ABSTRACT

ROBINSON, KEITH O'NEAL. Rhizobitoxine-induced chlorosis in soybean. (Under the direction of JOSEPH W. BURTON)

The purpose of this research was to understand the soybean response to rhizobitoxine-induced chlorosis. This study consisted of four objectives: (1) to study the inheritance of soybean susceptibility to rhizobitoxine, (2) to locate molecular markers that are associated with rhizobitoxine susceptibility in the soybean genome, (3) to screen important soybean ancestors to determine how common rhizobitoxine susceptibility might be in modern cultivars, and (4) to determine if rhizobitoxine-producing strains affect soybean yield in field grown soybeans.

Based on chi-square analysis, the F_2 progeny segregated in a 9 susceptible to 7 resistant ratio, indicating that there are two genes responsible for the soybean response to rhizobitoxine-induced chlorosis. This ratio was confirmed in the screening of the $F_{2:3}$ population. In addition, results from F_1 plants showed that genes for susceptibility to rhizobitoxine-induced chlorosis are dominant to genes for resistance to rhizobitoxine-induced chlorosis. SSR markers were used to locate the genes responsible for susceptibility to rhizobitoxine-induced chlorosis. A total of 455 markers were used in this study and 141 (31%) of them were polymorphic for the parents Brim (susceptible) and CNS (resistant). A gene was identified by marker Satt 657 on LG F that was found to be highly significant and explained 32% of the phenotypic variation among F_2 plants based on the p-value and R^2 of a single factor ANOVA. The other gene was not found and further work is needed to determine its location. The results of the ancestor soybean screening revealed that the frequencies of alleles that are susceptible to rhizobitoxine-induced chlorosis are more common in southern

soybean ancestors than in northern soybean ancestors. It was also shown that soybean ancestors that are resistant to rhizobitoxine-induced chlorosis can potentially pass on susceptible alleles to progeny. A field study on the effects of rhizobitoxine-producing strains on yield of soybean showed that these strains can reduce yields 8 – 13 % when compared to non-rhizobitoxine producing strains. These results are similar to earlier reports that rhizobitoxine-producing strains can lower yields in field grown soybean.

The results of this study can be of benefit to soybean breeders in the southeastern portion of the United States, as this is the area where rhizobitoxine-producing strains are found in the greatest abundance.

RHIZOBITOXINE-INDUCED CHLOROSIS IN SOYBEAN

By

KEITH O'NEAL ROBINSON

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Chair of Advisory Committee

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DEDICATION

I would like to dedicate this dissertation to my wife, Darilyn, and my two sons, Keion and Kendrick, for their patience and support during my tenure as a graduate student.

BIOGRAPHY

Keith O'Neal Robinson was born June 13, 1969 in Portsmouth, Virginia. He received his elementary and high school education in his home town of Ettrick, Virginia where he graduated from Matoaca High School in 1987. He attended Old Dominion University and received a B.S. in Biology in 1992.

After receiving his degree from Old Dominion University, he began working as a Quality Control Manager at Stewart Foods, Inc. in Norfolk, Virginia where he conducted microbial analysis of the company's product in addition to supervising quality control personnel and working with USDA and FDA inspectors for three and a half years.

In the fall of 1995, Keith was accepted as a graduate student at Virginia State University where he majored in Biology. It was during his time at Virginia State University that he was introduced to plant breeding and agricultural research by working with Dr. Harbans Bhardwaj. Under Dr. Bhardwaj, Keith evaluated lupin cultivars and bradyrhizobium strains to determine which cultivar-strain combinations were best at fixing nitrogen. The work that he did under the tutelage of Dr. Bhardwaj increased his interest in the field of plant breeding, and after graduating in 1997 with a Master of Science in Biology he decided that he would pursue a doctorate in plant breeding.

In the fall of 1998, Keith began working in the soybean breeding group of Dr. Joe Burton and Dr. Tommy Carter at North Carolina State University in Raleigh, North Carolina. During this time he applied for the graduate program in Crop Science at North Carolina State University and was accepted into the graduate program to begin his studies for a doctorate in

Plant Breeding with a minor in Biotechnology under the guidance of Dr. Joe Burton in the spring of 1999.

The author is a member of the American Society of Agronomy and Crop Science Society of America. He is married to Darilyn McKan Robinson and is the proud father of Keion and Kendrick Robinson.

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I would also like to thank Dr. H. Roger Boerma at the University of Georgia, for allowing me to conduct part of my research in his laboratory. Your help and kindness is greatly appreciated. Thanks also to his staff for their technical assistance.

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Finally, I would like to express my deepest appreciation to my wife, Darilyn, for carrying the load and taking care of our family as I pursued my doctorate degree. I could not have succeeded without her help and sacrifice.

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INTRODUCTION

Soybean (*Glycine max (L.)* Merrill) is a member of the legume family, Leguminosae. Like most legumes, soybean has a symbiotic relationship with certain bacterial strains that helps the plant by converting atmospheric nitrogen to ammonium that can be utilized by the plant. The bacterial strains that aid in this process belong to the genus *Bradyrhizobium.* Previously, all bacteria that fixed nitrogen in a symbiotic relationship with soybean were classified in the genus *Rhizobium* and strains were grouped as fast- or slow-growing. Both fast-growing and slow-growing strains fix nitrogen when associated with soybean. Jordan (1982) separated the slow-growing bacteria from the fast-growing bacteria based on morphology, physiology, and DNA homology. Based on his work, the slow-growing bacteria were put in a new genus called *Bradyrhizobium*. The symbiotic relationship between the plant and the bacteria gives rise to the development of nodules, which are the plant organs where nitrogen is fixed by bacteria. The plant host provides the bacteria with carbon and energy, and the bacteria in turn provide the plant with reduced nitrogen. Usually this symbiosis is beneficial to the plant as well as the bacteria. Occasionally, however, this symbiosis leads to the soybean plant developing a bacterial-induced chlorosis known as rhizobitoxine.

This chlorosis has been studied extensively, but much of this research has dealt with identifying symbiotic strains that induce chlorosis and characterizing the effects of rhizobitoxine-induced chlorosis on soybean development. Little is known about the genetics of the soybean response to rhizobitoxine. The objectives of this research were to (1) determine the inheritance of soybean susceptibility to rhizobitoxine, (2) locate molecular

markers that are associated with rhizobitoxine susceptibility in the soybean genome, (3) screen important soybean ancestors to determine how common this trait might be in modern cultivars, and (4) determine if rhizobitoxine-producing strains affect soybean yield in field grown soybeans.

REVIEW OF LITERATURE

Rhizobitoxine (2-amino-4-(2-amino-3-hydropoxy)-*trans*-but-3-enoic acid) is a phytotoxin that is synthesized by the legume symbiont *Bradyrhizobium elkanii* (Owens and Wright, 1964) and the plant pathogen *Burkholderia andropogonis* (Mitchell et al., 1986). This phytotoxin causes rhizobial-induced chlorosis of newly formed soybean leaves (Erdman et al., 1957; Johnson et al., 1959; La Favre and Eaglesham, 1986; Owens and Wright, 1964). Rhizobitoxine is an enol-ether amino acid, whose structure and configuration have been identified (Keith et al., 1975; Owens et al., 1972). The chlorosis caused by rhizobitoxine is a result of the synthesis of the toxin in the nodules and chlorosis usually does not appear prior to the second trifoliate leaf and sometimes may not appear until much later in the development of soybean (Johnson et al., 1958).

Johnson and Clark (1958) showed that rhizobitoxine originates within the root nodules of soybean. Using grafting procedures, they grafted top growth of chlorosis resistant cultivars onto chlorosis susceptible rootstock and observed chlorosis on leaves. However, when chlorosis susceptible cultivars where grafted onto chlorosis resistant rootstock, they did not observe any symptoms, thus showing that rhizobitoxine is derived in the nodules as opposed to the leaf. This also shows that resistance is probably not due to detoxification by the whole plant.

Rhizobitoxine has been isolated from nodules of susceptible soybean plants, but not from resistant plants. In a greenhouse study, Owens and Wright (1964) showed that susceptible varieties displayed a rhizobitoxine-induced chlorosis, and resistant varieties did not when inoculated with rhizobitoxine-producing strains USDA 76 and USDA 94. They

reported that as little as 20 μg of purified toxin caused chlorosis in susceptible varieties but not in resistant varieties. Resistant varieties maintained resistance even when inoculated with 80 μg of purified toxin; however, when these resistant varieties were inoculated with 200 μg of purified toxin they did develop chlorosis. The results from this study show that resistant varieties can display chlorosis if exposed to high levels of rhizobitoxine. They also reported that all susceptible varieties produced an unknown amino acid they called Y (now known as dihydrorhizobitoxine, an intermediate in the synthesis of rhizobitoxine). The resistant varieties, however, did not produce the unknown Y.

Using paper chromatographic analysis, Owens and Wright (1964) reported that neither rhizobitoxine nor the unknown Y (dihydrorhizobitoxine) were found in leaves of the non-chlorotic plants, but did find both substances in the leaves of chlorotic plants. In addition, they found that rhizobitoxine and the unknown Y (dihydrorhizobitoxine) were not found in the older, non-chlorotic leaves of susceptible plants. They concluded that resistance is derived from the soybean plant preventing the synthesis of rhizobitoxine or the accumulation of rhizobitoxine in the nodules.

Owens and Wright (1965) reported that rhizobitoxine as well as dihydrorhizobitoxine can also be synthesized by *Bradyrhizobium* strains in pure culture. In their study, the rhizobitoxine-producing strain USDA 94 (a strong rhizobitoxine-producing strain) was synthesized and accumulated the phytotoxin when it was cultured in synthetic yeast extract and yeast extract plus casamino acids media, but not when cultured in synthetic medium supplemented with casamino acids. Based on these results, the authors suggested that the proportion of nutrients in the medium is a critical factor in determining whether toxin is produced as opposed to the presence or absence of certain nutrients.

Rhizobitoxine-producing strains have been shown to be common in other parts of the world, such as Africa, Central America, and South America (Boddey and Hungria, 1997; La Favre and Eaglesham, 1986). Boddey and Hungria (1997) studied *in vitro* and *in vivo* characteristics of 40 soybean *Bradyrhizobium* strains to determine the phenotypic grouping of the most studied and most used Brazilian *Bradyrhizobium* strains as well as isolates from Cerrado soils. Cerrado soils comprise approximately 25% of the land in Brazil and were initially free of *Bradyrhizobium* strains. Soybean inoculation began in Brazil in the 1960s with many of the strains used coming from other countries, particularly the United States. This region has high temperatures and periods of extended water stress. The parameters studied *in vitro* included colony morphology, serological grouping, intrinsic resistance to antibiotics, synthesis of indole acetic acid (IAA), expression of hydrogenase activity, and growth in a medium enriched with asparagine. The *in vivo* analyses included the nodulation of *Rj4* soybean cultivar Hill and the detection of symptoms caused by rhizobitoxine. The *Rj4* gene gives an ineffective nodulation response with *Bradyrhizobium* strains USDA 61, USDA 62, USDA 83, USDA 94, USDA 238, and USDA 259 (Vest and Caldwell, 1972; Devine and O'Neill, 1986). Comparing the Brazilian strains with *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*, they showed that the Brazilian strains and isolates from the Cerrado region were similar to *B. elkanii* in antibiotic resistance, Hup^- (hydrogen uptake) phenotype, inhibition in the presence of asparagine, and rhizobitoxine-induced chlorosis of soybean. Based on these results, the authors determined that most of the Brazilian strains as well as the isolates from the Cerrado region were grouped in *B. elkanii*. La Favre and Eaglesham (1986) showed that out of ninety-three strains used in their study, fifty-six percent of these were chlorosis-producing strains. In the United States, rhizobitoxine-producing strains have been

observed in fields in Alabama, Arkansas, California, Florida, Georgia, Mississippi, North Carolina, and South Carolina (Erdman et al., 1957). Weber et al. (1989) analyzed nodules from 27 states of major soybean production areas to determine the serological composition of the nodules. The authors showed that the major serogroup was USDA 123 (a rhizobitoxine-nonproducing strain) which made up 24% of the nodules tested. However, USDA 31, a rhizobitoxine-producing strain, made up 14% of the nodules tested. These strains have been shown to occur in high frequencies in the southeastern United States and made up 37% of the nodules tested from the southern states. *B. elkanii* may tolerate higher soil temperatures than *B. japonicum* which may explain their higher frequencies in the southern portions of the United States. The authors did not find any rhizobitoxine-producing strains in the Midwest. Fuhrmann (1990) showed that 37% of the nodules sampled from 18 farms in Delaware had chlorosis-inducing *Bradyrhizobium* strains. A study by Streeter (1994) reported that 40% of the nodules sampled from sites in 25 states had *B. elkanii* strains. Keyser et al. (1984) conducted a survey of *Bradyrhizobium* strains from 12 states to determine Hup (hydrogen uptake system) phenotype and serogroup identity. A Hup^+ system is desirable in *Bradyrhizobium* because it increases the efficiency of symbiotic nitrogen fixation. Unfortunately, it has been shown that more than 75% of the soybeans sampled in a 28 state survey were Hup^- (Lim et al., 1981). Keyser et al. (1984) reported in their survey that only 20% of the isolates were Hup^+. In addition, they found that the most common serogroup in their survey was USDA 31. USDA 31 was the most common serogroup in five of the 12 states surveyed. These states included Arkansas, Delaware, Florida, Kansas, and Louisana. It was found in high frequencies in North Carolina and Mississippi as well. The high occurrence of USDA 31 in Kansas is interesting as it has been reported that rhizobitoxine-

producing strains are not common in the Midwest (Weber et al., 1989). USDA 76 was the third most common strain found in this study and it too is a rhizobitoxine-producing strain. This strain is a stronger rhizobitoxine-producer compared to USDA 31 and it was prevalent in Mississippi, North Carolina, and Florida which is consistent with other reports of rhizobitoxine-producing strains in higher frequencies in the southeastern portions of the USA (Caldwell and Hartwig, 1970; Weber et al., 1989).

Difference between *B. japonicum and B. elkanii*

Chlorosis causing bacterial strains that express rhizobitoxine production belong to the species *Bradyrhizobium elkanii*. Though both *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* are capable of nodulating soybean, they do differ. Hollis et al, (1981) reported that there are two species of *Bradyrhizobium* that are genetically distinct and divided them into two groups, Group I and Group II. Devine et al. (1988) proved that the strains in Group II caused chlorosis. They did this by testing 25 *Bradyrhizobium* strains with soybean line N53-3494, which is susceptible to rhizobitoxine-induced chlorosis. Sixteen of the strains tested belonged to DNA homology Group I and Ia and none of these strains caused chlorosis on N53-3494. Of the remaining nine strains used in this study, five caused chlorosis on N53-3494. A study by Minamisawa, (1990) reported that *B. japonicum* consisted of two highly, evolutionarily divergent lines that differed in phenotype, one of which was rhizobitoxine production. These strains were reported to have three similar characteristics: (1) the ability to nodulate the nodulation restrictive soybean genotype, *rj1*, which restricts nodulation by a broad range of rhizobia (2) the ability to cause rhizobitoxine symptoms on soybeans, and (3) the ability to cause nodule-like swellings on peanut roots

(Devine and Weber, 1977; Devine et al., 1983). La Favre and Eaglesham, (1984) found in their study that the ability to nodulate *rj1* was not linked to ability to produce rhizobitoxine.

Other than rhizobitoxine production, the separation of the two species is also based on other characteristics such as extracellular polysaccharide composition, *ex planta* nitrogenase activity, and hydrogenase phenotype (Minamisawa, 1989). They also differ in intrinsic antibiotic resistance, fatty acid composition (Kuykendall et al., 1988), and indole-3-acetic acid (IAA) production (Minamisawa and Fukai, 1991). Based on these differences and genetic studies (Kuykendall et al., 1992; Minamisawa, 1990), Group II was reclassified as a new species, *Bradyrhizobium elkanii* (Kuykendall et al., 1992). *B. elkanii* strains also differ from *B. japonicum* in host preferences (Minamisawa et al., 1997) and *B. elkanii* strains cause outer cortical swelling in soybean roots (Yuhashi et al., 1995). It was reprted that outer cortical swelling in soybean was shown to be caused by a specific response to inoculation with *B. elkanii* and was seen ten days after inoculation. In addition, high levels of serinol are found in nodules formed by *B. elkanii* (Minamisawa and Watanabe, 1986; Minamisawa and Kume, 1987).

The ability to synthesize rhizobitoxine appears to be limited to the slow-growing *B. elkanii* (Minamisawa et al., 1990) and *Burkholderia andropogonis* (a plant pathogen) (Mitchell et al., 1986). *B. elkanii* was found to be more competitive than *B. japonicum* for nodulation of *Macroptilium atropurpureum* (commonly known as siratro) in a multi-strain environment (Minamisawa et al., 1997). *B. elkanii* accumulates rhizobitoxine in cultures and in nodules, but *B. japonicum* does not (Devine et al., 1988; Kuykendall et al., 1992). Nodule occupancy is higher in *B. japonicum* than in *B. elkanii* with *G. max*, but *B. elkanii* is higher with *M. atropurpureum* (Minamisawa et al., 1997). *B. japonicum* was shown to have a higher

nitrogen fixation capacity and higher nodule efficiency (mg N mg^{-1} of nodules) when compared to *B. elkanii* (Hungria et al., 1998), but *B. elkanii* strains have been shown to produce a greater number of nodules than *B. japonicum* (Teaney III and Fuhrmann, 1992).

The serogroups of *B. japonicum* and *B. elkanii* also differ; serogroups 6, 110, 122, 123, and 135 belong to *B. japonicum* and serogroups 31, 46, 76, and 94 belong to *B. elkanii* (Hollis et al., 1981). In addition to the differences listed above, *B. japonicum* and *B. elkanii* also differ in nod factors they produce (Stacey et al., 1995). Nod factors are lipooligosaccharide nodulation signals required for nodulation (Carlson et al., 1993).

Inhibitory effects of rhizobitoxine

It has been reported that rhizobitoxine is a strong inhibitor of β-cystathionase in *Salmonella* (Owens et al., 1968). Using growth experiments, the authors showed that at a concentration of 0.25 mM, rhizobitoxine stopped the growth of *S. typhimurium* for approximately 25 minutes, after which time growth continued at 60% of its normal rate. When methionine or homocysteine was added, growth inhibition was prevented. When methionine-less *S. typhimurium* mutants, me-B and me-C, were studied they found that their growth was not affected by rhizobitoxine when homocysteine was added. However, one of the mutants, me-B, showed a two-fold increase in doubling time in the presence of rhizobitoxine and cystathionine. The mutant me-B showed toxin inhibition when the methionine source was cystathionine. However, it did not show toxin inhibition when the methionine source was homocysteine. Based on these results, the authors suggested that the toxin inhibited the cleavage of cystathionine to form homocysteine, suggesting a methionine deficiency.

Low levels of rhizobitoxine have also been shown to inhibit β-cystathionase in plants as well as bacteria (Giovanelli et al., 1971; Owens et al., 1968). Owens et al. (1971) wanted to determine the ability of rhizobitoxine to act as an *in vivo* inhibitor of ethylene biosynthesis from methionine and reported that rhizobitoxine inhibited ethylene production. The authors used sorghum seedlings and senescent apple tissue to show that rhizobitoxine inhibits ethylene biosynthesis by blocking the conversion of methionine to ethylene as methionine has been shown to be a precursor of ethylene in plant tissues (Lieberman et al., 1966). The sorghum seedlings were transplanted to Erlenmeyer flasks constructed with a side arm to collect CO_2. Three millimeters of gas were withdrawn for ethylene assay. The apple tissue samples were also placed in Erlenmeyer flasks that contained an aqueous solution and a small vial of KOH to absorb CO_2. The results showed that rhizobitoxine inhibited ethylene production about 75% in both the sorghum seedlings and the senescent apple tissue with moderately low levels of rhizobitoxine (2 μM for sorghum and 12 μM for the senescent apple). The addition of methionine did not completely prevent rhizobitoxine inhibition but did partially relieve rhizobitoxine inhibition of ethylene production by 75% in sorghum and 60% in the senescent apple. In addition, a precursor of methionine, homoserine, showed no effect on the inhibition of ethylene production by rhizobitoxine. Based on these results the authors concluded that the inhibition of ethylene production by rhizobitoxine was caused by a block in some step of the conversion of methionine to ethylene. However, inhibition of ethylene biosynthesis is not involved in the development of chlorosis. It has been reported that chlorosis is possibly caused by the inhibition of glutamyl-tRNA synthase (Mattoo et al, 1979), an enzyme involved in the synthesis of δ-amino-levulinic acid.

Biosynthetic pathway of rhizobitoxine

The biosynthetic pathway for rhizobitoxine has not been fully elucidated. Mithchell and Coddington (1991) studied the biosynthetic pathway of rhizobitoxine in *Pseudomonas andropogonis*. *P. andropogonis* is plant pathogen with a very wide host range that also produces rhizobitoxine. In their study, they studied the kinetics of production of rhizobitoxine and hydroxythreonine and showed that the appearance of hydroxythreonine in the medium precedes that of rhizobitoxine. It has been suggested that rhizobitoxine and hydroxythreonine are derived from aspartic acid and that hydroxythreonine is an intermediate in the biosynthetic pathway of rhizobitoxine.

Ruan et al. (1993) used mutants of rhizobitoxine-producing strain USDA 61 that differed in their ability to cause rhizobitoxine-induced chlorosis to determine the biosynthesis of rhizobitoxine. Each mutant contained a single Tn5 insertion (Tn5-17E and Tn5-18E) and the DNA sequence was determined for DNA surrounding the two Tn5 insertions. Based on their analysis of the DNA, they discovered two overlapping open reading frames, each interrupted by one of the Tn5 insertions. The open reading frame interrupted by Tn-18E was designated *rtxA* and the open reading frame interrupted by Tn-17E was designated *rtxB*. They suggested that *rtxA* is involved in serinol formation and dihydrorhizobitoxine synthesis. They also suggested that a translational frameshift is required for the expression of *rtxB*. Based on their findings they reported that *rtxA* plays a role in serinol biosynthesis and that *rtxB* plays a role in dihydrorhizobitoxine synthesis. They proposed that dihydrorhizobitoxine synthase would form dihydrorhizobitoxine from serinol and homoserine.

