MISSISSIPPI SOYBEAN PROMOTION BOARD
PROJECT NO. 32-2016 (YEAR 4)
2016 FINAL REPORT

Title: Phenotyping F_2 populations segregating for Frogeye resistance.

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NOTE: The Project was extended one year to complete specific experiments.

BACKGROUND AND OBJECTIVES

Frogeye Leaf Spot (FLS) is common in the southern and southeastern soybean production region of the US. Recently, FLS has spread further north into Midwestern soybean growing states, including Ohio, Indiana, Wisconsin, Michigan, Illinois, Iowa, and Missouri.

Under favorable environments, yield reductions ranging from 10 to 60% due to FLS infestations have been reported. Fungicides of the strobilurin class are widely used to control foliar diseases including FLS. However, recently a *C. sojina* isolate has been confirmed in soybean fields in 12 states (Alabama, Arkansas, Illinois, Indiana, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, Ohio, Tennessee and Virginia) with resistance to strobilurin chemistry. Reliance on fungicides could be reduced with the development and use of FLS resistant cultivars.

Several soybean genes conferring resistance to FLS have been identified, among which the *Rcs3* gene from cultivar Davis has provided the most durable resistance against all known isolates of FLS in the US. However, this single gene resistance presents a risk as it is only a matter of time before it is defeated by *C. sojina*. Additionally, *C. sojina* is a highly prolific fungus with more than 44 known races. The future development of *C. sojina* resistant cultivars may be dependent on developing race-specific resistance. Molecularly mapping of soybean resistance genes will allow the ability to differentiate and identify germplasm with new genes. The research described here provides the foundation for that process.

The objectives of this research project were to: (1) Analyze and couple *C. sojina* isolate pathogenicity and molecular data; (2) Phenotype soybean populations segregating for *C. sojina* resistance; and (3) Collect tissue for future DNA isolation and marker analysis.

SUMMARY

Over the course of this research we collected 227 *C. sojina* isolates from 16 geographic locations. For 83 of those isolates, we characterized their cultural characteristics and determined their pathogenicity on a set of 12 soybean cultivar differentials. The results indicated a wide range of variation for pathogenicity that could be characterized using the differentials.

Our previous research applying Simple Sequence Repeat (SSR) molecular markers to these 83 isolates showed wide-ranging genetic variation. In support of the molecular marker analysis, two *C. sojina* were

sequenced (including a fungicide-resistant isolate) to identify Single Nucleotide Polymorphic (SNPs) markers widely distributed across the *C. sojina*. The molecular analysis of the sequences is still under way.

Our other primary objective was to identify new resistance genes for Frogeye Leaf Spot disease. We focused on PI 458175B which we previously showed to have a resistance that appeared to be different than that of the original "Davis" type resistance (*Rcs3*). Reciprocal crosses were made between the resistant line PI 458175B and the highly susceptible cultivar "Blackhawk". Genetic analysis of segregating populations indicated that the resistance found in PI 458175B fit the expected segregation ratios for a single recessive resistance gene ($P > 0.05$).

We are unaware of any other recessive resistance genes for Frogeye Leaf Spot disease, although recessive resistance has been reported for other soybean diseases. Additionally, a polymorphic SNP located near the *Rcs3* locus was tested for association in one population. Analysis showed that the marker segregated independent of the resistance in PI 458175B, indicating the gene controlling its resistance is located elsewhere in the genome. Thus it is likely that the Frogeye Leaf Spot resistance exhibited by PI 458175B represents a new resistance locus.

We have initiated a breeding program between PI 458175B and a high-germination, high-yielding MG III breeding line and a similar MG IV breeding line. We are completing confirmation experiments and preparing publications of results.

REPORT OF PROGRESS/ACTIVITY

Objective 1. Analyze and couple *C. sojina* isolate pathogenicity and molecular data.

