Identification of QTL(s) Associated with Resistance to Sudden Death Syndrome (SDS) in Soybeans

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JoAnn E. Harren

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Abstract

Sudden Death Syndrome (SDS), caused by *Fusarium virguliforme* has been recognized to be one of the top four loss-causing diseases for soybeans (*Glycine max* L. Merr.) on a worldwide basis. By 2007, more than twenty QTLs providing resistance to SDS causing isolates were reported in eight different recombinant inbred line (RIL) populations grown in the Southern United States. This study was conducted to examine the Northern germplasm for SDS resistance. The objectives were to (1) establish a screening method for SDS of soybeans that provides informative phenotypes for both root resistance and foliar resistance (2) estimate the number, genetic positions and genetic effects of QTL involved in resistance under greenhouse and field conditions (3) determine the proportion of the genotypic variance explained by all detected QTL (4) to compare our results with those of other studies and (5) to draw conclusions about the prospects of utilizing marker-assisted selection (MAS) for increasing the level of resistance to SDS in the Northern germplasm. An RIL population of 230 F_5 derived F_{12} lines developed from 'Minsoy' x 'Noir1' was evaluated in the greenhouse and field with three varying procedures. Foliar and root rot SDS damage was assessed using a visual rating scale. Seven QTLS were detected for foliar resistance on chromosomes one, three, four, five, six and fifteen (D1a, N, C1, A1, C2, and E, respectively) with a total of 79.27% of genetic variance explained. Two QTLs were detected for root rot resistance on chromosomes two and three (D1b and N, respectively) with a total of 21.1% of genetic variance explained. A number of the intervals were associated with previous reported

SDS resistant QTLs; however, it appears that several of the QTLs are novel. Further research should be conducted to confirm the new locations.

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Chapter 1:
Literature Review

Soybean: Worldwide Crop

The soybean (*Glycine max* L. Merr.) is an economically important crop that is grown worldwide. In the United States, it is the second most cultivated crop with an average of 30 million hectares of production (Soy Stats, 2012), and in 2008 was reported to contribute approximately 17 billion dollars to the national economy (Radwan *et al.*, 2011). Soybeans originated in Southeastern Asia, and were thought to be domesticated between 1766 and 1125 BCE (Bilyeu *et al.* 2010). It is an annual, diploidized tetraploid species with a total of 20 chromosomes (Li *et al.* 2010). The estimated size of the soybean genome is 1,115 Mb (Cannon *et al.* 2009). The crop is grown for several different purposes. Besides being an ingredient in numerous food products, it is a major component in insecticides, disinfectants, printing inks, diesel fuel, animal feed, and much more (Pederson, 2007). When a crop of this magnitude suffers a significant yield loss from weather or pests, the economy is forced to adjust on a global scale.

Sudden Death Syndrome (SDS)

Sudden Death Syndrome (SDS), caused by *Fusarium virguliforme* (Akoi, O'Donnell, Homma & Lattanzi, 2003), formerly named *F. solani* (Mart.) Sacc. f.sp. *glycines* (referred to *F. virguliforme*), has been recognized to be one of the top four loss-causing diseases for soybeans on a worldwide basis (Wrather and Koenning 2006). The disease was first reported in the United States in the state of Arkansas, in 1971, and in 1982 M. C. Hirrelis was credited with establishing SDS as the disease name, based on the rapid appearance of the characteristic foliar symptoms (Roy *et al.* 1997). Since then, it has been reported in Tennessee, Missouri, Mississippi, Illinois, Kentucky, Kansas,

Indiana, Iowa, Nebraska, Minnesota (Navi *et al.*, 2008), Wisconsin (Bernstein *et al.*, 2007), and Michigan (Chilvers *et al.*, 2010). The disease has been reported in Canada (Anderson and Tenuta 1998), Argentina (Ploper 1993), Brazil (Nakajima *et al.* 1993, 1996), Thailand (Prathuangwong *et al.* 1996), Paraguay (Yorinori 1999), Bolivia (Yorinori 2002), and Uruguay (Ploper *et al.* 2003). Sudden Death Syndrome has been associated with yield losses typically ranging from 5 to 15% with up to 80% loss in individual fields (Roy *et al*., 1997). Estimated suppression of soybean yields (metric tons) due to SDS for the United States from 2004 to 2007 was 759,227 metric tons (Wrather and Koenning 2009). Yield loss is a result of the characteristic symptoms of SDS; root rot, crown necrosis, vascular discoloration of roots and stems, interveinal chlorosis and necrosis of leaves, premature defoliation, and pod abortion (Jin *et al.,* 1996).

Taxonomy of *Fusarium virguliforme*

The complexity of *F. virguliforme* has resulted in the taxonomy undergoing several revisions throughout the decades. SDS is the outcome of an infection initiated by the soil-borne hemibiotrophic fungi within a group (clade 2) of the *Fusarium solani* species complex. In North America, *Fusarium virguliforme* (Aoki *et al*., 2003), formerly *Fusarium solani* f. sp. g*lycines* (Roy *et al.*, 1997), is the causal agent of the disease. When it was first discovered in 1971, *Fusarium* spp., particularly *F. solani*, was repeatedly isolated from soybeans exhibiting SDS symptoms (Roy *et al.*, 1997; Roy *et al.*, 1989). From those isolations, two morphologically distinct *F. solani* strains were frequently reported. The two strains were referred to as FS-A and FS-B (Roy *et al.,* 1989). FS-A was isolated primarily from plants with SDS symptoms, whereas FS-B was

inconsistently isolated from plants with SDS symptoms. FS-B was isolated from soybean plants with SDS symptoms and from plants that appeared to be healthy. Koch's postulates were conducted with both strains, and the results revealed that only FS-A produced the SDS symptoms, confirming that the FS-A strain was the causal organism (Roy *et al.,* 1997; Roy *et al.,* 1989; Rupe 1989).

Genetic analysis using rDNA sequences showed that *F. solani* f. sp. *phaseoli* and FS-A were the same species. *F. solani* f. sp. *phaseoli* was previously reported, so it was concluded that it would be considered the official causal organism of SDS (Jackson 2005; O'Donnell and Gray, 1995; Roy 1997; Roy *et al.,* 1997). However, Roy (1997) determined that isolates of *F. solani* f. sp. *phaseoli* did not possess the same pathogenicity characteristics as FS-A. The *F. solani* f. sp. *phaseoli* isolates were unable to consistently produce foliar SDS symptoms. With the contrasting results, Roy suggested that FS-A had a different host range than *F. solani* f. sp. *phaseoli* and therefore FS-A isolates were renamed *F. solani* f. sp. *glycines* in 1997 (Roy 1997; Roy *et al.,* 1997).

It should be noted, that a number of the *F. solani* f. sp. *phaseoli* isolates have been reported to be incorrectly deposited in databases (Aoki and O'Donnell 2005). Some of the mistaken isolates have been identified as the soybean root rot pathogen, *F. cuneirostrum* sp. nov. (formerly considered to cause SDS in South America) (Aoki and O'Donnell 2005; O'Donnell 2010). Later, research revealed that the SDS pathogen in North America is genetically and morphologically distinct from other previously reported *Fusarium* spp. and is now classified as *F. virguliforme* (Aoki *et al.,* 2003). *F. virguliforme* remains the

current name of the SDS pathogen in North America. This research will concentrate on the United States pathogen, *F. virguliforme*, specifically the Midwest isolates (Minnesota isolate: Somerset #1A and Illinois isolate: Mont-1A).

In South America, *F. brasiliense* sp. nov., *F. cuneirostrum* sp. nov., *F. tucumaniae* (Aoki *et al.*, 2003), and *F. virguliforme* is the fungi that cause SDS symptoms (Aoki and O'Donnell 2005). The high genetic variability and/or speciation seen in South America are hypothesized to be the outcome of *F. tucumaniae* having both a teleomorph and anamorph stage (Covert *et al.*, 2007). This is because *Nectria haematococca*, the known teleomorph of *F. solani*, can serve as a compatible sexual mating type for *F. tucumaniae* (Covert *et al.* 2007, Webster and Weber, 2007). Presently in the U.S., *F. virguliforme* has been noted to be lacking the + mating type for a teleomorph stage, which may contribute to the low genetic variability and small potential for rapid evolution found among U.S. isolates compared to South American SDS pathogens (Aoki and O'Donnell 2005, Covert *et al.*, 2007).

Life Cycle of *Fusarium virguliforme*

The timing of *F. virguliforme* infection is a critical factor influencing which root tissues are colonized and subsequent development of foliar symptoms (Navi et al. 2008). The development of SDS is dependent on environmental factors such as rainfall, soil fertility and planting date (Ji *et al.*, 2006). Studies have shown that early soybean planting has been associated with increased SDS severity (Navi *et al.*, 2008). *F. virguliforme* is associated with infection of young soybean seedlings through the roots in the spring during cool, wet conditions. The fungus exogenously penetrates the root cell walls and

infects specific cells (Yuan *et al.*, 2008). The pathogen can infect as soon as the soybean seed germinates. The SDS pathogen germinates and produces germ tubes, appressorium, and an infection peg (Navi *et al.,* 2008). The point of entry of the infection peg is on the radicle through the base of the root hairs and/or root cap (Navi *et al.,* 2008). The hyphae then grow both intercellularly and intracellulary throughout the soybean root and eventually colonize the vascular tissue of the plant causing the development of the root rot. As the plant matures and if the environment remains favorable, rot continues to extend throughout the root and the pathogen begins production of the phytotoxins. If the xylem tissue becomes colonized, the phytotoxins are translocated to the leaves of the plant during the reproductive stages, causing the diagnostic foliar symptoms: leaf chlorosis, necrosis, and in severe cases, premature defoliation (Navi *et al.,* 2008). In the United States, *F. virguliforme* reproduces asexually via mass production of macro- and microconidia (predominately macroconidia), and through the formation of thick-walled resting spores called chlamydospores (Roy *et al.*, 1997). After the soybean plant is harvested, *F. virguliforme* persists and overwinters as chlamydospores or macroconidia in the plant residue or in the soil as chlamydospores for many years; similar to *F. oxysporum* (Roy *et al.*, 1997). Even with crop rotation, the pathogen can reemerge once soybeans are planted and the environment is favorable.

SDS Characteristic Symptoms

SDS of soybeans is accompanied by two different components that result from differing effects of the pathogen; root rot and leaf scorch. *F. virguliforme* is a soil borne root rot pathogen. It has a very wide host range. All legumes, most dicots and some

cereals are considered to be susceptible to the root rot colonization of *F. virguliforme* (Yuan *et al.*, 2008). The characteristic root symptoms found on a number of hosts are discolored taproots and basal stems (Navi *et al.*, 2008) and possible blue pigmentation around the base of the stem. The foliar symptoms, on the other hand, have only been observed in soybeans. The foliar symptoms first appear as scattered interveinal chlorosis and interveinal necrotic spots or blotches, usually on the leaves of the upper nodes. As the disease progresses, the chlorotic areas grow into streaks, causing the leaves to pucker and mottle (Navi *et al.*, 2008). The leaf scorch occurs during the reproductive stages when phytotoxin(s) produced in the root are translocated to the leaves of the plant.

Phytotoxicity associated with SDS

The phytotoxicity of culture filtrate from *F. virguliforme* was confirmed by Jin *et al*. (1996). The study concentrated on the relationship of calli sensitivity to culture filtrates and susceptibility of plants to inoculation by *F. virguliforme*. The results indicated that SDS causing isolates of *F. virguliforme* produce a phytotoxin in culture which contributes to pathogenicity, and is important in foliar symptom development. The role of the toxin in the root of the plant was not studied (Jin *et al.*, 1996).

Further studies have proven that *F. virguliforme* is a facultative pathogen releasing one or more phytotoxins into cultural media (Brar *et al.*, 2011). The number of phytotoxins and size of the molecules have varied from study to study. A phytotoxin was characterized from an SDS—causing isolate as a polypeptide with an estimated molecular weight of 17,000 by Jin *et al.*, 1996. It was described as heat unstable, negatively charged, absorbed by 10% charcoal, and destroyed by proteinase K. The

single protein was found in all the samples that causedbrowning of soybean calli, necrosis on detached soybean cotyledons and leaves, along with yellowing, curling, and drying of attached soybean cotyledons and leaves (Jin *et al.,* 1996). The gene encoding the 17,000 phytotoxin has not been isolated. In another report, an SDS—causing isolate was found to produce a low-molecular-weight phytotoxin, monorden. Monorden was noted to inhibit shoot and root growth as well as induce leaf and stem necrosis of the soybean plant (Baker, 1994). More recently, Brar *et al.* (2011) isolated the phytotoxin, FvTox1 and identified the single copy *FvTox1* gene. The 13.5-kDa acidic protein (phytotoxin) showed high similarity to hypothetical proteins of a number of pathogens. Specifically, its sequence was 91 % similar to *Fusarium verticillioides* (Brar *et al.,* 2011).

Studies have been conducted to determine how the phytotoxins induce the damaging results of SDS (Brar *et al.*, 2011). The pathogen *F. virguliforme* has never been isolated from diseased foliar tissue (Brar *et al.*, 2011). The causal agent has only been isolated from the roots and occasionally from above the crown (Jin *et al.,* 1996). In studying the effects of the toxin(s), Ji *et al*. (2006) established that light was essential for the activation of the phytotoxins that degrade the leaf tissue. The authors examined the changes in the protein profiles of diseased leaves infected with cell-free *F. virguliforme* culture filtrates prepared from *F. virguliforme* isolates. They reported that the diseased leaves did not contain a 55 kDa protein. Matrix-assisted laser desorption-ionization time of flight mass spectrometric analyses and a database search confirmed that the identity of the missing protein was the ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit. It was noted that Rubisco’s complete degradation occurred within 24 hours

of exposure to cell-free *F. virguliforme* culture filtrate-fed seedlings under light. The authors concluded that the disappearance of Rubisco was due to degradation rather than inhibition of its synthesis.

Rubisco is known to be involved in carbon assimilation and photorespiration (Ji *et al.*, 2006), and its degradation is associated with two important physiological processes: senescence and plant responses to environmental stresses (Ji *et al.*, 2006).When Rubisco carboxylation activity is disrupted, ATP and NADPH generation from photophosphorylation cannot be utilized for carbohydrate assimilation and ADP and $NADP^+$ are not formed for further photophosphorylation. The inhibition leads to the accumulation of reactive oxygen species (ROS) through the transfer of electrons from photosystem I to molecular oxygen rather than to ADP and $NADP^+$. The accumulation of ROS was reported in the study. ROS production associated with phytotoxins can lead to peroxidative breakdown of unsaturated fatty acids. The chlorophyll is then oxidized and bleached by the alkoxyl radicals produced by the peroxidation of fatty acids (Ji *et al.*, 2006). They suggested final result would be programmed cell death and presumably, leaf scorch. For confirmation, terminal deoxynucleotidyl transferase-mediated nick end labeling experiments were conducted. The results suggested that programmed cell death was initiated in the leaves of the seedlings fed with the cell-free *F. virguliforme* culture filtrates.

In addition, Ji *et al.* (2006) confirmed the important role of light in the activation of phytotoxins. The *F. virguliforme* culture filtrate-fed seedlings were first placed in the dark, and it was noted that the levels of Rubisco's large subunit did not change; whereas

in the light, rapid degradation occurred. They suggested that this confirms that light is essential for the production of ROS. Besides the necessity of light, Navi *et al.* (2008) determined that the severity and location of the root infection is a deciding factor in the expression of foliar symptoms. Soybeans were germinated, inoculated, and after 30 days the plants were washed and processed for microtome sectioning of resin blocks. Images were taken of the sections and were analyzed for colonization of *F. virguliforme* in the phloem and xylem. When the hyphae were located in the xylem, foliar symptoms were seen. However, they confirmed that the hyphae observed in the phloem of taproots did not cause the expression of foliar symptoms; only external discoloration of the basal stem and taproots was expressed. The authors proposed that the pathogen colonizes the vascular tissue by entering through the radical's root cap or the base of root hairs/epidermis to reach the xylem. Ultimately, the study concluded that the colonization and penetration of *F. virguliforme* in the xylem tissues is critical for soybean plants to display SDS leaf symptoms.

Brar *et al.* (2011) proposed that the role of the FvTox1 toxin that was identified, used similar mechanisms to the ones established by victorin, a cyclicized pentapeptide toxin produced by *Cochliobolus victoriae*, and Ptr ToxA, a toxin that has been transferred horizontally from *Stagonospora nodorum* to *Pytenophora*. They believe that *F. virguliforme* targets foliar tissues to reduce or eliminate the nutrient supply to roots, so that it can completely overcome possible partial root resistance (Brar *et al*., 2011). In order to confirm the role of FvTox1, the candidate *FvTox1* gene was expressed in the baculovirus-infected insect cell line Sf21. N-terminal sequencing was done. The

utilization of N-terminal sequencing determined the cleavage site. It appeared that the first 32 amino acids (aa) are cleaved off prior to the translocation through the endoplasmic reticulum membrane to the cytoplasm. It is known that the FvTox1 is processed from a pro-peptide. The pro-FvTox1 has the entire open reading frame along with the pro-toxin and pro-peptide, and the mature-FvTox1 only utilizes the secreted peptide that is found in the purified *F. virguliforme* culture filtrates. The first 32 aa were found to be a chloroplast transit peptide (cTP) in FvTox1. The recombinant FvTox1 that was isolated from the baculovirus-infected insect cells was infiltrated into disks of both susceptible and resistant soybean leaves. Chlorotic SDS symptoms were observed in the leaves of susceptible lines and no symptoms were noted in the leaves of resistant lines. Thus the authors concluded that FvTox1 is a major *F. virguliforme* pathogenicity factor involved in foliar SDS development in soybean.

Resistance

Resistance to SDS has been reported by some to be controlled by a single gene and others as several quantitative trait loci (QTL) (Chang *et al.,* 1996; Farias-Neto *et al.,* 2007; Hashmi, 2004; Hnetkovsky *et al.,* 1996; Iqbal *et al*., 2001; Kassem *et al*., 2006; Kazi, 2008; Lightfoot *et al*., 2001; Meksem *et al*., 1999; Njiti *et al*., 1998; Njiti *et al.,* 2002; Prabhu *et al.,* 1999; Sanitchon *et al*., 2004). The classical genetic studies predicted heterogeneity of resistance to SDS above 90%; with only a few major loci underlying resistance (Iqbal *et al.,* 2001). They reported that leaf scorch was controlled by a single dominant gene, *Rfs*1, (*Rfs*, is a loci that is associated with resistance to *Fusarium solani forma specialis glycines* (Triwitayakorn *et al.,* 2005)) in cv. Ripley (Stephens *et al.,*

1993) in greenhouse conditions, and two genes in cv. P9451 (Ringler, 1996). Others have reported (Njiti *et al.*, 1998, 2001, and 2002) that in different mapping populations resistance was conditioned by quantitative trait loci. A few major QTLs were determined to have partial and mulitgenic inheritance of SDS resistance (Hnetkovsky *et al.*, 1996). In 2007, QTL(s) for resistance to SDS-causing isolates were reported in eight different recombinant inbred line (RIL) populations with "logarithm of the odds" (LOD) scores at or exceeding 3.00 (Table 1.1, (Kazi *et al.*, 2008)). In genetics, the LOD score is a statistical estimate of whether two genes, or a gene and a disease gene, are likely to be located near each other on a chromosome and are therefore likely to be inherited. QTLs have been detected in overlapping intervals that might indicate a single locus. If the overlapping QTLs were taken into account, it is likely there are about 12 *qRfs* loci on nine chromosomes including chromosomes 5, 6, 17, 13, 18, 20, 16, 18, and 3 (A2, C2, D2, F, G, I, J, L, and N, respectively). A number of QTLs have been confirmed for SDS resistance and are suffixed with *cqRfs*- within Table 1 (Kazi *et al.*, 2008). The QTL confirmation was completed by being mapped to a similar location in separate populations or mapped for a second time in near isogeneic lines (NILs) derived from the RILs segregating across the regions that encompassed the QTL(s).

Conclusion

In addition to the numerous QTLs detected, the resistance to *F. virguliforme* is complicated. Several different soybean cultivars have displayed differences in resistance to both leaf scorch and root rot of SDS. Results suggest that separate selection for leaf scorch and root rot will be necessary to improve resistance to SDS. Various QTLs have

been specifically assigned to root rot resistance or leaf scorch resistance (Hnetkovsky *et al*. 1996; Iqbal *et al.* 2001); however, screening methods still need improvement to obtain accurate phenotypes for each symptom to map SDS resistant QTLs (Luckew *et al.*, 2012).

Table 1.1: Previously reported SDS QTLs found in different RIL populations by chromosome.