Yasuta et al. (2001) investigated the rhizobitoxine biosynthetic pathway of *B. elkanii* in culture by mutagenesis of the *rtxA* gene (serinol biosynthesis). They used an insertional

mutant of *rtxC* and showed that it produced serinol and dihydrorhizobitoxine but no rhizobitoxine. They also confirmed that dihydrorhizobitoxine is a key intermediate in rhizobitoxine biosynthesis. Their results showed that the *rtxA* gene is responsible for serinol formation and dihydrorhizobitoxine biosynthesis which are two important steps in rhizobitoxine biosynthesis. They hypothesized that serinol is possibly a precursor of dihydrorhizobitoxine due to the fact that the *rtxA* gene is involved in both serinol formation and dihydrorhizobitoxine biosynthesis. The authors concluded that *rtxC* encodes dihydrorhizobitoxine desaturase for the final step of rhizobitoxine production. Dihydrorhizobitoxine [O-(2-amino-hydroxypropyl) homoserine] has been found in cultures and nodules of *B. elkanii* (Owens et al., 1972). These results indicate that at least *rtxA* and *rtxC* are necessary for rhizobitoxine production.

Rhizobitoxine effect on soybean

Erdman et al (1957) conducted a study to determine the degree of susceptibility of 39 soybean varieties to bacterial-induced chlorosis. Their study was done in a greenhouse in pots with sand and seeds were inoculated with rhizobitoxine-producing strains USDA 31, USDA 76, and USDA 77. In this study, the researchers called USDA 31 a rhizobitoxine-nonproducing strain when in fact it is a rhizobitoxine producer. The soybeans used in this study had a wide range of photoperiod sensitivities. They reported a variety of symptoms ranging from plants that showed no chlorosis to plants that were highly susceptible. They reported that plants receiving the rhizobitoxine-producing strains had higher vegetative yields than those plants receiving the rhizobitoxine-nonproducing strains. However, these researchers were not comparing a rhizobitoxine-producing strain with a rhizobitoxine-nonproducing strain because the USDA 31 strain is a rhizobitoxine-producing strain.

Johnson and Means (1960) also conducted a greenhouse study on the relationship of rhizobitoxine-producing strains and soybean genotypes. Their study consisted of four phases: (1) 116 soybean varieties, in various maturity groups were screened with the rhizobitoxine-producing strain USDA 76; (2) four of these varieties (Hawkeye, Lee, Grant, and N53-3494) were screened with 105 bacterial strains; (3) eleven varieties that represented a range of susceptibility to USDA 76 were inoculated with eight strains known to stimulate chlorosis in one or more varieties; and (4) all resistant varieties were re-inoculated with USDA 76 as well as three other rhizobitoxine-producing strains. They reported 94 of the 116 (81%) soybean varieties were susceptible. They also screened *G. gracilis*, *G. ussuriensis*, *G. tomentosa*, and three introductions of *G. max*. All of these developed rhizobitoxine induced chlorosis when inoculated with the rhizobitoxine-producing strain USDA 76. Of the 105 bacterial strains used in this study, 46 were considered normal (i.e. does not produce rhizobitoxine) on all four varieties and 24 of these strains caused chlorosis on one or more of the soybean varieties (one was omitted). Thirty-four produced chlorosis on all four varieties. In addition, eleven of these 34 strains were isolated from plants in North Carolina and caused severe chlorosis on the four soybean varieties tested. The response of the 11 varieties to 8 strains showed that there was a significant amount of diversity ranging from some varieties being susceptible to all strains to varieties only susceptible to the most virulent strain. Finally, the resistant varieties that were re-inoculated with four chlorosis-inducing strains showed that of the original varieties that were resistant (22 in all); only seven were completely resistant to rhizobitoxine-induced chlorosis. This shows that susceptibility may be “normal” reaction of the soybean response to inoculation with rhizobitoxine-producing strains.

The earlier studies showed that there is much variability in the soybean response to rhizobitoxine. To determine the effects of environmental factors on the soybean response to rhizobitoxine, Johnson et al (1958) conducted a greenhouse study to test six soybean varieties (Blackhawk, Clark, Hawkeye, Lee, Harosoy, and CNS) that varied in rhizobitoxine susceptibility and phenotypic expression with rhizobitoxine-producing strain USDA 76. The environmental factors examined were modifications of nutrients in a mineral nutrient solution, three different sand substrates (unwashed quartz sand, unwashed quartz sand that had been thoroughly washed prior to use, and a washed river sand), comparison of plants watered with distilled water versus a nutrient solution, and cotyledon removal from seedlings at the unifoliate stage. The authors wanted to determine if any of these factors had an effect on the expression of rhizobitoxine-induced chlorosis of soybeans. They reported that factors such as modifications of nutrient solution and sand substrate, as well as cotyledon removal treatments significantly influenced the beginning and severity of chlorosis in soybeans. They showed that different compositions of nutrient solutions can cause rhizobitoxine-induced chlorosis and that some combinations of nutrients were shown to cause more rhizobitoxine-induced chlorosis in soybean than other combinations. They also reported that washed river sand was better at inducing rhizobitoxine. In addition they reported that when plants were cultured with water alone as opposed to nutrient solution that even the resistant varieties (CNS and Harosoy) displayed chlorosis. The removal of the cotyledons only affected one of the six soybean cultivars used in this study. The six soybean cultivars used in this study were Blackhawk, Clark, Hawkeye, Lee, Harosoy, and CNS. Of these, only Blackhawk was affected by cotyledon removal. This variety showed light chlorosis without the removal of the cotyledons or the removal of the cotyledons at the first trifoliate stage. When one

cotyledon was removed at the unifoliate stage, Blackhawk exhibited moderate chlorosis and when both cotyledons were removed it exhibited moderately severe chlorosis. The onset of chlorosis was also affected by cotyledon removal in Blackhawk. When cotyledons were removed at the unifoliate leaf stage, chlorosis appeared 21 to 26 days after planting and when the cotyledons were left intact chlorosis did not appear until 33 days after planting. Their results indicate that there is a genotype-environment interaction that may play a role in the induction of rhizobitoxine. It should be noted that in some instances, even resistant varieties showed signs of susceptibility depending on the variable being tested. That is, some plants that were reported resistant were scored as susceptible when certain nutrient solutions were used. The genotype-environment interaction can also be seen in the differences in the soybean response to the sand substrates. It shows that there may be minerals or nutrients in these sand substrates that play a role in the expression of susceptibility to rhizobitoxine. This is shown also in the fact that plants that were watered with only distilled water, even those considered resistant, displayed chlorosis. Again, this indicates that there may be certain nutrient combinations that enhance the expression of chlorosis due to rhizobitoxine production.

Teaney III and Fuhrmann (1992) conducted a study to determine if rhizobitoxine-producing strains had any effect on soybean response to nodulation. In a greenhouse study they evaluated the effects of rhizobitoxine on short term shoot productivity, nodulation, nitrogen fixation, and nodule protein production. In this study they used rhizobitoxine-producing strains USDA 31, 46, 76, and 94. In addition, rhizobitoxine-nonproducing strains USDA 110 and 123 were used as controls. Rhizobitoxine was detected in nodules of all rhizobitoxine-producing strains except for USDA 31(which is not as strong a rhizobitoxine-

producer compared to USDA 76 and USDA 94) and only USDA 76 and USDA 94 produced enough rhizobitoxine that could be quantified and showed symptoms. The authors did find that with moderate to severe chlorosis that there were reductions in chlorophyll concentrations, shoot and nodule dry weight, leaf protein, and total nitrogen fixation. Despite these results, they concluded that nodulation by rhizobitoxine-producing strains has a negligible effect on soybean productivity; however, Erdman et al. (1957) reported that rhizobitoxine-producing strain USDA 76 decreased vegetative yield as well as pod weight.

Studies have shown that rhizobitoxine-producing strains cause less severe chlorosis in the field than in the greenhouse. In the greenhouse environment, soybeans are grown in nitrogen-free media. In contrast, field-grown studies involving rhizobitoxine-producing strains are subjected to some nitrogen from the soil. Based on this, Teaney III and Fuhrmann (1993) conducted a study to determine the effects of nitrate on the expression of rhizobitoxine-induced chlorosis. In this greenhouse study, soybean plants were inoculated with rhizobitoxine-producing strains USDA 76 and USDA 94 and received nutrient solutions with various amounts of nitrate. Interestingly, they showed that the symptoms of chlorosis decreased when nitrate applications were increased. They concluded that nitrate can reduce the chlorosis on soybean plants and that this may be a reason why the symptoms of rhizobitoxine are not as severe in the field as they are in the greenhouse.

La Favre et al. (1988) studied the colony types of rhizobitoxine-producing strain USDA 76 with special reference to production of rhizobitoxine and to their symbiotic relationships with nodulating and non-nodulating soybean. Single colonies were obtained by inoculating soybeans with high doses of USDA 76 (rhizobitoxine-producing) or RCR 3410 (rhizobitoxine-nonproducing) and growing them in an environmentally controlled room. The

plants were then harvested and nodules were crushed and plated on yeast extract mannitol agar. Another way that single colonies were isolated was by growing the *Bradyrhizobium* strains in yeast extract mannitol broth. The broth was then diluted and streaked on yeast extract mannitol agar. After nine days, large and small colonies were individually isolated. The authors used isolines of Lee soybeans that differed in their nodulation (*Rj1Rj1* vs. *rj1rj1*) to evaluate the nodulation characteristics of colony-type derivatives. They showed that USDA 76 had two colony types; small and large. The small colony type produced chlorosis-inducing toxin in culture; however, the small and the large colony types both induced chlorosis as a result of their synthesis of the toxin in nodules. By using electron microscopy, they showed that the large colony type was encapsulated and that the small colony type was not encapsulated. The investigators tested the role of rhizobitoxine in nodulating non-nodulating soybean. Using Lee (*Rj1Rj1*) and Lee (*rj1rj1*), they inoculated them each with the small and large colony types. The toxin-producing small colony type was less effective on Lee (*rj1rj1*) than the large colony type. From this, they concluded that rhizobitoxine does not play an important role in nodulating non-nodulating soybean as described by Devine and Weber (1977).

The phenotypic expression of rhizobitoxine is clearly manifested in the greenhouse, but is rarely expressed in the field. In fact, many of the studies conducted on rhizobitoxine have been performed in the greenhouse. Though these studies can give useful information on the effects of rhizobitoxine on soybean in a controlled environment, it does not give much information on its effect on soybean in the field. One of the difficulties of studying the effects of rhizobitoxine in the field is the competition between native strains and introduced strains. Introduced *Bradyrhizobium* strains have been shown to occupy as little as 10% of the

nodules formed by soybean when grown in soil containing native *Bradyrhizobium* strains (Ellis et al., 1984).

Fuhrmann and Vasilas (1991) developed a method to overcome the problem of competition. Their method involves growing soybean seedlings inoculated with specific test strains in a greenhouse under controlled conditions and then transplanting these seedlings to the field. Using this method, they reported 97% to 99% of the nodules sampled from plants had the intended *Bradyrhizobium* strains when assayed. Fuhrmann and Vasilas (1993) used this technique to measure the response of the *Glycine-Bradyrhizobium* symbiosis to high initial levels of nodulation by selected strains of *Bradyrhizobium* when grown in soil containing native soybean bradyrhizobia. The plants were grown in the greenhouse and inoculated with USDA 122 (a superior nitrogen fixer), USDA 94 (a strong rhizobitoxine producer), or a soil suspension that contained a mix of the strains native to the location where the test was conducted. After 23 days, these plants were transplanted to the field. Nodules from the plants were sampled to determine if the intended strains were found in the nodules. They reported frequencies ranging from 94.5% to 99.5% occupancy of the intended strains, but the soil suspension showed substantial diversity in strain types. They reported significant increases in seed yield, the amount of nitrogen fixed, and total shoot nitrogen content when plants were inoculated with USDA 122 relative to the soil suspension. On the other hand, plants inoculated with USDA 94 showed a decrease in these variables. This study showed that the transplanting technique is very useful in measuring the effect of a given strain on soybean productivity.

Vasilas and Fuhrmann (1993) also used the technique developed by Fuhrmann and Vasilas (1991) to determine the response of soybean to nodulation by a rhizobitoxine-

producing strain. In their study, they inoculated the soybean cultivar Forrest with the USDA 94, a strong rhizobitoxine-producing strain, in the greenhouse and transplanted the seedlings to the field. Their results showed that the rhizobitoxine-producing strain not only caused chlorosis but also reduced plant nitrogen, reduced seed yields, decreased vegetative growth, delayed the onset of full pod, beginning seed and full seed stages. It also doubled the interval between beginning pod and full pod stages. Fuhrmann and Vasilas (1994) showed that there is variability among soybean genotypes in their response to nodulation by a rhizobitoxine-producing strain. The researchers conducted a greenhouse study to evaluate the response of soybean varieties to nodulation with USDA 94 as well as field study to determine if the genetic variability observed in the greenhouse would also manifest in the field. The greenhouse study showed that there was significant genotypic variability in soybean response to nodulation by USDA 94 and also that screening plants in the greenhouse could be used as a predictor for soybean response in the field.

In many studies that attempt to illustrate the role of rhizobitoxine production in *Bradyrhizobium*-soybean symbiosis, researchers have compared rhizobitoxine-producing strains and rhizobitoxine-nonproducing strains. One of the disadvantages of comparing *B. japonicum* strains with *B. elkanii* strains to determine if rhizobitoxine affects soybean production is that these are two genetically different species (Hollis et al., 1981). A better way to determine if rhizobitoxine is the cause of these results is to use genetically similar strains that differ only in rhizobitoxine production. Mutant strains have been developed for rhizobitoxine-producing strain USDA 61(Ruan and Peters, 1992), a weak rhizobitoxine-producing strain.

Ruan and Peters (1992) studied 11 rhizobitoxine mutants of USDA 61 that differed in their chlorosis phenotypes and rhizobitoxine production *in planta* to determine the role of rhizobitoxine in Bradyrhizobium-legume symbiosis. Their results showed that one group of mutants failed to make toxin and did not cause chlorosis on plants. They also reported a group of mutants that caused severe chlorosis on all cultivars of soybean tested. Compared to the wild type USDA 61, this mutant makes more rhizobitoxine in soybean nodules. The other mutants all produced rhizobitoxine *in planta* but the amount and severity of chlorosis varied. The authors showed that the amount of toxin in the nodules was proportionate to the severity of chlorosis. They also showed that resistant varieties could show chlorosis when inoculated with mutant strains that caused severe chlorosis in susceptible variety Lee. This result is interesting because it suggests that resistance may be due to the rhizobitoxine levels not accumulating to concentrations high enough to cause chlorosis in plants. In addition they tested the role of rhizobitoxine in nodulating *rj1* soybean using mutant strains of USDA 61 and reported that rhizobitoxine does not enhance nodulation of *rj1* soybeans. La Favre et al. (1988) also showed that rhizobitoxine does not play a role in nodulating *rj1* soybeans. The mutants used by Ruan and Peters (1992) are ideal in studying the affects of rhizobitoxine because these strains are genetically similar and only differ in their production of rhizobitoxine.

Using mutant strains of *B. elkanii* that lack the ability to produce rhizobitoxine, Xiong and Fuhrmann (1996) reported that plants nodulated with mutant strains had higher shoot weights than plants nodulated with the rhizobitoxine-producing wild type and concluded that these differences were likely due to rhizobitoxine production in the wild type. They also reported differences in shoot nitrogen content of soybean plants. Plants inoculated with the

mutant strain had higher shoot nitrogen concentrations than plants inoculated with rhizobitoxine-producing wild type.

In another study using mutant rhizobitoxine strains, Stokkermans et al (1992) reported that rhizobitoxine was involved in the ability of USDA 61 derivatives to overcome the *Rj4* nodulation restriction in soybean. USDA 61 is a rhizobitoxine-producing strain and soybeans with the *Rj4* gene have been shown to have ineffective nodulation by strain USDA 61 (Vest and Caldwell, 1972). The authors used an overproducing rhizobitoxine mutant strain of USDA 61 on BARC-2 soybean (*Rj4*) and reported that the overproducing mutant strains formed nodules at rate that was comparable to the rhizobitoxine-nonproducing strain USDA 110. However, when the BARC-2 soybean was inoculated with either a mutant strain that produced low levels of rhizobitoxine or the wild-type USDA 61, only a limited number of nodules were formed. The authors proposed that there is a positive relationship between the amount of rhizobitoxine made *in planta* and the ability of USDA 61 derivatives to nodulate *Rj4* soybeans.

Beneficial role of Rhizobitoxine

Despite the deleterious effects of rhizobitoxine on soybean, there have been a few studies that indicate that rhizobitoxine may have a beneficial role. *Macrophomina phaseolina* causes charcoal root rot and is a common disease of soybean in India. Chakraborty and Purkayastha (1983) conducted a study to determine if rhizobitoxine had an anti-fungal effect on charcoal root rot. They found that rhizobitoxine-producing strains did inhibit *M. phaseolina* in culture as well as *in planta*. When the roots were inoculated with *M. phaseolina* only, charcoal root rot was observed on soybean roots. However, when the roots

were inoculated with a rhizobitoxine-producing strain and then with *M. phaseolina* there was little to no charcoal root rot.

It has been hypothesized that rhizobitoxine plays a positive role in nodule development by inhibiting ethylene biosynthesis (Duodu et al., 1999). Using mungbean, the researchers screened for nodulation response to rhizobitoxine-producing strains and mutant strains that did not produce rhizobitoxine. They showed that when plants were inoculated with the mutant strains that they developed aborted nodules and that fewer mature nodules formed compared to the rhizobitoxine-producing strain. They also showed that adding other ethylene inhibitors, such as aminoethoxyvinylglycine and cobalt, that rhizobitoxine mutants were able to increase the number of mature nodules per plant. Some studies have shown that there are no significant differences in nodule number of plants inoculated with a mutant rhizobitoxine strain and a rhizobitoxine-producing strain (Ruan and Peters, 1992; Xiong and Fuhrmann, 1996).

Yuhashi et al (2000) suggested that rhizobitoxine might play a role in enhancing nodulation as well as competitiveness of B. elkanii on *Macroptilium atropurpureum*. *M. atropurpureum* is a leguminous plant known as siratro and is a nodulation host of both *B. elkanii* and *B. japonicum* (Kuykendall et al., 1992). Siratro is a perennial twining plant in the pea family and is native to Central and North America. When siratro seeds were planted in a multi-strain environment, the researchers reported that *B. elkanii* was more competitive than *B. japonicum* for nodulation. In another study these results were reported as well with another legume from the genus *Amphicarpaea*.

Using wild-type rhizobitoxine and mutant rhizobitoxine strains, Parker and Peters (2001) showed that when plants were inoculated with the wild type (i.e. rhizobitoxine-

producing strain) they had 150 or more nodules per plant. In contrast, those plants that were inoculated with mutant rhizobitoxine strains produced fewer than 10 nodules per plant. Minamisawa et al (1997) conducted a study to determine if *B. elkanii* and *B. japonicum* had host preferences in a multistrain environment. The hosts used in their study were *Glycine max*, *Glycine soja*, and *M. atropurepurum*. Their results showed that *B. japonicum* had a preference for nodulating *G. max* and that *B. elkanii* had a preference for nodulating *M. atropurepurum*. Both *B. japonicum* and *B. elkanii* were reported to have nodulated *G. soja*.

It has been suggested that rhizobitoxine may have potential as a herbicide (Owens, 1973). The author compared the herbicidal properties of rhizobitoxine with other herbicides. The herbicides used for comparison with rhizobitoxine were amitrole and metflurazone. The study consisted of post emergence test on various plant seedlings. The results showed that rhizobitoxine and amitrole were just about equal in their phytotoxicity and that both were much more phytotoxic than metflurazone.

CHAPTER I

INHERITANCE OF RHIZOBITOXINE-INDUCED CHLOROSIS IN SOYBEAN

INTRODUCTION

The relationship between soybean, (*Glycine max (L.)* Merrill), and bacterial strains belonging to the species *Bradyrhizobium japonicum* has been well established. This relationship is a symbiotic one in which the bacterial strains living in nodules on roots of soybean plants convert atmospheric nitrogen into ammonium that can be used by the soybean plant. In return, the plant provides sugars from photosynthesis that serve as an energy source for the bacteria.

In the 1950's a bacterial-induced chlorosis was found in the field on the soybean variety Lee (Erdman et al., 1956). This chlorosis was found in the newly developing trifoliates of soybean plants. By grafting top growth of plants resistant to rhizobitoxine-producing strains with rootstocks of plants susceptible to rhizobitoxine-producing strains, it was determined that the factor causing chlorosis was formed in root nodules of the soybean plants (Johnson and Clark, 1958). The top growth of chlorosis susceptible soybean varieties did not exhibit chlorosis when grafted to resistant soybean rootstock, but chlorosis was shown when the top growth of chlorosis resistant soybean varieties were grafted to susceptible soybean rootstock. In the early 1980's it was recognized that there are two distinct species of *Bradyrhizobium* which at that time were called Group I and Group II (Hollis et al, 1981). Later, Devine et al. (1988) demonstrated that the strains in Group II caused chlorosis. These strains were later reclassified as *Bradyrhizobium elkanii* (Kuykendall et al., 1992). It is now established that *Bradyrhizobium elkanii* produces rhizobitoxine, an

enol-ether amino acid (2-amino-4-(2-amino-3-hydropoxy)-*trans*-but-3-enoic acid), which causes chlorosis in susceptible soybean varieties.

Many studies have been conducted on rhizobitoxine and its effects on soybean growth. Research has shown that rhizobitoxine can have a negative effect on soybean. Based on greenhouse as well as field studies, rhizobitoxine has been shown to reduce chlorophyll concentrations, shoot and nodule dry weight, leaf protein, and total nitrogen fixation (Teaney III and Fuhrmann, 1992). In addition, Erdman et al. (1957) reported that rhizobitoxine-producing strains can decrease vegetative yield as well as pod weight. A genotype-environment interaction may play a role in expression of rhizobitoxine-induced chlorosis (Johnson et al., 1958). This was demonstrated in a greenhouse study in which modifications in nutrient solution and sand substrate as well as cotyledon removal treatments were used. The results of this study showed that the size and number of nodules were similar in all treatments. They also reported that all of the plants developed the most severe chlorosis when given nutrient solutions consisting of P, K Ca, Mg, S, and Cl at 31, 117, 47, 48, 57, 140 ppm, respectively. The results also indicate that expression of rhizobitoxine-induced chlorosis was greater with river-washed sand than unwashed quartz and that cotyledon removal had no effect on development of chlorosis. Vasilas and Fuhrmann (1993) showed that rhizobitoxine-producing strains not only cause chlorosis but also reduce plant nitrogen, reduced seed yields, decreased vegetative growth, delayed the onset of full pod, beginning seed and full seed stages. In addition it also doubled the interval between beginning pod and full pod stages.

