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Genetic and Phytopathological Studies on Charcoal Rot Resistance in Soybean [Glycine max (L) Merr.]

Marcos Paulo da Silva
University of Arkansas, Fayetteville

Genetic and Phytopathological Studies on Charcoal Rot Resistance in Soybean *[Glycine max (L) Merr.]*

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by

Marcos Paulo da Silva
ULBRA – Lutheran University of Brazil
Bachelor of Science in Agronomy Engineering, 2005
Iowa State University
Master of Science in Plant Pathology, 2010

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University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

Dr. Andy Pereira
Dissertation Director

Dr. Richard E. Mason
Committee Member

Dr. Burt H. Bluhm
Committee Member

Dr. John C. Rupe
Committee Member

Dr. Lisa Wood
Committee Member

ABSTRACT

Charcoal rot of soybean (*Glycine max* (L.), caused by *Macrophomina phaseolina* is a disease of economic significance in the United States. The identification and quantification of the resistance is difficult, and very little is known about the genetics and markers linked to the charcoal rot (CR) resistance genes. Current assay methods can be time consuming, and data may vary between tests. The objectives of this study were to 1) create a robust seed plate assay (SPA) for CR resistance by comparing results with cut-stem and CFUI assays; 2) correlate and compare field data disease assessments with SPA; 3) identify QTLs for CR resistance using SNP markers; 4) identify genetic loci and candidate genes for resistance using next-generation sequencing (NGS)-based bulked-segregant analysis. For objective one, seeds of eight differential soybean genotypes inoculated with an isolate of *M. phaseolina* on water agar plates were evaluated for germination, and showed correspondence to results from the cut-stem and CFUI assays. In the second objective, nineteen differential genotypes were used to assess disease resistance to *M. phaseolina* using the SPA, and results were correlated with field disease assessment data from Rohwer and Stuttgart, AR. Disease assessments were significant, and correlated with SPA between different years and locations. For the third objective, an F_{2-3} mapping population was developed from a cross between PI 567562A and PI 567437, the lines genotyped with 5403 single nucleotide polymorphism (SNP) markers covering 20 chromosomes, and the population phenotyped with the cut-stem assay. Composite Interval Mapping analysis indicated three QTLs for resistance to *M. phaseolina*. In the fourth objective, two extreme phenotypic bulks were generated from the same F_{2-3} population by pooling equal amounts of DNA from 10 plants of each bulk, and the bulks along with parents were sequenced using Illumina HiSeq™. A SNP-index was calculated at each SNP position for both bulks, and the average distributions of the

SNP-index and Δ SNP-index were estimated using sliding window analysis. Three genomic regions on chromosomes 5, 8 and 14 were identified with positive values of Δ SNP-index plots that potentially indicate QTLs governing the difference between the R-bulk and S-bulk.

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INTRODUCTION AND LITERATURE REVIEW

Charcoal rot of soybean (*Glycine max* (L.) Merr., caused by *Macrophomina phaseolina* (Tassi) Goid] (Goidanish, 1947) is a soil borne plant pathogen causative agent of disease in more than 500 plant species around the world, including economically important crops such as corn (*Zea mays* L.), sorghum (*Sorghum* Moench), fruits, legumes and cotton (*Gossypium* L.) (Dhingra and Sinclair, 1978; Wyllie, 1988; Su et al., 2001). For example, sunflower yields showed losses between 60% to 90% (Khan, 2007) in environments that favor the disease, and in strawberry cause a major impact in California as well as many countries (Koike et al.,2016). A special report that estimated total yield losses caused by different pathogens in the top ten soybean production countries classified *Macrophomina phaseolina* as one of the most yield impacting diseases (Wrather et al., 2001).

In the United States, charcoal rot was first observed in east Texas (Young, 1949), and has been reported throughout the country since then (Wyllie and Scott, 1988). Even though the disease has been described mostly in north central and north east (Wyllie and Scott, 1988; Yang and Navi, 2005; Cummings and Bergstrom, 2013), it is frequently most found in the southern soybean fields of the United States, because of the favorable environmental conditions in the region (Mengistu et al., 2007; Luna et al., 2017). Charcoal rot was ranked in 2003 and 2012 as the second most important soybean disease impacting yield in the country, with an estimated yield loss of 1.9 million to 2.0 million metric tons (Wrather et al., 2010; Luna et al., 2017).

Macrophomina phaseolina normally survives in the soil and host crop debris as microsclerotia for 2-15 years (Meyer et al., 1974; Short et al., 1980). The fungi can survive for

up to 3 years as mycelium in asymptomatic seeds or as microsclerotia in symptomatic seeds (Hartman et al., 1999). As a soil-borne pathogen, *M. phaseolina* can infect soybeans from seedlings to mature plants, although infections can remain latent until plants become stressed by environmental factors (Short and Wyllie, 1978; Bristow and Wyllie, 1986; Collins et al., 1991).

Disease life cycle

The disease life cycle of *M. phaseolina* is favored by dry soils and annual temperatures between 28º C and 35º C. Under these conditions, microsclerotia begin to germinate and produce germ tubes that penetrate plant epidermal cell walls or through natural openings (Dhingra and Sinclair, 1978; Bressano et al., 2010). During the early stages of infection, hyphae grow intercellularly and then intracellularly through the xylem in vascular tissues, where it can form microsclerotia that interfere with plant functions of transporting water and nutrients, resulting in disease symptoms, such as wilting and premature leaf death (Francl et al., 1988; Gupta and Chauhan, 2005; Khan, 2007). After harvest, the disease cycle starts over with the fungus surviving in the soil and soybean crop debris, generally as microsclerotia (Short et al., 1980).

Abiotic stresses, such as drought and high temperature play an important role in the weakening of plant defenses and enhancing susceptibility to diseases (Grodzki et al., 2004; Sandermann Jr, 2004). According to (Mittler, 2006), a combination of drought and heat stress increases severity of damage to soybean crops by *M. phaseolina*. Consequently, a conducive environment plays a significant role in the colonization of soybean plants by the pathogen, increasing the level of disease severity and infection as the crop develops mainly at the R5-R6, and R7 (Fehr et al., 1971) growth stages (Short and Wyllie, 1978; Wyllie, 1988). Besides soybean, drought stress increased charcoal rot development and stomatal resistance in common bean (*Phaseolus vulgaris*), resulting in higher transpiration rate and leaf temperature as

compared to plants exposed to only drought stress (Mayek-PÉrez et al., 2002). In addition to water and heat stresses, disease symptoms may increase under saline conditions (You et al., 2011). Damage caused by the pathogen can also increase under any type of these stressors acting separately (Hartman et al., 1999).

Disease symptoms

Charcoal rot symptoms mainly appear during hot and dry conditions and can be observed in soybean at all stages, although above-ground symptoms are more prevalent during reproductive stages of soybean development and are characterized by stunted growth, leaf chlorosis, early maturation, and incomplete pod filling (Hartman et al., 1999). Moreover, symptoms in the soybean fields can be easily recognized by premature yellowing in scattered patches, that can increase under severe disease conditions (Gupta et al., 2012).

Microsclerotia form in the vascular tissues and in the pith, and can result in plant wilting and flagging of branches as a result from the blockage of water flow (Hartman et al., 1999). Microsclerotia can grow aboveground and are first visible in stem nodes. As the disease progresses, it can infect and penetrate pods and grains. The belowground symptom, if the infection starts through the roots, is mainly a reddish-brown discoloration of the vascular elements (Gupta et al., 2012).

Control

Several strategies can be applied to mitigate damage caused by charcoal rot in soybean. For example, cultural methods such as staggering of planting dates, rotation with non-host crops, reducing plant densities, seed treatment, and irrigation (Francl, L. J. et al., 1988; Bowen and Schapaugh, 1989; Wrather, 2007) can be used in efforts to manage the disease. Soil fumigation

with different combinations of methyl bromide, chloropicrin, and sodium methyldithiocarbamate can reduce the population of microsclerotia of *M. phaseolina* in the soil (Watanabe et al., 1970; Kittle and Gray, 1982; Pearson et al., 1984). Unfortunately, these methods have not been fully adopted by farmers and have shown limited impact on mitigating disease severity (Mengistu et al., 2007; Twizeyimana et al., 2012a). Therefore, host resistance may be the best viable method to control charcoal rot in soybean, due to its reduced cost and eco-friendly management (Bowen and Schapaugh, 1989; Smith and Carvil, 1997b). A proposed notice of release of soybean lines DT98-7553, DT99-16864, DT99-17483, and DT99-17554 with resistance to charcoal rot and good yield potential was made by Alemu Mengistu (USDA-ARS-CGRU, Jackson, TN). These lines were evaluated for charcoal rot reaction in artificially infested fields in Stoneville, MS for two years and were rated as moderately resistant based on an index of colony forming units (CFU) (Mengistu et al., 2007). Likewise, Paris et al. (2006) released a soybean germplasm line DT97-4290, with moderate resistance to charcoal rot, however this resistance has not yet been incorporated into high yielding cultivars, and currently no commercial soybean cultivars are available with high levels of resistance to the pathogen (Mengistu et al., 2007).

Screening charcoal rot for resistance

In an effort to find new sources of resistance to charcoal rot, a reliable disease evaluation technique is necessary. Current approaches to identifying and quantifying resistance are difficult, time consuming and lack reliability and consistency across locations and seasons. Non-uniform inoculum distribution, soil characteristics, microflora, weather patterns, and plant maturity may all affect disease expression (Smith and Carvil, 1997; Mengistu et al., 2007; Radwan et al., 2013).

The primary disease screening methods to evaluate resistance to *M. phaseolina* were based on the colonization in the entire root system. Short et al. (1978) were the first to propose a method used to measure disease development in field and greenhouse studies of mycelial or sclerotial propagative units on infected soybean tissue. The density of microsclerotia of the pathogen, *M. phaseolina,* was most abundant in the roots following the death of soybean plants, and declined with height above the ground. Soybean cultivars varied in the amount of microsclerotia in the root, and in the height of colonization, suggesting that some cultivars were more resistant than others. Subsequently, Pearson et al. (1984) working in naturally infested, fumigated, and fumigated-infested soils, reported a screen technique similar to Short et al. (1978) to quantify *M. phaseolina* colonization, measured per gram of root (dry weight) for nine soybeans varieties, representing the maturity groups III, IV and V. Results showed that fumigation reduced initial soil population by 80%. However, it did not significantly reduce the subsequent disease incidence. Soybean genotypes differed on rates of colonization by the pathogen, and the disease was more severe at the end of the season, with plants approaching maturity (R7-R8).

Later, Smith and Carvil (1997) developed a new assessment criteria of host tissue colonization based on *M. phaseolina* microsclerotia densities in lower stem and taproot tissue at the growth stage R7, known as colony forming unit (CFU) assay. Even though this new method provided a better measure of the degree of host compatibility between soybean cultivars and *M. phaseolina*, it was still time-consuming, and the levels of colonization of *M. phaseolina* were affected by planting date and maturity group.

In a comparative study, Mengistu et al. (2007) developed a similar method of disease assessment in an effort to compare and establish a consistent and reliable field screen technique

to evaluate charcoal rot, and proposed a classification system based on a colony-forming unit index (CFUI). This method is based on the CFU technique by Smith and Carvil (1997) where the CFUI after disease treatment is calculated by dividing the CFU for each genotype by the CFU for the genotype with the highest CFU in the study. They claimed that a major advantage of using the CFUI over absolute CFU value is that CFU quantities can vary significantly over years and location, and a genotype with low CFU value may actually be susceptible but classified as resistant without a standard of susceptibility for comparison across environments. In contrast, CFUI provides criteria for rating soybean genotypes across years, locations, and experiments. Mengistu et al. (2007) also developed a screening method called root and stem severity (RSS) where plants at the R7 growth stage are scored by longitudinally splitting the stem and taproot of each one and visually rating the intensity of discoloration using a scale divided into four classes (Paris et al., 2006): where 1 = resistant, >1 to 2 = moderately resistant, >2 to <3 =moderately susceptible, and 3-5 = susceptible. They also created another disease screening classification method, percent height of internal stem discoloration (PHSD) that is comparable with RSS. However, PHSD is based on the percentage of stem height discoloration of plants at R7 stage, where the height from ground level of internal vascular discoloration is divided by the stem height and multiplied by 100 to determine the PHSD. Both methods though present a significant degree of variation between years.

The last screening method from Mengistu et al. (2007) was established using foliar symptoms (FS). The first one, was based on the Horsefall-Barrat scale (James, 1974) at the R7 growth stage, and is grounded on visual judgment that describes 12 grades of percentage disease assessment. Instead of using 12 grades, they used a scale from 0 to 11 for FS: 0 = no symptoms; 1 = 0 to 3%, 2 = 3 to 6%, 3 = 6 to 12%, 4 = 12 to 25%, 5 = 25 to 50%, 6 = 50 to 75%, 7 = 75 to

87%, 8 = 87 to 94%, 9 = 94 to 97%, 10 = 97 to 100%, and 11 = 100%. At the base of this scale, genotypes were classified into four classes as follows: resistant = 0, moderately resistant > 0 and < 5, moderately susceptible ≥ 5 and < 8, and susceptible ≥ 8. In the second method, plants were rated on a weekly basis beginning with the first onset of leaf symptoms up to the R7 growth stage. The disease was assessed on the percentage of plants in each plot that were affected as well as the intensity of infection, and the foliar symptoms over time were used to calculate area under the disease progress curve (AUDPC). Unfortunately, both foliar symptom ratings failed to determine moderately resistant genotypes, and visual results did not agree within and between tests. Therefore, all of these methods presented had some significant degree of variation within and between trials, lacking consistency to determine reactions to *M. phaseolina* across different environments of soybean cultivars under field condition.

Lastly, Twizeyimana et al. (2012) established a cut-stem assay to evaluate soybean genotypes under greenhouse conditions. This technique had previously been used to evaluate resistance *to Sclerotinia sclerotiorum* on soybean, dry bean and sunflower (Vuong et al., 2004), and also aggressiveness of *Phomopsis longicolla* and other *phomopsis* spp. on soybean (Li et al., 2010). Vuong and Hartman (2003) identified two soybean plant introductions (PI 194634 and PI 194639) with high levels of resistance to *S. sclerotiorum* using the technique. The cut stem inoculation technique involves the measurement of disease severity by the extent of necrosis of plants from the inoculation point. Mycelial plugs from the margin of an actively growing *M. phaseolina* culture growing on potato dextrose agar are taken using the open end of a 10 to 200 µl pipette tip (Fisher Scientific) and placed immediately over soybean plants at V2 stage, that were previously cut 25 mm above the unifoliate node with a sharp razor blade. Three days after the inoculation, the pipette tips are removed from each plant and discarded. Linear stem necrosis

in millimeters is measured on each plant with a ruler and recorded every 3 days until the end of the trial 13 to 15 days after inoculation. Linear extent of stem necrosis (mm) is used to calculate the area under the disease progress curve (AUDPC) (Campbell and Madden, 1990). With the advantage of quantifying the amount of inoculum in each plant, a controlled environment under greenhouse conditions as well as being precise and less time consuming, the cut-stem method presented less variation between and among trials when compared to results of the field tests. However, the technique still lacks consistency and reliability between trials.

With an effort to develop a consistent, reliable and less time consuming inoculation technique under controlled environment, our laboratory started to work with a petri dish assay that had been used to evaluate pathogenicity and aggressiveness of *Pythium* and *Rhizoctonia* spp. on both corn and soybean seed (Broders et al., 2007a). The assay is based on growing species of fungi on growth media (PDA) for a certain period of time and transferring a 3-mm plug to the center of a petri plate containing water agar, followed by colonization of the plate by the fungi, and planting seeds on it. Plates are scored by counting the number of seeds that successfully germinate. Similar petri plate assays were used before with the aim to identify resistance to *Pythium* spp. in alfafa (Altier and Thies, 1995) and also to evaluate fungicide seed treatment on corn seedlings to control *Fusarium* spp. (Munkvold and O'Mara, 2002). This method was used by Urrea Romero (2015) who identified two QTLs for resistance in soybean pathogen *Pythium aphanidermatum* using the seed plate assay.

Molecular markers and quantitative trait loci (QTL) mapping in soybean

Molecular markers

DNA markers have played an important role in plant genetics and breeding for decades. The first application of DNA polymorphism applying DNA markers in plant genotyping was described by Botstein et al. (1980), using restriction fragment length polymorphism (RFLP). Although the RFLP technique had been valuable in the construction of genetic linkage maps, it is complicated by use of hybridizations and radioactivity, is time consuming, and limited by the number of available probes (Bernatzky and Tanksley, 1986).

Although a low throughput method, RFLP mapping was used to construct the first soybean molecular genetic linkage map, based on F_2 lines (Keim et al., 1990). Subsequently, Young et al., (1999) constructed a linkage map with recombinant inbred lines (RILs) using RFLPs, PCR-based random amplified polymorphic DNA (RAPD) markers (Williams et al., 1990), and amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995). Even though RAPD and AFLPs have higher level of polymorphism when compared with RFLP, all of them still present complex polymorphic patterns and different primary applications (Jiang, 2013). Simple sequence repeats (SSRs), also known as microsatellites (Litt and Luty, 1989; Salimath et al., 1995), were proposed and integrated into the soybean linkage map (Akkaya et al., 1992, 1995). SSRs have a high level of allelic variation, with simple polymorphic banding patterns and appropriate for a range of application, such as diversity, genetics and breeding. Cregan et al. (1999), used SSRs, RFLPs, RAPDs, and other markers to create an integrated soybean linkage map using three RILs populations, and further created an updated integrated

soybean linkage map containing the same and other markers, but using five RILs (Song et al., 2004).

The advent and application of single nucleotide polymorphism (SNPs) as DNA markers in plant breeding has improved the potential to score more variation in precise DNA targets, as SNPs are considered to be the ultimate form of molecular marker, and besides that the most abundant in eukaryotic genomes (Lander, 1996; Brookes, 1999). Using DNA sequence of unigenes and expressed sequence tag (EST) from GenBank, Yoon et al. (2007) described a set of 23 informative SNPs distributed on 19 of the 20 soybean linkage groups and validated across diverse germplasm of soybean, to generate a third version of the soybean integrated linkage map. Subsequently, three RIL mapping populations were used to construct the fourth version of the soybean integrated linkage map using the GoldenGate (Illumina Inc., San Diego, CA) assay high-throughput analysis method (Hyten et al., 2010). In the same year, the whole genome shotgun sequence of *Glycine max* var. Williams 82, comprised 950 megabases (Mb) was reported (Schmutz et al., 2010). Song QiJian et al. (2013), selected a total of 52,041 SNPs to produce the SoySNP50K iSelect BeadChip. This new powerful resource has been used already to genotype 19,652 accessions in the USDA Soybean Germplasm Collection, and the genotypic information is available at Soybase (USDA, ARS Soybean Genetics and Genomics Database).

Today, molecular markers are widely used as a tool in plant breeding as a means to characterize plant germplasm, genetic mapping, marker-assisted selection (MAS) and many new applications. For instance, MAS has been improving the efficiency and precision of conventional plant breeding programs by accelerating process with selections at the seedling stage, single plants, and as a replacement for phenotypic screening, allowing fast, precise and more reliable

selection in off-season nurseries making it more cost-effective to grow more generations per year (Ribaut and Hoisington, 1998; Collard and Mackill, 2008).

QTL mapping

Based on the use of DNA markers, researchers can construct a linkage map for different crops in an effort to identify chromosomal regions that contain genes controlling simple qualitative as well as quantitative trait loci (QTL). To illustrate the construction of a linkage map, a parental set that differs in one or more traits of interest is needed, then crosses are made and a segregating population generated (Mohan et al., 1997; Collard et al., 2005). For mapping purposes numerous different populations may be used within a given plant species, each with its advantages and disadvantages, such as F_2 plants, recombinant inbred lines (RILs), backcross (BC) populations, and double haploids (DH) (Schneider, 2005). The simplest type is a F_2 population, resulting from F_1 hybrids and backcross (BC) populations, derived by crossing the F_1 hybrid to one of the parents. While they are easy to construct and only require a short time to produce, the drawback is that F_2 cannot be easily preserved, because they are not immortal, and F_3 progeny resulting from their selfing are genetically not identical. The disadvantage of RILs is the length of time needed to generate them; however, they are a perpetual resource, making them easy to replicate and share with many groups of the research community. A further advantage is that a RIL population shows a higher resolution for QTL mapping than using F_2 or BC populations. Lastly, double haploid lines constitute a permanent resource for mapping purposes due to their homozygosity, and the lines can be multiplied by selfing without any genetic change occurring, and like RILs they are also immortal lines (Burr and Burr, 1991; McCough and Doerge, 1995; Schneider, 2005; Collard et al., 2005).

The first quantitative trait locus (QTL) published in soybean was for resistance to soybean cyst nematode (SCN, *Heterodera glycines*) (*rhg*) that was identified in the early 1960s, and molecular markers linked to these QTL have been consistently used in a variety of soybean germplasm as a major source of resistance to SCN (Caldwell et al., 1960; Song et al., 2004). Moreover, QTL mapping has been reported for a different number of traits, such as protein (Kim et al., 2016), yield (Zhang et al., 2016), stress tolerance (Lee et al., 2004), and oil (Brummer et al., 1997). Besides soybean, QTL mapping has been applied in mapping disease resistance in a variety of crops, such as for rice blast fungus, late blight of potato, and gray leaf spot of maize and bacterial wilt of tomato (Young, 1996).