Chromosome Number	Linkage Group	Soybase QTL Name (Linked Markers) [a]	Populations	Probable Locus Names[b]	References
3	N	SDS 2-7 (0C01-650) SDS 8-3 (Satt 080) qSDS002 (Satt 631)	Essex x Forrest Pyramid x Douglas Ripley x Spencer	*qRfs6* *qRfs6* *cqRfs6* [d]	Chang *et al*., 1996 Njiti *et al*., 2002 Hashmi, 2004
6	C2	SDS 1-1 (0005-250) SDS 4–2 (K455_1) SDS 7–5 (Satt 371) SDS 8–2 (Satt 307) SDS 8–3 (Satt 316) SDS 9-2 (Satt277)	Essex x Forrest Essex x Forrest Essex x Forrest Pyramid x Douglas Pyramid x Douglas Flyer x Hartwig	*cqRfs4* *cqRfs4* *cqRfs4* *cqRfs4* *qRfs9* *cqRfs4*	Hnetkovsky *et al*., 1996 Njiti *et al*., 1998 Iqbal *et al*., 2001 Njiti *et al*., 2002 Njiti *et al*., 2002 Kazi, 2008
8	A2	SDS11-1 (Satt 187)	Ripley x Spencer	*qRfs7*	Hashmi, 2004
13	F	SDS10-1 (Satt 160)	Essex x Forrest	*qRfs10*	Kassem *et al*., 2006
16	J	SDS10-2 (Satt 285) SDSna(Satt183)	Essex x Forrest GC87018-12-2B-1 x GC89045-13-1	*qRfs8* *qRfs8*	Kassem *et al*., 2006 Sanitchon *et al*., 2004
17	D2	SDS11-2 (Satt 528) SDS 9-1 (Satt574) SDS 2-3 ($OZ19_{468}$)	Ripley x Spencer Flyer x Hartwig Pyramid x Douglas	*cqSDS001* *qRfs11* [c] *qRfs11*	Farias-Neto *et al*., 2007 Kazi, 2008 Lightfoot *et al*., 2001
18	G	SDS 6-1 (Bng 122_1) SDS 3-1 (0G13_490) SDS 6-2 (Satt	Essex x Forrest Essex x Forrest Essex x	*cqRfs1* [c] *cqRfs3* *cqRfs2*	Meksem *et al*., 1999 Chang *et al*., 1996 Meksem *et*

		309)	Forrest		*al*., 1999
		SDS8-1 (Satt309)	Pyramid x Douglas	*cqRfs1*	Njiti *et al*., 2002
		SDS 8-4 (Satt038)	Pyramid x Douglas	*cqRfs2*	Njiti *et al*., 2002
		SDS 7-1 (Satt 214)	Essex x Forrest	*cqRfs2*	Iqbal *et al*., 2001
		SDS 7-2 (Satt 309)	Essex x Forrest	*cqRfs2*	Iqbal *et al*., 2001
		SDS 7-3 (Satt 570)	Essex x Forrest	*cqRfs1* [c]	Iqbal *et al*., 2001
		SDS 7-4 (OEO2_1000)	Essex x Forrest	*cqRfs3*	Iqbal *et al*., 2001
		SDS 4-1 (0I03-512)	Essex x Forrest	*cqRfs1* [c]	Njiti *et al*., 1998
		SDS 4-3 (Bng122_1)	Essex x Forrest	*cqRfs1* [c]	Njiti *et al*., 1998
		SDS 5-1 (Satt 038)	Flyer x Hartwig	*cqRfs1* [c]	Prabhu *et al*., 1999
		SDS 8-1 (Satt 163)	Pyramid x Douglas	*cqRfs2*	Njiti *et al*., 2002
		SDS9-3 (Satt427)	Flyer x Hartwig	*cqRfs3*	Kazi, 2008
19	L	SDS12-1 (Sat 099)	Minsoy x Noir 1	*qRfs12*	Njiti and Lightfoot, 2006
		SDS12-1 (Sat 099)	Ripley x Spencer	*qRfs12*	Hashmi, 2004
20	I	SDS 7-6 (Satt 354)	Essex x Forrest	*qRfs5*	Iqbal *et al*., 2001

[a] QTL numbers are from Soybase 2004-2006 under the rules of the Soybean Genetics Committee 200-2006, where assigned.

[b] From 27 QTL detections there were 15 QTL counting each detection in a separate population once. Assuming QTL detected in common intervals in separate populations represents the alleles there were 11- 12 loci.

[c] QTL associated with resistance to root infection. QTL detected in common intervals in separate populations or derived NILs were considered confirmed and suffixed with c under Soybean Genetics Committee recommendations from 2000–2006 (http://soybase.agron.iastate.edu/nomenclature/QTL.html).

Chapter 2:
Revised Screening Protocols to obtain Phenotypes of *Fusarium virguliforme* Infection

Introduction

Decades of research have been conducted on SDS and various screening procedures have been published. However, screening methods still need improvement to obtain accurate phenotypes that will enable mapping of QTLs for resistance to SDS (Luckew *et al.,* 2012). In the past research screening for resistance to SDS has been conducted primarily in field trials (Chang *et al.,* 1996; Farias-Neto *et al.,* 2007; Kazi, 2008; Njiti *et al.,* 1998; Njiti *et al.,* 2002). Field research is complicated by unregulated temperature and moisture, which are important factors influencing disease development (Farias-Neto *et al.,* 2007; Roy *et al.,* 1997) and create challenges in evaluating cultivars in the field (Njiti *et al.,* 2001). Differences in inoculum density distribution in soils and unpredictable weather are some of the possible factors that could cause inconsistent cultivar rankings among field screening trials (Luckew *et al.,* 2012). To control these factors, placing inoculum in the soil in close proximity to the seed and irrigating during the reproductive growth stages has provided adequate disease pressure to distinguish partially resistant from susceptible genotypes (Farias-Neto *et al.,* 2007).

Greenhouse and growth chamber screening trials are frequently used to assess resistance to SDS. A variety of inoculation methods have been developed, especially with regards to the inoculum substrate. *F. virguliforme* infested oat (*Avena fatua* L.) seeds (Stephens *et al.,* 1993; Melgar and Roy, 1994), sorghum (*Sorghum bicolor* (L.) Monench ssp. Bicolor) 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) have been used as inoculum. A variety of inoculation methods have been

developed. Inoculum has been distributed in a layer planting method starting from the bottom with pasteurized soil, infested substrate, pasteurized soil, seeds, and pasteurized soil (Hashmi *et al*., 2005; Njiti *et al.*, 2001). Another method had soil mixed with ground infested sorghum seeds only on the outer edges of the planting pots to specifically infect the soybean's lateral roots while the taproot was allowed to grow in non-infested soil (Ortiz-Ribbing and Eastburn, 2004). A more recent method evenly mixed the pasteurized soil and infested substrate and the seeds were directly planted in the evenly inoculated soil (Luckew *et al.*, 2012).

Besides soil inoculation assays in the greenhouse, more recent screening protocols have used culture filtrates of *F. virguliforme* to induce SDS symptoms on soybean seedlings (Jin *et al.*, 1996b). Stems of cut soybean seedlings are placed into diluted *F. virguliforme* culture filtrates that contain the phytotoxins produced by the fungus. The method has been reported to be an effective approach to identifying resistance to SDS foliar symptoms (Li *et al*., 1999). Another method had leaf discs immersed in culture filtrate and then placed in Petri dishes for evaluation of SDS symptoms (Brar *et al.*, 2011).

In this study, 230 recombinant inbred lines (RIL) were evaluated that were developed from a cross of 'Minsoy' (PI 27890) by 'Noir1' (PI 290136) parental lines (Mansur *et al.*, 1996). The population was previously mapped for other agronomic traits such as lodging, height, flowering, maturity, oil, protein, seed weight, yield, leaf length, leaf width, and leaf area (Mansur *et al.*, 1996). The parents, 'Minsoy' and 'Noir1' demonstrated similar phenotypes for most traits; however, the range of values in the

progeny RI lines exceeded the parental values. It appeared that ‘Minsoy’ and ‘Noir1’ represent divergent lineages of the cultivated soybean containing different alleles at many loci (Mansur *et al.,* 1996). With the RIL population segregating for several traits, it was hypothesized that the population would segregate for a range of resistance to SDS.

This mapping population was tested in three different analyses: greenhouse soil inoculation analysis, phytotoxin cut-seedling-stem analysis, and field analysis for resistance to *F. virguliforme*, Minnesota isolate, Somerset #1A. In an effort to obtain a phenotype that positively correlates in two different conditions: greenhouse and field, experiments were conducted. Two separate protocols were used in the greenhouse: soil inoculation analysis and phytotoxin cut-seedling-stem analysis. One field trial was inoculated and irrigated in the 2012 growing season. The objectives of this chapter were to (1) establish reliable screening methods for SDS of soybeans that provided informative phenotypes for both root resistance and foliar resistance and (2) to compare our results with studies that have looked at the same RIL population.

Materials and Methods

Seed Acquisition

A recombinant inbred line (RIL) population of 230 soybean lines was developed from the cross of ‘Minsoy’ x ‘Noir1’ as described by Lark *et al.* (1995). At the F_2 stage, lines were advanced via single-seed descent (Brim 1966) and at the F_5 stage; individual plants were selected and maintained as an RIL to the F_{12} stage and then utilized in this study. Seven specialty lines: M06-235003 (M99-286149 x MN1606SP), M06-235007 (M99-286149 x MN1606SP), M06-235009 (M99-286149 x MN1606SP), M06-235039

(M99-286149 x MN1606SP), M06-235012 (M99-286149 x MN1606SP), M06-235028 (M99-286149 x MN1606SP), and M04R-543121 (M98-331009 x Ripley) were evaluated based on results seen in The Soybean Sudden Death Syndrome Regional Tests (Agronomy Research Center Southern Illinois University Carbondale, 2007) regarding SDS resistance. Four checks were grown throughout all of the experiments: 'Minsoy' (RIL parent), 'Noir1' (RIL parent), 'AG2107' (a Monsanto commercial cultivar, maturity 2.1) (susceptible check), and 'MN1606SP' (M90-764 x M90-2144) (resistant check). The lines are specific to the Northern germplasm of the United States maturity groups 0, I, and II.

Greenhouse Soil Inoculation Analysis

The greenhouse soil inoculation analysis was conducted in six separate plantings due to space and time constraints during the months of February, March, April, October, and November of 2012. Each planting was conducted in the same greenhouse environment and exposed to the same conditions. The number of lines tested per planting was 14, 46, 46, 46, 45, and 39.

The pathogen, *F. virguliforme,* Somerset #1A isolate was collected in the Minnesota township of Somerset, Steele County in 2002. The Somerset #1A isolate reproduced in inoculated soil was sprinkled on water agar with irradiated carnation leaves in the media and grown at room temperature for approximately one week. Five $1cm^3$ blocks of mycelium agar were transferred to 500mL of sterile red sorghum seed. When the inoculum began producing macroconidia, approximately 21 days after inoculation, it was combined in a one to twenty ration of inoculum to soil with a pasteurized soil

mixture (one-third agronomy mix (sandy loam, sand, peat and composted manure), one-third sand, and one-third Professional Growing Mix LC8 (with 70-80% Canadian Sphaghum peat moss, perlite, vermiculite, and dolomitic lime) distributed through Sun Gro Horticulture® (Seba Beach, Canada)). The soil was pasteurized for 45 minutes at 82.2ºC to eliminate most weed seeds, fungi, and insects (Nelson, 2003). Conetainers with sterile uninfested sorghum and pasteurized soil mixture combined in a one to twenty ratio were additionally prepared as controls with the parental lines. Twenty grams of Osmocote® 14-14-14 fertilizer was applied to the top of the soil per conetainer at the time of planting. Two seeds of each line were planted and after emergence thinned to a single plant per 200mL conetainer containing the soil/inoculum mixture.

The experiment was organized as a completely randomized design replicated five times. The plants were maintained at 22°C to 26 °C with 16 hours of daylight. Light intensity varied by season and the range of lux recorded was 3,750 to 32,000 over all of the plantings. Soil moisture in the conetainers was maintained near saturation for the 21 days duration of plant growth by daily watering and submerging the tips of the conetainers in a tray of water one to two cm deep. Foliar symptoms and root rot were assessed approximately 21 days after planting. The foliar symptoms were scored on the basis of a one to eight rating scale (Table 2.1), and root rot symptoms on a one to ten rating scale (Table 2.2) (Bowen and Slaminko, 2008). Plants were harvested after symptom development to measure stem and root length. Dry root and stem weights were obtained after three days of drying at 35ºC.

Phytotoxin Cut-Seedling-Stem Analysis

A phytotoxin cut-seedling-stem analysis was conducted in the greenhouse in two separate plantings due to time constraints in the months of October and November of 2012 based on the cut-seedling-stem assay (Hartman et al., 2004) with some minor modifications. A filtrate of *F. virguliforme* (Minnesota isolate, Somerset #1A) was produced from cultures grown for 21 days in the dark with shaking at 130 RPM (revolutions per minute); at 21 °C in potato dextrose broth (confirmed pH was between four and six). The liquid culture was filtered to remove any possible viable fungal structures with two separate filtration methods. The first method utilized a glass filtration apparatus with Whatman filter paper 0.22um (Whatman International Ltd, Maidstone, England). The second filtration was conducted in a disposable Nalgene Supor®machV Lot# 1063744 filter (Fisher Scientific Company, Ottawa, Ontario, Canada). The filtrate was diluted to a one to twenty ratio of filtrate to sterile distilled water. Each soybean line was evaluated in 30 mL of filtrate in three replications. Every planting was conducted in the same greenhouse environment and exposed to the same conditions.

The number of lines tested in planting one and two were 119 and 120, respectively. The plants were grown in the greenhouse for approximately 14 days in medium course vermiculite with approximately less than one percent crystalline silica (Sun Gro Horticulture® Seba Beach, Canada). Seedling stems were cut at the surface of the vermiculite and the stems of the cut soybean seedlings were immersed in 50 mL conical polypropylene centrifuge tubes (to a depth of three to five cm) containing 30 ml of the fresh, diluted *F. virguliforme* filtrate. Tubes containing 30 ml of non-inoculated liquid medium (diluted one to twenty in water) were additionally prepared as a control.

The cut- seedlings were placed in a dark chamber for four hours to prevent severe wilting that would occur if freshly cut plants were placed immediately under bright lights. Then the plants were placed in the greenhouse at 22°C to 26°C with 16 hours of daylight. The cut seedlings were monitored daily for foliar symptoms and approximately one week later foliar symptoms were scored on the one to eight rating scale (Table 2.1) (Bowen and Slaminko, 2008).

Field Analysis

In an attempt to eliminate some of the difficulties field research encounters, a method of artificially placing inoculum in the soil in close proximity to the seed and irrigation from planting to reproductive growth stages was used. Sorghum inoculum was prepared in mass quantities for soil inoculation in the field analysis. Pails were filled with dry sorghum and covered with distilled water and were left to sit overnight (ten liters maximum per pail). After the 12 hour soak, the water was poured off using cheesecloth secured with wire over the top of the pails. Approximately 1.5 liters of wet sorghum was separately placed in doubled “twelve by twenty-four” Clavies® autoclavable bags, (Cat#13185-1224 Scienceware, Wayne, New Jersey). The ends were cut off and caps removed from autoclavable 50 mL conical polypropylene centrifuge tubes. The shortened tubes were zip tied (20.32 cm miniature cable ties (McMaster Carr, Robbinsville, New Jersey)) ridge side down in the sorghum filled autoclavable bags. Each tube was filled with a foam plug, covered with foil, and secured with autoclave tape to avoid contamination. Each filled bag was autoclaved 60 minutes and cooled overnight. One fourth of a *F. virguliforme* (Minnesota isolate, Somerset #1A) cultured plate grown as

previously described for inoculum production was inserted in each bag and evenly distributed throughout. The bags were incubated at room temperature for approximately 21 days with regular kneading to ensure equal distribution of the pathogen over the sorghum.

Three replications of each line, consisting of five plants in each replication or hill plot, was planted 0.305 meters apart in 12.192 meter rows in a completely randomized design. The seed was planted directly into a 1,500 mL volume of soil that was mixed in a one to twenty ratio of infested sorghum to soil. Hill plots with sterile sorghum and field soil combined in a one to twenty ratio were additionally prepared as controls. The moisture of the soil was monitored and irrigated to maintain the necessary moisture for the optimal disease conditions for foliar severity scores and root rot scores. The experiment was planted on 14 May 2012, in Rosemount, MN. The soil at the site was Waukegan silt loam, well-drained (zero to one percent slope). The field plot was monitored weekly for foliar symptoms in both the vegetative and reproductive stages of the lines. When the lines reached the reproductive stages, root samples were taken on a subset of the population and scored for root rot using the one to ten rating scale (Table 2.2). A number of the roots were collected from both inoculated and control hill plots, cut into sections, and placed on potato dextrose agar (PDA) plates. The roots were plated for seven days and the media was examined for the growth of *F. virguliforme.* As the plants approached maturity, a weekly SDS foliar score (Table 2.1) was taken for each hill plot until all of the plants reached full senescence.

Baiting from Soil Samples: *Phytophthora sojae*

Soil samples were collected from the field during foliar ratings and tested for the presence of *Phytophthora sojae* following a protocol adapted from Dorrance *et al*. (2004). Large clods and pieces of vegetation were removed from the soil samples. Thirty grams of soil were taken directly from the sample without any drying and placed into a sterile Petri dish. The soil was saturated with autoclaved water for one hour. Excess water was siphoned off to allow the soil to be wet but not under standing water. Four replications were used with the samples. Each replication was covered with foil to keep the sample in the dark and stored on the lab bench at room temperature (23ºC). Simultaneously, 'McCall' (a susceptible cultivar to Phytophthora Root Rot) seeds were disinfected for one minute in a 10% sodium hypochlorite (NaClO) solution. The seeds were then rinsed twice in autoclaved water and placed/rolled on damp germination paper. Rubber bands were wrapped lightly around each end to secure seeds but not restrict growth. The secured germination paper was placed in a beaker with a small amount of water and covered with foil. The beaker was allowed to set on the lab bench at 23ºC for approximately three to four days to germinate the seeds. The germinated seedlings were disinfected in a 10% sodium hypochlorite (NaClO) solution for one minute and rinsed twice in autoclaved water. Each replicated Petri dish of soil received three to five sterilized seedlings gently pressed into the soil and were rewrapped with foil. Five days later, the seedlings were removed from the soil and examined for lesions. When lesions were present, the symptomatic seedlings were rinsed in water to remove any remaining soil and disinfected for thirty seconds in 10% sodium hypochlorite (NaClO) solution, then rinsed twice in autoclaved deionized water for one minute each rinse. Each lesion

was cut into five root segments with a sterilized scalpel and placed on selective media plates. The plates remained at room temperature (23ºC), whether in the light or dark. For the next three days, colonies growing from lesion segments were transferred onto fresh selective media plates. The resulting *P. sojae* cultures were then utilized to inoculate McCall plants in the greenhouse. Four days after the inoculation, the plants were checked for pathogenicity.

Statistical Analysis

The observed field foliar scores throughout the weeks of observations were averaged by line and the means were utilized for correlations and further analyses. Calculations of heritability (h^2) estimates represent the genetic variance over the genetic variance plus the confounded genetic by environmental variance and error. The h^2 coefficients were calculated with the variance components obtained through analysis of variance (ANOVA). Ninety percent confidence intervals around genetic variance were calculated as described by Bernardo (2010), and 90% confidence intervals around h^2 (Knapp *et al.*, 1985). Due to the low frequency of heterozygosity at the $F_{5:12}$, the genetic variance is almost entirely an additive and additive x additive interaction. As a result, the heritability estimate was considered narrow sense (Kazi *et al.*, 2008). Means, standard deviations, and frequency distributions of line response to inoculum were calculated. Spearman and Pearson correlation coefficients were calculated with 95% confidence intervals and statistical significance in R (version 2.13.2) (The R Foundation for Statistical Computing, 2011) between the greenhouse soil inoculation analysis, the phytotoxin cut-seedling-stem analysis, and the field analysis. Disease severity

distributions with skewness and kurtosis calculations for root rot and foliar symptoms were determined in R (version 2.13.2). Correlation coefficients were calculated to determine associations between foliar severity, root rot severity, root length and root dry weight, and shoot height and shoot weight in the greenhouse soil inoculation analysis with covariance estimates utilizing the equation: phenotypic correlation (τp) = phenotypic covariance between X and Y (cov (p)) / phenotypic standard deviation of X (σ p(x)) * phenotypic standard deviation of Y (σ p(y)) (Bernardo, 2010).

Results

Greenhouse Soil Inoculation Analysis

The results from the greenhouse soil inoculation analysis separated the two parents into two distinct classes with regard to resistance to SDS. In our results, 'Minsoy' was more susceptible to SDS and displayed both more severe symptoms with a foliar severity score and root rot score, 7.4 and 9.0, respectively (Table 2.3). In contrast, in our results 'Noir1' appeared to be more resistant with a less severe foliar severity score and root rot score of 1.9 and 8.5, respectively (Table 2.3).

In general, the foliar severity scores spanned a large range of values with the scores of one to seven in ratings (Table 2.3 and Table 2.1) for the progeny. The continuous distribution was skewed 0.84 from a normal distribution in the direction of resistance (Figure 2.1) and a distinct peak (kurtosis) of 3.76 was noted. The range observed in the progeny for root rot severity was not as broad, spanning the scores of four to ten (Table 2.3 and Table 2.2). The root rot severity scores displayed a continuous

distribution skewed -1.21 from normality in the susceptible direction with a peak (kurtosis 3.47) (Figure 2.2).

