Characterization and Survival of *Cercospora sojina* in Ohio

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### Abstract

Frogeye leaf spot (FLS) of soybean, caused by *Cercospora sojina*, has increased in incidence and severity during the last 3 years in Ohio and caused substantial economic losses for the first time during 2006. FLS is common in the southern United States but has only recently been reported to cause losses in the north-central region. This study evaluated several traits of the fungus which may have contributed to the increase in disease incidence such as: the ability of *C. sojina* to overwinter and sporulate on infested soybean debris; optimum temperatures for mycelial growth; races (pathotypes) present in Ohio; and pathogenicity on cultivars with *Rcs* gene(s) for FLS resistance. Conidia of *C. sojina* were recovered from soybean debris collected at two locations from December to May 2007-2008. The optimum temperature for mycelial growth of all isolates evaluated in this study including those from Louisiana and southern Illinois was 25°C. The 50 isolates of *C. sojina* collected in Ohio were able to infect a mean of 4.9 of 12 differentials and had a total of 20 different pathotypes. The *Rcs*3 gene conferred resistance to all of the Ohio isolates. Survival of *C. sojina* in the field during the winters of 2005 to 2007 contributed to the increase of *C. sojina* during the 2005, 2006, and 2007 production seasons in Ohio.

### Introduction

Frogeye leaf spot (FLS) is a common foliar pathogen of soybeans in the southern production regions of the United States (19), and is caused by the fungus *Cercospora sojina* Hara. Pathogen survival in crop residue is thought to be responsible for initiation and development of epidemics in the south (5). It was generally thought that *C. sojina* did not overwinter in northern areas (5), but the severity and prevalence of FLS in some north central states has increased during the last few years. For example, an outbreak of FLS occurred in central Iowa during 1999 (18) and the presence of FLS was reported for the first time in southern Wisconsin in 2000 (10). During 10 to 15 July 2005, Hurricane Dennis affected Florida, Alabama, Mississippi, Georgia, Tennessee and the Ohio Valley region (2). FLS was identified in a soybean sentinel plot in southern Ohio on 25 July 2005 (A. Dorrance, personal observation). In 2006, yield losses attributed to FLS reached 35% across 500,000 acres planted to FLS susceptible cultivars in Ohio (A. Dorrance, personal observation). Warmer winter temperatures, susceptible soybean germplasm, and conservation tillage practices have been proposed as potential causes for recent outbreaks of the disease (5).

FLS is managed primarily by planting resistant cultivars or by applying foliar fungicides to susceptible cultivars. Three dominant resistance genes have been identified in soybean, which confer resistance to *C. sojina* (15). *Rcs*1 identified in ‘Lincoln’ confers resistance to races 1 and 5, *Rcs*2 in ‘Kent’ confers resistance to race 2, and *Rcs*3 from ‘Davis’ confers resistance to all described races in the United States (3,12,13,16). *C. sojina* races 1, 2, 3, 4, and 5 have been described in the United States (1,13,17). Race designations for *C. sojina* races 5 through 15 on a standard set of 12 differential cultivars of soybean were recently proposed (11). More isolates of *C. sojina* have been characterized for virulence but the response on the soybean differentials does not fit the current list of races (5,11,15). Therefore, we have used the term pathotype which refers to the virulence formulae directly without the race code designation.
FLS can be found in Ohio, very late in the season on late maturing lines (Dorrance, personal observation). However, prior to 2006, there were no documented losses from FLS in Ohio. This study evaluated four traits of *C. sojina* which may have contributed to the increase in disease incidence: (i) overwintering survival in crop residue; (ii) optimum temperature(s) for mycelial growth; (iii) pathotypes of the fungus in Ohio; and (iv) pathogenicity of isolates on cultivars with *Rcs* gene for resistance to FLS.

**Winter Survival and Historical Winter Temperatures**

During October 2007, soybean leaves and stems heavily infected with *C. sojina* were collected at the Ohio Agricultural Research and Development Center (OARDC), Western Agricultural Research Station (Clark Co.). Procedures used to determine survival of *C. sojina* during the winter in Ohio were similar to those previously described (8,9). Ten grams of infected sun-dried leaves and 40 g of infected stem sections were placed inside mesh bags. Metal hardware screens were used to protect leaves from damage and off-site movement by wind after placement in the fields. Twenty samples each of leaves and stems were placed outdoors on the soil surface during October 2007 at two locations, OARDC Snyder Farm (Wayne Co.) and the OARDC Western Agricultural Research Station (Clark Co.). Each sample was anchored to the ground in a randomized complete block design with two replications at each location (Fig. 1). Two samples of leaves and stems were retrieved about every 30 days from each location from January through May 2008. For each sample, the plant material was rinsed with tap water for 15 sec to remove adhered soil, dried overnight, and placed inside 25.6-liter Sterilite containers (Sterilite Corp., Townsend, MA) which were lined with moist paper towels. Plant tissue was incubated at 25 ± 2°C with 12 h of light for 19 days. Plant tissue segments were observed under a dissecting scope (Leica S6D 6.3 to 40×) daily for the presence of newly developed conidiophores and conidia of *C. sojina*. Once conidia were detected, tissue was washed with 200 ml of sterile distilled water plus Tween 20 (20 ml in 500 ml of water) and the suspension was then filtered through four layers of cheesecloth. The conidia were counted with a hemocytometer and the number per gram of tissue was calculated. The number of conidia collected from the residue at each sampling time at each location was analyzed using PROC GLM of SAS (SAS Institute Inc., Cary, NC).