Although genetic sources of host resistance to charcoal rot may be the best approach to control the disease and facilitate selection of breeding materials, very little is known about molecular maker(s) linked to the charcoal rot resistance gene(s), and more research is needed to understand inheritance of charcoal rot resistance (Mengistu et al., 2007; Gupta et al., 2012). The specific question remains, is resistance controlled by one or a few major genes with high heritability, or as multiple genes with low heritability? It has been reported by Talukdar et al. (2009) that the expression of the disease reaction is continuous, starting from highly susceptible through moderately resistant to highly resistant. Therefore, it suggests that the disease resistance is influenced by more than one locus.

Working with molecular markers associated with resistance to *M. phaseolina* in common bean, it was reported that charcoal rot resistance in a common bean variety called BAT 477 is controlled by two dominant genes with double-recessive epistasis (Hernandez-Delgado et al., 2009). However, a larger sample size is needed for establishment of the association between the locus and genetic resistance to be statistically reliable.

Next generation sequencing

In the last decade with the advent of the genomic revolution that has been driving by the sequencing of many crop species reference genomes, plant breeding has shifted from phenotyping-based process to a high level of genotype based selection. This tendency has been increasing in the recent years with the introduction of Next-generation sequencing (NGS) technologies that have made high throughput DNA sequencing cost effective, enabling use of sequence-based trait mapping approaches to identify markers (Michael and Jackson, 2013; Varshney et al., 2014; Barabaschi et al., 2016).

Originally developed for high resolution association studies in maize, such as RAD-seq, the term Genotype-by-sequencing (GBS) was first introduced to plant science by Elshire et al., (2011). As a novel application of NGS protocols for discovering and genotyping SNPs in crop genomes and populations, GBS has been successfully used in the field of plant breeding in implementing genome-wide association study (GWAS), genomic diversity study, genetic linkage analysis, molecular marker discovery and large scale genomic selection of plant breeding programs (Baird et al., 2008; Poland and Rife, 2012; Narum et al., 2013). For instance, the technology has been applied in maize in order to investigate genetic diversity (Romay et al., 2013). In soybeans, GBS has been used for genomic prediction with potential to increase genetic gain (Jarquín et al., 2014) and also to develop GBS protocols to identify high quality SNPs among diverse lines (Sonah et al., 2013).

QTL-seq

As an alternative to the conventional QTL analysis, QTL-seg holds potential for rapid identification of QTLs. Combining bulked-segregant analysis (BSA) (Michelmore et al., 1991;

Giovannoni et al., 1991; Mansur et al., 1993; Darvasi and Soller, 1994) and next-generation sequencing (NGS), QTL-seg is faster, less labor-intensive, and relatively inexpensive when compared with regular QTL mapping, with no need to genotype a large number of individuals in segregating populations derived from bi-parental crosses (Lu et al., 2014). The new method is based on BSA, where two bulked DNA samples from a segregating population derived from a single cross are generated. Each DNA pool contains individuals with contrasting phenotypic trait values, for example resistance and susceptible to a particular plant disease. The DNA bulks are then screened for polymorphism using a specific molecular marker (Michelmore et al., 1991; Magwene et al., 2011). This QTL mapping technique was first reported by Giovannoni et al. (1991) where he described it as rapid and efficient method for isolation of molecular markers (RFLP) linked to any defined genomic interval. In the same year, Michelmore et al. (1991) developed BSA to identify RFLP and RAPD markers linked to disease resistance genes.

QTL-seq was first mentioned by Takagi et al. (2013), working with rapid mapping of quantitative trait loci in rice by whole genome resequencing of DNA bulks of phenotypic extremities. Their method relies on the estimation of SNP-index and Δ SNP-index to identify candidate genomic region (s) harboring the major QTL (s) associated with the trait of study. QTL-seq has been effectively applied to identify QTLs for flowering locus T in cucumber (Lu et al., 2014), fruit weight and locule number in tomato (Illa-Berenguer et al., 2015) and candidate gene underlying major trait-associated in chickpea (Das et al., 2015). With the recent rapid development in sequencing technology, QTL-seq strategy can be applied to rapidly identify QTLs. Further, different types of mapping populations, such as RILs and DH can be used.

Hypotheses

1) Development of an *in vitro* based seed plate screening assay for soybean charcoal rot resistance can reduce environmental variation and increase reliability compared to current methods of cut stem and CFUI assay techniques.

2) Seed plate assay disease assessment is accurate and reproducible with field disease assessment methods.

3) Resistance loci for charcoal rot in soybean can be identified from a biparental segregating population.

4) QTL-seq method can be used to determine the genetic basis of resistance to charcoal rot on soybean.

Objectives

The objectives of this study were to: 1) create a robust seed plate assay (SPA) for charcoal rot resistance by comparing results with cut-stem and CFUI assays, 2) correlate field-based disease assessments such as percent height of stem internal discoloration (PHSD), root and tem severity (RSS) and colony-forming units (CFUs) with SPA results on diverse soybean genotypes, 3) identify QTLs governing resistance to charcoal rot of soybean using a biparental population, 4) characterize the genetic basis of resistance to charcoal rot in soybeans, with a QTL-seq approach using next-generation sequencing (NGS) based bulked-segregant analysis (BSA).

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CHAPTER 2

Effectiveness of a Seed Plate Assay for Evaluating Charcoal Rot Resistance in Soybean

M. P. da Silva, Andy Pereira, Burt H. Bluhm, Richard E. Mason, Lisa S. Wood, Pengyin Chen, John C. Rupe*

M. P. da Silva, Andy Pereira, Richard E. Mason and Lisa Wood, Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR 72701; John C. Rupe and Burt H. Bluhm, Department of Plant Pathology, University of Arkansas, Fayetteville, AR 72701. Pengyin Chen, Fisher Delta Research Center, University of Missouri, Portageville, MO.

*Author for correspondence: John C. Rupe. jrupe@uark.edu

ABSTRACT

Charcoal rot of soybean, caused by *Macrophomina phaseolina*, is a disease of considerable economic significance in the United States. Although some soybean cultivars are moderately resistant, identifying and quantifying resistance is challenging. Existing assays, such as greenhouse-based cut-stem inoculations and field evaluations using root discoloration, height of colonization and colony-forming unit index (CFUI), are time consuming, and results can be variable or even irreproducible. The objectives of this research were to 1) create a robust seed plate assay (SPA) for charcoal rot resistance by comparing results with cut-stem and CFUI assays, and 2) correlate field-based disease assessments namely percent height of stem internal discoloration (PHSD), visual root and stem severity (RSS) and taproot colony-forming units (CFUs) with SPA results on diverse soybean accessions. To develop an SPA, surface-disinfected seeds from eight soybean genotypes (representing three sensitive and five resistant cultivars) were placed on water agar plates inoculated with *M. phaseolina*. After incubation at room temperature in darkness for seven days, germination ratios were calculated for each cultivar relative to germination on non-inoculated plates. Results from the SPA were in general agreement with the cut-stem and CFUI assays, although none of the soybean genotypes evaluated showed complete resistance to *M. phaseolina.* For the second objective, charcoal rot resistance in 19 soybean accessions was assayed with the SPA, and results were correlated with field disease assessments from two locations in two years. Data from the SPA quantitatively categorized soybean genotypes into different degrees of resistance, and results were consistent with previously published resistance determinations. PHSD assessment correlated significantly with SPA results for Stuttgart across years from 2011 to 2013 and year 2012 in Rohwer. Yield correlated significantly for Stuttgart in 2011, 2013 and 2014, and in 2011 and 2012 for Rohwer.

SPA was significantly correlated to Root and stem severity (RSS) at Rohwer in 2012, and with CFUs at Stuttgart for 2012. In conclusion, the SPA assay is a robust and efficient method to evaluate resistance to charcoal rot in soybean, given similar results as established procedures, but faster and with less work. Thus, the SPA provides soybean breeders a practical tool to screen large numbers of soybean accessions for resistance to charcoal rot.

INTRODUCTION

Macrophomina phaseolina (Tassi) Goid. (Goidanish, 1947) is a causative agent of disease in more than 500 plant species around the world, including economically important crops such as soybean [*Glycine max* (L.) Merr.], corn (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), fruits, legumes and cotton (*Gossypium hirsutum* L.) (Dhingra and Sinclair, 1978; Wyllie, 1988; Baird and Brock, 1999; Su et al., 2001). In the United States, charcoal rot in soybean was first observed in east Texas (Young, 1949), and has been reported throughout the country since then (Wyllie and Scott, 1988).

M. phaseolina can infect soybeans from seedlings to mature plants, although infections can remain latent until plants become stressed by environmental factors (Short and Wyllie, 1978; Bristow and Wyllie, 1986; Collins et al., 1991). Abiotic stresses, such as drought and heat, play an important role in the weakening of plant defenses, and enhance susceptibility to charcoal rot (Grodzki et al., 2004; Sandermann Jr, 2004). Accordingly, symptoms of charcoal rot mainly appear during hot and dry conditions, and are more prevalent during reproductive stages of soybean development. Symptoms are variable, and include stunted growth, leaf chlorosis, early maturation, and incomplete pod filling (Hartman et al., 1999).

Several strategies can mitigate the impact of charcoal rot on soybean production, including cultural methods such as staggering of planting dates, rotation with non-host crops, reduced plant densities, and irrigation (Francl et al., 1988; Bowen and Schapaugh, 1989; Wrather, 2007). Additionally, soil fumigation with various combinations of methyl bromide, chloropicrin, and sodium methyldithiocarbamate can reduce, but not eliminate, populations of *M. phaseolina* microsclerotia in the soil (Watanabe et al., 1970; Kittle and Gray, 1982; Pearson et

al., 1984). However, host resistance is widely considered to be the most viable strategy to control charcoal rot in soybean, due to economic and environmental considerations (Bowen and Schapaugh, 1989; Smith and Carvil, 1997b). To this end, Paris et al. (2006) released a soybean germplasm line DT97-4290 with moderate resistance to charcoal rot.

Historically, developing assays to quantify charcoal rot resistance in soybean has been challenging, and existing assays often produce contradictory results. Initially, assays to identify charcoal rot resistance in soybean relied on quantifying pathogen colonization of the entire root system (Short, 1978). Subsequently, a colony forming unit (CFU) assay was developed by Smith and Carvil (1997) to quantify microsclerotia from soybean stems and roots. Although this technique represented an advancement, the assay was still laborious, and results were somewhat inconsistent among studies (Mengistu et al., 2007; Luna et al., 2017). More recently, Mengistu et al. (2007) proposed a colony-forming unit index (CFUI) to standardize resistance evaluations across cultivars and field conditions. However, this approach was still difficult, and results were not consistent across locations and years. Mengistu et al. (2007) also developed additional screening assays, namely root and stem severity (RSS) and percent of internal stem discoloration (PHSD), based on internal stem discoloration caused by *M. phaseolina*. Both methods are subject to significant variation between years and sensitivity to environmental conditions (Twizeyimana et al., 2012a). Furthermore, Mengistu et al. (2007) explored two foliar assays to evaluate charcoal rot resistance. One was based on the Horsefall-Barrat scale (James, 1974). In the second, plants were rated weekly from the onset of leaf symptoms until the R7 growth stage. Unfortunately, both foliar rating systems failed to identify moderately resistant genotypes and were insufficiently robust across replications and repetitions (Mengistu et al., 2007). Therefore, all existing methods to quantify charcoal rot resistance in soybean produce higher than desired

levels of variation within and between trials, which complicates effort to identify and deploy effective genetic resistance in commercial cultivars.

Given the complications associated with field-based assays for charcoal rot resistance, potential alternatives include assays in controlled conditions such as greenhouses or growth chambers. To this end, Twizeyimana et al. (2012a) developed a cut-stem assay to evaluate soybean cultivars for resistance to charcoal rot in greenhouse conditions. The cut-stem assay provided quicker results and less variation between and among trials than in field assays. However, the technique still lacks consistency and reliability between trials. To date, growth chamber based assays have not been reported for charcoal rot resistance in soybean.

Seed plate assays have been developed to evaluate resistance for a variety of plant diseases (Broders et al., 2007a). For example, seed plate assays successfully identify resistance to *Pythium* spp. in alfafa (Altier and Thies, 1995), as well as *Pythium* and *Rhizoctonia* spp. on corn and soybean (Broders et al., 2007a), and are also used to evaluate fungicide seed treatment on corn seedlings to control *Fusarium* spp. (Munkvold and O'Mara, 2002). Urrea Romero (2015) identified two QTLs for resistance in soybean to *Pythium aphanidermatum* using a seed plate assay modified from Broders et al. (2007a). However, a seed plate assay for charcoal rot resistance has not yet been described in soybean or other agronomically important crop species.

The objectives of this research were to 1) create a robust seed plate assay (SPA) for charcoal rot resistance by comparing results with cut-stem and CFUI assays, and 2) correlate field-based disease assessments such as percent height of stem internal discoloration (PHSD), root and stem severity (RSS) and colony-forming units (CFUs) with SPA results on diverse soybean accessions.

MATERIALS AND METHODS

Seed plate assay development

All inoculation procedures mentioned below used the same *M. phaseolina* isolate; named Conway (collected in Arkansas and obtained from Dr. John Rupe, University of Arkansas, Fayetteville).

In the first iteration of the assay, *M. phaseolina* isolate was grown on potato dextrose agar (Difco Laboratories) in 100x15 mm petri dishes (VWR International Corp) at 28ºC for five days. Then, ten soybean seeds from two different genotypes, including susceptible Pharaoh, and resistant genotype DT97-4290 (Mengistu et al., 2007; Twizeyimana et al., 2012a), were surface-sterilized by immersing in 70% ethanol for 3 minutes. Next, seeds were poured onto a dry autoclaved paper towel to air-dry until all the alcohol had evaporated. After that, ten soybean seeds, were placed on each plate, equally spaced approximately 1 cm from the edge. Plates were covered with aluminum foil to exclude light, and incubated at room temperature 22ºC for seven days. Results were evaluated by determining the number of seeds that successfully germinated. Additionally, the procedure outlined above was performed as described, but with 0.5-cm of sterile vermiculite (Medium vermiculite, Sun Gro®, Belleve Washington, USA) placed on top of the agar before transferring soybean seeds to plates.

In the second iteration of assay development, inoculum of *M. phaseolina* was prepared in potato dextrose broth (PDB: Neogen Corporation). Flasks (250 ml) containing 50 ml of PDB were inoculated with a 5 mm agar plug of *M. phaseolina* from 5 days old cultures grown on potato dextrose agar. Flasks were shaken at 130 rpm for five days at 22ºC. Fungal tissue was then harvested by filtration through Whatman Nº 1 filter paper and subsequently dried at 45ºC for two days. Dry weight of fungal tissue was calculated as follows: Dry weight = (weight of

filter paper + mycelium) – (weight of filter paper). Dried fungal tissue was ground with a Wiley Mini-Mill (Thomas Scientific) and mixed with autoclaved vermiculite at rates of 0.01g, 0.03g, 0.05g and 0.06g per petri dish. Then, a 0.5-cm layer of infested vermiculate was placed in empty petri dishes. Ten surface-sterilized soybean seeds were placed in each petri dish, and incubated at 22ºC for seven days.

In the third and last iteration of the seed plate assay, isolate was grown on potato dextrose agar (Difco Laboratories), and maintained in an incubator at 28ºC for five days. A 3-mm plug was then transferred to the center of a 9-cm petri dish, containing 2% water agar (Agar gelidium, Moor Agar, Incorporated) and incubated for 5 days. Following fungal growth, a 0.5-cm layer of autoclaved vermiculite (Medium vermiculite, Sun Gro®, Belleve Washington, USA) was placed on top of the agar.

Sterilization and seed screening

Soybean seeds were surface-sterilized by immersing in 70% ethanol for 3 minutes, followed by air-drying on autoclaved paper towel until the alcohol evaporated. Ten soybean seeds were then evenly spread across the surface of each plate containing 2% water agar and previously inoculated with *M. phaseolina*, approximately 1 cm from the edge. Plates were covered with aluminum foil to exclude light, and incubated at room temperature 22ºC for seven days.

Laboratory experiments

Two separate experiments were conducted in the Department of Plant Pathology laboratory at the University of Arkansas, Fayetteville, AR in 2015. The experimental design was a completely randomized design with five replicates, plus a check, where each plate with 10 seeds was an experimental unit. The first experiment was conducted twice with ten surface

disinfested seeds from eight differential soybean genotypes, including the susceptible genotype LS94-3207 identified by Twizeyimana et al. (2012), LS98-0358 and Pharaoh evaluated by Twizeyimana et al. (2012) and Mengistu et al. (2007), and the resistant genotypes DT97-4290, DT98-7553, DT99-16864 and DT99-17554 also identified by Twizeyimana et al. (2012) and Mengistu et al. (2007), and DT99-17483 evaluated by Mengistu et al. (2007). The second experiment was conducted twice with nineteen different genotypes (Table 1).

Data collection (SPA)

Plates were read after seven days by scoring the number of seeds that had successfully germinated on inoculated plates compared with the number that germinated on non-inoculated plates (Figures 8 and 9). Seeds were considered successfully germinated if the radicle was >1 cm long and was not visible colonized by the pathogen (Broders et al., 2007b).

Statistical analysis (SPA)

Data for the two repeat trials of the SPA experiment were analyzed separately due to the variable effects of *M. phaseolina* infestation between the trials. Due to the ordinal rating scale that was used to evaluated pathogenicity (Broders et al., 2007b) and also in order to meet the ANOVA assumptions for normality and homogeneity of variance, ANOVA was performed on the proportion of germination using a general linear mixed model with PROC GLIMMIX (SAS version 9.4, SAS Institute Inc. Cary, NC. USA).

Field inoculum and fungal infestation

Inoculum of *M. phaseolina* (isolate Pinetree) was cultivated on sterile millet seed for 2-3 weeks at room temperature. After that, the inoculum was air dried and added to the seed packet at a rate of 0.5 g/30 cm. The planting dates were June 1, 2011 and June 1, 2012 in Rohwer, AR, and June 1, 2011, May 23, 2012, May 31, 2013 and June 17, 2014 in Stuttgart, AR.

Field plot design and treatments

Field experiments with soybean were established in 2011 and 2012 at the Southeast Research and Extension Center in Rohwer, AR, and in 2011 through 2014 at the Rice Research and Extension Center in Stuttgart, AR. The experimental design was a randomized complete block with four replications. Each plot consisted of four rows 6.09 meters long, with 0.75 meter row spacing in Stuttgart, and 0.95 meters in Rohwer (seeding rate was 200 seeds per row, using a four-row Almaco plot planter in both locations, equipped with John Deere XP row units). The research plots have been under long-term tillage management. Nineteen soybean genotypes were selected based on previous observation (Table 1).

Disease assessment (field plot experiments)

Three different methods of disease assessment were used at the growth stage R7 in this study. The first method was root stem severity (RSS) (Paris et al., 2006) where ten plants (five from each border row) were randomly selected and gently uprooted per plot and RSS scored by longitudinally splitting the stem and taproot of each plant and visually rating the intensity of discoloration as well as the microsclerotia load covering the vascular and cortical tissue. The ratings were on a scale of 1 to 5, where 1 = resistant (no discoloration), >1 to 2 = moderately resistant, > 2 to < 3 = moderately susceptible, and 3 – 5 = susceptible (highly discolored). The second method was the percent height of stem internal discoloration (PHSD) (Mengistu et al., 2007), that was also based on microsclerotial stem discoloration. PHSD was determined by the height from ground level of internal vascular discoloration and divided by the stem height X 100. The third disease assessment was based on colony forming unit (CFU) (Mengistu et al., 2007). The samples that were uprooted for the RSS and PHSD assessment were also used to determine CFUs. Samples were taken from the lower stem and root, washed and rinsed to remove soil.

They were ground, and 0.005 g per sample blended with NaOCl and collected to add on PDA plates incubated at 30º C for 3 days. CFUs were counted and genotypes classified as follows: resistant 0 to < 10, moderately resistant 10 to < 30, moderately susceptible > 30 to 60, and susceptible > 60.

Yield data

Soybean yield was determined by harvesting the two center rows of each subplot, with an Almaco Plot Combine. Harvested seeds were weighed to determine yield, and then the weight was adjusted to 13% moisture content.

Statistical analysis (field plot experiments)

Analysis of variance (ANOVA) for a randomized complete block design for combined years were performed using PROC GLIMMIX of SAS ver. 9.4 (SAS Institute, Cary, NC). In order to meet ANOVA assumptions regarding normality and homogeneity of variance, ANOVA was performed on transformed (log10) data for CFU and proportion for PHSD disease measurement. RSS and yield were not transformed. Mean comparisons were made using Fisher's least significant differences LSD (P<0.05). The PROC CORR procedure of SAS was used to compute Pearson's correlation coefficients between the mean of soybean varieties under different field disease screening methods with the mean of the same soybean varieties using seed plate assay method. Broad sense heritability by entry means (H^2) was calculated for each disease assessment by location, with the following equation (Greene et al., 2008): $H^2 = \sigma^2g / (\sigma^2g + \sigma^2gy)/y + \sigma^2error/(y*r)$, where σ^2g is the genetic variance, r is the number of replications, and y the number of years.

RESULTS

Seed plate assay experiments

Seed plate assay exploration stage. Results from the first iteration using *M. phaseolina* growing on PDA did not differed between genotypes due to abundant growth of the isolate Conway, and all of the seeds were completely colonized by the pathogen. The second iteration of the assay using one layer with different rates of infested vermiculite, results were comparable with previous research, and all seeds were colonized by the pathogen (could not distinguish charcoal rot resistant from susceptible soybean genotypes). As the quantity of pathogen inoculum influence host reaction during disease establishment, even in the lower treatment, microsclerotia germination and infectivity of *M. phaseolina* colonized all the seeds, with no distinguish between resistant and susceptible soybean genotypes. As a result, we changed the media, and started to use water agar with a layer of vermiculite to plant the seeds.

