# Genetic Interactions Between *Glycine max* and *Sclerotinia sclerotiorum* Using a Straw Inoculation Method

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**ABSTRACT**

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Genetic interactions for disease response between cultivars of *Glycine max* and isolates of *Sclerotinia sclerotiorum* were evaluated in controlled-environment inoculations of five soybean cultivars with four genetically unique isolates of *S. sclerotiorum*. The objective of this study was to identify host–pathogen interactions using isolates of the pathogen which had different geographical and crop-wise distribution as well as a different DNA-based fingerprint. To do so, 4-week-old soybean plants were inoculated with individual isolates of *S. sclerotiorum* using a straw inoculation method. Inoculated plants were incubated for 48 h in continuous leaf wetness and rated for disease severity 1 and 2 weeks after inoculation. Significant differences in disease severity were detected among the soybean cultivars, and NK S08-80 consistently had the lowest disease severity among the five cultivars tested. No significant differences in disease severity were observed among pathogen isolates and no significant interactions were detected between soybean cultivars and pathogen isolates. These results suggest the following interpretations: (i) either the clonal genotype of the four pathogen isolates as determined by mycelial compatibility and DNA fingerprint was not associated with level of virulence on the five soybean cultivars or (ii) the soybean cultivars themselves were not capable of revealing any differences in virulence among isolates that would be related to their genetic fingerprint or regional distribution. The results of this study are consistent with the practice of considering different isolates of *S. sclerotiorum* sampled from soybean in the same geographical region as equivalent for the evaluation of soybean cultivars for resistance to Sclerotinia stem rot.

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is an important disease in many soybean-growing areas (38–41). In North America, Sclerotinia stem rot has been problematic mostly for the more temperate regions (North Dakota, South Dakota, Wisconsin, Iowa, Illinois, Minnesota, Indiana, and Ontario;(41). It has been ranked as the second to fourth most important soybean disease in the United States during 1994 to 1998 (38,41) and as the third most important disease in Ontario during 1999 to 2002, despite drought conditions during 2001 that resulted in an absence of the disease in Ontario (40).

Partial disease resistance has been reported among selected soybean genotypes to *S. sclerotiorum* from controlled-environment and field studies. Boland and Hall (4–6) reported that cv. Ozzie was more susceptible than Maple Arrow in controlled-environment and field inoculations, whereas Nelson et al. (31) found Ozzie to be of comparable resistance to Maple Arrow in both controlled-environment and field evaluations. In several studies, the response of cultivars to *S. sclerotiorum* has been variable. For example, the response of Ozzie was stable across different environmental conditions within each study but different between two studies (4,6,31). Similarly, the soybean cv. Evans was rated as susceptible in several controlled-environment and field studies (4,6,12,16,31) but was found to have one of the lowest disease incidences in an unrelated 2-year field study (11). Wegulo et al. (37) also observed variations in the ranking among field and controlled environments. These findings have prompted an interest in looking at potential causes of obtaining differential disease evaluations for the same cultivars in different studies.

Variations in the response of soybean cultivars to *S. sclerotiorum* in field environments has been correlated with several plant architecture and development variables, such as cultivar height, maturity, and lodging (4,6). Cultivars with shorter height, earlier maturity, or reduced lodging were associated with lower numbers of apothecia and lower disease incidence than cultivars with greater height, later maturity, or more lodging (6). Therefore, variables such as these appear to influence the development of the pathogen or disease, or both, by modifying environmental conditions within the crop canopy.

The type of inoculation method used also can affect the response of soybean cultivars to *S. sclerotiorum*. Kim et al. (18–20) used three inoculation methods to evaluate soybean genotypes for response to disease and found variable responses using these methods in different environments. For example, disease ratings for cv. BSR 101 ranged from the highest to the lowest across years and locations in field tests (18) and were not constant using any of the three inoculation methods (19). Cvs. NK S19-90 and Corsoy79 displayed consistently lower disease severity indices (DSIs), whereas cvs. Faribault and Resnik displayed consistently higher DSI using artificial inoculation and field methods (19,20).

The genotype of the pathogen used for inoculation also may affect the response of the host to *S. sclerotiorum*. There have been experimental data indicating that an isolate representing a genotype from a wild population of *S. sclerotiorum* associated with the woodland plant *Ranunculus ficaria* was significantly less aggressive on canola (*Brassica napus* L.) than isolates recovered from canola (15). However, no experimental information has been published on the potential of isolates recovered from agricultural locations to cause differential levels of disease among genotypes of other hosts.