Erdman et al., (1957) reported variability in the degree of susceptibility of 40 soybean varieties to rhizobitoxine-induced chlorosis. The degree of susceptibility in their study ranged

from highly resistant to highly susceptible. Fuhrmann and Vasilas (1994) also showed variability among soybean genotypes in response to rhizobitoxine. They evaluated 38 soybean varieties in the greenhouse for their response to rhizobitoxine and reported that varieties ranged from tolerant to very sensitive. They suggested that greenhouse results are a good predictor of rhizobitoxine susceptibility or resistance in the field.

Though much is known about how these strains affect soybean, the genetics of soybean response to rhizobitoxine has not been elucidated. Some cultivars are known to have both resistant and susceptible types, but the number of genes and their mode of action is unknown. The purpose of this study was to investigate the inheritance of soybean susceptibility to rhizobitoxine.

MATERIALS AND METHODS

The populations used in this study were derived from crosses CNS x Brim, Haberlandt x Ogden, CNS x Volstate, and Davis_S x Davis_R (Table 1-1). The resistant parents were CNS, Haberlandt, and Davis_R. The Davis_R parent was selected from a bulk population of the variety Davis which had both resistant and susceptible plants in a preliminary screening (data not shown). The susceptible parents were Brim, Ogden, and Davis_S. The Davis_S parents were selected from the same bulk population of the variety Davis in which Davis_R was selected and showed susceptibility to rhizobitoxine-producing strain CD2-5. Parents were selected on the basis of rhizobitoxine resistance and susceptibility and on the basis of flower color. Plants with white colored flowers were used as the female and plants with purple colored flowers were used as the males in order to successfully identify F1 plants and eliminate plants that were produced as a result of self-pollination. All purple F1 plants that were inoculated with the rhizobitoxine-producing strain were evaluated for susceptibility or resistance. This technique, however, could not be done with the Davis_S x Davis_R cross since both parents have white flowers; therefore all the F1 plants from this cross were evaluated for their response to the rhizobitoxine-producing strain. The F2 seeds used for this study were derived from crosses of rhizobitoxine susceptible and rhizobitoxine resistant plants. The crosses were made at Clayton, NC, August 2001. The F1 seeds were grown in a Puerto Rico winter nursery in 2001 to produce F2 seed. The study was conducted in a greenhouse and planted on May 17, 2002. All populations were planted on the same day. There were 150 F_2 plants evaluated in each of the four populations. In addition, 18 plants of each of the parents, which were used as checks, and 10 F_1 plants (except for the Davis_S x Davis_R population which only had 2 F1 plants) were evaluated for rhizobitoxine-induced

chlorosis with each of the F_2 populations. Plants were scored based on their phenotype as either resistant or susceptible when inoculated with a rhizobitoxine-producing strain (Figure 1-1).

Bacterial strain preparation

Two strains were used in this study: the rhizobitoxine-producing strain CD2-5, which was isolated from the soils of North Carolina (Ramirez et al, 1997b) and the non-rhizobitoxine producing strain MN-110 (Mathis et al, 1986). The cells of these bacterial strains were each streaked on separate Yeast Extract Mannitol (YEM) agar plates to generate inoculant for initiation of liquid culture. Several loops of inoculant from plates were transferred to 100 ml of YEM liquid media. When cultures reached stationary phase 5 ml was transferred to 500 ml of fresh YEM liquid media. The flasks were then put into a shaker at 28° C at a shaking speed of 150 rpm until stationary phase was attained (5-6 days). The seeds used in this study were inoculated with 0.5 ml of stationary phase culture from either the rhizobitoxine-producing strain (CD2-5) or the rhizobitoxine-nonproducing strain (MN 110) which contained 10^9 colony forming units (CFU) per ml.

Seed preparation and planting

Seed of the parent, remnant F1 seed, and F2 seed were sterilized by soaking them for one minute in 95% ethanol then decanting the ethanol. The seeds were then soaked in a 1:5 Clorox:water solution for 3 minutes. After decanting the 1:5 Chlorox:water solution, the seeds were then rinsed five times with sterile water. The seeds were kept in a beaker and covered with aluminum foil until time of planting to prevent desiccation.

Seeds were planted in 32 ounce cups that were filled with horticultural grade vermiculite. Prior to dispensing seeds, all cups were watered to saturation early in the

morning and given N free nutrient solution later that morning (usually two hours before planting). Seeds were planted in the afternoon with one seed planted in one cup. 0.5 ml of CD2-5 was directly placed on each F_1 and F_2 seed. Half of the parents of all crosses were inoculated with CD2-5 and the other half was inoculated with MN 110 as a control. The vermiculite was kept moist, but the cups were not watered to saturation as to not allow dripping of the water which may have washed away the bacterial strains. After five days, cups were thoroughly watered everyday and given N free nutrient solution twice a week (McClure and Israel, 1979). Symptoms began developing 25-30 days after planting and plants were scored as either resistant or susceptible. Hypothesized Mendelian ratios were tested with chi-square test for goodness of fit using PROC FREQ with the TESTP option in SAS.

RESULTS AND DISCUSSION

All of the resistant parents (CNS, Haberlandt, and Davis_R) that were inoculated with CD2-5 did not show the chlorotic phenotype and were considered resistant based on this phenotype. These resistant parents also did not show a chlorotic phenotype when inoculated with MN 110, a non-rhizobitoxine producing strain. All of the susceptible parents did display the chlorotic phenotype when inoculated with the rhizobitoxine-producing strain CD2-5, but with the non-rhizobitoxine producing strain, MN 110, did not show the chlorotic phenotype.

F_1 Results

The F_1 seed for each population was evaluated to determine if the genes for rhizobitoxine susceptibility in soybean were dominant or recessive (Table 1-2). Only purple flowered F_1 plants were evaluated for each population except the Davis_S x Davis_R population which had all its surviving F_1 plants evaluated. All of the 15 F_1 plants were susceptible to rhizobitoxine-induced chlorosis when inoculated with CD 2-5. The phenotype of these plants was similar to the phenotypes of the parents that were susceptible to chlorosis. Thus it appears that rhizobitoxine-susceptibility is dominant to resistance.

F_2 Results

The F_2 plants from each population were scored as either resistant or susceptible (Table 1-3). The results showed that three of the four populations segregated in a 9:7 (susceptible:resistant) ratio and the fourth population segregated in a 7:9 (susceptible:resistiant) ratio. Based on these results, it was hypothesized that the populations were segregating in a 9:7 (susceptible:resistant) ratio. This type of segregation pattern would suggest that there are two genes involved in rhizobitoxine susceptibility in soybean.

Such a segregation pattern in a dihybrid F_2 population is termed duplicate recessive epistasis (Fehr, 1993). To facilitate interpretation of results we designated *Rts1* and *Rts2* as alleles for rhizobitoxine susceptibility and *rts1* and *rts2* as alleles for resistance. Based on this proposed genotypic distribution two dominant alleles, one from each of two independent gene loci, are needed to express rhizobitoxine susceptibility as would be the case for plants with the *Rts1_Rts2_* genotype. Resistant genotypes would be double recessive at one (*Rts1_rts2rts2* and *rts1rts1Rts2_*) or both loci (*rts1rts1rts2rts2*).

As stated earlier, three of the four populations tested were consistent with a 9:7 (susceptible:resistant) ratio (Table 1-3). However, the Brim x CNS population did not fit the hypothesized 9:7 ratio. One possible reason for this discrepancy may have been misclassification. At the time of phenotypic evaluation, plants that were scored as resistant may not have been growing long enough to exhibit chlorosis so that some susceptible plants were misclassified as resistant. This population along with the Volstate x CNS population was tested further to verify their F_2 results.

$F_{2:3}$ results of the Brim x CNS and Volstate x CNS populations

Based on the proposed two gene, 9:7 (susceptible:resistant) model, the segregation ratio of the families is expected to be 7:8:1 (All resistant:Segregating:All susceptible) ratio. The resistant genotypes, *Rts1Rts1rts2rts2, rts1rts1Rts2Rts2,* and *rts1rts1rts2rts2*, when self-pollinated will not segregate and are the same phenotype (resistant) as their respective parents. The *Rts1rts1rts2rts2* and the *rts1rts1Rts2rts2* genotypes will segregate but all progeny will be resistant. The resistant genotypes cannot be distinguished from each other as all families that are derived from resistant genotypes will be completely resistant. The frequencies of these resistant families add up to 7/16 of the total. When considering the

susceptible genotypes, *Rts1Rts1Rts2Rts2* plants do not segregate and account for 1/16 of the total. The segregating susceptible genotypes are *Rts1Rts1Rts2rts2, Rts1rts1Rts2Rts2,* and *Rts1rts1Rts2rts2*, making up 8/16 of the total. Families derived from plants with the genotypes *Rts1Rts1Rts2rts2* and *Rts1rts1Rts2Rts2* will segregate 3:1 (susceptible:resistant). The families derived from plants with genotypes *Rts1rts1Rts2rts2* will segregate 9:7 (susceptible:resistant). When chi-square analysis was used to determine if the $F_{2:3}$ families fit the proposed 7:8:1 (All R : H : All S) ratio, it showed that neither of the $F_{2:3}$ populations differed significantly from the hypothesized ratio (Table 1-4). The results of the $F_{2:3}$ family data of the Brim x CNS population were used to re-evaluate the F_2 data to determine if some of the F_2 plants had been misclassified. Based on the $F_{2:3}$ data, 27 of the plants that were previously considered resistant when scoring the F_2 data were found to be susceptible. A total of 85 plants were re-evaluated after the F_2 data was reclassified based on the $F_{2:3}$ results. Of these 85 plants, 51 were scored as susceptible and 34 were scored as resistant. Chi-square analysis confirmed that the re-evaluated Brim x CNS did not differ significantly from the 9:7 (susceptible:resistant) ratio ($p = 0.4575$) (Table 1-6).

In conclusion, the results of this study suggest to a two gene model for rhizobitoxine-induced susceptibility in soybean with susceptibility showing complete dominance to resistance. Owens and Wright (1964) reported that rhizobitoxine as well as dihydrorhizobitoxine (an intermediate in the synthesis of rhizobitoxine) were found in soybean varieties that were susceptible to rhizobitoxine-induced chlorosis. Yasuta et al (2001) also reported that dihydrorhizobitoxine is a key intermediate in rhizobitoxine biosynthesis. The two genes controlling the susceptible response may condition the synthesis of dihydrorhizobitoxine and rhizobitoxine. If we consider the proposed genotypes (Table 1-5)

used in this study, it shows that for susceptibility to occur there must be at least one dominant allele for each locus. Therefore *Rts1_Rts2_* may both be needed for expression of susceptibility to rhizobitoxine-induced chlorosis in the plant. One locus may condition a factor in the nodules of the plant that prevents the synthesis of dihydrorhizobitoxine and the other locus may condition a factor in the nodules of the plant that prevents synthesis of rhizobitoxine. If we allow *Rts1* to be the locus that is responsible for prevention of dihydrorhizobitoxine synthesis in the nodules and *Rts2* to be the locus that is responsible for prevention of rhizobitoxine synthesis in the nodules we can see how these loci may interact. An *rts1rts1Rts2Rts2* genotype would not permit synthesis of dihydrorhizobitoxine in the nodules. Since dihydrorhizobitoxine is considered an intermediate step in the synthesis of rhizobitoxine, it would not be possible for the bacterial strain to produce rhizobitoxine when these alleles are recessive. Also, an *Rts1Rts1rts2rts2* genotype would allow dihydrorhizobitoxine synthesis in the nodules, but would not allow rhizobitoxine synthesis. The *rts1rts1rts2rts2* genotype would prevent both dihydrorhizobitoxine synthesis and rhizobitoxine synthesis in the nodules since it has recessive alleles at both loci. The *Rts1_Rts2_* genotype is the only one that permits both dihydrorhizobitoxine synthesis and rhizobitoxine synthesis to occur in the nodules.

The fact that some soybean varieties can have both a susceptible and resistant phenotype is of interest. Johnson and Means (1960) reported that the soybean varieties Blackhawk, Jackson, JEW 45, and Patoka were moderately susceptible, but Erdman et al (1957) reported that these soybean varieties were resistant. Both of these studies used the rhizobitoxine-producing strain USDA 76. In the current study, we used a resistant Davis and a susceptible Davis. These plants were derived by selecting resistant and susceptible varieties

of Davis in a preliminary greenhouse study of 93 plants (data not shown). In this preliminary study, 53% of the plants were resistant to rhizobitoxine-induced chlorosis and 47% were susceptible (data not shown). The author has observed this in other soybean varieties as well. Though most soybean varieties observed have been completely resistant or completely susceptible, there are instances where a soybean variety has plants with both phenotypes such as seen in the Davis variety. The Davis variety was derived by an F_5 plant selection which means that it is about 94% homozygous at all loci. If the plants selected from this generation are homozygous at all loci it would be expected that all plants should be either resistant or susceptible. However, if there is variation observed then it can be concluded that the plants selected from this generation are heterozygous at one or both loci. This would rule out *Rts1rts1rts2rts2* and *rts1rts1Rts2rts2* because these would produce all resistant plants (Table 3-5 of ancestor chapter). It is more likely that the genotype of the soybean variety Davis is derived from either *Rts1Rts1Rts2rts2* or *Rts1rts1Rts2Rts2* as these genotypes segregate to give both susceptible and resistant plants.

Figure 1-1. Phenotype of soybean plants that are susceptible and resistant to rhizobitoxine-induced chlorosis. The plant on the left is susceptible to rhizobitoxine-induced chlorosis and shows the chlorotic phenotype in the newly formed trifoliate. The plant on the right is resistant to rhizobitoxine-induced chlorosis and does not show the chlorotic phenotype.

Table 1-1. Parents used in crosses for inheritance study and their phenotype when inoculated with rhizobitoxine-producing strain CD2-5 and non-rhizobitoxine producing strain MN 110.

Parent[a]	CD2-5[b]	MN 110[c]
Brim	S	R
CNS	R	R
Ogden	S	R
Haberlandt	R	R
Volstate	S	R
Davis_R	R	R
Davis_S	S	R

[a] Davis_R and Davis_S, selected from a bulk population of Davis and are resistant and susceptible, respectively, when inoculated with rhizobitoxine-producing strain CD2-5.

[b] Symptom of parent when inoculated with rhizobitoxine-producing strain CD2-5; S, susceptible (chlorotic phenotype); R, resistant (non-chlorotic phenotype).

[c] Symptom of parent when inoculated with non-rhizobitoxine producing strain MN 110; R, resistant (non-chlorotic phenotype).

Table 1-2. F_1 phenotype of progeny from crosses between susceptible and resistant genotypes inoculated with rhizobitoxine-producing strain CD2-5.

			Number of Plants	
Cross	Generation	Total	Susceptible	Resistant
Brim x CNS	F1	5	5	0
Haberlandt x Ogden	F1	4	4	0
Volstate x CNS	F1	4	4	0
Davis_S x Davis_R†	F1	2	2	0

† Davis_S is susceptible to rhizobitoxine-producing strain CD2-5 and was selected from a bulk population of Davis that showed segregation after inoculation with the rhizobitoxine-producing strain CD2-5. Davis_R is resistant to rhizobitoxine-producing strain CD2-5 and also selected from the same bulk population of Davis.

Table 1-3. Chi-square analysis of F_2 generations for four populations inoculated with rhizobitoxine-producing strain CD2-5.

Cross	Segregating Generation	Total	S†	R	Expected Ratio‡	df	χ^2	Prob.
Brim x CNS	F_2	134	62	72	9:7	1	5.15	0.023*
Haberlandt x Ogden	F_2	139	85	54	9:7	1	1.49	0.210
Volstate x CNS	F_2	135	73	62	9:7	1	0.20	0.652
Davis_S x Davis_R	F_2	116	72	44	9:7	1	1.73	0.188

† S, Susceptible; R, Resistant.

‡ Expected ratio of 9 susceptible : 7 resistant.

* Significant at P = 0.05.

Table 1-4. Segregation of $F_{2:3}$ soybean families from Brim x CNS and Volstate x CNS crosses.

	----------Number of families---------						
Cross	All R†	H	All S	Expected Ratio‡	df	χ^2	P
Brim x CNS	35	47	3	7:8:1	2	1.61	0.4467
Volstate x CNS	34	47	6	7:8:1	2	0.77	0.6793

† R, resistant; H, segregating; S, susceptible

‡ Expected ratio 7 R : 8 H : 1 S

Table 1-5. Proposed genotypes of resistant and susceptible soybean plants based on the 9:7 (susceptible:resistant) ratio and two gene model found in four F_2 populations derived from crosses of parents that were resistant and susceptible to rhizobitoxine-producing strain CD2-5.

Genotype	Frequency	Symptom†
Rts1Rts1Rts2Rts2	1	S
Rts1Rts1Rts2rts2	2	S
Rts1rts1Rts2Rts2	2	S
Rts1rts1Rts2rts2	4	S
Rts1Rts1rts2rts2	1	R
Rts1rts1rts2rts2	2	R
rts1rts1Rts2Rts2	1	R
rts1rts1Rts2rts2	2	R
rts1rts1rts2rts2	1	R
Total	16	9 S : 7 R

† S, susceptible; R, resistant.

Table 1-6. Re-evaluation of Brim x CNS F_2 population using results of the Brim x CNS $F_{2:3}$ families.

Cross	Total	S†	R	Expected Ratio‡	df	χ^2	P
Brim x CNS	85	51	34	9:7	1	0.5519	0.4575

† S, susceptible; R, resistant

‡ Expected ratio 9 S : 7 R

CHAPTER II

MOLECULAR MAPPING AND IDENTIFICATION OF MARKERS ASSOCIATED WITH RHIZOBITOXINE-INDUCED CHLOROSIS IN SOYBEAN

INTRODUCTION

DNA marker technology is being widely used in plant breeding. Of particular importance, in soybean (*Glycine max* (L.) Merr.) has been the development of DNA markers and the integrated genetic linkage map of the soybean genome (Cregan et al., 1999). These developments have made it possible to genetically map qualitative and quantitative traits in soybean. One class of markers, simple sequence repeats (SSR), has been especially useful. Simple sequence repeats, also known as mircosatellites or short tandem repeats, are made up of two to five nucleotide repeat units usually no greater than 100 base pairs (bp). Other markers that were based on restriction fragment length polymorphism (RFLP) were once the major type of marker used for mapping soybean. A drawback to using RFLPs in soybean is that they have a low level of polymorphism detection. SSR markers, in contrast, have a higher level of polymorphism detection (Morgante and Olivieri, 1993). Akkaya et al (1992) conducted a study in which they searched GenBank, a sequence database, to determine the incidence and magnitude of simple sequence repeats that were found in soybean. Their search identified 33 sequences in soybean with at least 5 repeat units. Over half of these sequences had SSRs that were comprised of $(AT/TA)_n$ repeats with n ranging from 5 to 27. Rafalski and Tingey (1993) compared different molecular marker techniques and found that SSRs not only had a higher level of polymorphism, but also showed codominance when compared with RFLPs and RAPDs (Random Amplification of Polymorpic DNA), respectively. Other advantages of SSR markers is that the amount of DNA that is required for

analysis is very small and SSR markers do not require radioactive materials for visualization of the DNA. In addition, SSR markers are PCR (polymerase chain reaction) based and commercially available to research laboratories (Saghai-Maroof et al. 1994). Because of these advantages, many plant breeders have incorporated them in their breeding programs to help facilitate their research.

One use for molecular markers in soybean is to determine the location of genes in the soybean genome. Iqbal et al (2001) used SSR markers to identify genes for resistance to soybean sudden-death syndrome (SDS). In this study they used an F_5 derived population of 100 recombinant inbred lines (RILs) derived from a cross of Essex and Forrest to map genes associated with resistance to SDS. They found six loci that were involved in resistance to SDS. Four of these genes were on linkage group G (LG G) and explained 50% of the variation in SDS disease occurrence. The remaining two genes were located on LG C2 and LG I and explained roughly 40% of the variation found in SDS occurrence. Arahana et al (2001) used SSR markers to determine which loci are associated with Sclerotinia stem rot. They studied five RIL populations and found that there were seven genes that were associated with resistance to Sclerotinia stem rot and that by using markers they could screen segregating breeding populations. Mian et al (1999) used SSR markers to map the *Rcs3*gene for resistance to frogeye leaf spot in soybean. In their study, bulk segregant analysis was used to evaluate the progeny (Michelmore et al, 1991). The bulk segregant analysis involves selecting individuals from a single cross that differ in a particular trait and grouping together the DNA of those that have the same phenotype, such as a resistant group versus a susceptible group. The result is two bulks that are genetically different for a specific trait.

These bulks can then be used with molecular markers to determine which markers are associated with the trait of interest.

Mian et al (1999), evaluated the progeny from the cross of Blawkhawk (susceptible) x Davis (resistant) to determine markers associated with resistance to frogeye leaf spot. They mapped the gene near a disease resistant gene cluster found on LG J and suggested that these results could help soybean breeders by allowing them to screen for frogeye leaf spot resistance in their segregating populations. Li et al (2002) mapped the genes conditioning reduced palmitic acid content in soybean. Using SSR markers, they studied four near-isogenic lines with normal and reduced palmitic acid content as well as the F_2 and $F_{2:3}$ generations of a population derived from the cross of Cook x N87-2122-4. They reported a major gene with an allele for reduced palmitic acid contributed by N87-2122-4 on LG A1 that explained 38% of the variation in palmitic acid content in the F_2 generation and 31% in the $F_{2:3}$ generation. In addition, they found a minor gene on LG M that explained 8% and 9% of the variation in the F_2 and $F_{2:3}$ generations, respectively.

In soybean, a rhizobitoxine-induced chlorosis has been reported in several soybean genotypes (Erdman et al., 1957; Devine et al., 1988; Teaney III and Fuhrmann, 1992; Fuhrmann and Vasilas, 1994). Rhizobitoxine is a phytotoxin that is produced in the nodules of soybeans (Owens and Wright, 1964) and is reported to cause yield reductions in soybean genotypes (Fuhrmann and Vasilas, 1991). Based on an inheritance study conducted by the author it has been determined that two genes are involved in the soybean susceptibility of rhizobitoxine-induced chlorosis; however, these genes have not been molecularly identified in the soybean genome. Locating these genes within the genome could assist soybean breeders in identifying genotypes susceptible to rhizobitoxine-induced chlorosis using

marker assisted selection instead of waiting for visual field or greenhouse observations. The objective of this study was to identify SSR markers associated with the soybean response to rhizobitoxine-induced chlorosis of an F_2-derived soybean population from the cross of Brim (susceptible) x CNS (resistant) and to verify these markers with the cross of Volstate (susceptible) x CNS (resistant).