Comparing the SPA with cut-stem and CFUI assays from published data. There was a statistically significant (P =0.0012) difference among soybean genotypes for the first trial (Figure 1), and (P <0.0001) for the second trial (Figure 2), indicating different levels of resistance to *M. phaseolina* infection, as mentioned previously by Twizeyimana et al. (2012) and Mengistu et al. (2007). In addition, there was a statistically significant interaction between trials (P=0.007), showing some degree of variation between three genotypes, with different levels of resistance for DT97-4290 (R) and susceptibility for Pharaoh and LS98-0358 (S).

Evaluating resistance to *M. phaseolina* in soybean genotypes. Analysis of variance indicated that there was a statistically significant difference (P <0.0001) among soybean genotypes for both trials (Figures 3 and 4), indicating different levels of resistance to *M. phaseolina* infection. There was a statistically significant interaction between trials (P <0.0001),

showing some degree of variation to resistance to *M. phaseolina* among genotypes, such as Pharaoh, K07 1544, NK BRAND539-A3, LS98-0358, and DT97-4290.

Field data experiments

Location Rohwer. Analysis of variance for CFUs indicated that there was a statistically significant difference (P=0.03) within maturity groups (Figure 5), and among soybean genotypes (P=0.0009) (Table 2). Data for CFUs were not collected for the year of 2012. There was no statistically significant difference within maturity groups and among soybean genotypes for PHSD. The RSS screening method showed no statistically significant difference within maturity groups, but there was a statistically significant difference among soybean genotypes (P=0.01) (Table 3). Yield was statistically significant different (P=0.01) within maturity groups (Figure 6), and not statistically significant different among soybean genotypes. The correlation between the PHSD and SPA was not statistically significant for the year of 2011, however was statistically significant for the year of 2012 (Table 5). In the same way, correlation between RSS and SPA was only statistically significant for the year 2012 (Table 5). No significant correlation was found for CFUs and SPA in 2011 and data was not collected in 2012. Correlation between yield and SPA was statistically significant for both years (Table 5). The broad-sense heritability for CFU was 0.69, PHSD 0.60, RSS 0.83 and yield 0.45.

Location Stuttgart. Analysis of variance for the PHSD screening method indicated that there was no statistically significant difference within maturity groups, however there was a statistically significant difference (P=0.0008) among soybean genotypes (Table 4). The RSS method presented a statistically significant difference (P=0.04) within maturity groups (Figure 7), and (P=0.008) among soybean genotypes (Table 4). The CFU screening method showed no statistically significant difference within maturity groups, however there was a statistically

significant difference (P=0.01) among soybean genotypes (Table 4). Yield presented no statistically significant difference within maturity group and among soybean genotypes. The correlation between PHSD and SPA was statistically significant and most consistent across years from 2011 to 2013 (Table 6). Correlation between RSS and SPA was not statistically significant across years from 2011 to 2014 (Table 6). Correlation between CFUs and SPA was only significant in 2012 (Table 6), and yield and SPA were statistically significant correlated across the years of 2011, 2013 and 2014 (Table 6). The broad-sense heritability for CFU was 0.61, PHSD 0.75, RSS 0.64 and yield 0.37.

DISCUSSION

SPA versus cut-stem and CFUI assays

Germination results from the SPA were in agreement with the cut-stem and CFUI assays, for the same eight genotypes evaluated in the field and greenhouse. Germination of resistant lines ranged from 58% to 84% and susceptible lines from 46% to 54%. None of the genotypes evaluated with the SPA were completely resistant to *M. phaseolina*. However, data from the SPA distinguished genotypes based on differing degrees of resistance and susceptibility, as previously reported (Mengistu et al., 2007; Twizeyimana et al., 2012a). Results from the SPA confirmed that genotypes DT97-4290, DT98-7553, DT99-16864, DT99-17554, and DT99-17483 have high levels of resistance to *M. phaseolina* as confirmed previously (Mengistu et al., 2007; Twizeyimana et al., 2012a). However, the genotype DT97-4290, rated as moderately resistant (Paris et al., 2006; Mengistu et al., 2007; Twizeyimana et al., 2012a), presented a significant degree of variation for resistance (Figures 1 and 2) between experimental trials. In a similar manner, the genotype LS98-0358 and Pharaoh, rated as susceptible according to Mengistu et al. (2007), working with colony-forming unit index (CFUI), presented a significant degree of

variation for susceptibility between trials (Figures 1 and 2). Similarly, Mengistu et al. (2007) reported a significant degree of variation, between moderately susceptible and susceptible for the genotypes LS98-0358 and Pharaoh, among different disease assessment methods, including root and stem severity (RSS), percent height of stem discoloration (PHSD), and foliar symptoms (FS).

The SPA method to assess the disease response, therefore presented a comparable genotype ranking to the other assays, such as CFUI ratings and the cut-stem inoculation technique.

Furthermore, the SPA can shorten the cycle for disease assessment in comparison to others assays, such as cut-stem that takes 8 weeks for disease evaluation (Twizeyimana et al., 2012a), and much longer with PHSD, RSS and CFUI methods that rely on a complete field test (Mengistu et al., 2007). In addition, all field methods of screening are based on the degree of colonization at the end of the season, without assessing how and at which stage plants are infected by the pathogen. In contrast, each cycle of evaluation using SPA can be accomplished in about 8 days, 7 days for seed germination and 1 day for disease evaluation, which makes the method less sensitive to environmental changes.

SPA evaluating of resistance to *M. phaseolina* in soybean genotypes

Except for the genotypes DT97-4290 (moderately resistance), Pharaoh (susceptible), LS98-0358 (susceptible), K07-1544 and NKBrand539-A3, the results from the repeated experiments in this study showed consistent levels of resistance to *M. phaseolina* (Figures 3 and 4). In fact, ten genotypes showed similar ranking of resistance between trials, with a clear difference between susceptible and resistant genotypes.

As described in the first experiment with 8 lines, the genotypes DT97-4290, LS98-0358 and Pharaoh also indicated a significant degree of variation in resistance to *M. phaseolina*.

Correlating of field data with SPA

The purpose of correlating different field disease assessment methods, such as PHSD, RSS and CFUS with SPA was to validate the assay by demonstrating its accuracy and reproducibility. When genotypes are evaluated in the field for disease resistance, a natural environment is more unpredictable to the development of the disease. A synergistic interaction between the pathogen and other microorganisms may also increase disease severity. Greenhouse and laboratory disease assessments are more efficient than field assays due to better environmental control such as temperature, moisture, and photoperiod. Moreover, genotypes can be evaluated all year long using precise amounts of inoculum. An important question for a new disease assessment method like the SPA is how well it correlates with published methods.

Rohwer. Disease pressure for PHSD and RSS was higher in 2011 when compared to 2012, indicating that the environmental conditions were favorable for the pathogen, despite having the same planting date of June 1 for both years. However, correlations between PHSD and RSS were not statistically significant with SPA for the year of 2011, instead correlation was statistically significant in 2012 when disease incidence was lower (Table 5). This may indicate that the SPA can even estimate disease severity in the field with lower levels of disease incidence. Yield shows higher correlation (Table 5) in 2012, when the disease less impacted yield loss. Furthermore, the statistically significant correlation across years between yield and SPA demonstrates the accuracy of SPA compared to PHSD, RSS and CFU within different years.

Cultivar response among plant maturity groups was statistically significant for CFU in 2011, showing higher levels of disease colonization rate for lately maturity groups, such as IV and V and lower for MG III and Late III (Figure 5). In contrast to Pearson et al. (1984) working with a range of MG III to MG V, concluded that later maturity cultivars have an advantage if host development is not considered as a covariate in assessments of resistance. Likewise, Mengistu et al. (2013) and Twizeyimana et al. (2012b) pointed out that differences in plant maturity may affect disease evaluation results. However, for yield the disease did not affect MG IV and V (Figure 6).

Stuttgart. Disease incidence varied by year and method used, with different cultivar responses for resistance and susceptibility among years for PHSD, RSS and CFUs (Table 4). Plant maturity presented higher levels of disease colonization for the late MGs, such as Late IV, V and Early IV. However, MG II also presented higher levels of disease colonization. PHSD values showed higher correlation with the SPA in 2012 and 2013 (Table 6), where levels of disease were higher compared with 2011 and 2014, in contrast to the correlation between SPA and CFUs in the year of 2012, when the disease pressure was not too high. Besides that, as showed in the data from Rohwer (Table 6) the correlation between SPA and yield (Table 7) was statistically significant among years, demonstrating that SPA is a comparable and reliable method to assess disease.

In conclusion, our results showed the SPA screening to be a reliable, consistent, and effective method for evaluating soybean genotypes for resistance to charcoal rot. The SPA screening technique could be further improved by using a growth chamber with different sets of temperature, because room temperature may favor soybean seed rather than pathogen growth. This study has also formed a foundation for a simplified and faster disease assessment that may

help researchers measure large numbers of breeding lines for resistance to charcoal rot. However, similar to other assays, SPA cannot predict field performance of soybean genotypes under stress conditions.

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Table 1. Genotypes used to assess disease resistance to *M. phaseolina.*

Cultivar	Maturity group
Jack	II
K07-1544	III
NK 539-A3	III
Exp1_Stine39LA02	late III
Exp2_XC3810	late III
Spencer	early IV
DT97-4290	IV
DK4866	IV
JTN-4307	IV
Pharaoh	late IV
LS98-0358	late IV
RO1581F	V
CPL_RC5663	V
CPL_RC5007	V
JTN-5208	V
JTN-5308	V
MorSoy RT5388N	V
Osage	V
Hutcheson	V

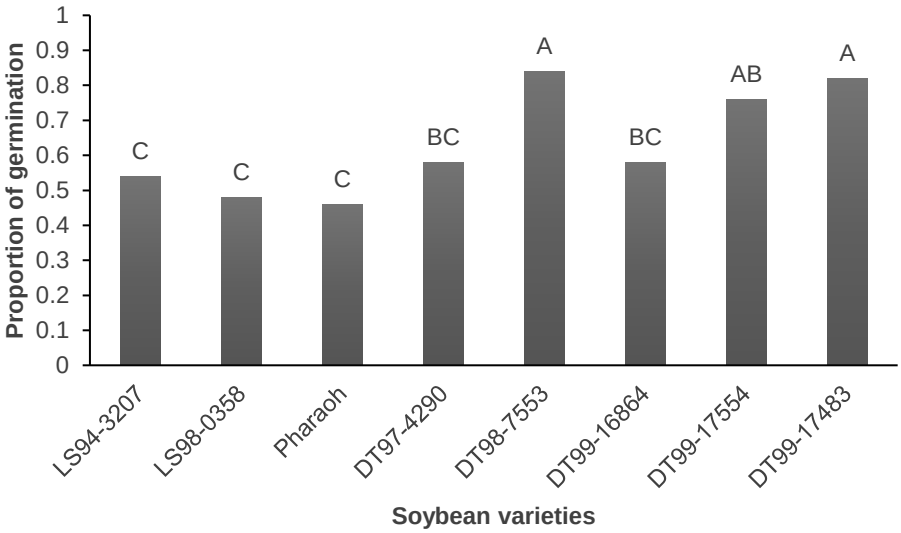

Figure 1. Seed plate assay first experiment means for the first trial. Means with the same letter are not significantly different according to the LSD test, $\alpha = 0.05$.

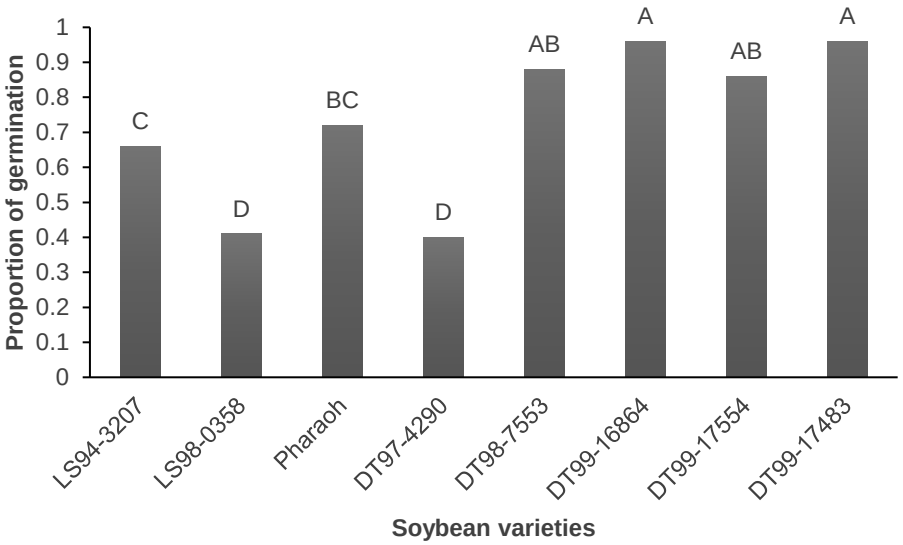

Figure 2. Seed plate assay first experiment means for the second trial. Means with the same letter are not significantly different according to the LSD test, $\alpha = 0.05$.

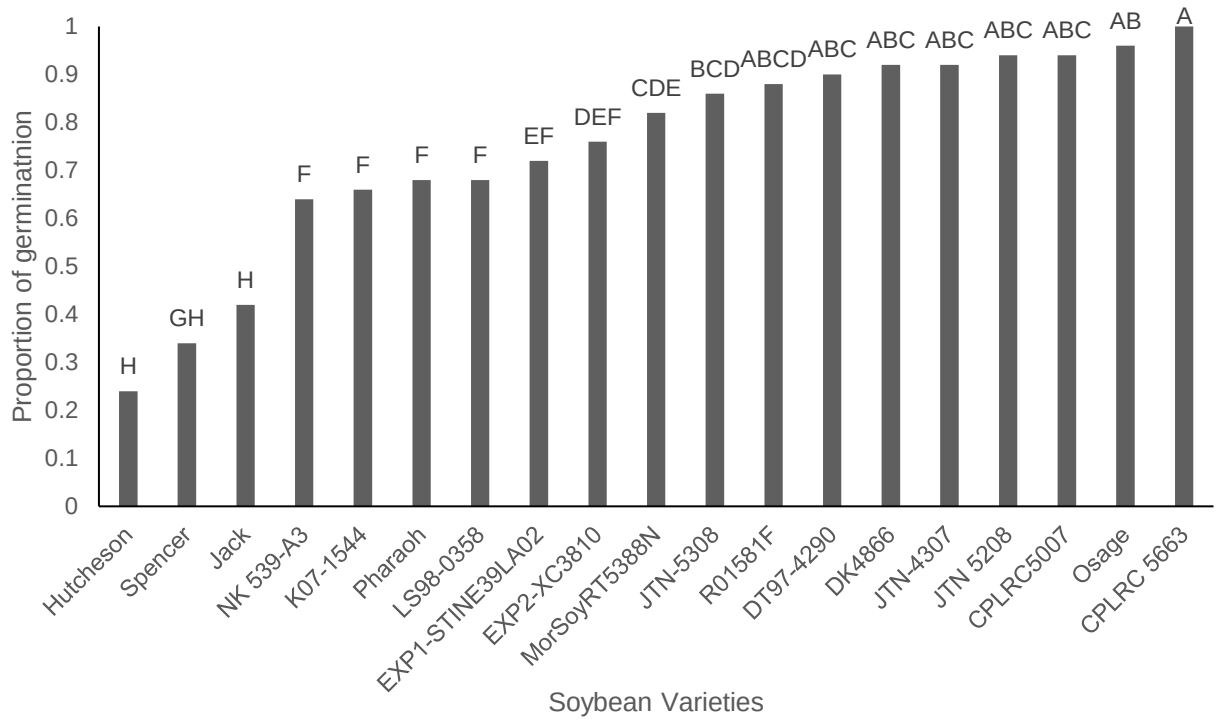

Figure 3. Seed plate assay second experiment means for the first trial. Means with the same letter are not significantly different according to the LSD test, $\alpha = 0.05$.

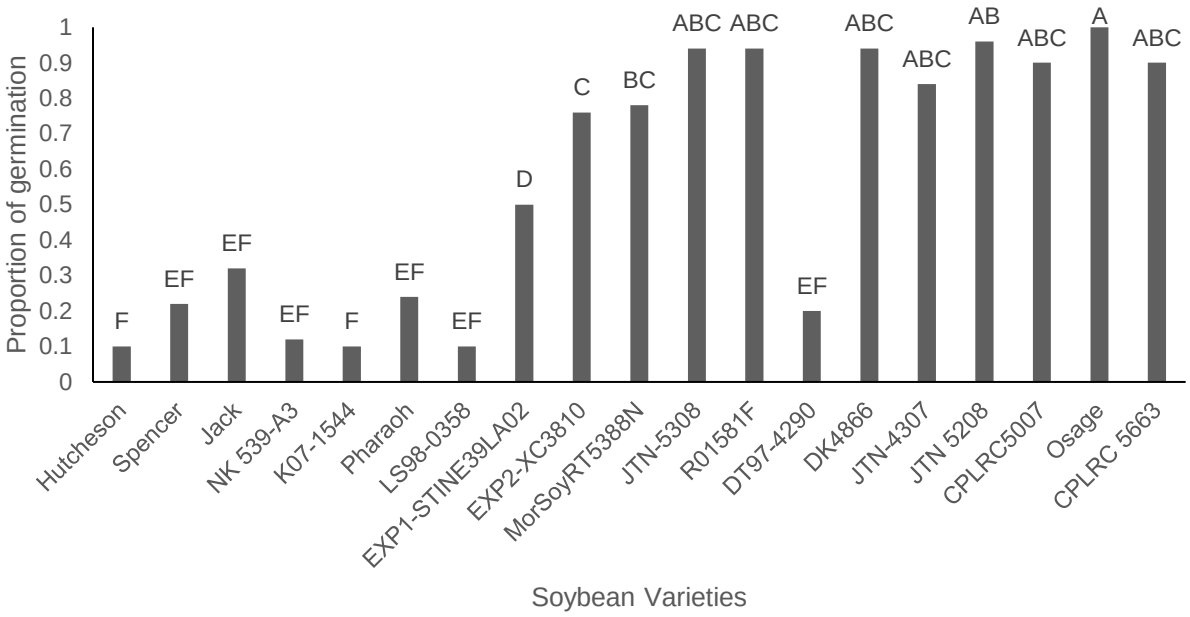

Figure 4. Seed plate assay second experiment means for the second trial. Means with the same letter are not significantly different according to the LSD test, $\alpha = 0.05$.

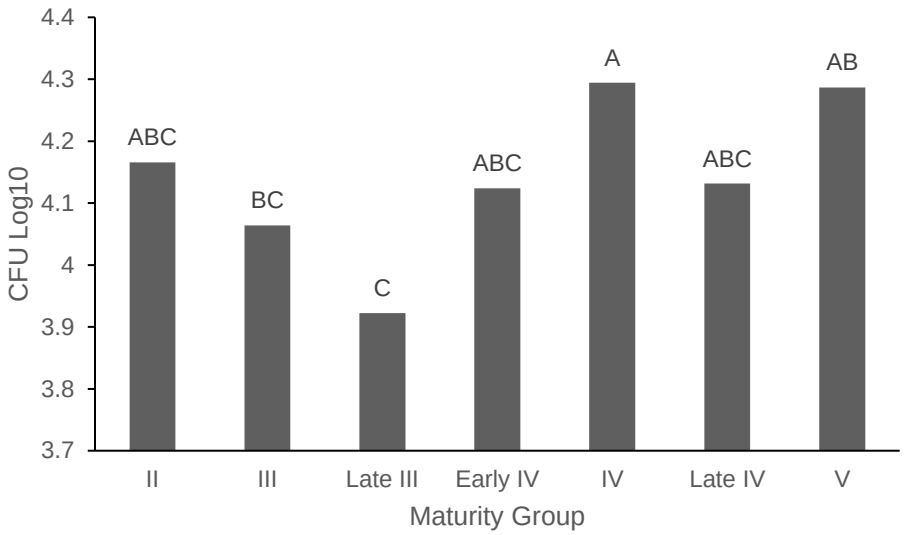

Figure 5. Colony forming unit (CFU) means Log10 for maturity group in Rohwer 2011. Means with the same letter are not significantly different according to the LSD test, $\alpha = 0.05$.

Table 2. Colony forming unit (CFU) means in Log10 scaled values of 14 soybean genotypes measured at Rohwer in 2011. Means with the same letter are no significantly different according to the LSD test, $\alpha = 0.05$.

Cultivar	MG	CFU log10
Jack	II	4.16ABC
K07-1544	III	3.76E
NK539-A3	III	4.35ABC
Exp1_Stine39LA02	Late III	4.03CDE
Exp2_XC3810	Late III	3.81ED
Spencer	Early IV	4.12BCD
DK4866	IV	4.27ABC
JTN-4307	IV	4.17ABC
DT97-4290	IV	4.42AB
LS98-0358	Late IV	4.36ABC
Pharoah	Late IV	3.89CDE
RO1581F	V	4.51A
CPL_RC5007	V	4.49A
MorSoyRT5388N	V	3.85CDE

Table 3. Measures of the intensity of root and stem severity (RSS) of 14 soybean genotypes measured at Rohwer in 2011/2012. Means with the same letter are no significantly different according to the LSD test, $\alpha = 0.05$.

