Soybean improvement strategies: Insect-pollinator attraction and genetic resistance to whitefly and to brown stem rot

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Soybean improvement strategies: Insect-pollinator attraction and genetic resistance to whitefly and to brown stem rot

by

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Breeding

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CHAPTER 1: GENERAL INTRODUCTION

INTRODUCTION

Soybean \( Glycine \textit{max} \) (L.) Merr.] is one of the most important crop plants cultivated worldwide. It is a source of oil and protein and is used for livestock feed, human food, and also for industry. The major producers are the United States, which accounts for 34% of world production, Brazil with 27%, Argentina with 22%, and China with 6%, (FAO Statistics, 2010). Research goals are related to increases in grain yield along with desirable quality characteristics such as high seed protein and seed oil content. The majority of farmers in the U.S. derive their income on the basis of seed yield, which is the final plant expression of all metabolic processes and interactions with environment (biotic and abiotic factors). Because of this, yield is the main trait in breeding improved cultivars, both in the public and private sector.

Over the years, yield increases have been accomplished due to genetic improvement and enhancement of agronomic practices. These increases have been cited extensively in the literature. Boerma (1979) reported average yield increases of 0.7% per year for cultivars that were released from 1914 to 1973 in the southern U.S. In a similar study, Luedders (1977) reported a 26% yield increase of cultivars released from 1940 to 1950 compared to plant introductions, and a 16% yield increase from cultivars released from 1960 to 1970 compared with those released from 1940 to 1950. Specht and Williams (1984) reported a soybean yield increase of 21 kg ha\(^{-1}\) annually in the period from 1924 to 1980, with the majority of the
yield increase attributed to breeding. Wilcox (2001) estimated that public soybean breeders in the northern U.S. soybean production region have increased seed yield approximately 60% over the past 60 years. Specht et al. (1999) estimated that similar progress in breeding also has been made in the private sector, although these results were obtained during the last two to three decades. In general, yield improvement may be achieved by breeding directly for the trait, i.e. crossing high-yielding parents to bring together yield genes and selecting at every stage of testing the highest producing individuals. Additionally, improved yield can be obtained indirectly by improving other aspects of plant production, i.e. physiological aspects and plant health. Soybean breeding efforts for yield have been made by applying different strategies related to selection methods (Piper and Fehr, 1987; Sumarno and Fehr, 1982; Uphoff et al., 1997; Voigt and Weber, 1960) and to population development (Cerna et al., 1997; Thompson and Nelson, 1998).

Soybean breeding programs have been focused on the development of inbred lines, which represent the totality of cultivars available in the market. However, increases in soybean production may be possible through the development of hybrids (Brim and Cockerham, 1961; Burton and Brownie, 2006; Cerna et al., 1997; Lewers, 1996; Manjarrez-Sandoval et al., 1997; Nelson and Bernard, 1984; Ortiz-Perez, et al., 2007; Palmer et al., 2001, 2003; Pandini et al. 2002; Perez, et al., 2009a; Sun et al., 1999). Breeding for resistance to diseases and pests is an extremely important objective to improve soybean yield. Soybean is threatened by insect pests (i.e. whitefly) that impact soybean yield in commercial plantings, or in research plantings under confined conditions, such as greenhouses. In
addition, several diseases [i.e. brown stem rot (BSR)] lowers yield, which decreases production and results in less income (Wrather et al., 2001).

The objectives of this study were: 1) to determine if four plant introductions (PI) from south-central China, identified as resistant to the disease brown stem rot (BSR), represent new sources of BSR resistance genes, 2) to identify quantitative trait loci (QTL) for whitefly resistance in soybean, and 3) to determine nectar composition (sucrose, glucose, fructose, and total carbohydrates) and flower size of greenhouse-grown wild perennial plants.

LITERATURE REVIEW

Brown stem rot (BSR)

BSR is an economically important disease prevalent in soybean producing regions in the north-central U.S. and Canada (Gray and Grau, 1999). Under conditions that favor disease development, yield losses of up to 38% have been reported (Gray, 1972). *Phialophora gregata* f. sp. *sojae* is the causal organism, infecting plants through the roots, colonizing the pith and vascular system and moving up the stem into the leaves of susceptible genotypes (Allington and Chamberlain, 1948; Schneider et al., 1972). Two types of *Phialophora gregata* f. sp. *sojae* have been identified. Type I, causes leaf necrosis and defoliation, and stem vascular discoloration, and Type II, causes vascular discoloration only (Gray, 1972; Harrington et al., 2003).

The most effective way to manage the disease is by planting BSR-resistant soybean cultivars (Bachman et al., 1997; Mengistu et al., 1986). To date, three BSR resistance genes
(Rbs1, Rbs2, and Rbs3) have been found and these genes map to the same general region on chromosome 16 (formerly molecular linkage group J) (Bachman et al. 2001; Klos et al., 2000; Lewers et al., 1999; Patzoldt et al, 2005; Webb, 1997). The identification of new sources of resistance to BSR is important for soybean breeding programs. Plant introductions (PI) have been valuable sources of genetic diversity in soybean improvement because they can carry genes different than those found in commercial cultivars (Carter et al., 2004).

**Whitefly**

Whitefly, *Bemisia tabaci* (Gennadius) (order: Homoptera, family: Aleyrodidae), is a common economic pest on many crops throughout the world. Historically, whiteflies have been major pests in vegetable crops, cotton, and greenhouse plants. In addition, they have a wide range of hosts, including wild plant species, which makes it difficult to control once they have infested a crop (Perring, 2001). The whitefly species complex is formed by highly cryptic sibling species (Campbell et al., 1996; Perring, 2001). To date, 41 distinct populations of *B. tabaci* have been studied; 24 of these populations have been given a specific biotype designation (Perring, 2001). Adults and nymphs have piercing-sucking mouthparts and feed on the lower leaf surfaces where they lay their eggs (Byrne and Bellows, 1991; Perring, 2001). Whiteflies damage crops by extracting large quantities of phloem sap (Jones, 2003), and heavy infestations may result in development of chlorotic spots on leaves, wilting, and stunting of plants. In addition, these insects excrete a sticky material called honeydew, which in high concentrations promotes the growth of sooty mold fungi, which interferes with photosynthesis. Some species of the whitefly complex serve as vectors of several economically important viral plant pathogens (Byrne and Bellows, 1991).
Host plant resistance to whitefly can be due to physical characteristics of the leaf surface, e.g. hairiness vs. glabrousness, sticky glandular trichomes, leaf shape, microclimate due to foliage density; and chemical characteristics, such as pH of leaf sap (Berlinger, 1986). However, the main reason for the resistance to whitefly is unknown.

