Evaluating usefulness of 10 genomic regions associated with Sudden Death Syndrome resistance in soybean

By

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TABLE OF CONTENTS

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ABSTRACT

Soybean sudden death syndrome (SDS) is one of the most important soybean diseases in the Midwestern United States soybean producing region, leading to losses of $190 million per year from 1996 to 2007. The causal agent of SDS is the soilborne fungus, *Fusarium virguliforme.* The main management practice used to reduce the impact of SDS is planting resistant soybean cultivars. Resistance to SDS is known to be quantitative with 12 known quantitative trait loci (QTL). To identify the QTL with the largest effects on reducing disease severity recombinant inbred lines (RIL) were crossed among themselves and their progeny screened for resistance to the pathogen. The current greenhouse screening method, the layer method, proved to not be sensitive enough to evaluate the progeny of the RILs, which could possess up to 12 quantitative trait loci (QTL) resistant alleles, leading to a skewed distribution towards the highly resistant side. In order to increase the sensitivity of the current greenhouse screening method, three experiments were conducted using sorghum (*Sorghum bicolor* (L.) Moench) infested with *F. virguliforme* as inoculum to modify it. The first experiment was conducted to identify the optimum inoculum density to distinguish genotypic levels of resistance to SDS when mixed homogeneously throughout the pot. The second experiment was conducted to study the association between the modified greenhouse screening method and SDS ratings from the field using eight different soybean genotypes. In the third experiment, the modified mixed method was compared to a layer method with an equivalent level of inoculum to see potential negative effects. A 1:20 inoculum:soil ratio was found to be the most effective to differentiate among resistant soybean genotypes. The modified method correlated well (r =

0.82, P<0.05) with field ratings at 36 days after planting (dap). It was also determined that the best time to evaluate genotypes was from 30 to 36 dap. This modified mixed method was capable of shifting the frequency of the SDS scores of the progeny from the RILs to approach a normal curve.

Using this modified method, the 12 QTL from five resistant parents (EF23, FH13, FH33, FH35, and PD98) obtained from the recombinant inbred line (RIL) populations 'Essex' X 'Forrest', 'Flyer' x 'Hartwig', and 'Pyramid' x 'Douglas' were evaluated. A total of 321 $F_{2:3}$-derived lines were evaluated for four disease assessment criteria, eg. disease incidence (DI), disease severity (DS), area under the disease progress curve (AUDPC), and root rot severity. Five QTL (*qRfs4, qRfs5, qRfs7, qRfs12,* and *Rfs12)* were shown to be associated with at least one disease assessment criteria across multiple populations. Two of the QTL also showed an association with more than one disease assessment criteria across populations, eg. *qRfs4* was associated with DI, AUDPC, and root rot severity and *qRfs12* was associated with AUDPC and root rot severity. These findings provide breeders with new tools to evaluate germplasm for resistance to SDS and show the 12 QTL have differences in SDS resistance. This information is of importance to develop new and improved SDS resistant cultivars.

CHAPTER 1.

GENERAL INTRODUCTION

Thesis Organization

This thesis is divided into four chapters. Chapter 1 includes a general introduction, literature review of the importance of soybean sudden death syndrome (SDS) resistant germplasm and cultivars, the history of screening methods for SDS resistance, and analysis of the genetic control of resistance traits. Chapter 2 describes the modification of a widely used greenhouse screening method to improve the ability to differentiate different levels of resistance in progeny from resistant by resistant crosses. Chapter 3 evaluates 10 resistance alleles (QTL) and their relative association with four disease assessment criteria. Chapter 4 presents the general conclusion of this research work.

General Introduction

Sudden death syndrome (SDS) of soybean (*Glycine max* L. Merr.) is caused by the soilborne fungus *Fusarium virguliforme* (formerly *Fusarium solani* f. sp. g*lycines*) (Aoki et al., 2003; Roy, 1997a). The fungus infects soybean roots causing a light brown to black discoloration leading to a reduction in root mass and root nodules (Rupe, 1989; Stephens et al., 1993b). A toxin produced by the fungus is taken up by the soybean plant and translocated to

the foliage leading to expression of leaf chlorosis and necrosis, premature defoliation and finally to pod abortion (Jin et al., 1996; Rupe et al., 1989).

The impact that premature defoliation and pod abortion have on yield are responsible for SDS being ranked second to fifth disease in importance, compared to all other soybean diseases which cause yield impact in the Midwestern US soybean producing region. From 1996 to 2007 losses due to SDS averaged $190 million a year (Robertson and Leandro, 2010; Roy et al., 1997b; Wrather and Koenning, 2009; Wrather et al., 2010).

SDS is managed through cultural practices that improve soil drainage and reduce soil compaction, but is mainly focused on planting resistant cultivars (Hershman et al., 1990; Roy et al., 1997b; Wrather et al., 1995). Resistant cultivars are primarily identified through field screens that are variable due to environmental factors (Farias Neto et al., 2006; Roy et al., 1997b; Rupe et al., 1994). Field tests are also expensive and time consuming (Hashmi et al., 2005) and unpractical at early stages in breeding programs, when large numbers of genotypes need to be evaluated. To remediate the situation, greenhouse and growth chamber screening methods have been developed using a wide array of inoculation techniques (Gray and Achenbach, 1996; Hartman et al., 1997; Hashmi et al., 2005; Klingelfuss et al., 2002; Melgar and Roy, 1994; Mueller et al., 2002; Njiti et al., 2001; Ortiz-Ribbing and Eastburn, 2004; Stephens et al., 1993a). The method that is most widely used and was first developed (Hartman et al., 1997) is known as the layer method. The layer method (Hartman et al., 1997) has been the method adopted by the soybean breeding community for its ease of use and the possibility to also screen for root infection. It correlates well to field data. The reported literature has the correlations ranging from $r = 0.43$ (Hartman et al., 1997) to $r = 0.84$ (Hashmi et al., 2005). The

latter correlation increased when use of water baths was incorporated to facilitate the maintenance of soil temperature.

Inheritance of resistance to SDS is quantitative in nature (Chang et al, 1996; Hnetkovsky et al., 1996; Iqbal et al, 2001; Kassem et al, 2007; Kazi et al., 2008; Lightfoot et al., 2005; Meksem et al, 1999; Njiti et al., 1997; Njiti et al., 2002; Prabhu et al., 1999), therefore it is assumed that accumulation of multiple resistance genes in a single soybean genotype would allow for resistance to be expressed even at high inoculum density levels in the soil. Currently, 12 QTL associated with SDS resistance have been identified in three recombinant inbred line (RIL) populations, 'Essex' x 'Forrest' (EF), 'Pyramid' x 'Douglas' (PD), and 'Flyer' x 'Hartwig' (FH) (Kazi et al., 2008). Two of the QTL are common across the three populations; one is on linkage group (LG) C2 and the other one on LG G (Iqbal et al, 2001; Kazi et al., 2008; Lightfoot et al, 2005; Njiti et al., 2002; Prabhu et al., 1999). Another two QTL that are common to RIL populations EF and FH, one on LG G and one on LG I (Chang et al, 1996; Kassem et al., 2007; Kazi et al., 2008; Prabhu et al., 1999). The RIL populations PD and FH have two QTL in common, one on LG D2 and one on LG N (Kazi et al., 2008; Njiti et al., 2002; Prabhu et al., 1999). The other six QTL are unique to the corresponding populations in which they were detected, therefore they could be considered as population-specific for the three populations mentioned. Up to date, there is no published information on relative importance of the 12 QTL compared to one another, even if they introgressed in similar genetic backgrounds.

The first objective of the study was to determine if a modified inoculation method for evaluating SDS foliar and root rot severity could be developed for screening soybean genotypes derived from crosses of two resistant parents, without disrupting seed germination. This

objective was accomplished by evaluating genotypic response of soybeans to a range of *F. virguliforme* inoculum density levels created by homogeneously mixing infested sorghum seed with soil in different proportions.

The second objective of the study reported herein was to determine if the known QTL previously determined as associated with SDS resistance and detected in three different RIL populations may all be required to confer SDS resistant which would be superior to that on existing commercial cultivars. The importance of this objective is that it would be challenging for breeding programs to introgress the 12 QTL in just one genotype. With this being an important limitation in breeding for resistance, research was needed to determine how many and which of the 12 QTL have the largest and most important contribution to SDS resistance in soybean.

Literature Review

Economic impact of SDS in soybean production

SDS reduces soybean yields in four of the top eight soybean producing countries in the world, Argentina, Brazil, Canada, and the USA. In the year 2006 alone, yield was reduced by 1.849 million metric tons worldwide (Wrather, 2010). In the USA, SDS is ranked from second to fifth as the most important soybean disease compared to other soybean diseases in relation to yield impact. Yield suppression of SDS in the USA increased from 3.7 million bushels in 1996 to 34.5 million bushels in 2009 with two years, 2000 (75.7 million bushels lost) and 2004 (42.3 million bushels lost), as outliers (Koenning and Wrather, 2010; Wrather and Koenning, 2009). From 1996 to 2007, losses averaged $190 million a year in the Midwestern US soybean

producing region and in 2010 losses were expected to exceed 20% in some production fields (Robertson and Leandro, 2010).

Several studies have attempted to estimate the incremental yield loss in relation to different disease assessment criteria. Gibson et al. (1994) found that every 1% increase in SDS disease incidence (DI) resulted in a 0.1 to 0.5 bu/A increase in yield loss. Njiti et al. (1994) considered SDS foliar leaf scorch severity and found that each unit increase equaled a yield loss of 12 to 22%. Using SDS foliar disease index, Luo et al. (2000) claimed each unit increase was equivalent to 18 to 29 kg/ha or 0.26 to 0.43 bu/A in yield loss.

Screening methods for SDS resistance

Selection of resistant germplasm or cultivars has been done primarily by field screenings. Southern Illinois University has performed SDS variety trials since 2005. However, environmental factors, such as ambient temperature and moisture, play important roles in disease expression (Farias Neto et al., 2006; Roy et al., 1997b; Rupe et al., 1994), creating challenges to evaluation of cultivars under field conditions (Njiti et al., 2001). Unpredictable weather and field distribution of the inoculum density can lead to inconsistent cultivar rankings among field screening trials. These challenges were overcome by placing inoculum in the soil in close proximity to the seed and irrigating during the reproductive growth stages. The practice resulted in disease pressure high enough to distinguish partially resistant from susceptible genotypes (Farias Neto et al., 2006).

Field testing may be the primary way to screen for SDS resistant germplasm and cultivars, however, this is expensive and time consuming (Hashmi et al., 2005), especially at

early stages of breeding when large numbers of genotypes need to be screened. Also, due to weather conditions and geographic location, field tests at certain locations can only be performed once a year during the summer.

These limitations lead to the development of greenhouse and growth chamber assays to evaluate soybean germplasm and cultivars for resistance to SDS under controlled conditions. Many different inoculation techniques were developed. Stephens et al. (1993a) looked at the correlation of a technique developed by Lim (1991) which places three to five infested oat seed around the crown of soybean roots at V3 stage and found values ranging from $r = 0.60$ to $r = 0.91$. Hartman et al. (1997) used 5 cm^3 infested red sorghum kernels placed in a furrow 2 cm below the soybean seeds in large trays in the greenhouse and found a significant ($P = 0.05$) but low correlation, $r = 0.43$ with field microplots. Njiti et al. (2001) transplanted two week old seedlings into sand soil treated with one of three spore suspensions rates, low (3.3×10^3), moderate (5×10^3), and high (10^4) and found correlations with the field to range from $r = 0.40$ to $r = 0.60$. A method allowing the tips of toothpicks to become infested and then inserted into the center of the hypocotyl of 12 to 15 day old soybean plants was shown by Klingelfuss et al. (2002) and Melgar and Roy (1994) to reproduce SDS foliar leaf scorch. Huang and Hartman (1998) used a culture filtrate method developed by Jin et al, (1996) where soybeans cut at the crown of the stem were placed into diluted culture filtrate. The authors correlated the method to the Hartman et al. (1997) method and found the two were highly correlated ($r = 0.94$). Hashmi et al. (2005) built off of the layer method developed by Hartman et al. (1997) by using 15 white sorghum kernels in a layer, in cones incubated in water baths at 24°C. The correlation with field results was high and significant ($r = 0.84$). The layer method has been the most

commonly used method to screen for SDS resistance due to its ease of use, high correlation to the field, and ability to screen for root infection.

Inheritance of resistance to SDS

Genetic resistance to SDS has been found to be polygenic or quantitative(Chang et al, 1996; Hnetkovsky et al., 1996; Iqbal et al, 2001; Kassem et al, 2007; Kazi et al., 2008; Lightfoot et al., 2005; Meksem et al, 1999; Njiti et al., 1997; Njiti et al., 2002; Prabhu et al., 1999). In the recombinant inbred lines (RIL) developed from the cross of the partially resistant parent 'Forrest' (Hartwig and Epps, 1973) and the susceptible parent 'Essex' (Smith and Camper, 1973), nine quantitative trait loci (QTL) were identified associated with SDS resistance (Chang et al, 1996; Hnetkovsky et al., 1996; Iqbal et al, 2001; Kassem et al, 2007; Lightfoot et al., 2005; Meksem et al, 1999). Forrest has been shown to be partially resistant to both foliar leaf scorch and root infection (Njiti et al., 1997). Four of the eight QTL were placed on linkage group G, with the resistant allele coming from the partially resistant parent Forrest. Linkage groups C2 and I each contained one QTL with the resistant allele coming from the susceptible parent Essex (Iqbal et al, 2001; Lightfoot et al, 2005). Additional Forrest resistant QTL alleles were also identified on linkage groups I, J, and N (Chang et al, 1996; Kassem et al., 2007).