A total of 227 isolates were collected from 16 different geographical locations, of which 83 isolates (Table 1) were tested on 12 differential soybean cultivars. Pathogenicity data for all 83 isolates were collected in addition to cultural characteristics of each isolate. Isolate cultural characteristics were measured based on growth and sporulation on three different media (Potato Dextrose Agar; Lima Bean Agar, and Soybean Stem and Pod Agar). Over all isolates, there was clear variation in cultural characteristics and the degree of sporulation between isolates (data not shown).

Table 1. Geographical locations where *C. sojina* isolates were collected and the number of isolates from each location evaluated.

State	# of Isolates	# of isolates tested
AL	10	7
AR	6	5
Brazil	16	8
China	11	3
FL	1	1
GA	29	15
IA	4	2
IL	31	2
IN	1	0
KY	6	0
LA	6	3
MO	11	0
MS	16	9
SC	6	1
TN	62	26
WI	1	1
Total	**227**	**83**

Pathogenicity of the isolates was measured on 12 differential cultivars (Davis, Peking, Kent, CNS, Palmetto, Tracy, Blackhawk, S-100, Hood, Lincoln, Lee and Richland). Disease severity ratings were taken two weeks apart and lesion numbers counted from all leaves on each inoculated differential soybean line in the greenhouse.

Results showed a wide range of responses over the differentials and between isolates. For example, isolate TN40 was highly pathogenic on differentials Tracy, S-100, and Lee compared to TN10 (control isolate). TN40 was even pathogenic on the resistant cultivar Davis. The second rating two weeks after the first rating also provided evidence of the aggressiveness of the isolates. Blackhawk, the susceptible control, had a maximum lesion number of 1089 for TN10 but had a lower number for TN40, indicating differences in severity response. Based on media comparisons, TN40 produced a greater number of spores on the SP (Stem and Pod media) compared to the sporulation of TN10 on the same media.

Figure 1 shows the relative response of all 83 isolates tested on the 12 differential genotypes. On the relative scale, positive values indicate the isolates are more pathogenic on the differential than average and negative values which indicate less pathogenicity than average. For example, Blackhawk (left most differential) is known to be highly susceptible and almost all isolates have some degree of pathogenicity (although clearly some more than others). Compare this to the Davis differential (third from left), where almost none of the isolates are pathogenic (or at least have very low pathogenicity). Davis is well-known for its tolerance to Frogeye Leaf Spot disease.

Other differentials ranged between these two extremes. The purpose of using differentials is to measure variation in pathogenicity among isolates. Figure 1 shows that this set of 12 soybean genotypes was able to differentiate isolates. We are now analyzing these data to determine if we can reduce the number of differentials while maintaining the information. Fewer differentials would reduce the time and cost of measuring individual isolate responses.

Using the differential measurements, we were able to statistically group isolates into categories based on differential responses (i.e. number of lesions, lesion size, etc.).