MATERIALS AND METHODS

Plant Genetic Materials

The susceptible soybean cultivar Brim (Burton et al., 1994) was crossed with the resistant soybean cultivar CNS to produce an F_2 population of 132 individuals. A second mapping population of 125 F_2 individuals from the cross Volstate (susceptible) x CNS (resistant) was used as a reference population to determine if markers found linked to rhizobitoxine-induced chlorosis would locate the genes in this population as well.

Rhizobitoxine-induced chlorosis scoring

All F_2 plants and the parents were screened for rhizobitoxine-induced susceptibility. The plants were grown in the greenhouse May 2002 and were scored as resistant or susceptible based on the absence or presence of a chlorotic phenotype after inoculation with the rhizobitoxine-producing strain, CD2-5, 25 – 30 days after planting. For purposes of this study, the phenotypic scores (susceptible and resistant) were arbitrarily changed to numeric scores (4 and 5, respectively).

DNA isolation

Soybean plant leaf tissue was stored at -80º C until used. The DNA of soybean leaf tissue was extracted according to the protocol described in the DNeasy Plant Mini Kit (QIAGEN Inc. Valencia, CA). The DNA concentrations were measured by a spectrometer at 260 nm (Beckman DU-600) and diluted to a 1:10 dilution for genotypic analysis.

SSR amplifications

Based on the results of a chi-square analysis from an inheritance study conducted by the author, F_2 populations from the above two crosses were predicted to segregate 9 susceptible : 7 resistance. Such a segregation pattern in a dihybrid F_2 population is termed

duplicate recessive epistasis (Fehr, 1993). Because of this segregation pattern, bulk segregant analysis was not feasible and each F_2 plant was genotyped separately.

SSR markers from all 20 linkage groups of soybean were selected at approximately 20 cM intervals from the soybean genetic map (Cregan et al. 1999). The primer pairs were supplied by Dr. Roger Boerma, University of Georgia. The PCR reaction mix contained 10X PCR buffer, 2.5 mM dNTP mix, 25 mM $MgCl_2$, 5 U/μL of Taq DNA polymerase, and 0.5 μM of primer (forward and reverse primers combined). The PCR reaction consisted of 25 sec of denaturation at 94º C, 25 sec annealing at 46º C, and 25 sec of extension at 68º C for 32 cycles on a 96-well GeneAmp PCR System 9700 Perkin Elmer Applied Biosystems (PE-ABI, Foster City, CA) thermocylcer. For each soybean parent and progeny, the PCR products of 3-4 SSR markers with different fluorescent labels and/or different allele sizes were pooled together. This sample was loaded in a polyacrylimide gel which was placed on an ABI Prism 377 DNA sequencer (AB-PEC, Foster City, CA) for separation of the DNA. A loading sample was prepared that consisted of 500 μL of formamide, 300 μL of loading buffer, and 50 μL of Genescan Rox-500 (PE-ABI, Foster City, CA). The Genescan Rox-500 is an internal size standard. In each well of the 96-well plate, there were 3 μL of the PCR DNA added to 3 μL of the loading buffer. This mixture was then denatured at 95º C for 5 minutes and approximately 1.0 μL of sample was loaded on each of the 96 lanes on a polyacrylamide gel. Electrophoresis was run 1.5 hours on ABI PRISM 377 DNA Sequencer at 0.75 kV. The gel was then analyzed using GeneScan software (AB-PEC, Foster City, CA) and Genotyper software (AB-PEC, Foster City, CA) was utilized to differentiate alleles.

Markers associated with soybean response to rhizobitoxine-induced susceptibility

All available markers for each of the 20 soybean linkage groups were screened with the parents, Brim and CNS, to determine which markers were polymorphic. All polymorphic markers were then screened with the F_2 progeny. To determine the loci associated with susceptibility to rhizobitoxine-induced chlorosis in soybean, marker data (genotypic data) was compared with rhizobitoxine scores (phenotypic data) by a single factor analysis of variance (ANOVA) performed with SAS (SAS Institute Inc., Cary, NC). The dependent variable was the phenotypic data and the independent variable was the genotypic data. Genotypic scores were arbitrarily set at 1, 2, or 3 which were associated with the Brim, heterozygote, and CNS alleles, respectively and phenotypic scores were arbitrarily set at 0 and 1 which were associated with chlorotic phenotype and non-chlorotic phenotype, respectively. A CONTRAST statement was used to determine if gene action was additive or dominant. The use of the single factor analysis of variance yields similar results as that of the Chi-square contingency table; however, the single factor analysis of variance was used in this study because of the use of the CONSTRAST statements. The probability of association of each marker (genotypic data) with each rhizobitoxine score (phenotypic data) was determined and a significant relationship was confirmed if $p \leq 0.05$.

RESULTS AND DISCUSSION

A total of 455 markers were used in this study (Table 2-1) and were distributed among the 20 linkage groups (LG) found in the public linkage map of soybean (Cregan et al., 1999). SSR markers were chosen to divide each linkage group into segments not longer than 20 cM. SSR analysis was conducted on the parents Brim (susceptible to rhizobitoxine-induced chlorosis) and CNS (resistant to rhizobitoxine-induced chlorosis)) with these markers to determine which markers were polymorphic for the parents. The results showed that there were 141 polymorphic markers (31%) (Table 2-1). The total number of markers screened for each linkage group ranged from12 to 35 depending on the size of the linkage group (Table 2-1). The polymorphic markers were then used to screen the F_2 population from the cross of CNS x Brim. Some linkage groups had a high number of markers that did not amplify with the parents in the polymorphism test. On linkage group A1, 76% (19 of 25) of the markers used did not amplify. On linkage group D1a, 43% (12 of 28) of the markers used did not amplify. Other linkage groups that had a high percentage of non-amplifying markers were H, I, K, L, and M (54%, 55%, 72%, 50%, and 46%, respectively). The markers that were polymorphic in the 7 linkage groups that had a high percentage of non-amplification were not significantly associated with the phenotype (Appendix 1). This, however, can not rule out these linkage groups as potentially having a marker associated with rhizobitoxine-induced susceptibility in soybean because there are areas in these linkage groups that have gaps where markers did not amplify or were simply not available. In some instances these areas were greater than the 20 cM selection criterion that was used. For example, linkage group I, which had 10 markers out of 18 that did not amplify, has an area from Sat_268 to

Sat_299 (approximately 48 cM) based on map by Cregan et al., (1999) with markers that did not amplify, were unlabeled, or were not available.

The markers from the other 13 linkage groups had fewer markers that did not amplify and were within the 20 cM selection criterion. There were two linkage groups that appeared to be significant based on their p-value in the single factor ANOVA (Appendix 1). On linkage group A2, SSR marker GMENOD2B had a p-value of 0.0124. However, this marker was not considered linked to alleles for rhizobitoxine-induced susceptibility because the genotypic means were not additive (data not shown). Furthermore, markers within a 20 cM range either side of this marker were not significant based on their p-value from the one-way ANOVA. Marker Satt 301 on linkage group D2 had a slightly significant p-value of 0.0526. The genotypic means were not additive for this marker (data not shown). In addition, the markers surrounding Satt 301 were either not significant or monomorphic. There were also 14 progeny that did not receive a genotypic score due to non-amplification of the marker and these missing points could have caused a false significance. These two linkage groups should be investigated further before it can be determined with certainty that they do not have significant markers associated with rhizobitoxine-induced chlorosis in soybean.

On the basis of the single factor ANOVA, four SSR markers were detected on LG F that had p-values that were significant (based on $p < 0.05$) for susceptibility to rhizobitoxine-induced chlorosis (Table 2-2). A gene was identified by marker Satt 657 on LG F that was highly significant and had a R^2 value of 0.318 (Table 2-2). This indicates that the marker explains almost 32% of the phenotypic variation among F_2 plants. Therefore, differences in genotype at this locus can account for approximately 32% of the total phenotypic differences found among plants with the rest of the variation coming from other markers and error. The

gene action for this marker was additive (Table 2-2) and the Brim allele was associated with rhizobitoxine susceptibility. In addition, Satt 554, Satt 362, and Satt 490 accounted for 22%, 20%, and 21% of the variation in the F_2 generation, respectively (Table 2-2). The gene action for these markers was additive except for marker Satt 362. A linkage map with these markers was constructed with QTX Mapmanger (Manly et al., 2001). A LOD score of 3.45 was used to determine the presence of a QTL (Figure 2-1). The LOD (logarithm of the odds favoring linkage) score is used in the statistical analyses of linkage. Of these four significant markers, three had LOD scores that were higher than the significant level (Table 2-2). The high LOD score and the height of the peak position indicates that one of the genes for susceptibility to rhizobitoxine-induced chlorosis in soybean is located on LG F possibly in the region between marker Satt 657 and AW756935. Because no polymorphic SSR marker was found distal to Satt 657, it was not possible to determine the precise location of the QTL for soybean susceptibility to rhizobitoxine-induced chlorosis. Satt 218 and Satt 522, which are approximately 0.7 and 2.3 cM distal to Satt 657 (Cregan et al., 1999), were monomorphic when screened in the parental polymorphism test. These two markers are 0.7 and 1.5 cM distal to Satt 657. Markers Sat_090 and Satt 656 were also monomorphic when screened in the parental polymorphism test and are approximately 13 and 17.5 cM distal of marker Satt 657. Marker AW756935 was not available for screening. This marker is approximately 8 cM distal of marker Satt 657. Therefore, it is suggested that the QTL associated with susceptibility to rhizobitoxine-induced chlorosis in soybean may be located between Satt 657 and AW756935 (Figure 2-2).

The Satt 657 marker was used to screen a second F_2 soybean population from the cross of Volstate x CNS. This marker was highly significant with this population (Table 2-2).

The Satt 657 marker also explained 20.4% of the variation and had an additive gene action. This is further evidence that SSR marker Satt 657 is associated with one of the genes that controls rhizobitoxine-induced chlorosis susceptibility in soybean. This population was not mapped and therefore no LOD score is given.

Based on the evidence from this study, we conclude that one of the genes for rhizobitoxine-induced chlorosis in soybean is located on LG F at or very near SSR marker Satt 657. Results from an inheritance study conducted by the author shows that there are two genes involved in the soybean response to rhizobitoxine-induced chlorosis. However, the other gene was not found in this molecular study. As stated earlier, there were some LG that had markers that did not amplify well with the parents used in this study when screened for polymorphisms. The markers in these LG should be investigated further before it can be concluded that they are not associated with rhizobitoxine-induced chlorosis. Also, the markers found in LG A1 and LG D1a that appear to be false significant values should be re-evaluated and possibly screened with the confirmation population to determine if further investigation should be done. The fact that these genes involved in the soybean response to rhizobitoxine-induced chlorosis segregate in a 9 susceptible to 7 resistant fashion makes it impossible to use bulk segregant analysis. Because of this, each individual plant must be screened with a desired marker, thus making the procedure more time consuming than if bulks were used. Appendix 1 lists all the markers that were used in this study and reports the results of the parental polymorphism tests. This will be useful to anyone trying to locate the second gene as it will give them an idea of what LG need more investigation. If markers for both genes involved in rhizobitoxine-induced chlorosis are known it could make it possible to screen soybean lines to determine which plants are resistant or susceptible to

rhizobitoxine-induced chlorosis. This would be advantageous because based on the ancestor study (Chapter III) even soybean plants with a non-chlorotic (i.e. resistant) phenotype, can still possess susceptible alleles that can be passed to their progeny. It would particularly benefit southern soybean breeders as the strains responsible for rhizobitoxine-induced chlorosis are found in the Southeast. By using markers associated with rhizobitoxine-induced chlorosis, those plants that possess the susceptible alleles could be eliminated thus making it easier to select for soybean lines that are resistant to rhizobitoxine-induced chlorosis; however, if susceptibility is a positive trait one could select for susceptibility.

Table 2-1. Results of the markers used to screen the F_2 population from the cross of Brim x CNS.

Linkage Group	No Amp†	Monomorphic‡	Polymorphic§	Total¶
A1	19	3	3	25
A2	4	13	12	29
B1	3	5	4	12
B2	7	1	5	13
C1	2	9	10	21
C2	6	10	12	28
D1a	12	9	7	28
D1b	4	7	8	19
D2	6	8	11	25
E	6	6	9	21
F	12	13	10	35
G	9	9	6	24
H	12	4	6	22
I	10	5	3	18
J	5	5	11	21
K	18	2	5	25
L	8	3	5	16
M	12	8	6	26
N	4	15	6	24
O	6	14	3	23
Total#	165	149	141	455

† Marker did not amplify.

‡ Markers were the same size (bp) for both parents (Brim and CNS).

§ Markers were different sizes for the parents.

¶ Total amount of markers screened for the respective linkage groups.

Cumulative totals for results of markers used in parent polymorphic screen.

Table 2-2. The probability, R^2, LOD score, and mean allelic score for SSR markers from linkage group (LG) F that were significant for the genes responsible for rhizobitoxine-induced chlorosis based on the data from the F_2 population from the Brim x CNS cross. The SSR marker Satt 657 was screened with the confirmation population from the F_2 progeny of the Volstate x CNS cross

Marker	LG	Probability†	R^2	LOD score‡	Mean allelic score§		
					AA	AB	BB
Satt 554	F	0.000152815	0.219	3.97	0.2	0.5	0.8
Satt 657	F	0.000000001	0.318	7.48	0.1	0.6	0.9
Satt 362	F	0.00104	0.201	3.12	0.4	0.4	0.8
Satt 490	F	0.000000537	0.214	3.95	0.3	0.4	0.9
Satt 657¶	F	0.000002603	0.204	----	0.1	0.4	0.8

†, Significant at the 0.05 probability level.

‡, A LOD score of 3.45 was used to determine the presence of a QTL.

§, AA= homozygous for the Brim allele (susceptible), AB= heterozygotes, BB= homozygous for the CNS allele (resistant).

¶, SSR marker Satt 657 screened with Volstate x CNS population. The LOD was not scored.

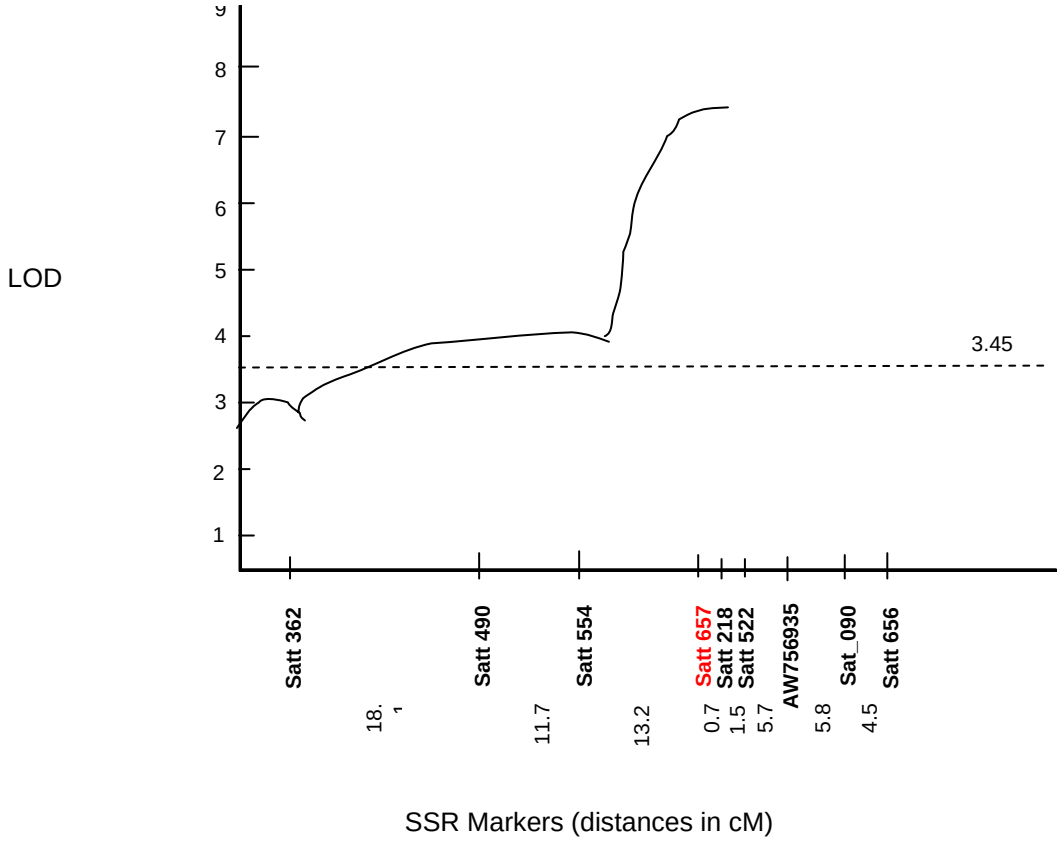

Figure 2-1. QTL-likelihood plot for soybean susceptibility to rhizobitoxine-induced chlorosis on Linkage Group F for the F_2 population of Brim x CNS based on interval mapping. The significance threshold is indicated by a line at LOD = 3.45.

Note: Satt 218, Satt 522, Sat_090, and Satt 656 are Monomorphic.

CHAPTER III

EVALUATION OF THE SOYBEAN GENETIC BASE FOR SUSCEPTIBILITY TO RHIZOBITOXINE-INDUCED CHLOROSIS

INTRODUCTION

Several studies have shown variable responses to rhizobitoxine-producing strains by soybean varieties. Erdman et al., (1957) studied the degree of susceptibility to rhizobitoxine-producing strains on 40 soybean cultivars and reported that 10 did not show bacterial-induced chlorosis (Blackhawk, Harosoy, Virginia, Tanner, CNS, Palmetto, FC 33123, Improved Pelican, Otootan, and Yelnando); 7 were scored as negligibly susceptible (Patoka, Laredo, Rokusun, Jackson, Biloxi, J.E.W. 45, and Seminole); 11 were lightly susceptible (Grant, Norchief, Chippewa, Hawkeye, Dunfield, Lincoln, Clark, Perry, Wabash, Luthy, and Arksoy); 6 were moderately susceptible (Aoda, Chief, Dorman, Dortchsoy 31, C-1068, and S-100); and 6 were highly susceptible (Lee, Roanoke, Gibson, Ogden, PI 54619-5-1, and D51-4888). Johnson and Means (1960) screened 116 soybean varieties against bacterial strain USDA 76, which is a rhizobitoxine-producing strain. They reported that 94 of the varieties developed rhizobitoxine-induced chlorosis that ranged from light to severe. These varieties included one selection of *G. gracilis*, two selections of *G. ussuriensis*, two selections of *G. tomentella*, and three introductions of *G. max*; all of which were susceptible to rhizobitoxine-induced chlorosis caused by strain USDA 76. From their results the authors speculated that genes for rhizobitoxine susceptibility may be more common than genes for rhizobitoxine resistance in US soybean germplasm.

Delannay, et al., (1983) examined the pedigrees of 158 US and Canadian soybean varieties and reported that the gene pool of soybean varieties of hybrid origin could be traced to 50 plant introductions. In their study they evaluated the genetic contribution of both

southern and northern soybean varieties that were released from 1951 to 1981. Their results showed that 80% of the northern soybean varieties could be traced back to ten soybean introductions (Mandarin (Ottawa), Manchu, Mandarin (Illinois), Richland, A.K. (Harrow), CNS, Mukden, Strain 171, Tokyo, and PI 54610). In addition, they reported that 80% of the southern soybean varieties could be traced to seven soybean introductions (CNS, S-100, Roanoke, Tokyo, PI 54610, PI 240664, and Palmetto). Gizlice, et al., (1994) evaluated the genetic base of North American public soybean varieties that were released between 1947 and 1988. They reported that over a half of the genetic base of North American soybean is made up of six ancestors (Mandarin (Ottawa), CNS, Richland, S-100, and the unknown parents of Lincoln). They showed that for both the southern and northern soybean genetic base, each is made up of 10 ancestors that make up to 80% of the genes contributed, respectively. From their analysis, they demonstrated that 35 varieties of soybean ancestors and first progeny contributed approximately 95% of the genes found in varieties released between 1947 and 1988. This soybean genetic base is very useful in that it can be used to screen for the existence of a trait and by using pedigree analysis it can help plant breeders determine the probability of an undesirable (or desirable) allele being present in their breeding populations. Based on this list of ancestors that are known to contribute genes to modern soybean varieties, the current study was conducted to determine the susceptibility of these 35 ancestors to the bacterial-induced phytotoxin, rhizobitoxine. In addition, 25 other soybean cultivars were screened for rhizobitoxine-induced chlorosis. This group of soybean cultivars represented a mix of soybean ancestors that made a very small contribution to modern northern and southern soybean cultivars.

MATERIALS AND METHODS

35 soybean ancestors (Table 3-2) and 25 soybean cultivars (Table 3-5) were screened for rhizobitoxine susceptibility under greenhouse conditions in December 1999 in Raleigh, North Carolina. The 25 soybean cultivars were a mix of soybean ancestors that contributed a small percentage of genes to modern soybean cultivars. The 35 soybean ancestors were selected because they are major contributors of genes to modern day soybean cultivars (Gizlice et al., 1994). Soybean seed used for this study were obtained from Dr. Randy Nelson, curator of the USDA soybean germplasm collection, and the USDA-ARS soybean breeding program at North Carolina State University.

Bacterial strain preparation

Two strains were used in this study: CD2-5 (a rhizobitoxine-producing strain that was isolated from the soils of North Carolina) (Ramirez et al, 1997b) and MN-110 (a rhizobitoxine-nonproducing strain) (Mathis et al, 1985). The cells of these bacterial strains were each streaked on separate Yeast Extract Mannitol (YEM) agar plates and allowed to grow. They were then each transferred to 100 ml flask of modified YEM liquid media. The flasks were put into a shaker at 28° C at a shaking speed of 150 rpm.

Seed Sterilization

Seed of each entry in the test were placed in separate 250 ml beakers and covered with 95% ethanol and stirred for one minute. After the ethanol was discarded, seeds were covered with a 1:5 (chlorox:sterile water) chlorox solution and stirred for five minutes. The solution was carefully decanted and the seeds were washed five times with sterile water, decanting the water after each washing. Seeds were left in the beakers and kept moist to prevent desiccation until time of planting.

Planting of seed in the greenhouse

Seed were planted in 32 ounce cups that were filled with horticultural grade vermiculite. Each cup had three holes in the bottom to allow drainage. Cups were watered the day before planting with tap water and the day of planting with tap water and 50 ml of N-minus nutrient solution. Four seed were added to each cup and each genotype had 9 cups. The experimental design was a randomized complete block design. Four seeds were planted per cup, there were 3 cups in each replication. There were three replications in this study. Thus, a total of 36 seed per genotype were evaluated for rhizobitoxine susceptibility. Each seed was inoculated with 0.5 ml of rhizobitoxine-producing strain CD2-5 (10^9 CFU/ml) . After inoculation, the seed were covered with vermiculite, and the soil was kept moist by watering the tops with tap water, but not enough to allow them to drain. After five days, the cups were watered as needed with tap water and with nutrient solution twice a week.