RIL derived from the cross of the susceptible parent 'Douglas' (Nickell et al., 1982) and the partially resistant parent 'Pyramid' (Myers and Schmidt, 1988), identified four QTL associated with SDS resistance (Njiti et al., 2002). Similarly to Forrest, Pyramid has been shown to be partially resistant to foliar leaf scorch and susceptible to root rot (Gibson et al., 1994). Two QTL were mapped to linkage group G, and one QTL to linkage group N with the resistant

alleles coming from Pyramid (Njiti et al., 2002). In this RIL population, there also was one QTL that mapped to linkage group C2, in this case Douglas provided the resistant allele (Njiti et al., 2002).

An additional RIL mapping population developed at Southern Illinois University by crossing the partially resistant 'Hartwig' (Anand, 1992) cultivar and the susceptible parent 'Flyer' (McBlain et al., 1990), also mapped four QTL associated with SDS resistance (Kazi et al., 2007; Prabhu et al., 1999). As in previous populations, Hartwig has been shown to be partially resistant to both foliar leaf scorch and root infection (Hartman et al., 1997; Njiti et al., 1997). Two QTL mapped to linkage group G with the beneficial allele coming from Hartwig (Kazi et al,. 2008; Prabhu et al., 1999). One QTL mapped to linkage group C2 and another mapped to linkage group D2, both having the beneficial allele coming from Flyer (Kazi et al., 2008).

These results confirm the quantitative nature of the inheritance of soybean resistance to SDS. They also suggest possible difficulties in breeding for improved resistance to SDS, particularly if all the QTL identified turn out to have significant contributions to the final expression of SDS resistance.

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CHAPTER 2.

SCREENING METHOD FOR DISTINGUISHING SOYBEAN RESISTANCE TO *FUSARIUM VIRGULIFORME* IN RESISTANT BY RESISTANT CROSSES

Abstract

Current greenhouse screening methods for soybean (*Glycine max* (L.) Merr.) sudden death syndrome (SDS) are not sensitive enough to evaluate progeny of resistant by resistant crosses, which possess up to 12 quantitative trait loci (QTL). The objective of the study was to modify the current greenhouse screening method, the layer method, to increase its sensitivity in distinguishing SDS resistant lines. Three experiments were conducted using infested sorghum (*Sorghum bicolor* (L.) Moench) as inoculum. In experiment 1, five different inoculum densities (1:1, 1:5, 1:10, 1:15, 1:20 inoculum:soil ratio), were compared, and the optimum inoculum density to distinguish genotypes was identified. In experiment 2, the association between SDS field ratings and greenhouse severity scores was determined. In experiment 3, the inoculum layer method was compared to the proposed modified mixed method. Inoculum ratios 1:15 and 1:20 showed the largest difference in SDS ratings between soybean genotypes. The best time to assess disease severity was 30 to 36 days after planting (dap), with the highest correlation with field ratings at 36 dap ($r^2 = 0.82$, $P<0.05$). The proposed modified method shifted the frequency distribution of SDS scores towards a normal curve. The findings indicate that the modified screening method using a 1:20 inoculum:soil ratio correlated well with field

data, and provided adequate screening of lines possessing up to 12 SDS resistant QTLs, without negatively impacting germination.

Introduction

Sudden death syndrome (SDS) of soybean (*Glycine max* L. Merr.) is caused by the soilborne fungus *Fusarium virguliforme* (formerly *Fusarium solani* f. sp. g*lycines*) (Aoki et al., 2003; Roy, 1997a). SDS reduces yield in most of the major soybean producing countries (Wrather, 2010). In the U.S. it ranked second to fifth compared to all other soybean diseases regarding yield impact in the Midwestern soybean producing region from 1996 to 2007 (Roy et al., 1997b; Wrather and Koenning, 2009; Wrather et al., 2010). The pathogen infects soybean roots causing a light brown to black discoloration and leading to a reduction in root mass and root nodules (Rupe, 1989; Stephens et al., 1993a). Above ground symptoms of foliar chlorosis and necrosis are caused by the translocation of toxins released by the pathogen, and can cause premature defoliation and pod abortion (Jin et al., 1996a; Rupe et al., 1989). Management of this disease focuses on planting resistant cultivars and using cultural practices that improve soil drainage and reduce compaction (Hershman et al., 1990; Roy et al., 1997b; Wrather et al., 1995).

Identification of resistant cultivars has been done primarily in field screenings. However, environmental factors, such as temperature and moisture, play important roles in disease expression (Farias Neto et al., 2006; Roy et al., 1997b; Rupe et al., 1994), creating challenges in the evaluation of cultivars in field conditions (Njiti et al., 2001). Differences in inoculum density distribution in soils and unpredictable weather are some of the factors that may cause

inconsistent cultivar rankings among field screening trials. Artificially placing inoculum in the soil in close proximity to the seed and irrigating during reproductive growth stages has helped to overcome some of these challenges, resulting in adequate disease pressure to distinguish partially resistant from susceptible genotypes (Farias Neto et al., 2006).

While field tests have been the primary method used to screen soybean cultivars for SDS resistance, they are expensive and time consuming (Hashmi et al., 2005), particularly if large numbers of genotypes are screened as it occurs at early stages of breeding. Additionally, in the northern and southern hemispheres, field tests are only planted during summer, which only allows a single field evaluation per year.

Greenhouse and growth chamber assays are also commonly used to evaluate soybean cultivars for resistance to SDS (Farias Neto et al., 2008; Hashmi et al., 2005). An array of different inoculation methods have been developed, such as soil amendments with oat seeds (Stephens et al., 1993b; Melgar and Roy, 1994), sorghum seeds (Hartman et al., 1997; Mueller et al., 2002), cornmeal (Gray and Achenbach, 1996; Njiti et al., 2001) and toothpicks (Klingelfuss et al., 2002; Melgar and Roy, 1994) infested with *F. virguliforme*. In some methods, inoculum is homogeneously mixed with the growing plant media prior to planting of soybean seeds and applied in a layer below the seeds (Hashmi et al., 2005; Njiti et al., 2001). In contrast, Ortiz-Ribbing and Eastburn (2004), placed soil mixed with ground infested sorghum seeds on the outer edges of pots to selectively infect soybean lateral roots while allowing the taproot to grow into non-infested soil.

Culture filtrates of *F. virguliforme* have also been used to induce SDS symptoms on soybean seedlings (Jin et al., 1996b). Stems of cut soybean seedlings are placed into diluted *F.*

virguliforme culture filtrates containing phytotoxins produced by the fungus. This protocol has proven to be an effective method for identifying resistance to foliar symptoms (Li et al., 1999). Another method using culture filtrate involves leaf discs immersed in culture filtrate and then placed on petri dishes for evaluation of SDS reactions (Brar et al., 2011).

Screening methods using soybean seeds planted in soil amended with sorghum infested with *F. virguliforme* are, however, the most widely used in evaluating soybean resistance to SDS. Reliability of the screening methods is of key importance for development of improved resistance to SDS, so greenhouse methods have been compared to field screening tests. Farias Neto et al. (2008) determined that application of infested sorghum in a layer in planting cones correlated better with field results (r^2 ranged from 0.61 to 0.74) than the application of inoculum in furrows in trays. Hashmi et al. (2005) compared three greenhouse assays, and found that the application of a layer of inoculum in cones incubated in water baths showed the greatest correlation to field symptoms ($r^2 = 0.84$).

Since SDS resistance is quantitatively inherited (Hnetkovsky et al., 1996; Iqbal et al., 2001; Njiti et al., 2002), accumulation of multiple resistance genes in a soybean genotype may lead to superior levels of resistance. For this reason, Iowa State University (ISU) breeders are interested in screening soybean progeny from resistant by resistant parent crosses. In preliminary screenings, the infested-sorghum layer method used to evaluate soybean lines at ISU lead to a distribution of SDS frequency ratings skewed to the left of the curve, i.e., to the resistant side of the disease symptom expression curve. These results suggest the method may not be able to distinguish resistance levels determined by single QTL in progeny from crosses of two resistant parents. To improve assay sensitivity, it was hypothesized that an increase in

inoculum density might be necessary. Based on prior work conducted in Cianzio lab, however, this increase in inoculum raised concerns about a negative impact on soybean seed germination (Peter Lundeen, personal communication, 2011).

The objective of our study was to determine if a modified inoculation method for evaluating SDS foliar and root rot severity could be developed for screening soybean genotypes derived from crosses of two resistant parents, without disrupting seed germination. The objective was accomplished by evaluating genotypic response of soybeans to a range of *F. virguliforme* inoculum levels created by homogeneously mixing infested sorghum seed with soil in different proportions.

Materials and Methods

Screening of Resistant x Resistant (RxR) breeding population using layer method

Nine populations with different parentage, each composed of 6 to 62 soybean lines (overall total of 324 lines plus the parents) were screened for foliar and root rot reaction to SDS. Parents of the nine populations were combinations of lines derived from 'Essex' (Smith and Camper, 1973) by 'Forrest' (Hartwig and Epps, 1973), 'Pyramid' (Myers and Schmidt, 1988) by 'Douglas' (Nickell et al., 1982), and 'Flyer' (McBlain et al., 1990) by 'Hartwig' (Anand, 1992) crosses. According to molecular analysis conducted at the Lightfoot lab (David Lightfoot, personal communication, 2011), resistant parents contained 12 of the 18 currently identified QTL for SDS resistance.

The lines were screened for SDS foliar and root rot reactions applying the method used at the ISU soybean resistance breeding program. The method, which is a modification by Peter Lundeen (personal communication, 2011) of the layer method developed by Hartman et al. (1997) and patented by David Lightfoot (Lightfoot et al., 2007), consists of mixing ground *F. virguliforme* infested sorghum (*Sorghum bicolor* (L.) Moench) kernels with sterile soil:sand mix in a 1:5 sorghum to soil ratio. Styrofoam cups, 240 ml size, were filled with 150 ml of sterile soil mix, covered with 30 ml of the inoculum mix, and followed by 30 ml of soil mix. A soybean seed was then placed on top of the soil and covered with an additional 30 ml of soil mix. Cups were placed in the greenhouse at 23°C ±4 with a 16 hour photoperiod, and watered once daily to maintain high soil moisture. After 28 days, soybean plants (lines) were assessed for leaf scorch severity as percentage of leaf area showing typical SDS symptoms.

Development of modified screening method

Plant material

A set of eight soybean genotypes, representing resistant, moderately resistant, moderately susceptible and susceptible reactions based on the SDS commercial variety trials (http://soybean.siuc.edu/), were used to develop a modified screening method for SDS resistance. The resistant genotypes were 'Ripley', maturity group (MG) IV (Cooper et al., 1990), and 'MN1606SP', MG I, identified in SDS screening tests (Cathy Schmidt, personal communication, 2010). The 'MN 1606' line had been selected as food grade soybean by the Minnesota breeding project (Dr. Jim Orf, personal communication, 2010), and had never been tested for SDS resistance. The moderate resistant varieties included 'AR10-SDS', MG I (Cianzio et al., 2008) and 'MN1805SP', MG I (Dr. Jim Orf, personal communication, 2010; SDS variety

trials, http://soybean.siuc.edu/). Moderate susceptible varieties included 'MYC5171' MG I (SDS variety trials, http://soybean.siuc.edu/) and '299N' MG II (SDS variety trials, http://soybean.siuc.edu/). 299N was developed by Beck's Hybrid and MYC5171 by Mycogen Seeds, both used with permission. Susceptible varieties were 'Spencer', MG IV (Wilcox et al., 1989) and 'MAC02-4757' MG IV developed by Dr. Sleper and Kerry Clark at the University of Missouri (Cathy Schmidt, personal communication, 2011). Field performance of the eight genotypes along with the respective foliar classification is shown in Table 2.

Inoculum preparation and planting

F. virguliforme isolate NE305 obtained in 2006 from roots of a SDS symptomatic plant from a production field in Nevada, IA was used in all experiments. The isolate was grown on PDA amended with 0.150 g per liter of streptomycin sulfate and chlortetracycline hydrochloride for six weeks under continuous light at room temperature. Five hundred grams of sterile white sorghum seeds in quart mason jars were autoclaved for one hour on two consecutive days, and inoculated with five mycelia plugs (7 mm diameter) of a *F. virguliforme* culture. Jars were incubated at room temperature for two weeks, and shaken daily for 1 to 2 minutes to ensure uniform fungal growth.

To create five inoculum concentrations, the infested sorghum inoculum was homogeneously mixed with pasteurized 2:1 sand to soil mixture at the densities of 1:1, 1:5, 1:10, 1:15, and 1:20 inoculum to sand/soil (v/v). Sterile sorghum mixed with pasteurized sand/soil at the same ratios was used for controls. Seeds of each of the eight soybean genotypes were planted into the infested and sterile soil mixtures in 240 ml styrofoam cups.

Two soybean seeds were planted in the center of the cup at a depth of 2 cm, and seedlings were thinned to one plant per cup after emergence. The cups were incubated in a growth chamber at 23°C ±1 with a 16 hour photoperiod. The plants were watered once daily to maintain soil moisture.

Experimental design

Three experiments were performed to determine if a modified inoculation method could be used to screen soybean genotypes derived from crosses of resistant by resistant parents. Experiment 1 was established to identify an increased inoculum density capable of distinguishing a resistant from a susceptible cultivar without being detrimental to seed germination. The experiment was established according to a randomized complete block design, with genotype and inoculum density as factorial treatments. The experiment was repeated twice. In the first two runs, five inoculum density treatments, 1:1, 1:5, 1:10, 1:15, and 1:20 inoculum to sand/soil (v/v), and two genotype treatments, Ripley (resistant) and Spencer (susceptible), were used, with three replicate cups per treatment combination. In the third run, the 1:1 inoculum treatment was not included due to low seed germination. In addition, a third soybean genotype, the moderately resistant germplasm line AR10-SDS, was added, and the number of replicate cups was increased to five.