Little information is available on the influence of genetic variability among isolates of *S. sclerotiorum* on the response of soybean cultivars and breeding lines to disease incidence or severity. Agricultural populations of *S. sclerotiorum* have been characterized as predominantly clonal, with little evidence of outbreeding and recombination. This clonality is the result of both asexual reproduction by means of sclerotia and sexual but self-fertilized reproduction in this haploid species (7,23). It is important to note that all members of a clone are not necessarily genetically identical and that clonal lineages are not evolutionarily static. Each clonal lineage can evolve by means of mutations, which are passed on in mitosis and which can accumulate over time. Individual isolates are classified in clonal lineages through the

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use of two or more unlinked markers such as mycelial compatibility groups (MCGs), DNA fingerprinting, or microsatellites (7,17,25,36). In infested soils with a history of disease, including crops such as canola, cabbage, and soybean, there is strong regional partitioning of clones (13,17,33), which may be consistent with differences in fitness among clones under regional patterns of selection or may reflect patterns of pathogen dispersal. Infested soils with a history of disease typically comprise a mosaic of clonal genotypes, where several clones often predominate in frequency. Distinct regional patterns of clonal distribution have been observed (7,10,13,21,25,26,29,33), and some clones are distributed over thousands of kilometers and persist over 10 or more years (23). All of these patterns recently have been confirmed in samples of soybean from Ontario, Québec, and Pennsylvania (17,22), and clones frequently sampled in previous years from canola (*B. napus*) and cabbage (*B. oleracea*) were recovered at similar frequencies from soybean (17,22).

There has been little consideration of genotypic variation in *S. sclerotiorum* in studies of the responses of soybean cultivars to this pathogen. In controlled-environment experiments, most authors have recovered a representative isolate from diseased plant materials into sterile culture. In field experiments, naturally occurring populations of the pathogen are relied upon to incite disease or, in some case, natural populations are supplemented with sclerotia from other uncharacterized sources such as seed-cleaning plants. No study of the levels of virulence on different soybean genotypes using clonal groups characterized through DNA-based techniques has been published to date. The goal of this study was to address this issue by testing three null hypotheses, as follows. The first null hypothesis was that there is no difference among cultivars for resistance to *S. sclerotiorum*. The second hypothesis was that there is no difference among genetically unique isolates of *S. sclerotiorum* for virulence on soybean. The third hypothesis was that there is no interaction in disease severity between cultivars of soybean and isolates of *S. sclerotiorum*.

## MATERIALS AND METHODS

**Plant material.** Five soybean cultivars (OAC Shire, OAC Bayfield, OAC Salem, NK S08-80, and Nattosan) were selected for evaluation for resistance to Sclerotinia stem rot in controlled-environment experiments. The cultivars were selected because they are known to differ in resistance to Sclerotinia stem rot. Two seeds were sown in 10-by-10-cm pots containing LA4 aggregate growing mix #4 (Sun Gro Horticulture Inc., Bellevue WA) topped with 1 cm of vermiculite. Pots were arranged in trays, each tray holding 14 pots of each cultivar. The plants were thinned to one plant per pot once seedlings had their unifoliate leaves open. The plants were maintained in a growth room at 21°C with a photoperiod of 16 h for 2 weeks, followed by a shorter photoperiod of 12 h until the end of the experiment. Photosynthetic photon flux density (PPFD) was maintained at approximately 300 mol $m^{-2}$ $s^{-2}$. Plants were inoculated 4 weeks after planting.