Scoring of Plants

Symptoms began appearing 25-30 days after inoculation. Plants that had chlorotic newly formed leaves at the top of the plant were considered susceptible and plants that did not have chlorotic newly formed leaves at the top of the plant were considered resistant. Even though the soybean ancestors used in this study have been inbred for many decades, many of them displayed variation in their response to the rhizobitoxine-producing strain. The fact that these soybean ancestors are highly inbred suggests that they are homozygous at all loci. Based on the results of an inheritance study conducted by the author, the segregation of inbred soybean cultivars found in this study can be explained. Biparental F_2 populations derived in the inheritance study showed that the genes responsible for rhizobitoxine-induced chlorosis display a segregation pattern termed duplicate recessive epistasis (Fehr, 1993). To

facilitate interpretation of results, we designated *Rts1* and *Rts2* as alleles for rhizobitoxine susceptibility and *rts1* and *rts2* as alleles for resistance. Two dominant alleles from each of two independent gene loci are required to express rhizobitoxine susceptibility. Therefore, the *Rts1_Rts2_* genotype would be susceptible to rhizobitoxine-induced chlorosis. The resistant genotypes would be *Rts_rts2rts2*, *rts1rts1Rts2_*, and *rts1rts1rts2rts2*. A description of all possible genotypes in a highly inbred line or cultivar is given in Table 3-1.

Plants were scored based on their segregation patterns or lack thereof. Highly susceptible (HS) soybean cultivars were soybean cultivars that had 100% susceptibility to the rhizobitoxine-producing strain and highly resistant (HR) soybean cultivars were soybean cultivars that had 100% resistance. Moderately susceptible (MS) soybean cultivars were soybean cultivars that displayed a segregation pattern similar to 50% susceptible and 50% resistant. Slightly susceptible (SS) soybean cultivars were soybean cultivars that displayed a segregation pattern similar to 25% susceptible and 75% resistant. It should be noted that HS, HR, MS, and SS are not phenotypic descriptions of the chlorosis. These notations are examples of the degree of susceptibility (percentage susceptible) that were observed in this study and are used as a means to discuss the data.

RESULTS AND DISCUSSION

Screening of the 35 soybean ancestors against the chlorosis-producing strain showed that there was variation for rhizobitoxine-induced chlorosis. Thus, if one considers the frequency of these soybean ancestors in the pedigrees of modern soybean cultivars, it is possible to estimate the frequency of susceptible alleles among modern soybean cultivars. There are four possible genotypes that the soybean ancestors can have: homozygous at both loci for susceptibility (*Rts1Rts1Rts2Rts2*), homozygous at both loci for resistance (*rts1rts1rts2rts2*), homozygous at one locus for susceptibility (*Rts1Rts1rts2rts2*) or homozygous at the other locus for susceptibility (*rts1rts1Rts2Rts2*). Those soybean ancestors that are homozygous at one or both loci for susceptibility to rhizobitoxine-induced chlorosis can contribute susceptible alleles. Therefore when considering the potential contribution of susceptible alleles by soybean ancestors to modern day soybean cultivars, these variables have to be considered and are discussed in this section.

Soybean ancestor contribution to modern soybean cultivars and response to rhizobitoxine

Of the 35 soybean ancestors screened for rhizobitoxine susceptibility 14 were scored as highly resistant, 7 were scored as slightly susceptible, 8 were scored as highly susceptible, and 6 were scored as moderately susceptible (Table 3-2). Their total contributions to the genetic base of modern soybean cultivars were 57.59%, 8.13%, 23.42%, and 6.81%, respectively, and together they make up 95.95% of the genes contributed to modern soybean cultivars. However, as stated earlier, even those genotypes that are scored as highly resistant have the potential to pass along susceptible alleles as well as the moderately susceptible and the slightly susceptible genotypes. When this is taken into consideration, the frequency of individuals that are homozygous for susceptible alleles at both loci totals 28.84% and is the

known frequency of *Rts1* and *Rts2* alleles contributed to modern day soybean cultivars (Table 3-2). The total maximum frequency of possible individuals homozygous for susceptible alleles at one locus is 36.23% and is the maximum frequency of *Rts1* and *Rts2* alleles contributed to modern day soybean cultivars. The total minimum frequency is 2.03%. So the potential frequency of susceptible alleles that are contributed to modern soybean cultivars ranges from 2.03 – 36.23% depending on the genotypes of the contributing soybean ancestors. When considering the resistant alleles that could be potentially passed to modern day soybean cultivars, the maximum frequency is 59.62% and the minimum frequency is 28.78% (Table 3-2). The range of potential contributions of resistant alleles is higher than that of susceptible alleles. If we assume that all highly susceptible genotypes are homozygous at both loci and that all highly resistant genotypes are homozygous at both loci, then the soybean ancestors have a higher frequency of resistant alleles than susceptible alleles (Table 3-2).

The bulk of the resistance genes came from four ancestors (Lincoln, Mandarin (Ottawa), CNS, and Richland) that are highly resistant to rhizobitoxine-induced chlorosis which collectively made up 47.64% of the genes contributed to modern soybean cultivars. Most of the genes from highly susceptible ancestors came from three soybean ancestors (S-100, Ogden, and AK Harrow) which collectively accounted for 17.31% of the genes contributed to modern soybean cultivars. Even though genotypes that are homozygous dominant at one locus can contribute susceptible alleles to rhizobitoxine-induced chlorosis, these genotypes will always display a non-chlorotic phenotype. Therefore, of the 35 soybean ancestors, 21 of these are capable of exhibiting rhizobitoxine-induced chlorosis (i.e. 60%). These results are consistent with the results of Johnson and Means (1960) who reported that

94 out of 116 soybean varieties (i.e. 81%) used in their study were susceptible when inoculated with a rhizobitoxine-producing strain. In addition, a study conducted by Erdman et al (1957) reported 58% susceptibility to rhizobitoxine-induced chlorosis of 40 soybean varieties.

Soybean ancestor contribution to southern varieties and response to rhizobitoxine

There were 21 soybean ancestors that contributed genes to modern southern soybean cultivars. Of these 21 soybean ancestors, 9 were highly resistant, 5 were slightly susceptible, 5 were highly susceptible, and 2 were moderately susceptible (Table 3-3). Collectively, they contribute 95.17% of the genes found in southern soybean ancestors. The frequency of individuals that are homozygous for susceptible alleles at both loci totals 43.22% and is the frequency of the *Rts* alleles contributed to modern day soybean cultivars (Table 3-3). The total maximum frequency of possible individuals homozygous for susceptible alleles at one locus is 28.27% of the genes contributed to modern day soybean cultivars and the total minimum frequency is 3.85%. The potential frequency of susceptible alleles that are contributed to modern soybean cultivars ranges from 3.85 – 43.22% depending on the genotypes of the contributing soybean ancestors. When considering the resistant alleles that could be potentially passed to modern day soybean cultivars, the maximum frequency is 43.54% and the minimum frequency is 19.84% (Table 3-3). If we assume that all highly susceptible genotypes are homozygous at both loci and that all highly resistant genotypes are homozygous at both loci, then the southern soybean ancestors have an equal frequency of resistant and susceptible alleles (43.54% vs. 43.22%) (Table 3-3). The majority of the genes from the highly resistant soybean ancestors came from CNS which contributed 24.71% of the genes found in modern southern soybean cultivars. The majority of the genes from the highly

susceptible soybean ancestors came from S-100 which contributed 21.31% of the genes found in modern southern soybean cultivars.

Based on the proposed genotypes in Table 3-1, 12 of the 21 southern soybean ancestors that contribute genes to modern day soybean can display rhizobitoxine-induced chlorosis when inoculated with a rhizobitoxine-producing strain (i.e. 60%). It has been reported that symptoms of rhizobitoxine are predominately found in the southeastern portion of the United States, such as Alabama, Arkansas, Florida, Georgia, Mississippi, North Carolina, and South Carolina (Erdman et al., 1957). Keyser et al (1984) conducted a study that involved surveying *Bradyrhizobium* strains from 12 states and found that the most common *Bradyrhizobium* strain was a rhizobitoxine-producing strain (USDA 31). It should be noted that rhizobitoxine-producing strains are not common in the Midwest (Weber et al., 1989). Distribution of susceptible genes in modern cultivars is potentially high based on the genetic contribution of susceptible ancestors and those ancestors that contribute susceptible genes to modern cultivars. This fact in conjunction with the fact that rhizobitoxine-producing strains are very common in the southern United States explains the occurrence of rhizobitoxine-induced chlorosis in this region.

Soybean ancestor contribution to northern varieties and response to rhizobitoxine

There were a total of 32 ancestors that contributed genes to modern northern soybean cultivars of which 12 were highly resistant, 7 were slightly susceptible, 7 were highly susceptible, and 6 were moderately susceptible (Table 3-4). Collectively, they contribute 96.31% of the genes found in modern day northern soybean cultivars. The frequency of individuals that are homozygous for susceptible alleles at both loci totals 22.83% and is the known frequency of *Rts* alleles contributed to modern day northern soybean cultivars (Table

3-4). The total maximum frequency of possible individuals homozygous for susceptible alleles at one locus is 39.58% of the genes contributed to modern day soybean cultivars and the total minimum frequency is 1.26%. The potential frequency of susceptible alleles that are contributed to modern soybean cultivars ranges from 1.26 – 39.58% depending on the genotypes of the contributing soybean ancestors. When considering the resistant alleles that could be potentially passed to modern day soybean cultivars, the maximum frequency is 66.35% and the minimum frequency is 32.50% (Table 3-4). If we assume that all highly susceptible genotypes are homozygous at both loci and that all highly resistant genotypes are homozygous at both loci, then the northern soybean ancestors have a higher frequency of resistant alleles than susceptible alleles (66.35% vs. 22.83%) (Table 3-4). Three soybean ancestors (Lincoln, Mandarin (Ottawa), and Richland) make up the bulk of the total contribution to northern soybean cultivars and all three are highly resistant to rhizobitoxine-induced chlorosis. Collectively, they contribute 55.71% of the genes contributed to northern soybean cultivars. The highly susceptible soybean ancestors combine to contribute 17.05% of the genes found in modern northern soybean cultivars. Most of this contribution is from two soybean ancestors, AK Harrow and Ogden. These two soybean ancestors make up 66% of the highly susceptible soybean ancestor total. Of the 32 soybean ancestors that contribute genes to modern northern soybean cultivars, 20 will display rhizobitoxine-induced chlorosis in the phenotype (i.e. 63%). However, the soybean cultivars that make up the majority of the contribution to northern soybean cultivars are made up of highly resistant soybean ancestors. It was stated previously that the symptoms of rhizobitoxine do not occur in the Midwest. This may be due to the fact that rhizobitoxine-producing strains are not found in this region as stated by Weber et al. (1989). It could also be explained by the fact that there are a higher

percentage of resistant genes being contributed by soybean ancestors in this region. Both of these factors may play a role in the extremely low occurrence of rhizobitoxine-induced chlorosis found in the Midwestern region which has used these ancestors in pedigrees for northern varieties.

Soybean response to rhizobitoxine-producing strains with 25 soybean cultivars

Of the 25 non-ancestral soybean cultivars screened, 10 were highly resistant, 3 were slightly susceptible, 3 were highly susceptible, and 9 were moderately susceptible to rhizobitoxine-induced chlorosis (Table 3-5). Ten of these soybean cultivars would not show rhizobitoxine-induced chlorosis based on the proposed genotypes in Table 1. However, the other 15 soybean cultivars could produce genotypes that had the chlorotic phenotype (i.e. 60%). It appears that the more modern soybean cultivars show susceptibility to rhizobitoxine-producing strains. From this list, there are 14 soybean cultivars that were released from the 1940s to the 1960s. There were 8 highly resistant soybean cultivars, 4 moderately susceptible soybean cultivars, and 2 slightly susceptible soybean cultivars. There were not any highly susceptible soybean cultivars. However, of the 9 soybean cultivars released after 1970 one of these was highly resistant, 2 were highly susceptible, 5 were moderately susceptible, and 1 was slightly susceptible. There were two soybean cultivars that were not used because their date of release could not be verified. The fact that susceptibility seems to increase with the release of more modern soybean cultivars shows that the genes for susceptibility to rhizobitoxine-induced chlorosis are not linked to poor agronomic genes. These genes may in fact be neutral as they do not appear to be preferentially selected during cultivar development. One way to determine if the genes responsible for rhizobitoxine production in soybean are linked to favorable genes would be to screen modern day soybean

cultivars for rhizobitoxine-induced chlorosis. Modern soybean varieties are selected for favorable agronomic traits such as yield and lodging. If the genes associated with rhizobitoxine-induced chlorosis were linked to genes selected for favorable agronomic traits one would expect to see more or less rhizobitoxine-induced chlorosis in modern soybean cultivars.

The total maximum frequency of individuals homozygous for susceptible alleles at both loci shows that the ancestor contribution to modern southern soybean cultivars is higher than those for modern northern soybean cultivars, 43.22% and 22.83%, respectively (Table 3-3 and 3-4). However, when the total maximum frequency of individuals homozygous for resistant alleles is considered, the total frequency for southern soybean cultivars is lower than that of northern soybean cultivars, 43.54% and 66.35%, respectively (Table 3-3 and 3-4). This shows that there is a higher frequency of susceptible alleles in the southern ancestor contribution to modern day soybean cultivars and a higher frequency of resistant alleles in the northern ancestor contribution to modern day soybean cultivars. When the minimum and maximum frequencies of individuals homozygous for susceptible alleles at one locus is considered, the southern ancestor contribution is lower than the northern contribution, 3.85-28.27% and 1.26-39.58%, respectively (Table 3-3 and 3-4). This shows that there is a potentially high frequency of susceptible alleles for rhizobitoxine-induced chlorosis in the northern soybean ancestors. When the total frequencies of all genotypes that can produce alleles susceptible to rhizobitoxine-induced chlorosis are added together (i.e., genotypes that are homozygous at both loci for susceptibility to rhizobitoxine-induced chlorosis and the maximum and minimum frequencies of the genotypes that are homozygous at one locus), the

southern soybean ancestors are shown to contribute more susceptible alleles than the northern soybean ancestors (75.34% and 63.67%, respectively).

Pedigree of a southern soybean cultivar and the response to rhizobitoxine-induced chlorosis

Many of the soybean cultivars used in this study were soybean ancestors or cultivars that had been used decades ago. The soybean cultivar Brim is a more modern soybean cultivar that has shown rhizobitoxine-induced chlorosis. The pedigree of this soybean cultivar was used to determine how the genes for rhizobitoxine-induced chlorosis were passed on to this modern soybean cultivar (Figure 3-1). The great great grandparents of Brim are Perry, S-100, CNS, Ogden, Ralsoy, Roanoke, and Hill. Only CNS and Hill are highly resistant to rhizobitoxine-induced chlorosis. Ralsoy is slightly susceptible to rhizobitoxine-induced chlorosis (i.e. it segregated 75% resistant and 25% susceptible to rhizobitoxine-induced chlorosis). Perry, S-100, Ogden, and Roanoke were all highly susceptible to rhizobitoxine-induced chlorosis. The only great grandparent in this pedigree that was screened for rhizobitoxine-induced chlorosis was the soybean cultivar Lee. Lee is slightly susceptible to rhizobitoxine-induced chlorosis. Only one of the four grandparents (Tracy) of Brim was resistant to rhizobitoxine-induced chlorosis and this soybean cultivar is highly resistant. The three remaining grandparents were slightly susceptible (Essex), moderately susceptible (Davis), and highly susceptible (Ransom) to rhizobitoxine-induced chlorosis. Even though only one great grandparent from this pedigree was screened, the fact that three of the four grandparents were susceptible to rhizobitoxine-induced chlorosis to some degree shows that the great grandparents that were not screened have also passed on genes for rhizobitoxine-induced chlorosis to the grandparents. Both of the parents, Young and N73-1102, are moderately susceptible (i.e. it segregated 50% resistant and 50% susceptible to

rhizobitoxine-induced chlorosis) and highly susceptible, respectively, to rhizobitoxine-induced chlorosis. Of the 15 soybean cultivars that were screened in this pedigree, 13 of them exhibited some degree of rhizobitoxine-induced chlorosis. When it was possible to select a highly resistant type, the line selected always had some level of susceptibility. The results from the pedigree helps verify the results that were shown in this study in that the genes for rhizobitoxine-induced chlorosis are prevalent in southern varieties.

It has been well established that there is variability in soybean response to rhizobitoxine (Erdman et al, 1957; Johnson and Means, 1960; Fuhrmann and Vasilas, 1994). This study confirms this as well, in addition to screening important soybean ancestors that have contributed a large portion of the genes found in modern soybean varieties. The results of this study are interesting in that there appears to be a relationship with soybean ancestor contribution and rhizobitoxine susceptibility. The greatest contributors to southern soybean cultivars are ancestors that contribute genes that are susceptible to rhizobitoxine-induced chlorosis, with the exception of CNS which makes up more than half of the resistant ancestor contribution. However, resistant soybean ancestors can potentially contribute susceptible genotypes (Table 3-1). Knowledge of the rhizobitoxine susceptibility of these important soybean ancestors is important because it can give the soybean breeder a better understanding of what to expect from lines that are derived from these varieties. The fact that 21 of the 35 (60%) soybean ancestors had susceptible genotypes, 15 of the 25 (60%) other soybean cultivars had susceptible genotypes, and that 13 of the 15 soybean cultivars screened in the pedigree of Brim had susceptible genotypes suggest that alleles for rhizobitoxine susceptibility in the soybean germplasm are being passed down and are not being selected against in southern soybean cultivars. If this is the case, then one would have to wonder if

being susceptible to rhizobitoxine-induced chlorosis is beneficial to the plant in someway, thus giving them a selective advantage. Chakroborty and Purkayastha (1984) reported that rhizobitoxine producing strains could protect soybean roots from charcoal rot (*Macrophomina phaseolina*). In their study, they showed that by adding rhizobitoxine-producing bacterial strains to *M. phaseolina in vitro*, that the growth of *M. phaseolina* was inhibited. In addition, they measured the disease intensity of soybean plants that were inoculated with rhizobitoxine-producing strains and introduced to *M. phaseolina*. Their results showed that disease was significantly decreased.

Rhizobitoxine has also been shown to suppress ethylene (Owens et al, 1971; Duodu et al., 1999). Ethylene is known to cause senescence of nodules, but it has been reported that soybean nodulation is not sensitive to ethylene (Hunter, 1993). In a growth chamber experiment, they inoculated soybean plants with *Bradyrhizobium japonicum* and measured ethylene formed by the roots using gas chromatography. The results of this study showed that nodule numbers did not decrease and that soybean nodulation was not affected by low and intermediate levels of ethylene. Using *Amphicarpaea*, an herbaceous annual related to soybean, Parker and Peters (2001), showed that the plants inoculated with rhizobitoxine-producing strains produced more nodules per plant compared to those plants that were not inoculated with rhizobitoxine-producing strains. They also used acetylene reduction assays to show that there was considerable nitrogenase activity in plants with abundant nodulation. Duodu et al (1999) showed that rhizobitoxine is beneficial for nodule development in mungbean. In their study, the researchers used two mutant strains of rhizobitoxine-producing strain USDA 61that did not produce rhizobitoxine and compared these with the wild type USDA 61 to see if rhizobitoxine had a role in nodule development. They reported that

mungbean plants that were inoculated with the wild-type USDA 61 (rhizobitoxine-producing) had 21.7 mature nodules per plant and that the two mutant strains (non-rhizobitoxine producing) had 2.9 and 1.3 nodules per plant, respectively. Their results suggest that rhizobitoxine production has a positive role in some legumes, mungbean in particular, in terms of nodulation. The effect on total nitrogen fixation has not been determined. Rhizobitoxine susceptibility may have played a role in soybean some time during its evolutionary development, but this role may have been lost as soybean became domesticated. The fact that Johnson and Means (1960) reported that *G. gracilis*, two selections of *G. ussuriensis*, and two selections of *G. tomentella* were all susceptible to rhizobitoxine-induced chlorosis shows that rhizobitoxine production in soybean may have had a function in pre-domesticated soybean, possibly increased nodulation.

In conclusion, this study shows that the frequency of alleles that confer susceptibility to rhizobitoxine-induced chlorosis is more common in southern soybean ancestors than in northern soybean ancestors. Even ancestors that are resistant to rhizobitoxine-induced chlorosis can possess susceptible alleles without displaying a chlorotic phenotype and can pass these alleles down to future generations. The symbiosis between the rhizobitoxine-producing strains and the soybean plant has been shown to produce lower yields in many soybean cultivars. Judging from the pedigree of Brim it suggests that the genes for rhizobitoxine-induced chlorosis may be linked to agronomically important loci since all cultivars with the exception of Tracy have some degree of susceptibility. If they were linked to an agronomically unfavorable locus one would not expect to see rhizobitoxine-induced chlorosis passed on to the modern soybean cultivar Brim. However, if they are linked to agronomically important loci, then one would expect to see a higher incidence of

rhizobitoxine-induced chlorosis in this study of the northern soybean cultivars that were screened with the rhizobitoxine-producing strain. The low incidence of the occurrence of rhizobitoxine-induced chlorosis found in the fields of northern varieties can be explained by the fact that the strains that produce rhizobitoxine are not commonly found in these regions. The fact that the northern soybean ancestors screened in this study did not have a higher frequency of susceptibility to rhizobitoxine-induced chlorosis makes it difficult to suggest that the genes for susceptibility to rhizobitoxine-induced chlorosis can be linked to agronomically important loci. If rhizobitoxine-producing strains do play a role in preventing fungi from infecting soybean roots as it was previously stated, one would have to look at the incidence of fungal root diseases on soybean and determine if these incidences are higher in the southern portions of the US than in the northern portions. The hypothesis that rhizobitoxine-producing strains may be beneficial to soybean cultivars may be plausible, but this study did not investigate this and further work is needed for verification.

Table 3-1. Description of all possible genotypes for fully inbred soybean ancestors based on possible genetically variable parent combinations.