The means and standard deviations for the progeny in response to inoculation were calculated for stem length, stem weight, root length, and root weight (Table 2.3). For all four traits, the progeny averaged higher values than the two parental lines. For example, ‘Minsoy’ and ‘Noir 1’ average 6.6 cm and 6.8 cm respectively for root length, and the mean of the progeny was 7.5 cm (Table 2.3). The heritability coefficients were calculated for each trait (Table 2.3). The lowest heritability was calculated for root weight at 34% (Table 2.6). The remaining traits had heritability coefficients ranging from 69% associated with root rot score to 87% associated with foliar severity score.

Root rot severity score was negatively correlated with the root weight and root length, -0.20 and -0.62, respectively (Table 2.9). Foliar severity score was negatively correlated with stem weight and stem length, -0.42 and -0.39 (Table 2.9). Negative correlations were associated with the measured stem traits of stem length/stem weight and root rot severity scores, -0.58 and -0.47 respectively (Table 2.9). The measured root traits of root length/root weight were negatively correlated with the foliar severity score, -0.20 and -0.12 respectively (Table 2.9). Root rot severity scores and foliar severity scores were positively correlated 0.34 (Table 2.9).

Phytotoxin Cut-Seedling-Stem Analysis

Results from the phytotoxin cut-seedling-stem analysis confirmed the two distinct classes that were observed with the two parents with foliar severity scores to SDS noted in the greenhouse soil inoculation analysis. In our results, ‘Minsoy’ was more susceptible

to SDS and displayed more severe SDS symptoms with an overall mean foliar severity score of 5.7 (Table 2.4). ‘Noir1’ was more resistant with less severe foliar severity scores, overall mean foliar score of 3.0 (Table 2.4). The cut-seedling-stem inoculated plants data spanned a range that covered the SDS foliar scale from two to eight for foliar severity (Table 2.4 and Table 2.1). The continuous distribution (kurtosis, 2.31) for the inoculated plants was negatively skewed (-0.15) with a slight favor for susceptibility (Figure 2.1). The means and standard deviations for the progeny and the four checks in response to inoculation were calculated for foliar severity scores (Table 2.4). The inoculated progeny had a mean of 4.4 that was between the foliar severity score mean of the two parental lines: ‘Minsoy’ and ‘Noir 1’, 5.7 and 3.0, respectively (Table 2.4). The heritability estimate associated with the foliar severity trait was 20% (Table 2.7).

Field Analysis

The presence of *F. virguliforme* in the field was confirmed with the plating of the inoculated roots on PDA, where *F. virguliforme* identification was based on published morphological characteristics (Aoki *et al.*, 2003). Soil samples plated for *P. sojae* indicated that *P. sojae* was not present in the field plot. The foliar disease ratings of the two inoculated parental lines in the field were not significantly different. Both ‘Minsoy’ and ‘Noir1’ had less severe foliar severity scores than the greenhouse with an overall mean of 1.2 and 1.3, respectively. The RIL progeny had a range of foliar severity scores from one to six on the foliar scale (Table 2.5 and Table 2.1). The continuous distribution (kurtosis 7.18 defining a very distinct sharp peak spreading from the foliar score of two) was skewed at 1.43 in the direction of lower severity (Figure 2.1). The means and

standard deviations for the foliar severity scores for the progeny and the four checks in response to inoculation were calculated (Table 2.5). The RIL progeny and the four checks did not express a foliar severity score of more than two (Table 2.5). The heritability estimate was 32% (Table 2.8). The selected sample of roots collected from the field plot had a larger range from one to nine for root rot severity scores compared to four to ten range observed in the greenhouse soil inoculation analysis (Figure 2.3). The root rot severity distributions were not continuous across the root rot severity range and no significant correlation was noted between the two data sets (Table 2.10).

Analysis Comparison

Correlation coefficients were calculated across all three analyses: greenhouse soil inoculation analysis, phytotoxin cut-seedling-stem analysis, and field analysis comparing both the root rot and foliar symptoms separately and together. Small but insignificant correlations were calculated when comparing the foliar symptoms between the analyses: foliar symptoms in phytotoxin cut-seedling-stem analysis/foliar symptoms in field analysis and the foliar symptoms greenhouse soil inoculation analysis/foliar symptoms field analysis (Table 2.11). However, a significant negative Pearson coefficient at -0.13 was calculated when comparing the foliar symptoms of phytotoxin cut-seedling-stem analysis to the foliar symptoms of greenhouse soil inoculation analysis. All of the coefficients calculated in association with the root rot and foliar symptom comparisons were nonsignificant with large confidence intervals (Table 2.12). It should also be noted that a large negative correlation, even though insignificant, was determined between the field root rot and greenhouse soil inoculation foliar symptoms.

Discussion

In our study, 230 lines were evaluated that were developed from a cross of previously reported partially resistant ('Minsoy') and relatively susceptible ('Noir1') parental lines to *F. virguliforme* using the Illinois isolate ST-90. The foliar severity ratings were 1.9 and 3.5, respectively in the Njiti and Lightfoot (2006) study. The results from the greenhouse soil inoculation analysis and the phytotoxin cut-seedling-stem analysis had the same conflicting results with the foliar resistance of the parental lines from the conclusions reported by Njiti and Lightfoot (2006). In our study, the foliar symptoms of 'Minsoy' indicated it was more susceptible than 'Noir 1' to SDS. The results of our greenhouse soil inoculation analysis and phytotoxin cut-seedling-stem analysis resulted in 'Minsoy' expressing mean foliar severity scores of 7.4 and 5.7, respectively (Table 2.3 and Table 2.4). In contrast 'Noir1', expressed less severe foliar symptoms with mean foliar severity scores of 1.9 and 3.0 for the greenhouse soil inoculation analysis and phytotoxin cut-seedling-stem analysis, respectively.

There are a number of possible explanations as to why contrasting results were seen. Njiti and Lightfoot utilized a very different greenhouse screening method that could affect the sensitive environmental conditions necessary for accurate *F. virguliforme* infection. The Njiti and Lightfoot (2006) procedure used a mixture of cornmeal and sand for inoculum substrate compared to our study which used sterile red sorghum seed. In addition, the Njiti and Lightfoot inoculum rate was a one to forty mix of inoculum to soil and our procedure used a one to twenty mix of inoculum to soil. The soybean plant age at which infection occurred was different between the two procedures. The Njiti and

Lightfoot method inoculated the plants 14 days after planting or at approximately the V1 stage of soybean. Our study planted seeds into inoculated soil in an attempt to establish infection at germination or VE stage of soybean. The Illinois procedure grew the plants to 35 days instead of 21 days in the Minnesota protocol. A pronounced difference between the Njiti and Lightfoot study and our procedure was the method of maintaining soil moisture. Njiti and Lightfoot immersed perforated Styrofoam cups with two week old seedlings into a water bath to the depth of 2.5 cm to maintain soil saturation for 21 days. Our study did not utilize a water bath instead soil moisture was maintained by placing the tips of the conetainers in a tray of water with daily watering over the top, throughout the 21 day duration of the study. The last and perhaps the most significant difference is the fact that two different *F. virguliforme* isolates were applied as inoculum. The Illinois isolate, ST-90, was used to inoculate Njiti and Lightfoot's experiment, and the Minnesota isolate, Somerset #1A, was used in this study.

In the past, studies have been conducted to examine the genetic relatedness of several *F. virguliforme* isolates and found genetic homogeneity throughout all the isolates (Achenbach and Patrick, 1996; Achenbach *et al.*, 1997; Aoki *et al.*, 2003). However, the homogeneity is still being questioned, for many studies have shown considerable variation in aggressiveness among *F. virguliforme* isolates in both greenhouse and field studies (Roy *et al.*, 1989; Malvick and Bussey, 2008). It has been suggested there are different races of *F. virguliforme* based on deviations in aggressiveness (Lim and Jin, 1991), but there are no soybean differentials established for race determination. The conflicting parental grouping for SDS resistance that was noted in our study when

compared with the Njiti and Lightfoot analysis is most likely linked to the methods of inoculation, but it maybe as a result of varying levels of aggressiveness displayed by the two isolates. Further research will need to be conducted to understand the differences observed in the Njiti and Lightfoot (2006) and our study.

The means and standard errors for the prepared controls with the parental lines are shown in Table 2.3. Overall, the measured agronomic traits of the controls showed the effects of *F. virguliforme* infection by having considerably lower values than the inoculated parents. The foliar severity scores for both parents were 1.0 indicating SDS foliar symptoms characteristics were completely absent. The root rot severity scores of the control parents were 2.4 for 'Minsoy' and 2.5 for 'Noir1' (Table 2.3). It has been hypothesized that the presence of root rot is associated with an allelopathic effect from the sterile sorghum inoculum substrate (Personal communication with James Kurle, University of Minnesota). Recently, a research study with sorghum and sunflower (*Helianthus annuus* L. aap. *Jaegeri* (Heiser) Heiser) extracts reported that when water drained from the seeds of sorghum was applied to the seeds of *Eleusine indica* (L.) Gaertn. and *Trianthema portulacastrum* (L.) inhibitory effects were observed on the time taken to 50% germination (Mubeen *et al*., 2012). Further research of the allelopathic effects associated with sterile sorghum seed on soybeans is presently being conducted in the Kurle Soybean Pathology Laboratory at the University of Minnesota (Personal communication).

Results of the controls in the field evaluation for the two parental lines had the same foliar severity score with the overall mean of 'Minsoy' at 1.2 and 'Noir1' at 1.3.

The data obtained from the field analysis was not as robust as desired. In the past, field trials have been reported to be complicated by environmental factors, such as temperature and moisture, which are vital for disease expression (Farias-Neto *et al.,* 2007; Roy *et al.,* 1997). The field plot in 2012 at Rosemount, MN, experienced flooding pressure and hail damage during the soybean growth stages of VC to V1. We hypothesize that the hail damage wounded the plants and provided entry points for *Pseudomonas savastanoi pv. glycinea*, and as a result Bacteria Blight was seen throughout the entire plot. Deer browsing occurred during the reproductive stages randomly dispersed through the plot. With these challenges listed above, it was difficult to distinguish the true presence of SDS symptoms in the hill plots. Yet, the presence of *F. virguliforme* in the field was confirmed with the plating of the inoculated roots on PDA, and the identification of *F. virguliforme* based on published morphological characteristics (Aoki *et al.,* 2003). The root rot could not be associated with the pathogen *Phytophthora sojae,* because the soil baiting results were negative. It is believed that the SDS symptoms that were seen at the early vegetative stages and the later reproductive stages were correctly identified as characteristic SDS symptoms, allowing for the data reported to be considered symptoms of SDS. The high peak at the foliar score of two maybe explained by the uncertainty that SDS was present, since Bacteria Blight damage was present on the leaf surface (Figure 2.3). Ultimately, SDS was confirmed in the field analysis, but prominence necessary to obtain robust and confident data was lacking.

The heritability coefficients calculated for each measured trait across the different analyses: greenhouse soil inoculation analysis, phytotoxin cut-seedling-stem analysis, and

field analysis were all reasonable from a breeding perspective. The most highly heritable traits were seen in greenhouse soil inoculation analysis (Table 2.6), making the data for QTL mapping the most robust out of the three analyses that were conducted in the greenhouse and field environments. The greenhouse soil inoculation analysis heritability coefficients for the root rot severity trait and the foliar severity trait were 69% and 87%, respectively. However, the greenhouse soil inoculation analysis only provided early symptom assessment at 21 days or the V1-V2 stage of soybean growth. Late foliar and root rot symptoms were not collected because the plants were not maintained beyond the late V2 soybean vegetative plant growth stage.

The same situation occurred with the phytotoxin cut-seedling-stem analysis which only assessed the SDS foliar symptoms at 21 days. The heritability of the foliar severity score trait was 32% (Table 2.7) and the continuous distribution (no high peaks, kurtosis 2.31) across the scale can be considered to be better than what was observed with the greenhouse soil inoculation analysis with a skewness of -0.15 (Figure 2.1). The distribution can be considered to be normal allowing us to hypothesize the trait will be able to be utilized in breeding in a reasonably heritable manner for foliar severity resistance. Arguably, the phytotoxin cut-seedling-stem analysis would provide a better phenotype in relation to the early foliar symptoms, because the results had a greater distribution and it concentrates solely on the foliar reaction to the phytotoxin, making the analysis extremely useful. Without the presence of roots, there is no possibility of confounding the results of the foliar trait with root rot resistance. It is possible that the lack of roots can explain why a significant negative correlation of -0.13 was calculated

between the greenhouse soil inoculation analysis and the phytotoxin cut-seedling-stem analysis (Table 2.11). Another hypothesis regarding the phytotoxin cut-seedling-stem analysis was proposed for the normal distribution on the severe end of the foliar severity scale. We believe the plants were able to utilize fully functional vascular tissue (without the presence of rotted roots) that allowed transpiration to occur without any complications. The undamaged tissue allowed the phytotoxin(s) to travel to the leaves more effectively (Figure 2.1) resulting in higher foliar severity scores.

The field analysis was the only method that allowed the plants to grow to maturity in order to evaluate the later symptoms expressed at reproductive stages of the soybean. The heritability estimate that was determined for the field foliar trait was the smallest at 20% (Table 2.8). The lower heritability estimate confirms our lack of high confidence in the data.

All three analyses provided varying results, but it confirms the complexity that is seen in phenotyping SDS symptoms. Each analysis had a different set of challenges and the results concentrated on a different trait that might be of interest. Greenhouse soil inoculation was intended to obtain both foliar and root rot symptoms expressed at the early stages of soybean growth. The phytotoxin cut-seedling-stem analysis exclusively concentrated on the early foliar symptoms without any confounding results from the possible root resistance that could have affected the foliar symptoms noted in the greenhouse soil inoculation analysis. The field analysis may not have been as robust as desired as a result of the 2012 growing conditions, but it is still an essential method that should be utilized to observe the late foliar and possible root rot symptoms. It is very

likely that different QTLs will provide resistance to the SDS symptoms that were made in the analyses, making each one essential for a researcher in order to obtain an accurate phenotype associated with the infection of *F. virguliforme*.

Table 2.1: Foliar rating scale for plants exhibiting SDS symptoms

Score	Symptoms
1	Symptoms of SDS are not present
2	Upper leaves displaying some yellowing. Yellowing may appear in blotched throughout the leaf.
3	Leaves begin to display interveinal chlorosis.
4	Leaves have interveinal chlorosis in addition to necrosis starting along the outer margin of the leaf.
5	Leaves begin to show cupping with interveinal chlorosis along with increasing marginal necrosis.
6	Leaves have interveinal necrosis. More than half of the leaf is necrotic and most will be severely cupped. The plants might be stunted and defoliation has begun on all leaves of the plant.
7	Almost if not all of the leaves on the plant are displaying 100% necrosis. The plant is obviously dying.
8	A dead plant that is fully defoliated. The stem remains with potentially a few fully necrotic leaves attached.

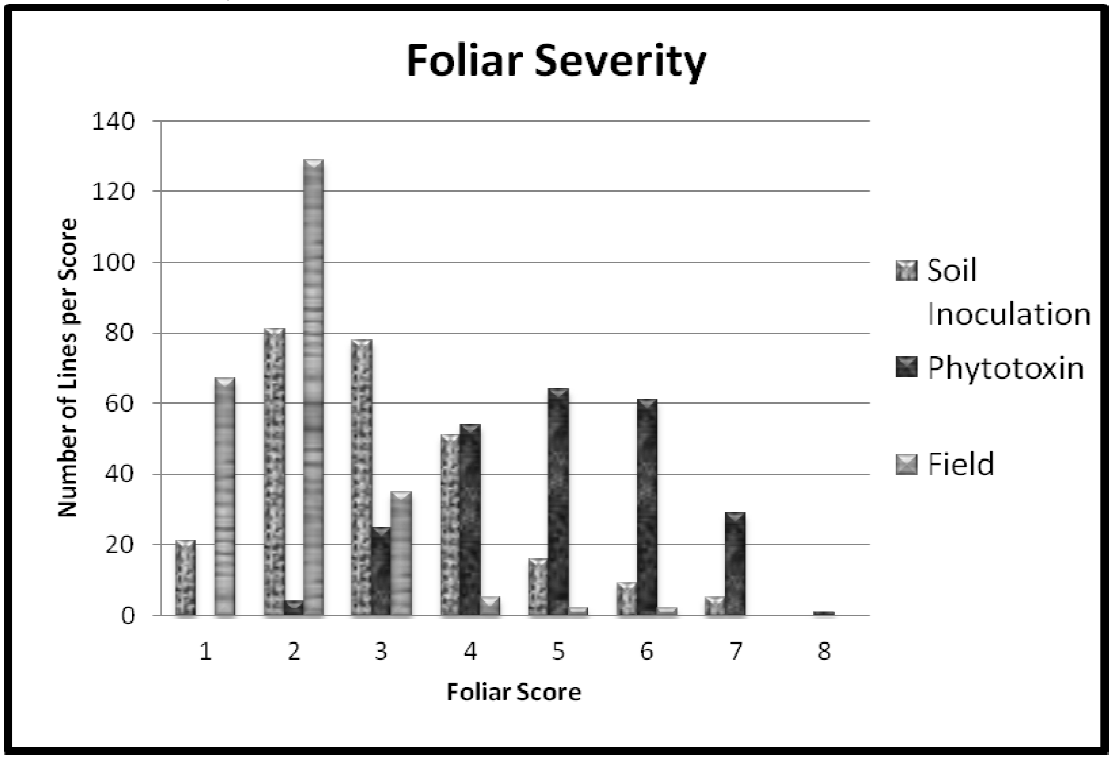
(Bowen and Slaminko, 2008)

Table 2.2: Root rot rating scale for plants exhibiting SDS symptoms

Score	Symptoms
1	0% root rot
2	10% root rot
3	20% root rot
4	30% root rot
5	40% root rot
6	50% root rot
7	60% root rot
8	70% root rot
9	80% root rot
10	90-100% root rot

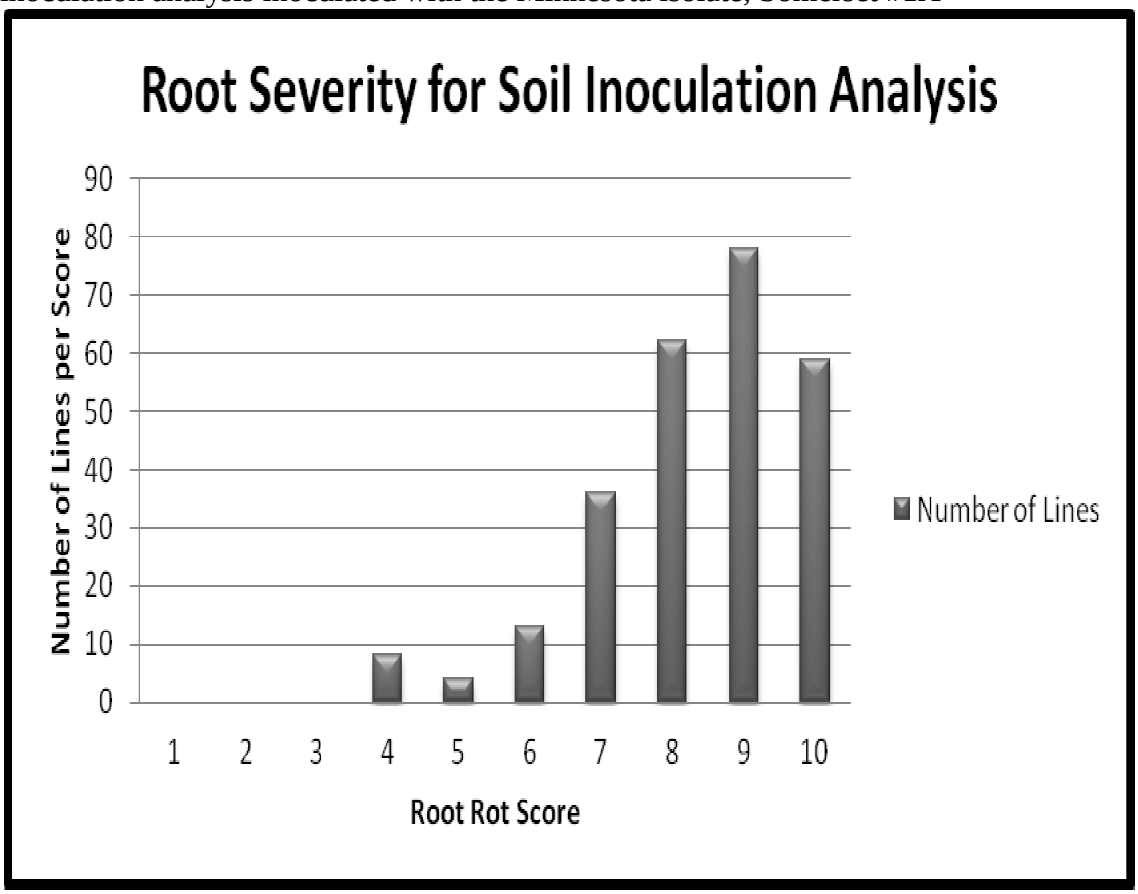
(Bowen and Slaminko, 2008)

Figure 2.1: Distribution of foliar severity associated with the greenhouse soil inoculation analysis, phytotoxin cut-seedling-stem analysis and field analysis inoculated with the Minnesota isolate, Somerset #1A