Cultivar	MG	RSS
Jack	II	2.91ABC
Exp1_Stine39LA02	III	2.57CD
NK539-A3	III	2.92ABC
Exp2_XC3810	III	3.04ABC
K07-1544	III	3.33AB
Spencer	Early IV	3.41A
DK4866	IV	2.98ABC
DT97-4290	IV	2.79ABC
JTN-4307	IV	1.9D
LS98-0358	Late IV	3.11ABC
Pharaoh	Late IV	2.68CB
CPL_RC5007	V	2.83ABC
MorSoyRT5388N	V	2.69ABC
RO1581F	V	2.61C

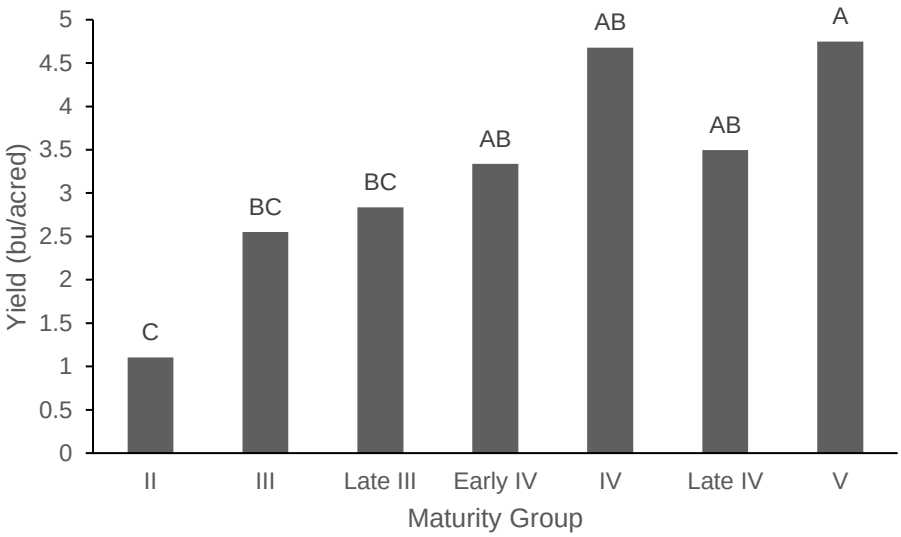

Figure 6. Yield means for different maturity groups measured in Rohwer during 2011/2012. Means with the same letter are not significantly different according to the LSD test, $\alpha = 0.05$.

Table 4. *M. phaseolina* disease assessment methods evaluated in Stuttgart between 2011/2014. Means with the same letter are not significantly different according to the LSD test, $\alpha = 0.05$.

Cultivar	MG	PHSD[a]	RSS[b]	CFU log10[c]
Jack	II	0.13BC	3.93AB	4.49AB
NK539-A3	III	0.20ABC	3.70ABCDEFG	4.63A
K07-1544	III	0.21ABC	3.37EFG	4.52AB
Exp1_Stine39LA02	Late III	0.103C	3.48CDEFG	4.55AB
Exp2_XC3810	Late III	0.17BC	3.59BCDEFG	4.53AB
Spencer	Early IV	0.19ABC	3.71ABCDEFG	4.47ABC
DT97-4290	IV	0.15BC	3.33G	4.33BCD
JTN-4307	IV	0.15BC	3.40DEFG	4.43ABC
DK4866	IV	0.17BC	3.81ABC	4.46ABC
LS98-0358	Late IV	0.19BC	4.03A	4.57AB
Pharaoh	Late IV	0.21ABC	3.79ABCD	4.48ABC
RO1581F	V	0.33A	4.00A	4.52AB
Osage	V	0.25AB	3.66ABCDEFG	4.32BCD
MorSoy RT5388N	V	0.23ABC	3.76ABCDE	4.20DC
CPL_RC5007	V	0.20ABC	3.45CDEFG	4.35ABC
CPL_RC5663	V	0.20ABC	3.75ABCDEF	4.31BCD
JTN-5308	V	0.16BC	3.37FG	4.11D
JTN-5208	V	0.14BC	3.34G	4.21DC

[a] Percent height of stem internal discoloration means in proportion
[b] Root and stem severity
[c] Colony forming unit means in Log10 values

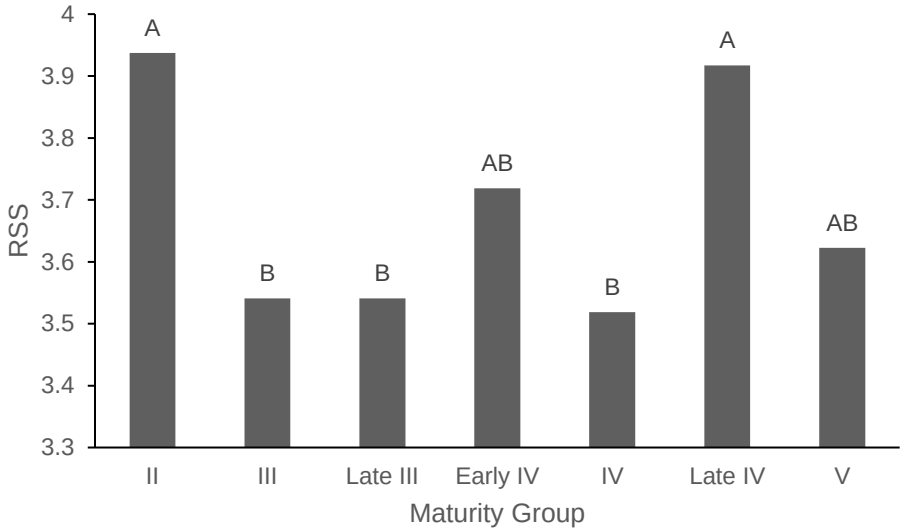

Figure 7. Measures of the intensity of internal discoloration (RSS) for different maturity groups in Stuttgart from 2011/2014. Means with the same letter are not significantly different according to the LSD test, $\alpha = 0.05$.

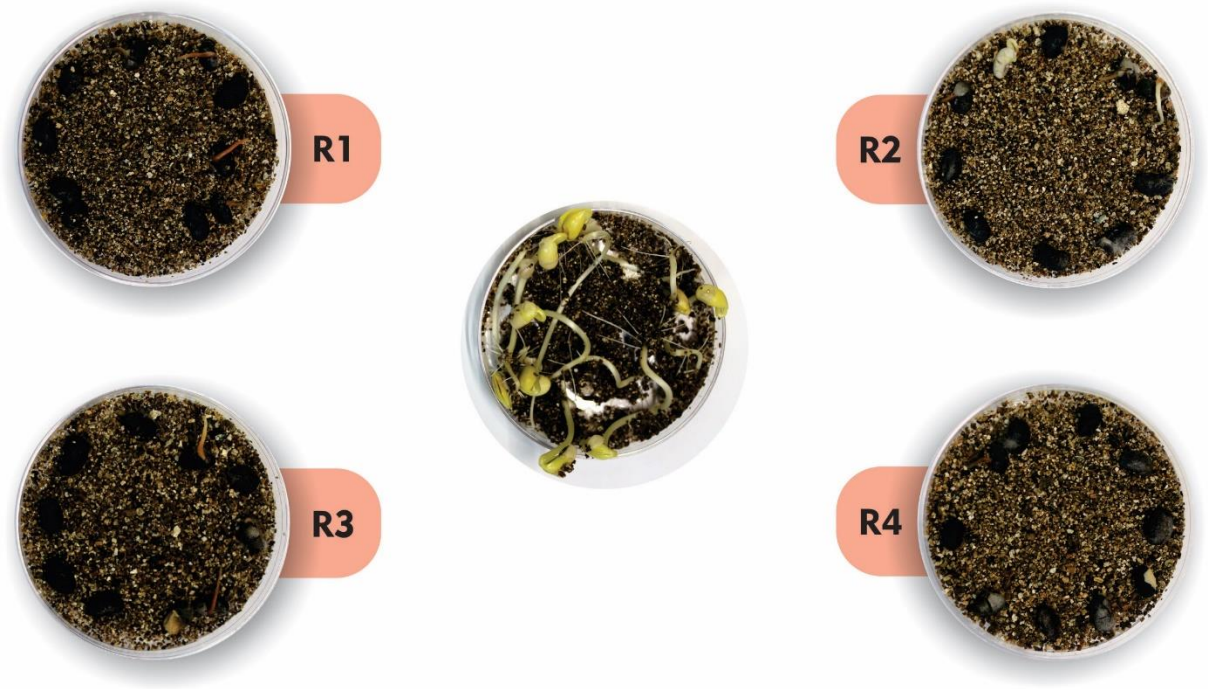

Figure 8. Seed plate assay (SPA) disease assessment showing results for the susceptible genotype LS94-3207, and non-inoculated plate.

Figure 9. Seed plate assay (SPA) disease assessment showing results for the resistant genotype DT99-17483, and non-inoculated plate.

Table 5. Correlation matrix of three *M. phaseolina* disease assessment methods and yield with Seed plate assay (SPA), evaluated in 2011 and 2012.

			Parameters				
Location	**Year**		**YIELD**	**PHSD**	**RSS**	**CFUS**	**SPA**
ROHWER	**2011**	**PHSD[a]**	-0.04NS	1	0.52NS	0.10NS	-0.45NS
		RSS[b]	-0.20NS	0.52NS	1	-0.05NS	-0.32NS
		CFUS[c]	0.10NS	0.10NS	-0.05NS	1	0.24NS
		YIELD[d]	1.00	-0.04NS	-0.20NS	0.10NS	0.65*
		SPA[e]	0.65*	0.45NS	-0.32NS	0.24NS	1
ROHWER	**2012**	**PHSD[a]**	-0.39NS	1	0.69**	ND	-0.69**
		RSS[b]	-0.06NS	0.69**	1	ND	-0.60*
		CFUS[c]	ND	ND	ND	ND	ND
		YIELD[d]	1.00	-0.39NS	-0.06NS	ND	0.70***
		SPA[e]	0.70***	-0.69*	-0.60*	ND	1

[a] Percent height of stem internal discoloration
[b] Root and stem severity
[c] Colony-forming unit
[e] Seed plate assay
* Significant at the 0.05 probability level
** Significant at the 0.01 probability level
*** Significant at the 0.001 probability level
ND no data for CFUS in 2012
NS, not significant (*P>0.05*)

Table 6. Correlation matrix of three *M. phaseolina* disease assessment methods and yield with Seed plate assay (SPA), evaluated in 2011 to 2014.

			Parameters				
Location	**Year**		**YIELD**	**PHSD**	**RSS**	**CFUS**	**SPA**
STU	**2011**	**PHSD[a]**	-0.27NS	1	0.47NS*	0.75***	-0.56**
		RSS[b]	-0.17NS	0.47*	1	0.61*	-0.12NS
		CFUS[c]	-0.22NS	0.75***	0.61*	1	-0.31NS
		YIELD[d]	1.00	-0.27NS	-0.17NS	-0.22NS	0.65***
		SPA[e]	0.65***	-0.56**	-0.12NS	-0.31NS	1
STU	**2012**	**PHSD[a]**	0.36NS	1	0.46*	0.67**	-0.59**
		RSS[b]	-0.08NS	0.46*	1	0.34NS	-0.44NS
		CFUS[c]	0.31NS	0.67**	0.34NS	1	-0.64**
		YIELD[d]	1.00	0.36NS	-0.08NS	0.31NS	-0.09NS
		SPA[e]	-0.09NS	-0.59**	-0.44NS	-0.64**	1
STU	**2013**	**PHSD[a]**	0.50*	1	-0.02NS	0.41NS	0.79***
		RSS[b]	0.11NS	0.02NS	1	0.61*	-0.17NS
		CFUS[c]	-0.40NS	-0.41NS	0.61*	1	-0.40NS
		YIELD[d]	1.00	0.50*	0.11NS	-0.40NS	0.48*
		SPA[e]	0.48*	0.79***	-0.17NS	-0.40NS	1
STU	**2014**	**PHSD[a]**	0.31NS	1	0.60*	0.59*	0.52NS
		RSS[b]	0.05NS	0.60*	1	0.79***	-0.08NS
		CFUS[c]	-0.16NS	0.59*	0.79***	1	0.12NS
		YIELD[d]	1.00	0.31NS	0.05NS	-0.16NS	0.57**
		SPA[e]	0.57**	0.52NS	-0.08NS	0.12NS	1

[a] Percent height of stem internal discoloration
[b] Root and stem severity
[c] Colony-forming unit
[e] Seed plate assay
* Significant at the 0.05 probability level
** Significant at the 0.01 probability level
*** Significant at the 0.001 probability level
NS, not significant (*P>0.05*)

CHAPTER 3

QTL Mapping of Charcoal Rot Resistance in PI 567562A Soybean Accession

M. P. da Silva, Andy Pereira, Burt H. Bluhm, Richard E. Mason, Lisa Wood, Pengyin Chen, John C. Rupe, Leandro A. Mozzoni*

M. P. da Silva, Andy Pereira, Richard E. Mason, Leandro A. Mozzoni and Lisa Wood, Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR 72701; John C. Rupe and Burt H. Bluhm, Department of Plant Pathology, University of Arkansas, Fayetteville, AR 72701. Pengyin Chen, Fisher Delta Research Center, University of Missouri, Portageville, MO.

*Author for correspondence: Leandro A Mozzoni (lmozzon@uark.edu)

Abbreviations: ANOVA, analysis of variance; LG, linkage group; LOD, logarithm of odds; MAS, marker-assisted selection; MQM, multiple-QTL modeling; PI, plant introduction; SNP, single nucleotide polymorphism; QTL, quantitative trait locus.

ABSTRACT

Charcoal rot of soybean (*Glycine max* (L.) Merr.), caused by the soilborne fungus *Macrophomina phaseolina* (Tassi) Goid., has ranked among the most important soybean diseases in the United States. Disease management is typically conducted in a multi-faceted approach through crop rotation, tillage, irrigation, and seed treatments aimed at minimizing damage caused by the pathogen. Development of genetic resistance to charcoal rot appears to be the most efficient strategy to control the disease; however, there are no reports of genetic regions associated with tolerance or resistance to the disease. The objective of this study was to identify quantitative trait loci (QTL) governing resistance to charcoal rot in soybean using a bi-parental population of PI 567562A (R, resistant) × PI 567437 (S, susceptible). A total of 140 F_2-derived lines were genotyped with 5403 single nucleotide polymorphism (SNP) markers covering 20 chromosomes, of which 2283 were polymorphic. Resistance to charcoal rot was evaluated in the $F_{2:3}$ lines using the cut-stem inoculation technique under greenhouse conditions. QTL mapping analysis indicated one major QTL for resistance to *M. phaseolina* on Chr. 15 explaining 29.4% of phenotypic variation, and two minor QTL on Chr. 16 explaining 25.4% and 8.4% of phenotypic variation. To our knowledge, this is the first report of genomic regions harboring resistance to charcoal rot in soybean, and may facilitate breeding and molecular engineering progress to combat charcoal rot disease in the future.

INTRODUCTION

Charcoal rot of soybean (*Glycine max* (L.) Merr.) is caused by the soilborne plant pathogen *Macrophomina phaseolina* (Tassi) Goid (Goidanish, 1947), and was first observed in the United States in 1949 (Young, 1949). The disease has been reported throughout the country ever since (Wyllie and Scott, 1988), causing significant yield losses that have been estimated at 1.9 and 2.0 million metric tons in 2003 and 2012, respectively (Wrather et al., 2010; Luna et al., 2017). Infection of *M. phaseolina* is favored by hot and dry conditions, and symptoms, characterized by stunted growth, leaf chlorosis, premature yellowing and early maturation, or incomplete pod filling, could be expressed at any soybean physiological stage (Hartman et al., 1999; Gupta et al., 2012).

An integrated management approach including crop rotation, tillage, irrigation, and seed treatments is used to minimize charcoal rot damage in soybeans, although, none of these practices have been sufficient for controlling the disease (Mengistu et al., 2007; Twizeyimana et al., 2012a). Although moderately resistant cultivars are currently commercially available, significant progress has been made to identify soybean germplasm resistant to charcoal rot. Among 698 screened soybean accessions lines, 13 were identified with higher levels of resistance to *M. phaseolina* than the standard released germplasm, DT97-290 (Mengistu et al. 2007, 2013; Pawlowski et al. 2015). As a result, efforts have focused on the development of genetic resistance to charcoal rot, including reliable and efficient procedures to characterize resistance, and most important, the development of resistant cultivars (Mengistu et al., 2007; Pawlowski et al., 2015; Luna et al., 2017).

Although genetic sources of host resistance to charcoal rot may be the best approach to control the disease, very little is known about genomic regions and molecular makers linked to the charcoal rot resistance gene(s) and more research is needed to understand the inheritance of

charcoal rot resistance (Mengistu et al., 2007; Gupta et al., 2012). The specific question remains: is resistance controlled by one or a few major genes with high heritability or as multiple genes with low heritability? It was reported by Talukdar et al. (2009) that the disease reaction shows a continuous distribution, ranging from highly susceptible through moderately resistant, to highly resistant. This, therefore, suggests that disease resistance is influenced by multiple loci. Correspondingly, two dominant genes with double-recessive epistasis were reported for resistance in common bean ((Hernandez-Delgado et al., 2009).

The objective of this study was to identify QTL associated with resistance to charcoal rot in the resistant soybean plant introduction (PI) 567562A using a biparental population, and identify molecular markers that could be used for marker-assisted selection (MAS) to facilitate incorporation of genetic resistance to charcoal rot in to breeding programs. Towards this goal, the population was genotyped using the Illumina 6K Infinium BeadChip, for genetic mapping of the disease resistance trait.

MATERIALS AND METHODS

Plant Material

A population derived from a cross between resistant PI 567562A (MG IV) and susceptible PI 567437 (MG IV) was used to map QTL associated with resistance to *M. phaseolina*. Parent PI 567562A was selected based on Mengistu et al. (2013) identification as resistant to charcoal rot. The cross was made in 2014 at La Uruca (San Jose, Costa Rica). The F_1 seeds were grown at the Rosen Alternative Pest Control Center, University of Arkansas. A total of 140 F_2 single plants were pulled and single-plant threshed to form the $F_{2:3}$ recombinant inbred lines for mapping study.

Phenotypic Evaluation and Statistical Analysis

Phenotypic evaluation was conducted in the Rosen Center greenhouse at the University of Arkansas in Fayetteville, AR, between September and November of 2016 using the cut-stem inoculation technique (Twizeyimana et al., 2012a). Inoculation was performed using the *M. phaseolina* isolate Conway collected in Arkansas. Prior to inoculation, the isolate was grown on potato dextrose agar (Difco Laboratories) in an incubator at 28ºC for five days. Fungal inoculum was transferred by pipette tips to V2-stage (Fehr et al., 1971) soybean plants, and removed three days after inoculation. Disease severity was assessed based on the linear extent of stem necrosis (mm) after twelve days post inoculation.

The 140 $F_{2:3}$ lines were divided into three groups of 1680 plants, where 12 plants were replicated 3 times per line. Each replication was evaluated in an incomplete block design, and consisted of 36 plants per $F_{2:3}$ line with parental genotypes used as checks in each individual assay. Each line was planted in 48-pot plastic inserts (number 1204; Hummert International) filled with autoclaved soil-less mix (Sunshine Mix, LC1; Sun Gro Horticulture Inc.), placed inside a flat tray with drainage holes (number T1020; Hummert International), and fertilized at planting with slow-release pellets (Osmocote 19-6-12; 1 to 2 pellets/cm2). The greenhouse temperature was maintained at 28 ± 2 ºC day/night temperature regime, 60/65% day night relative humidity, with 14 h photoperiod.

The stem necrosis lesions (mm), caused by *M. phaseolina* infection, were analyzed using the PROC GLIMMIX (SAS version 9.4, SAS Institute Inc. Cary, NC. USA), and PROC UNIVARIATE was used to test the normality of the disease stem necrosis distribution for CR lines. Broad sense heritability (H^2) of disease response was calculated with the following equation (Greene et al., 2008): $H^2 = \sigma^2g/ \sigma^2g+ \sigma^2e/r$, where σ^2g is the genetic variance, σ^2_e is the error, and

r is the number or replications. Analysis of variance was used to determine phenotypic differences between parents PI 567562A and PI 567437, and the derived lines.

Genotyping and Linkage Map Construction

Genomic DNA was extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Single nucleotide polymorphism (SNP) genotyping was performed using the Illumina Infinium® Genotyping HD BeadChip (6K SNPs) on Illumina iScan (Illumina, San Diego, CA) at the genotyping core facility of Michigan State University, East Lansing, MI. Each 4 µl sample with >200 ng/µl genomic DNA was used for SNP analysis. Intensities of the bead fluorescence were detected using the Illumina iScanTM Reader and the allele call for each SNP locus were performed using llumina's BeadStudioTM software 28 (Illumina, San Diego, CA, v3.2.23). Linkage map was constructed using JoinMap 4.0 (Van and J.W., 2006) and the threshold for logarithm of odds (LOD) for linkage group construction was set as 3.0. Regression mapping of each chromosome/linkage group (LG) was performed with a Kosambi mapping function (Kosambi, 1944).

QTL Analysis

Quantitative trait loci mapping was performed using MapQTL 5.0 (Van, 2004). Permutation tests were conducted in analyzed lines for 1000 times, and initial LOD threshold of 3.0 was used under type I error 0.05. Interval mapping at 1-cM intervals along the chromosomes was used to detect QTL based on the LOD threshold. Markers closely linked to positions with the highest LOD scores were taken as cofactors for multiple-QTL modeling (MQM) analysis. Graphical presentation of the QTL were drawn using MapChart 2.30 (Voorrips, 2002). Selection of candidate genes for each detected QTL interval was annotated in Glyma 2 assembly

(Wm82.a2.v1) of 'Williams 82' gene models in SoyBase (www.soybase.org). Further predictions were based on the genes of known function in soybean related to plant disease resistance.