Parents	F_1	F_α	Symptom	Score
Rts1Rts1Rts2Rts2 x *Rts1Rts1rts2rts2*	*Rts1Rts1Rts2rts2*	½ *Rts1Rts1Rts2Rts2*	S	MS
		½ *Rts1Rts1rts2rts2*	R	
Rts1Rts1Rts2Rts2 x *rts1rts1Rts2Rts2*	*Rts1rts1Rts2Rts2*	½ *Rts1Rts1Rts2Rts2*	S	MS
		½ *rts1rts1Rts2Rts2*	R	
Rts1Rts1Rts2Rts2 x *rts1rts1rts2rts2*	*Rts1rts1Rts2rts2*	¼ *Rts1Rts1Rts2Rts2*	S	SS
		¼ *Rts1Rts1rts2rts2*	R	
		¼ *rts1rts1Rts2Rts2*	R	
		¼ *rts1rts1rts2rts2*	R	
Rts1Rts1rts2rts2 x *rts1rts1Rts2Rts2*	*Rts1rts1Rts2rts2*	¼ *Rts1Rts1Rts2Rts2*	S	SS
		¼ *Rts1Rts1rts2rts2*	R	
		¼ *rts1rts1Rts2Rts2*	R	
		¼ *rts1rts1rts2rts2*	R	
Rts1Rts1rts2rts2 x *rts1rts1rts2rts2*	*Rts1rts1rts2rts2*	½ *Rts1Rts1rts2rts2*	R	HR
		½ *rts1rts1rts2rts2*	R	
rts1rts1Rts2Rts2 x *rts1rts1rts2rts2*	*rts1rts1Rts2rts2*	½ *rts1rts1Rts2Rts2*	R	HR
		½ *rts1rts1rts2rts2*	R	
rts1rts1rts2rts2 x *rts1rts1rts2rts2*	*rts1rts1rts2rts2*	all *rts1rts1rts2rts2*	R	HR
Rts1Rts1Rts2Rts2 x R*ts1Rts1Rts2Rts2*	*Rts1Rts1Rts2Rts2*	all R*ts1Rts1Rts2Rts2*	S	HS

F_α, Possible genotypes after selection of single plant from the F_1 generation followed several generations of inbreeding; Symptom, S= susceptible to rhizobitoxine-induced chlorosis; R= resistance to rhizobitoxine-induced chlorosis; Score, MS= moderately susceptible, SS= slightly susceptible, HR= highly resistant; HS= highly susceptible.

Table 3-2. Ancestor total contribution to North American soybean cultivars, rhizobitoxine score, and maximum and minimum frequency when inoculated with rhizobitoxine-producing strain CD2-5.

Ancestor†	% contribution‡	Rhizobitoxine symptom§	P(S)¶	RR#	Max Rr††	Min Rr‡‡	Max rr§§	Min rr¶¶
Lincoln	17.90	HR	0	0.00	8.95	0.00	17.90	8.95
Mandarin (Ottawa)	12.15	HR	0	0.00	6.08	0.00	12.15	6.08
CNS	9.38	HR	0	0.00	4.69	0.00	9.38	4.69
Richland	8.21	HR	0	0.00	4.11	0.00	8.21	4.11
S-100	7.52	HS	1	7.52	0.00	0.00	0.00	0.00
Ogden	4.94	HS	1	4.94	0.00	0.00	0.00	0.00
AK (Harrow)	4.85	HS	1	4.85	0.00	0.00	0.00	0.00
Dunfield	3.62	HR	0	0.00	1.81	0.00	3.62	1.81
Mukden	3.46	MS	½	1.73	1.73	0.00	0.00	0.00
Jackson	3.25	SS	¼	0.81	1.62	0.81	0.81	0.00
Illini	2.20	SS	¼	0.55	1.10	0.55	0.55	0.00
Roanoke	2.10	HS	1	2.10	0.00	0.00	0.00	0.00
Perry	2.07	HS	1	2.07	0.00	0.00	0.00	0.00

Table 3-2 (con't).

Ancestor[†]	% contribution[‡]	Rhizobitoxine symptom[§]	P(S)[¶]	RR[#]	Max Rr[††]	Min Rr[‡‡]	Max rr[§§]	Min rr[¶¶]
Capital	1.67	HR	0	0.00	0.83	0.00	1.67	0.83
Manitoba Brown	1.06	HR	0	0.00	0.53	0.00	1.06	0.53
Anderson	1.04	MS	½	0.52	0.52	0.00	0.00	0.00
Haberlandt	0.83	HR	0	0.00	0.41	0.00	0.83	0.41
840-7-3	0.78	SS	¼	0.19	0.39	0.19	0.19	0.00
Bansei	0.78	MS	½	0.39	0.39	0.00	0.00	0.00
Kanro	0.73	HS	1	0.73	0.00	0.00	0.00	0.00
Flambeau	0.68	HR	0	0.00	0.34	0.00	0.68	0.34
Mejiro	0.68	HS	1	0.68	0.00	0.00	0.00	0.00
Ralsoy	0.62	SS	¼	0.16	0.31	0.16	0.16	0.00
Strain 18	0.53	HR	0	0.00	0.26	0.00	0.53	0.26
Korean	0.53	MS	½	0.26	0.26	0.00	0.00	0.00

Table 3-2 (con't).

Ancestor[†]	% contribution[‡]	Rhizobitoxine symptom[§]	P(S)[¶]	RR[#]	Max Rr[††]	Min Rr[‡‡]	Max rr[§§]	Min rr[¶¶]
Jogun	0.53	HS	1	0.53	0.00	0.00	0.00	0.00
Arksoy	0.52	SS	¼	0.13	0.26	0.13	0.13	0.00
Improved Pelican	0.51	HR	0	0.00	0.25	0.00	0.51	0.25
Fiskeby III	0.51	MS	½	0.25	0.25	0.00	0.00	0.00
PI 88788	0.49	MS	½	0.24	0.24	0.00	0.00	0.00
Bilomi 3	0.48	HR	0	0.00	0.24	0.00	0.48	0.24
Peking	0.40	SS	¼	0.10	0.20	0.10	0.10	0.00
FC 31745	0.38	HR	0	0.00	0.19	0.00	0.38	0.19
Fiskeby V	0.36	SS	¼	0.09	0.18	0.09	0.09	0.00
PI 71506	0.19	HR	0	0.00	0.09	0.00	0.19	0.09
Total	95.95			28.84	36.23	2.03	59.62	28.78

† Soybean ancestors and first progeny that make up the soybean genetic base and contribute at least 95% of the genes found in public cultivars (Gizlice et al., 1994).

‡ Total contribution to genes found in public cultivars (Gizlice et al., 1994).

§ Symptom of ancestor when inoculated with rhizobitoxine-producing strain CD2-5; HR, highly resistant (all plants resistant); SS, slightly susceptible (75% plants resistant, 25% plants susceptible); HS, highly susceptible (all plants susceptible); MS, moderately susceptible (50% plants susceptible, 50% plants resistant). Resistant, plants do not exhibit chlorosis; Susceptible, plants exhibit chlorosis.

¶ Probability of displaying a chlorotic phenotype.

Frequency of individuals homozygous for susceptible alleles at both loci.

†† Maximum frequency of individuals homozygous for susceptible alleles at one locus.

‡‡ Minimum frequency of individuals homozygous for susceptible alleles at one locus.

§§ Maximum frequency of individuals homozygous for resistant alleles at both loci.

¶¶ Minimum frequency of individuals homozygous for resistant alleles at both loci.

Table 3-3. Ancestor contribution to southern cultivars, rhizobitoxine score, and maximum and minimum frequency when inoculated with rhizobitoxine-producing strain CD2-5.

Ancestor[†]	% contribution[‡]	Rhizobitoxine score[§]	P(S)[¶]	RR[#]	Max Rr[††]	Min Rr[‡‡]	Max rr[§§]	Min rr[¶¶]
CNS	24.71	HR	0	0.00	12.35	0.00	24.71	12.35
S-100	21.31	HS	1	21.31	0.00	0.00	0.00	0.00
Jackson	10.61	SS	¼	2.65	5.31	2.65	0.00	0.00
Roanoke	6.54	HS	1	6.54	0.00	0.00	0.00	0.00
Ogden	6.44	HS	1	6.44	0.00	0.00	0.00	0.00
Dunfield	3.86	HR	0	0.00	1.93	0.00	3.86	1.93
Lincoln	2.90	HR	0	0.00	1.45	0.00	2.90	1.45
Haberlandt	2.50	HR	0	0.00	1.25	0.00	2.50	1.25
Mejiro	2.30	HS	1	2.30	0.00	0.00	0.00	0.00
Perry	2.06	HS	1	2.06	0.00	0.00	0.00	0.00
Ralsoy	1.93	SS	¼	0.48	0.97	0.48	0.48	0.00
Improved Pelican	1.75	HR	0	0.00	0.88	0.00	1.75	0.88
Arksoy	1.67	SS	¼	0.42	0.84	0.42	0.42	0.00
Bilomi 3	1.64	HR	0	0.00	0.82	0.00	1.64	0.82

Table 3-3 (con't).

Ancestor[†]	% contribution[‡]	Rhizobitoxine score[§]	P(S)[¶]	RR[#]	Max Rr[††]	Min Rr[‡‡]	Max rr[§§]	Min rr[¶¶]
FC 31745	1.19	HR	0	0.00	0.59	0.00	1.19	0.59
Peking	1.14	SS	¼	0.29	0.57	0.29	0.29	0.00
Richland	0.81	HR	0	0.00	0.41	0.00	0.81	0.41
PI 88788	0.74	MS	½	0.37	0.37	0.00	0.00	0.00
Anderson	0.70	MS	½	0.35	0.35	0.00	0.00	0.00
PI 71506	0.33	HR	0	0.00	0.16	0.00	0.33	0.16
Illini	0.04	SS	¼	0.01	0.02	0.01	0.01	0.00
Total	95.17			43.22	28.27	3.85	43.54	19.84

† Soybean ancestors and first progeny that make up the soybean genetic base and contribute at least 95% of the genes found in public cultivars (Gizlice et al., 1994).

‡ Total contribution to genes found in public cultivars (Gizlice et al., 1994).

§ Symptom of ancestor when inoculated with rhizobitoxine-producing strain CD2-5; HR, highly resistant (all plants resistant); SS, slightly susceptible (75% plants resistant, 25% plants susceptible); HS, highly susceptible (all plants susceptible); MS, moderately susceptible (50% plants susceptible, 50% plants resistant). Resistant, plants do not exhibit chlorosis; Susceptible, plants exhibit chlorosis.

¶ Probability of displaying a chlorotic phenotype.

Frequency of individuals homozygous for susceptible alleles at both loci.

†† Maximum frequency of individuals homozygous for susceptible alleles at one locus.

‡‡ Minimum frequency of individuals homozygous for susceptible alleles at one locus.

§§ Maximum frequency of individuals homozygous for resistant alleles at both loci.

¶¶ Minimum frequency of individuals homozygous for resistant alleles at both loci.

Table 3-4. Ancestor contribution to northern cultivars, rhizobitoxine score, and maximum and minimum frequency when inoculated with rhizobitoxine-producing strain CD2-5.

Ancestor†	% contribution‡	Rhizobitoxine symptom§	P(S)¶	RR#	Max Rr††	Min Rr‡‡	Max rr§§	Min rr¶¶
Lincoln	24.17	HR	0	0.00	12.08	0.00	24.17	12.08
Mandarin (Ottawa)	17.23	HR	0	0.00	8.61	0.00	17.23	8.61
Richland	11.31	HR	0	0.00	5.65	0.00	11.31	5.65
AK (Harrow)	6.88	HS	1	6.88	0.00	0.00	0.00	0.00
Mukden	4.91	MS	½	2.45	2.45	0.00	0.00	0.00
Ogden	4.31	HS	1	4.31	0.00	0.00	0.00	0.00
Dunfield	3.51	HR	0	0.00	1.75	0.00	3.51	1.75
Illini	3.10	SS	¼	0.77	1.55	0.77	0.77	0.00
CNS	2.98	HR	0	0.00	1.49	0.00	2.98	1.49
Capital	2.37	HR	0	0.00	1.18	0.00	2.37	1.18
Perry	2.08	HS	1	2.08	0.00	0.00	0.00	0.00
S-100	1.75	HS	1	1.75	0.00	0.00	0.00	0.00
Manitoba Brown	1.50	HR	0	0.00	0.75	0.00	1.50	0.75
Anderson	1.18	MS	½	0.59	0.59	0.00	0.00	0.00

Table 3-4 (con't).

Ancestor†	% contribution‡	Rhizobitoxine symptom§	P(S)¶	RR#	Max Rr††	Min Rr‡‡	Max rr§§	Min rr¶¶
840-7-3	1.10	SS	¼	0.27	0.55	0.27	0.27	0.00
Bansei	1.10	MS	½	0.55	0.55	0.00	0.00	0.00
Kanro	1.03	HS	1	1.03	0.00	0.00	0.00	0.00
Flambeau	0.97	HR	0	0.00	0.48	0.00	0.97	0.48
Korean	0.76	MS	½	0.38	0.38	0.00	0.00	0.00
Jogun	0.76	HS	1	0.76	0.00	0.00	0.00	0.00
Strain 18	0.75	HR	0	0.00	0.37	0.00	0.75	0.37
Fiskeby III	0.72	MS	½	0.36	0.36	0.00	0.00	0.00
Fiskeby V	0.52	SS	¼	0.13	0.26	0.13	0.13	0.00
PI 88788	0.38	MS	½	0.19	0.19	0.00	0.00	0.00
Roanoke	0.24	HS	1	0.24	0.00	0.00	0.00	0.00
Jackson	0.18	SS	¼	0.04	0.09	0.04	0.04	0.00
PI 71506	0.14	HR	0	0.00	0.07	0.00	0.14	0.07
Haberlandt	0.13	HR	0	0.00	0.06	0.00	0.13	0.06

Table 3-4 (con't).

Ancestor†	% contribution‡	Rhizobitoxine symptom§	P(S)¶	RR#	Max Rr††	Min Rr‡‡	Max rr§§	Min rr¶¶
Peking	0.10	SS	¼	0.02	0.05	0.02	0.02	0.00
Ralsoy	0.08	SS	¼	0.02	0.04	0.02	0.02	0.00
Arksoy	0.04	SS	¼	0.01	0.02	0.01	0.01	0.00
FC 31745	0.03	HR	0	0.00	0.01	0.00	0.03	0.01
Total	96.31			22.83	39.58	1.26	66.35	32.50

† Soybean ancestors and first progeny that make up the soybean genetic base and contribute at least 95% of the genes found in public cultivars (Gizlice et al., 1994).

‡ Total contribution to genes found in public cultivars (Gizlice et al., 1994).

§ Symptom of ancestor when inoculated with rhizobitoxine-producing strain CD2-5; HR, highly resistant (all plants resistant); SS, slightly susceptible (75% plants resistant, 25% plants susceptible); HS, highly susceptible (all plants susceptible); MS, moderately susceptible (50% plants susceptible, 50% plants resistant). Resistant, plants do not exhibit chlorosis; Susceptible, plants exhibit chlorosis.

¶ Probability of displaying a chlorotic phenotype.

Frequency of individuals homozygous for susceptible alleles at both loci.

†† Maximum frequency of individuals homozygous for susceptible alleles at one locus.

‡‡ Minimum frequency of individuals homozygous for susceptible alleles at one locus.

§§ Maximum frequency of individuals homozygous for resistant alleles at both loci.

¶¶ Minimum frequency of individuals homozygous for resistant alleles at both loci.

Table 3-5. List of 25 soybean cultivars, year of release, and rhizobitoxine symptom when inoculated with rhizobitoxine-producing strain CD2-5.

Cultivar	Year of release	Rhizobitoxine Symptom[†]
Adams	1948	HR
Blackhawk	1950	HR
Clark	1953	HR
D49-2491		HR
Dorman	1952	HR
Dyer	1967	HR
Forrest	1972	MS
Harosoy	1951	MS
Hawkeye	1947	MS
Hill	1959	HR
Hood	1958	MS
Lee	1954	SS
Pagoda		HR
Scott	1959	SS
Volstate		HS
Ware	1978	MS
Wayne	1964	HR
PI 416937		HS
PI 471938		MS
Hutcheson	1987	MS
Davis	1965	MS

Table 3-5 (con't).

Cultivar	Year of release	Rhizobitoxine Symptom†
Young	1984	MS
Essex	1972	SS
Brim	1994	HS
Tracy	1973	HR

† Symptom of cultivar when inoculated with rhizobitoxine-producing strain CD2-5; HR, highly resistant (all plants resistant); SS, slightly susceptible (75% plants resistant, 25% susceptible); HS, highly susceptible (all plants susceptible); MS, moderately susceptible (50% plants susceptible, 50% plants resistant).

Resistant, plants do not exhibit chlorosis; Susceptible, plants exhibit chlorosis.

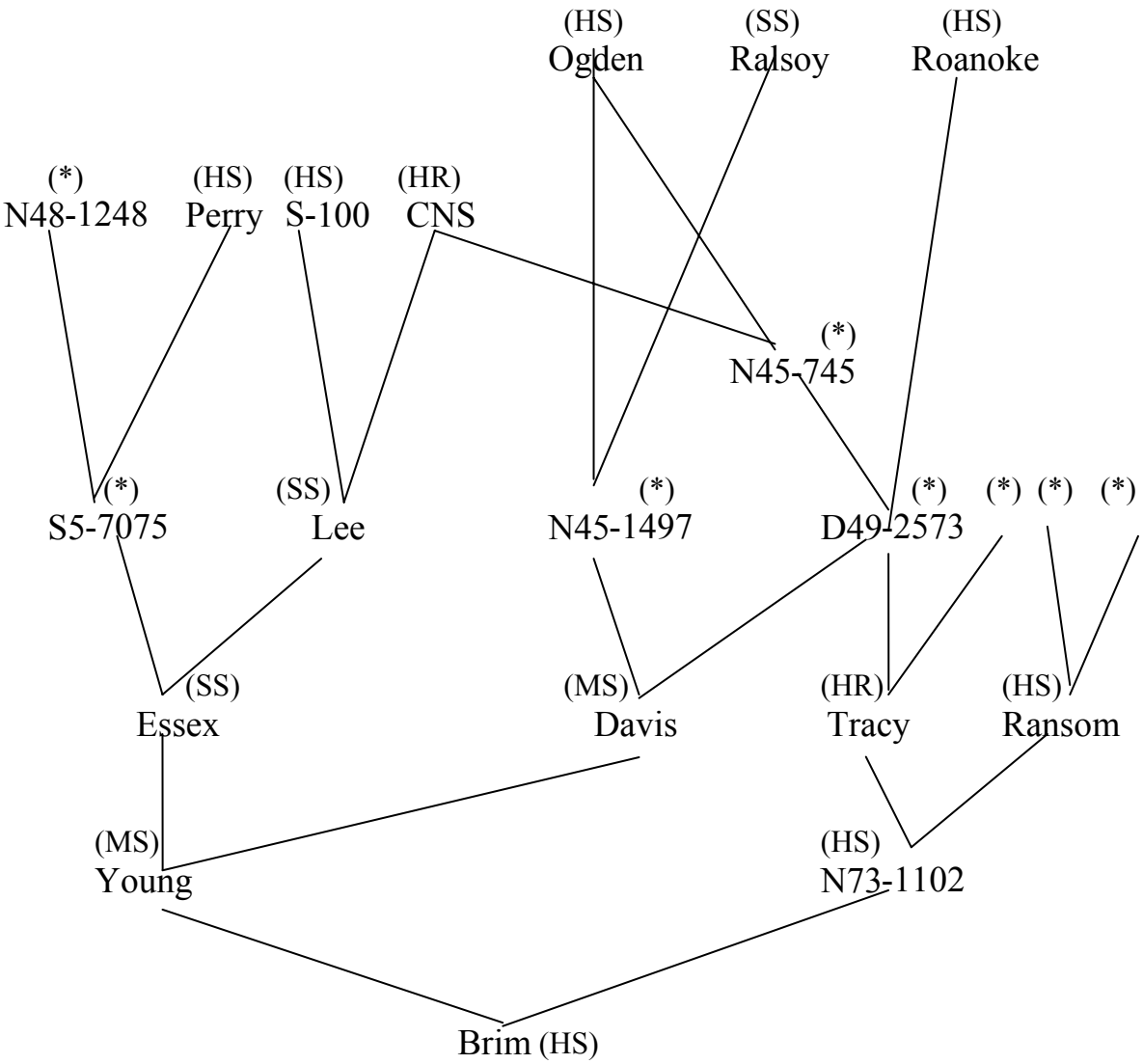

Figure 3-1. Pedigree of southern soybean cultivar Brim and the soybean response to rhizobitoxine-induced chlorosis.

HS, highly susceptible; SS, slightly susceptible (75% resistant:25% susceptible); MS, moderately susceptible (50% resistant : 50% susceptible); HS, highly susceptible; *, no data

CHAPTER IV

THE EFFECTS OF RHIZOBITOXINE-INDUCED CHLOROSIS ON SOYBEAN YIELD

INTRODUCTION

The relationship between soybean, (*Glycine max* (L.) Merr.), and bacteria that belong to the genus *Bradyrhizobium* is a symbiotic relationship. In short, the bacteria provide the soybean plant with nitrogen converted from atmospheric nitrogen to ammonium and in turn the soybean plant provides the bacteria with access to carbon, micronutrients, and protection from desiccation. However, there is a species of *Bradyrhizobium*, known as *Bradyrhizobium elkanii* (Kuykendall et al., 1992) which can effectively nodulate soybean but causes a chlorosis on some varieties of soybean (Owens et al., 1964). This chlorosis was first observed in the soybean cultivar Lee by Erdman et al (1956) and has been seen in other soybean cultivars that are grown in the southeastern region of the United States. It is now known that this chlorosis is caused by rhizobitoxine, a chemical produced in the nodules by *Bradyrhizobium elkanii*.