Skewness = 0.84 associated with the greenhouse soil inoculation analysis
Kurtosis = 3.76 associated with the greenhouse soil inoculation analysis
Skewness = -0.15 associated with the phytotoxin cut-seedling-stem analysis
Kurtosis = 2.31 associated with the phytotoxin cut-seedling-stem analysis
Skewness = 1.43 associated with the field analysis
Kurtosis = 7.18 associated with the field analysis

Figure 2.2: Distribution of root rot severity associated with the greenhouse soil inoculation analysis inoculated with the Minnesota isolate, Somerset #1A



Skewness = -1.21
Kurtosis = 3.47

Figure 2.3: Distribution of root rot severity for the selected lines collected in the field associated with the greenhouse soil inoculation analysis inoculated with the Minnesota isolate, Somerset #1A compared to the root severity in the field analysis

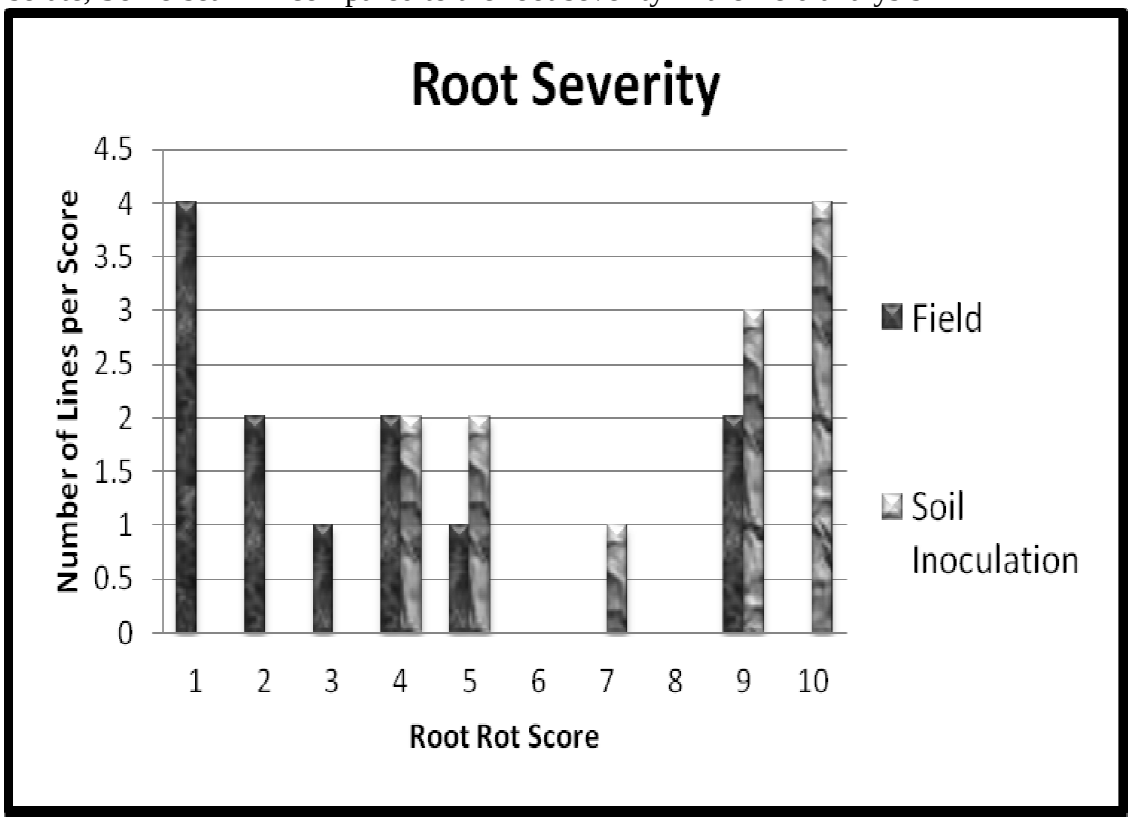


Table 2.3: Means plus or minus standard errors, range of line means and heritability coefficients (h^2) results from the greenhouse soil inoculation analysis inoculated with Somerset #1A and the noninoculated controls

	#[b]	**Root Length (cm)**	**Root Weight (g)**	**Root Rot Score (1-10)**	**Stem Length (cm)**	**Stem Weight (g)**	**Foliar Score (1-8)**
Minsoy	25	6.6 +/- 2.19	0.06 +/- 0.03	9.0 +/- 0.81	14.1 +/- 1.39	0.19 +/- 0.05	7.4 +/- 1.92
Noir 1	23	6.8 +/- 3.47	0.08 +/- 0.03	8.5 +/- 1.10	13.5 +/- 1.83	0.23 +/- 0.06	1.9 +/- 0.27
AG2107[c]	23	7.1 +/- 3.12	0.07 +/- 0.02	8.5 +/- 0.99	14.3 +/- 2.22	0.21 +/- 0.04	7.0 +/- 1.42
MN1606SP[d]	19	9.8 +/- 3.48	0.11 +/- 0.02	6.9 +/- 1.03	14.7 +/- 1.60	0.29 +/- 0.04	1.4 +/- 0.96
Progeny	230	7.5 +/- 3.87	0.09 +/- 0.08	8.3 +/- 1.40	14.6 +/- 2.81	0.24 +/- 0.10	3.0 +/- 1.27
Range in RILs		2.4-17.4	0.02-1.03	4.0-10.0	8.5-25.8	0.05-0.58	1.0-7.0
h^2 %[a]		79	34	69	84	83	87
Controls							
Minsoy	11	17.4 +/- 4.88	0.19 +/- 0.08	2.4 +/- 1.27	19.1 +/- 3.33	0.54 +/- 0.18	1.0 +/- 0.00
Noir 1	10	18.9 +/- 2.59	0.23 +/- 0.10	2.5+/- 1.52	18.6 +/- 1.00	0.78 +/- 0.23	1.0 +/- 0.00

[a]h^2 represents the heritability coefficient = genetic variance / genetic variance + (confounded genetic by environment variance and error variance)
[b]# represents number of replications the calculations were developed from
[c]Susceptible check line
[d]Resistant check line

Table 2.4: Means plus or minus standard errors, range of line means and heritability coefficient (h^2) results from the phytotoxin cut-seedling-stem analysis for the Somerset #1A inoculation of the parents and checks and the noninoculated controls

	#[b]	Foliar Score (1-8)
Minsoy	6	5.7 +/- 1.86
Noir 1	5	3.0 +/- 1.79
AG2107[c]	6	5.0 +/- 2.00
MN1606SP[d]	5	4.7 +/- 2.19
Progeny	230	4.4 +/- 1.98
Range		2.0-8.0
h^2 %[a]		20
Controls		
Minsoy	6	1.0 +/- 0.00
Noir 1	6	1.0 +/- 0.00

[a]h^2 represents the heritability coefficient = genetic variance / genetic variance + (confounded genetic by environment variance and error variance)
[b]# represents number of replications the calculations were developed from
[c]Susceptible check line
[d]Resistant check line

Table 2.5: Means plus or minus standard errors, range of line means and heritability coefficient (h^2) results from the field analysis for the Somerset #1A inoculation of the parents and checks and the noninoculated controls

	#[b]	Foliar Score (1-8)
Minsoy	9	1.2 +/- 0.93
Noir 1	7	1.3 +/- 0.93
AG2107[c]	6	1.0 +/- 0.93
MN1606SP[d]	8	1.5 +/- 0.93
Progeny	230	1.8 +/- 0.93
Range		1.0-6.0
h^2 %[a]		32
Controls		
Minsoy	4	1.0 +/- 0.00
Noir 1	5	1.0 +/- 0.00
AG2107	8	1.0 +/- 0.00
MN1606SP	5	1.0 +/- 0.00

[a]h^2 represents the heritability coefficient = genetic variance / genetic variance + (confounded genetic by environment variance and error variance)
[b]# represents number of replications the calculations were developed from
[c]Susceptible check line
[d]Resistant check line

Table 2.6: Heritability coefficients (h^2) and 90% confidence intervals (CI) coupled with genetic variance (Vg) and h^2 for measured agronomic traits in the soil inoculum analysis associated with *F. virguliforme*, Minnesota isolate, Somerset #1A, inoculation

	Root Length	**Root Weight**	**Root Score**	**Stem Length**	**Stem Weight**	**Foliar Score**
h^2%[a]	79	34	69	84	83	87
90% CI: Vg	8.9202 ≤ μ ≤ 13.0916	0.0010 ≤ μ ≤ 0.0027	1.0778 ≤ μ ≤ 1.6736	5.2027 ≤ μ ≤ 7.4635	0.0065 ≤ μ ≤ 0.0094	1.2478 ≤ μ ≤ 1.7667
90% CI: h^2	0.7576 ≤ μ ≤ 0.8210	0.2266 ≤ μ ≤ 0.4381	0.6459 ≤ μ ≤ 0.7390	0.8131 ≤ μ ≤ 0.8619	0.7998 ≤ μ ≤ 0.8521	0.8485 ≤ μ ≤ 0.8880
90% CI: h^2 [b]	0.7505 ≤ μ ≤ 0.8223	0.2127 ≤ μ ≤ 0.4395	0.6359 ≤ μ ≤ 0.7407	0.8074 ≤ μ ≤ 0.8629	0.7938 ≤ μ ≤ 0.8532	0.8439 ≤ μ ≤ 0.8888

[a]h^2 represents the heritability coefficient = genetic variance / genetic variance (Vg) + (confounded genetic by environment variance and error variance)

[b]Another method of calculation by Knapp *et al.*, 1985

Table 2.7: Heritability coefficients (h^2) and 90% confidence intervals (CI) coupled with genetic variance (Vg) and h^2 for measured agronomic traits in the phytotoxin cut-seedling-stem analysis associated with *F. virguliforme*, Minnesota isolate, Somerset #1A, inoculation

	Foliar Score
h^2%[a]	32
90% CI: Vg	$0.2415 \leq \mu \leq 0.7269$
90% CI: h^2	$0.1989 \leq \mu \leq 0.4277$
90% CI: h^2 [b]	$0.1830 \leq \mu \leq 0.4365$

[a]h^2 represents the heritability coefficient = genetic variance / genetic variance (Vg) + (confounded genetic by environment variance and error variance)
[b]Another method of calculation by Knapp *et al.*, 1985

Table 2.8: Heritability coefficients (h^2) and 90% confidence intervals (CI) coupled with genetic variance (Vg) and h^2 for measured agronomic traits in the field analysis associated with *F. virguliforme*, Minnesota isolate, Somerset #1A, inoculation

	Foliar Score
h^2%[a]	20
90% CI: Vg	$0.0078 \leq \mu \leq 0.1251$
90% CI: h^2	$0.0291 \leq \mu \leq 0.3249$
90% CI: h^2 [b]	$0.0215 \leq \mu \leq 0.3349$

[a]h^2 represents the heritability coefficient = genetic variance / genetic variance (Vg) + (confounded genetic by environment variance and error variance)
[b]Another method of calculation by Knapp *et al.*, 1985

Table 2.9: Phenotypic correlation coefficients comparing the measured agronomic traits and SDS characteristic scoring with the greenhouse soil inoculation analysis results

		Root			**Stem**		
		Length	Weight	Rot Score	Length	Weight	Foliar Score
Root	Length		0.04	-0.62	0.58	0.34	-0.2
	Weight	0.04		-0.2	28.63	0.43	-0.12
	Rot Score	-0.62	-0.2		-0.58	-0.47	0.34
Stem	Length	0.58	28.63	-0.58		0.59	-0.39
	Weight	0.34	0.43	-0.47	0.59		-0.42
	Foliar Score	-0.2	-0.12	0.34	-0.39	-0.42	

[a]Utilized the equation: phenotypic correlation (τp) = phenotypic covariance between X and Y (cov(p)) / phenotypic standard deviation of X (σ p(x)) * phenotypic standard deviation of Y (σ p(y)) (Bernardo, 2010).

Table 2.10: Root rot score correlation coefficients with 95% confidence interval (CI) in association with the inoculation of the Minnesota isolate, Somerset #1A, for the field analysis and greenhouse soil inoculation analysis results

	Field/Soil Inoculation
Spearman	-0.18
Pearson	-0.14
p-value	0.67
95% CI	$-0.6595 \leq \mu \leq 0.4737$

Table 2.11: Foliar score correlation coefficients with 95% confidence interval (CI) in association with the inoculation of the Minnesota isolate, Somerset #1A, for the phytotoxin cut-seedling-stem analysis, greenhouse soil inoculation analysis, and field analysis results

	Phytotoxin/Soil Inoculation*	**Phytotoxin/Field**	**Soil Inoculation/Field**
Spearman	-0.09	0.00	0.01
Pearson	-0.13	-0.01	0.01
p-value	0.05	0.92	0.92
95% CI	2.50E-01≤ μ ≤6.67E-05	-0.1331≤ μ ≤0.1207	-0.1207≤ μ ≤0.1336

*Significant

Table 2.12: Root rot score and foliar score correlation coefficients with 95% confidence interval (CI) in association with the inoculation of the Minnesota isolate, Somerset #1A, for greenhouse soil inoculation analysis, phytotoxin cut-seedling-stem analysis, and field analysis results

	Field root rot/Soil inoculation foliar	**Field root rot/Phytotoxin foliar**	**Field root rot/Field foliar**
Spearman	-0.43	0.20	0.06
Pearson	-0.40	-0.01	-0.02
p-value	0.32	0.98	0.96
95% CI	$-0.8627 \leq \mu \leq 0.4211$	$-0.7099 \leq \mu \leq 0.6994$	$-0.7152 \leq \mu \leq 0.6938$

Chapter 3:
Variation in Aggressiveness in *Fusarium virguliforme* in Two Midwest Isolates

Introduction

In previously published research conducted to identify QTL markers for resistance to SDS, the soybean cultivars ‘Minsoy’ and ‘Noir’ differed significantly in their susceptibility to Sudden Death Syndrome (SDS) (Njiti and Lightfoot, 2006). In a greenhouse resistance assay that included ‘Minsoy’, ‘Noir 1’, ‘Ripley’, ‘Forrest’, and 200 recombinant inbred lines resulting from a cross of ‘Minsoy’ x ‘Noir 1’ all cultivars and RILs developed symptoms characteristic of SDS following inoculation of a sand-soil growth media with a mixture of cornmeal and silicon dioxide infested with *Fusarium virguliforme* (Strain ST-90). The parental lines ‘Minsoy’ and ‘Noir 1’ were reported as partially resistant and relatively susceptible to SDS with mean foliar severity rating of 1.9 and 3.5 for ‘Minsoy’ and ‘Noir 1’, respectively (Njiti and Lightfoot, 2006).

In this study, 230 RI lines that were developed from the same cross of ‘Minsoy’ by ‘Noir1’ parental lines were evaluated. Beyond the research conducted by Njiti and Lightfoot (2006), the population was previously mapped for other agronomic traits such as: lodging, height, flowering, maturity, oil, protein, seed weight, yield, leaf length, leaf width, and leaf area (Mansur *et al.,* 1996). The parents, ‘Minsoy’ and ‘Noir 1’ demonstrated similar phenotypes for most traits; however, transgressive segregants that exceeded the parental values occurred in the progeny RI lines. It appeared that ‘Minsoy’ and ‘Noir1’ represent divergent lineages of the cultivated soybean containing different alleles at many loci (Mansur *et al.,* 1996). With the RIL population segregating for several traits, it was hypothesized that the population would segregate for resistance to SDS.

Resistance of the two parental lines, ‘Minsoy’ and ‘Noir 1’, and various combinations of the 230 RI lines to SDS was evaluated in three experiments. In the first experiment resistance of the two parental lines, ‘Minsoy’ and ‘Noir 1’, and the 230 RI lines were evaluated using two assays for resistance, greenhouse soil inoculation with sorghum infested with either of the *F. virguliforme* isolates Mont-1A or Somerset #1A and phytotoxin cut-seedling-stem assay using *F. virguliforme* toxin produced on liquid media inoculated with the Minnesota isolate, Somerset #1A. In these experiments ‘Minsoy’ was more susceptible than ‘Noir 1’ when either assay was used. Foliar disease severity resulting from greenhouse soil inoculation was greater for ‘Minsoy’ than for ‘Noir 1’ (7.4 vs. 1.9 respectively). Foliar disease symptoms occurring in the phytotoxin cut-seedling-stem assay were more severe on ‘Minsoy’ than on ‘Noir 1’ (5.7 vs. 3.0 respectively) (Table 2.3 and Table 2.4). There are a number of possible explanations why conflicting results were seen when our results are compared with those of the earlier study. The *F. virguliforme* isolates, Mont-1A and Somerset #1A, were used in our study. In contrast the Illinois isolate, ST-90, was used in the Njiti and Lightfoot research. Mont-1A, Illinois isolate, was used in our study to replace ST-90, Illinois isolate, because the ST-90 isolate has fallen out of favor for SDS research use. Mont-1A was suggested to be used as an alternative by Lightfoot (Personal communication). In addition, the methodology used to characterize the resistance phenotype was different from that used by Njiti and Lightfoot (2006) which utilized a different inoculum substrate, cornmeal and silicon dioxide, rather than the sorghum used in our experiments and maintained soil

moisture by submerging the plant containers in a water bath throughout the experiment (Njiti and Lightfoot, 2006).

In an effort to explain the contrasting phenotypes and conflicting results seen in both the greenhouse soil inoculation and phytotoxin cut-seedling-stem analysis, two more analyses were conducted with the Mont-1A and Somerset #1A isolates. The two protocols were carried out in the greenhouse: sorghum inoculum analysis and cornmeal and silicon dioxide analysis. The objectives of this chapter were to (1) determine if there was a difference in virulence or aggressiveness between the two Midwest isolates and (2) to contrast the results with those of the earlier study.

Materials and Method

Cornmeal and Silicon Dioxide Inoculum Analysis

Three separate attempts of the cornmeal and silicon dioxide inoculum analysis were conducted in an effort to replicate the results reported by Njiti and Lightfoot (2006). The first attempt of the cornmeal and silicon dioxide inoculum analysis was conducted with the four checks that were grown throughout all of the experiments: ‘Minsoy’ (RIL parent), ‘Noir1’ (RIL parent), ‘AG2107’ (susceptible check), and ‘MN1606SP’ (resistant check). Five replications were conducted with each isolate. Due to time and space constraints the second and third attempts were conducted with only the parental lines of ‘Minsoy’ and ‘Noir1’ with three replications of each line for each isolate.

Two isolates of *F. virguliforme* were used in the three attempts: Minnesota isolate, Somerset #1A and the Illinois isolate, Mont-1A. The Somerset #1A isolate was

collected in the Minnesota township of Somerset, Steele County in 2002. Mont-1A was collected in central Illinois in Montgomery County in1991 (Li *et al*., 2009).

The analyses were conducted during the months of September 2012 through January 2013. The two *F. virguliforme* isolates were grown on separate potato dextrose agar (PDA) medium at room temperature for 14 days. Two $1cm^3$ sections of PDA for each isolates containing actively growing fungus were transferred onto 25 g of sterile one to one mixture of cornmeal and silicon dioxide in a 250 mL Wheaton Glass Media Bottle. Upon inoculation, 10 mL of sterile water was added and the mixture was mixed with a sterile glass rod. The inoculum for each isolate was allowed to incubate at room temperature for 14 days. Seeds were planted in the greenhouse, simultaneously with inoculum preparation, and allowed to grow for 14 days in medium course vermiculite (Chapter 2 p21). A one to one to one soil mixture (Chapter 2 p19) was utilized in the ratio of one part inoculum to forty parts soil. The inoculated soil mixture for Mont-1A and Somerset #1A was used to fill individual perforated Styrofoam cups, and another set of cups was filled with the pasteurized soil and sterile one to one mixture of cornmeal and silicon dioxide for controls. Two-week-old seedlings were transplanted into the cups and were placed in a water bath to maintain soil saturation. The lines were subjected to temperatures between 22°C and 26 °C with 16 hours of daylight. The plants were grown for 21 additional days before the foliar symptoms were scored on the one to eight rating scale (Table 2.1) ((Bowen and Slaminko, 2008)). The experiments were set up in a completely randomized design.

Sorghum Inoculum Analysis

A subset of the RIL population was evaluated for resistance to SDS when inoculated with infested sorghum inoculum. This group included the four check lines: ‘Minsoy’ (RIL parent), ‘Noir1’ (RIL parent), ‘AG2107’ (susceptible check), and ‘MN1606SP’ (resistant check) and eighteen lines selected on the basis of previous greenhouse results obtained from the first experiment (Chapter 2) conducted with Minnesota isolate, Somerset #1A. The results obtained in this assay enabled us to rank the lines for resistance to both root rot and foliar symptoms. Nine lines were chosen for root rot symptoms because their resistance characteristic spanned the range of the rating scale: three lines receiving the highest ratings for resistance, three lines receiving the average ratings for resistance, and three lines receiving the lowest ratings for resistance. Nine lines were selected in the same fashion for foliar symptoms (Table 3.1).