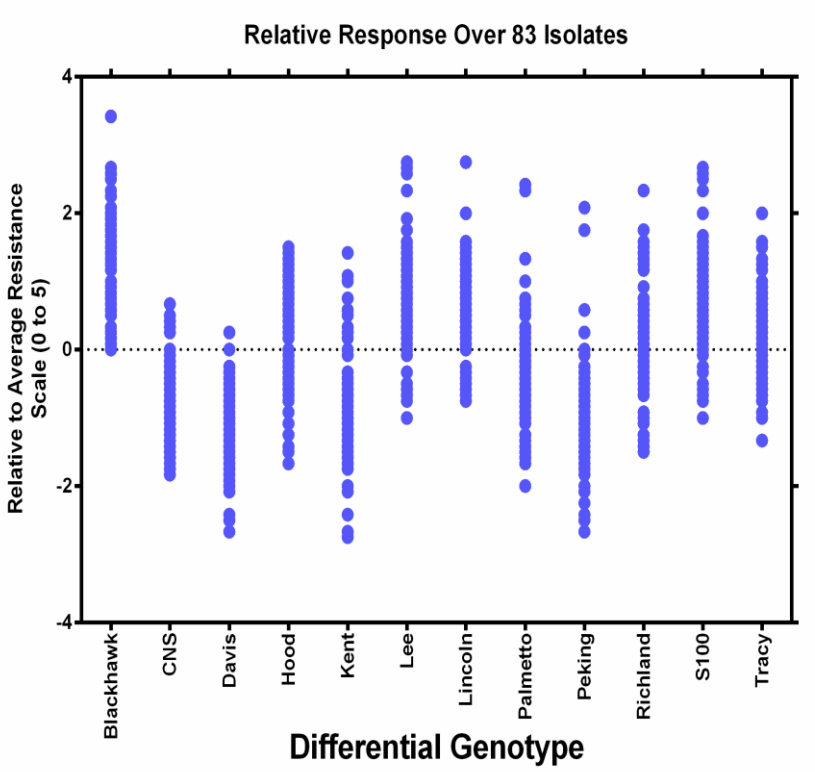

Figure 1. Relative pathogenicity responses of 83 *C. sojina* isolates on 12 soybean differentials.

In our previous research we genotyped these 83 isolates with a set of Simple Sequence Repeat (SSR) markers we developed. Using the SSRs we were able to cluster the isolates based on the markers. Those results indicated a high degree of genetic variation among the isolates [which is supported by the isolates responses to the set of soybean differentials (Figure 1)]. However, our ultimate goal was to link the isolate differential responses to the marker data. After preliminary analysis, we concluded we did not have a great enough number of markers to fully accomplish the task. We decided that a more recently developed molecular marker, Single Polymorphic Nucleotides (SNP), would be better suited. However, very few SNPs have been developed.

In order to generate additional SNP markers, we sequenced two *C. sojina* isolates and are now comparing the sequences to identify SNPs to use on these data. Two things worth noting about the sequencing: 1) one of the isolates we sequenced is a fungicide-resistant isolate and 2) we provided the sequence information to a Mississippi Valley State Master's student who is analyzing the data for SNPs as part of her graduate research (expected to graduate in May, 2017). We anticipate applying the SNP information from her thesis to our isolate data in future research.

Objectives 2 and 3 (Discussed together). (2) Phenotype soybean populations segregating for *C. sojina* resistance; and (3) Collect tissue for future DNA isolation and marker analysis.

A number of reciprocal crosses between PI 458175B and Blackhawk were made and advanced to the F_2 generation, with the F_1 generation grown in the USDA-ARS winter nursery in Puerto Rico. Five separate F_2 populations (a total of 821 F_2 plants plus parents and checks) were phenotyped for Frogeye resistance in high humidity-controlled environment chambers. In each test, Frogeye inoculum generated from isolate TN213 was applied to the plants. Figure 2 shows the appearance of Frogeye leaf spot on soybean leaves. We noted that the lesions on Blackhawk had a larger diameter than those on the F_2 plants. At the end of each test, leaf tissue was collected and processed in anticipation of DNA extraction and marker analysis.

Figure 2. An example of *C. sojina* infection

Of the five tests conducted, we concluded that disease severity was not high enough to ensure accurate disease measurement in three tests and therefore these were dropped from the analysis. The remaining two tests had average disease severity ratings of 38 and 42 % for the Blackhawk parental plants (at least 15 plants each of Blackhawk and PI 458175B were included in each test as a control).

All of the susceptible Blackhawk parental control plants had Frogeye lesions and none of PI 458175B resistant parental plants had any lesions. Considering plants with no lesions as resistant and any plant with lesions to have some degree of susceptibility, this produced susceptible:resistant (S:R) ratios of 103:47 for the first population and 130:37 for the second population. In both cases, these data statistically fit the ratios expected for a single recessive gene (Table 2).

Table 2. Frequency of F_2 plants with and without Frogeye lesions and comparison to a 3:1 ratio (with and without lesions).

	No. of F_2 Plants				
Cross	**Total**	**With Lesions**	**Without Lesions**	**Ratio**	**Prob.[1]**
PI 458175B x Blackhawk	150	103	47	3:1	P = 0.0897
PI 458175B x Blackhawk	167	130	37	3:1	P = 0.4476
Combined	**317**	**233**	**84**	**3:1**	**P = 0.5815**

[1] Probability of being different than the tested ratio, with Yate's correction factor applied.