In soybean, whitefly has become an important pest problem in tropical locations and in greenhouse conditions (Costa, 1976; Vaishampayan et al. 1975). The sweetpotato whitefly, *Bemisia tabaci* (Gennadius), and the bandedwinged whitefly, *Trialeurodes abutiloneus* (Haldeman) have been recorded colonizing field-grown soybean (Vaishampayan et al. 1975). In greenhouse conditions, soybean is colonized by *Trialeurodes vaporariorum* (Westwood). Viruses such as soybean crinkle mosaic, and soybean dwarf mosaic can be transmitted by whiteflies (Costa, 1976). Breeding of soybean cultivars resistant to this pest is the most effective management alternative (Arioglu et al., 1989).

**Soybean hybrids**

The interest in hybrid soybean developed after the identification of the first male-sterile, female-fertile mutant (Brim and Young, 1971). Its use in recurrent selection breeding programs (Brim and Stuber, 1973), increased the awareness of the potential to produce commercial hybrid soybean. According to Palmer et al. (2001) there are five components that are crucial for the successful development of commercial hybrid soybean:

1. Parental combinations that produce heterosis levels superior to the best pure-line cultivars.
2. A stable male-sterile, female-fertile sterility system.
3. A selection system to obtain 100% female (pod parent) plants that set seed normally and can be harvested mechanically.
4. An efficient pollen transfer mechanism from pollen parent to pod parent.

5. An economical level of seed increase for the seedsman and growers that ultimately benefits the consumer.

Regarding the first requirement for hybrid soybean production, several heterosis studies have showed that heterosis levels, above the high parent, are possible (Brim and Cockerham, 1961; Burton and Brownie, 2006; Cerna et al., 1997; Lewers, 1996; Manjarrez-Sandoval et al., 1997; Nelson and Bernard, 1984; Ortiz-Perez et al., 2007; Palmer et al., 2001; Pandini et al. 2002; Perez et al., 2009a, 2009b; Sun et al., 1999). In order to meet the second requirement for soybean hybrid production (a stable male-sterile, female-fertile sterility system), mutations affecting male cell and organ development have generated male-sterile, female-fertile lines that can be used as female parents for hybrid seed production (Palmer, 2000). The third requirement is to have a selection system to obtain 100% female plants because in hybrid seed production fields, female rows will be segregating for the male sterility (ms) mutation. According to Palmer et al. (2003), any of the selection systems employed with the soybean nuclear male-sterile genes, seed size differential (Carter et al., 1984), linkage between genes controlling the green cotyledon trait and the Ms5 locus (Burton and Carter, 1983), as well as the WI flower color locus and the Ms6 locus (Lewers and Palmer, 1997; Lewers, 1996; Lewers et al., 1998a, 1998b) are suitable to identify male-sterile, female-fertile plants for hybrid seed production.

Upon obtaining a stable male sterility system, it is necessary to transfer the pollen from the male parent to the female parent. An efficient pollen transfer mechanism is the most limiting factor for soybean hybrid production (Brim, 1975; Davis, 1975; Palmer et al., 2001, 2003). In soybean, manual cross-pollination to produce large quantities of hybrid seed is
difficult and time consuming. The small size of the soybean flowers, the low success rate and the few seeds obtained per hybrid pod contribute to the difficulty of manually producing large quantities of hybrid seed (Fehr, 1991). Insect cross-pollination of male-sterile soybean plants facilitates the production of hybrid seed (Lewers, 1996; Nelson and Bernard, 1984; Ortiz-Perez et al., 2007). Pollinator insects such as honeybees *Aphis melliphera* and alfalfa leaf cutter bee, *Megachile rotundata* F. are used in hybrid soybean production. Also, some wild native bees primarily from families Megachilidae, Halictidae, Anthophoridae, and Andrenidae could be efficient pollinators of soybean flowers (Ortiz-Perez et al., 2007).

Soybean is an obligate self-pollinated crop with less than 1% natural cross-pollination (Palmer et al., 2001). Before soybean hybrids can become a reality, it is necessary to understand some of the mechanisms for insect attraction and reward. Wild relatives of the cultivated soybean represent a germplasm reservoir for agronomic improvement of the cultivated species (Singh and Hymovitz, 1999).

**Wild perennial *Glycine* species**

The genus *Glycine* (Willd.) is composed of two subgenera: *Soja* (Moench) F. J. Herm. and *Glycine*. The subgenus *Soja* contains the cultivated soybean *Glycine max* (L.) Merr. and the wild annual *Glycine soja* (Sieb. and Zucc.). The subgenus *Glycine* has approximately 25 defined wild perennial species distributed throughout Australia and islands of the Pacific Ocean, extending to Taiwan. The unexploited valuable pool of genetic diversity of the wild perennial species is largely unexplored by soybean breeders (Singh and Hymowitz, 1999). Traits of interest that could be incorporated into the cultivated soybean
include resistance to soybean rust, soybean brown spot, powdery mildew, phytophthora root rot, white mold, sudden death syndrome, tobacco ring spot virus, yellow mosaic virus, soybean cyst nematode, and tolerance to certain herbicides and to salt (Palmer and Hymowitz, 2004).

*Glycine* species are predominantly self-pollinated, although they possess chasmogamous flowers capable of outcrossing. Within the wild perennial species *Glycine argyrea* (Tind.), Brown et al. (1986) recorded outcrossing rates among chasmogamous flowers from zero to complete outcrossing, with an average of 40%. The identification and characterization of sources of insect attraction among the wild perennial species of the genus *Glycine* could be useful in soybean breeding programs for hybrid production, because in contrast with the low natural out-crossing in the cultivated soybean, out-crossing in the wild perennial soybean species can exceed 50%.

**DISSERTATION ORGANIZATION**

This dissertation is organized into five chapters. Chapter one is the general introduction. Chapter two is a manuscript submitted to *Crop Science* entitled "Genetic analysis of new sources of soybean resistance to brown stem rot". Chapter three is a manuscript to be submitted to *Journal of Crop Improvement* entitled "QTL mapping of whitefly resistance in soybean". Chapter four is a manuscript to be submitted to *Journal of American Botany* entitled "Nectar composition and flower characteristics of wild perennial *Glycine* species". Chapter five is the general conclusion of this study. Different objectives will be addressed in chapters two, three, and four, each of them with the ultimate purpose to
contribute to improve yield in soybean. Chapters two and three each have objectives that relate to plant health, as a means to improve yield potential through genetic resistance to a fungal disease and an insect pest. Chapter two describes the search for new sources of resistance to brown stem rot in four plant introductions from south-central China. Chapter three describes quantitative trait loci mapping of whitefly resistance in two segregating populations. Chapter four evaluates factors that affect insect-pollinator attraction and reward in the wild perennial Glycine species, which could be used to improve pollen transfer in the cultivated soybean for hybrid production.

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maturity groups VI, VII, and VIII. Crop Sci. 19:611-613.