Experiment 2 was conducted to verify effectiveness of the optimum inoculum density determined in Experiment 1 using soybean genotypes with a wider range of SDS foliar resistance classifications. The experiment was established according to a randomized complete block design, and repeated twice. The 1:20 inoculum density and the eight soybean genotypes

described above (two for each of the resistant, moderate resistant, moderate susceptible, and susceptible reaction classifications) were used, with three replicate cups planted in each of the three runs.

In Experiment 3, the ISU inoculum layer method was compared to the inoculum mixing method of Experiment 2 using the eight genotypes. For the layer method, styrofoam cups were filled with 150 ml of 2:1 soil mix, with 12 ml of infested sorghum placed in a layer, and covered with 30 ml of the soil mix. Three soybean seeds were then placed in the cup, and covered with 30 ml of soil mix. The layer method used 12 ml of infested sorghum, to match the inoculum density in the mixing method. Soybean germination rate was recorded 21 days after planting (dap). The experiment had three replications and was conducted once. In addition, the 324 lines and parents from the R x R breeding population were planted in a randomized complete block design using the 1:20 mixed method described above with two repetitions.

Disease assessment

In Experiment 1, SDS symptoms were evaluated 21, 28, and 35 dap. In Experiment 2 and 3, symptoms were evaluated at 21, 24, 27, 30, 33, and 36 dap. Disease severity (DS), scored as a percentage of leaf area showing typical SDS symptoms, and shoot height in cm were evaluated at each assessment time. In addition, root rot, root dry weight, root length, leaf area, and shoot dry weight were collected 35 dap in Experiment 1 and 36 dap in Experiment 2. Root rot was scored as the percentage of root area showing brown to black discoloration. To determine plant dry weights, individual shoots and roots were dried in an oven at 75°C for 24

hours, and recorded in g to two decimal places. Area under the disease progress curve (AUDPC) was calculated in Experiment 2 using the midpoint rule (Campbell and Madden, 1990).

Statistical analysis

For Experiment 1 and 2, runs were combined and analysis of variance was performed using the PROC MIXED procedure of SAS version 9.1 (SAS Institute, Cary, NC) on foliar DS, root rot, root dry weight, leaf area, and shoot dry weight. Genotype and inoculum density were considered fixed effects in Experiment 1, and genotype was considered a fixed effect in Experiment 2. In both experiments, run, replication and their interactions were considered random effects. The LSMEANS procedure and PDIFF function were used to detect differences in means for foliar and root rot DS with $\alpha = 0.05$. To calculate the rank correlation between data collected and the field data collected by the SDS commercial variety trials, the Spearman correlation analysis was conducted using PROC CORR procedure of SAS. A PROC UNIVARIATE procedure from SAS to test for normality distribution was run.

Results

Identification of optimum inoculum density (Experiment 1)

Overall means for severity of SDS foliar symptoms increased as inoculum density increased for both the resistant and moderate resistant genotype, but foliar severity did not differ among inoculum densities in the susceptible genotype (Figure 1a). Root rot severity increased as inoculum density increased for all genotypes (Figure 1b). Soybeans grown in the

1:1 inoculum density had a 3% germination rate in all runs of the experiment. Germination rate for the three genotypes increased as the inoculum densities decreased from 1:1 to 1:20 (Table 1).

There was a significant effect of genotype on disease severity at each inoculum density, with the resistant showing lower ($P<0.05$) foliar severity than the susceptible at all inoculum densities. The largest difference in foliar severity between the resistant and susceptible genotype was observed at the 1:20 ratio. At this ratio, the susceptible genotype showed significantly less root rot compared to the resistant genotype ($P<0.01$) (Figure 1b), but a greater ($P<0.05$) loss in root dry weight than the resistant genotype (Figure 1c). Root dry weight loss was inversely related to inoculum density for the resistant genotype, but the susceptible genotype did not show differences in root dry weight loss among the inoculum densities. No foliar symptoms were developed on control plants but root rot was observed in some control plants (data not shown). The largest difference in foliar disease severity was observed at 35 dap on the 1:20 ratio treatment.

Validation of the modified method using multiple genotypes (Experiment 2)

At 24 and 27 dap, the resistant and moderately resistant genotypes had lower ($P<0.05$) foliar severity than the moderately susceptible and susceptible genotypes (Figure 2a). By 30 dap, the resistant genotypes had a lower ($P<0.05$) foliar severity than the other three genotypes (Figure 2a) and the moderately resistant genotypes also showed lower ($P<0.05$) foliar severity than the susceptible genotypes. At 30, 33 and 36 dap foliar severity correlated to the field data with r^2 values of 0.77, 0.82, and 0.82 ($P<0.05$).

The area under the disease progress curve (AUDPC) was smaller ($P<0.01$) for the resistant and moderately resistant genotypes compared to the susceptible and moderately susceptible genotypes (Figure 2b). However, genotypes classified as moderately resistant and moderately susceptible did not differ statistically from their respective groups (resistant and susceptible) for AUDPC. Spearman rank correlations run on all disease and plant growth measures showed no significant correlations except for foliar severity and AUDCP ($r^2= 0.95$, $P<0.001$), foliar severity and root dry weight ($r^2= -0.83$, $P<0.05$), and AUDPC and root dry weight ($r^2 = -0.74$, $P<0.05$). Foliar symptom severity was not correlated ($P=0.35$), with root rot severity. Nevertheless, genotypes classified as resistant generally had lower root rot ($P<0.01$) than the susceptible genotypes (Figure 3a). Foliar severity at 30 to 36 dap was correlated with root dry weight ($r^2 = -0.83$, $P<0.05$) (Figure 3b) and shoot dry weight ($r^2 = 0.74$, $P<0.05$) (Figure 3c).

Comparison of layer method with mixing method (Experiment 3)

At 21 dap, seed germination was significantly ($P<0.05$) lower in the layer method (47%) than in the mixed method (100%). Controls in both methods did not significantly differ from 100% germination. Also, SDS foliar severity did not differ between the two methods (data not shown). With the layer method used at ISU, the distribution of SDS severity ratings (Figure 4a) on the 324 lines generated from resistant by resistant crosses was skewed towards the low severity side of the curve in each of the three runs of the experiment, with a mean foliar severity of 11%. With the modified method, the 324 lines had a mean foliar severity of 18% (Figure 4b). While both methods curves were non-normal, the mixed method curve approached

normality (P=0.003) better than the layer method (P<0.0001). The layer method had a kurtosis of 0.80 and skewness of 1.05. The mixed method had a kurtosis of 0.05 and skewness of 0.52.

Discussion

This work identified a screening method capable of distinguishing soybean lines derived from populations in which both parents were resistant to SDS. The method differs from previously published protocols by using a higher inoculum level that is mixed homogeneously with soil. Using this method, resistant and moderately resistant cultivars could be distinguished at 30 dap with no negative effects on germination. The mixed method was also capable of shifting the SDS severity frequency curve of a population of soybean lines derived from resistant by resistant crosses to the right, i.e., to higher mean foliar severity, compared to the previously used layer method. The increased inoculum pressure used to screen the populations will improve the ability to phenotypically identify the most resistant lines and draw conclusions about different QTL effects present in the lines/populations.

The increase in inoculum density in soil increased SDS foliar severity, in a similar way as previously reported (Gongora-Canul et al., in press; Gray and Achenbach, 1996; Njiti et al., 2001). In the present study, the quantitative resistance traits in the resistant cultivar Ripley were overcome at the highest inoculum densities (1:1, 1:5, and 1:10), leading to similar foliar reaction as the susceptible cv. Spencer. This breakdown in resistance was also seen by Njiti et al. (2001) with progeny from an Essex x Forrest cross. With high soil inoculum rate (wt/wt), Gray and Achenbach (1996) also saw a breakdown in resistance in the cultivar Ripley. These

observations may be explained by the quantitative nature of SDS resistance, in which even lines classified as resistant may develop symptoms under high inoculum levels (Parlevliet, 1979). Since quantitative resistance is controlled by several genes, it may be possible to develop resistant lines possessing higher number of beneficial alleles, which will maintain the resistance trait even at high levels of inoculum density. The susceptible cultivars used in the study did not show significant changes in phenotype in response to increasing inoculum densities.

Ranking of the eight genotypes consistently followed the same trend in the mixed treatment compared to the classical layer method; the resistant varieties had the lowest severity, with severity increasing towards the most susceptible genotypes. This result indicates that the mixed method with a higher inoculum density than traditionally used is reliable. Ranking of the eight genotypes also correlated well with the field data, showing the method could be used to predict SDS foliar reaction in the field. Similar correlations between greenhouse and field phenotypic results have also been observed by others using a variety of assays (Farias Neto et al., 2008; Gray and Achenbach, 1996; Hashmi et al., 2005; Klingelfuss et al., 2002; Li et al., 1999; Mueller et al., 2002; Njiti et al., 2001; Stephens et al., 1993b). While the genotype ranking does not match up identically with field ratings, the observed rank changes are not of concern since the genotypes known to be resistant still performed the best.

At 30 dap resistant, moderately resistant, and susceptible lines are capable of being distinguished by the mixed method, suggesting that this date might be the optimum time to screen for foliar resistance. This distinction is also seen at 33 and 36 dap, however, screening at 30 dap will allow for results 3 to 6 days earlier. This is different from most other screening methods, but similar to Mueller et al. (2002). Typically plants are assessed once at 21 dap or

days after inoculation for foliar leaf scorch (Farias Neto et al., 2008; Gray and Achenbach, 1996; Klingelfuss et al., 2002; Li et al., 1999; Njiti et al., 2001). Although the 30 dap assessment, identified as the best evaluation time in the mixed method, is 9 days later than the layer method this difference is offset by the fact the mixed method allows efficient differentiation among resistant lines possessing large numbers of QTL associated with resistance to SDS.

Root rot severity evaluated 36 dap on the eight varieties did not correlate with foliar severity classifications, with most genotypes showing high levels of root rot. In contrast, root dry weight correlated well (r^2 = -0.83,$P<0.05$) with foliar severity. This lack of correlation between root rot and foliar severity has been previously observed (Gongora-Canul et al., in press; Gray and Achenbach, 1996; Luo et al., 1999; Luo et al., 2000). Gongora-Canul et al. (in press), for example, observed this lack of correlation when root rot was assessed at the end of the experiment, but found strong correlation (r^2 = 0.96, $P<0.01$) when roots were assessed earlier than leaves. It is worthy to note that soybean variety Beck 299N showed the highest foliar severity and the second lowest root rot severity. This may confirm results by Triwitayakorn et al. (2005), who demonstrated the existence of QTL for foliar resistance and others associated with root rot resistance.

One of the major problems with increasing inoculum level is the negative effect this may have on seed germination. In the study reported herein, comparison of the layer and mixed methods using equal inoculum densities showed that germination was reduced by approximately 50% in the layer method when compared to the mixed method. A possible explanation for this reduced germination in the layer method may be that the mixed method delays infection at early stages of plant development, thus allowing healthier root formation in

the soybean seedlings. This is supported by findings by Gongora-Canul and Leandro (2011), showing that inoculation of older soybean seedlings resulted in less foliar and root rot severity than when plants were inoculated at germination.

Evaluation of the 324 progeny lines from the resistant by resistant crosses using the mixed method showed an increase in SDS severity symptoms compared to the ISU layer method. By being a discriminator test, the mixed method changed the distribution curve, showing an approach to normality.

A normal distribution curve allows for an ANOVA test to be run to evaluate differences in QTL, and associations between QTL and phenotypic information. The improvement observed in the distribution curve achieved by the mixed method will permit use of additional statistical techniques, i.e. transformation of data, to significantly reduce the effect of non-normality. In the layer method, 117 lines showed no disease symptoms, not allowing for the distinction of QTL in these lines. The higher inoculum density mixed method reduced the 117 lines to 1 line showing no disease symptoms. The changes observed by using a screening method that has increased discriminate power will allow for a clearer distinction of different QTL in the most resistant soybean lines.

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Tables

Table 1. Germination rates (%) for three soybean genotypes planted into five different inoculum densities of *Fusarium virguliforme* averaged across three repetitions grown in the growth chamber at 23°C ±1 with a 16 hour photoperiod.

	Inoculum density				
Genotype	1:1	1:5	1:10	1:15	1:20
AR10-SDS	N/A[†]	20%	28%	84%	96%
Ripley	0%	16%	80%	91%	92%
Spencer	7%	41%	70%	95%	92%

[†]AR10-SDS was planted in the third repetition only and the 1:1 inoculum density was only used in the first two repetitions

Table 2. *Fusarium virguliforme* foliar severity disease index (DX) from previous field data collected in the SDS commercial variety trials, disease severity (DS) data collected from the 1:20 inoculum density mixed method grown in the growth chamber at 23°C ±1 with a 16 hour photoperiod and evaluated at 36 days after planting and the corresponding rank of eight soybean genotypes.

