacobsen 1983). The method described in this article with *C. glycines* on soybean worked consistently, was reproducible and quantifiable, and does not rely on production and collection of conidia or other spore types to generate inoculum.

The concentration of inoculum which resulted in the highest disease levels was 1.94 cm³ of ground agar inoculum (containing mycelium as well as abundant sclerotia) per 10 ml of water.

Table 2. Analysis of variance for percent Williams 82 soybean leaf area infected by *Coniothyrium glycines* as a function of temperature (20 and 25°C) and inoculum density

Source	DF	P value
Experiment ^a	2	0.504
Temperature (Temp)	1	0.076
Inoculum density	4	0.000
Temp × inoculum density	4	0.545
Error	528	...
Total	539	...

^a Eighteen leaflets were inoculated with *C. glycines* isolate IMI 294986/treatment/replicate experiment, and 54 leaflets/treatment total for the three experiments performed. Five different inoculum densities were used, including the control. After 5 days of incubation in dew chambers at 20 or 25°C, infected leaves were removed and scanned on a flatbed scanner. Percent leaf area infected was calculated as (lesion area/leaf area) × 100.

Increasing the inoculum level to 3.87 cm³ of inoculum per 10 ml of water resulted in a slight decline in disease levels compared with the 1.94 cm³ amount, perhaps due to saturation of infection sites on the soybean leaves or other inhibitory effects due to the increased volume of agar present in the liquefied inoculum.

Hartman and Sinclair (1992) performed cultural studies with *C. glycines* at both 20 and 25°C and showed that pycnidiospore germination occurred almost equally at these two temperatures. They also conducted detached-leaf inoculation studies at 25°C. Levy (1987) performed numerous cultural studies with the fungus at a temperature of 25°C. Thus, incubation temperatures (20 versus 25°C) were compared for inoculation of soybean with *C. glycines* because both temperatures had been used in previous studies. No significant difference was observed in percent leaf area infected for Williams 82 soybean incubated at 25 versus 20°C, indicating that *C. glycines* can infect over a range of temperatures. In the studies of Hartman and Sinclair (1992), it was found that pycnidiospore germination of *C. glycines* was nearly equal at 20 versus 25°C and even though many of their studies, including detached-leaf inoculation studies, were performed at 25°C, other studies such as growth on various NaCl concentrations amended in Bacto agar and studies of pycnidia and sclerotia production on detached-leaf disks were performed at 20°C. Because the pathogen can grow and infect soybean over a range of temperatures, it has the potential to become established in more diverse regions of the United States compared with pathogens having a narrower temperature range.

Table 3. Mean percent infected leaf area for whole plants of soybean cultivars inoculated with *Coniothyrium glycines* in a containment greenhouse^w

Cultivar name ^x	PI ^y	Origin	Maturity group	Mean leaf area infected (%) ^z
Ogden	548477	China, Japan	VI	20.1 a
Haberlandt	548456	North Korea	VI	19.9 a
Williams 82	518671	Unknown	III	19.6 a
Roanoke	548485	China	VII	19.6 a
Anderson	33243	Unknown	IV	19.5 a
Illini	548348	China	III	19.3 a
Jackson	548657	China, Japan	VII	19.3 a
Ralsoy	548484	North Korea	VI	19.3 a
Mandarin (Ottawa)	548379	China	0	19.2 a
A.K. (Harrow)	548298	China	III	19.1 a
Bansei	548302	Japan	II	19.1 a
Perry	548603	China	IV	18.9 a
Mukden	548391	China	II	18.7 a
CNS	548445	China	VII	18.6 a
Dunfield	548318	China	III	18.4 a
Capital	548311	China	0	18.3 a
Lincoln	548362	China	III	18.2 a
Bossier	567789	Unknown	VIII	17.9 a
Kanro	548356	North Korea	II	17.9 a
S-100	548488	China	V	17.9 a
Fiskeby 840-7-3	438477	Sweden	00	17.8 a
Manitoba Brown	548382	Canada	00	17.8 a
Richland	548406	China	II	17.3 a
Flambeau	548325	Russia	00	17.2 a

^w Soybean lines consisted of 23 ancestral U.S. soybean genotypes plus Williams 82.