CHAPTER 2: GENETIC ANALYSIS OF NEW SOURCES OF SOYBEAN RESISTANCE TO BROWN STEM ROT

A paper submitted to Crop Science

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ABSTRACT

Brown stem rot (BSR) of soybean [Glycine max (L.) Merr.], caused by Phialophora gregata, is an economically important disease prevalent in soybean producing regions of the north-central U.S. and Canada. To date, all BSR resistant genes identified are located on chromosome 16 (formerly molecular linkage group J). The objective of this study was to determine if four plant introductions (PI) from south-central China identified as BSR-resistant have resistance genes mapping to the same location on chromosome 16 as previously mapped BSR resistance genes. The four plant introductions, PI 594637, PI 594638B, PI 594650A, and PI 594858B were crossed to the BSR-susceptible cultivar ‘Century 84’ to develop four F$_2$ populations. Each segregating population and the parental lines were screened for BSR resistance in growth chamber conditions. The F$_{2:3}$ individual plants of each population were tested with the simple sequence repeat (SSR) markers Satt431
or Satt547, which map closely to BSR resistance quantitative trait loci (QTL) on chromosome 16. Associations between molecular data and phenotypic data used to validate QTL were analyzed using single factor analysis of variance. Three of the four populations had markers on chromosome 16 significantly associated with BSR resistance with $R^2$ values from 24 to 48%. However, when marker Satt547 was regressed on BSR resistance in population PI 594637 X Century 84, no significant association was observed. This result suggests that PI 594637 could have a new BSR resistance gene. Transgressive segregation also was observed in this population, and highly BSR resistant progeny could be used in the development of BSR resistant cultivars. Additional research and testing in this population will be conducted to identify resistance QTL(s) from this source.

**Abreviations:** BSR, brown stem rot • LSMEANS, Least square means • MAS, marker assisted selection • MG, maturity group • PCR, polymerase chain reaction • PI, plant introduction • QTL, quantitative trait loci • SSR, simple sequence repeat.

**INTRODUCTION**

Brown stem rot (BSR) of soybean is an economically important disease prevalent in soybean producing regions in the north-central U.S. and Canada (Hartman et al., 1999; Sinclair and Backman, 1989). Under conditions that favor disease development, yield loses of up to 38% have been reported (Bachman et al., 1997; Gray, 1972). *Phialophora gregata* f. sp. *sojae* is the causal organism, infecting plants through the roots, colonizing the pith and vascular
system and moving up the stem and into the leaves of susceptible genotypes (Allington and Chamberlain, 1948; Schneider et al., 1972). Two types of Phialophora gregata f. sp. sojae have been identified. Type I, causes leaf necrosis and defoliation, and stem vascular discoloration, and Type II, only causes vascular discoloration (Gray, 1971; Harrington et al., 2003). The most effective way to manage the disease is by planting BSR-resistant soybean cultivars (Bachman et al., 1997; Mengistu et al., 1986).

Resistant genes have been identified and utilized in cultivar development and germplasm enhancement (Bachman et al., 2001; Chamberlain and Bernard, 1968; Tachibana and Card, 1972). Genetic analyses indicated that BSR resistance is conferred by dominant alleles at three independent loci, Rbs1, Rbs2 and Rbs3. The Rbs1 gene was identified in PI 84946-2 and L78-4094 (Bachman et al., 2001; Hanson et al., 1988; Klos et al., 2000; Lewers, et al., 1999; Sebastian et al., 1985), Rbs2 in PI 437833 (Bachman et al., 2001; Hanson et al., 1988; Klos et al., 2000; Patzoldt et al., 2005b), and Rbs3 in PI 437970, PI 84946-2, 'BSR 101', 'BSR 201', 'BSR 301', 'BSR 302' and 'IA 2008' (Klos et al., 2000; Lewers, et al., 1999; Willmot and Nickell, 1989). Other resistance sources have been identified, but their genetic control has yet to be determined (Lewers, et al., 1999; Nelson et al., 1989).

BSR disease expression exhibits a wide range of continuous variation in segregating populations. This may be an indication of environmental effects on the phenotypic disease expression and possible evidence of polygenic inheritance of BSR resistance (Bachman and Nickell, 2000; Bachman et al., 1997), in addition to the major genes identified. These characteristics lead to low heritability of resistance, making development of high-yielding resistant cultivars difficult. Marker assisted selection (MAS) might be an alternative for
selection of traits such as BSR resistance. Once precise and accurate screening methods are achieved, environmental effects would less influence selection for BSR resistance.

To identify quantitative trait loci (QTL) that confer BSR resistance, mapping studies with various types of molecular markers have been conducted. Webb (1997) reported that the \textit{Rbs3} gene was associated with BSR resistance on molecular linkage group J (chromosome 16, Schumtz et al., 2010) using restriction fragment length polymorphism (RFLP) markers on 328 recombinant inbred lines (RIL) from a cross between the resistant cultivar BSR 101 and the susceptible accession PI 437654. Lewers et al. (1999) identified one major and one minor QTL on chromosome 16 responsible for BSR resistance in BSR101 using RFLP and amplified fragment length polymorphism (AFLP) markers. Klos et al. (2000) evaluated 17 resistant and 29 susceptible cultivars and plant introductions (PI) as well as RIL derived from the cross of BSR 101 with PI 437654. These authors used nine new DNA markers derived from RFLP, SSR, and bacterial artificial chromosome sequences, which mapped to chromosome 16. Bachman et al. (2001) observed in a population of F\textsubscript{2:3} lines from the cross of L78-4094, a resistant line with the \textit{Rbs1} gene, to the susceptible cultivar Century, SSR markers Satt215 and Satt431 were linked to \textit{Rbs1}. In the same study, the authors also identified SSR markers Satt244 and Satt431 linked to \textit{Rbs2} in a F\textsubscript{2:3} population developed from crossing PI 437833 to Century. Patzoldt et al. (2005b) localized a BSR resistant QTL from ‘Bell’, a BSR resistant cultivar, close to molecular markers Satt431, Satt547, 21E22.sp1, 21E22.sp2, K375, and 35E22.sp1. These studies placed the BSR resistance genes \textit{Rbs1}, \textit{Rbs2}, \textit{Rbs3} and a gene from Bell to the same region on chromosome 16.

New sources of resistance are important in breeding soybeans due to its narrow genetic
base (Gizlice et al., 1994). Plant introductions have been particularly valuable to soybean improvement because they can contribute different genes than those found in adapted genotypes (Carter et al., 2004; Patzoldt et al., 2005a; Thompson and Nelson, 1998; Thorne and Fehr, 1970; Vello et al., 1984). In an effort to expand the genetic base of resistance to BSR, Patzoldt et al. (2005a) mapped BSR resistance QTL in five accessions from central China. In all five accessions, they mapped a QTL for BSR resistance to the same chromosome 16 region as previously reported for the three BSR resistant genes. Our study continues the search in soybean for sources of new BSR resistance genes. Our objective was to determine if four BSR resistant plant introductions from south-central China had resistance genes different from those previously reported on chromosome 16.