The experiment was organized as a completely randomized design replicated five times. The parental lines were planted in conetainers containing autoclaved sorghum and pasteurized soil mixture combined in a one to twenty ratio as established controls. The analysis was conducted during the months of November 2012 and December 2012. The plants were grown in the same greenhouse environment with the same conditions and protocol as the greenhouse soil inoculation analysis inoculated with the Minnesota isolate, Somerset #1A, conducted previously (Chapter 2 p19-20), except the inoculum was developed with the *F. virguliforme* Illinois isolate, Mont-1A. Plants were destructively sampled for disease symptoms and plant components were measured as described in Chapter 2 p19-20.

Statistical Analysis

Means, standard deviations, and frequency distributions of RIL response to the sorghum inoculum analysis were calculated. Spearman and Pearson correlation coefficients were calculated with 95% confidence intervals and statistical significance in R (version 2.13.2) (The R Foundation for Statistical Computing, 2011). The correlation analysis were conducted to determine associations between foliar symptom severity, root rot severity, root length, root dry weight, shoot height, and shoot weight resulting from inoculation with Mont-1A and Somerset #1A isolates in the greenhouse soil inoculation analysis.

Results

Cornmeal and Silicon Dioxide Inoculum Analysis

The results for the first attempt were inconclusive, since no SDS symptoms were present at the end of the 35 day period. The second attempt resulted in perplexing symptoms. There was no range of symptoms or differences between the severities of symptoms expressed by the two parents ‘Minsoy’ and ‘Noir 1’. The plants were unable to be rated by the previously described foliar scale (Table 2.1) because the plants did not display any of the characteristic SDS foliar symptoms. The displayed symptoms were either a damped off seedling that was fully defoliated where the stem remained with a few fully necrotic leaves attached, or a healthy plant with uniform chlorosis throughout the leaves from the lack of nutrients (Figures 3.3 and 3.4). The 35 day old plants were not fertilized for the duration of the experiment. It should be noted that all of the plants inoculated with the Somerset #1A isolate exhibited the damped off seedling, and all of the plants inoculated with the Mont-1A isolate displayed a uniform chlorosis on healthy

plants. Results of the third attempt repeated the uncharacteristic SDS symptoms found with this method. All of the plants inoculated with Somerset #1A showed the uniform chlorosis associated with nutrient deficiency. The 'Minsoy' plants inoculated with the Mont-1A isolate displayed the foliar symptoms associated with nutrient deficiency, not SDS. The 'Noir1' plants that were inoculated with Mont-1A exhibited the damped off seedling symptom. However, the plants did not display any of the characteristic SDS symptoms such as interveinal or marginal necrosis that are displayed in Figure 3.5. Root rot was present randomly throughout all of the plants in the second and third analyses; however, the rot was not confirmed to be caused by the infection of *F. virguliforme*.

Sorghum Inoculum Analysis

The sorghum inoculum analysis using Mont-1A was evaluated approximately 21 days after planting. Characteristic foliar and root rot symptoms were distinguished and evaluated. A small distribution of foliar severity scores was rated across the RIL subset population with the scores of two to four (Table 3.2). An even smaller distribution of root rot scores were assessed with the scores of eight to ten (Table 3.2). Both distributions associated with the characteristic symptoms were considerably less than the distribution associated with the Minnesota isolate, Somerset #1A. The RIL subset population had been selected to cover the root rot symptom scores in the range of four to ten (Figure 3.1) and foliar severity scores in the range of one to seven (Figure 3.2). Spearman and Pearson correlation coefficients calculated for each measured trait (Table 3.2) showed no significant correlation between the root rot scores and foliar severity scores associated

with the two separate isolates. The correlation coefficients (Spearman at 0.34 and Pearson at 0.50) calculated for the trait of stem length was significant at 0.018 (Table 3.2).

'Minsoy' was more susceptible than Noir 1 when inoculated with the Illinois isolate, Mont-1A. The overall mean in foliar severity scores and root rot scores in the sorghum inoculum analyses were 4.4 and 8.4 respectively (Table 3.1). 'Noir1'appeared to be more resistant when either foliar severity score or root rot score were evaluated with overall mean of 2.3 and 7.0, respectively, in the sorghum inoculum analysis (Table 3.1). The follow-up sorghum inoculated assay produced results that were consistent with the trends established in Chapter 2 with the greenhouse soil inoculation and phytotoxin cut-seedling-stem analyses (Table 2.3 and Table 2.4). This is displayed in Table 3.4, which compared the results of the infection of the two isolates with the sorghum infested experiments.

Discussion

A subset of the RIL population was chosen based on the results obtained from the sorghum inoculum analysis with a total of nine lines to cover the range of each disease symptom: (1) three lines chosen which expressed the highest disease ratings for resistance (2) three lines chosen which expressed the average disease ratings for resistance and (3) three lines chosen which expressed the lowest disease ratings for resistance (Table 3.1). The subset was tested with the same greenhouse soil inoculation analysis that was utilized with the Minnesota isolate, Somerset #1 A, with the exception of the sterile sorghum substrate being infested with the Illinois isolate, Mont-1A. The analysis was completed to gain a better understanding of aggressiveness of the two

isolates. Aggressiveness components of *F. virguliforme* include sporulation rate, infection efficiency (Lou *et al.*, 1999), ability to colonize the xylem (Navi and Yang, 2008), and ability to produce toxin that cause foliar symptoms (Li *et al.*, 2009). Disease assessment and measurements of traits were conducted for all the traits and only the stem length trait associated with SDS infection was significant with a Spearman correlation coefficient of 0.34, p-value of 0.018 (Table 3.1). The lack of significant correlation between the disease severity ratings produced by the two isolates indicates the fact that there are differences in aggressiveness between the Illinois and Minnesota isolates. The range of foliar scores over the subset population that was chosen as most resistant was considerably smaller when inoculated with the Mont-1A isolate that when inoculated with the Somerset #1A isolate (Disease severity (DS) two to four vs. one to seven respectively). It can be hypothesized from this comparison that the ability to colonize the xylem or the ability to produce toxin(s) that cause foliar symptoms is less aggressive in the Illinois isolate, Mont-1A. Root rot severity ranges displayed by the contrasting inoculation of Somerset #1A and Mont-1A were the scores of four to ten and the scores of eight to ten, respectively. These results suggest that the sporulation rate and infection efficiency of the root is more aggressive in the Mont-1A isolate. The Mont-1A isolate was only tested on twenty-two lines with the sorghum inoculation method, whereas 240 lines were evaluated with the Somerset #1A isolate. Further research will need to be conducted to confirm which component(s) of the isolates is contributing to the distinct aggressiveness levels that are being displayed.

A recent study analyzed the genetic homogeneity of seventy-two *F. virguliforme* isolates collected from the states of Arkansas, Illinois, Iowa, and Minnesota (Mbofung *et al.*, 2012). With the methods of CAT_5 hybridization and 12 random amplified polymorphic DNA (RAPD) primers, this study identified twenty-five different genotypes within *F. virguliforme* revealing that there is a greater amount of genetic diversity than was previously known. It grouped all the Minnesota isolates in separate genotypes in comparison with the Mont-1A, Illinois isolate (Mbofung *et al.,* 2012). Mbofung *et al.* (2012) identified a major genotype among the *F. virguliforme* isolates from Iowa and Minnesota suggesting that a single genotype may have been introduced into the region (Minnesota and Iowa), and that the minor genotypes may have risen from mutations. It should be noted that the Minnesota isolate, Somerset #1A, was not included in the Mbofung *et al.* study. With regards to the Minnesota isolates in the study, there were contrasting results in grouping between the CAT_5 hybridization and 12 RAPD primers results. The CAT_5 probe placed the isolates into three genotypes, whereas the RAPD analysis grouped them all together. As a result, the authors felt it was plausible that a larger sample from Minnesota might show a more varied population than could be detected with the relatively small number of isolates analyzed (Mbofung *et al.,* 2012). Therefore, it is possible that the isolate, Somerset #1A is unique. The analysis examined the levels of aggressiveness associated with 30 *F. virguliforme* isolates with the soybean cultivars: ‘Spencer’ previously reported to be susceptible (Wilcox *et al*., 1989) and ‘92M91’(DuPont Pioneer, USA) previously reported to be moderately resistant (Schmidt *et al.,* 2008). The study was unable to find any associations between the genetic markers

and differences in aggressiveness detected among the isolates. However, the study evaluated the Mont-1A isolate among the moderately aggressive isolates. Based on the data presented in this study, it can be suggested that the Minnesota isolate, Somerset #1, is among the more aggressive isolates for foliar severity symptoms that have been collected across the Midwest.

The parental lines were previously reported partially resistant (‘Minsoy’) and relatively susceptible (‘Noir1’) to *F. virguliforme* when inoculated with Illinois isolate, Mont-1A, with mean in foliar severity of 1.9 and 3.5 for ‘Minsoy’ and ‘Noir1’, respectively (Njiti and Lightfoot, 2006) with a cornmeal and silicon dioxide inoculum analysis. However, conflicting results were reported in the current sorghum inoculum analysis inoculated with the Illinois isolate, Mont-1A. ‘Minsoy’ expressed more severe foliar symptoms and root rot symptoms when inoculated with the Illinois isolate, Mont-1A. Mean foliar and root rot severity were 4.4 and 8.4, respectively (Table 3.2). In contrast‘Noir1’expressed less severe foliar and root rot symptoms 2.3 and 7.0, respectively (Table 3.2). When evaluated using the sorghum inoculum method inoculation with either isolates produced results that conflicted with those previously reported in Njiti and Lightfoot (2006). The discrepancy between resistance expressed by ‘Minsoy’ and ‘Noir1’ in our study when compared to the results obtained by Njiti and Lightfoot (2006) could have been caused by an error in interpretation of foliar symptoms attributed to SDS. The cornmeal and silicon dioxide analyses that were conducted based on the Njiti and Light (2006) method were inconclusive. In our results, plants in the first attempt were grown for 35 days, but no root rot or foliar symptoms were noted. The first

attempt was grown in greenhouse conditions in the month of September, and temperatures above the desired 22°C to 26 °C were noted a couple of times. Since environmental factors, such as temperature and moisture, are vital for SDS disease expression (Farias-Neto *et al.,* 2007; Roy *et al.,* 1997), there was potential that the lack of characteristic symptoms was linked to the high temperatures. However, the following two cornmeal and silicon dioxide analyses did not produce characteristic SDS symptoms. The symptoms that did appear were similar to those of flooded or water logged plants. These plants had been maintained in saturated conditions in a water bath for 21 days. The conflicting results suggest that this method produced a phenotype for SDS mistakenly interpreted as symptoms of SDS, and the difference between the earlier research and our experiments is based on a phenotyping error.

Table 3.1: Means plus or minus standard errors for the selected subset population based on the greenhouse soil inoculation analysis with the Minnesota isolate, Somerset #1A (Chapter 2)

Lines	Root Length (cm)	Root Weight (g)	Root Rot Score (1-10)	Stem Length (cm)	Stem Weight (g)	Foliar Score (1-8)
Mn-1	13.0 +/- 4.92	0.04 +/- 0.02	9.0 +/- 1.41	10.3 +/- 0.95	0.08 +/- 0.04	7.0 +/- 0.81
MN-5	12.6 +/- 4.33	0.07 +/- 0.06	8.0 +/- 1.67	13.3 +/- 3.18	0.14 +/- 0.10	4.0 +/- 2.0
MN-9	13.0 +/- 6.44	0.09 +/- 0.04	6.2 +/- 1.71	14.9 +/- 4.50	0.17 +/- 0.09	4.2 +/- 2.63
MN-18	11.5 +/- 2.19	0.04 +/- 0.04	9.4 +/- 0.89	13.7 +/- 1.13	0.12 +/- 0.03	7.0 +/- 0.71
MN-64	4.6 +/- 1.00	0.05 +/- 0.05	6.6 +/- 1.82	13.7 +/- 2.89	0.16 +/- 0.08	1.2 +/- 0.45
MN-110	4.1 +/- 0.92	0.24 +/- 0.43	8.0 +/- 2.12	9.9 +/- 5.00	0.14 +/- 0.05	4.0 +/- 2.34
MN-156	10.9 +/- 9.63	0.05 +/- 0.04	7.7 +/- 4.04	15.8 +/- 5.45	0.22 +/- 0.20	4.0 +/- 3.61
MN-161	3.24 +/- 1.22	0.02 +/- 0.03	10.0 +/- 0.00	10.4 +/- 2.88	0.05 +/- 0.05	5.8 +/- 2.59
MN-192	8.1 +/- 4.39	0.16 +/- 0.05	6.4 +/- 2.19	14.2 +/- 2.14	0.43 +/- 0.07	1.2 +/- 0.45
MN-203	10.3 +/- 1.18	0.19 +/- 0.05	4.0 +/- 0.71	20.2 +/- 1.74	0.56 +/- 0.12	1.8 +/- 0.84
MN-231	4.6 +/- 3.95	0.10 +/- 0.06	10.0 +/- 0.00	15.7 +/- 2.39	0.25 +/- 0.13	4.8 +/- 2.05
MN-247	17.2 +/- 2.17	0.11 +/- 0.03	3.6 +/- 2.51	25.8 +/- 2.59	0.40 +/- 0.11	1.6 +/- 0.89
MN-249	17.2 +/- 4.87	0.11 +/- 0.03	3.6 +/- 1.14	18.6 +/- 2.51	0.33 +/- 0.08	1.6 +/- 0.89
MN-258	13.0 +/- 4.58	0.09 +/- 0.04	6.3 +/- 2.08	19.3 +/- 0.58	0.24 +/- 0.01	1.3 +/- 0.58
MN-281	12.2 +/- 6.02	0.07 +/- 0.02	7.2 +/- 2.49	16.6 +/-2.30	0.22 +/- 0.05	1.2 +/- 0.45
MN-306[a]	3.7 +/- 0.93	0.03 +/- 0.01	10.0 +/- 0.00	10.6 +/- 2.08	0.09 +/- 0.06	7.3 +/- 0.96
M06-235028	6.4 +/- 4.37	0.13 +/- 0.06	6.3 +/- 2.08	15.5 +/- 0.98	0.25 +/- 0.09	1.7 +/- 0.58

[a]MN-306 was selected for both the root rot and foliar symptoms

Table 3.2: Means plus or minus standard errors, range of line means from the sorghum inoculum analysis results for the Illinois isolate, Mont-1A inoculation of the parents and checks and the noninoculated controls

Information	#[c]	Root Length (cm)	Root Weight (g)	Root Rot Score (1-10)	Stem Length (cm)	Stem Weight (g)	Foliar Score (1-8)
Minsoy	5	7.2 +/- 5.59	0.06 +/- 0.02	8.4 +/- 1.34	12.8 +/- 1.64	0.15 +/- 0.05	4.4 +/- 1.67
Noir 1	4	12.5 +/- 4.65	0.07 +/- 0.04	7.0 +/- 2.94	10.5 +/-1.73	0.18 +/- 0.03	2.3 +/- 0.50
AG2107[a]	4	3.3 +/- 0.58	0.03 +/- 0.02	10.0 +/- 0.00	15.3 +/- 3.21	0.15 +/- 0.04	3.0 +/- 0.50
MN1606SP[b]	3	5.0 +/- 2.83	0.06 +/- 0.04	8.8 +/- 1.89	10.3 +/- 3.21	0.19 +/- 0.07	1.3 +/- 1.00
Progeny	18	4.9 +/- 1.32	0.05 +/- 0.05	9.4 +/- 0.57	13.1 +/- 1.97	0.15+/- 0.04	2.7 +/- 0.86
Range in RILs		3.0-7.2	0.02-0.22	8.0-10.0	10.6-16.8	0.07-0.21	2.0-4.0
Controls							
Minsoy	2	9.0 +/- 8.49	0.06 +/- 0.04	5.5 +/- 4.95	12.5 +/- 0.71	0.24 +/- 0.08	1.0 +/- 0.00
Noir1	2	4.0 +/- 1.41	0.06 +/- 0.03	6.0 +/- 4.24	11.0 +/- 0.00	0.28 +/- 0.10	1.0 +/- 0.00

[a] Susceptible check line
[b] Resistant check line
[c]# represents number of lines the calculations were developed from

Table 3.3: Correlation coefficients and 95% confidence intervals (CI) calculated in association with the inoculated plants from the greenhouse soil inoculation analysis comparing the results between the two *F. virguliforme* isolates, Illinois isolate, Mont-1A and Minnesota isolate, Somerset #1A

Information	Root Length	Root Weight	Root Rot Score	Stem Length*	Stem Weight	Foliar Score
Spearman	-0.08	-0.11	-0.29	0.34	0.25	0.37
Pearson	-0.12	-0.10	-0.24	0.50	0.22	0.35
p-value	0.610	0.667	0.276	0.018	0.328	0.106
95% CI: Lower	-0.5118	-0.4983	-0.6027	0.1010	-0.2236	-0.0792
95% CI: Upper	0.3222	0.3383	0.1992	0.7619	0.5862	0.6750

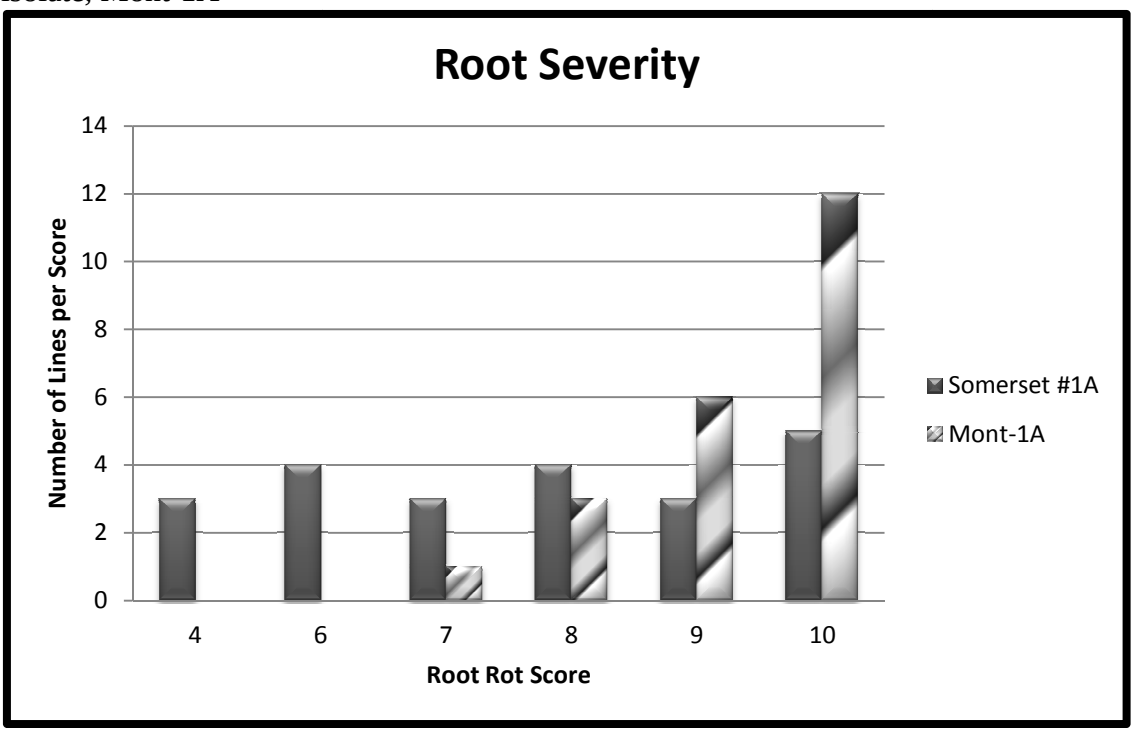
*Significant

Table 3.4: Means plus or minus standard errors, range of line means from the sorghum inoculum analysis and greenhouse soil inoculation analysis (Chapter 2) results for the Illinois isolate, Mont-1A (IL) and the Minnesota isolate, Somerset #1A (MN) inoculation, respectively

	Root Length (cm)		**Root Weight (g)**		**Root Rot Score (1-10)**		**Stem Length (cm)**		**Stem Weight (g)**		**Foliar Score (1-8)**	
Information	MN	IL	MN	IL	MN	IL	MN	IL	MN	IL	MN	IL
Minsoy	6.6 +/- 2.19	7.2 +/- 5.59	0.06 +/- 0.03	0.06 +/- 0.02	9.0 +/- 0.81	8.4 +/- 1.34	14.1 +/- 1.39	12.8 +/- 1.64	0.19 +/- 0.05	0.15 +/- 0.05	7.4 +/- 1.92	4.4 +/- 1.67
Noir 1	6.8 +/- 3.47	12.5 +/- 4.65	0.08 +/- 0.03	0.07 +/- 0.04	8.5 +/- 1.10	7.0 +/- 2.94	13.5 +/- 1.83	10.5 +/- 1.73	0.23 +/- 0.06	0.18 +/- 0.03	1.9 +/- 0.27	2.3 +/- 0.50
AG2107[a]	7.1 +/- 3.12	3.3 +/- 0.58	0.07 +/- 0.02	0.03 +/- 0.02	8.5 +/- 0.99	10.0 +/- 0.00	14.3 +/- 2.22	15.3 +/- 3.21	0.21 +/- 0.04	0.15 +/- 0.04	7.0 +/- 1.42	3.0 +/- 0.50
MN1606SP[b]	9.8 +/- 3.48	5.0 +/- 2.83	0.11 +/- 0.02	0.06 +/- 0.04	6.9 +/- 1.03	8.8 +/- 1.89	14.7 +/- 1.60	10.3 +/- 3.21	0.29 +/- 0.04	0.19 +/- 0.07	1.4 +/- 0.96	1.3 +/- 1.00
Progeny	9.4 +/- 4.70	4.9 +/- 1.32	0.09 +/- 0.06	0.05 +/- 0.05	7.3 +/- 2.11	9.4 +/- 0.57	15.0 +/- 4.17	13.1 +/- 1.97	0.21 +/- 0.13	0.15 +/- 0.04	3.72 +/- 2.32	2.7 +/- 0.86
Range	2.4-17.4	3.0-7.2	0.02-1.03	0.02-0.22	4.0-10.0	8.0-10.0	8.5-25.8	10.6-16.8	0.05-0.58	0.07-0.21	1.0-7.0	2.0-4.0