There are three reported Frogeye resistance genes (*Rcs1*, *2*, and *3*), but only *Rcs3* has been mapped. For all three genes, resistance was reported to be dominant. The Frogeye resistance found in PI 458175B in our study appears to be recessive (Table 2) and is therefore likely to be a new gene or an alternative allele of one of the known genes. Although recessive resistance for Frogeye leaf spot has not been previously identified, it has been reported for other diseases.

For the first population, we analyzed the segregating plants with a Single Nucleotide Polymorphic (SNP) marker located near the genomic location of the *Rcs3* locus. The SNP showed good distributions between alleles (Figure 3), but showed no significant association between the resistance in the F_2 population and the *Rcs3* locus (P = 0.9442). This result indicates that the resistance found in PI 458175B is located elsewhere in the soybean genome and is therefore not likely an alternative allele for *Rcs3*. The SNP is similarly being applied to the second population for confirmation, but this work was not completed in time for this report.

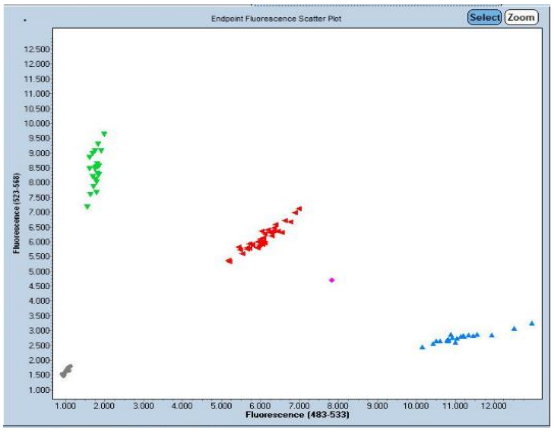

Figure 2. Distribution of SNP alleles.

FUTURE WORK

We are continuing with the analysis of the DNA sequence information and we will use that information to select SNPs covering the whole genome. When we have a sufficient amount of genomic data we will complete the analysis of the pathogenicity as related to genetic information.

For the gene discovery, we are in the process of confirming the marker analysis in the second population. We also plan one more growth chamber experiment to further confirm the recessive nature of the resistance. Recessive resistance is somewhat unusual, and in this case, is clearly indicative of a new gene/allele. The possibility of a new gene is also highlighted in that the resistance found in the first population does not map to the same locus as *Rcs3*. Given the importance of identifying new Frogeye resistance genes we need to confirm these results.

We have already initiated a new breeding program designed to incorporate the resistance found in PI 458175B into improved, better adapted germplasm. To accomplish this, PI 458175B is being crossed to a high germination-high yielding MG III breeding line and a similar MG IV breeding line, both developed at Stoneville. Additionally, when funds are available, we will determine the genomic location of the gene which will be useful in pyramiding resistance. We have generated a tremendous amount of data and are the processes of confirming results and preparing scientific reports.

IMPACTS AND BENEFITS TO MISSISSIPPI SOYBEAN PRODUCERS

As Growers in more and more States encounter fungicide-resistant *C. sojina* isolates, host-plant resistance becomes increasingly important. The results of this MSPB funded research strongly support the conclusion that the Frogeye resistance evident in PI 458175B is the result of a gene imparting recessive resistance. Recessive resistance to Frogeye Leaf Spot disease has not been previously identified.
PI 458175B originated in South Korea and has low yield as well as high lodging and high shattering. Therefore, we have initiated a breeding program to transfer the resistance into MG III and IV germplasm better adapted for the Mid-South.

END PRODUCTS–COMPLETED OR FORTHCOMING

Pathologists will be able to use the differentials developed and tested as part of this research to characterize *C. sojina* isolates collected from any location. The genomic sequence information and SNPs will be submitted to an open database maintained by the National Center for Biotechnology Information (NCBI). After we confirm the genetic results indicating recessive resistance, we will publish a report in a peer-reviewed scientific journal. At the end of the breeding program we expect a germplasm releases incorporating the resistance of PI 458175B into better adapted germplasm.