**MATERIALS AND METHODS**

**Plant material**

Four plant introductions (PI) from south-central China previously identified as BSR-resistant (Patzoldt et al., 2003) were crossed to the maturity group (MG) II susceptible cultivar Century 84 (Walker et al., 1986). PIs were PI 594637, PI 594638B, PI 594650A of MG IV, and PI 594858B from MG V.

The F$_1$ seed was produced in Urbana, IL, and the F$_{2:3}$ mapping populations were developed at Isabela, PR. The F$_{2:3}$ lines along with the parental lines were evaluated for BSR resistance in Ames, IA. One hundred and fifty five F$_{2:3}$ individuals from each segregating population and their parental lines were grown in a growth chamber in a randomized
complete block design with three replications. One seed of each individual genotype was planted in a mix of soil, sand, and perlite in 4-by-21-cm plastic, cone-shaped containers. Temperature in the growth chamber was constant at 19°C, with 16 hours of light. Two-week old plants were inoculated with *P. gregata* by introducing inoculum (described below), into the stems.

**Inoculation protocol**

Inoculum of *P. gregata f. sp. sojae* isolate Oh2-3, was prepared in cultures as described by Tabor et al. (2003). Cultures were started on green bean agar (GBE) medium (ground frozen *Phaseolus vulgaris* L. green pods at 35 g/liter) supplemented with ampicillin at 50 mg/liter and were incubated for 44 to 64 days at room temperature (21 to 23°C) in the dark until abundant sporulation was observed. Mycelium of *P. gregata* was harvested from the agar plates and conidia were suspended in 0.8% water agar (2.7 x 10^7 conidia/ml). The conidial suspension was mixed into a paste, then, the bevel of an 18-gauge needle was filled with this inoculum paste. Stems of two-week-old plants were punctured approximately 2 cm above the soil line to introduce inocula. After inoculation, plants were grown under 16-h light-photoperiod, at a constant temperature of 19 °C, watered daily, and fertilized weekly.

**Severity assays**

Five weeks after inoculation, BSR severity was evaluated using both a BSR severity or symptom scale and a measurement of the recovery of *P. gregata* from the stem after plating stem pieces on green bean agar (Tabor et al., 2003). For the first method, disease severity was accessed by evaluating each plant for its vegetative stage (*V*-stage, Fehr et al., 1971) and for its vigor (overall plant health condition) (Table 1). For each individual plant in
the three replications, vigor was measured on a scale from 1 to 7. In this scale, a score of 1 corresponds to a dead plant; 2 to a plant with a green stem and no leaves; 3 when chlorotic and necrotic leaves are prominent; 4 when some stunting, mosaic chlorosis and necrosis on leaves is observed; 5 when leaf area is normal except for some yellowing; 6 when leaf area is normal, and plants are small but healthy; and 7 when plants are completely healthy (P. Lundeen, unpublished).

For the plating method, the procedure described by Tabor et al. (2003) was used. After vigor scores were taken, plants were defoliated and stems were cut at the soil line, immersed for 3 min in 70% ethanol, followed by 5 min in 10% sodium hypochlorite and finally rinsed with sterile water. Incidence of BSR was estimated by cutting 1-cm stem segments at the inoculation point and at the top of the plants. The two stem segments were plated on green bean agar, supplemented with ampicillin. After plating stem segments, cultures were allowed to grow in the dark at 5 °C for two weeks. Following this period, plates were evaluated for P. gregata infection. A plant was considered colonized if P. gregata was recovered from the stem segment corresponding to the inoculation point. Plants were considered susceptible if P. gregata also was recovered from the top portion of the stem in addition to the inoculation point. Conversely, a plant was considered resistant if the fungus was recovered only from the inoculation point and was not present at the top segment (Figure 1). The variable plate susceptibility was recorded as 0 for a resistant plant, which showed infection only at the inoculation point; and as 1 for a susceptible plant, infected at the inoculation point and plant top.
DNA marker analysis

DNA extraction and genotyping was performed in Urbana, IL. For each mapping population, DNA was extracted from leaves of 10 plants in each F$_{2:3}$ line. DNA was extracted according to Keim and Shoemaker (1988) with modifications outlined by Patzoldt et al. (2005b). Each line was genotyped with SSR markers Satt431 or Satt547 from chromosome 16 (Song et al., 2004) according to Patzoldt, et al. (2005b). SSR markers were amplified through polymerase chain reaction (PCR). Amplified samples were run on 6% non-denaturing acrylamide (Wang et al., 2003) or 3% Metaphor agarose gels (Patzoldt et al., 2005b). Ethidium bromide was used to stain the gels and bands were visualized under UV light.

Data analysis

Phenotypic data were analyzed using PROC MIXED of SAS v. 9.1 (SAS Institute, 2003). Broad-sense heritabilities were estimated for each F$_2$ population with the formula $H^2 = \sigma^2_G / ((\sigma^2_E/r) + \sigma^2_G)$, where $r$ is the number of replications. All effects were treated as random in the model and variance components were estimated with the COVTEST option. Subsequently, F$_{2:3}$ lines and parental lines were considered fixed effects. Least square means (LSMEANS) were used to estimate average BSR resistance level for each F$_{2:3}$ individual and parental lines. The ESTIMATE statement was used to test the difference between average of the F$_{2:3}$ individuals and the average of each parental line. Transgressive segregation was estimated according to the method used by Shahid et al. (2008). The BSR resistance level of each F$_{2:3}$ line was compared with the resistance exhibited by each parent. Transgressive segregants for resistance were defined as F$_{2:3}$ lines which exceed the most resistant parent by two standard deviations. Similarly, the number of F$_{2:3}$ lines with transgressive segregation for
BSR susceptibility was calculated as the number of F\textsubscript{2:3} individuals with susceptibility higher than that of the most susceptible parent by two standard deviations. Single-factor analysis of variance using PROC GLM of SAS v.9.1 was performed to detect associations between SSR markers and BSR resistance in segregating populations. A significant association was declared if P<0.05.

**RESULTS**

**Phenotypic analysis**

The broad-sense heritability calculated for vigor ranged from 22% to 60%, and from 35% to 70% for plate susceptibility (data not shown). Each segregating population, along with the susceptible and resistant parents, was evaluated for plate susceptibility and vigor to BSR infection (Table 2). The resistant parents, PI 594638B, PI 594650A, and PI 594658B, showed zero plate susceptibility, meaning that *P. gregata* was not recovered from the top of the plants. The PIs also showed high vigor. In contrast, PI 594637 exhibited greater BSR susceptibility and was not statistically different from Century 84. The susceptible parent, Century 84, also showed susceptibility for plate susceptibility and vigor scores. In all F\textsubscript{2} populations, segregation was observed for visual symptoms and plate susceptibility (Figures 2-3).