RESULTS

Phenotypic Variation of Parental Lines and $F_{2:3}$ Population

A significant (P<0.0001) phenotypic difference between the two parents was detected after screening against the *M. phaseolina* (Table 1). The resistant PI 567562A averaged 26 mm lesions, whereas susceptible PI 567437 showed 57 mm lesions. Analysis of variance (ANOVA) indicated that there was a significant (P <0.0001) genotype variation among the $F_{2:3}$ lines derived from PI 567562A x PI 567437 (Table 2), indicating different levels of resistance to *M. phaseolina* infection. All soybean lines developed stem necrosis, ranging from 22 mm to 59 mm (Table 2, Fig. 1). The normality test using the Kurtosis statistical numerical method showed that the resistance for *M. phaseolina* was normally distributed (Figure 1). The broad-sense heritability (H^2) was estimated to be 0.45.

Quantitative Trait Loci Identification

QTL conferring resistance to *M. phaseolina* were detected using 6K SNPChip genotyping. A total of 2,283 SNP (38%) were polymorphic, and allowed to generate a linkage map with an average coverage of 0.98 cM per marker (Table 3). Three QTL conferring resistance to *M. phaseolina* were identified using MQM mapping analysis: one major QTL on chromosome (Chr.) 15 (LG E) and two minors on QTL on Chr. 16 (LG J) (Table 4). The major QTL on chromosome 15 was mapped within a 1,209 kb confidence interval between SNPs Gm15_01842053 and Gm15_03051337, with a peak closer to Gm15_03051337. The confidence interval of Gm15:01842053-03051337 contained 155 candidate genes as shown in SoyBase

(www.soybase.org). This QTL displayed a LOD score of 5.25, and explained 29.4 % of total phenotypic variance (R^2) and had an additive effect of -7.7 mm (Table 4, Figure 2).

Two minors QTL were mapped on Chr. 16. The first one located in a 1,533 kb interval positioned between SNPs Gm16_28961127 and Gm16_30493887, with the peak appearing close to Gm16_30493887. The LOD value was 4.32 and the QTL explained 25.4% of phenotypic variation with an additive effect of -8.8 mm (Table 4, Figure 2). The second QTL on Chr. 16 was mapped into a 1,105 kb region between Gm16_35973543 and Gm16_37078478. The QTL peak was located at the Gm16_36809255 marker. The LOD peak showed value of 3.6, and explained 8.84% of the phenotypic variation, and the additive effect was -4.8 (Table 4, Figure 2). The confidence intervals of these QTL Gm16:28961127-30493887 and Gm16:35973543-37078478 included 114 and 138 genes, respectively, as shown in SoyBase (www.soybase.org). The resistance alleles for all QTL were contributed by PI 567562A (Table 3).

DISCUSSION

No complete or vertical resistance to charcoal rot (also known as Charcoal Rot Drought Complex) has been identified to date. However, several cultivars were reported as possessing partial or horizontal resistance (Smith and Carvil, 1997a). Soybean accession PI 567562A (MG IV) was collected from Shandong province in China and it was classified as resistant to charcoal rot among 628 evaluated accessions (Mengistu et al., 2013).

In this research, some of the lines exhibited higher levels of resistance than the resistant parent (PI 567562A), and also higher levels of susceptibility when compared to the susceptible parent (PI 567437). Lower broad sense heritability (H^2 = 0.45) values indicated that disease resistance to charcoal rot is greatly influenced by environmental factors, making selection less effective for breeding purposes.

In this study three QTL were detected in PI 567562A: one on Chr. 15 with a confidence interval of Gm15:01842053-03051337, and two on Chr. 16 with confidence intervals of Gm16:28961127-30493887 and Gm16:35973543-37078478, respectively**.** Each of these intervals contained over one hundred candidate genes that included transcriptional activation, signal transduction and defense-related genes. Determining which of these genes was responsible for resistance is difficult due to our lack of knowledge about molecular mechanisms of *M. phaseolina* infection. Further work will be needed to closely analyze these genomic regions, and determine the physiological and molecular mechanisms determining the resistance response. Although there were no reports on QTL conditioning charcoal rot resistance in soybean, several reports describe mapping in common bean (Hernandez-Delgado et al., 2009), sesame (Wang et al., 2017), cowpea (Muchero et al., 2011), and sorghum (Adeyanju et al., 2015; Kumar et al., 2017). However, no relationship/synteny between these QTL is known as yet.

Charcoal rot QTL on Chr. 15 is located downstream of the *Fusarium graminearum* resistance QTL mapped close to BARC-042629-08331 (Gm15:01276087) in soybean cultivar Conrad (Ellis et al., 2012), and Soybean Mosaic Virus (SMV) strain SC7 resistance mapped close to BARC-018959-03045 (Gm15:01303462) in Kefeng No.1 (Yan et al., 2015). The same QTL partially overlaps with the resistance QTL to *Sclerotinia sclerotiorum* which causes Sclerotinia stem rot, mapped between Satt411 and Satt369 (Gm15:02517404-49011508) in PI 391589B (Guo et al., 2008).

The region on Chr. 16 between the two newly-identified QTLs for charcoal rot include QTLs for resistance to Soybean Mosaic Virus strain SC7, mapped near BARC-041267-07957 (Gm16:31944251) in Kefeng No.1 (Yan et al., 2015), and to Soybean cyst nematode cqSCN-003 (SCN, *Heterodera glycines* Ichinohe) race 3 (Hg type 7), mapped between Satt244 and Satt547

(Gm16:33819094-34035391) (Glover et al., 2004; Guo et al., 2005; Chang et al., 2011). Also, one QTL on Chr. 16 overlapped with SCN race 2 (Hg type 1.2.5.7.), 3 (Hg type 0) and 14 (Hg type 1.3.5.6.7.) resistance (Jiao et al., 2015).

On December 2017, Pioneer Hi-Bred International, Inc (Johnston, IA) patented the use of Satt512 marker (Gm15: 11240351-11240647) to identify, select, and construct tolerant plants to charcoal rot. The charcoal rot QTL on Chr. 15 detected in this study overlaps with the patented chromosomal region that was marked by Pioneer as closely linked to the Satt512 marker (http://www.freepatentsonline.com/20170354106.pdf), with chromosomal interval flanked by and including markers Satt575 (Gm15:00877198) and Sat_136 (Gm16:632923). In the present research, this QTL explained the highest phenotypic variation among all three detected QTL.

Despite notable progress in research directed towards enhanced tolerance to charcoal rot, improved high yielding soybean lines that are tolerant to this disease are still in high demand (Luna et al., 2017). This study presents the first report on mapping QTL controlling partial resistance to *M. phaseolina* in soybean. However, additional phenotyping on other backgrounds is needed to confirm the stability of the identified QTLs. In addition, field experiments replicated across sites and over years are necessary to investigate the environmental impact on charcoal rot resistance QTL. Furthermore, creating recombinant inbred lines (RILs) from diverse tolerant genotypes would be useful for accurate detection of QTL and to study epistatic interactions at a more precise genetic basis. The findings herein reported have important implications for breeding programs aimed at improving productivity under presence of the pathogen in the soil. Identified molecular markers could be used as diagnostic tool to accelerate Marker Assisted Selection (MAS) to select parents, populations and breeding material with the tolerant phenotype.

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Table 1. Analysis of variance between the two parental lines (PI 567562A x PI 567437) using the cut-stem inoculation assay with *Macrophomina phaseolina*.

Effect	df	F value	Pr>F
Block	2	0.09	0.9136
Genotype	1	120.81	<.0001
Block*Genotype	2	0.13	0.8816
Residual	208		

Table 2. Analysis of variance in the cut-stem inoculation assay with *Macrophomina phaseolina* screen of 142 F_{2-3} lines and the parents, PI 567562A x PI 567437.

Effect	df	F value	Pr>F
Replication	2	2.12	0.1202
Genotype	141	4.30	<.0001
Residual	3548		

Table 3. Summary of single nucleotide polymorphism markers used in the initial screen of the parental genotypes and $F_{2:3}$ population derived from PI 567562A x PI 567437.

Chr.	Length†	Nº. All SNPs‡	All SNPs Coverage§	Nº P-SNPs*	P-SNPs coverage#
1	97.29	263	0.37	113	0.86
2	135.54	328	0.41	150	0.90
3	96.07	280	0.34	110	0.87
4	112.2	266	0.42	100	1.12
5	86.75	294	0.30	130	0.67
6	136.51	313	0.44	154	0.89
7	132.49	319	0.42	129	1.03
8	144.35	380	0.38	113	1.28
9	95.02	261	0.36	109	0.87
10	132.89	317	0.42	118	1.13
11	115.97	279	0.42	92	1.26
12	109.78	259	0.42	98	1.12
13	118.3	381	0.31	140	0.85
14	100.27	275	0.36	96	1.04
15	98.11	305	0.32	96	1.02
16	90.46	238	0.38	99	0.91
17	118.32	265	0.45	73	1.62
18	107.09	380	0.28	143	0.75
19	101.14	326	0.31	100	1.01
20	112.77	271	0.42	120	0.94
Mean	**110.99**	**287**	**0.38**	**111.5**	
Total		**6000**		**2283**	

† Chromosome length in cM based on GmConsensus 4.0 map on SoyBase (http://www.soybase.org).

‡ Number of markers screened for each chromosome: All SNPs (all 6K SNPs); P-SNPs (polymorphic SNPs).

§ Distribution of SNP markers on chromosomes (total chromosome length/number of SNP markers screened): All SNPs (all 6K SNPs); P-SNPs (polymorphic SNPs).

***** Number of polymorphic SNP markers screened for each chromosome.

Chromosome length per polymorphic SNP marker (total chromosome length/number of polymorphic SNP markers).

Table 4. Summary of QTL mapping, marker intervals, LOD scores, R^2 values, and additive effects of QTL resistance to *Macrophomina phaseolina,* using 142 individuals from an $F_{2:3}$ population derived from PI 567562A (R) x PI 567437 (S).

Chr. (LG)	Confidence Interval†	Interval [kb]	Peak	LOD	R^2[%]	Add‡
15 (E)	Gm15_01842053 - Gm15_03051337	1,209	Gm15_03051337	5.25	29.4	-7.7
16 (J)	Gm16_28961127 - Gm16_30493887	1,533	Gm16_30493887	4.32	25.4	-8.8
16 (J)	Gm16_35973543 – Gm16_37078478	1,105	Gm16_36809255	3.60	8.4	-4.8

† Physical position of interval markers in base pairs based on Williams 82 reference genome (Wm82.a2.v1).
‡ Additive effect on the specific QTL. (A negative additive effect indicates that the resistance allele is contributed by PI 567562A.

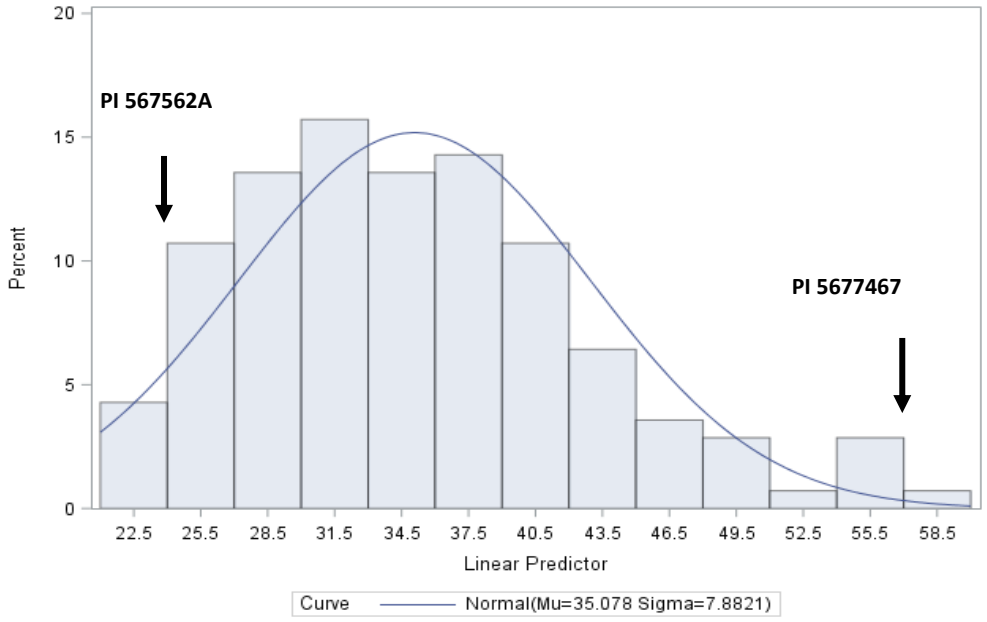

Figure 1. Frequency distribution of *M. phaseolina* resistance scores of 140 $F_{2:3}$ lines derived from PI 567562A x PI 567437, evaluated using a cut-stem inoculation technique.

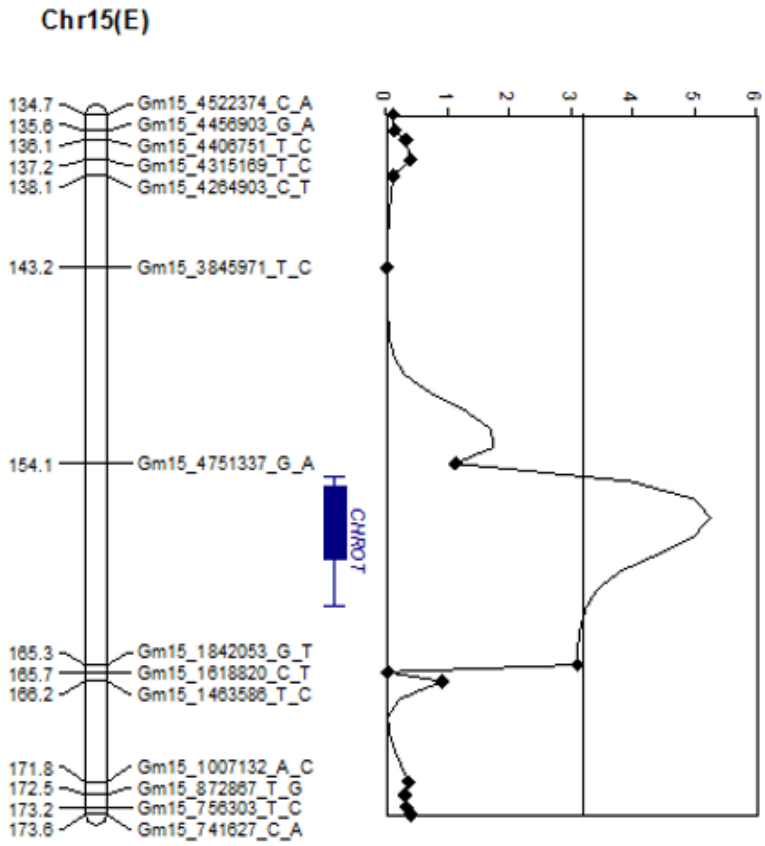

Figure 2. Quantitative trait loci conferring resistance to *Macrophomina phaseolina*, detected on Chr. 15 using 140 individuals of an $F_{2:3}$ population from the cross of PI 567562A (R) × PI 567437 (S) using a genome-wide threshold logarithm of odds (LOD) of 3.2.

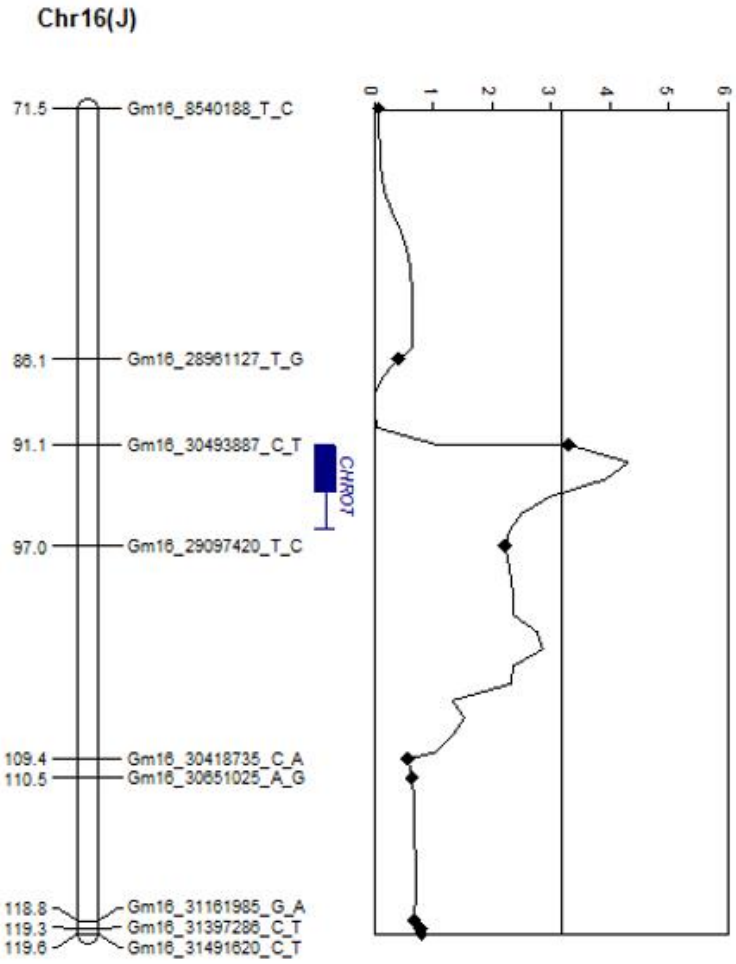

Figure 3. Quantitative trait loci conferring resistance to *Macrophomina phaseolina*, detected on Chr. 16 using 140 individuals of an $F_{2:3}$ population from the cross of PI 567562A (R) × PI 567437 (S) using a genome-wide threshold logarithm of odds (LOD) of 3.2.

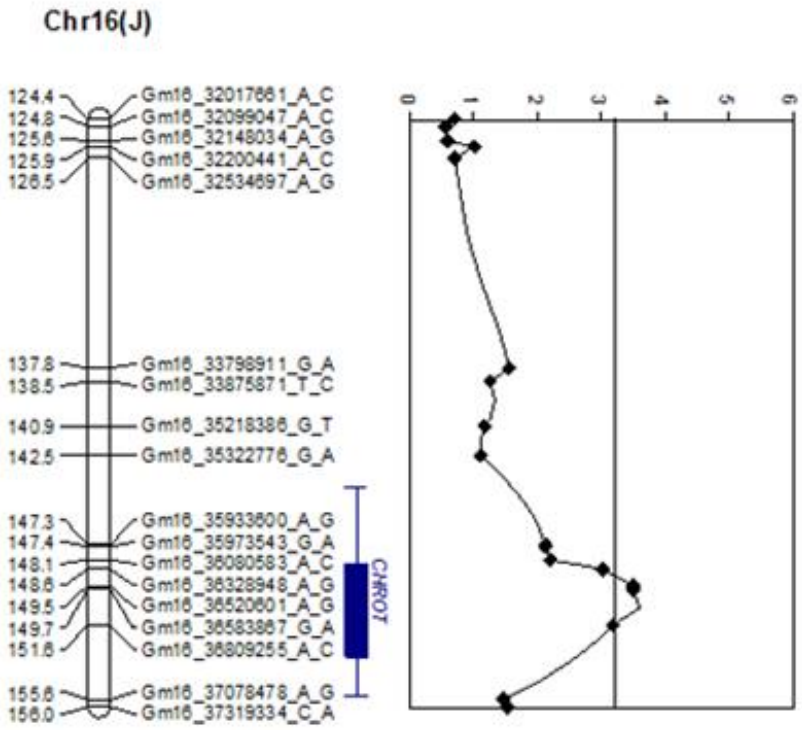

Figure 4. Quantitative trait loci conferring resistance to *Macrophomina phaseolina*, detected on Chr. 16 using 140 individuals of an $F_{2:3}$ population from the cross of PI 567562A (R) × PI 567437 (S) using a genome-wide threshold logarithm of odds (LOD) of 3.2.

CHAPTER 4

Bulked Segregation Analysis Using Next-Generation Sequencing for Identification of Genetic Loci for Charcoal Rot Resistance in Soybean

M. P. da Silva, Richard E. Mason, John C. Rupe, Burt H. Bluhm, Lisa Wood, Pengyin Chen, A ndy Pereira*

M. P. da Silva, Andy Pereira*, Richard E. Mason and Lisa Wood, Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR 72701; John C. Rupe and Burt H. Bluhm, Department of Plant Pathology, University of Arkansas, Fayetteville, AR 72701. Pengyin Chen, Fisher Delta Research Center, University of Missouri, Portageville, MO.