Genotype	Classification	Previous Field Data		Mixed Method Data	
		DX[†]	Rank[§]	DS[‡]	Rank[§]
Ripley	Resistant	2	1	44	2
MN1606SP	Resistant	6	3	25	1
AR10-SDS	Mod. Resistant	2	1	48	3
MN1805SP	Mod. Resistant	6	3	48	4
MYC5171	Mod. Susceptible	16	5	56	5
Beck 299N	Mod. Susceptible	26	6	70	8
Spencer	Susceptible	43	8	69	7
MAC02-4757	Susceptible	40	7	56	6

[†]DX is averaged over multiple environments: Ripley 58, MN1606SP 14, AR10-SDS 5, MN1805SP 5, MYC5171 21, Beck 299N 18, Spencer 52, and MAC02-4757 24

[‡]DS is averaged over three runs with foliar severity data collected at 36 DAP

[§]Rank is based on the DX for the previous field data or DS for the mixed method data

Figures

Figure 1. Reaction to *Fusarium virguliforme* of genotypes Ripley and Spencer grown in four inoculum densities (1:5, 1:10, 1:15, and 1:20) averaged over three runs and assessed at 35 days after planting. Plants were grown in a growth chamber at 23°C ±1 with a 16 hour photoperiod. (**A**) Foliar leaf scorch severity (%) (**B**) Root rot severity (%) (**C**) Percent loss of root dry weight relative to control.

Figure 2. Reaction to *Fusarium virguliforme* reaction at the 1:20 inoculum density for eight genotypes (MN1606SP, Ripley, AR10-SDS, MN1805SP, Beck 299N, MYC5171, MAC024757, Spencer) **(A)** Disease progress curve of foliar leaf scorch severity (%) assessed at six time points (21, 24, 27, 30, 33, and 36 days after planting) and (**B**) Area under the disease progress curve (AUDPC). Plants were grown in a growth chamber at 23°C ±1 with a 16 hour photoperiod.

Figure 3. Reaction to *Fusarium virguliforme* of eight genotypes (MN1606SP, Ripley, AR10-SDS, MN1805SP, Beck 299N, MYC5171, MAC024757, Spencer) at the 1:20 inoculum density assessed at 36 days after planting. Plants were grown in a growth chamber at 23°C ±1 with a 16 hour photoperiod. (**A**) Root rot severity (%) (**B**) Root dry weight (g) and (**C**) Shoot dry weight (g)

Figure 4. Distribution of 324 soybean lines generated from resistant by resistant crosses based on foliar leaf scorch severity (%) to *Fusarium virguliforme* averaged over three runs (**A**) using the ISU layer method assessed at 28 days after planting and (**B**) using the 1:20 inoculum density mixed method assessed at 30 days after planting. Plants were grown in the greenhouse at 23°C ±4 with a 16 hour photoperiod.

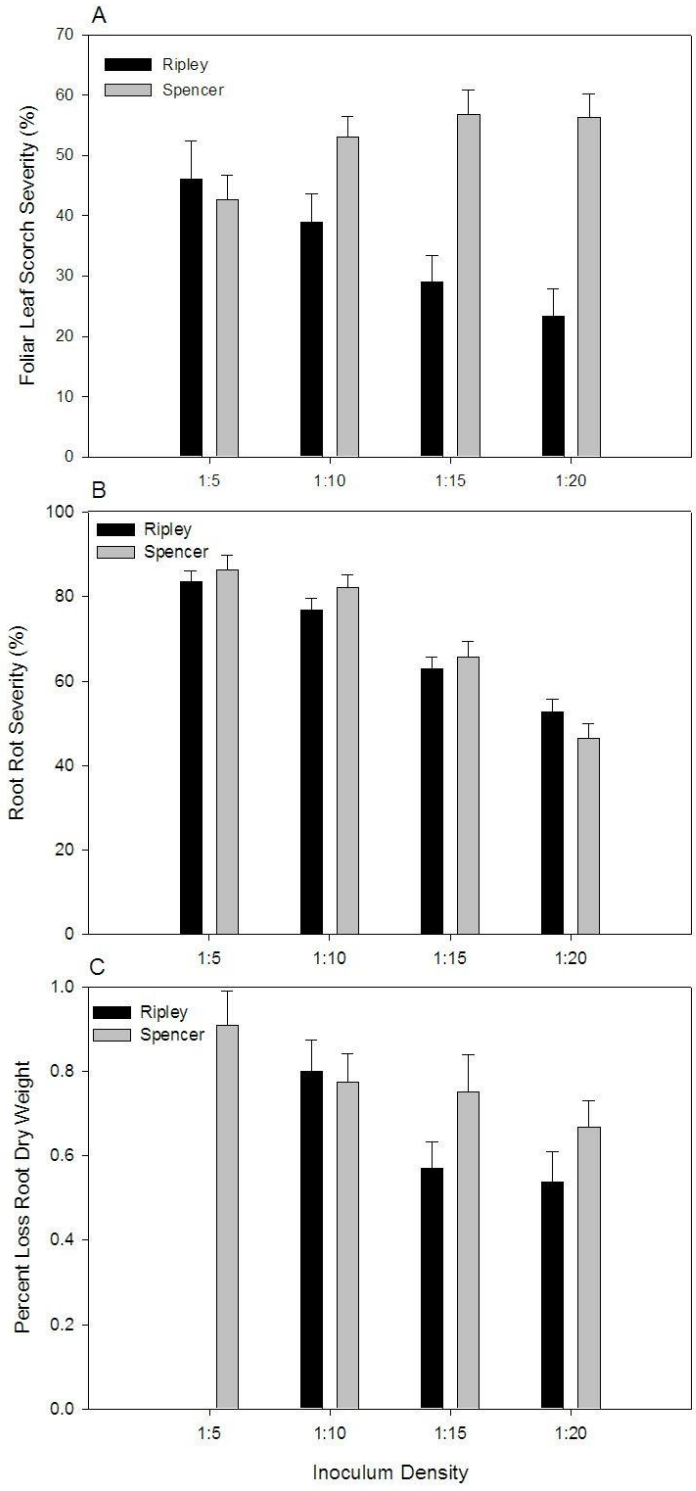
A
Ripley
Spencer
Foliar Leaf Scorch Severity (%)
70
60
50
40
30
20
10
0
1:5
1:10
1:15
1:20
B
Ripley
Spencer
Root Rot Severity (%)
100
80
60
40
20
0
1:5
1:10
1:15
1:20
C
Ripley
Spencer
Percent Loss Root Dry Weight
1.0
0.8
0.6
0.4
0.2
0.0
1:5
1:10
1:15
1:20
Inoculum Density

Figure 1.

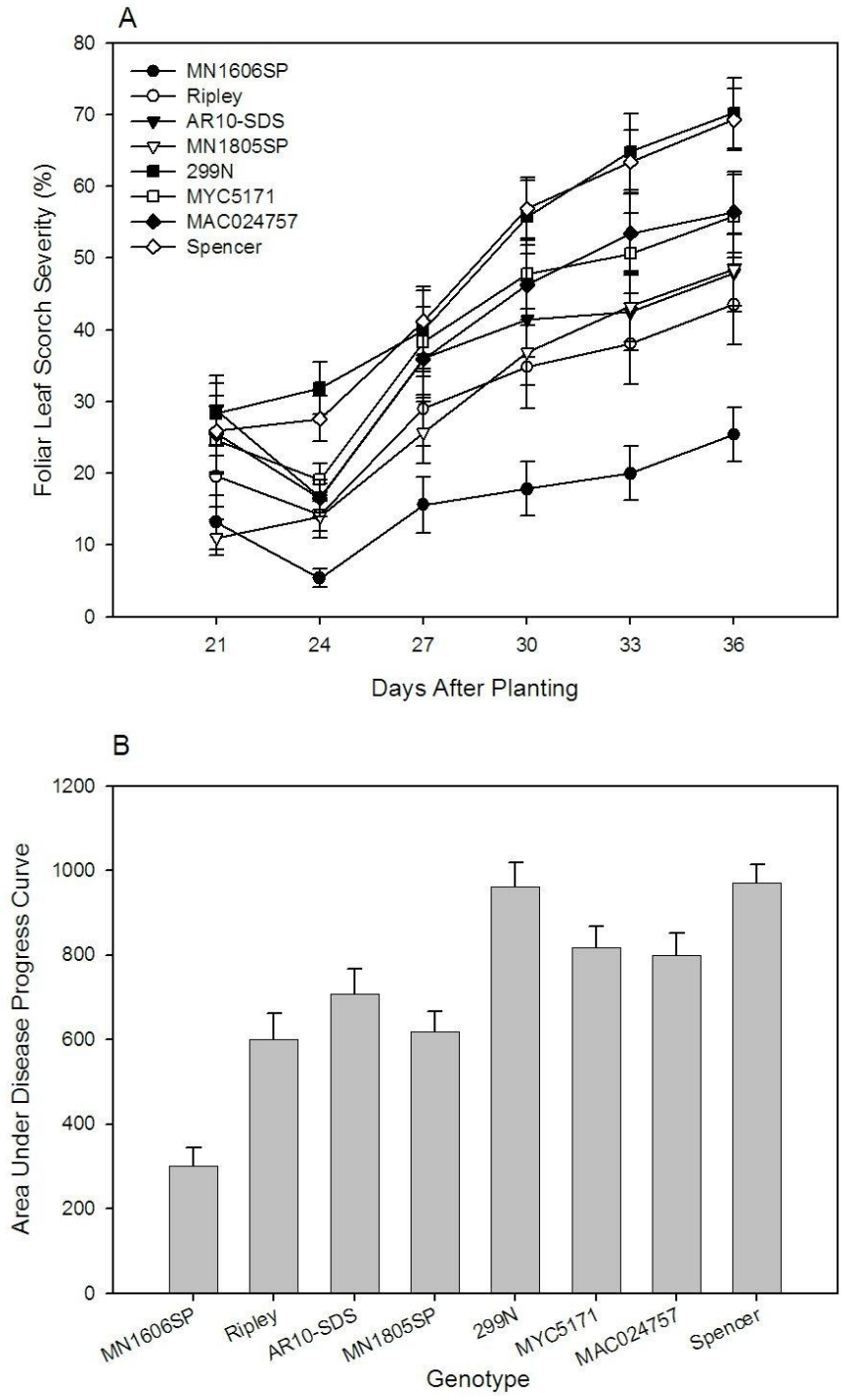
A
MN1606SP
Ripley
AR10-SDS
MN1805SP
299N
MYC5171
MAC024757
Spencer
Foliar Leaf Scorch Severity (%)
0
10
20
30
40
50
60
70
80
21
24
27
30
33
36
Days After Planting
B
Area Under Disease Progress Curve
0
200
400
600
800
1000
1200
MN1606SP
Ripley
AR10-SDS
MN1805SP
299N
MYC5171
MAC024757
Spencer
Genotype

Figure 2.

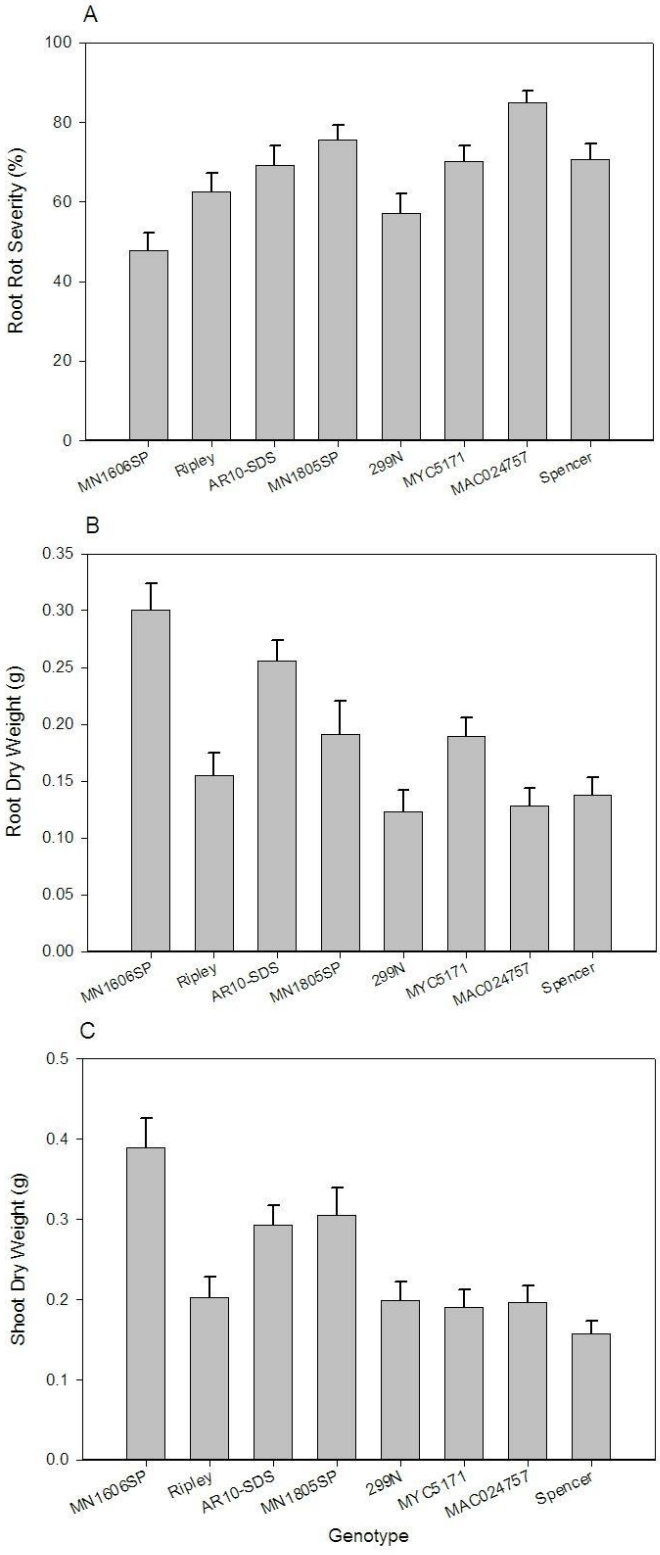
A
Root Rot Severity (%)
100
80
60
40
20
0
MN1606SP
Ripley
AR10-SDS
MN1805SP
299N
MYC5171
MAC024757
Spencer
B
Root Dry Weight (g)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MN1606SP
Ripley
AR10-SDS
MN1805SP
299N
MYC5171
MAC024757
Spencer
C
Shoot Dry Weight (g)
0.5
0.4
0.3
0.2
0.1
0.0
MN1606SP
Ripley
AR10-SDS
MN1805SP
299N
MYC5171
MAC024757
Spencer
Genotype

Figure 3.