The average of F\textsubscript{2:3} lines in the PI 594637 X Century 84 population showed intermediate values of BSR susceptibility, the average for plate susceptibility was 0.48 and 3.63 for vigor (Table 2). Average value of the F\textsubscript{2:3} lines for plate susceptibility was not statistically different from both parental lines. However, the average of F\textsubscript{2:3} lines showed
significantly higher average vigor than PI 594637 (Table 2; Figures 2-3). Although PI 594637 was used in these tests as a resistant parent based on previous screening (parents alone), when the screening was repeated for the current tests, it showed higher susceptibility to BSR symptoms than those of the F2 population. This population showed transgressive segregants in both directions (Table 3). There were 8 transgressive segregants two standard deviations more resistant than the most resistant parent, and 24 transgressive segregants two standard deviations more susceptible than the most susceptible parent.

Population PI 594638B X Century 84 showed segregation for the two resistance traits (Table 2; Figures 2-3). F2:3 individuals exhibited averages of 0.34 and 5.54 for plate susceptibility and vigor, respectively (Table 2). The average of the F2:3 lines was significantly (P-value < 0.05) more resistant than Century 84 for plate susceptibility. No statistical differences were detected between the average of the F2:3 lines and PI 594638B for plate susceptibility and vigor. Only three transgressive segregants for BSR resistance were observed (Table 3).

For Population PI 594650A X Century 84, averages of F2:3 individuals were 0.26 and 5.15 for plate susceptibility and vigor, respectively (Table 2; Figure 2-3). The population average was significantly (P-value < 0.01) more BSR resistant than the susceptible parent Century 84 for plate susceptibility and vigor (Table 2). Conversely, no significant differences were detected between the average of the F2:3 individuals and the resistant parent PI 594650A. Only one transgressive segregant was observed and it was for BSR susceptibility (Table 3).
Population PI 594658B X Century 84 showed segregation for the two variables (Table 2; Figure 2-3). Averages of plate susceptibility and vigor were 0.29 and 5.94, respectively. The average of the F$_{2:3}$ lines was significantly (P-value <0.01) different from the susceptible parent, with lower values for plate susceptibility and higher values of vigor. No statistical differences were detected between the mean of the F$_{2:3}$ lines and PI 594638B for vigor. This population had 20 transgressive segregants more susceptible than the susceptible parent (Table 3).

**Single marker analysis**

Marker Satt431 showed polymorphism for population PI 594650A x Century 84, and Satt547 was polymorphic for populations PI 594637 X Century 84, PI 594638B X Century 84, and PI 594658B X Century 84. Phenotypic data from each segregating population was used to test for associations between BSR resistance and molecular markers Satt431 and Satt547, which are in the region where B$_{SR}$ resistance was previously mapped, using single marker analysis. In three of the four populations, F$_{2:3}$ lines homozygous for the marker allele from the PI showed significantly greater BSR resistance than lines homozygous for alleles from Century 84 (Table 4). For the three populations in which significant associations were detected between the markers and the phenotypes, lines segregating for the marker exhibited average resistance levels that were intermediate between the two classes of homozygous lines.

Single marker regression revealed a significant association between BSR resistance and the SSR markers on chromosome 16 for three of the four populations (Table 4). Molecular marker Satt547 was significantly associated with BSR resistance in population PI
594638B X Century 84, with $R^2$ values of 44% for plate susceptibility and 33% for vigor. In population PI 594650A X Century 84, Satt431 was significantly associated with BSR resistance, with $R^2$ values of 34 and 37% for plate susceptibility and vigor, respectively. Satt547 was significantly associated to BSR resistance in population PI 594658B X Century 84, with $R^2$ values of 48% for plate susceptibility and 25% for vigor. Conversely, population PI 594637 X Century 84 did not show significant association with molecular marker Satt547. These results are suggestive of a possible new source of resistance in PI 594637, mapping to a different region from those previously identified on chromosome 16, where $Rsb1$, $Rsb2$, and $Rsb3$ map.

**DISCUSSION**

The study was conducted to determine if BSR resistance from PIs from south-central China is governed by genes mapping to genetic regions different from the previously reported resistance genes, $Rbs1$, $Rbs2$, and $Rbs3$. We hypothesized that association between BSR resistance and SSR molecular makers Satt431 or Satt547, in F$_2$ segregating populations, could indicate the presence of any of the previously identified resistance genes, which map close to these SSR markers on chromosome 16.

Given the high association between the SSR markers used and BSR resistance in populations PI 594638B X Century 84, PI 594650A X Century 84, and PI 594658B X Century 84, the BSR resistance genes present in these PIs could be allelic to previously identified BSR resistance genes on chromosome 16. This is because the three BSR resistance genes, $Rbs1$, $Rbs2$, and $Rbs3$ were confirmed to map to a region on chromosome
16 that the SSR markers Satt431 and Satt537 map (Bachman et al., 2001; Klos et al., 2000; Lewers et al., 1999; Patzold et al., 2005b; Webb, 1997). BSR resistance in population PI 594637 X Century 84 showed non-association with SSR Satt547. This could be indicative of the presence of a new resistance gene not associated with the molecular marker Satt547 on chromosome 16. However, in order to test this assumption, allelism tests between PI 594637 and genotypes possessing Rsb1, Rbs2, and Rbs3 genes need to be performed to confirm if PI 594637 carries a gene non allelic to any of the three genes. This research is currently in progress.

In contrast to the other three PIs used, which were highly resistant to BSR, PI 594637 showed intermediate resistance. Highly resistant individuals, however, were present in the F2:3 population derived from this PI crossed with susceptible cultivar Century 84 (Table 3; Figure 2-3), and transgressive segregant individuals for BSR resistance also were detected. Transgressive segregation of traits is a common phenomenon in segregating hybrid populations, especially in plants, where transgressive segregants might be the rule rather than the exception (Rieseberg et al., 1999; Rieseberg et al., 2003). A number of different mechanisms that could be responsible for transgressive segregation in hybrids are: elevated mutation rate, reduced developmental stability, epistasis, overdominance, unmasking of rare recessive alleles, chromosome number variation, and complementary gene action (Lynch and Walsh, 1998; Rieseberg et al., 1999; Rieseberg et al., 2003). In plants, results from classical and QTL studies have provided evidence to conclude that complementary gene action is the most likely explanation for transgressive segregation (Li et al., 1995; Vega and Frey, 1980; de Vicente and Tanksley, 1993). Vega and Frey (1980) working in oats concluded parents
might have contributed one or more useful genes to traits probably with a cumulative effect on phenotype. de Vicente and Tanksley (1993) in tomatoes, reported transgressive segregation was directly linked to the presence of complementary QTL alleles in the parental cultivars. Transgressive segregants in soybean also have been observed (Fehr et al., 1991; Alt et al., 2005).