*C. sojina* survived during the winter of 2007-2008 in naturally-infested soybean leaves and stems on the surface of field plots. Conidia were produced on infested crop residue collected during the months of January to May from both locations (Fig. 2). On tissue collected from January through March, conidia were observed 19 days after incubation, and 8 to 10 days after incubation on tissue collected in April and May. Sporulation on overwintering leaves peaked by the end of April with a mean of 59,218 conidia/g of leaf tissue (Table 1). There were fewer FLS lesions on infected soybean stems and low numbers of conidia were produced on stem lesions (data not shown).
Table 1. Mean number of *C. sojina* conidia per g of leaf tissue collected from overwintering leaves at OARDC Agricultural research stations in Ohio.*

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Snyder Farm Wayne Co.</th>
<th>Western Clark Co.</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>19,778</td>
<td>15,714</td>
<td>18,947</td>
</tr>
<tr>
<td>February</td>
<td>18,644</td>
<td>11,940</td>
<td>15,079</td>
</tr>
<tr>
<td>March</td>
<td>49,914</td>
<td>30,769</td>
<td>40,310</td>
</tr>
<tr>
<td>April</td>
<td>69,689</td>
<td>49,636</td>
<td>59,218</td>
</tr>
<tr>
<td>May</td>
<td>6,667</td>
<td>34,807</td>
<td>21,250</td>
</tr>
</tbody>
</table>

* Conidia were washed from 10 g leaf tissue and counted with a hemocytometer.

The mean daily and monthly air temperatures from the winters of 2005-2006, 2006-2007, and 2007-2008 were compared to historical winter temperatures to determine if these winter temperatures were among the coldest. The number of days when winter temperatures were below -8°C, between -8°C and -2.9°C, -3°C, and 1.9°C, and above 2°C, were compared. For each location the mean monthly temperature for December, January, and February were calculated from historical weather data which was obtained from the OARDC central storage facility at www.oardc.ohio-state.edu/newweather. In Ohio, the lowest winter air temperatures in the past three decades occurred during 1983 to 1986 and these were compared to 2005 to 2008 based on the number of days when temperatures were below -8°C. There were 17 to 20 days where temperatures were below -8°C during the mid 1980s compared to 6 to 14 days during 2005 to 2008 (Table 2). In addition, the mean monthly temperatures were also higher during 2005 to 2008 compared to 1983 to 1986. It is possible that *C. sojina* can survive in Ohio when the monthly mean winter temperature reaches -4.2°C (Fig. 3). More studies are needed to determine if *C. sojina* can survive when the mean monthly winter temperatures are below -4.2°C and if this method can be used as a predictor to develop a risk assessment model for FLS.

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Fig. 2. Conidia of *C. sojina* produced on an overwintering soybean leaf (A) and a stem lesion (B). Plant tissues were collected from the field during April 2008.
Fig. 3A. Mean monthly air temperature for December, January, and February during 2007-2008 at the OARDC Western Agricultural Research Station compared to 1983-1986.
Isolate Collection and Optimum Temperature for Growth

A total of 50 isolates of *C. sojina* were collected from soybean leaves with characteristic lesions of FLS from 25 locations across 22 counties in Ohio during 2007 (Fig. 4). Many of the samples were submitted as part of the effort to monitor for soybean rust. Leaves with large circular lesions, gray to brown
centers, and reddish to purple margins were incubated in a moist chamber (ziplock bag with moist paper towel) for 24 to 48 h. Conidia were collected with a sterile toothpick from sporulating lesions on the underside of leaves and transferred onto clarified V8 media. Each plate was touched five or six times with the toothpick in different locations. Plates were incubated at 22°C with 12 h of light, followed by two more transfers of single conidia at 10-day intervals to obtain pure cultures. Once the pathogen was isolated, cultures were maintained on dilute V8 juice agar (5) at 25°C with no light, and transferred every 30 days to maintain sporulation (adapted from J. Bond, personal communication). All isolates were examined for the size and shape of conidia and colony morphology to verify that they were *C. sojina* (7).