***Sclerotinia sclerotiorum.*** Four isolates of *S. sclerotiorum* (O-CEF-31, O-CEF-33, O-CEF-35, and O-CEF-74) were used to inoculate the soybean cultivars. All isolates were collected in the fall of 1999 by S. Hambleton (17). Isolates O-CEF-31, O-CEF-33, and O-CEF-35 were collected from the soybean cv. Nattosan at the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada in Ottawa, Ontario, Canada. Isolate O-CEF-74 was collected at Le Centre de recherche sur les grains (CÉROM) Inc. in St-Bruno-de-Montarville, Québec, Canada from cv. Hasgro 1123. All isolates were differentiated into distinct clonal groups using MCGs (24) and DNA fingerprinting, as follows. O-CEF-31 was fingerprint 752, MCG D (15), a clone previously sampled from New York State cabbage (isolate B9 I, haplotype 14) (5); O-CEF-33 was fingerprint 1, MCG C (17), a frequently sampled clone in a 1989 canola sample from Ontario (clone 1) (25), and a clone previously sampled from New York State cabbage (isolate B3); O-CEF-35 was fingerprint 835, MCG F, a clone only sampled in Ontario in 1999 (17); and O-CEF-74 was fingerprint 2, MCG A (17), one of the most frequently sampled and widely dispersed clones across Canada and the northeastern United States (clone 2) (21,25). Axenic cultures were prepared from sclerotia surface disinfected in 2.5% sodium hypochloride solution and then transferred to fresh potato dextrose agar (PDA; 200 g of peeled potatoes, 1 liter of water, 20 g of dextrose, 20 g of Bacto agar; Difco Laboratories, Sparks, MD). The initial cultures were subcultured onto fresh PDA and the first subcultures were used as a source of inoculum.

**Experimental design.** The five cultivars of soybean and four isolates of *S. sclerotiorum* were arranged in a split-plot experimental design with two replicates (blocks). The main plot factor was isolate and the split-plot factor was cultivar. The experiment was repeated once. Experimental units were single trays with 14 plants of each cultivar inoculated with each isolate. Means of 14 plants were used for the analysis of variance.

**Inoculation procedure.** Four-week-old plants were inoculated using the straw inoculation method (32), as follows. One end of a plastic straw, approximately 5 mm in diameter and 2 cm long, was stapled and the other end was used to bore into the leading edge of a growing culture of *S. sclerotiorum*. The stems were cut just below the most newly expanded trifoliolate leaf node and the straw bearing the agar plug was inserted over the cut. The plants were misted in the growth room for 48 h after inoculation. Three misting nozzles per block maintained continuous leaf wetness for the first 48 h after inoculation. Lesions were evaluated 1 and 2 weeks after inoculation on a scale from 1 to 9, where: 1 = no lesion; 2 = lesion just under the straw; 3 = lesion beyond the straw, but not extending to the first node from the straw; 4 = lesion extending to the first node from the straw; 5 = between the first and second node from the straw; 6 = at the second node from the straw; 7 = between the second and third node from the straw; 8 = at the third node from the straw; and 9 = beyond the third node from the straw.

**Data analysis.** The means of the 14-plant experimental units were computed and analyzed using the SAS procedure PROC MIXED (SAS 8.2, 1999–2001; SAS Institute Inc., Cary, NC). Analysis of residuals and PROC UNIVARIATE confirmed the assumptions that experimental errors were random, independent, normally distributed around a zero mean, and had a common variance. The data were analyzed as a split-plot experimental design combined over experiments for the lesion ratings 1 and 2 weeks after inoculation. Fixed effects were the main plot effect (isolate), subplot effect (cultivar), and cultivar–isolate interaction. Random effects were experiments, experiment–isolate, and isolate–block (experiment). Type I error was set at 0.05.

## RESULTS

The analyses of variance from the disease ratings at 1 and 2 weeks after inoculation (Tables 1 and 2) in each of the two experiments indicated significant differences for disease severity among the five soybean cultivars. No significant effects were detected among the four isolates or the interaction between cultivar and isolates. Furthermore, no significant effects were detected between the two experiments or for the interactions between experiment and isolate 1 and 2 weeks after inoculation (Tables 1 and 2).

OAC Shire was the most susceptible cultivar for the disease rating 1 and 2 weeks after inoculation. NK S08-80 and OAC Salem were the most resistant cultivars for the disease ratings at 1 and 2 weeks after inoculation (Table 3). The analysis of variance showed that, on average, isolates did not differ significantly in aggressiveness 1 week after inoculation. However, taking into account the associated standard error of 0.30, the isolate O-CEF-35 was significantly more virulent (mean of 4.7) than isolate O-CEF-74 (mean of 4.4.). The isolates did not differ at all 2 weeks after inoculation.

No significant difference was found for cultivar–isolate interaction 1 and 2 weeks after inoculation (*data not shown*).

## DISCUSSION

Three null hypotheses were tested in this study. The first null hypothesis was that there was no difference among cultivars for response to Sclerotinia stem rot. The second null hypothesis was that there was no difference among isolates of *S. sclerotiorum* for virulence on soybean. The third null hypothesis was that there was no interaction between the response of soybean cultivars and the virulence of *S. sclerotiorum* isolates.