For BSR resistance in soybean, Bachman and Nickell (2000) proposed a genetic model in which resistance is the result of epistatic interaction between pairs of loci. It is plausible that the high levels of resistant observed in some F₂:₃ progeny of population PI 594637 X Century 84 could be due to epistatic or additive interaction between resistance loci from PI 594637 and Century 84. Although Century 84 was considered as the susceptible parent, some degree of resistance was also observed in it for the variables evaluated (Table 2). In the cross PI 594637 X Century 84, the highly BSR resistant progeny identified can be used for development of BSR resistant cultivars. This alternative could even be more attractive if the PI is confirmed as possessing a new source of resistance for BSR.

Acknowledgements

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REFERENCES


Table 1. Visual assessment scale of brown stem rot (BSR) severity in soybean plants five weeks after inoculation.

<table>
<thead>
<tr>
<th>Vigor†</th>
<th>Plant Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dead plant</td>
</tr>
<tr>
<td>2</td>
<td>Green stem and no leaves</td>
</tr>
<tr>
<td>3</td>
<td>Chlorotic and necrotic leaves are prominent</td>
</tr>
<tr>
<td>4</td>
<td>Some stunting, mosaic chlorosis and necrosis on leaves</td>
</tr>
<tr>
<td>5</td>
<td>Leaf area is normal, with some yellowing</td>
</tr>
<tr>
<td>6</td>
<td>Leaf area is normal, plants are small but healthy</td>
</tr>
<tr>
<td>7</td>
<td>Healthy plants.</td>
</tr>
</tbody>
</table>

†Vigor is a measure of overall plant health.
Table 2. Brown stem rot (BSR) resistance ratings for plate susceptibility and vigor in four F$_2$ populations and their parental lines.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean values</th>
<th>P-value of the difference between F$_2$ population and parental lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F$_2$</td>
<td>PI</td>
</tr>
<tr>
<td>PI 594637 X Century 84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate susceptibility</td>
<td>0.48</td>
<td>0.45</td>
</tr>
<tr>
<td>Vigor</td>
<td>3.63</td>
<td>2.95</td>
</tr>
<tr>
<td>PI 594638B X Century 84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate susceptibility</td>
<td>0.34</td>
<td>0.00</td>
</tr>
<tr>
<td>Vigor</td>
<td>5.54</td>
<td>5.71</td>
</tr>
<tr>
<td>PI 594650A X Century 84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate susceptibility</td>
<td>0.26</td>
<td>0.00</td>
</tr>
<tr>
<td>Vigor</td>
<td>5.15</td>
<td>5.87</td>
</tr>
<tr>
<td>PI 594658B X Century 84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate susceptibility</td>
<td>0.29</td>
<td>0.00</td>
</tr>
<tr>
<td>Vigor</td>
<td>5.94</td>
<td>6.57</td>
</tr>
</tbody>
</table>

† Plate susceptibility: 1 = infection at inoculation point and at plant top; 0 = infection only at inoculation point.
‡ Vigor: Overall plant health, measured on a scale from 1 to 7; 1 = dead plant; 7 = healthy plant.
Table 3. Number of F$_{2;3}$ lines with higher brown stem rot (BSR) resistance than the most resistant parent or lower than the most susceptible parent by two standard deviations.

<table>
<thead>
<tr>
<th>Transgressive segregation</th>
<th>Number of F$_{2;3}$ lines showing transgressive segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plate Susceptibility</td>
</tr>
<tr>
<td>PI 594637 X Century 84</td>
<td></td>
</tr>
<tr>
<td>For resistance †</td>
<td>2</td>
</tr>
<tr>
<td>For susceptibility ‡</td>
<td>23</td>
</tr>
<tr>
<td>PI 594638B X Century 84</td>
<td></td>
</tr>
<tr>
<td>For resistance</td>
<td>0</td>
</tr>
<tr>
<td>For susceptibility</td>
<td>0</td>
</tr>
<tr>
<td>PI 594650A X Century 84</td>
<td></td>
</tr>
<tr>
<td>For resistance</td>
<td>0</td>
</tr>
<tr>
<td>For susceptibility</td>
<td>1</td>
</tr>
<tr>
<td>PI 594658B X Century 84</td>
<td></td>
</tr>
<tr>
<td>For resistance</td>
<td>0</td>
</tr>
<tr>
<td>For susceptibility</td>
<td>20</td>
</tr>
</tbody>
</table>

† Transgressive segregants for BSR resistance. Lines with resistance higher than that of the most resistant parent by 2σ.
‡ Transgressive segregants for BSR susceptibility. Lines with susceptibility higher than that of the most susceptible parent by 2σ.
Table 4. Means of genotypic classes and $R^2$ values of molecular markers on chromosome 16 (formerly molecular linkage group J) associated with brown stem rot (BSR) resistance.

<table>
<thead>
<tr>
<th>Variable</th>
<th>SSR marker</th>
<th>Mean values for genotypic classes</th>
<th>$R^2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Homozygous for alleles from PI</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homozygous for alleles from Century 84</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Segregating for alleles from both parents</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PI 594637 X Century 84</strong></td>
<td>Satt547</td>
<td>0.50</td>
<td>2.1</td>
</tr>
<tr>
<td>Plate susceptibility</td>
<td></td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Vigor</td>
<td></td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PI 594638B X Century 84</strong></td>
<td>Satt547</td>
<td>0.05</td>
<td>44.4***</td>
</tr>
<tr>
<td>Plate susceptibility</td>
<td></td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Vigor</td>
<td></td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PI 594650A X Century 84</strong></td>
<td>Satt431</td>
<td>0.06</td>
<td>34.3***</td>
</tr>
<tr>
<td>Plate susceptibility</td>
<td></td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Vigor</td>
<td></td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PI 594658B X Century 84</strong></td>
<td>Satt547</td>
<td>0.03</td>
<td>48.1***</td>
</tr>
<tr>
<td>Plate susceptibility</td>
<td></td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Vigor</td>
<td></td>
<td>0.21</td>
<td></td>
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</tbody>
</table>