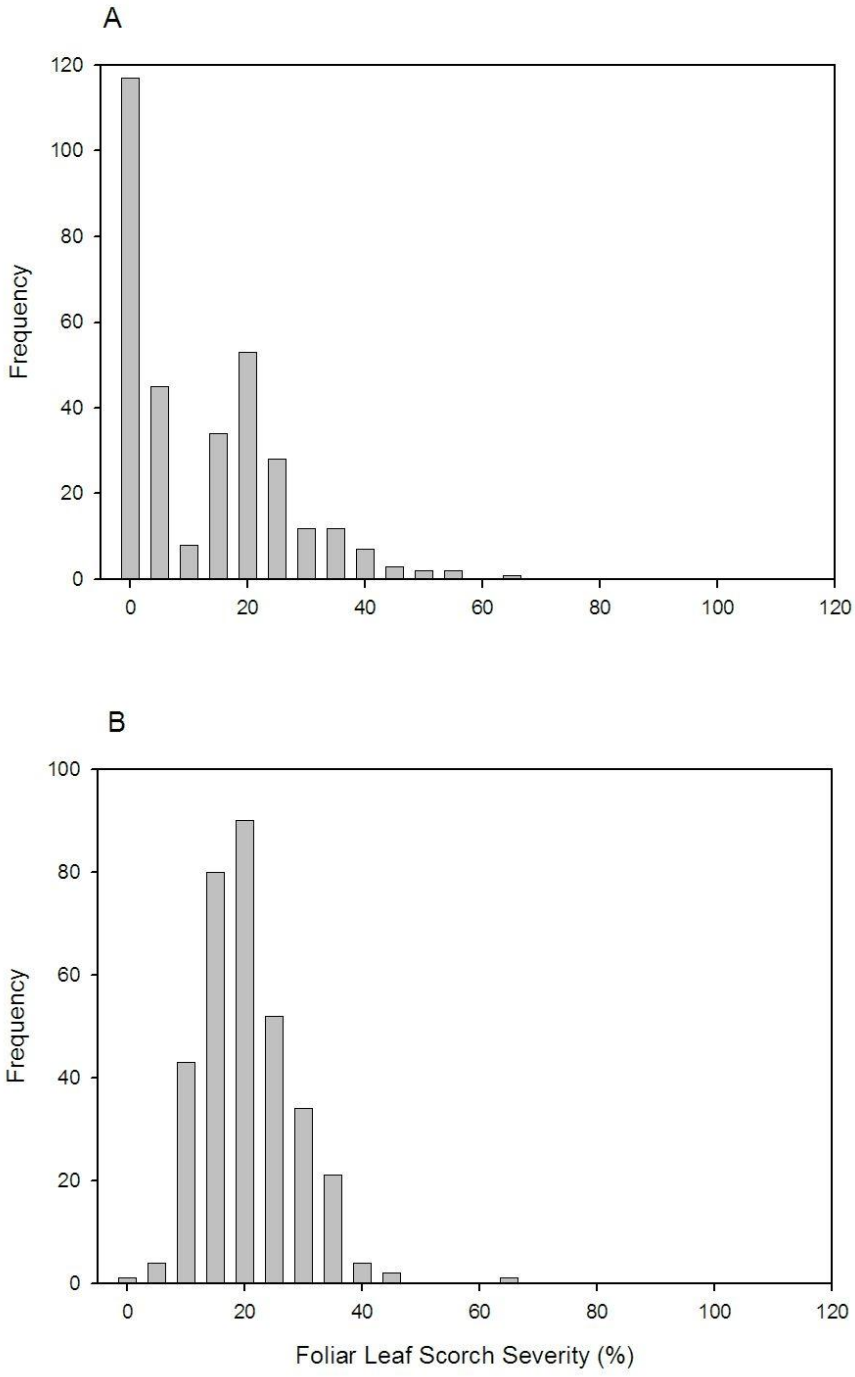
A
120
100
80
60
40
20
0
Frequency
0
20
40
60
80
100
120
B
100
80
60
40
20
0
Frequency
0
20
40
60
80
100
120
Foliar Leaf Scorch Severity (%)

Figure 4.

CHAPTER 3.

EVALUATING USEFULNESS OF 10 GENOMIC REGIONS ASSOCIATED WITH SUDDEN DEATH SYNDROME RESISTANCE IN SOYBEAN

Abstract

Currently, 12 quantitative trait loci (QTL) have been confirmed to be associated with resistance to soybean sudden death syndrome (SDS), an important disease caused by the fungus *Fusarium virguliforme*. The objective of this study was to evaluate usefulness of informative QTL from crosses made among five soybean parents obtained from recombinant inbred line (RIL) populations developed from the crosses of 'Essex' x 'Forrest' (EF), 'Flyer' x 'Hartwig' (FH), and 'Pyramid' x 'Douglas' (PD). Sorghum (*Sorghum bicolor* (L.) Moench) kernels infested with *F. virguliforme* were mixed with soil at a 1:20 ratio (v/v inoculum:soil) and was used to screen 321 F_2-derived lines for resistance to SDS based on four disease assessment criteria - foliar disease incidence (DI), foliar leaf scorch disease severity (DS), area under the disease progress curve (AUDPC), and root rot severity. Five QTL (*qRfs4, qRfs5, qRfs7, qRfs12,* and *Rfs12)* were identified as being associated with at least one of the disease assessment criteria across multiple populations. Of the five QTL, *qRfs4* was associated with DI, AUDPC, and root rot severity, and *qRfs12* was associated with AUDPC and root rot severity. The findings suggest it may be possible for plant breeders to focus on stacking fewer QTL in improved lines than those identified up to date. By introgressing some of these QTL it might be possible to develop SDS resistant cultivars capable of overcoming the disease even when high disease pressure might be present in infested soils.

Introduction

Sudden death syndrome (SDS) of soybean (*Glycine max* L. Merr.) is caused by the soilborne fungus *Fusarium virguliforme* (formerly *Fusarium solani* f. sp. g*lycines*) (Aoki et al., 2003; Roy, 1997a). The pathogen infects soybean roots causing a light brown to black discoloration, leading to reductions in root mass and root nodules (Rupe, 1989; Stephens et al., 1993). Above ground symptoms of foliar chlorosis and necrosis result from the translocation of toxins released by the pathogen, and can cause premature defoliation and pod abortion (Jin et al., 1996; Rupe et al., 1989).

SDS reduces yield in most of the major soybean producing countries (Wrather, 2010). In the United States it ranked second to fifth compared to all other soybean diseases regarding yield impact in the Midwestern soybean producing region from 1996 to 2007, leading to average losses of $190 million a year (Roy et al., 1997b; Wrather and Koenning, 2009; Wrather et al., 2010). In 2010, SDS was seen at high levels in Iowa and soybean yield loss was expected to exceed 20% in some production fields (Robertson and Leandro, 2010), with a yield loss estimate of 70 million bushels for the US (Wrather and Koenning, 2011).

Management of the disease focuses on planting resistant cultivars and using cultural practices that may improve soil drainage and reduce soil compaction (Hershman et al., 1990; Roy et al., 1997b; Wrather et al., 1995). Resistant cultivars are primarily identified through field screens planted on SDS infested soils. However, environmental factors such as temperature and moisture, play important roles in disease expression (Farias Neto et al., 2006; Roy et al., 1997b; Rupe et al., 1994), creating challenges in the evaluation of cultivars in field conditions (Njiti et

al., 2001). To remediate this limitation, greenhouse screening methods have been developed to evaluate genotypic resistance. In general, correlations between greenhouse and field tests are significant but weak, with an approximate correlation coefficient (*r)* of 0.60 (Farias Neto et al., 2008; Hashmi et al., 2005). Molecular markers and marker–assisted selection may provide additional tools for plant breeders to overcome some of the challenges in identifying SDS resistant cultivars.

Genetic resistance to SDS has been found to be polygenic or quantitative (Chang et al, 1996; Hnetkovsky et al., 1996; Iqbal et al, 2001; Kassem et al, 2007; Kazi et al., 2008; Lightfoot et al., 2005; Meksem et al, 1999; Njiti et al., 1997; Njiti et al., 2002; Prabhu et al., 1999). In recombinant inbred lines (RIL) developed from crossing the partially resistant parent 'Forrest' (Hartwig and Epps, 1973) and the susceptible parent 'Essex' (Smith and Camper, 1973), nine quantitative trait loci (QTL) were identified associated with SDS resistance (Chang et al, 1996; Hnetkovsky et al., 1996; Iqbal et al, 2001; Kassem et al, 2007; Lightfoot et al., 2005; Meksem et al, 1999). Forrest has been shown to be partially resistant to both foliar leaf scorch and root infection (Njiti et al., 1997). Four of the nine QTL were placed on linkage group G, with the resistant allele coming from the partially resistant parent Forrest. Linkage groups C2 and I each contained one QTL with the resistant allele contributed by the susceptible parent Essex (Iqbal et al, 2001; Lightfoot et al, 2005). Additionally, resistant QTL alleles from Forrest were also identified on linkage groups I, J, and N (Chang et al, 1996; Kassem et al., 2007).

RILs derived from the cross of susceptible parent 'Douglas' (Nickell et al., 1982) and the partially resistant parent 'Pyramid' (Myers and Schmidt, 1988), allowed identification of four QTL associated with SDS resistance (Njiti et al., 2002). Similar to Forrest, Pyramid has been

shown to be partially resistant to foliar leaf scorch and susceptible to root rot (Gibson et al., 1994). Two QTL were mapped to linkage group G, and one QTL to linkage group N with the resistant alleles from donor parent Pyramid (Njiti et al., 2002). In this RIL population, there also was one QTL that mapped to linkage group C2, with the resistant allele provided by Douglas (Njiti et al., 2002).

An additional RIL mapping population developed at Southern Illinois University by crossing the partially resistant 'Hartwig' (Anand, 1992) cultivar and the susceptible parent 'Flyer' (McBlain et al., 1990), also mapped four QTL associated with SDS resistance (Kazi et al., 2007; Prabhu et al., 1999). As in previous populations, Hartwig has been shown to be partially resistant to both foliar leaf scorch and root infection (Hartman et al., 1997; Njiti et al., 1997). Two QTL mapped to linkage group G with the beneficial alleles coming from Hartwig (Kazi et al,. 2008; Prabhu et al., 1999). One QTL mapped to linkage group C2 and another mapped to linkage group D2, both having the beneficial allele from Flyer (Kazi et al., 2008).

Since resistance is quantitative (Kassem et al., 2007; Kazi et al., 2008; Njiti et al., 2002), breeding SDS resistant cultivars requires accumulation of multiple resistance genes. The molecular work conducted with RIL and discussed previously, suggested common QTL in the Essex x Forrest (ExF), Pyramid x Douglas (PxD), and Flyer x Hartwig (FxH) populations. Kazi et al. (2008), noted *cqRfs4* on linkage group C2 and *cqRfs1* on linkage group G to be found in all three populations, ExF, PxD, and FxH, hinting these two QTL may be of importance in breeding for SDS resistance. Populations PxD and FxH are hypothesized to share QTL *qRfs11* on linkage group D2 and ExF and FxH are hypothesized to share the QTL *cqRfs3* on linkage group 3 (Kazi et

al., 2008). Ultimately, Kazi et al. (2008) suggested there were 12 unique QTL across the three RIL populations of ExF, FxH, and PxD.

With the present state of technology, it would be impractical for plant breeders to try to accumulate the 12 novel QTL from the three populations into a single genetic background. Stacking a large number of those QTL would require unusually large population sizes that may not always be feasible to obtain. A related question may be how many of the QTL and which ones may be needed to confer higher levels of SDS resistance compared to current cultivars. The objective of this study was to determine if the known QTL previously associated with SDS resistance and detected in three different RIL populations would be required to improve SDS resistance to levels superior to those of existing commercial cultivars.

Material and Methods

Plant material

Populations were developed with different combinations of RIL parents possessing the 12 QTL previously identified (Chang et al, 1996; Hnetkovsky et al., 1996; Iqbal et al, 2001; Kassem et al, 2007; Kazi et al., 2007; Lightfoot et al., 2005; Meksem et al, 1999; Njiti et al., 2002; Prabhu et al., 1999) and summarized by Kazi et al. (2008). Parents used in crossing were lines previously identified from the RIL mapping populations of 'Essex' x 'Forrest' (EF-13), 'Pyramid' x 'Douglas' (PD-98), and 'Flyer' x 'Hartwig' (FH-13, FH-33, FH-35).The plant material used in the study included the five parental lines (EF-13, PD-98, FH-13, FH-33, and FH-35), 'Ripley' (Cooper et al., 1990) as a resistant control, 'Spencer' (Wilcox et al., 1989) as a

susceptible control, and 321 $F_{2:3}$-derived lines ($F_{2:3}$) developed from nine different crosses. The parental lines had been previously genotyped at the Lightfoot lab and QTL molecular information was published by Kazi et al., (2008). Parent information is summarized in Table 4.