*Author for correspondence: Andy Pereira (apereira@uark.edu)

Abbreviations: Quantitative trait loci, QTL-seq analysis, single nucleotide polymorphisms, breeding, whole genome sequencing, next generation sequencer, marker assisted selection

ABSTRACT

Charcoal rot, caused by *Macrophomina phaseolina* in soybean (*Glycine max* (L.) is a disease of economic significance in the United States. Due to the lack of effective chemical and cultural control, host resistance could be a potential method to control charcoal rot in soybean. However, the identification and quantification of resistance is difficult, and very little is known about molecular marker(s) linked to charcoal rot resistance loci. The objective of this study was to characterize the genetic basis of resistance to charcoal rot in soybean genotype PI 567562A, which is resistant to charcoal rot, using a QTL-seq approach with next-generation sequencing (NGS) based bulked-segregant analysis (BSA). An F_{2-3} mapping population was developed from a cross between PI 567562A (resistant) and PI 567437 (susceptible) (Mengistu et al., 2013), and the two extreme phenotypes (resistant vs susceptible) of 10 plants each were used to isolate genomic DNA separately that was equally pooled to make the resistant and susceptible DNA pools. Both bulks along with parents were sequenced using Illumina HiSeq™ (PE150, Q30≥80%). The sequence reads were aligned to the PI567562A reference genome and used to calculate the SNP-index at each SNP position for both the Resistant (R) and Susceptible (S) bulks. Subsequently, the average distributions of the SNP-index and Δ SNP-index across the genome were estimated for genomic intervals using a sliding window analysis with 2-Mb window size and a 10-kb step. Following this analysis, three genomic regions on chromosomes 5, 8 and 14 were identified with positive values in Δ SNP-index plots that potentially correspond to QTLs governing the difference between the R-bulk and S-bulk sub-populations. The current study is the first attempt employing QTL-seq to identify genomic regions that may contain major QTLs controlling charcoal rot disease resistance, and provide information on the underlying genetic mechanisms that regulate disease resistance to *Macrophomina phaseolina* in soybean.

INTRODUCTION

Soybean (*Glycine max* (L.) is a worldwide planted legume crop that ranks as the largest source of animal protein feed and second as source of vegetable oil. The United States ranks as the leading producer with a total of 89.5 million acres harvested area in 2017 (USDA Reports, 2017). Soybean production can be affected by several diseases and pests caused by biotic factors, which include nematodes, viruses and fungi (Hartman et al., 1999). As one of the important fungal diseases, charcoal rot (Goidanish, 1947), is a soil-borne plant pathogen, caused by *Macrophomina phaseolina* [(Tassi) Goid], that has been shown to decrease soybean yields in experimental plots by 15% (Mengistu et al., 2011). In the United States, the disease was ranked in 2003 and 2012 as the second most important disease impacting yield in the country, with an estimated yield loss of 1.9 million to 2.0 million metric tons (Wrather et al., 2010; Luna et al., 2017).

Due to the absence of effective chemical and cultural control, host resistance appears as an alternative method to control charcoal rot in soybean. However, the quantification and identification of resistance is difficult, and very little is known about molecular marker(s) linked to the charcoal rot resistance loci (Mengistu et al., 2007; Gupta et al., 2012). Moreover, no QTL's have been reported till present for charcoal rot resistance in soybean.

As an alternative to conventional QTL analysis, QTL-seq (QTL-sequencing) holds great potential for rapid identification of QTLs. Combining next-generation sequencing (NGS) and bulked-segregant analysis (BSA) (Michelmore et al., 1991; Giovannoni et al., 1991; Mansur et al., 1993; Darvasi and Soller, 1994), the QTL-seq strategy is faster, less labor-intensive, and relatively inexpensive when compared to regular QTL mapping. This is mainly because it is not necessary to genotype a large number of individual plants in segregating populations derived

from bi-parental crosses (Lu et al., 2014). QTL-seq was first termed by Takagi et al., (2013), working with rapid mapping of quantitative trait loci in rice using whole genome resequencing of DNA bulks of phenotypic extremes. Later, the technique was applied for the identification of QTLs for the flowering locus T in cucumber, tomato fruit weight, candidate genes underlying major trait-associated in chickpea, and two qualitative trait genes controlling cotyledon color of seed in soybean (Mansur et al., 1993; Lu et al., 2014; Das et al., 2015; Illa-Berenguer et al., 2015; Song et al., 2017).

As introduced before, this new method is based on BSA, where two bulked DNA samples from a segregating population derived from a single cross are generated. Each DNA pool contains individuals with extremes in contrasting phenotypic trait values, for example resistance and susceptible to a particular plant disease. Subsequently, the DNA bulks are then screened for polymorphisms using molecular markers that distinguish the DNA bulk samples (Michelmore et al., 1991; Magwene et al., 2011). The application of DNA bulk samples for mapping genes in a chromosomal interval was first proposed by Giovannoni et al., (1991), where they described a rapid and efficient method for isolation of molecular markers in any defined genomic interval. The same year, Michelmore et al., (1991) demonstrated the application of BSA to identify markers linked to disease resistance genes. Based on these concepts, QTL-seq uses next generation sequencing markers to capitalize on the same genetic principles.

The objective of this study was to characterize the genetic basis of resistance to charcoal rot in soybeans, with a QTL-seq approach using next-generation sequencing (NGS) based bulked-segregant analysis (BSA).

MATERIALS AND METHODS

Plant material and population development

In 2014, crosses were made between resistant PI 567562A (MG IV) and susceptible PI 567437 (MG IV) (Mengistu et al., 2013) (Table 1) at the Agricultural Experiment Station, University of Arkansas in Fayetteville, AR (U of A). The F_1 seeds were grown at the Rosen Alternative Pest Control Center (U of A). The F_1 plants were confirmed as true hybrids using forty SSR markers covering the entire genome of soybean (two markers per chromosome). In the summer of 2015, the F_2 population was grown at the Agricultural Experiment Station (U of A), where 140 F_2 plants were individually harvested to form the segregating population. Subsequently, the 140 F_{2-3} lines were planted in the greenhouse for tissue sample collection and disease screening.

Fungal infestation

Inoculation was performed using the Conway isolate of *M. phaseolina* (collected in Arkansas by Dr. John Rupe, University of Arkansas, Fayetteville, AR). The isolate was grown on potato dextrose agar (Difco Laboratories), and maintained in an incubator at 28ºC for five days.

Disease screening

A total of 140 F_{2-3} lines derived from the PI 567562A x PI 567437 cross, along with the parental genotypes were screened for disease resistance in the greenhouse (28 ± 2 ºC**,** 14 h photoperiod) at the Rosen Center at U of A, from September to November of 2016 using the cut-stem inoculation technique (Twizeyimana et al., 2012a). The 140 F_{2-3} lines were evaluated in an incomplete block design which consisted of 36 plants per line that were divided in 3 replications of 12 plants and, the two parents of the mapping population as checks in each individual assay.

Each line was sown in 48-pot plastic inserts (number 1204; Hummert International), with 12 seeds per row in each insert, and 36 seeds per F_{2-3} line were evaluated. Each insert was filled with autoclaved soil-less mix (Sunshine Mix, LC1; Sun Gro Horticulture Inc.), placed inside a flat with drainage holes (number T1020; Hummert International), and fertilized at planting with slow-release pellets (Osmocote 19-6-12; 1 to 2 pellets/cm2). At the end of the screening, 5112 plants were assessed for charcoal rot resistance. Four WatchDog B-Series Data Logger (Spectrum Technologies, Inc) were placed strategically at different points in the greenhouse to collect temperature and humidity data. The average temperature was 29º C with 65% of humidity.

Construction of pools

Genomic DNA was isolated from 20 lines along with parental genotypes, from young leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The DNA solutions were stored at -80ºC. DNA samples were quantified using a NanoDrop 2000 spectrophotometer for measurement of DNA quality and for assessing the OD_{260}/OD_{280} ratio. DNA concentration was measured with the Qubit 2.0 fluorometer (Invitrogen-Molecular Probes, Eugene, OR). Two DNA bulks of the population were generated for analysis by pooling equal amounts of DNA from 10 plants featuring extreme values for the trait (Figure 1).

Construction of sequencing libraries and Illumina Sequencing

About 5 µg of DNA from the two bulks with 10 lines per bulk (resistant and susceptible), was used for next generation sequencing. The DNA pools were used to construct paired-end sequencing libraries, which were sequenced on an Illumina HiSeq^{TM} (PE150, Q30≥80%), to generate 200K raw tags/sample (data on hard drive 1TB) and 100K raw tags/sample from the two parental lines (data on hard drive 128G). The high-quality sequences were aligned and

mapped to the *Glycine max* Wm82.a2.v1 reference genome from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) using BWA with default parameters (Li and Durbin, 2009). The duplicates were removed by SAMTOOLS (parameters: rmdup) (Li et al., 2009).

Data Analysis

SNP-Index Analysis

A SNP-index was calculated at each SNP position for both the Resistance (R) and Susceptible (S) bulks using the PI567562A genome as the reference. In this, a SNP-index of 1 indicates that reads in the population are derived only from the PI567562A (R), whereas a SNP-index of 0 indicates the reads are derived only from PI567437 (S), and SNP-index of 0.5 indicates an equal genome contribution from both parents (Abe et al., 2012; Takagi et al., 2013; Singh et al., 2016). A significant deviation from a SNP-index of 0.5 could indicate contribution of that SNP to the phenotypic difference observed in the bulks (Abe et al., 2012). The Δ SNP-index of each SNP position was calculated by subtraction of the SNP-index of resistance bulk from SNP-index of susceptible bulk (Takagi et al., 2013; Fekih et al., 2013; Das et al., 2015; Singh et al., 2016). Only SNP positions with Δ SNP-index = 1 (i.e. with allele called from the resistance bulk, that was the same as that of PI PI567562A, while absent or lacking in the susceptible bulk) were considered as the causal SNPs responsible for the phenotypic difference observed in the bulks.

Sliding-Window Analysis

The average distributions of the SNP-index and Δ SNP-index across the genome were estimated in a given genomic interval using sliding window analysis (Tajima, 1991), with 2-Mb window size and a 10-kb step using an R script that we developed for this purpose. The SNP-

index graphs of Resistance and Susceptible pools, as well as the corresponding Δ SNP-index graph were plotted. According to Takagi et al., (2013) the Δ SNP-index value should not be significantly different from 0 in a genomic region with no major QTL of the target gene. Besides that, a substantial deviation from SNP-index 0.5 (Abe et al., 2012), could indicate a significant contribution of the SNP to the phenotypic difference observed in the bulks.

Phenotyping data Analysis

The stem necrosis (mm), caused by *M. phaseolina* infection was analyzed using the PROC GLIMMIX (SAS version 9.4, SAS Institute Inc. Cary, NC. USA), and PROC UNIVARIATE was used to test the normality of the disease stem necrosis distribution for CR lines. Broad sense heritability (H^2) of disease response was calculated with the following equation: $H^2 = \sigma^2g/ \sigma^2g+ \sigma^2e/r$ (Greene et al., 2008), where σ^2g is the genetic variance, σ^2_e is the error, and r is the number or replications.

RESULTS

Phenotypic Data

Analysis of variance indicated that there was a significant difference (P <.0001) among the 140 F_{2-3} lines derived from PI 567562A x PI 567437 (Table 3), indicating different levels of resistance to *M. phaseolina* infection. All soybean lines developed stem necrosis, ranging from 22 mm to 59 mm, exceeding the stem necrosis range of the parental genotypes, which indicated the presence of transgressive segregation, where the progeny display phenotypic trait values that arise by segregation of genes for a quantitative character that falls outside the range of the parents (Poehlman, 1994). The average disease score for *M. phaseolina* was 23 mm for resistant bulk (R-bulk) and 53 mm for susceptible bulk (S-bulk). The normality test using the Kurtosis statistical numerical method showed that the resistance for *M. phaseolina* is a quantitative trait

controlled by multiple genes or QTLs (Figure 1). However, the accuracy to identify superior transgressive segregants relies on how the expression of the quantitative trait is affected by the environmental variation, and this holds true for the expression of charcoal rot disease in the field conditions. The heritability estimate for *M. phaseolina* infection was 0.45.

Sequencing and mapping of reads to the genome

Illumina HiSeq^{TM} sequencing resulted in 4,015,296 short reads from the R-bulk (99.3% coverage) and 4,217,604 short reads from S-bulk (99.39%), respectively (Table 2). The short reads were aligned to the *Glycine max* Wm82.a2.v1 reference genome from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) using BWA with default parameters (Li and Durbin, 2009), and 30,615 SNPs were identified between R-bulk and S-bulk alignments. A SNP-index was calculated for each identified SNP, and an average SNP-index was computed with 2-Mb window size and 10-kb step using an R script that we developed for this purpose. The SNP-index graphs of Resistant (Figure 2) and Susceptible (Figure 3) pools, as well as corresponding Δ SNP-index graph were plotted for each chromosome (Figure 4).

Candidate genomic region(s) for charcoal rot resistance

The SNP-index represents the frequency of parental alleles in the population of bulked samples, in which a SNP-index = 0.5 will signify contribution of both parents, and any significant deviation from this frequency indicate the potential existence of more alleles of one parent than the other for a particular genomic position. The SNP-index graphs presented highly contrasting patterns for R-bulk and S-bulk for chromosomes 01, 02, 03, 05, 07, 08, 09, 11, 12, 13, 14, 17, 18, 19 and 20 (Figures 2 and 3). Following these estimates, Δ SNP-index was calculated to enable detection of variation in SNP-indices between R-bulk and S-bulk. Most of the genomic regions show uniform distribution along the 20 chromosomes with Δ SNP-index

around 0, which indicates both parents have the same SNP-indices at the genomic regions (Figure 4). However, Δ SNP-index value should be significantly different from 0 in order to have a genomic region harboring a major QTL of a target gene (Lu et al., 2014). The results of the SNP mapping identified genomic regions on chromosomes 05, 08 and 14 that exhibit positive values of Δ SNP-index that may correspond to QTLs governing the difference between the R-bulk and S-bulk (Figures 5, 6 and 7). The region on chromosome 1 (between 25 to 25.6 Mb) has a peak of Δ SNP-index higher than 0.5 (Figure 5), the chromosome 8 segment between 73.5 to 76.5 Mb has a peak of Δ SNP-index higher than 0.6 (Figure 6), and the region on chromosome 14 segment between 24.7 to 25.5 Mb with a peak of Δ SNP-index higher than 0.4 (Figure 7).

DISCUSSION

Using next generation sequencing based methods of QTL mapping with QTL-seq (Takagi et al., 2013), in this study we identified 3 significant genomic regions on different chromosomes (Figure 5, 6 and 7) that could harbor QTLs governing disease resistance to charcoal rot in soybean. The QTLs regions were recognized as peaks of the SNP-index plots and confirmed with quantitative Δ SNP-index for each chromosome. All chromosomes presented a range of Δ SNP-index values (peaks), with highest being 0.6 in a 3 Mb interval on chromosome 8 (Figure 6), followed by chromosome 5 (Figure 6) and chromosome 14 (Figure 7). As the Δ SNP-index value was obtained by subtraction of SNP-index of R-bulk from S-bulk, Δ SNP-index values close to a value = 1 were considered as the causal SNPs responsible for phenotypic differences observed in the bulks, representing that their alleles were derived from the resistant parent 567562A (Table 1), even though no confidence intervals were calculated for the Δ SNP-index. In a like manner, Takagi et al., (2013), working with QTL-seq from two bulked populations, proposed that QTLs could be identified as peaks or valleys of the SNP-index plot.

Therefore, the genomic region on chromosome 8 from 73.5 to 76.5 Mb with Δ SNP-index value higher than 0.6 has a higher probability of having a major QTL controlling charcoal rot disease resistant in soybeans. Also, according to Takagi et al., (2013) the power of QTL-seq for detection QTLs is higher in RILs than in F_2 population because of the additive affects, although Takagi et al., (2013) conclude that QTL-seq application to the F_2, would be a reasonable choice to quickly detect QTLs.

The current study is the first attempt using QTL-seq to identify genomic regions that may have major QTLs controlling charcoal rot disease resistance, and may provide a basis for the underlying genetic mechanisms that regulate disease resistance to *Macrophomina phaseolina* of soybean. However, the statistical confidence interval of Δ SNP-index for all the SNP positions under the null hypothesis of no QTLs should be calculated to facilitate detection of differences in SNP-indices between the R-bulk and S-bulk. Next, identification of candidate genes for charcoal rot resistance following by validation of identified genomic regions, using CAPS/dCAPS marker assays to genotype SNPs in the population can assist genetic dissection and map-based cloning. Besides the molecular analysis, it would be useful to conduct replicated experiments across sites and over time to investigate the environmental impact of the identified QTLs affecting disease resistance in charcoal rot. Furthermore, the creation of recombinant inbred lines (RILs) for a mapping population would help in the accurate detection of QTLs, by evaluation of the homozygous lines that can be increased and replicated to reduce the environmental variation in the analysis.

By taking advantage of the NGS based method, using GBS and bulked-segregant analysis (BSA), QTL-seq has the potential over traditional QTL analysis for sequence-based high-resolution genome mapping and subsequent fine mapping of target candidate genomic regions

harboring a major trait-associated QTL. Furthermore, the QTL-seq method can rapidly detect genomic region(s) controlling the target trait and candidate genes in the region, with no necessity to genotype a large population. Finally, this approach is a cost-effective and successful method when applied to a RIL population (Takagi et al., 2013; Das et al., 2015; Singh et al., 2016)

In conclusion, much more research is needed to understand the genetic basis of charcoal rot resistance in soybean, and a QTL-seq approach with greater depth of sequencing and subsequent phenotyping of a RIL population has the potential to identify shorter precise genomic regions that may be associated with charcoal rot resistance than classical QTL mapping methods.

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Table 1. Summary of genotypes and corresponding phenotypes

MG	Accession	Disease severity[x]	Resistance reaction	FC*	PUB**	Pod Color	Hilum Color
IV	567562A	1	resistant	white	gray	Brown	buff
IV	567437	4.4	susceptible	white	light tawny	Brown	brown

[x] Disease severity was based on root and stem severity on a scale of 1 to 5 (Mengistu et al., 2013).
* Flower color
** Pubescence color

Table 2. Summary of Illumina sequencing data for PI 567562A x PI 567437 F_{2-3} populations.

Mapping population	Sample[a]	Mapped reads[b]	Mapping rate (%)[c]	Mean depth[d]
567562A x 567437	R-bulk	4,015,296	99.3	20.095
	S-bulk	4,217,604	99.39	20.811

[a] DNA from 10 individuals sampled and bulked in each pool.
[b] The number of clean reads mapped to the reference assembly, including both single-end reads and reads in pairs.
[c] Mapping rate: The ratio of the reference genome mapped reads to the total sequenced clean reads.
[d] Average depth for tags within specified range (289~315), only calculated for 300,000 tags with greatest depths.

Table 3. Analysis of variance of cut-stem inoculation assay with *Macrophomina phaseolina* of 142 F_{2-3} lines and the parents, PI 567562A x PI 567437.

Effect	df	F value	*P*-value
Genotype	141	2.12	<.0001
Replication	2	4.30	0.1202
Residual	3548		

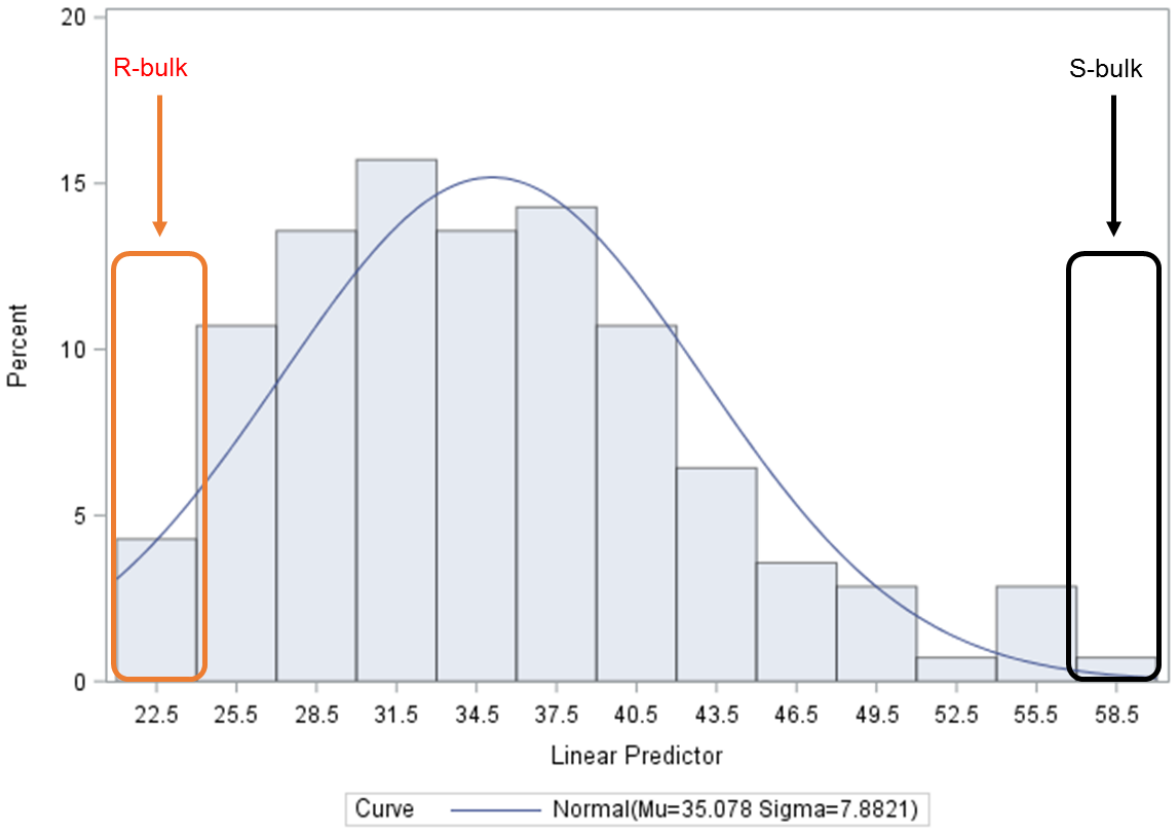

Figure 1. Frequency distribution of *M. phaseolina* resistance of 140 F_{2-3} lines from PI 567562A x PI 567437, evaluated using a cut-stem inoculation technique in the greenhouse. The selection of lines used for making resistant (R) and susceptible (S) bulk pools for DNA isolation and analysis are shown.