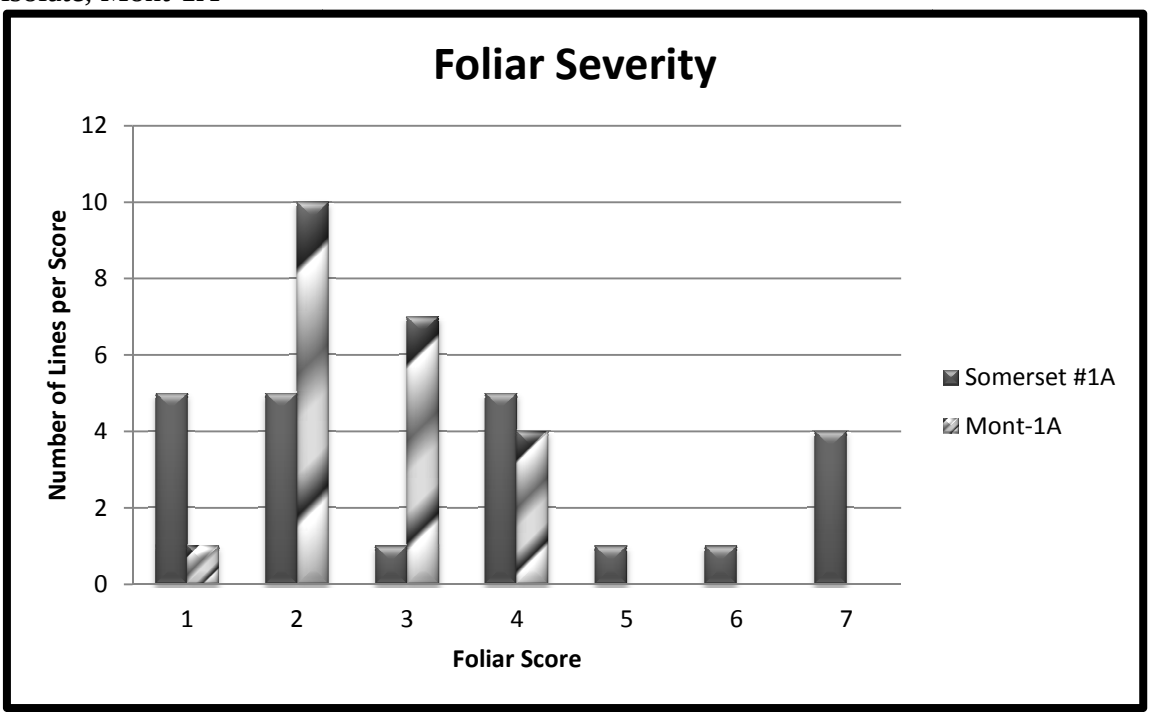
[a] Susceptible check line
[b] Resistant check line

Figure 3.1: Root severity distribution over the RIL subset population associated with the infection of the two Midwest isolates: Minnesota isolate, Somerset #1A and Illinois isolate, Mont-1A



Skewness = -0.34 associated with Minnesota isolate, Somerset #1A
Kurtosis = 2.04 associated with Minnesota isolate, Somerset #1A
Skewness = -1.07 associated with Illinois isolate, Mont-1A
Kurtosis = 3.17 associated with Illinois isolate, Mont-1A

Figure 3.2: Foliar severity distribution over the RIL subset population associated with the infection of the two Midwest isolates: Minnesota isolate, Somerset #1A and Illinois isolate, Mont-1A



Skewness = 0.22 associated with Minnesota isolate, Somerset #1A
Kurtosis = 1.46 associated with Minnesota isolate, Somerset #1A
Skewness = 0.28 associated with Illinois isolate, Mont-1A
Kurtosis = 2.20 associated with Illinois isolate, Mont-1A

Figure 3.3: The uncharacteristic SDS symptoms (uniform chlorosis and dead/damped-off seedling) displayed by the parental line ‘Minsoy’ after the cornmeal/silicon analysis. From left to right: group of three plants infested with Illinois isolate, Mont-1A, group of three plants infested with Minnesota isolate, Somerset #1A

Figure 3.4: The uncharacteristic SDS symptoms (uniform chlorosis) displayed by the parental line ‘Noir 1’ after the cornmeal/silicon analysis. From left to right: group of three plants infested with Minnesota isolate, Somerset #1A, group of three plants infested with Illinois isolate, Mont-1A

Figure 3.5: Soybean plant displaying characteristic SDS symptoms with interveinal chlorosis and necrosis displayed in the sorghum inoculated soil

Chapter 4:
Identifying Potential Quantitative Trait Loci linked with SDS Resistance

Introduction

Recombinant inbred lines are direct descendants of F_2 plants, which have been inbred to near homozygosity, resulting in lines that have special attributes thus making them particularly useful for mapping research. During the inbreeding process, the RI lines have undergone several rounds of meiosis allowing for more opportunity for segregation and recombination (Mansur *et al.,* 1996). It has been shown that, for small linkage distances, the proportion of recombination among self-pollinated inbreds is about twice the conventional rate observed in F_2 segregants (Haldane and Waddington, 1931). In conclusion, spurious QTL-marker associations due to linkage errors are less likely with RI lines than with F_2 or backcross populations (Mansur *et al.,* 1996).

Numerous QTL studies have been conducted to search for SDS resistance in multiple populations that are representative of the Southern United States germplasm(Chang *et al.,* 1996; Farias-Neto *et al.,* 2007; Hashmi, 2004; Hnetkovsky *et al.,* 1996; Iqbal *et al*., 2001; Kassem *et al*., 2006; Kazi, 2008; Lightfoot *et al*., 2001; Meksem *et al*., 1999; Njiti *et al*., 1998; Njiti *et al.,* 2002; Prabhu *et al.,* 1999; Sanitchon *et al*., 2004).

In the present study, the RIL population of 230 F_5 derived F_{12} lines (Mansur *et al.,* 1996) were utilized in QTL mapping, in addition seven specialty lines based on results seen in The Soybean Sudden Death Syndrome Regional Tests (Agronomy Research Center Southern Illinois University Carbondale, 2007) regarding SDS resistance. The RIL population utilized is representative of the Northern United States germplasm covering the maturity groups of 0, I, and II that are grown in parts of Iowa, Minnesota,

Nebraska, North Dakota, South Dakota, and Wisconsin. The population was phenotyped with three different analyses: greenhouse soil inoculation analysis, phytotoxin cut-seedling-stem analysis and field analysis for resistance to SDS and were mapped using simple sequence repeat (SSR) and single-nucleotide polymorphism (SNP) markers. The objectives of this chapter were to (1) estimate the number, genetic positions and genetic effects of QTL involved in resistance to SDS occurring in greenhouse and field environments (2) determine the proportion of the genotypic variance explained by all detected QTL (3) to compare the results with those of other studies and (4) to draw conclusions about the prospects of marker-assisted selection (MAS) for increasing the level of resistance to SDS in the Northern germplasm.

Material and Methods

Seed Acquisition

In this study, the RIL population developed from 'Minsoy' by 'Noir1' parental lines described in Chapter 2 p17 were utilized. The previously described (Chapter 2 p17) seven specialty lines: M06-235003, M06-235007, M06-235009, M06-235039, M06-235012, M06-235028, and M04R-543121 were genotyped and examined. The phenotypic data collected in the three analyses: greenhouse soil inoculation analysis, phytotoxin cut-seedling-stem analysis, and field analysis conducted in Chapter 2 was used to identify potential QTLs that were specific to the measured traits: root rot severity, root weight, root length, foliar severity greenhouse soil inoculation, foliar severity phytotoxin cut-seedling-stem, foliar severity field, stem weight and stem length.

Composite Interval Maps of QTL

For accurate location of QTL among sets of linked markers, the composite interval map (CIM) function of WinQTL Cartographer (version 2.5_011) was used (Jansen and Stam 1994; Basten *et al.*, 2001). The Model Six: Standard Model was selected with the forward regression method at a walk speed of one cM. A potential QTL was inferred when LOD score peaks exceeded 3.0 for the traits studied, considering a $P < 0.05$ corrected for two separate analyses: 323 SSR markers on eighty-eight RI lines and 634 SNP markers on 227 RI lines and seven specialty lines. The SSR marker analysis examined data generated from 734 polymorphic markers and was narrowed down to 323 markers based on the 25% rate for missing data. An analysis was run with 140 SSR markers based on a five percentage rate for missing data; however, the overall coverage of the soybean genome was insufficient to obtain potential QTLs. To confirm linkage, experiment-wise threshold was calculated from 1,000 permutations of each genotype marker against the phenotype in the population.

Linkage Map and Statistical Analysis

A linkage map was developed in WinQTL Cartographer (version 2.5_011) to display all the linkage groups with potential QTLs reported in the two CIM analyses (Figures 4.1 and 4.2). Allelic means and standard deviations were estimated for the parental lines for each marker that was associated with a potential interval in R (version 2.13.2) (The R Foundation for Statistical Computing, 2011).

Results

Potential QTLs associated with Characteristic SDS Symptoms

The combined results of the two CIM analyses revealed nine potential QTLs associated with the foliar and root rot symptoms associated with the *F. virguliforme*, Minnesota isolate Somerset #1A, and infection across the three different phenotyping methods. Seven different QTLs were predicted for foliar resistance. The greenhouse soil inoculation analysis foliar trait was associated with three varying intervals on three different chromosomes, chromosome four, five, and fifteen (C1, A1, and E respectively) (Table 4.1). The total explained genetic variance for the early expressed foliar trait established with the greenhouse soil inoculation was 33.9% (Table 4.1) by combining all three QTLs. Three additional potential intervals were calculated from the phytotoxin cut-seedling-stem analysis; however, the three QTLs were associated with only two chromosomes, one on chromosome three (N) and two on chromosome six (C2) (Table 4.1). By combining the genetic variances of the three identified QTLs associated with the foliar symptoms established solely by phytotoxin infection, 34.2% of variance connected to SDS infection was explained with the majority being contributed by the QTL with the marker BARC062759n1804 on chromosome three with an R^2 value of 0.21 and a LOD score of 6.55 (Table 4.1). The field analysis had one QTL associated with the foliar trait on chromosome one (D1a) (Table 4.1). The one QTL linked with the late foliar symptom observed in the field analysis explained 11.1% of the variance associated with SDS (Table 4.1). The greenhouse soil inoculation analysis was the only phenotyping procedure that provided enough root rot data in order to run a CIM analysis. The analysis suggested two probable intervals associated with the early root rot symptom established from the greenhouse phenotypic data collected with the root rot severity score at the

soybean plant age of 21 days. The two potential QTLs for the early root rot symptom are on chromosomes two and six (D1b and C2, respectively) (Table 4.1). The genetic variance that is explained by the two QTLs totals 21.1% (Table 4.1). The allelic means and standard deviations that were estimated for the parental lines for each marker that was associated with the potential intervals are displayed in Table 4.3.

Potential QTLs associated with Agronomic Traits

With the combination of the two CIM analyses, four QTLs were identified to be associated with the 21 day old greenhouse plant growth traits of root length and stem weight in relation to SDS infection. Three separate loci were found to be associated with root length on chromosomes four and eighteen (C1 and G, respectively) (Table 4.2). The total genetic variance explained by the three QTLs was 26.2% (Table 4.2). One QTL was predicted for the stem weight on chromosome ten with the marker BARC051149n11016 with the total genetic variance of 17.7% (Table 4.2). The allelic means and standard deviations that were estimated for the parental lines for each marker associated with the potential intervals are shown in Table 4.4.

Discussion

Dense coverage was observed with the SNP analysis with markers distributed on average less than ten cM apart across the soybean genome. Eighty-eight RI lines were run with the phenotypic data and seven different QTLs were tentatively identified on chromosomes one, two, three, five, fifteen, and eighteen (D1a, D1b, N, A1, E, and G, respectively) (Table 4.1 and 4.2). The total QTL variance explained by this analysis for the greenhouse foliar trait was 59.6% (Table 4.1). The majority of the QTL variance was

contributed by the potential QTL associated with the marker BARC062759n1804 on chromosome three with the R^2 value of 0.21 and a LOD score of 6.55 (Table 4.1). The identified location was derived from the phenotypic data provided by the phytotoxin cut-seedling-stem analysis. This finding emphasizes the importance of this phenotypic screening method. The method is absent of roots, which allows the leaves to display only foliar symptoms. The results were not confounded by any potential root resistance. The total QTL variance that was explained for the root rot trait was 13.7% (Table 4.1).

A majority of the variance was accounted for by the foliar characteristics, but a substantial portion is left unexplained by the root rot trait. A possible reason for the large amount of unexplained variance for root rot is that the analysis was only run with eighty-eight lines, a population size less than ideal. The sample size for many linkage analysis studies in plants involves about 250 individuals (F_2, BC_1, RIL, etc) with a homogenous, bi-parental genetic background (Bernardo, 2002). The small size might allow some false positives to be observed with this particular analysis; however, a number of the same peaks observed in this analysis were noted in the SSR analysis but did not reach the LOD threshold of 3.0. It is a possibility that the QTLs identified in this analysis are minor in affect and are seen as a majority in the 88 lines compared to the remaining RIL population.

Other QTLs were identified in the same regions for the intervals on the chromosomes two, three, ten, and eighteen (D1b, N, O, and G, respectively). The marker BARC047372n12911 representing the position of 56.6 cM on chromosome two (D1b) associated with the root rot trait (Table 4.1) is approximately the same region of the

chromosome: Phytoph 1-2 and Phytoph 3-2 (Cregan et al. 1999; Burnham *et al*., 2003). These previous QTLs (Phytoph 1-2 and Phytoph 3-2) provide resistance to the pathogen *Phytophthora sojae* which is associated with another root rot disease, Phytophthora rot (Burnham *et al*., 2003). Marker BARC062759n18042 at the position of 0.00 cM on the end of chromosome three (N) was identified with the phytotoxin foliar trait (Table 4.1). It is in approximately the same region as the previously reported SDS resistant QTL, q*Rfs*6 (Chang *et al.,* 1996, Njiti *et al.,* 2002; Hashmi, 2004). The stem weight trait identified with the marker BARC051149n11016 at the position 43.1 cM on chromosome ten (O) (Table 4.2) was in the region of the previously reported QTL, Reprod 5-5 (Cregan et al. 1999; Cheng *et al.,* 2011). Reprod 5-5 is a QTL that has been found to be associated with reproductive period and post-flowering photoperiod (Cheng *et al.,* 2011). Root length was identified as linked to the marker BARC055551n13421 at position 8.2 cM on chromosome eighteen (G) (Table 4.2). This region has already been reported to be associated with several QTLs associated with soybean cyst nematode (SCN) resistance and plant morphological traits such as Plant height 23-3, Pod maturity 22-6, Lodging 20-7, and Protein 26-12 (Cregan et al. 1999). The remaining QTLs located on chromosomes one, five, and fifteen (D1a, A1, and E, respectively) were identified with the traits of late foliar symptoms (foliar symptoms observed in the field analysis), early foliar symptoms (foliar symptoms observed in the greenhouse soil inoculation analysis), and early foliar symptoms (foliar symptoms observed in the greenhouse soil inoculation analysis), respectively, (Table 4.1) appear to be novel. Four out of the seven loci identified in this

study have previously been reported with other traits, which strengthens the confidence of the overall analysis.

Almost the entire RIL population genotypic data, including the 88 lines run with the SNP analysis, were utilized in the SSR analysis. When a whole population is mapped the likelihood of false positive associations are avoided due to the lack of population structure and familial relatedness that can be seen in other approaches such as association mapping (Myles *et al.,* 2009; Zhu *et al.* 2008).The coverage associated with the SSR analysis was not as extensive, but on average the distribution was less than twenty cM apart across the soybean genome. Six QTLs were identified in the CIM on chromosomes of four and six (C1 and C2, respectively) (Table 4.1 and 4.2). The QTL variance explained across for the foliar symptom trait in the SSR analysis totaled 19.7% (Table 4.1) and the total QTL variance for the root rot symptom was 7.4% (Table 4.1). Both traits only explained a small amount of the total phenotypic variance for the two SDS characteristic traits, which suggests further research is needed to account for what this study was unable to identify. Three QTLs were identified on chromosome four (C1) with approximately the same position (85.37 cM greenhouse soil inoculated foliar analysis (Table 4.1), 87.3 cM root length (Table 4.2), and 94.62 cM root length (Table 4.2)). These were in close proximity to a QTL identified by Njiti and Lightfoot (2006) associated with SDS, SDS 9-3 positioned between 89.72 cM and 91.72 cM with the LOD score of 2.04. A QTL was reported at the position 87.3 cM, Flower number 1-3, which is considered to be associated with yield (Song *et al.,* 2004). In this study, the marker Satt670 with the position of 84.5 cM was identified with the early foliar trait with the

greenhouse soil inoculation analysis (Table 4.1). The marker Sat_207 at the position of 87.5 and the marker Sat_235 at the position 93.5 were associated with the root length trait (Table 4.2). Three QTLs were identified on chromosome six (C2) for the greenhouse soil inoculation analysis root rot trait and the phytotoxin cut-seedling-stem analysis foliar trait. The root rot QTL was identified with the marker Sat_076 at the position of 94.4 cM (Table 4.1), and no QTL have been associated with the marker previously. The markers Satt079 at 118.0 cM and Sct_028 at 122.0 cM are both associated with the early foliar trait. It is likely that these two separate peaks are identifying the same potential QTL position, being in such close proximity of each other. Two SDS resistant QTLs were previously reported around this area with the positions of 107.58 for cq*Rfs*4 which has been reported to provide resistance to root rot (Hnetkovsky et al. 1996; Njiti et al. 1998; Iqbal et al. 2001; Njiti et al. 2002; Kazi, 2008) and 127.66 for q*Rfs*9 (Njiti et al. 2002) for foliar resistance. It is possible that all of the three QTLs located on chromosome six (C2) are confirming the location of the previously reported resistant SDS intervals.

In a recent study, the most common R proteins contain a nucleotide-binding site and a leucine-rich repeat (NBS-LRR) domain have been found to be co-localized with an number of disease resistance QTLs (Kang *et al.*, 2012). NBS-LRR domains have been reported on seventeen of the twenty chromosomes in the soybean genome. The positions of the potential QTLs identified in this study were compared with the reported NBS-LRR clusters. Four individual QTLs were confirmed to be in close proximity of the reported R proteins on chromosomes one, six, and ten (D1a, C2, and O) (Table 4.5) (Kang *et al.*, 2012).

The results of the two separate CIM analyses produced new potential QTLs. The two different sets of markers (634 SNP vs. 323 SSR) and RI lines (88 lines vs. 227 lines) did not result in the same positions, which maybe emphasizing the differences seen with SDS even further. Both of the mapping studies had reasonable marker coverage of the entire soybean genome, but the varying number of lines for each study could account for part of the variation that is seen between the two. Overall the two studies had similar identified peaks across the chromosomes, but the LOD scores varied for the peaks which resulted in the diverse potential QTLs. Beyond the discrepancy of markers and size of populations, the phenotypes exhibited with SDS infection are quite inconsistent, perhaps with the numerous symptoms that are expressed with SDS. It has been noted in the past, the continuous variation in phenotypes observed for many important traits in agriculture are caused by the segregation of independent polygenes of small effect (Paterson *et al.,* 1990). It is possible the two different CIM analyses are able to detect various independent polygenes of small effect that could prove to be useful in breeding for SDS resistance in the future.

Further research will be necessary for any substantial use of the new QTLs to be utilized in Northern breeding programs. Polygenes can be detected and mapped within ten to twenty cM intervals as QTL, particularly with Deoxyribonucleic acid (DNA) markers (Meksem *et al.,* 1999). Such localization is sufficient for the initial screening of a population for a certain trait as was seen in this particular study. However, highly accurate estimates of the QTL map location, with less than one to two cM, are necessary for effective marker-assisted selection (Prabhu *et al.,* 1999), the dissection of genes

within complex loci (Chase *et al.*, 1997; Graham *et al.,* 1997), and the physical mapping of genes underlying QTLs (Meksem *et al*., 1998).