Population development was conducted at the Iowa State University (ISU) research site of the University of Puerto Rico, at Isabela, Puerto Rico. F_1 seed was obtained in November 2006. F_1 seeds were planted and F_2 plants were individually harvested during April 2007. F_1 plants were harvested individually, and identity of plants was preserved, even if morphological markers were available to confirm the hybrid nature of the crosses. F_1 plant identity was maintained throughout generation advances.

$F_{2:3}$ lines used in the study were randomly selected from all lines available in each population, for which seed numbers on an individual plant basis were adequate to facilitate conducting several runs of the experiment with appropriate number of replications. Population 1 (Pop1) was formed by 46 lines from the cross of 'FH-13' x 'EF-23'. Population 2 (Pop2) had 6 lines from the reciprocal cross of Pop1, 'EF-23' x 'FH-13'. Population 3 (Pop3) was formed by 62 lines obtained from the cross of 'FH-13' x 'PD-98'. Population 4 (Pop4) consisted of 45 lines derived from the cross of 'FH-33' x 'EF-23'. Population 5 (Pop5) consisted of 59 lines from the cross of 'FH-33' x 'PD-98'. Population 6 (Pop6) consisted of 8 lines from the cross 'PD-98' x 'FH-35'. Population 7 (Pop7) was the reciprocal cross of Pop6 and consisted of 30 lines. Population 8 (Pop8) consisted of 34 lines from the cross 'PD-98' x 'EF-23', and Population 9 (Pop9) consisted of 31 lines derived from the reciprocal cross of Pop8.

Inoculum preparation

F. virguliforme isolates Clinton1b and Scott obtained in 1996 from roots of SDS symptomatic plants from production fields in Clinton and Scott counties in Iowa were used in the screening experiments. The two isolates were used in combination and mixed in equal proportion as determined by Sanogo et al. (2000) to prepare inoculum for the experiment. The experiment consisted of three runs conducted at three different dates. Inoculum was prepared independently for each run, following the same protocol.

Isolates were grown on antibiotic-amended potato dextrose agar (PDA) (0.150 gL^{-1} of streptomycin sulfate, and chlortetracycline hydrochloride, 39 g Difco PDA per liter) for 6 weeks under natural day length light cycles, maintained at room temperature. Sterile white sorghum (*Sorghum bicolor* (L.) Moench) kernels (500 g) were placed in quart mason jars filled with water for 24 h. The water was then drained off and the flasks were autoclaved for one hour on two consecutive days. After cooling, sorghum kernels were inoculated with five mycelia plugs (7 mm diameter) of a single *F. virguliforme* culture. Mason jars were incubated at room temperature for two weeks, shaken daily by hand for 1 to 2 minutes to ensure uniform fungal growth. Mason jars were visually inspected to verify uniform fungal growth and were emptied on racks in a fume hood for 24 h to allow infested sorghum kernels to dry. After drying of infested kernels with each *F. virguliforme* isolate, kernels infested with each isolate were combined in equal parts into a single plastic biohazard bag. The isolates were combined in equal proportions to better represent field conditions.

Greenhouse Experiment

The 321 $F_{2:3}$-derived lines, parents, and the resistant control Ripley and the susceptible control Spencer were screened for foliar leaf scorch and root rot resistance to *F. virguliforme* at the ISU Plant Pathology Greenhouses, in Ames, IA. The infested sorghum inoculum was homogeneously mixed with pasteurized 2:1 sand: soil mixture at the ratio of 1 part inoculum to 20 parts sand/soil (v/v) according to Luckew et al., (2012). Sterile sorghum mixed with pasteurized sand/soil at the same ratio as the infested sorghum was used as additional controls for the resistant and susceptible check cultivars.

A replication was considered a 240 ml Styrofoam cup with five seeds planted at a depth of 2 cm. There were four replicate cups per each soybean genotype. The two checks were planted into sterile soil mixtures and infested soil mixtures to verify contamination was not present. Cups were incubated in the greenhouse at 23 ±5°C with a 16 h photoperiod. Plants were watered once daily to maintain soil moisture.

Experimental design was a randomized complete block design (RCBD) with genotypes as treatments with four replications. The experiment was conducted three times (runs) planted on May 2010 (Run 1), December 2010 (Run 2), and April 2011 (Run 3). Three of the 321 genotypes did not germinate in Run 3.

Disease assessment

SDS foliar leaf scorch symptoms were evaluated 21, 24, 27, 30, 33, and 36 days after planting (dap). Foliar leaf scorch disease severity (DS) was scored as a percentage of leaf area

showing typical SDS symptoms, and foliar disease incidence (DI) was calculated as the total number of plants in the cup showing typical SDS foliar symptoms, divided by the total number of plants in each cup. In addition, root rot was assessed at 36 dap and scored as the percentage of root area showing brown to black discoloration. Following the midpoint rule of Campbell and Madden (1990), the area under the disease progress curve (AUDPC) was calculated.

Molecular marker analysis

For all lines, including parents, DNA was isolated from leaf samples of ten individual plants collected 36 dap, corresponding to soybean plant growth stage V3-V4 (Fehr and Caviness, 1977). Leaves of each of five plants were collected from two of the three runs. DNA was isolated following a modification of the method described in the CIMMYT laboratory protocols manual, section entitled 'Small scale extraction of high quality DNA' (CIMMYT, 2005). Leaves from each line were combined over the four replications, keeping the runs separate, and were finely ground with mortar and pestle. Leaf tissue in the amount of 0.5 ml of the finely ground leaves of each genotype combined over replications was placed in 2 ml Eppendorf tubes. At room temperature, 1.25 ml of mixed alkyltrimethyl-ammonium bromide (CTAB) was combined with 0.5 ml of the leaf tissue and placed in a water bath for 90 min at 65°C. Afterwards, 0.625 ml of chloroform:octanol (24:1) was added, shaken for 5 min and centrifuged at 4000 rpm for 20 min. The aqueous layer was removed and placed in a new 2 ml Eppendorf tube to which a 5 µl of RNAse was added and mixed gently at room temperature for 30 min. DNA was precipitated by adding 700 µl isopropanol and mixing gently. The mixture was centrifuged at 4°C at 12000 rpm for 10 min and the supernatant was removed. DNA pellet was

washed by adding 500 µl of 70% ethanol and centrifuging at 4°C at 12000 rpm for 5 min. After removing the ethanol, the remaining pellet was allowed to dry for 1 hr at room temperature.

The DNA pellet was resuspended in 100µl of TE buffer. Simple sequence repeat (SSR) markers (Table 1) were used to test the isolated DNA for the known QTL. A step down polymerase chain reaction (PCR) was performed. An initial 2 min at 94°C was followed by five cycles of 94°C for 30 s, then 60°C for 30 s with a step of -2°C every cycle, and finished with 72°C for 1 min. The last 35 cycles mimicked the first 5 cycles starting with 30 s at 94°C followed with 50°C for 30 s, then 72°C for 45 s. After all cycles finished, the products were kept at 72°C for 10 min followed by 15°C for 10 min. Products from the PCR were run on 8% agarose gel for 3 h and visually scored for presence or absence of the corresponding marker associated with SDS resistance using the known base pair size of the marker.

Data analysis

The three runs of the greenhouse experiment were combined and an analysis of variance was performed using the PROC GLM statement in SAS version 9.2 (SAS, 2008) on foliar DS and DI at 36 dap, root rot severity, and AUDPC, for each population separately.

The PROC GLM statement was used to perform contrasts analysis of the 10% most and the 10% least resistant categories for DS and root rot severity. A Spearman rank correlation was performed on DI, DS, AUPDC, and root rot using the PROC CORR statement. PROC UNIVARIATE was run to obtain a Shapiro-Wilk test for normality of the curves for each of the disease assessment criteria, DI, DS, AUDPC, and root rot severity.

Main effects tested were run, replication, and genotype, and interactions, all considered random effects. Control genotypes were considered fixed. Of the 12 QTL identified in the RILs used to develop the populations for the study, *qRfs1* and *qRfs10*, were not informative and were not considered in the analysis. The remaining ten QTL were polymorphic and co-dominant allowing identification and scoring of heterozygous individuals. Allele frequencies of the ten QTL were calculated within two defined groups, the 10% most resistant and 10% least resistant for each population and the five parents. The allele frequencies were compared to each other to identify differences in frequencies for the disease assessment criteria.

Results

The three disease assessment criteria, DI, DS, and root rot severity, and the calculated AUDPC curve, had frequency distribution curves that approached normal uni-modal distributions (Figure 1). Over runs, DI distribution had a normal curve ($P=0.1665$) with kurtosis of 0.49, skewness of 0.12, and mean DI of 56%. DS curve also approached normality ($P=0.0006$), with kurtosis and skewness both at similar value of 0.52, and mean DS averaged over repetitions of 26% (Figure 1a). Distribution of root rot severity approached normality ($P=0.0037$), with kurtosis of 0.74, skewness of 0.46, and a mean root rot severity averaged over the three runs of 37% (Figure 1c). Disease progress data plotted as AUDPC was the only disease assessment criteria which had a distribution that did not approach normality ($P<0.0001$). For AUDPC, kurtosis was 1.38, skewness 0.65, and the mean AUDPC averaged over runs was 239 (Figure 1b).

Of the five parents, four had a calculated DI over runs that approximated the general combined average of 56% calculated for the 321 $F_{2:3}$-derived lines. For parent PD98, DI was 42%, and was 53%, 60% and 67% for EF23, FH13, and FH33, respectively. Parent FH35 had a DI of 91%, which was higher ($P<0.05$) than the combined mean (56%). For DS, four of the five parents averaged close to the overall mean of 26%, with a DS of 24%, for PD98 and EF23, 30% for FH13, and 39% for FH35 (Figure 1a). Parent FH33 had a lower ($P<0.05$) DS (8%) than the average mean (%). For root rot severity, four parents had values that approached the overall mean, i.e. PD98 31%, EF23 47%, FH13 42%, FH33 37%. For FH35, however, root rot severity was higher ($P<0.05$) than the overall mean, with a value of 57% (Figure 1c). For AUDPC, four of the parents (PD98, EF23, FH13, and FH35) had averages similar to the overall AUDPC mean (Figure 1b), with FH33 being lower ($P<0.05$) than the mean.

Mean squares for run, replicate, and their interaction were significant ($P<0.05$) in all populations for DS, AUDPC, and root rot severity, except for populations FH33xEF23, PD98xFH35 and PD98xEF23 (Table 2). For the majority of the disease assessment criteria, mean squares due to genotypes (321 F_2-derived lines plus the five parents), were not significant suggesting that resistance in genotypes was similar. Mean squares due to genotypes assessed by DS showed significant differences in two of the populations, FH13 x PD98 ($P=0.0037$) and FH33xPD98 ($P=0.0491$). For AUDPC, only one population showed a significant mean square due to genotypes, FH13xPD98 ($P=0.0268$). The other assessment criteria and populations did not show significant differences among genotypes when the population was considered as a whole. Mean squares of each interaction term were all non-significant.

Contrasts of the 10% most and least resistant categories showed significant differences (P<0.0001) for each of the four disease assessment criteria. The Spearman rank correlation among the four disease assessment criteria were significant (P<0.0001), with *r* values ranging from 0.48 to 0.89 (Table 3). The highest correlation value was obtained between AUDPC and DS recorded at 36 dap, *r* = 0.89.

Allele frequencies for the ten QTL in each of the six populations were obtained from the contrasts between the 10% most and least resistant genotypes for each of the four disease assessment criteria, and per population (Appendix tables 1 thru 4). With this information, frequency of populations having a QTL in the most resistant and least resistant categories was calculated (Table 5).

For DI, the QTL *qRfs4* was consistently present in the 10% most resistant group in five of the six populations (Table 5). The DI assessment also showed that QTL *Rfs* and *qRfs3* were present in four of the six populations within the 10% least resistant group. Focusing on DS, a different set of QTL common across the six populations was observed (Table 5). QTL *qRfs7* was identified in five of the populations in the 10% most resistant group and *qRfs11* in four populations in the 10% least resistant category. Root rot severity had three common QTL across four populations, *qRfs5, qRfs12*, and *qRf4*. The 10% most resistant groups had *qRfs5* and *qRfs12* present in five of the six populations while *qRfs4* was present in the10% least resistant group in four of the populations.

Although AUDPC is related to DS, AUDPC showed a different set of QTL present across populations (Table 5), *qRfs4* and *qRfs12* were common in the 10% most resistant in four populations as well as *Rfs12* in five of the populations. AUDPC also had two QTL common

across populations in the 10% least resistant group, *Rfs2* in four populations and *qRfs11* in five populations.

QTL common presence was also observed over populations and disease assessment criteria (Table 5). One QTL, *qRfs4*, was present across three disease assessments, DI, AUDPC, and root rot severity. While the QTL is common in all three disease criteria, *qRfs4* is not in the same resistant/least resistant category in the three populations. The QTL was present in the 10% most resistant group for DI and AUDPC, while in the case of root rot severity, the common QTL for the two disease assessment criteria was in the 10% least resistant group. QTL *qRfs11* was also common across two disease assessments, DS and AUDPC. The QTL was present in the 10% least resistant group in both DS and AUDPC. Also *qRfs11* had a higher frequency in the 10% least resistant group of root rot severity. The last QTL common across disease assessment criteria was *qRfs12* for both AUDPC and root rot severity. For both criteria *qRfs12* was in the 10% most resistant group. Also, in both DI and DS, *qRfs12* is present in the 10% most resistant group at a higher frequency than in the 10% least resistant group.