Studies which determine the effects of rhizobitoxine-producing strains on soybean have shown that these strains can have a negative effect on soybean production. Erdman et al (1957) reported that rhizobitoxine-producing strains did reduce yields under greenhouse conditions, but under field conditions these strains did not cause any quantifiable yield reductions. Teaney III and Fuhrmann (1992) studied rhizobitoxine-producing strains (USDA strains 31, 46, 76, 94, and 130) that differed in their abilities to cause rhizobitoxine-induced foliar chlorosis on the soybean cultivar Forrest. They examined the effects of these rhizobitoxine-producing strains on chlorophyll content, leaf protein and biomass

accumulation, nodular contents of leghemoglobin, soluble protein and rhizobitoxine, and total shoot nitrogen content under greenhouse conditions. They found rhizobitoxine in the nodules of all of the rhizobitoxine-producing strains except for USDA 31, but only USDA 76 and USDA 94 had measurable concentrations of rhizobitoxine and produced rhizobitoxine-induced chlorosis. They also showed that plants exhibiting moderate to severe visual chlorosis symptoms also had reductions in chlorophyll concentrations, shoot and nodule dry weight, leaf protein, and total N_2 fixation (measured by Kjeldahl procedure). Reductions in leghemoglobin and soluble nodular protein were observed when long periods of severe chlorosis were reported. In addition, they showed that plants inoculated with non-rhizobitoxine-producing strains were more productive than plants that were inoculated with rhizobitoxine-producing strains. Even so, the authors concluded that the impact of nodulation by rhizobitoxine-producing strains was nominal on short term soybean productivity in the absence of observable visual symptoms because only in the occurrence of severe rhizobitoxine-induced chlorosis did reductions in total plant productivity occur. Rhizobitoxine-producing strains have been shown to be affected by nitrate (NO_3^-). Teaney III and Fuhrmann (1993) examined the effects of nitrate on rhizobitoxine production by using rhizobitoxine-producing strains USDA 76 and USDA 94 and the soybean cultivar Forrest in a greenhouse study. They reported decreased rhizobitoxine production with increased nitrate application and concluded that rhizobitoxine production can be reduced by applying nitrate to soybeans. It has been documented that some bradyrhizobial strains are more efficient than others at fixing nitrogen in soybean. *Bradyrhizobium elkanii* tend to be less efficient than *B. japonicum*. Israel (1981) reported that the rhizobitoxine-nonproducing USDA 110 (*B. japonicum*) fixed over 100% more nitrogen than the rhizobitoxine-producing strain USDA 31

(*B. elkanii*) in a greenhouse study. He also reported that the amount of nitrogen stored in vegetative tissues for remobilization to the developing seed was limited by the rhizobitoxine-producing strain USDA 31.

Rhizobitoxine-producing strains have not been studied as extensively under field conditions. While studies conducted on rhizobitoxine-producing strains under greenhouse conditions have shed some light on how rhizobitoxine affects soybean productivity, a disadvantage has been that these studies were usually carried out in sterile soils. Bacterial strains which would be typical under field conditions were not present. One of the obstacles that prevent researchers from studying strains under field conditions is the fact that introduced strains must compete with indigenous strains. Fuhrmann and Vasilas (1991) developed a method that allowed researchers to study strains of interest under field conditions. This method involves pre-nodulation of soybean seedlings under greenhouse conditions in containers that can be used to produce transplants. Soybean seedlings are inoculated with a desired strain of *Bradyrhizobium* and grown in the greenhouse until the time of transplantation (usually 3 weeks after inoculation). At 34 days after transplanting, they dug up three plants from each plot to determine the success with which the strains were established in the nodules. They reported that 97-99% of the nodules sampled had the initial bacterial strain that was used at the time of inoculation. They recommended using this technique for microplot research. Vasilas and Fuhrmann (1993) used this technique to study the field response of soybean to nodulation by a rhizobitoxine-producing strain. In their study, they inoculated the soybean cultivar Forrest with rhizobitoxine-producing strain USDA 94 to determine if rhizobitoxine-producing strains had any effects on N_2 fixation, seed yield, and plant development. They reported decreased seed yields, vegetative growth,

delayed onset of full pod, beginning seed and full seed stages, and the doubling of the interval between beginning pod and full pod stages of plants inoculated with the rhizobitoxine-producing strain USDA 94. This is in contrast with the earlier report by Erdman et al (1957) in which they stated that rhizobitoxine-producing strains did not measurably reduce yield of soybean grown under field conditions. In North Carolina, Ramirez et al (1997) showed that up to 50% of the bacterial strains found in nodules at one of the two locations were associated with rhizobitoxine production; however, they did not find any negative effects on plant growth or yield due to these rhizobitoxine-producing strains. In addition, they reported a low nitrogen-fixing capacity for approximately 20% of the isolates from bradyrhizobial populations of two soils. A low nitrogen-fixing capacity was based on isolates that fixed lower amounts of nitrogen than the rhizobitoxine-producing strain USDA 31.

The objective of this study is to examine the effects of rhizobitoxine on seed yield, protein concentration, and total protein by comparing soybean plants nodulated by rhizobitoxine-producing strains with those nodulated by non-rhizobitoxine producing strains grown under field conditions.

MATERIALS AND METHODS

Preparation of seed and bacterial strain and transplantation protocol

Five soybean varieties were used in this study Brim (Burton et al, 1994), CNS (an ancestor from China), Lee (Hartwig, 1954), Young_Susceptible (YS), and Young_Resistant (YR)) conducted in the summer of 2001. The YS and YR soybean varieties were selected from a preliminary greenhouse study in which the soybean variety Young (Burton et al, 1987) was screened for rhizobitoxine susceptibility. The results of this preliminary screen produced Young soybean plants that were resistant and plants that were susceptible to rhizobitoxine induced chlorosis. Resistant and susceptible plants were then grown separately to produce seed. The seed from each were used in this study.

The soybean seed were soaked in 95% ethanol for one minute. The liquid was decanted and the seed were then soaked in a 1:5 (chlorox:water) chlorox solution for four minutes. The liquid was then decanted and the seeds were rinsed five times with sterile water. The seeds were then stored in beakers and covered with aluminum foil in order to not allow the seeds to desiccate. Seed were not allowed to sit in water. The cells of the CD2-5 and MN 110 bacterial strains were cultured on separate Yeast Extract Mannitol (YEM) agar plates to allow them to grow. They were then each transferred to 100 ml flasks of modified YEM liquid media. The flasks were then put into a shaker at 28° C at a shaking speed of 150 rpm. 2x2x3 inch planting trays with 64 cells per tray were filled with vermiculite and watered to saturation with an N free nutrient solution prior to planting of the soybean seed. After the watering, trays were allowed to drain. A single soybean seed was placed in each cell and then inoculated with 0.5 ml of either MN-110 or CD2-5 (10^9 CFU/ml). The seeds were covered with vermiculite and kept damp for the first five days. After five days, the trays were watered

daily and given N free nutrient solution twice a week. Plants were kept in the greenhouse for approximately 21 days after planting and then transplanted in the field. The plants were transplanted to 10 foot plots in trenches that were dug with a hoe on June 25, June 29, and July 3, 2001 at Clayton, Sandhills, and Clinton, respectively. The trenches were approximately 5 inches deep and 4.5 inches wide. The plants were placed in the trenches and water was added to the trenches until the trench was full. The trenches were then covered with soil and the soil was packed around the plant. Plants were planted in a randomized complete block with 3 reps and each plot had plants planted at 3 plants per foot. Plots were watered as needed with 2.5 gallons of water per 10 foot plot for the Clayton and Clinton locations for the first two weeks. The borders were mechanically planted and were watered by rainfall. After that, there was no irrigation other than rainfall. The Sandhills location was irrigated with one inch of water following transplanting and plants were irrigated as needed. This location had 4 plants per foot per plot. Two plants per plot were later dug up with a spade from each end of the plot; nodules were separated as either crown or lateral root nodules. Nodules were collected 71, 74, and 73 days after planting at Clayton, Sandhills, and Clinton, respectively. The nodules were placed in separate tubes and stored on ice. After nodule collection was completed, the nodules were placed in a freezer until analysis. When plants reached maturity, the middle row of each three row plot was harvested and seed weight was later recorded in grams per plot. In addition, flower date, flower color, maturity date, pubescence color, height, and lodging were recorded. Seed yield, percent seed protein, and total protein were tested for significance at the $P < 0.05$ level based on analysis of variance using PROC GLM in SAS with the MEANS statement and LSD option to

determine differences between genotypes (SAS Institute Inc., Cary, NC). The locations in this study are random.

Preparation of nodules for microplate analysis

For each soybean/bacterial strain combination, 24 tap and 24 lateral nodules were selected for analysis. Individual nodules were placed in 80 mm x 10 mm test tubes (one nodule per tube) and 0.5 mL of saline with Azide solution was added to each tube. The nodules were crushed with glass stirring rods and then steamed for 10 minutes in a water bath at 90° to 100° C. After incubation, each test tube had 1 mL of coating buffer added and 100 µL of the nodule solution (henceforth referred to as antigen) was added to 96 round well plate. Plates were incubated at 28° C for 1 hour (or overnight at 5° C). After incubation, the plates were washed 3 times with phosphate-buffered saline with Tween (PBST), with the second wash standing for 5 minutes before rinsing the third time. The PBST was shaken from each plate and 100 µL of antisera was added to the appropriate wells and incubated for 1 hour at 28° C. The antisera were used to test for the presence of the rhizobitoxine-producing strains USDA 31, USDA 76, and USDA 94. After incubation, plates were washed 3 times with PBST. After the final washing, 100 µL of goat anti-rabbit globulin (GARG) was added and allowed to incubate overnight at 5° C followed by 3 washes with PBST.

Analysis of nodules for rhizobium content

Each well had 100 µL of substrate solution added and after 30 – 60 minutes a yellow color began to develop indicating that the plates were ready for analysis. Plates were analyzed using an ELISA microplate reader (Bio-Tek EL-307) at an absorbance of 405nm. Microplate readings of 0.50 or more were considered positive. Analysis of soybean nodules

showed that the introduced strains used in this study were recovered 80 – 96% of the nodules tested depending on the location and the genotype.

RESULTS AND DISCUSSION

Combined analysis showed significant differences for yield ($p = 0.0110$) among genotypes, but the genotype x location interaction was not significant (Table 4-1). When genotypes were partitioned into strain, line, and strain x line, strain and lines were significant for yield ($P < 0.05$) (Table 4-1). The strain x line interaction was not significant. The overall mean yield for this study was 1386 kg ha^{-1}. To determine if rhizobitoxine-producing strains caused lower yields compared to non-rhizobitoxine producing strains, the yields of the genotypes with each strain were compared (Table 4-2). The table shows that when a genotype is inoculated with a rhizobitoxine-producing strain that overall it has a lower yield than when the same genotype is inoculated with a non-rhizobitoxine producing strain. The only exception was the comparison of the Young_Resistant genotypes. These genotypes had a higher yield when inoculated with the rhizobitoxine-producing strain compared with the non-rhizobitoxine producing strain (1510 versus 1423 kg ha^{-1}, respectively). This comparison shows that as a nitrogen-fixing strain, the rhizobitoxine-producing strain (CD2-5) is just as good as the non-rhizobitoxine producing strain for the Young_Resistant genotypes. Though there were differences in yield for the genotype strain comparisons, none of these differences were significantly different except for the genotype strain comparison for Lee (Table 4-3). In order to determine if yield differences between the genotypes inoculated with MN 110 and CD2-5 are due to rhizobitoxine-production in the CD2-5, the comparison of the yields of the Young_Resistant and Young_Susceptible when inoculated with the rhizobitoxine-producing strain CD2-5 were analyzed. These genotypes are essentially isolines that differ only in the genes responsible for the soybean response to rhizobitoxine-induced chlorosis. The mean yield for Young_Resistant was higher than the mean yield for Young_Susceptible when

inoculated with the rhizobitoxine-producing strain (1510 versus 1338 kg ha^{-1}, respectively) (Table 2). However, these differences were not significantly different (Table 4-3). On average, the susceptible genotypes (Brim and Young_Susceptible) had lower yields than the resistant genotypes (Lee, and Young_Resistant) when inoculated with the rhizobitoxine-producing strain (CD2-5). The resistant genotype CNS had a lower yield than the susceptible genotypes, but this is partly due to this genotype having a poor stand. In addition, in the presence of MN 110 the susceptible genotypes, on average, did not have lower yields than the resistant genotypes. The comparison of the Young_Resistant genotype with the Young_Susceptible genotype when inoculated with the non-rhizobitoxine producing strain MN 110 shows that the Young_Susceptible genotype has a higher yield than the Young_Resistant (1516 versus 1423 kg ha^{-1}, respectively). This difference was not significant and this comparison shows that the Young_Resistant genotype and the Young_Susceptible genotype have similar yield potentials. This finding in conjunction with the finding that the Young_Susceptible had a lower yield than the Young_Resistant in the presence of the rhizobitoxine-producing strains gives strong evidence that rhizobitoxine is responsible for the low yields when genotypes that are susceptible to rhizobitoxine are inoculated with rhizobitoxine-producing strains.

Overall, genotypes with the rhizobitoxine producing strain had lower mean yields than genotypes with the non-rhizobitoxine producing strain, with the exception (as stated earlier) of the Young_Resistant genotype. However, this comparison was not significantly different (Table 4-3). The significant difference found among strains indicates that they have different effects on yield and this was shown based on the results of this study. In a similar transplant study, Vasilas and Fuhrmann (1993) also reported decreased seed yields in

soybean cultivars that were inoculated with a rhizobitoxine-producing strain (USDA 94). Even though the other soybean genotype comparisons did not have significant differences in mean yield, they were different and seem to show that rhizobitoxine-induced chlorosis can cause soybean seed yield reductions. Conversely, the fact that the Young_Resistant genotype in the presence of CD2-5 (rhizobitoxine-producer) had a higher mean yield than the Young_Resistant genotype in the presence of MN 110 (non-rhizobitoxine producer) shows that rhizobitoxine-producing strains may not affect the yield of soybean cultivars that are resistant to rhizobitoxine-induced chlorosis and that these two strains are both good nitrogen-fixers for this genotype.

For percent protein, the combined analysis showed that the differences between strains in percent protein were not significant (Table 4-1). There was a significant difference between lines; however only CNS in the presence of CD2-5 and MN 110, respectively, were different from the other genotypes (Table 4-2). Based on these results, it appears that rhizobitoxine-induced chlorosis does not have a significant effect on the percent protein of soybean.

Total protein is a measure of how the strain may have affected the nitrogen accumulation of the plant throughout the year. The combined analysis shows that there were significant differences in total protein with regards to genotype, but not for genotype x location (Table 4-1). When the genotype was partitioned into strain, lines, and strain x lines, only the strains were significant (Table 4-1). Overall, the total protein was higher for the genotypes that were inoculated with the non-rhizobitoxine producing strain (MN 110) (Table 4-2). As with yield, the genotype Young_Resistant had a higher total protein in the presence of the rhizobitoxine-producing strain (CD2-5) (Table 4-2). The only significant differences

among genotypes were with the genotype Lee, which had a higher total protein when inoculated with the non-rhizobitoxine producing strain (MN 110) (Table 4-2). The total protein of all soybean cultivars in this study were negatively affected when inoculated with the rhizobitoxine-producing strain, with the exception of the Young_resistant, which had a higher total protein when inoculated with the rhizobitoxine-producing strain compared to the non-rhizobitoxine producing strain (Table 4-2).

Overall, the rhizobitoxine-producing strain did reduce yield and total protein (with the exception of Young_Resistant) even though it was not significant (with the exception of Lee). The fact that, on average, the resistant genotypes had a higher yield mean and a higher total protein mean when inoculated with the rhizobitoxine-producing strain compared to the susceptible genotypes indicates that resistant genotypes may be preventing rhizobitoxine production or metabolizing it so that it does not affect yield. This is evidence that rhizobitoxine-induced chlorosis does lower soybean yield and that susceptible soybean cultivars lack a mechanism to prevent this from happening.

Field studies are different than greenhouse studies with bacterial strains because in the greenhouse there is no competition with other strains. The fact that this study did not find significant differences in yield and total protein (with the exception of the genotype Lee) for rhizobitoxine-induced chlorosis in soybean may have been due to the fact that the soybean cultivars compensated in some way by using indigenous strains as well as the introduced strains. A greenhouse study conducted by Teaney III and Fuhrmann (1993) concluded that nitrate can reduce chlorosis on soybean plants thus minimizing the symptoms of rhizobitoxine. They reported that this may be a reason that symptoms of rhizobitoxine are not as severe in the field as they are in the greenhouse. In the current study, chlorosis was

observed to have decreased after the soybean plants were established in the field. However, when analyzed using an ELISA mircoplate reader, the introduced strains were observed in 80-96% of the nodules of plants sampled 72 days after planting. Though this is lower than the 97-99% occupancy reported by Fuhrmann and Vasilas (1991), it does show that the intended strain was recovered at a high rate.

With the soybean cultivar Lee, there was a significant 13% reduction in yield in the presence of the rhizobitoxine-producing strain. This shows that there is the potential for yields of soybean cultivars to be significantly affected when inoculated with a rhizobitoxine-producing strain. More field studies are needed to further quantify the effect rhizobitoxine-producing strains have on soybean yield. This study used five soybean cultivars and only one rhizobitoxine-producing strain. It is known, however, that there are many rhizobitoxine-producing strains in nature, but it is not known if these different strains would affect soybean in a similar manner. In a study of 767 isolates of root nodule bacteria from 79 locations in 27 states, Weber et al (1989) reported that 37% of the nodules had the rhizobitoxine-producing strain USDA 31. In a study conducted in North Carolina, Ramirez et al (1997) reported that a high percentage of the nodules were occupied by several rhizobitoxine-producing strains.

Given the high incidence of rhizobitoxine-producing strains found in southeastern soils, they may play a role in preventing soybean from reaching its full yield potential. The results of this study are similar to the results reported in previous studies, in that, rhizobitoxine-producing strains reduce soybean yield in some soybean genotypes but not others (Vasilas and Fuhrmann, 1993; Fuhrmann and Vasilas, 1993). To understand the soybean response to rhizobitoxine-induced chlorosis and its affect on yield, more field studies are needed to help explain the role that rhizobitoxine-producing strains play in

soybean yield and to determine what effect, if any, that competition has on yield of soybeans inoculated with rhizobitoxine-producing strains.

Table 4-1. Combined analysis of variance, p-value of genotype, r^2, CV, and mean for yield, percent protein, and total protein from a one year study at 3 locations (Clayton, Sandhills, and Clinton) with 10 genotypes per location, and 3 reps per variety.

		Mean Square		
Source	df	Yield	Percent Protein	Total Protein
Loc	2	852464 *	21.33 *	189960 *
Rep(Loc)	6	118919 *	2.69 *	18781 *
Genotype†	9	235194 *	6.72 *	29373 *
Strain	*1*	*227673 **	*0.00005 ns*	*37765 **
Lines	*4*	*412486 **	*15.05 **	*47168 ns*
Strain x lines	*4*	*59782 ns*	*0.08 ns*	*9479 ns*
Gen x Loc	18	66685 ns	1.18*	9992 ns
Error	54	42477	0.45	6773
p-value‡		0.0110	0.0009	0.0246
r^2		0.7143	0.8528	0.7192
CV		14.866	1.6528	14.659
mean		1386	40.6	561

*, Significant at the 0.05 probability level.

ns, Not significant at the 0.05 probability level.

†, There were five genotypes used in this study and each was inoculated with a rhizobitoxine-producing strain and a non-rhizobitoxine-producing strain. Each combination was used as a separate genotype. The italicized sources represent a partitioning of the genotype.

‡, The p-value of the genotype.

Table 4-2. Yield, protein, and total protein means of genotype x strain interaction from a one year study at 3 locations (Clayton, Sandhills, and Clinton).

		Mean		
Genotype†	Strain‡	Yield (kg ha^{-1})	Seed Protein (%)	Total Protein (kg ha^{-1})§
Brim	110	1477	40.21	595
Brim	25	1368	40.29	551
CNS	110	1168	42.09	491
CNS	25	1074	42.27	453
Lee	110	1599	40.04	641
Lee	25	1391	39.97	556
Young Resistant	110	1423	40.20	573
Young Resistant	25	1510	40.03	607
Young Susceptible	110	1516	40.23	610
Young Susceptible	25	1338	40.21	538
$LSD_{0.05}$		195	0.63	78
Overall Mean		1386	40.55	561

†, Young Resistant genotypes do not exhibit rhizobitoxine-induced chlorosis when inoculated with rhizobitoxine-producing strains and Young Susceptible genotypes do exhibit rhizobitoxine-induced chlorosis when inoculated with rhizobitoxine-producing strains.

‡, 110 refers to MN110 (non-rhizobitoxine producing strain) and 25 refers to CD2-5 (a rhizobitoxine-producing strain).

§, Total protein = (Yield * % protein/100).

Table 4-3. Comparison of genotype-strain interactions partitioned by genotype for yield, protein, and total protein from a one year study at 3 locations (Clayton, Sandhills, and Clinton).

		Mean Square		
Source†	df	Yield	Seed Protein	Total Protein
Brim25 vs. Brim110	1	54108 ns	0.0272 ns	8661 ns
CNS25 vs. CNS110	1	40122 ns	0.1440 ns	6488 ns
Lee25 vs. Lee110	1	195187 *	0.0214 ns	32024 *
Young_R25 vs. Young_R110	1	34509 ns	0.1318 ns	5062 ns
Young_S25 vs. Young_S110	1	142879 ns	0.00269 ns	23448 ns
Young_R25 vs. Young_S25	1	39156 ns	0.00435 ns	6141 ns
Young_R110 vs. Young_S110	1	133867 ns	0.142222 ns	21288 ns
CD2-5 vs. MN 110	1	227673 *	0.00005 ns	37765 *

†, 25 and 110 refer to the two strains, CD2-5 (rhizobitoxine producing) and MN110 (non-rhizobitoxine producing). For example, Brim25 is the soybean cultivar Brim inoculated with CD2-5 and Brim110 is the soybean cultivar Brim inoculated with MN110.

ns, Not significant at the 0.05 probability level.

*, Significant at the 0.05 probability level.

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APPENDIX

Appendix 1. Linkage group, polymorphism result, marker size, and p-value of markers used in this study.

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 042	A1	No amplification	-	-	-
Satt 073	A1	No amplification	-	-	-
Satt 050	A1	No amplification	-	-	-
Satt 625	A1	No amplification	-	-	-
Satt 165	A1	No amplification	-	-	-
Satt 200	A1	No amplification	-	-	-
Satt 155	A1	No amplification	-	-	-
Sat_368	A1	No amplification	-	-	-
Sat_271	A1	No amplification	-	-	-
Satt 364	A1	No amplification	-	-	-
Satt 648	A1	No amplification	-	-	-
Satt 526	A1	No amplification	-	-	-
Satt 382	A1	No amplification	-	-	-
Satt 572	A1	Monomorphic	?	?	-
Satt 511	A1	No amplification	-	-	-
Satt 225	A1	No amplification	-	-	-
Satt 454	A1	Polymorphic	251	254	0.442
Satt 593	A1	No amplification	-	-	-
Satt 471	A1	No amplification	-	-	-
SOYNOD26A	A1	No amplification	-	-	-
Satt 236	A1	Monomorphic	215	215	-
Satt 276	A1	Monomorphic	323	323	-
Satt 300	A1	Polymorphic	124	109	0.312

Appendix 1 (con't).