In order to further this research of SDS resistance in Northern breeding programs, a fine mapping analysis would need to be conducted with the potential QTLs for confirmation and refined localization. Fine mapping relies on the analysis of derived sub-populations in which the QTLs can be further localized. Some of the methods would include: substitution mapping in BC_2F_1 lines (Paterson *et al.*, 1990), mapping in advanced intercross lines (Darvasi and Seller, 1995), mapping in recombinant backcross inbred lines (Eshed and Zamir, 1995), and mapping in recombinant inbred sub-line populations (Haley *et al.*, 1994). Each of these related methods involves isolation of the QTLs to a 30 to 40 cM region using molecular markers, followed by the analysis of recombination events within this region in an otherwise homogeneous genetic background. A single cycle of recombination analysis can place QTLs within intervals less than one cM. Subsequent cycles generated by intercrossing novel recombination events will reduce the interval size still further, given a sufficient marker density and population size (Meksem *et al.*, 1999). Two recombinant inbred sub-line populations have been bred with the parental lines: ‘Minsoy’ by ‘Archer’ and ‘Noir1’ by ‘Archer’. These populations could be utilized in further research to fine map the potential QTLs that were reported in this study.

Table 4.1: Potential QTLs associated with resistance to *F. virguliforme*, Minnesota isolate, Somerset #1A, in regards to root rot and foliar symptoms based on the greenhouse soil inoculation analysis, the phytotoxin cut-seedling-stem analysis, and the field analysis

Locus	Chromosome	LG[a]	Composite Position (cM)	Marker Type	LOD[d] Score	Additive	R^2	Trait
BARC047699n10383	1	D1a	17.33	SNP[b]	3.09	0.3077	0.11	Field Foliar
BARC047372n12911	2	D1b	55.56	SNP	3.58	0.6353	0.14	Root Rot Score
BARC062759n18042	3	N	4.08	SNP	6.55	-0.6078	0.21	Phytotoxin Foliar
Satt670	4	C1	85.37	SSR[c]	3.20	0.3200	0.06	Soil Inoculated Foliar
BARC019415n03923	5	A1	17.65	SNP	4.20	0.5093	0.14	Soil Inoculated Foliar
Satt079	6	C2	117.87	SSR	3.00	0.2900	0.07	Phytotoxin Foliar
Sct_028	6	C2	122.01	SSR	3.00	0.3000	0.07	Phytotoxin Foliar
Sat_076	6	C2	99.18	SSR	3.52	-0.4502	0.07	Root Rot Score
BARC042937n08466	15	E	92.37	SNP	4.26	-0.5200	0.14	Soil Inoculated Foliar

[a]LG represents linkage groups
[b]SNP represents single nucleotide polymorphisms markers
[c]SSR represents single sequence repeat markers
[d] LOD stands for the "logarithm of the odds" and is a representation of the likelihood of a linkage between two genetic traits. In this study the score of 3 or higher means that there is a high probability of genetic linkage.

Table 4.2: Potential QTLs associated with *F. virguliforme*, Minnesota isolate, Somerset #1A, infection in regards to root length and stem weight based on the greenhouse soil inoculation analysis

Locus	Chromosome	LG[a]	Composite Position (cM)	Marker Type	LOD[d] Score	Additive	R^2	Trait
Sat_207	4	C1	87.3	SSR[b]	3.90	-1.0380	0.07	Root Length
Sat_235	4	C1	94.62	SSR	3.61	-1.0130	0.07	Root Length
BARC051149n11016	10	O	47.348	SNP[c]	4.96	-0.0548	0.18	Stem Weight
BARC055551n13421	18	G	8.153	SNP	3.90	1.6046	0.13	Root Length

[a]LG represents linkage groups
[b]SSR represents single sequence repeat markers
[c]SNP represents single nucleotide polymorphisms markers
[d] LOD stands for the "logarithm of the odds" and is a representation of the likelihood of a linkage between two genetic traits. In this study the score of 3 or higher means that there is a high probability of genetic linkage.

Table 4.3: Potential QTLs associated with the resistance to *F. virguliforme*, Minnesota isolate, Somerset #1A, in regards to root rot and foliar symptoms based on the greenhouse soil inoculation analysis, the phytotoxin cut-seedling-stem analysis, and the field analysis with the means plus or minus standard errors, range of parental line means

Locus	Chr[a]	LG[b]	Composite Position (cM)	Marker Type	LOD Score	Trait	#[e] of Noir1	Mean +/- STDEV[f]	# of Minsoy	Mean +/- STDEV
BARC047699n10383	1	D1a	17.33	SNP[c]	3.09	Field Foliar	49	2.0 +/- 0.86	39	2.0 +/- 0.86
BARC047372n12911	2	D1b	55.56	SNP	3.58	Root Rot Score	37	8.2 +/- 1.65	51	8.2 +/- 1.64
BARC062759n18042	3	N	4.08	SNP	6.55	Phytotoxin Foliar	49	5.2 +/- 1.31	39	5.1 +/- 1.32
Satt670	4	C1	85.37	SSR[d]	3.20	Soil Inoculated Analysis Foliar	100	3.0 +/- 1.23	106	3.0 +/- 1.26
BARC019415n03923	5	A1	17.65	SNP	4.20	Soil Inoculated Foliar	37	2.9 +/- 1.32	51	2.7 +/- 1.12
Satt079	6	C2	117.87	SSR	3.00	Phytotoxin Foliar	104	5.1 +/- 1.25	100	5.1 +/- 1.24
Sct_028	6	C2	122.01	SSR	3.00	Phytotoxin Foliar	112	5.1 +/- 1.24	103	5.1 +/- 1.24
								8.3 +/-		8.3 +/-

Sat_076	6	C2	99.18	SSR	3.52	Root Rot Score	92	1.45	104	1.44
BARC042937n08466	15	E	92.37	SNP	4.26	Soil Inoculated Foliar	44	2.8 +/- 1.25	44	2.9 +/- 1.33

[a]Chr represents chromosomes
[b]LG represents linkage groups
[c]SNP represents single nucleotide polymorphisms markers
[d]SSR represents single sequence repeat markers
[e]# represents the number of lines
[f]STDEV represents the standard deviations

Table 4.4: Potential QTLs associated with *F. virguliforme*, Minnesota isolate, Somerset #1A, infection in regards to root length and stem weight based on the greenhouse soil inoculation analysis with means plus or minus standard errors, range of parental line means

Locus	Chr[a]	LG[b]	Composite Position (cM)	Marker Type	LOD Score	Trait	#[e] of Noir1	Mean +/- STDEV[f]	# of Minsoy	Mean +/- STDEV
Sat_207	4	C1	87.30	SSR[c]	3.90	Root Length	98	7.51 +/- 3.88	103	7.49 +/- 3.87
Sat_235	4	C1	94.62	SSR	3.61	Root Length	112	7.51 +/- 3.86	105	7.49 +/- 3.87
BARC051149n11016	10	O	47.35	SNP[d]	4.96	Stem Weight	55	0.24 +/- 0.11	33	0.25 +/- 0.10
BARC055551n13421	18	G	8.15	SNP	3.90	Root Length	45	7.87 +/- 4.22	43	7.66 +/- 4.16

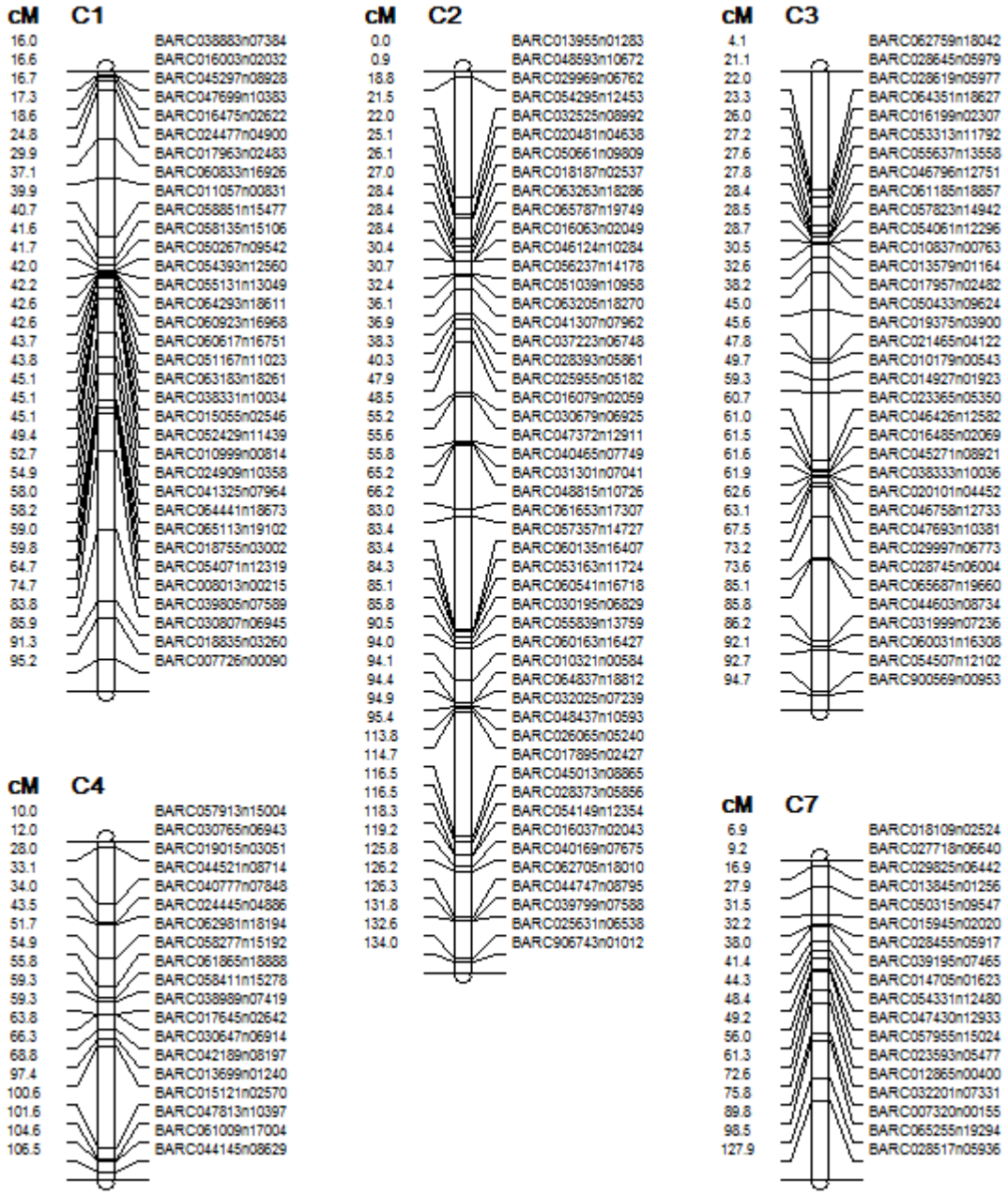
[a]Chr represents chromosomes
[b]LG represents linkage groups
[c]SSR represents single sequence repeat markers
[d]SNP represents single nucleotide polymorphisms markers
[e]# represents the number of lines
[f]STDEV represents the standard deviations

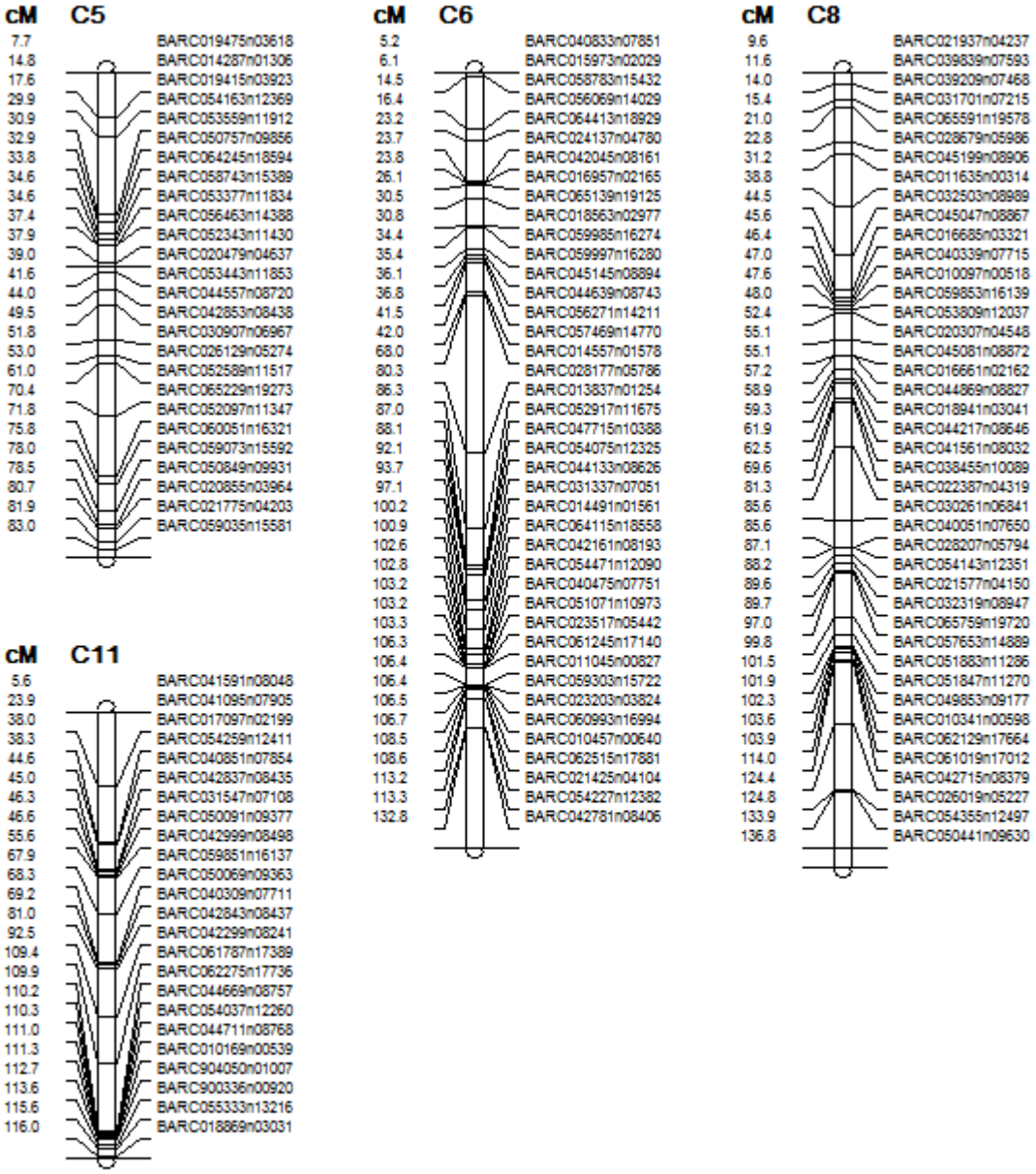
Table 4.5: Potential QTLs associated with *F. virguliforme*, Minnesota isolate, Somerset #1A, infection that are in close proximity of NBS-LRR domains and the previously reported QTLs co-localized with the regions

Locus	Chr[a]	LG[b]	Composite Position (cM)	Marker Type	Trait	Genes in close proximity of NBS-LRR[e] domain	Previous QTL
BARC047699n10383	1	D1a	17.33	SNP[c]	Field Foliar	Glyma01g01400, Glyma01g01420	SCN 19-3, SCN 20-3, SCN 21-2
Sat_076	6	C2	99.18	SSR[d]	Root Rot Score	Glyma06g17560	SCN 9-6
Sct_028	6	C2	122	SSR	Phytotoxin Foliar	Glyma06g41240, Glyma06g41290, Glyma06g41330, Glyma06g41380, Glyma06g41380, Glyma06g41430, Glyma06g43850	SDS 8-2
BARC051149n11016	10	O	47.35	SNP	Stem Weight	Glyma10g32780, Glyma10g32800	Sclero 2-23, Sclero 3-18, Sclero 4-10, Sclero 5-15, Sclero 6-12

[a]Chr represents chromosomes
[b]LG represents linkage group
[c]SNP represents single nucleotide polymorphism
[d]SSR represents single sequence repeat
[e]NBS-LRR represents nucleotide-binding site and a leucine-rich repeat

Figure 4.1: Single nucleotide polymorphism (SNP) linkage map for the chromosomes one through twenty