Fig. 4. Locations in Ohio where soybean leaves were collected with characteristic frog eye leaf spot lesions. Leaves were collected predominately from soybean sentinel plots for monitoring of soybean rust.
Mycelial growth of isolates was compared to growth of an isolate from southern Illinois (obtained from J. Bond, Southern Illinois University) and from Louisiana (obtained from R. Schneider, Louisiana State University) over a 24-h period in darkness. In the first experiment three plates of each isolate were incubated at 17, 21, 25, 28, and 32°C and the study was repeated. In a second experiment, a subset of 10 isolates collected in Ohio representing different rates of mycelial growth and one isolate each from southern Illinois and Louisiana were evaluated at the same temperatures in a randomized complete block design with three replicates of one plate per replicate. In both experiments, isolates were grown on clarified V8 medium for 20 days and a 5-mm plug taken from the margin of the actively growing colony was transferred to the center of each plate containing the same medium. Three plates per isolate were used, and mycelial growth measured (mm) in two perpendicular directions at 6, 14, 22, and 30 days after the plugs were transferred. Variation in mycelial growth among isolates and temperatures as well as within temperatures at each time point were analyzed using PROC GLM. Growth curves generated from each isolate were then compared.

After 6 days at 25°C, the average colony diameter ranged from 4 to 5.5 mm. At the end of 20 days, colonies were gray to olive brown in color with concentric ridges and sporulation was abundant in most of the isolates. The optimum temperature for mycelial growth of *C. sojina* was approximately 25°C (Fig. 5) for all isolates collected from Ohio as well as those from southern Illinois and Louisiana. Since the optimum temperature for growth was the same among all these isolates, this suggests that conditions which favor infection in the south would be the same as those in the north central region.
Fig. 5. Comparison of mycelial growth of 10 isolates of *C. sojina* from Ohio, 1 from southern Illinois, and 1 from Louisiana at various temperatures on dilute V8 agar medium.

**Isolate pathotypes**

Soybean differential cultivars Davis, Peking, Kent, CNS, Palmetto, Tracy, Hood, Lincoln, Lee, Richland, S-100, and Blackhawk (11) were used to characterize the pathotype of 50 isolates of *C. sojina* from Ohio. Six seeds of each cultivar were planted in 15.2-cm-diameter pots and emerged plants were thinned to 3 plants per pot. One pot of each differential and two of the universal susceptible cultivar Blackhawk were used for inoculation with each isolate, and each of the 50 isolates was inoculated onto plants in two separate experiments. Inoculum of *C. sojina* was prepared by flooding two, 28-day-old cultures grown on dilute V8 agar with 20 ml of sterile water. Conidia were gently harvested with a rubber policeman and large mycelial fragments were removed by passing the suspension through two layers of cheesecloth. Conidia were collected in a 50 ml test tube and counted using a hemocytometer. The final inoculum concentration was adjusted to $6 \times 10^4$ conidia/ml and approximately 0.3 ml was sprayed onto the youngest fully expanded trifoliate of each plant (11). The inoculated plants were placed in a mist chamber for 72 h at 22 to 23°C and 100% relative humidity, and then returned to the greenhouse at 25 ± 4°C with 12 h of light from 6 am to 6 pm. Disease reactions were evaluated 15 days after inoculation, and differentials were scored as susceptible when 2- to 5-mm-diameter lesions developed with light gray to brown centers, purple margins and abundant...
sporulation (12). These inoculations, which were under highly favorable conditions, did produce a few small lesions (<1 mm) with minimal sporulation on some differentials and these were classified as resistant (D. Phillips, personal communication). Six to 10 isolates, chosen at random, were evaluated at one time due to mist chamber space limitations. For each isolate, only the susceptible responses which were the same for both experiments were entered into the Habgood-Gilmour spreadsheet. This spreadsheet was used to summarize the virulence data and to obtain tables of frequency distribution for isolate complexity and pathotypes (6).

Twenty different pathotypes were identified among the C. sojina isolates collected in Ohio (Fig. 6). Pathotype 0463 (race 12) was the most common, followed by pathotypes 0673 (race 15), 0663, and 0263 whose reactions have not previously been described. Among these 50 isolates of C. sojina there was a susceptible response following inoculation on a mean of 4.9 of 12 differentials. Differentials, Lincoln, Lee, Richland, and S100 had the highest frequency of susceptible responses to these isolates. The Rcs3 gene in Davis (3,12,14) conferred resistance to all of the Ohio isolates of C. sojina which is similar to that reported from the southern United States (14) (Fig. 7).

Fig. 6. Pathotypes of C. sojina identified in Ohio and their frequency distribution.
Conclusion

The widespread planting of highly susceptible cultivars from 2005 through 2007 combined with a high proportion of fields with reduced tillage and warmer winter temperatures that allowed survival of *C. sojina* on plant residue in the field may have contributed to the increased incidence of FLS in Ohio. Based on winter air temperatures from 2005 through 2008, mean monthly temperatures greater than -4.2°C during December, January, and February may indicate a higher probability for survival of *C. sojina* in the field and higher potential for FLS development the following year. However, more research is needed to refine this threshold temperature and to add other forecasting variables. High levels of inoculum present at the end of the season (A. E. Dorrance, personal observation) and *C. sojina* survival during the winter allowed for high levels of primary inoculum the following spring. The *Rcs*3 gene was effective against all pathotypes isolated from Ohio and host resistance remains the most effective management strategy for this disease. Soybean cultivars targeted for the north central region should now be rated for resistance to *C. sojina* in order to prevent further widespread epidemics.

Acknowledgments

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