*** Significant at the 0.001 probability level.
Figure 1. Plate susceptibility. A) Susceptible plant with infection of *P. gregata* in stem fragments from the inoculation point and from the plant top. B) Resistant plant with infection only at the inoculation point.
Figure 2. Distribution of plate susceptibility (1 = infection at inoculation point and at plant top; 0 = infection only at inoculation point) for four segregating populations and their parental lines. Arrows indicate parental mean values (continuous arrows for Century 84 and dashed arrows for PI 594637, PI 594638B, PI 594650A, and PI 594658B).
Figure 3. Distribution of vigor (measured on a scale from 1 to 7; 1= dead plant; 7 = healthy plant) for four segregating populations and their parental lines. Arrows indicate parental mean values (continuous arrows for Century 84 and dashed arrows for PI 594637, PI 594638B, PI 594650A, and PI 594658B).
CHAPTER 3: QTL MAPPING OF WHITEFLY RESISTANCE IN SOYBEAN

A paper to be submitted to Journal of Crop Improvement

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ABSTRACT

Whitefly Bemisia tabaci (Gennadius) is one of the most damaging insects attacking crops in the world. In soybean [Glycine max (L.) Merr.], in addition to causing plant stress and reduced seed size, they also can be vectors of viruses, e.g. soybean crinkle mosaic, and soybean dwarf mosaic. Resistance to whitefly in soybean has been reported; however, whitefly resistance genes have not been identified. The objective of the study was to identify quantitative trait loci (QTL) for whitefly resistance in soybean. Two F2 segregating populations, derived from crosses between whitefly resistant lines ‘Cajeme’ and 'Corsoy 79' and the susceptible line ‘Williams 79’, were developed to determine inheritance of resistance to B. tabaci. Parental lines were screened with simple sequence repeats (SSR). Segregating F2 populations and parents were evaluated for whitefly infestation for two years at one
location in Northwest Mexico, where whitefly is a recurrent pest of soybean. QTL analyses were performed using standard interval mapping (SIM), and composite interval mapping (CIM). Results indicate that whitefly resistance is a polygenic trait, controlled by QTL on different chromosomes. The significant QTL detected in this study were located on chromosomes 12, 18, and 19 (formerly molecular linkage groups H, G, and L, respectively). In addition, there was evidence that other QTL could be involved in resistance, such as those detected on chromosomes 1, 7, 10, 16, and 17 (formerly molecular linkage groups D1a, M, O, J, and D2, respectively). Fine-mapping of the large effect QTL detected in this study would be helpful to identify tightly linked markers for use in marker-assisted selection (MAS) for whitefly resistance in soybean.

Abbreviations: • CIM, composite interval mapping • LOD, likelihood of odds • LSMEANS, Least square means • MAS, marker-assisted selection • PCR, polymerase chain reaction • QTL, quantitative trait loci • SIM, standard interval mapping • SSR, simple sequence repeat.

INTRODUCTION

Whitefly, *Bemisia tabaci* (Gennadius) (order Homoptera, family Aleyrodidae), is a common pest in many crops throughout the world. Historically, whiteflies have been major pests in vegetable crops, cotton, and greenhouse plants. In addition, they have a wide range of hosts, including wild plant species, which makes it difficult to control once they have infested a crop (Perring, 2001). In the United States, annual yield losses exceed US $200 million, and occur in cotton, peanuts, soybean, vegetables, and ornamentals (Faust, 1992). Damage can be caused by whiteflies directly feeding on the crop and also by virus
transmission while feeding (Costa, 1976; Kogan and Herzog, 1980; Vaishampayan et al. 1975).

Infestations of whiteflies in soybeans have been recorded in Brazil, India, Japan, Turkey, Australia, and Mexico (Arioglu, 1987; Costa, 1976; Vaishampayan et al. 1975). In certain regions of Mexico, soybean plantings have been banned due to heavy whitefly infestations that could also spread to other crop species (Morales and Anderson, 2001). In Puerto Rico, whiteflies have caused losses to soybean planted in off-season nurseries. In the U.S., losses have been reported in Florida and Georgia (McPherson and Douce, 1992). Recently, the insect has been reported in soybean fields in Illinois (Gray, 1999) and in Iowa (O’Neal and Rice, 2006; Rice, 2007).

Whitefly, *B. tabaci*, is a species complex formed by highly cryptic sibling species (Campbell, 1993; Campbell et al., 1996; Perring, 2001). To date, 41 distinct populations of *B. tabaci* have been studied; 24 of these populations have been given a specific biotype designation (Perring, 2001). Adults and nymphs have piercing-sucking mouthparts and feed on the lower leaf surfaces where they lay their eggs (Byrne and Bellons, 1991; Perring, 2001). The life cycle averages 32 days, depending on temperature, i.e. temperatures of about 25 ºC favor development of the insect on soybean (University of Illinois - Integrated Pest Management Bulletin, 2004). Whiteflies damage crops by extracting large quantities of phloem sap (Jones, 2003), and heavy infestations may result in development of chlorotic spots on leaves, wilting, and stunting of plants. In addition, the insects excrete a sticky material called honeydew, which in high concentrations promotes the growth of sooty mold fungi, which interferes with the host plant photosynthesis. Some species of the whitefly
complex also serve as vectors of several economically important viral plant pathogens (Byrne and Bellons, 1991). Their high reproduction rate and short generation interval during the growing season favor development of resistance to insecticides. Under this scenario, management of the pest is complex and chemical controls are usually ineffective. Breeding resistant cultivars is the most effective management alternative.

In soybean, viruses such as soybean crinkle mosaic, and soybean dwarf mosaic can be transmitted by whiteflies (Costa, 1976). Two biotypes of whiteflies have been recorded that colonize field-grown soybean: the sweetpotato whitefly, *B. tabaci*, and the bandedwinged whitefly, *Trialeurodes abutiloneus* (Haldeman) (Vaishampayan et al. 1975). In greenhouse conditions, soybean is colonized by a different species, *Trialeurodes vaporariorum* (Westwood).

In the search for genetic resistance that could be used in breeding soybean, systematic screenings of germplasm collections have been done. In 1987, 109 soybean cultivars were screened in Turkey (Arioglu, 1987) for resistance to *B. tabaci* during the pod-filling stage. Variation among genotypes was observed, with the indication that one factor that could be affecting whitefly resistance was pubescence type (long, medium, short, acute, prostrate, erect) and density (glabrous, sparse, dense). Soybean genotypes with medium to long pubescence have, in general, lower whitefly infestation. Conversely, cultivars with dense pubescence have higher whitefly infestation than glabrous cultivars (Arioglu et al., 1989; Kilen and Lambert, 1993; Lamberton et al., 1995; McAuslane, 1996; McAuslane et al., 1995). Partial whitefly resistance of glabrous soybean cultivars is due in part, by reduced ovipositional preference related to lack of foliar pubescence (McAuslane, 1996).
The wide adaptation of the insect and its increase in spread into new regions are of utmost concern. This is especially true due to the limited information about whitefly on soybean in the Midwest, and the lack of information about the inheritance of resistance to whitefly in soybean. To date few inheritance studies of whitefly resistance in soybean have been reported. Xu et al. (2009) studied inheritance of whitefly resistance by analyzing different genetic models. The authors evaluated mean nymph whitefly infestation per leaflet of F1 and F2:3 plant populations from the cross between a highly susceptible variety 'Qihuang' and a highly resistant variety 'Huapidou'. They found that the model with a major gene + polygene(s) had the best fit to explain whitefly resistance in soybean. These findings indicate the complex inheritance of whitefly resistance in soybean. In the study, it was not determined, at the molecular level, how many and where the quantitative trait loci (QTL) were located. For a trait of such complexity, incorporation of molecular marker information in inheritance studies is an important requirement to identify and locate the resistance genes that could be linked to molecular markers. The objective of our study was therefore, to identify number and location of QTL for whitefly resistance in soybean.