Significant differences were detected among soybean cultivars in their response to inoculation with *S. sclerotiorum*. Differences in cultivar response clearly were present throughout the study and were expected. The cultivars had been selected for differences in resistance to Sclerotinia stem rot, based on field data (J. Auclair and I. Rajcan, *unpublished data*). OAC Shire was the most susceptible cultivar, and NK S08-80 and OAC Salem were the most resistant, which was in accordance with our previous field evaluations.

No significant differences were detected among the isolates of *S. sclerotiorum* for aggressiveness on soybean 2 weeks after inoculation (Table 3). However, 1 week after inoculation, isolate O-CEF-74 seemed to approach significant difference to isolate O-CEF-35, having obtained a disease severity rating of 4.4 versus 4.7 for O-CEF-35 (Table 3), at standard error of 0.30 (4.4 + 0.30 = 4.70). Perhaps a more relaxed type I error (0.10) rate would have found more isolates significantly different for virulence on the five soybean cultivars 1 week post inoculation.

It should be noted that only four isolates were used in this study. Riddle et al. (35) and Ekins (14) used a greater number of isolates and observed large overlaps of significant differences in aggressiveness. Morrall et al. (30) described differences in virulence in *S. sclerotiorum* as a "continuum" where significant differences among the most aggressive and the least aggressive could be observed but no real categorization could be detected. The absence of significant differences among isolates of *S. sclerotiorum* in our study agrees with previous work, which used a larger number of isolates (28,32,34). To our knowledge, significant isolate–cultivar interactions for Sclerotinia stem rot using a cut-stem method has been reported only once and quite recently (27).

In the present study, the four isolates were chosen to represent four genetic lineages of known frequency in Ontario and Québec soybean field samples. Isolates were assigned to clonal lineages by mycelial compatibility grouping and DNA fingerprinting. For DNA fingerprinting,

**Table 1.** Analysis of variance combined over two experiments of 14-plant mean arranged in split-plot design with disease severity ratings 1 week after straw-method inoculations in a controlled environment of five soybean cultivars as subplot factor and four isolates of *Sclerotinia sclerotiorum* as main-plot factor

| Effects | Estimate | Standard error | *Z* value | *P* > *Z* | Numerator df | Denominator df | *F* value | *P* > *F* |
|---|---|---|---|---|---|---|---|---|
| Random effects | | | | | | | | |
| Experiment | 0.1339 | 0.2053 | 0.65 | 0.2572 | … | … | … | … |
| Experiment × isolate | 0.008306 | 0.04077 | 0.20 | 0.4192 | … | … | … | … |
| Isolate × block (experiment) | 0.03909 | 0.03702 | 1.06 | 0.1455 | … | … | … | … |
| Residual | 0.1683 | 0.03436 | 4.90 | <0.0001 | … | … | … | … |
| Fixed effects | | | | | | | | |
| Isolate | … | … | … | … | 3 | 3 | 0.58 | 0.6664 |
| Cultivar | … | … | … | … | 4 | 48 | 17.67 | <0.0001 |
| Isolate × cultivar | … | … | … | … | 12 | 48 | 0.67 | 0.7734 |

**Table 2.** Analysis of variance combined over two experiments of 14-plant mean arranged in split-plot design with disease severity ratings 2 weeks after straw-method inoculations in a controlled environment of five soybean cultivars as subplot factor and four isolates of *Sclerotinia sclerotiorum* as main-plot factor

| Random effects | Estimate | Standard error | *Z* value | *P* > *Z* | Numerator df | Denominator df | *F* value | *P* > *F* |
|---|---|---|---|---|---|---|---|---|
| Random effects | | | | | | | | |
| Experiment | 0.6651 | 1.0282 | 0.65 | 0.2588 | … | … | … | … |
| Experiment × isolate | 0.1291 | 0.2075 | 0.62 | 0.2670 | … | … | … | … |
| Isolate × block (experiment) | 0.1347 | 0.1167 | 1.15 | 0.1243 | … | … | … | … |
| Residual | 0.4776 | 0.09750 | 4.90 | <0.0001 | … | … | … | … |
| Fixed effects | | | | | | | | |
| Isolate | … | … | … | … | 3 | 3 | 0.61 | 0.6518 |
| Cultivar | … | … | … | … | 4 | 48 | 29.61 | <0.0001 |
| Isolate × cultivar | … | … | … | … | 12 | 48 | 0.45 | 0.9356 |