Discussion

Using RILs developed in the populations of Essex x Forrest, Flyer x Hartwig, and Pyramid x Douglas, and progeny from crosses among the RILs, this study was able to identify certain QTL showing consistent levels of association to SDS resistance under high disease pressure. The high disease pressure in screening was attained by using the protocol developed by Luckew et al. (2012). It has been suggested that for common beans, screening genotypes for resistance

under high levels of disease pressure may predict cultivar performance in the field (Duncan et al., 2012). Although similar information is not yet available for SDS in soybean, it is expected that screening under high disease pressure in controlled conditions as it was performed in this research, using the Luckew et al. (2012) modified protocol, will also be predictive of SDS resistance in outbreak years similar to 2010 (Robertson and Leandro, 2010). Screening and testing under this high disease pressure may allow for the discrimination of QTL, providing indications on which and how many number of desirable QTL might be important to consider in a breeding program to improve SDS resistance in soybean. Most importantly, with this information it may be possible to reduce the number of QTL important for resistance to a level that will be practical to use by soybean breeders.

The results indicated that of the ten QTL for which the lines were polymorphic, five of them, *qRfs4, qRfs5, qRfs7, qRfs12,* and *Rfs12* may have a potentially greater effect on SDS resistance, particularly in each of the four disease assessment criteria DI, DS, root rot severity and AUDPC. Effects of these QTL in disease resistance expression were particularly evident when comparisons between the 10% most and least resistant lines were done in each of the populations.

Previously, *qRfs5* was identified as conferring resistance to SDS in relation to the DI assessment criteria and it was stated to explain 11.5% of the total variation associated with SDS DI in the Essex x Forrest population (Iqbal et al., 2001). In our study, the finding of the association of QTL *qRfs5* with root rot severity is a novel result. The association with resistance to DI may be a byproduct of resistance acting at the root rot level. The association of QTL *qRfs7* with DS observed in this research is also a somewhat new observation. Kazi et al. (2008)

showed the QTL explained 25% of the variation in root rot severity but only a weak association with DS. The difference among reports and our observations may be explained in part by the higher disease pressure we used in the study reported herein, in which rotted roots were observed even when the foliar resistance mechanism was not overcome.

A different result was observed in relation to *qRfs4* and previous reports. For DI, the *qRfs4* QTL was observed in the 10% most resistant category, whereas for AUDPC it was observed in the 10% least resistant category for root rot severity. QTL *qRfs4* was first identified as a QTL associated with resistance for DI (Iqbal et al., 2001) and later shown to also be associated with the calculated DX resistance (Kazi et al., 2008). Both disease assessment criteria, AUDPC and DX, result from calculations in which DS is part of the equation. The association between the criteria is also shown in the correlation observed in this study of r = 0.89. The observation of *qRfs4* being in the least resistant category for root rot is not new, previous research has shown QTL for foliar resistance and root resistance may differ (Kazi et al., 2008; Triwitayakorn et al., 2005).

It has been hypothesized that resistance to SDS in soybean may be governed by two different genetic systems, one system acting at the foliar level, and the other at root level (Kazi et al., 2008; Triwitayakorn et al., 2005). Nevertheless, recent work in the Meksem Lab has suggested that some QTL may have significant association with resistance expressed both at the foliar and root levels (Meksem, Southern Illinois University – Carbondale, personal communication). In this study, QTL *qRfs12* could be one of the QTL associated with resistance at the foliar and root levels, since no distinction was observed between foliar and root resistance disease assessment criteria. These observations may be indicative of the QTL being

associated with both DS and root rot severity. This is the first time a dual type of association in SDS resistance is observed and reported for *qRfs12.* A previous report had only identified the association with DS (Kassem et al. 2006). It is necessary to clarify that Kassem et al. (2006) did not consider root rot severity in their research.

Five QTL, *Rfs, Rfs2, qRfs3, qRfs4,* and *qRfs11,* were consistently observed in the 10% least resistant category. Although the QTL have been previously reported as associated with SDS resistance, our observations do not fully contradict the previous reports. The genetic backgrounds used in this study and in previous reports are different, which in itself may explain the different contribution of the QTL to the final expression of resistance.

A significant aspect of the study was to identify QTL that were common across populations and disease assessment criteria. We identified three QTL common to more than one disease assessment criteria across populations. In addition, two of the QTL were also observed to be in the 10% most resistant category, *qRfs4* and *qRfs12*. It is possible that the two QTL could become a starting point for plant breeders to improve SDS resistance in soybean. Both QTL confer resistance to the foliar leaf scorch associated with the disease and *qRfs12* also appears to favor root rot resistance.

While there is nothing definitively known about the relationship between the root rot caused by *F. virguliforme* and soybean yield, it might be advantageous to search for SDS resistance QTL and determine if there is also a yield association. Marker-assisted selection practiced for these QTL in combination with classical breeding methods and appropriate disease pressure screening protocols will allow for resistant cultivars to be selected and

withstand natural disease pressure even in unusual years when disease symptoms may be different than expected.

It is important however, that results of this study be interpreted with caution. The results were based solely on greenhouse screening tests, even though, the screening protocol used was purposely designed to create increased disease pressure as compared to classical greenhouse screening methods. For a complete description of the classical greenhouse layer method readers may refer to patent #7,288,386 issued to Lightfoot et al. (2007) and to previous literature (Hartmann et al., 1997; X.B. Yang, Plant Pathology Dept., ISU, personal communication). No field screening tests were conducted in the study reported here, and this may be an important limitation to confirm the results, particularly when the QTL tested were identified in previous research through field screens. Counteracting this observation, however, Luckew et al. (2012) has reported that genotypic rankings between greenhouse and field screenings can also be highly correlated. Another important limitation is the study was not planned to break the genetic variance components. This precludes the consideration of QTL association with the components of the genetic variance, and the potential interactions among them, such as additive x additive, and other higher interactions. However, on the basis of previous research, this fact may not be relevant enough, since contradictory results on higher order genetic interactions have been reported in the literature (Iqbal et al. 2001; Njiti et al. 2002). Further research is in progress for the evaluation of the lines under field conditions, both on SDS-infested and non-infested soil conditions to establish a possible association between important resistant QTL and yield expression.

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Tables

Table 1. Twelve quantitative trait loci (QTL) for resistance to *Fusarium virguliforme* described by the name of the QTL, the simple sequence repeat marker (SSR) name, the linkage group (LG) of the QTL, and the primer sequences.

QTL	SSR	LG	Upper Primer Sequence (5'→3')	Lower Primer Sequence (5'→3')
Rfs	Satt_403	G	GCGGCGTCATGTTAGTTGGAACC	GCGAGCCATTTTTCTCTTTTAGACAAT
Rfs1	Satt570	G	CTCATGTGGTCCTACCCAGACTCA	CGCTATCCCTTTGTATTTTCTTTTGC
Rfs2	Satt309	G	GCGCCTTCAAATTGGCGTCTT	GCGCCTTAAATAAAACCCGAAACT
qRfs3	Satt163	G	GCGGCACGAGAAAAGGAGAGAGAG	GCGGGGGAAAAACTATGTTCT
qRfs4	Satt371	C2	TGCAAACTAACTGGATTCACTCA	GAGATCCCGAAATTTTAGTGTAACA
qRfs5	Satt354	I	GCGAAAATGGACACCAAAAGTAGTTA	GCGATGCACATCAATTAGAATATACAA
qRfs6	Satt80	N	CCATAAAATAATAAAGGTCAAT	TAATCAGTGGAAAAAAAGTTAT
qRfs7	Sat_001	D2	GCGGATACGACCAAAAATTGTT	GCGAACTGCGAAGATACTACCC
qRfs10	Satt183	J	TAGGTCCCAGAATTTCATTG	CACCAACCAGCACAAAA
Dt2/qRfs11	Satt138	G	GACATTTTTCCACGGATATTGAAT	AACGGGCGATTTATGGCTAT
Rfs12	Satt353	H	CATACACGCATTGCCTTTCCTGAA	GCGAATGGGAATGCCTTCTTATTCTA
qRfs12	Satt160	F	TCCCACACAGTTTTCATATAATATA	CATCAAAAGTTTATAACGTGTAGAT

Table 2. Degrees of freedom (df) and mean squares (MS) from the ANOVA tables for four disease assessment criteria (disease incidence (DI), foliar disease severity (DS), area under the disease progress curve (AUDPC), and root rot severity) and six populations (FH13xEF23, FH13xPD98, FH33xEF23, FH33xPD98, PD98xFH35, and PD98xEF23)

Main Effect	Disease Assessment Criteria							
	DI		DS		AUDPC		Root Rot	
	df†	MS	df	MS	df	MS	df†	MS
	Population 1, FH13xEF23							
Run	1	0.736	2	34452***	2	2863402***	1	30685***
Rep	3	0.158	3	5179.8***	3	263607.3**	3	7415.0***
Run*Rep	3	0.934*	5	8381.7***	5	466360.3***	3	16018***
Genotype	51	0.290	51	690.19	51	53548.49	51	839.24
Error	305	0.259	423	634.95	424	49916.93	304	655.00
	Population 2, FH13xPD98							
Run	1	8.794***	2	59407***	2	6029312***	1	90339***
Rep	3	0.297	3	2759.9*	3	177498.4*	3	5140.1***
Run*Rep	3	1.727***	5	6397.9***	5	454922.5***	3	18054***
Genotype	61	0.152	61	943.62*	61	86399.91*	61	830.09
Error	388	0.157	538	588.30	537	61282.19	388	618.09
	Population 3, FH33xEF23							
Run	1	6.923***	2	40767***	2	4182412***	1	53358***
Rep	3	0.528*	3	738.98	3	144273.7	3	3986.9**
Run*Rep	3	0.396	5	673.88	5	93220.52	3	8245.9***
Genotype	43	0.152	44	465.69	44	40226.83	43	662.92
Error	271	0.170	343	578.67	343	65369.22	270	578.16
	Population 4, FH33xPD98							
Run	1	11.01***	2	45258***	2	3470430***	1	56847***
Rep	3	0.397*	3	4216.7***	3	176313.6*	3	7805.2***
Run*Rep	3	1.805***	5	6596.5***	5	265332.0***	3	19218***
Genotype	58	0.103	58	623.15*	58	62299.13	58	510.24
Error	376	0.126	470	459.95	472	48135.21	379	554.80
	Population 5, PD98xFH35							
Run	1	5.515***	2	33539***	2	1985523***	1	19969***
Rep	3	0.067	3	2472.4**	3	113916.3	3	9325.5***
Run*Rep	3	1.028**	5	1919.1*	5	96552.26	3	6650.4***
Genotype	37	0.176	37	800.74	37	70671.16	37	962.20
Error	215	0.148	287	610.07	289	60693.95	215	671.99
	Population 6, PD98xEF23							
Run	1	3.567***	2	47801***	2	3272560***	1	54173***
Rep	3	0.427*	3	2905.3**	3	117508.9	3	7913.9***
Run*Rep	3	3.027***	5	8354.1***	5	417585.5***	3	18283***
Genotype	62	0.166	64	810.55	64	77277.52	62	771.98
Error	331	0.146	431	627.52	432	69453.76	331	702.21

*Significant at alpha of 0.05

**Significant at alpha of 0.01

***Significant at alpha of 0.001

†DI and Root rot severity data was only collect in two runs of the experiment.

Table 3. Spearman rank correlation of foliar disease incidence (DI) at 36 dap, foliar leaf scorch disease severity (DS) at 36 dap, area under the disease progress curve (AUDPC), and root rot severity of soybean sudden death syndrome for 321 $F_{2:3}$-derived lines grown in the greenhouse with a 16 h photoperiod at 23 ±5°C.

	DI	DS	AUDPC	Root Rot
DI		0.74***	0.64***	0.48***
DS			0.89***	0.59***
AUDPC				0.54***

*Significance at P<0.05
**Significance at P<0.01
***Significance at P<0.0001

Table 4. Presence or absence of ten quantitative trait loci (QTL) associated with resistance to soybean sudden death syndrome (SDS) in the five parents used to create the 321 $F_{2:3}$-derived lines evaluated for resistance to SDS in the greenhouse at 23 ±5°C with a 16 h photoperiod.

QTL	Parent				
	ExF23	FxH13	PxD98	FxH33	FxH35
Rfs	+	+	-	+	+
Rfs2	+	+	+	+	+
qRfs3	+	-	-	-	-
qRfs5	+	+	-	+	+
qRfs4	+	+	+	+	+
qRfs7	-	+	+	+	+
Rfs12	+	-	-	-	-
qRfs12	+	-	-	-	-
qRfs6	-	+	+	+	+
qRfs11	-	-	+	-	-

Table 5. Population frequency for ten quantitative trait loci (QTL) from the 10% most resistant and least resistant lines from six populations,FH13 x EF23, FH13 x PD98, FH33 x EF23, FH33 x PD98, PD98 x FH35, and PD98 x EF23 assessed by disease incidence (DI), foliar disease severity (DS) at 36 days after planting (dap), area under the disease progress curve (AUDPC), and root rot severity.

Criteria	Category	*Rfs*	*Rfs2*	*qRfs3*	*qRfs4*	*qRfs5*	*qRfs6*	*qRfs7*	*qRfs11*	*Rfs12*	*qRfs12*
DI	Most Res.	1/6	2/6	1/6	5/6	2/6	1/6	3/6	3/6	3/6	3/6
	Least Res.	4/6	2/6	4/6	1/6	1/6	3/6	0/6	3/6	1/6	3/6
DS	Most Res.	3/6	2/6	2/6	3/6	1/6	1/6	5/6	2/6	3/6	3/6
	Least Res.	2/6	2/6	2/6	3/6	1/6	1/6	0/6	4/6	2/6	1/6
AUDPC	Most Res.	2/6	1/6	1/6	4/6	1/6	1/6	2/6	1/6	4/6	5/6
	Least Res.	3/6	4/6	2/6	2/6	2/6	2/6	0/6	5/6	2/6	1/6
Root rot severity	Most Res.	3/6	1/6	2/6	1/6	5/6	1/6	3/6	2/6	5/6	1/6
	Least Res.	1/6	2/6	2/6	4/6	0/6	3/6	3/6	3/6	1/6	1/6

Figures

Figure 1. Distribution of 321 $F_{2:3}$-derived soybean lines and the five parents averaged over three runs based on symptoms resulting from infection of *Fusarium virguliforme* (**A**) foliar leaf scorch severity (%), (**B**) area under the disease progress curve, and (**C**) root rot severity (%). Plants were grown in the greenhouse at 23±5°C with a 16 hour photoperiod. The vertical arrows represent the position of the parents in each of the three histograms.