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61.0 BARC052589n11517
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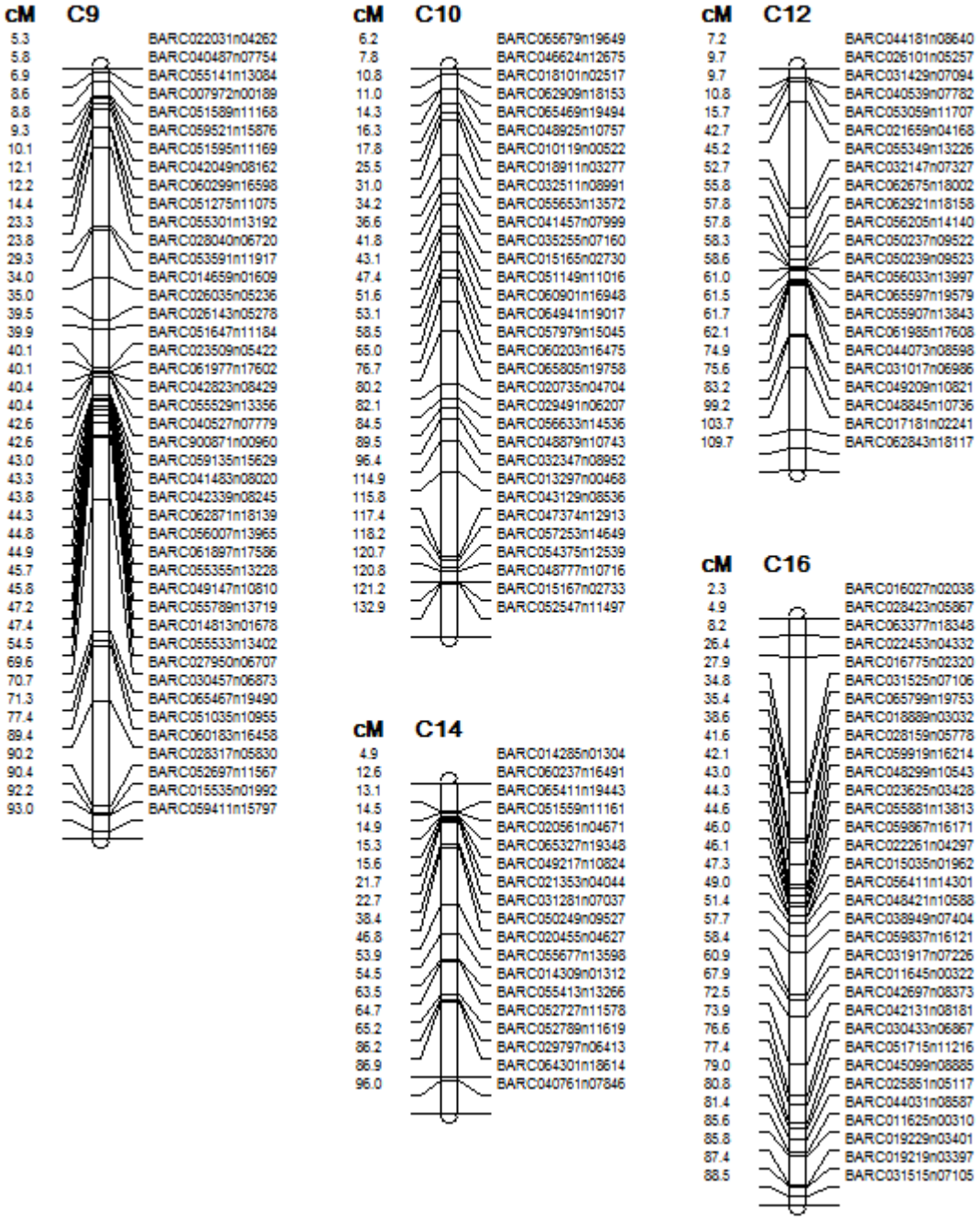
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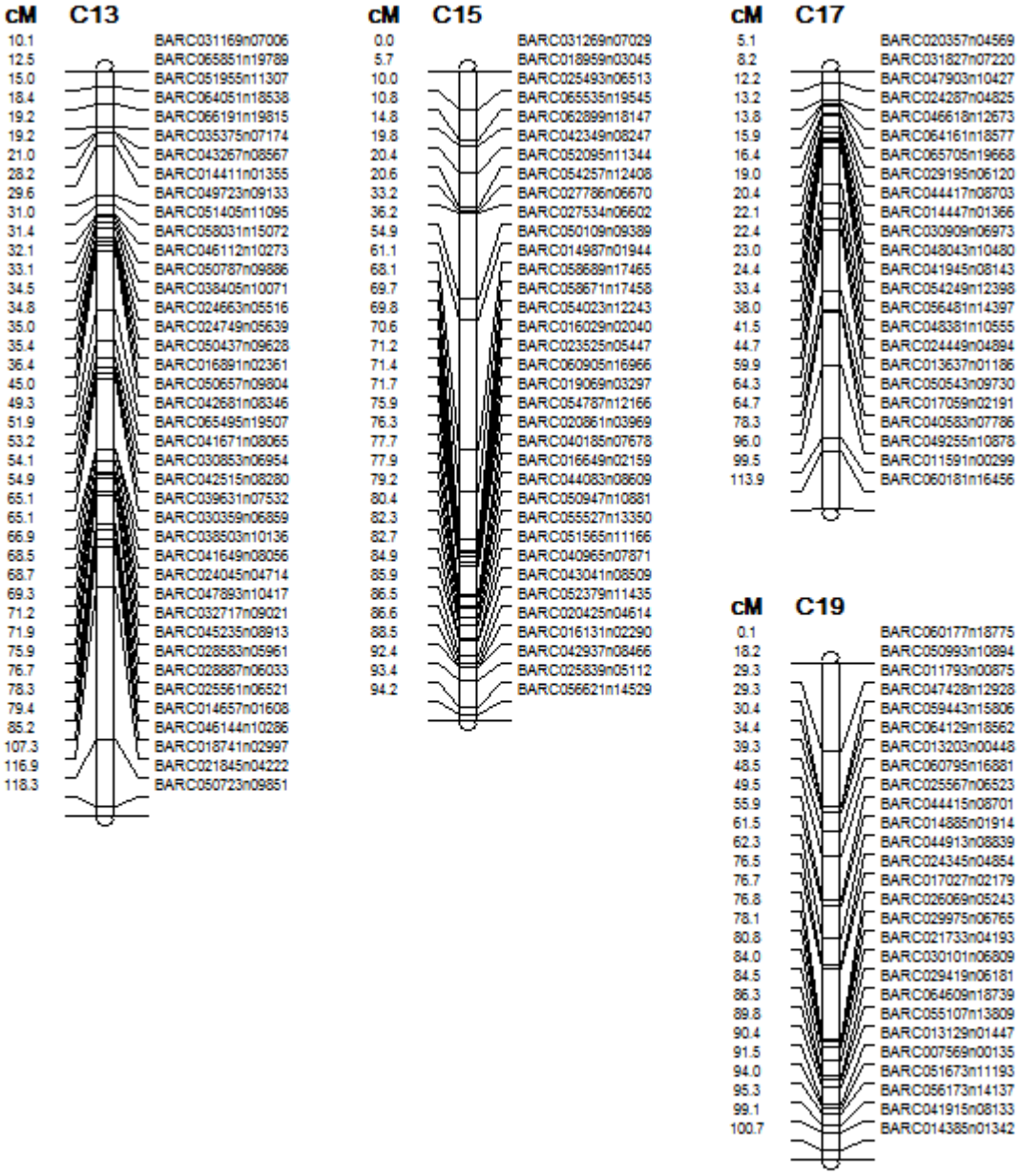
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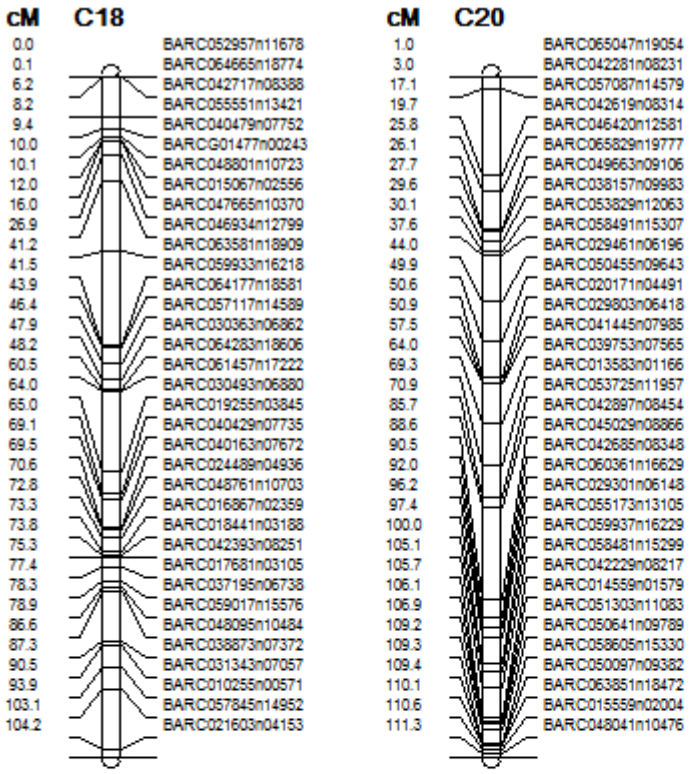
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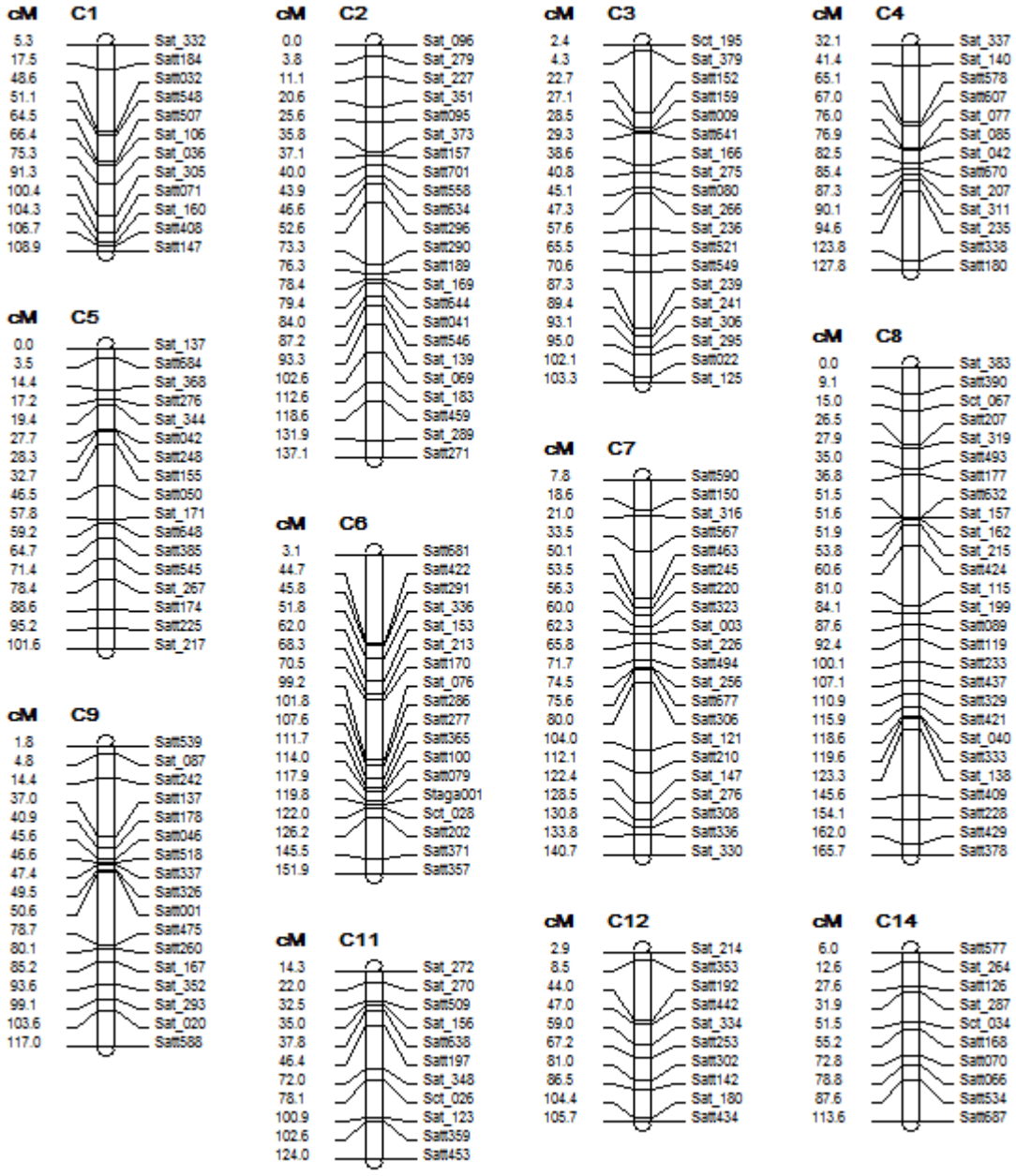
109.3 BARC058605n15330
109.4 BARC050097n09382
110.1 BARC063851n18472
110.6 BARC015559n02004
111.3 BARC048041n10476

Figure 4.2: Single sequence repeat (SSR) linkage map for the chromosomes one through twenty



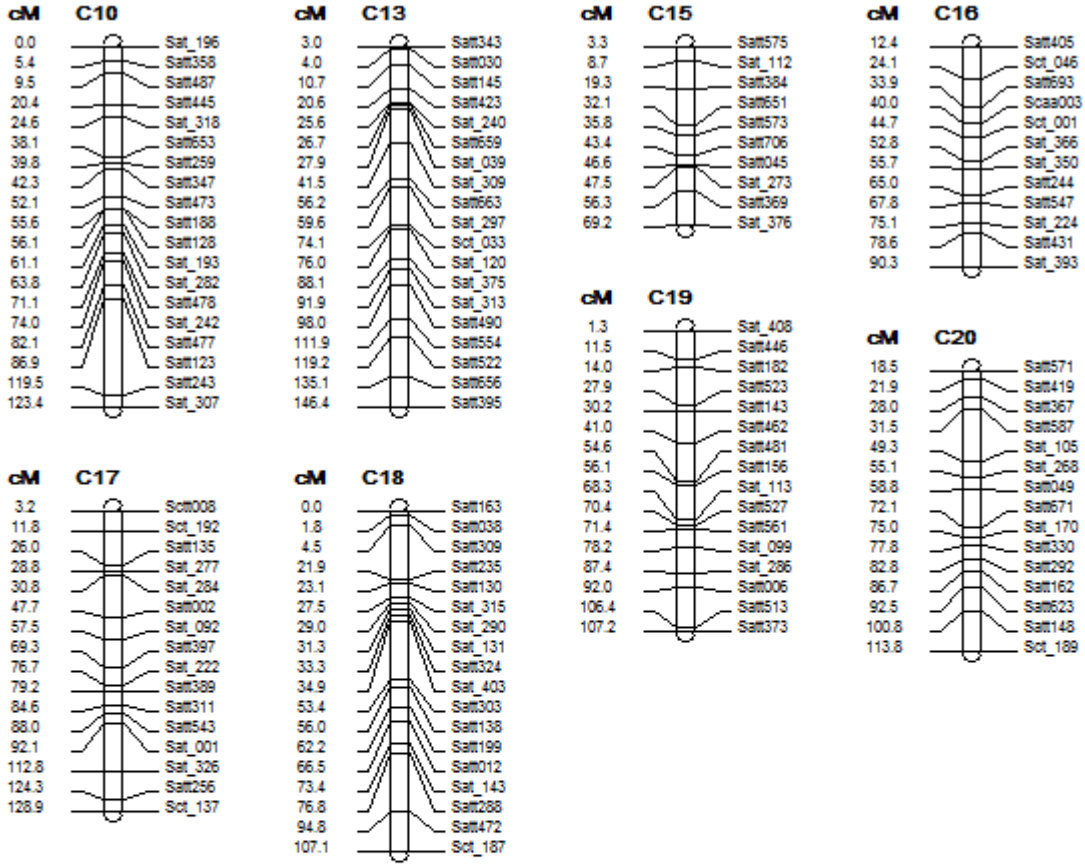


Figure 4.3: Linkage map of the potential intervals from the single nucleotide polymorphism (SNP) composite interval map (CIM) analysis associated with *F. virguliforme*, Minnesota isolate, Somerset #1A, infection on chromosome one, two, three, and five (D1a, D2b, N, and A1, respectively) identified with late foliar symptoms, root rot symptoms, phytotoxin foliar symptoms, and early foliar symptoms, respectively

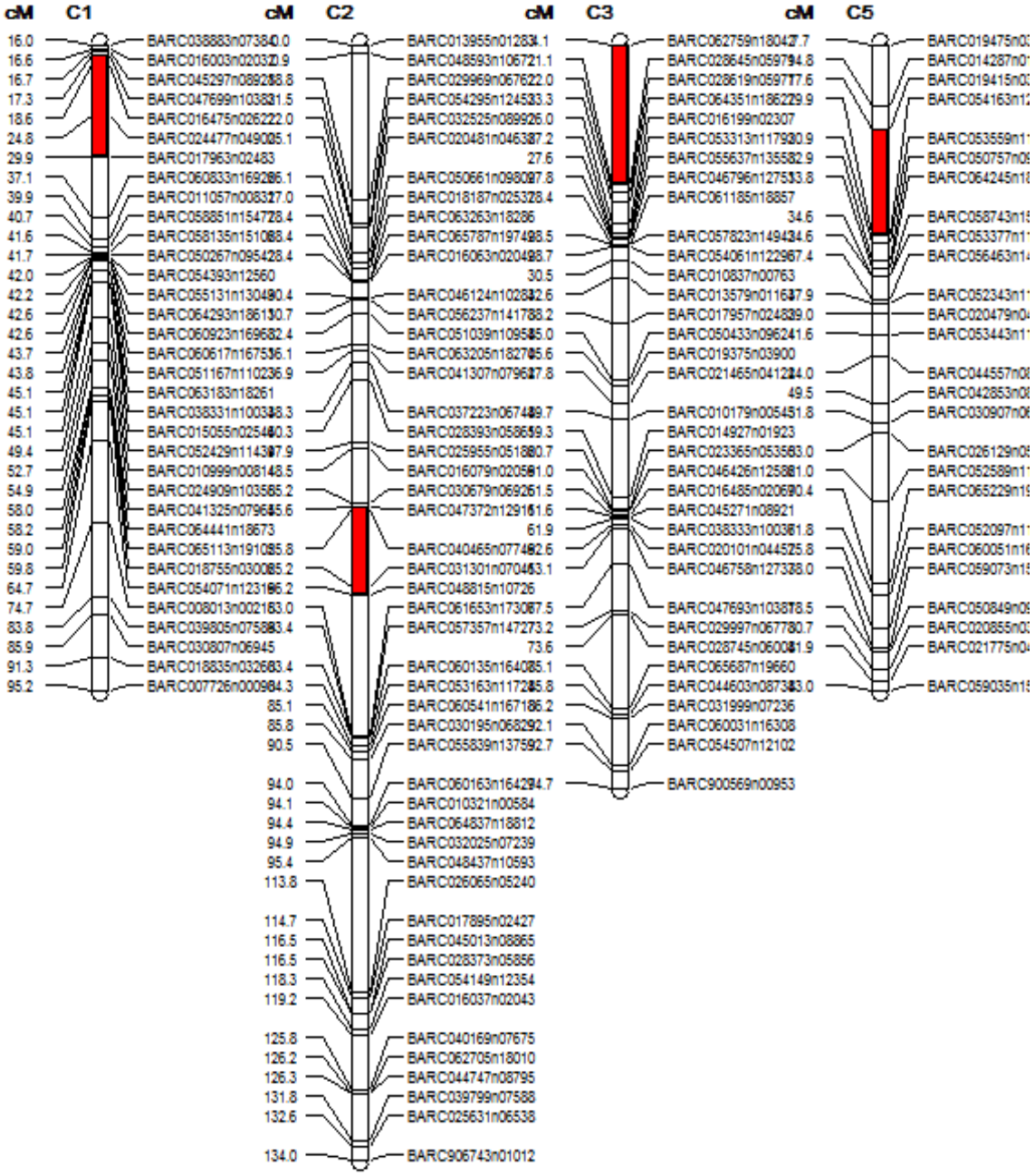


Figure 4.4: Linkage map of the potential intervals from the single nucleotide polymorphism (SNP) composite interval map (CIM) analysis associated with *F. virguliforme*, Minnesota isolate, Somerset #1A, infection on chromosome ten, fifteen, and eighteen (O, E, and G, respectively) identified with stem weight, early foliar symptoms, and root length, respectively

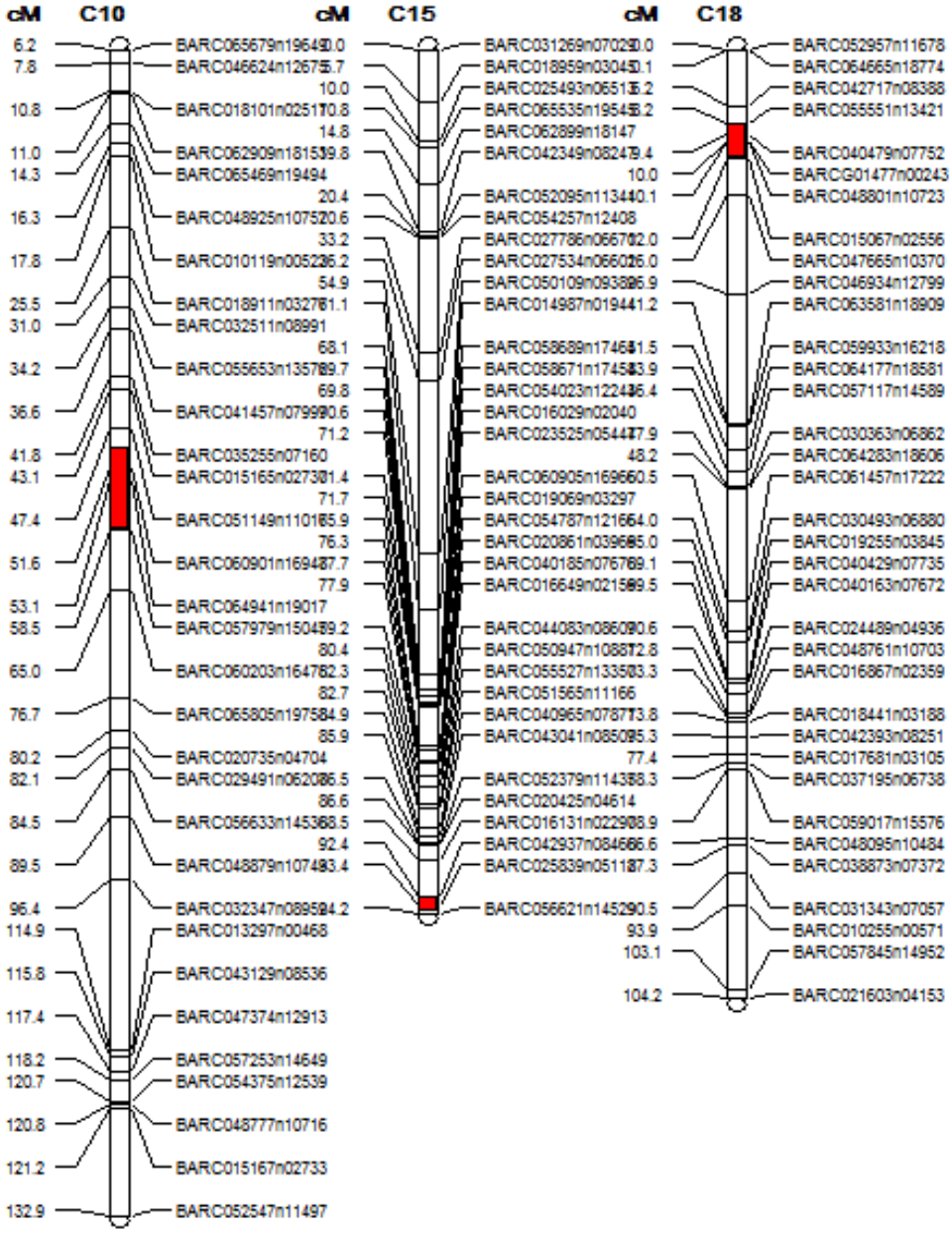
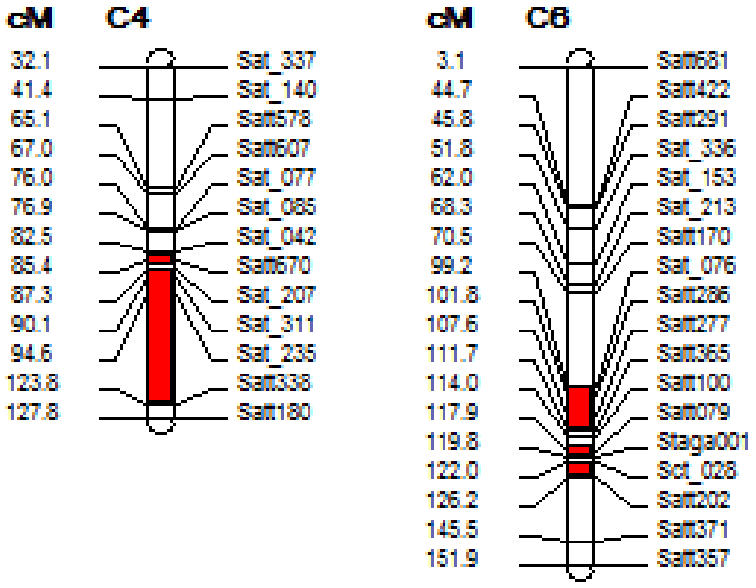


Figure 4.5: Linkage map of the potential intervals from the SSR CIM analysis associated with *F. virguliforme*, Minnesota isolate, Somerset #1A, infection on chromosome four, and six (C1 and C2, respectively) identified with early foliar symptoms, root length, root length, phytotoxin foliar symptoms, phytotoxin foliar symptoms, and root rot symptoms, respectively



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