**Table 3.** Least squares means for the fixed effects from the analysis of variance combined over two experiments of 14-plant mean arranged in split-plot design with disease severity ratings 1 and 2 weeks after straw method inoculations in controlled environment

| | | Least squares means estimate at weeks post inoculation[z] | | | | | |
|---|---|---|---|---|---|---|---|
| Effect | Isolate or cultivar | 1 week | SE | df | 2 weeks | SE | df |
| Isolate | O-CEF-31 | 4.5 ab | 0.30 | 3 | 6.4 a | 0.67 | 3 |
| Isolate | O-CEF-33 | 4.6 ab | 0.30 | 3 | 6.5 a | 0.67 | 3 |
| Isolate | O-CEF-35 | 4.7 b | 0.30 | 3 | 6.4 a | 0.67 | 3 |
| Isolate | O-CEF-74 | 4.4 a | 0.30 | 3 | 5.9 a | 0.67 | 3 |
| Cultivar | NKS08-80 | 4.2 a | 0.28 | 48 | 5.1 a | 0.62 | 48 |
| Cultivar | Nattosan | 4.5 b | 0.28 | 48 | 6.4 b | 0.62 | 48 |
| Cultivar | OAC Bayfield | 4.7 b | 0.28 | 48 | 6.7 b | 0.62 | 48 |
| Cultivar | OAC Salem | 4.2 a | 0.28 | 48 | 5.7 a | 0.62 | 48 |
| Cultivar | OAC Shire | 5.2 c | 0.28 | 48 | 7.6 c | 0.62 | 48 |

[z] SE = standard error. Means followed by a common letter do not differ ($P \leq 0.05$).

whole-cell genomic DNAs were digested with *Bam*H1 and hybridized to a cloned probe, pLK44.20, containing a 4.5-kb dispersed element of nuclear DNA from *S. sclerotiorum* (25). This is a highly repeatable method for identifying clones in major crop production areas where reproduction has been shown to be predominantly asexual or selfed in this haploid organism. Distinct regional patterns of spatial distribution of these clones have been demonstrated, with some clones recovered over large geographical areas and repeatedly since 1989 (7,10,13,21,23,25,27,29,33). Under the conditions of the present study, clonal lineages were not associated with differences in virulence on the soybean cultivars tested in these experiments. A logical extension of this study would be testing for variation in virulence among several isolates from a clonal lineage, perhaps comparing isolates from one geographic area with a sample from more than one area. Multiple isolates from a clone could be further characterized by amplified fragment length polymorphisms or other methods that screen the genome more widely than the restriction fragment length polymorphism approach.

With expanded efforts at breeding soybean cultivars with higher levels of partial resistance to *S. sclerotiorum,* it may become easier to distinguish between resistant and susceptible reactions. Quantitative trait loci (QTL) mapping with an ever-increasing resolution may dissect the resistance trait into its component QTL (1,2,18), which can then be tested individually against a panel of pathogen isolates. In the study of complex disease resistance, many factors must be considered, from selection of inoculation method to the choice of the best way to quantify disease resistance (3). Although QTL mapping of resistance loci may help to clarify whether individual loci are plant genotype or cultivar specific (42), poorly executed mapping actually could obscure genotype-specific interactions. Our study has attempted to address the specific question of whether or not the genetic variation in the pathogen itself is affecting the phenotyping of disease resistance to Sclerotinia stem rot in soybean. The straw method was able to separate the cultivars into different phenotypic classes while being challenged with genotypically different isolates of *S. sclerotiorum*.

In this study, a limited number of soybean cultivars and *S. sclerotiorum* isolates were used to test the three hypotheses. The cultivars were significantly different in the level of resistance to the isolates using the straw method of inoculation. Further studies are needed to determine the amount of pathogen variability observed. Using a wider selection of cultivars and isolates from other populations of *S. sclerotiorum,* as revealed by genealogical analysis of multi-locus DNA sequence typing (8,9,33) or a more accurate mapping of virulence genes, may help to characterize the genetic basis of pathogen virulence factors that are most important to the incidence of Sclerotinia stem rot in soybean. The results of our study are consistent with the practice of considering all pathogenic isolates as similar for the evaluation of resistance of soybean cultivars to Sclerotinia stem rot.

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