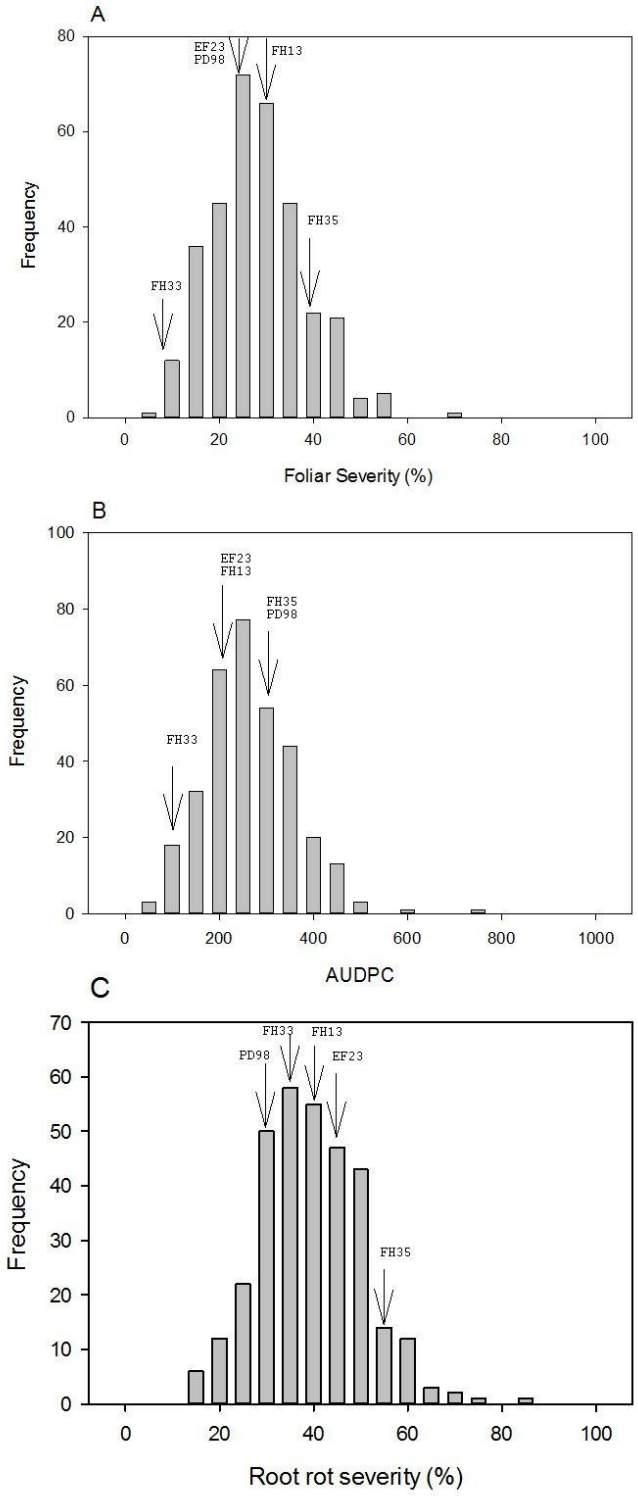
A
Frequency
Foliar Severity (%)
EF23
PD98
FH13
FH35
FH33
B
Frequency
AUDPC
EF23
FH13
FH35
PD98
FH33
C
Frequency
Root rot severity (%)
FH33
FH13
PD98
EF23
FH35

Figure 1.

Appendix

Appendix 1. Allele frequency of ten quantitative trait loci (QTL) from the 10% most resistant and 10% least resistant lines from six populations, FH13xEF23, FH13xPD98, FH33xEF23, FH33xPD98, PD98xFH35, and PD98xEF23, assessed by disease incidence (DI) of soybean sudden death syndrome (SDS).

QTL	FH13xEF23		FH13xPD98		FH33xEF23		FH33xPD98		PD98xFH35		PD98xEF23	
	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant
Rfs	0.20	0.40	0.42	0.33	0.25	0.25	0.10	0.40	0.17	0.33	0.50	0.67
Rfs2	0.30	0.30	0.33	0.58	0.00	0.25	0.20	0.00	0.67	0.33	0.00	0.00
qRfs3	0.20	0.30	0.33	0.42	0.00	0.00	0.00	0.30	0.17	0.50	0.08	0.00
qRfs5	0.20	0.00	0.33	0.33	0.75	0.75	0.80	0.80	0.67	0.00	0.50	0.83
qRfs4	0.40	0.30	0.58	0.33	0.63	0.38	0.70	0.50	0.83	0.67	0.17	0.33
qRfs7	0.10	0.10	0.17	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.33	0.00
Rfs12	0.60	0.50	0.25	0.17	0.13	0.25	0.40	0.30	0.00	0.33	0.08	0.17
qRfs12	0.20	0.20	0.33	0.67	0.88	0.00	0.80	0.50	0.33	0.17	0.33	0.33
qRfs6	0.00	0.00	0.17	0.33	0.75	0.00	0.20	0.40	0.00	0.67	0.00	0.00
qRfs11	0.30	0.40	0.33	0.25	0.00	0.50	0.00	0.20	0.67	0.00	1.00	0.67

Appendix 2. Allele frequency of ten quantitative trait loci (QTL) from the 10% most resistant and 10% least resistant lines from six populations, FH13xEF23, FH13xPD98, FH33xEF23, FH33xPD98, PD98xFH35, and PD98xEF23, assessed by foliar disease severity (DS) of soybean sudden death syndrome (SDS).

QTL	FH13xEF23		FH13xPD98		FH33xEF23		FH33xPD98		PD98xFH35		PD98xEF23	
	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant
Rfs	0.60	0.40	0.50	0.33	0.38	0.38	0.60	0.20	0.17	0.50	0.58	0.75
Rfs2	0.20	0.30	0.42	0.50	0.00	0.00	0.40	0.00	0.50	0.17	0.00	0.00
qRfs3	0.30	0.20	0.50	0.25	0.00	0.13	0.00	0.30	0.17	0.17	0.00	0.00
qRfs5	0.20	0.20	0.42	0.42	0.50	0.50	0.90	0.80	0.33	0.33	0.33	0.75
qRfs4	0.40	0.60	0.25	0.50	0.50	0.13	0.60	0.40	0.17	0.67	0.33	0.08
qRfs7	0.10	0.00	0.08	0.00	0.00	0.00	0.10	0.00	0.50	0.33	0.17	0.00
Rfs12	0.30	0.30	0.58	0.50	0.38	0.63	0.40	0.30	0.33	0.33	0.25	0.00
qRfs12	0.60	0.20	0.33	0.33	0.63	0.75	1.00	0.50	0.67	0.50	0.00	0.17
qRfs6	0.00	0.00	0.17	0.17	0.75	0.50	0.40	0.40	0.33	0.67	0.00	0.00
qRfs11	0.50	0.30	0.00	0.17	0.25	0.63	0.00	0.20	0.50	0.33	0.83	1.00

Appendix 3. Allele frequency of ten quantitative trait loci (QTL) from the 10% most resistant and 10% least resistant lines from six populations, FH13xEF23, FH13xPD98, FH33xEF23, FH33xPD98, PD98xFH35, and PD98xEF23 assessed by the area under the disease progress curve (AUDPC) of soybean sudden death syndrome (SDS).

QTL	FH13xEF23		FH13xPD98		FH33xExF23		FH33xPD98		PD98xFH35		PD98xEF23	
	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant
Rfs	0.80	0.40	0.33	0.42	0.25	0.63	0.50	0.10	0.17	0.17	0.67	0.83
Rfs2	0.20	0.60	0.33	0.50	0.00	0.00	0.20	0.00	0.50	0.50	0.00	0.00
qRfs3	0.20	0.30	0.42	0.33	0.00	0.00	0.00	0.30	0.17	0.17	0.00	0.00
qRfs5	0.80	0.40	0.42	0.42	0.50	0.75	0.80	0.80	0.33	0.33	0.50	0.75
qRfs4	0.70	0.50	0.42	0.58	0.63	0.38	0.90	0.40	0.17	0.83	0.33	0.08
qRfs7	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.50	0.33	0.00	0.00
Rfs12	0.50	0.30	0.58	0.33	0.38	0.63	0.50	0.40	0.33	0.00	0.17	0.00
qRfs12	0.80	0.60	0.33	0.17	0.38	0.75	0.80	0.40	0.67	0.33	0.00	0.17
qRfs6	0.00	0.00	0.17	0.17	0.50	0.75	0.40	0.20	0.33	0.67	0.00	0.00
qRfs11	0.40	0.50	0.08	0.17	0.00	0.25	0.30	0.60	0.50	0.33	0.83	1.00

Appendix 4. Allele frequency of ten quantitative trait loci (QTL) from the 10% most resistant and 10% least resistant lines from six populations, FH13xEF23, FH13xPD98, FH33xEF23, FH33xPD98, PD98xFH35, and PD98xEF23, assessed by root rot severity of soybean sudden death syndrome (SDS).

QTL	FH13xEF23		FH13xPD98		FH33xEF23		FH33xPD98		PD98xFH35		PD98xEF23	
	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant	10% Most Resistant	10% Least Resistant
Rfs	0.20	0.20	0.25	0.25	0.25	0.13	0.60	0.30	0.17	0.00	0.50	0.58
Rfs2	0.20	0.30	0.58	0.42	0.00	0.25	0.20	0.20	0.67	0.67	0.00	0.00
qRfs3	0.30	0.20	0.33	0.33	0.13	0.00	0.10	0.20	0.17	0.33	0.00	0.00
qRfs5	0.40	0.00	0.50	0.42	1.00	0.63	0.90	0.40	0.33	0.00	0.83	0.83
qRfs4	0.30	0.50	0.33	0.33	0.25	0.50	0.40	0.60	0.67	0.83	0.33	0.08
qRfs7	0.10	0.00	0.00	0.08	0.00	0.25	0.10	0.00	0.33	0.00	0.00	0.08
Rfs12	0.30	0.40	0.33	0.33	0.50	0.50	0.30	0.30	0.17	0.00	0.00	0.00
qRfs12	0.60	0.40	0.50	0.67	0.38	0.25	0.80	0.10	0.67	0.00	0.33	0.17
qRfs6	0.00	0.00	0.00	0.17	0.50	0.75	0.40	0.20	0.67	1.00	0.00	0.00
qRfs11	0.60	0.20	0.00	0.08	0.25	0.50	0.20	0.20	0.50	0.33	0.67	1.00

CHAPTER 4.

GENERAL CONCLUSIONS

This thesis describes a new modified method to screen for resistance to soybean sudden death syndrome (SDS), caused by *Fusarium virguliforme.* The modified method was devised to distinguish progeny from resistant by resistant crosses, used to evaluate ten QTL with known association with SDS resistance. The main goal of the research was to determine how and which of the QTL previously determined could impact disease incidence (DI), disease severity (DS), area under the disease progress curve (AUDPC) and root rot severity for SDS in soybean.

During the process of developing the modified screening method for SDS resistance, the research also suggested an effect of inoculum rate on SDS symptom expression. In the study, five inoculum densities (1:1, 1:5, 1:10, 1:15, 1:20 inoculum:soil) homogeneously mixed with soil were compared to identify the appropriate density for screening use. A trend for decreasing foliar severity as inoculum density decreased was observed, agreeing with previous research. This observation is consistent with the quantitative nature of soybean SDS resistance, as the resistant control cultivar Ripley had its resistance broken at the highest inoculum. The study also suggests that root dry weight could be a better indicator (r^2 = -0.74) of foliar severity than root rot severity. The inoculum density most appropriate for screening lines derived from crosses of resistant parents was found to be 1:20 inoculum:soil. This rate allowed differentiation of resistant and partially resistant genotypes at 30 to 36 days after planting (dap) without negatively impacting soybean seed germination.

The modified protocol method provided the means to distinguish small differences in levels of resistance in progeny derived from crosses of resistant by resistant parents. The new modified method when compared to the layer method, shifted distributions of the four disease assessment criteria (DI, DS, AUDPC, and root rot severity) to normal distributions from the skewed distribution observed with the layer method. The normal distributions in this study were also used to contrast the 10% most and 10% least resistant categories to compare QTL present in each category. With this technique, the study identified five QTL, *qRfs4, qRfs5, qRfs7, qRfs12,* and *Rfs12* that may have important effects on SDS resistance. These five QTL were associated with at least one disease assessment criteria across multiple populations in the 10% most resistant category. Of the five QTL, *qRfs4* and *qRfs12* had effects associated with more than one disease assessment criteria. Five other QTL, *Rfs, Rfs2, qRfs3, qRfs4,* and *qRfs11* were also identified to be in the 10% least resistant category across multiple populations, which may suggest a potentially low value for breeding purposes. These results will help soybean breeders select for the more impactful SDS resistance QTL, which ultimately may lead to cultivars with higher levels of resistance than presently available commercial cultivars.