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 385	A1	Polymorphic	265	303	0.254
Sat_356	A1	No amplification	-	-	-
Satt 390	A2	Monomorphic	230	230	-
Satt 207	A2	Polymorphic	240	235	0.856
Satt 177	A2	Polymorphic	113	107	0.125
Satt 315	A2	Monomorphic	245	245	-
Sat_215	A2	No amplification	-	-	-
Satt 187	A2	Polymorphic	270	245	0.736
GMENOD2B	A2	Polymorphic	160	190	0.012
Satt 424	A2	Polymorphic	269	148	0.352
Satt 341	A2	Polymorphic	225	220	0.207
Sat_129	A2	Polymorphic	210	250	?
Sat_199	A2	Monomorphic	250	250	-
Satt 089	A2	Monomorphic	129	129	-
Satt 525	A2	Monomorphic	200	200	-
Satt 233	A2	Monomorphic	250	250	-
Satt 437	A2	Monomorphic	260	260	-
Satt 327	A2	Monomorphic	250	250	-
Satt 329	A2	Monomorphic	250	250	-
Satt 158	A2	Polymorphic	?	?	-
Satt 421	A2	No amplification	-	-	-
Sat_382	A2	Polymorphic	250	210	?
Sat_378	A2	Monomorphic	210	210	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 707	A2	No amplification	-	-	-
Sat_377	A2	Monomorphic	210	210	-
Satt 133	A2	No amplification	-	-	-
Satt 209	A2	Monomorphic	205	205	-
Satt 409	A2	Polymorphic	265	274	?
Satt 228	A2	Polymorphic	250	220	?
Satt 429	A2	Polymorphic	245	280	0.656
Satt 378	A2	Monomorphic	200	200	-
Satt 197	B1	Monomorphic	186	186	-
Satt 332	B1	Monomorphic	?	?	-
Satt 251	B1	Monomorphic	205	205	-
Satt 509	B1	Monomorphic	?	?	-
Satt 519	B1	Monomorphic	?	?	-
Satt 415	B1	Polymorphic	168	156	0.944
Satt 484	B1	No amplification	-	-	-
Satt 453	B1	No amplification	-	-	-
Satt 665	B1	Monomorphic	303	303	-
Sat_270	B1	Polymorphic	190	205	0.711
Sct_026	B1	Monomorphic	125	125	-
Sat_123	B1	No amplification	-	-	-
Satt 577	B2	Polymorphic	113	110	0.241
Satt 126	B2	Polymorphic	148	121	0.302

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Sat_287	B2	No amplification	-	-	-
Satt 168	B2	No amplification	-	-	-
Satt 601	B2	No amplification	-	-	-
Satt 272	B2	Polymorphic	?	?	0.654
Satt 070	B2	No amplification	-	-	-
Satt 556	B2	No amplification	-	-	-
Satt 066	B2	No amplification	-	-	-
Sct_064	B2	Monomorphic	?	?	-
Satt 063	B2	No amplification	-	-	-
Satt 534	B2	Polymorphic	260	242	0.591
Satt 577	B2	Polymorphic	113	110	0.241
Satt 578	C1	Monomorphic	?	?	-
Satt 161	C1	Polymorphic	?	?	0.339
Satt 139	C1	Polymorphic	160	260	?
Satt 396	C1	Polymorphic	170	180	0.139
Satt 646	C1	Polymorphic	180	200	0.349
Satt 164	C1	Polymorphic	240	237	0.333
Satt 399	C1	Monomorphic	?	?	-
Satt 338	C1	Polymorphic	260	225	0.779
Sat_337	C1	Polymorphic	260	240	?
Satt 524	C1	Monomorphic	?	?	-
Sat_085	C1	Monomorphic	?	?	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 195	C1	Monomorphic	?	?	-
Sct_186	C1	Monomorphic	?	?	-
SOYGPATR	C1	Monomorphic	?	?	-
Sct_191	C1	Polymorphic	125	110	0.091
Sat_140	C1	No amplification	-	-	-
Sat_077	C1	No amplification	-	-	-
Satt 194	C1	Monomorphic	?	?	-
Satt 180	C1	Polymorphic	245	269	0.828
Satt 294	C1	Polymorphic	285	294	0.361
Satt 565	C1	Monomorphic	348	348	-
Sat_062	C2	Polymorphic	?	?	-
Satt 432	C2	Monomorphic	270	270	-
Satt 281	C2	Polymorphic	280	318	?
Satt 422	C2	Monomorphic	250	250	-
Satt 291	C2	Monomorphic	215	215	-
Sat_336	C2	Polymorphic	275	272	?
Satt 457	C2	Polymorphic	280	260	0.450
Satt 305	C2	No amplification	-	-	-
Satt 170	C2	Monomorphic	195	195	-
Satt 322	C2	No amplification	-	-	-
Satt 376	C2	Polymorphic	249	220	0.936
Satt 363	C2	Polymorphic	235	255	0.633

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 286	C2	Monomorphic	230	230	-
Satt 277	C2	Polymorphic	180	175	0.112
Satt 365	C2	Monomorphic	270	270	-
Satt 557	C2	Monomorphic	200	200	-
Satt 289	C2	Monomorphic	?	?	-
Satt 489	C2	Monomorphic	190	190	-
Satt 134	C2	Polymorphic	305	315	?
Satt 100	C2	No amplication	-	-	-
Satt 708	C2	Polymorphic	230	245	?
Satt 079	C2	No amplification	-	-	-
Satt 307	C2	Polymorphic	127	139	0.289
Sct_028	C2	No amplification	-	-	-
Satt 316	C2	Polymorphic	205	200	?
Satt 433	C2	No amplification	-	-	-
Satt 371	C2	Monomorphic	275	275	-
Satt 357	C2	Polymorphic	215	227	0.918
Satt 147	D1a	Polymorphic	174	210	0.894
Satt 408	D1a	Polymorphic	180	190	0.232
Satt 071	D1a	No amplification	-	-	-
Sat_036	D1a	No amplification	-	-	-
Satt 436	D1a	Polymorphic	200	260	0.668
Satt 198	D1a	No amplification	-	-	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Sat_106	D1a	Monomorphic	120	120	-
Satt 507	D1a	Monomorphic	220	220	-
Sat_110	D1a	Polymorphic	220	170	?
Satt 580	D1a	Monomorphic	120	120	-
Satt 203	D1a	No amplification	-	-	-
Satt 402	D1a	Monomorphic	320	320	-
Satt 267	D1a	Monomorphic	240	240	-
Satt 383	D1a	Polymorphic	270	220	0.897
Satt 254	D1a	No amplification	-	-	-
Satt 179	D1a	Monomorphic	139	139	-
Satt 295	D1a	No amplification	-	-	-
Satt 603	D1a	No amplification	-	-	-
Satt 548	D1a	Polymorphic	170	205	0.438
Satt 169	D1a	Polymorphic	180	177	?
Satt 321	D1a	Monomorphic	230	230	-
Satt 532	D1a	No amplification	-	-	-
Satt 032	D1a	No amplification	-	-	-
Satt 482	D1a	No amplification	-	-	-
Satt 531	D1a	Monomorphic	245	245	-
Sat_353	D1a	No amplification	-	-	-
Satt 184	D1a	Monomorphic	150	150	-
Sat_332	D1a	No amplification	-	-	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Sat_096	D1b	Monomorphic	175	175	-
Sat_351	D1b	Polymorphic	260	275	?
Satt 157	D1b	Polymorphic	253	272	0.667
Satt 558	D1b	Polymorphic	230	225	0.875
Satt 296	D1b	Polymorphic	230	235	?
Satt 412	D1b	No amplification	-	-	-
Satt 141	D1b	Polymorphic	149	187	0.803
Satt 290	D1b	Polymorphic	230	235	?
Satt 005	D1b	No amplification	-	-	-
Satt 600	D1b	No amplification	-	-	-
Satt 537	D1b	Monomorphic	150	150	-
Satt 189	D1b	No amplification	-	-	-
Satt 350	D1b	Monomorphic	255	255	-
Satt 428	D1b	Monomorphic	245	245	-
Satt 041	D1b	Monomorphic	300	300	-
Satt 172	D1b	Polymorphic	233	224	0.290
Satt 274	D1b	Polymorphic	200	195	?
Satt 459	D1b	Monomorphic	185	185	-
Satt 271	D1b	Monomorphic	115	115	-
Sctt_008	D2	Monomorphic	100	100	-
Satt 458	D2	Polymorphic	160	175	0.116
Satt 135	D2	Monomorphic	200	200	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§]		p-value[¶]
			Brim	CNS	
Satt 372	D2	Monomorphic	250	250	-
Satt 154	D2	No amplification	-	-	-
Sat_092	D2	No amplification	-	-	-
Satt 669	D2	Monomorphic	150	150	-
Satt 208	D2	Polymorphic	200	190	0.911
Satt 397	D2	Polymorphic	165	180	0.134
Satt 389	D2	No amplification	-	-	-
Satt 226	D2	Polymorphic	324	306	0.335
Sat_362	D2	Polymorphic	250	220	0.224
Sat_194	D2	Polymorphic	210	165	0.117
Satt 082	D2	Polymorphic	115	120	?
Sat_365	D2	Polymorphic	205	195	0.791
Satt 574	D2	No amplification	-	-	-
Sat_354	D2	Monomorphic	?	?	-
Satt 301	D2	Polymorphic	260	200	0.052
Satt 186	D2	Polymorphic	227	230	?
Sat_326	D2	Polymorphic	250	220	?
Sat_086	D2	No amplification	-	-	-
Sat_022	D2	No amplification	-	-	-
Satt 386	D2	Monomorphic	200	200	-
Satt 256	D2	Monomorphic	190	190	-
Sat_220	D2	Monomorphic	220	220	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 483	E	Polymorphic	280	250	0.961
Satt 045	E	Polymorphic	139	150	?
Satt 263	E	Polymorphic	230	250	0.900
Satt 117	E	No amplification	-	-	-
Satt 452	E	Polymorphic	210	225	0.953
Satt 212	E	Monomorphic	?	?	-
Satt 384	E	Monomorphic	?	?	-
Satt 204	E	Polymorphic	210	225	?
Sat_124	E	No amplication	-	-	-
Sat_172	E	Monomorphic	?	?	-
Satt 369	E	No amplification	-	-	-
Satt 651	E	Monomorphic	?	?	-
Satt 268	E	Polymorphic	355	312	?
Satt 699	E	Polymorphic	190	170	0.721
Satt 706	E	No amplification	-	-	-
Sat_235	E	No amplification	-	-	-
Satt 598	E	Monomorphic	?	?	-
Sat_381	E	No amplification	-	-	-
Satt 231	E	Polymorphic	217	244	0.256
Satt 185	E	Polymorphic	225	250	?
Satt 411	E	Monomorphic	100	100	-
Satt 114	F	Monomorphic	97	97	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 510	F	Polymorphic	374	390	0.174
Satt 146	F	Polymorphic	311	287	?
Satt 334	F	No amplification	-	-	-
Satt 145	F	Polymorphic	150	155	0.420
Satt 362	F	Polymorphic	270	250	0.00104
Sct_033	F	No amplification	-	-	-
Satt 160	F	No amplification	-	-	-
Sat_120	F	Monomorphic	340	340	-
Sat_197	F	No amplification	-	-	-
Sct_188	F	Monomorphic	290	290	-
Satt 072	F	No amplification	-	-	-
Satt 335	F	Polymorphic	180	160	?
Satt 149	F	No amplification	-	-	-
SOYHSP176	F	No amplification	-	-	-
Sat_133	F	No amplification	-	-	-
Sat_317	F	Monomorphic	170	170	-
Sat_154	F	No amplification	-	-	-
Satt 423	F	Polymorphic	230	250	0.301
Satt 218	F	Monomorphic	230	230	-
Satt 516	F	Monomorphic	220	220	-
Satt 144	F	Monomorphic	210	210	-
Sat_074	F	No amplification	-	-	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 395	F	Monomorphic	280	280	-
Satt 490	F	Polymorphic	260	280	0.000000537
Satt 554	F	Polymorphic	255	250	0.000152815
GMRUBP	F	No amplification	-	-	-
Satt 269	F	Polymorphic	270	260	0.499
Satt 522	F	Monomorphic	240	240	-
Satt 325	F	Monomorphic	220	220	-
Satt 348	F	Monomorphic	220	220	-
Sat_090	F	Monomorphic	170	170	-
Satt 657	F	Polymorphic	270	265	0.00000001
Satt 656	F	Monomorphic	145	145	-
Satt 374	F	No amplification	-	-	-
Satt 199	G	Polymorphic	159	162	0.849
Satt 191	G	Polymorphic	224	191	?
Satt 038	G	Monomorphic	170	170	-
Satt 130	G	Monomorphic	240	240	-
Satt 131	G	Monomorphic	170	170	-
Satt 235	G	Monomorphic	130	130	-
Satt 472	G	Polymorphic	200	230	0.723
Sat_372	G	No amplification	-	-	-
Satt 566	G	Polymorphic	260	240	0.723
Sat_088	G	No amplification	-	-	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 570	G	Monomorphic	100	100	-
Sat_117	G	No amplification	-	-	-
Satt 594	G	Polymorphic	140	150	0.441
Satt 288	G	No amplification	-	-	-
Satt 612	G	Monomorphic	240	240	-
Satt 324	G	Monomorphic	230	230	-
Satt 356	G	Monomorphic	240	240	-
Sat_064	G	No amplification	-	-	-
Satt 517	G	No amplification	-	-	-
Satt 303	G	Polymorphic	260	240	0.932
Sat_131	G	No amplification	-	-	-
Sct_187	G	No amplification	-	-	-
Sat_210	G	Monomorphic	220	220	-
Satt 564	G	No amplification	-	-	-
Satt 666	H	No amplification	-	-	-
Sat_214	H	No amplification	-	-	-
Satt 635	H	Monomorphic	180	180	-
Satt 353	H	Polymorphic	111	96	0.127
Sat_127	H	No amplification	-	-	-
Sctt 009	H	No amplification	-	-	-
Satt 192	H	Monomorphic	378	378	-
Satt 442	H	No amplification	-	-	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 541	H	Polymorphic	170	160	0.362
Satt 469	H	No amplification	-	-	-
Sat_118	H	Polymorphic	200	210	0.699
Satt 052	H	No amplification	-	-	-
Satt 253	H	Monomorphic	137	137	-
Sat_206	H	No amplification	-	-	-
Satt 279	H	Polymorphic	190	180	0.743
Sat_175	H	No amplification	-	-	-
Satt 142	H	Polymorphic	150	155	0.318
Satt 293	H	Polymorphic	220	200	0.667
Satt 317	H	No amplification	-	-	-
Satt 181	H	No amplification	-	-	-
Sat_218	H	No amplification	-	-	-
Satt 434	H	Monomorphic	317	317	-
Sat_170	I	No amplification	-	-	-
Satt 571	I	No amplification	-	-	-
Satt 700	I	Monomorphic	?	?	-
Sat_299	I	No amplification	-	-	-
GMLPS12	I	No amplification	-	-	-
Sct_189	I	No amplification	-	-	-
Satt 419	I	Monomorphic	295	295	-
Satt 354	I	No amplification	-	-	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 614	I	Monomorphic	?	?	-
Satt 367	I	Monomorphic	?	?	-
Satt 440	I	Polymorphic	190	210	0.234
Satt 270	I	Polymorphic	225	240	0.668
Satt 162	I	No amplification	-	-	-
Satt 127	I	No amplification	-	-	-
Satt 148	I	Polymorphic	300	297	0.706
Satt 049	I	No amplification	-	-	-
Satt 292	I	Monomorphic	178	178	-
Satt 330	I	No amplification	-	-	-
Satt 596	J	Monomorphic	260	260	-
Satt 529	J	Polymorphic	210	225	0.303
Sat_366	J	Monomorphic	190	190	-
Satt 431	J	Polymorphic	175	187	0.638
Satt 414	J	Polymorphic	307	298	0.674
Satt 215	J	Polymorphic	136	139	0.072
Sct_065	J	Monomorphic	160	160	-
Satt 456	J	Polymorphic	290	280	0.511
Satt 380	J	Polymorphic	142	139	0.636
Sct_046	J	Monomorphic	160	160	-
Satt 406	J	No amplification	-	-	-
Satt 547	J	Polymorphic	210	225	0.000114913

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§]		p-value[¶]
			Brim	CNS	
Sat_350	J	No amplification	-	-	-
Satt 132	J	No amplification	-	-	-
Satt 249	J	Polymorphic	255	250	0.846
Satt 183	J	Polymorphic	250	260	0.545
Satt 244	J	Polymorphic	150	170	?
Satt 280	J	No amplification	-	-	-
Sct_001	J	Polymorphic	190	200	0.416
Satt 405	J	Monomorphic	300	300	-
Satt 287	J	No amplification	-	-	-
Satt 539	K	Monomorphic	160	160	-
Sat_087	K	No amplification	-	-	-
Satt 441	K	Polymorphic	270	243	0.193
Satt 242	K	Polymorphic	156	150	?
Sat_119	K	No amplification	-	-	-
Satt 055	K	No amplification	-	-	-
Satt 137	K	No amplification	-	-	-
Satt 349	K	No amplification	-	-	-
Satt 247	K	No amplification	-	-	-
Satt 381	K	No amplification	-	-	-
Satt 046	K	No amplification	-	-	-
Satt 167	K	No amplification	-	-	-
Satt 124	K	No amplification	-	-	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 326	K	No amplification	-	-	-
Sat_363	K	No amplification	-	-	-
Satt 710	K	No amplification	-	-	-
Satt 240	K	Monomorphic	250	250	-
Satt 273	K	No amplification	-	-	-
Sat_043	K	No amplification	-	-	-
Satt 499	K	No amplification	-	-	-
Satt 475	K	No amplification	-	-	-
Satt 260	K	No amplification	-	-	-
Satt 168	K	Polymorphic	227	230	0.596
Satt 196	K	Polymorphic	190	180	0.471
Satt 588	K	Polymorphic	170	173	?
Satt 495	L	No amplification	-	-	-
Satt 232	L	Polymorphic	250	270	0.326
Satt 182	L	Monomorphic	195	195	-
Sat_071	L	No amplification	-	-	-
Satt 388	L	No amplification	-	-	-
Satt 143	L	Monomorphic	301	301	-
Satt 398	L	No amplification	-	-	-
Satt 313	L	Polymorphic	250	225	0.466
Sat_340	L	Polymorphic	240	270	?
Satt 156	L	Polymorphic	207	222	0.968

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Sat_113	L	No amplification	-	-	-
Satt 527	L	Polymorphic	200	180	0.795
Satt 229	L	No amplification	-	-	-
Satt 513	L	No amplification	-	-	-
Satt 373	L	Monomorphic	100	100	-
Sat_245	L	No amplification	-	-	-
Satt 404	M	No amplification	-	-	-
Satt 590	M	Polymorphic	320	315	0.602
Satt 150	M	No amplification	-	-	-
Satt 567	M	No amplification	-	-	-
Satt 540	M	Polymorphic	150	160	?
Satt 435	M	No amplification	-	-	-
Satt 463	M	Monomorphic	125	125	-
Satt 245	M	Monomorphic	210	210	-
Satt 220	M	Monomorphic	250	250	-
Satt 323	M	Monomorphic	170	170	-
Satt 702	M	Polymorphic	290	150	?
Satt 536	M	No amplification	-	-	-
Sat_003	M	Monomorphic	120	120	-
Satt 175	M	Monomorphic	174	174	-
Sat_256	M	No amplification	-	-	-
Satt 677	M	No amplification	-	-	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Satt 655	M	No amplification	-	-	-
Sat_288	M	No amplification	-	-	-
Satt 551	M	Monomorphic	220	220	-
Sat_121	M	No amplification	-	-	-
Satt 250	M	Monomorphic	180	180	-
Satt 346	M	No amplification	-	-	-
Satt 210	M	Polymorphic	250	245	0.401
Satt 308	M	Polymorphic	174	156	0.404
Satt 336	M	Polymorphic	180	190	0.993
Sat_359	M	No amplification	-	-	-
Sat_033	N	No amplification	-	-	-
Satt 125	N	Monomorphic	200	200	-
Satt 234	N	Monomorphic	220	220	-
Satt 312	N	Monomorphic	160	160	-
Satt 255	N	Monomorphic	139	139	-
Satt 237	N	Monomorphic	275	275	-
Sat_166	N	Polymorphic	270	250	?
Satt 339	N	Monomorphic	210	210	-
Sat_084	N	Polymorphic	145	150	0.335
Satt 393	N	No amplification	-	-	-
GMABAB	N	Monomorphic	150	150	-
Satt 080	N	Monomorphic	180	180	-

Appendix 1 (con't)

Marker	Linkage Group[†]	Result[‡]	Size[§] Brim	CNS	p-value[¶]
Sat_304	N	No amplification	-	-	-
Satt 009	N	Monomorphic	184	184	-
Satt 257	N	Monomorphic	230	230	-
Satt 631	N	Monomorphic	160	160	-
Satt 530	N	Polymorphic	250	25	0.086
Sat_125	N	Monomorphic	125	125	-
Sct_195	N	Monomorphic	150	150	-
Satt 521	N	Polymorphic	255	230	0.488
Satt 387	N	Polymorphic	210	190	0.314
Satt 549	N	Monomorphic	210	210	-
Sat_236	N	No amplification	-	-	-
Satt 022	N	Monomorphic	172	172	-
Satt 487	O	Monomorphic	230	230	-
Satt 500	O	Monomorphic	300	300	-
Satt 492	O	Polymorphic	230	227	-
Satt 445	O	Monomorphic	205	205	-
Satt 259	O	Monomorphic	225	225	-
Satt 420	O	Monomorphic	225	225	-
Sat_291	O	Monomorphic	210	210	-
Satt 188	O	Monomorphic	240	240	-
Satt 128	O	Monomorphic	275	275	-
Satt 608	O	No amplification	-	-	-

Appendix 1 (con't)

Marker	Linkage Group†	Result‡	Size§		p-value¶
			Brim	CNS	
Satt 633	O	Monomorphic	129	129	-
Satt 345	O	Monomorphic	200	200	-
Satt 241	O	Monomorphic	220	220	-
Satt 563	O	No amplification	-	-	-
Satt 478	O	No amplification	-	-	-
Satt 477	O	Monomorphic	140	140	-
Satt 123	O	Monomorphic	200	200	-
Satt 592	O	No amplification	-	-	-
Sat_038	O	Polymorphic	250	210	?
Satt 153	O	Polymorphic	200	220	0.081
Satt 243	O	No amplification	-	-	-
Sat_108	O	No amplification	-	-	-
Scaa_001	O	Monomorphic	120	120	-

† Linkage Group of soybean based on Cregan et al (1999).

‡ Results of polymorphism test on the parents Brim and CNS.

§ Size of markers for Brim and CNS in base pairs.

¶ p-value at the 0.05 significant level for the F_2 progeny of the cross of Brim x CNS.