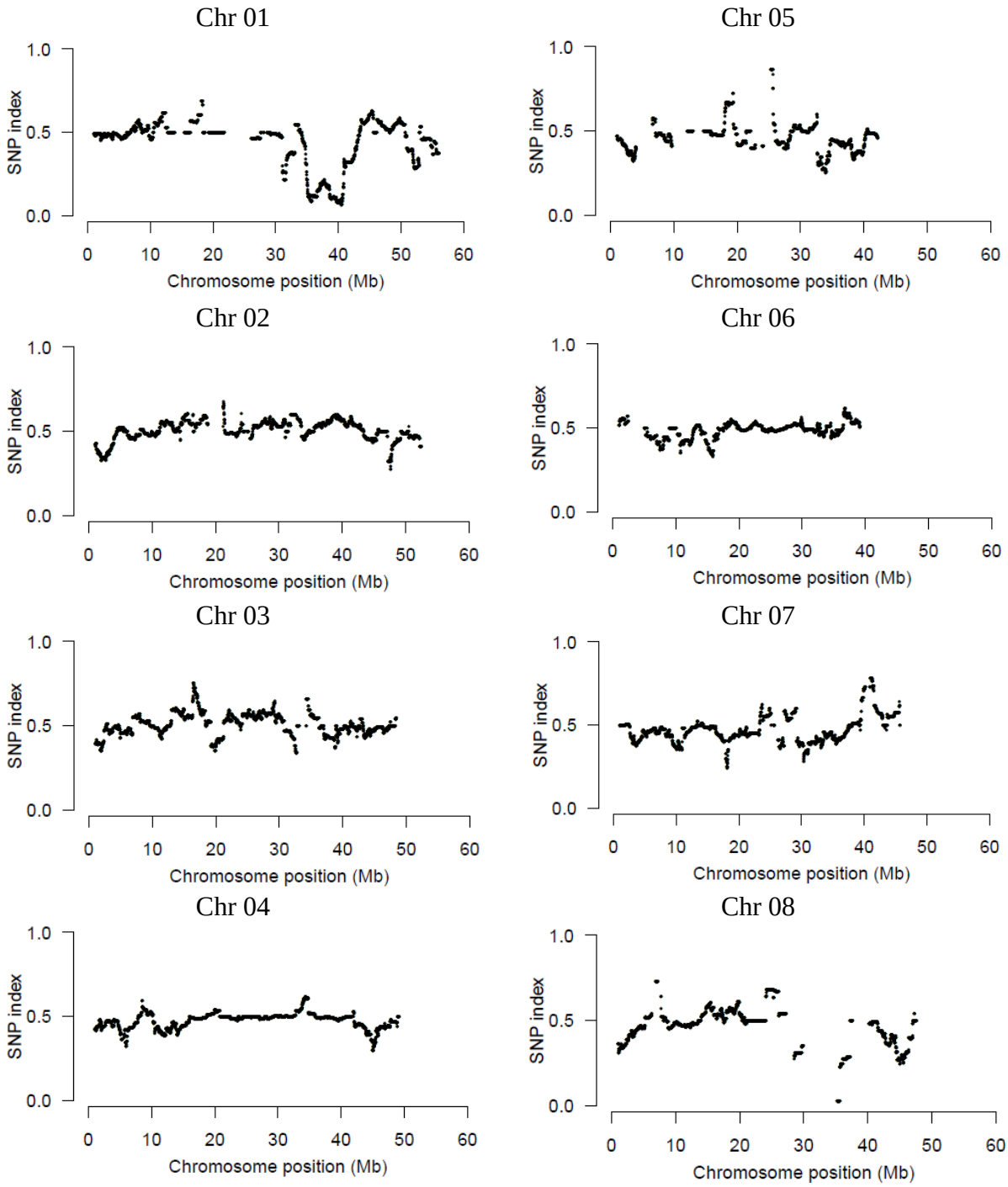

Figure 2. SNP-index plots for bulked DNA of the resistant $F_{2\text{-}3}$ lines from 20 chromosomes derived from PI 567562A x PI 567437 (Cont.)

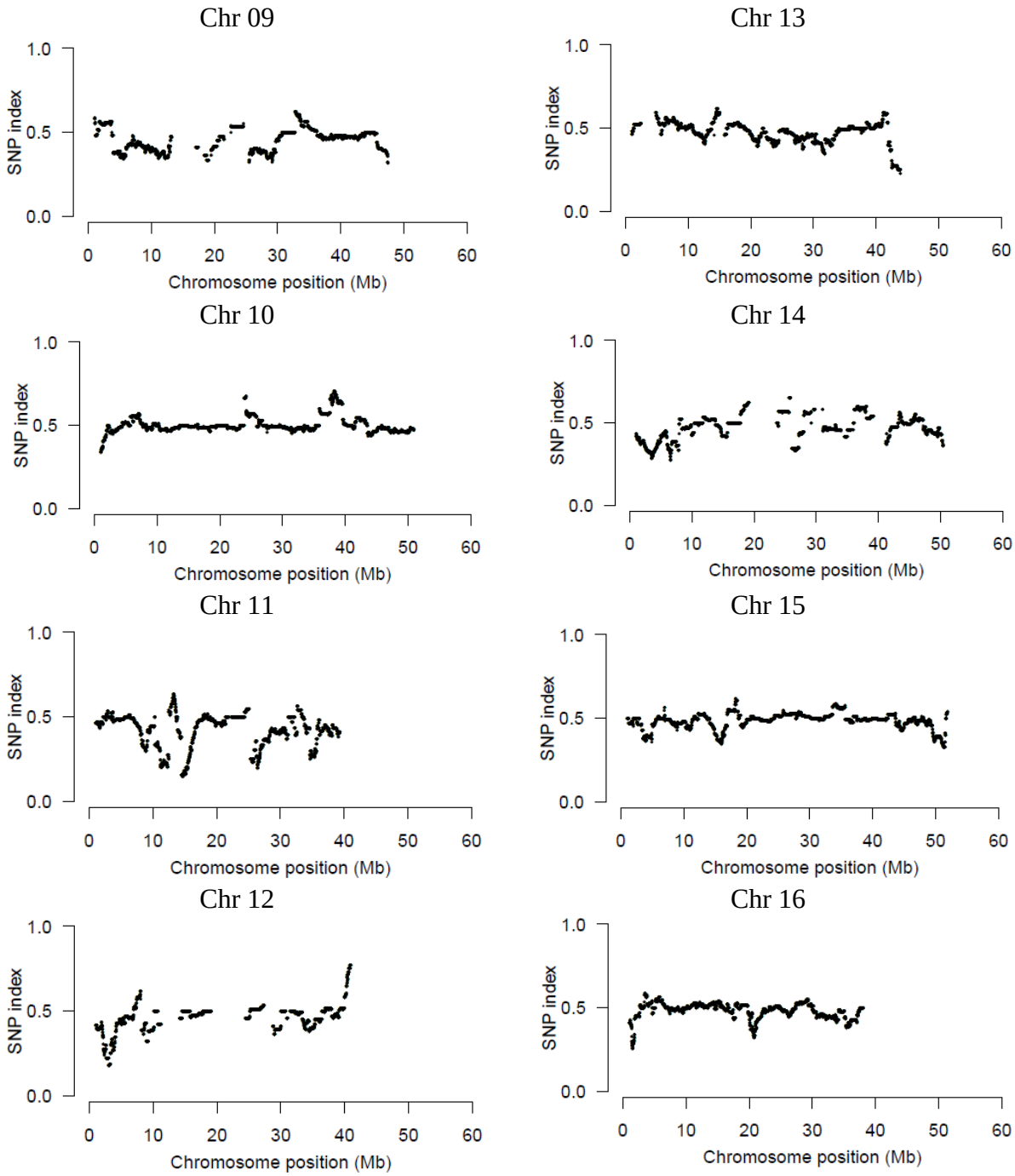

Figure 2. SNP-index plots for bulked DNA of the resistant $F_{2\text{-}3}$ lines from 20 chromosomes derived from PI 567562A x PI 567437 (Cont.)

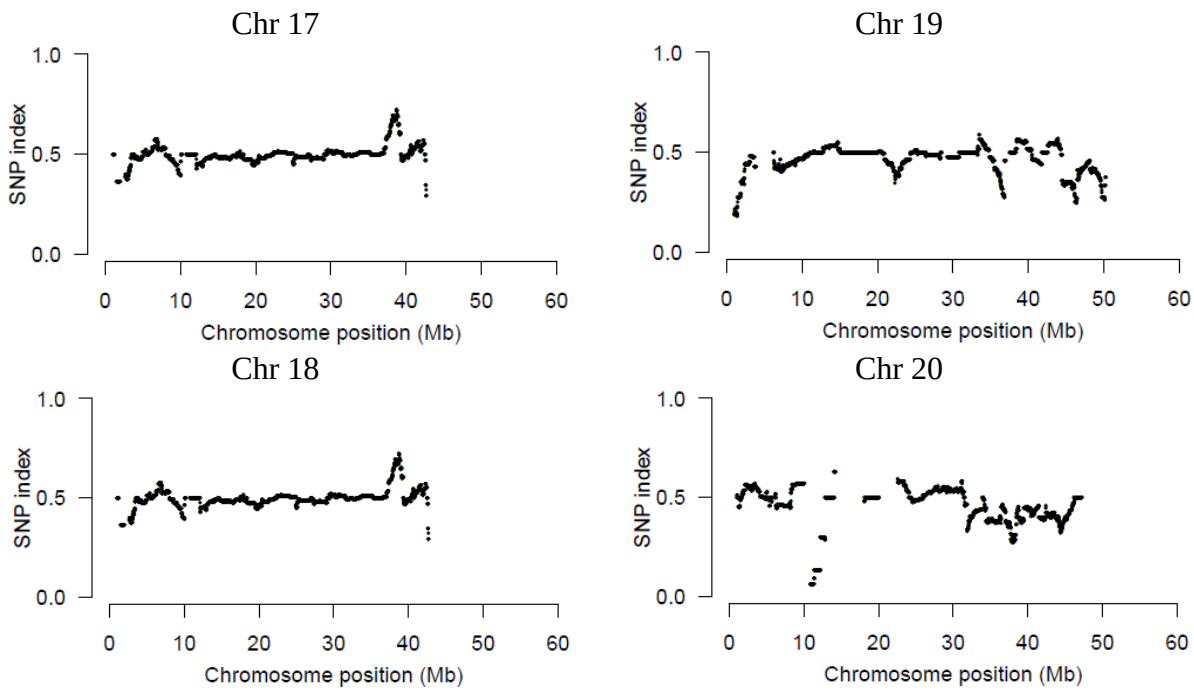

Figure 2. SNP-index plots for bulked DNA of the resistant $F_{2\text{-}3}$ lines from 20 chromosomes derived from PI 567562A x PI 567437 (Cont.).

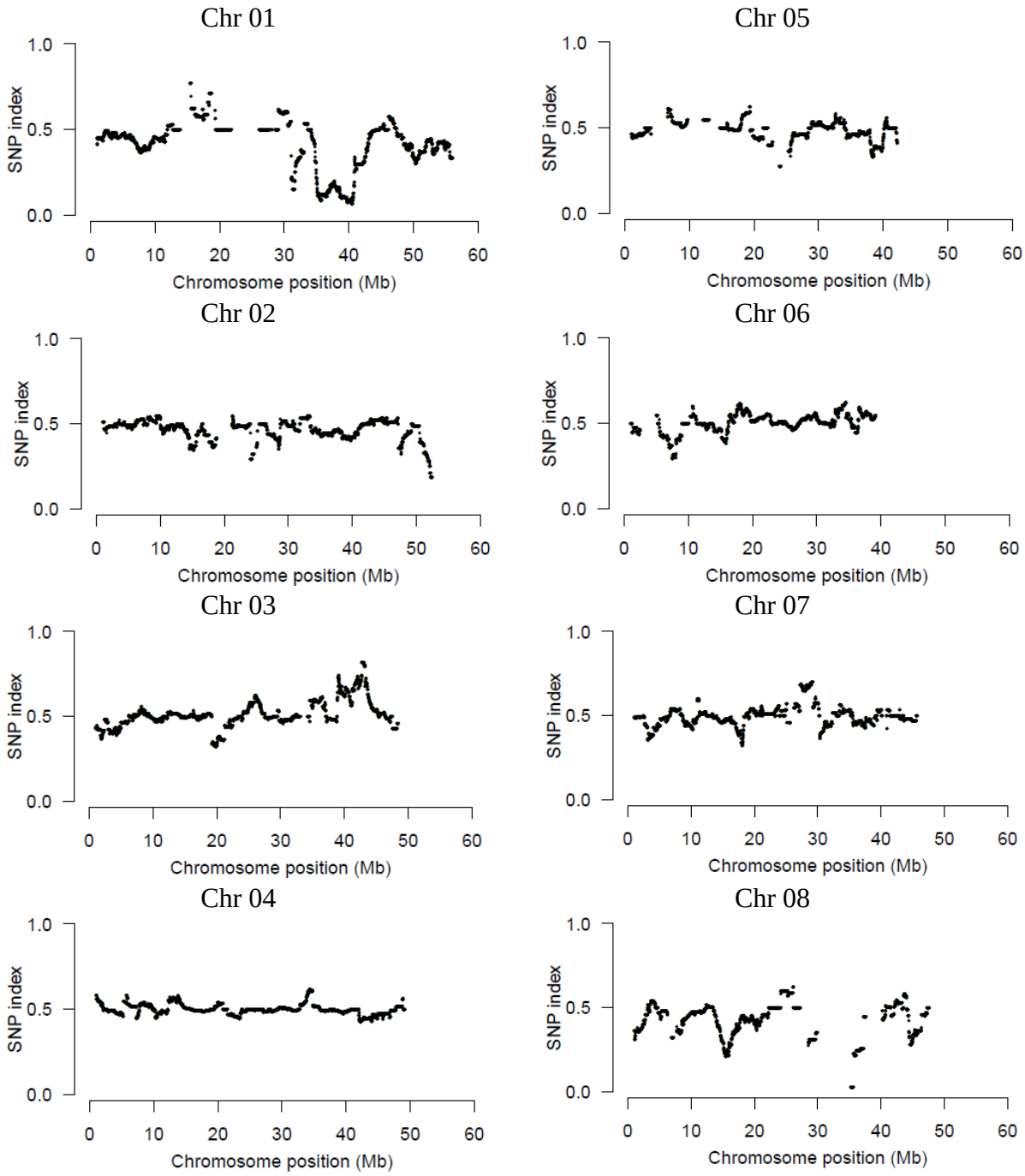

Figure 3. SNP-index plots for bulked DNA of the susceptible $F_{2\text{-}3}$ lines from 20 chromosomes derived from PI 567562A x PI 567437 (Cont.)

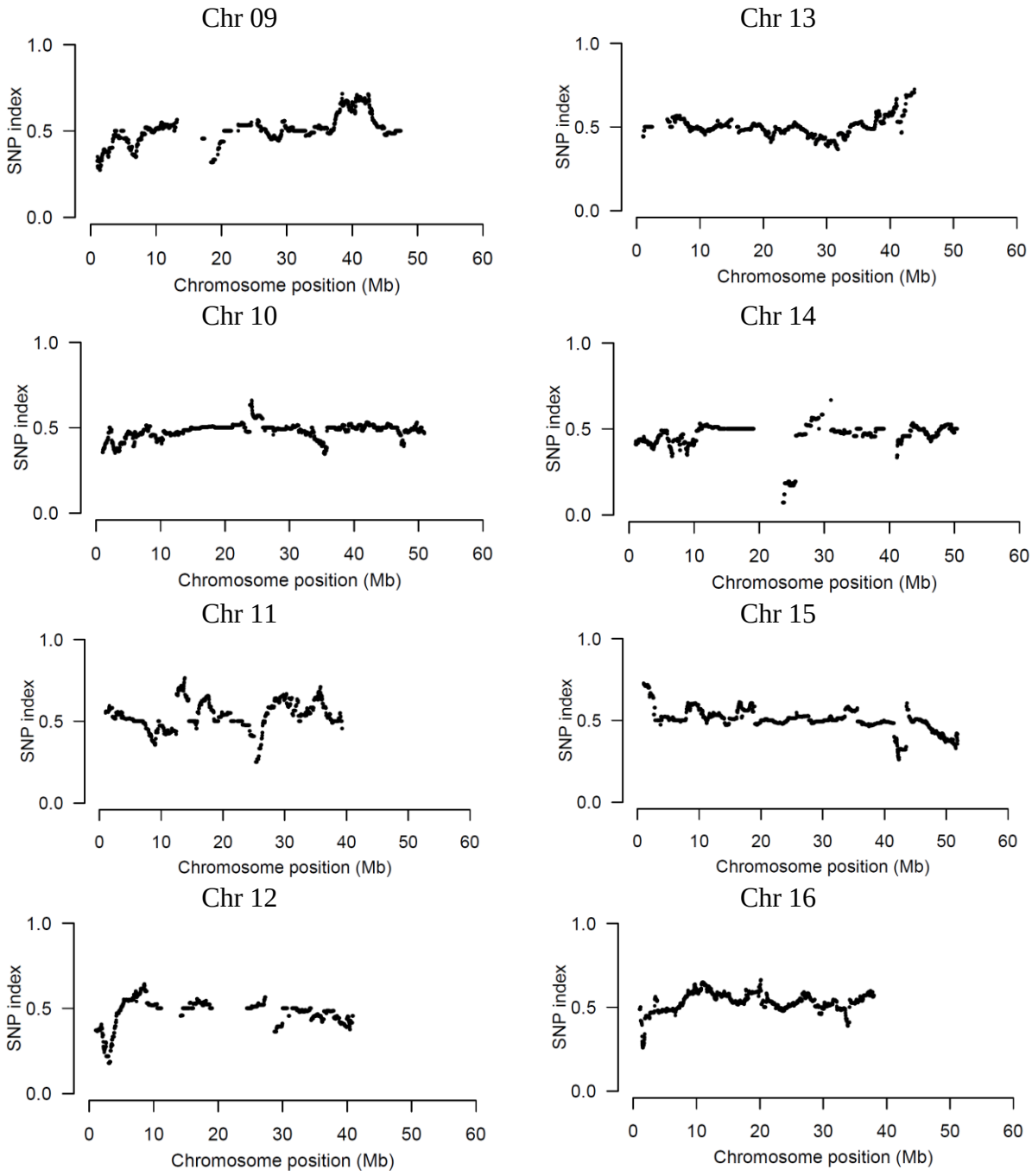

Figure 3. SNP-index plots for bulked DNA of the susceptible F_{2-3} lines from 20 chromosomes derived from PI 567562A x PI 567437 (Cont.)

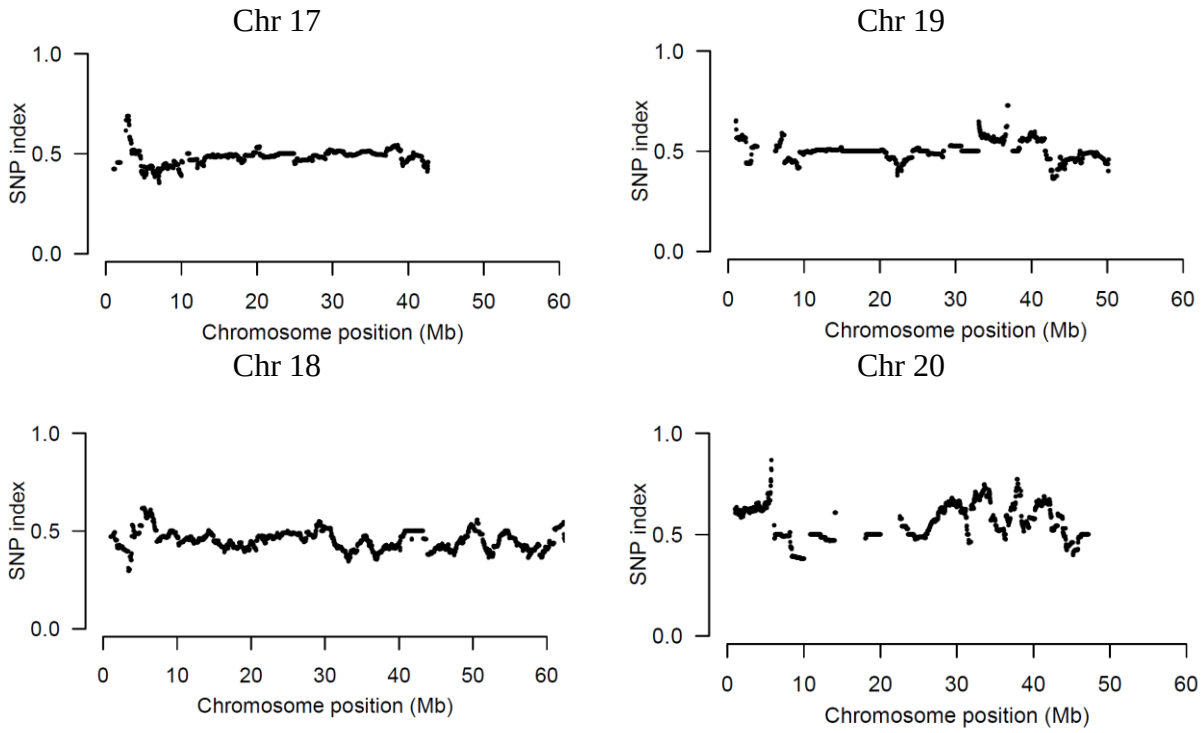

Figure 3. SNP-index plots for bulked DNA of the susceptible $F_{2\text{-}3}$ lines from 20 chromosomes derived from PI 567562A x PI 567437 (Cont.).