^x Whole plants were inoculated with ground mycelium and sclerotia at a density of 1.94 cm³ of agar inoculum per 10 ml of water and placed in a moist chamber at 100% relative humidity and 25°C for 5 days. Experiments were performed in a Biosafety Level 3 containment greenhouse, United States Department of Agriculture–Agricultural Research Service, Fort Detrick, MD.

^y Plant introduction number.

^z Means within a column followed by the same letter do not differ significantly according to the Tukey method using a 95% joint confidence level. Data are means of three replicate experiments, each containing 10 inoculated trifoliates per cultivar.

Datnoff et al. (1987) reported 'Jupiter' and 'Tunia' soybean to have some resistance to red leaf blotch in the field in Zambia. Even though they were reported to show lower disease severity, vertical disease progress, and area under the disease progress curve compared with 'Geduld', 'Magoye', 'Oribi', and 'Sable', disease severity (expressed as the percentage of the total leaf area diseased at growth stages R5 to R6) was still quite high and was reported as 21% for Jupiter and 16% for Tunia. A later study (Hartman and Sinclair 1996) reported that Tunia was susceptible, with defoliation percentages of 43 to 57% in unsprayed plots in two growing seasons in Zambia. Reports of resistance to red leaf blotch had not been confirmed in additional studies and Sinclair (1989) reported that all commercial U.S. cultivars tested in Zambia and Zimbabwe between 1982 and 1984 were susceptible. The U.S. soybean germplasm tested was uniformly susceptible to red leaf blotch as determined by the described seedling inoculation method. This result is consistent with results obtained from field studies performed in regions of the world where red leaf blotch is endemic (Datnoff et al. 1987; Levy et al. 1990; Sinclair 1989). Hopefully, some level of resistance can be identified in additional soybean genotypes, for there exist over 32,500 soybean germplasm accessions (USDA-ARS 2017), most of which have not been evaluated for their susceptibility to red leaf blotch (Hartman 2014; Hartman et al. 2009).

In the detached-leaf studies of Hartman and Sinclair (1992), percent leaf area infected ranged from 2 to 98% for the various host species tested whereas, in our studies, mean percent leaf area infected was generally less than 20%. The differences in the studies that could account for the varying percentages are that, in the studies of Hartman and

Sinclair (1992), leaves were incubated for periods of 2 weeks for determination of leaf infection whereas, in our studies, leaf infection was assessed after 5 days. If plants would have been incubated longer following the 5-day treatment at 100% relative humidity, lesions would have expanded and higher percentages of infected leaf area would have undoubtedly been obtained. However, in preliminary studies, it was observed that leaf curling and dropping tended to occur if plants were left to incubate longer in the greenhouse following high-humidity treatment; therefore, the most consistent and reliable results were to be obtained by determining infection percent following removal from dew or humidity chambers.

No differences were found among isolates from Zambia and Zimbabwe in their ability to infect Williams 82 soybean at 25°C. Isolates from additional geographic regions from which *C. glycines* has been reported to infect soybean (such as Nigeria and Bolivia) should be tested as well, and an evaluation of the genetic variability present within the pathogen performed using molecular markers to determine whether the pathogen population is homogeneous or contains genetic variation for pathogenicity and other characteristics.

Additional studies are necessary to evaluate genetic variability within the pathogen population from different countries where it occurs, and to evaluate additional soybean accessions in the search for resistance to red leaf blotch to be deployed should the pathogen become established in the United States. Further studies are also required to determine the temperature limits for infection and survival of *C. glycines* so that its threat potential can be more accurately determined.

Fig. 3. A, Culture of *Coniothyrium glycines* (14 days old) growing on 20% V8-juice agar (scale bar represents 2 mm); B, humidity chamber set up in containment greenhouse for inoculation of soybean with *C. glycines*. C and D, Symptoms from inoculation of Williams 82 (C) and Fiskeby 840-7-3 (D) soybean using humidity chamber in a containment greenhouse.

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