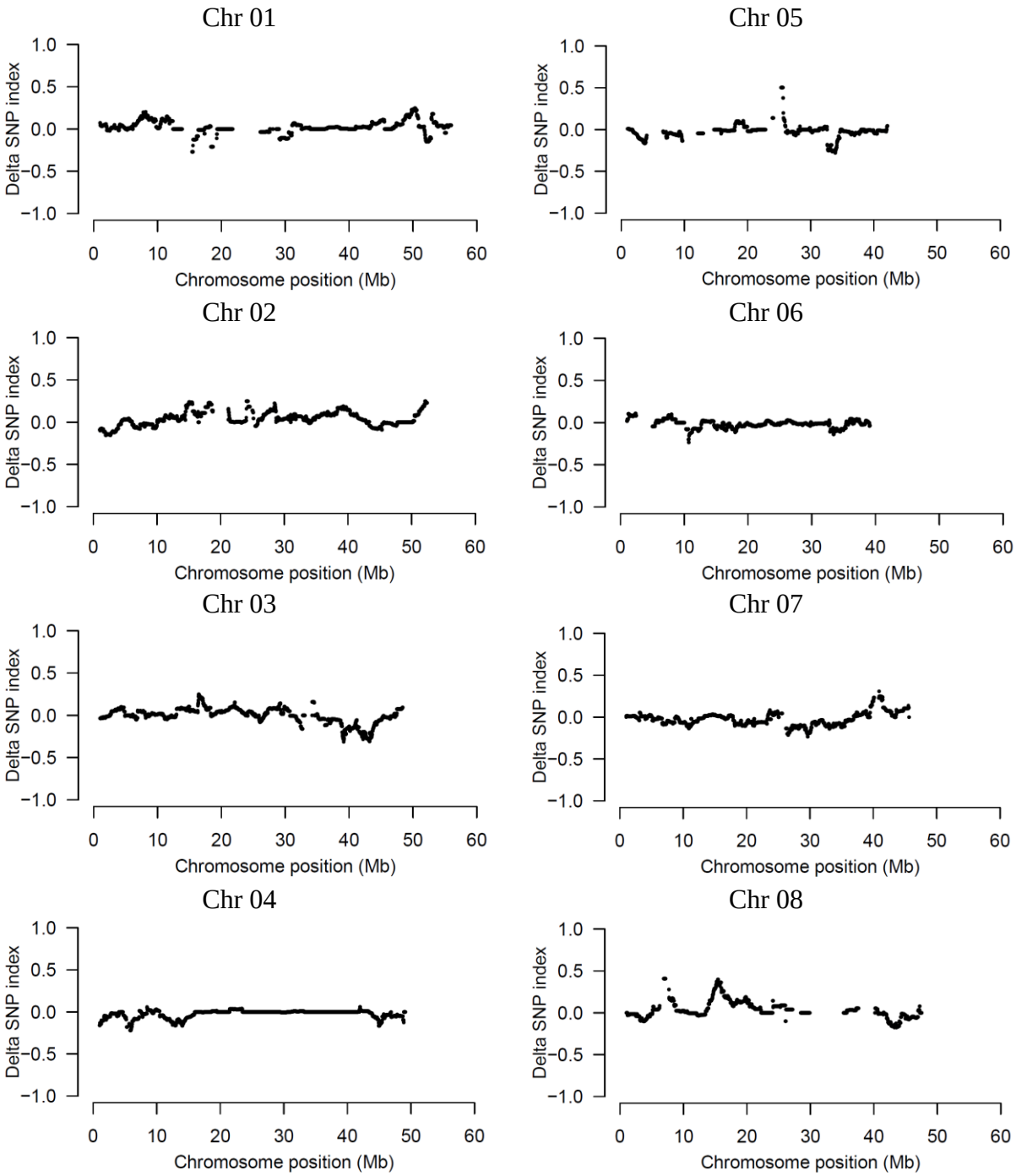

Figure 4. Δ SNP-index plots obtained by subtraction of resistant SNP-index from susceptible SNP-index of the $F_{2\text{-}3}$ lines from 20 chromosomes derived from PI 567562A x PI 567437 (Cont.

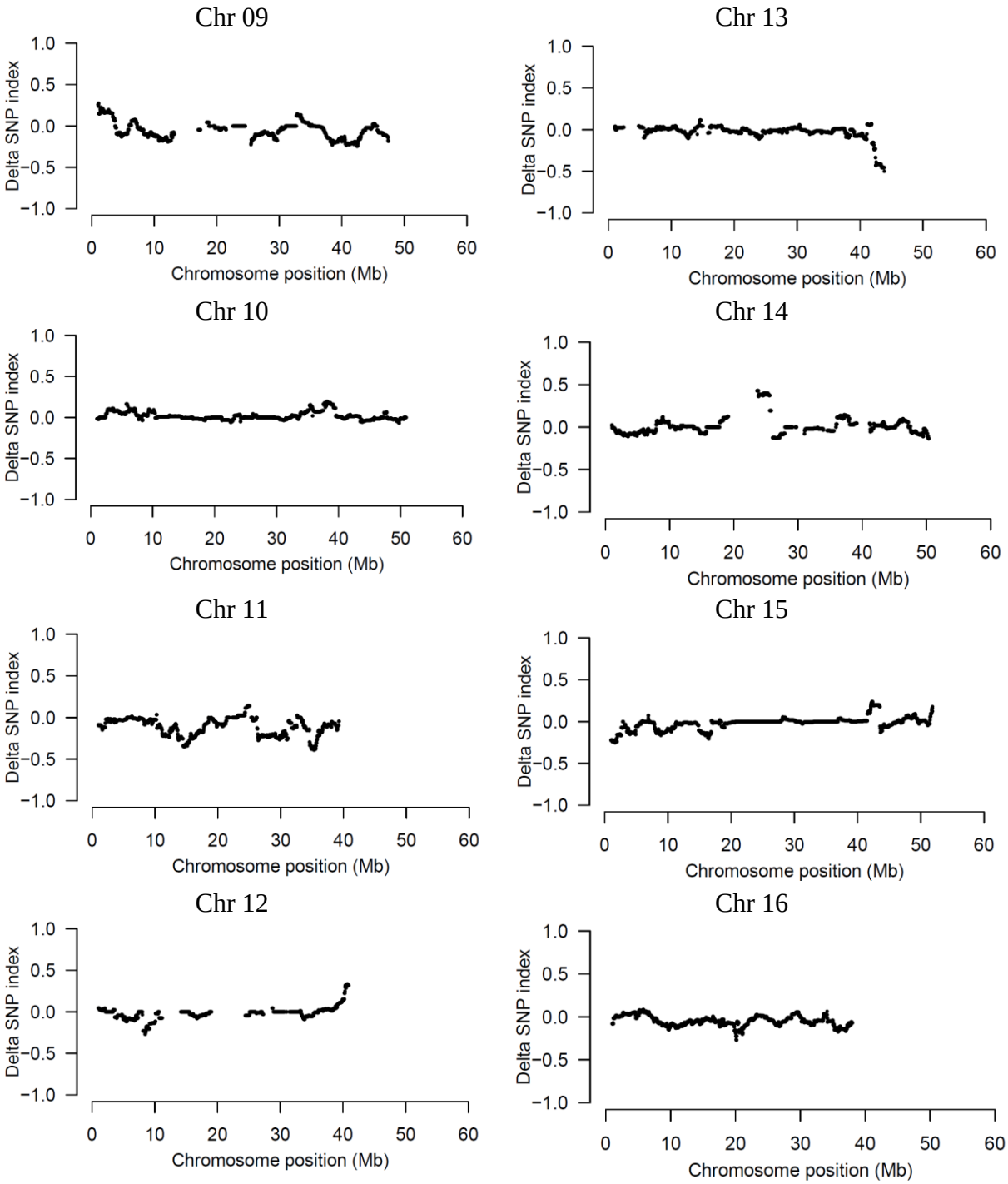

Figure 4. Δ SNP-index plots obtained by subtraction of resistant SNP-index from susceptible SNP-index of the $F_{2\text{-}3}$ lines from 20 chromosomes derived from PI 567562A x PI 567437 (Cont.)

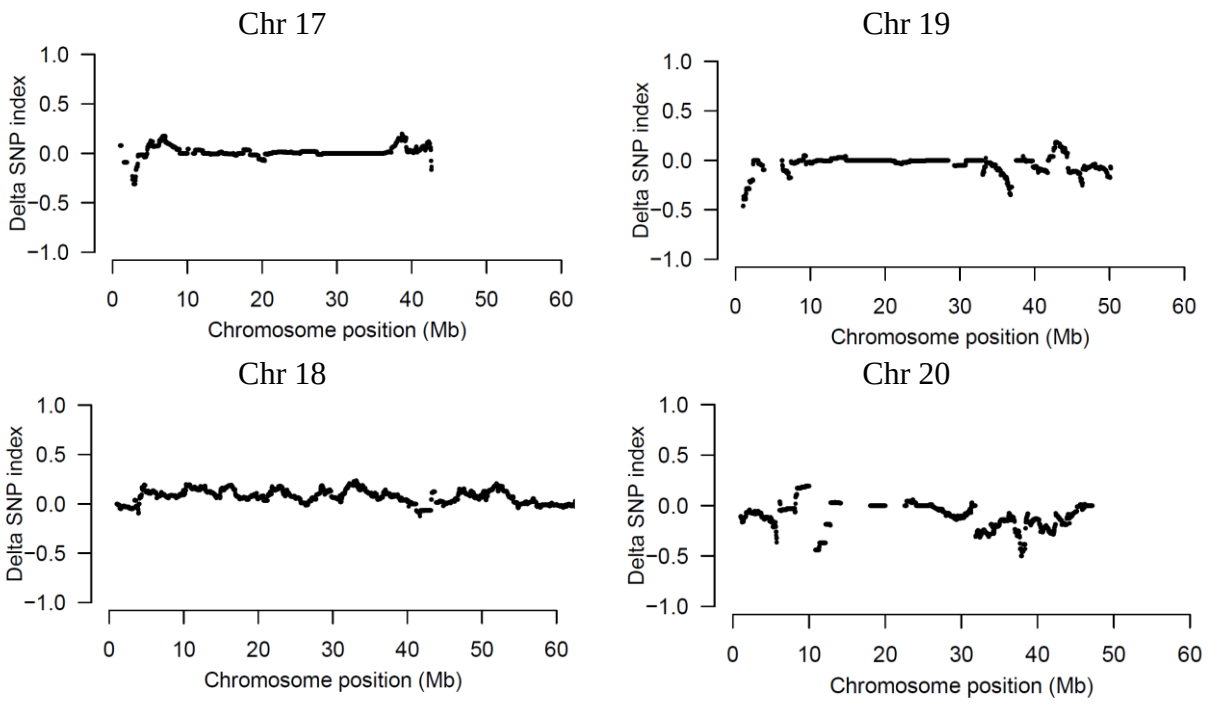

Figure 4. Δ SNP-index plots obtained by subtraction of resistant SNP-index from susceptible SNP-index of the $F_{2\text{-}3}$ lines from 20 chromosomes derived from PI 567562A x PI 567437 (Cont.).

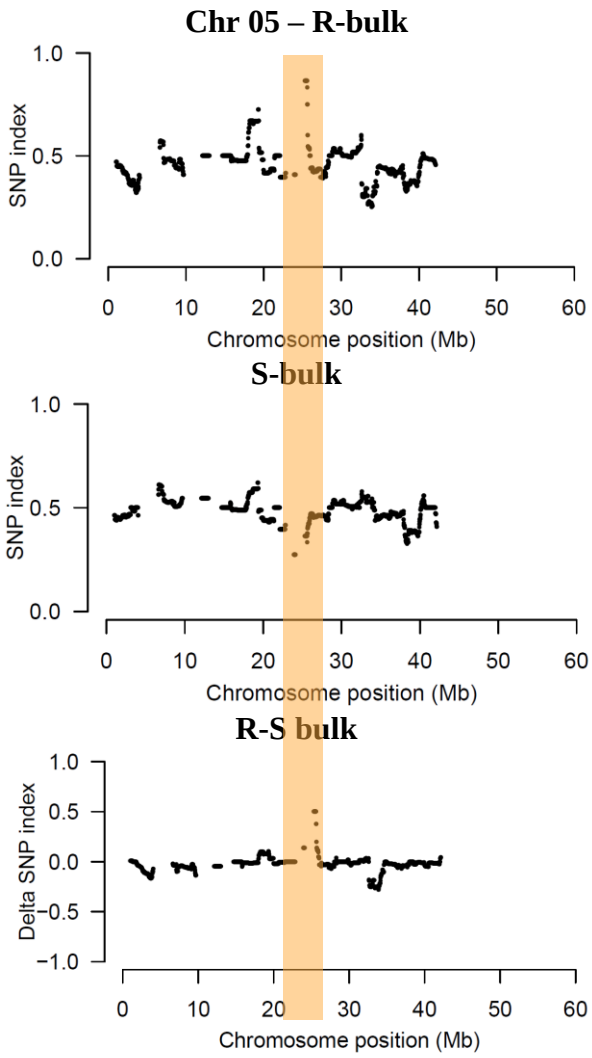

Figure 5. SNP-index plot of R-bulk (top), S-bulk (middle) and Δ SNP-index plot (bottom) of chromosome 05. The significant genomic regions are highlighted in shaded color (25 to 25.6 Mb) with a peak higher than 0.5.

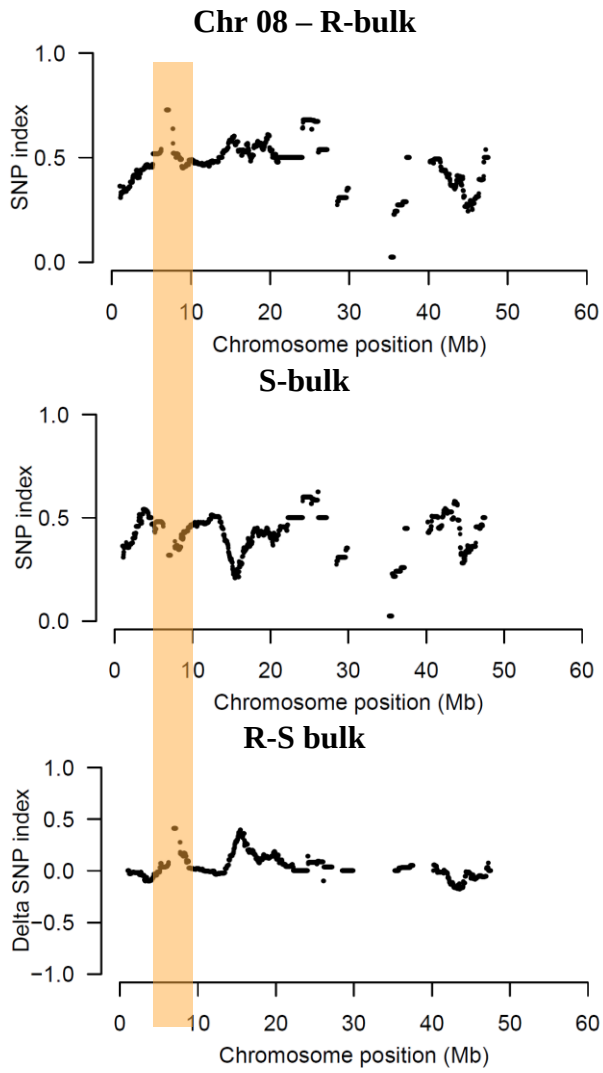

Figure 6. SNP-index plot of R-bulk (top), S-bulk (middle) and Δ SNP-index plot (bottom) of chromosome 08. The significant genomic regions are highlighted in shaded color (73.5 to 76.5 Mb) with a peak higher than 0.6.

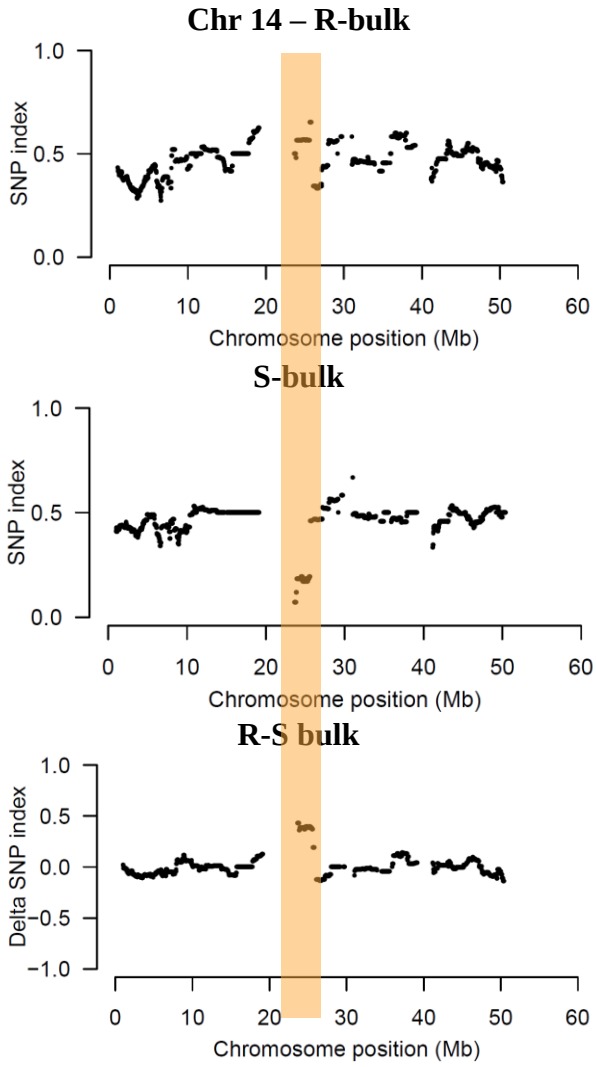

Figure 7. SNP-index plot of R-bulk (top), S-bulk (middle) and Δ SNP-index plot (bottom) of chromosome 14. The significant genomic regions are highlighted in shaded color (24.7 to 25.5 Mb) with a peak higher than 0.4.

OVERALL CONCLUSION

In this research two different aspects of charcoal rot (CR) resistance in soybean, genetics and phytopathological, were studied in order to characterize the complex mechanisms underlying disease resistance. The first aspect investigated was related to evaluation of screening methods, mainly because current assay methods, such as greenhouse based cut-stem inoculation and field evaluations using colony-forming unit index (CFUI) can be time consuming, and data may vary between tests. To achieve this goal, a reproducible Seed plate assay (SPA) for CR resistance was developed and results compared with the cut-stem and CFUI assays, field data disease assessments PHSD (percent height of stem internal discoloration), RSS (root and stem severity) and CFUs (colony-forming unit) across different genotypes. Germination results from the SPA were in agreement with the cut-stem and CFUI assays, although none of the genotypes evaluated with SPA showed complete resistance to *M. phaseolina*. However, data from the SPA could quantitatively separate genotypes into different degrees of resistance and susceptibility, as showed in previous reports. PHSD assessment correlated significantly with SPA results for Stuttgart from 2011 to 2013 and 2012 in Rohwer. Yield correlated significantly for Stuttgart in 2011, 2013 and 2014, and in 2011 and 2012 for Rohwer. Root and stem severity (RSS) was significantly correlated only Rohwer for 2012, as well as CFUs significantly correlated only in Stuttgart for 2012.

The second goal of this research was to investigate and characterize the inheritance of tolerance to charcoal rot in soybean. Towards this, two different genetics strategies including bi-parental quantitative trait loci (QTL) mapping, and next-generation sequencing (NGS) from bulked-segregant analysis (BSA) based on QTL-seq, were used to identify markers and genomic regions for resistance to CR.

Traditional QTL mapping with MQM mapping (composite interval mapping) analysis indicated one major QTL for resistance to *M. phaseliona* on Chr. 15 explaining 29.4% of phenotypic variation, and two minor QTL's on Chr. 16 explaining 25.4% and 8.4% of phenotypic variation.

The QTL-seq strategy includes construction of two extreme phenotypic bulks selected from the F_{2-3} population used for QTL mapping and pooling equal amounts of DNA from 10 plants of each bulk. The two bulk DNA pools along with DNA of parents were sequenced using Illumina HiSeq™ (PE150, Q30≥80%). A SNP-index was calculated at each SNP position for both Resistant (R) and Susceptible (S) bulks using the PI567562A soybean genome as the reference. Subsequently, the average distributions of the SNP-index and Δ SNP-index across the genome were estimated in a given genomic interval using sliding window analysis, with 2-Mb window size and a 10-kb step. The analysis resulted in identification of three genomic regions on chromosomes 5, 8 and 14 with positive values of Δ SNP-index plots that potentially correspond to resistance QTLs governing the difference between the R-bulk and S-bulk.

In conclusion, the results supported SPA screening as a reliable, consistent and effective method for evaluating soybean genotypes for resistance to charcoal rot. Furthermore, the present study is the first attempt to characterize QTLs for resistance to CR, and likewise, employing QTL-seq analysis to identify genomic regions that may have major QTLs controlling CR disease resistance, and provide the basis for further characterization of the underlying genetic mechanisms that determine disease resistance to *Macrophomina phaseolina* in soybeans.