MATERIALS AND METHODS

Population development

Two F2 segregating populations for whitefly resistance were developed. On the basis of Arioglu’s (1987) results, two cultivars 'Corsoy 79' (Bernard and Cremmens, 1988a) and 'Cajeme', a Mexican cultivar, were identified as whitefly resistant. The cultivars were crossed to the susceptible cultivar 'Williams 79' (Bernard and Cremmens, 1988b), which was used as female in both crosses.
The mapping populations Williams 79 x Corsoy 79, and Williams 79 x Cajeme consisted of 181 and 156 F$_2$ plants, respectively. The F$_1$ seed was obtained at Ames, IA, and was sent to Isabela, Puerto Rico, where the F$_2$ plants and their F$_{2:3}$ families were developed. Leaves were collected from F$_2$ plants for DNA extraction, which afterwards were grown to maturity. Each F$_2$ plant was single-plant threshed to generate the F$_{2:3}$ families. From the Williams 79 x Corsoy 79 population, 150 F$_{2:3}$ families were obtained, and from the Williams 79 x Cajeme population, 90 F$_{2:3}$ families.

**Phenotypic evaluation**

Phenotyping of F$_{2:3}$ families was done in Isabela, Puerto Rico in 2001, however, the environmental conditions that year did not favor whitefly infestation and use of this location was discontinued. In 2003 and 2004 phenotypic evaluations of F$_{2:3}$ families were done at the International Maize and Wheat Improvement Center (CYMMIT) - Ciudad Obregon, Sonora, Mexico. The location was selected because whitefly is a common and recurrent pest of soybean in this region. Parents and F$_{2:3}$ lines were assigned to plots, arranged in a randomized complete block design with three replications, with each population grown as a separate test. The lines were grown in 4-row plots, 3 m long, 0.6 m between rows, planted at 8-seeds/0.3048 m.

Assessment of whitefly infestation was done weekly (7-10 times) during the pod-filling period, which is usually when the heaviest infestation occurs in soybean (Arioglu, 1987). From five random plants in each plot, five fully expanded leaflets were cut from near the top of the plant and the number of nymphs in an area of 6.5 cm$^2$ (1 square inch) was
recorded using a magnifying glass. This number represented the variable nymph density. Nymph density at the peak of whitefly infestation was used for QTL analysis.

**DNA marker analysis**

From each F$_2$ population and the parental lines, genomic DNA was extracted from leaf tissue. Both populations were genotyped with polymorphic simple sequence repeat (SSR) markers covering the 20 chromosomes (Schmutz et al., 2010) of the soybean genome, according to the soybean consensus map (Cregan et al., 1999; Song et al., 2004). Genotyping was performed by the Monsanto molecular laboratory in Huxley, IA. The SSR marker fragments were amplified in a 10 µl reaction mixture consisting of 10x polymerase chain reaction (PCR) buffer, 1 µl containing 1.25 mM of each dNTP, 1 µl forward and reverse primers (20 µM), 20 ng DNA, 0.3 µl Taq DNA polymerase (5 U/µl), and sterile deionized H$_2$O to volume. Amplification conditions were: 95°C for 2 min, 35 cycles of 95°C for 45s; 53°C for 45 s; 72°C for 1 min; and a 10 min extension at 72°C. PCR products were size separated on 4% SFR agarose (Amresco) gel.

**Statistical analysis**

Statistical analyses for phenotypic data were performed using SAS 9.1 (SAS Institute, 2003). A combined analysis was performed for the years 2003 and 2004. Homogeneity of variances across years was evaluated using the Bartlett test (Snedecor and Cochran, 1983). A mixed-effects linear model was used to estimate nymph density values using PROC MIXED in SAS 9.1 (SAS Institute, 2003). The model for both populations was $Y_{ijk} = \mu + G_i + Y_j +$
\[ R(Y)_{jk} + GY_{ij} + \epsilon_{ijk} \]

where \( \mu \) is the overall mean for nymph density; \( G_i \) is the effect of genotypes \((i = 1, 2, 3, \ldots, n, \) where \( n \) is the number of \( F_{2:3} \) lines plus parental lines); \( Y_j \) is the effect of year \((j=1,2)\); \( R(Y)_{jk} \) is the effect of the \( k \) replication within each year \((k=1,2)\); \( GY_{ij} \) is genotype x year interaction, and \( \epsilon_{ijk} \) is the error term. Lines were considered fixed effects and the other effects were considered random. Mean whitefly infestation averaged across all replications and years were calculated with PROC LSMEANS in SAS 9.1 (SAS Institute, 2003). Differences among the parents and the mean of the \( F_{2:3} \) lines were assessed using Tukey’s multiple-comparison test. The whitefly resistance level of each \( F_{2:3} \) line was compared with the resistance exhibited by the resistant parent. Transgressive segregants for whitefly resistance were defined as \( F_{2:3} \) lines which exceeded the most resistant parent by at least two standard deviations.

Genotypic information was used to construct a linkage map with the polymorphic SSR markers for each population, using Mapmaker 3.0 (Lander et al., 1987). Linkage was declared with minimum likelihood of odds (LOD) of 3.0 and maximum recombination frequency of 40 cM using the Kosambi’s mapping function (Kosambi, 1944).

Genomic regions significantly associated with nymph density in both populations, in 2003, 2004, and in the combined analysis across years, were detected using standard interval mapping (SIM), and composite interval mapping (CIM). QTL analysis was conducted with R/qtl version 1.14-2 (Broman and Sen, 2009; Broman et al., 2003), an add-on package for the general statistical software R (The R Project for Statistical Computing, 2005). The Haley-Knott regression methods were used to identify QTL. This method uses the regression of phenotypes on multi-point genotype probabilities (Haley and Knott, 1992). The statistical
significance of the results was evaluated by permutation tests. One thousand permutation replicates were used to calculate genome-wide significant LOD thresholds (Lander and Botstein, 1989). QTL with LOD values higher than the genome-wide threshold were considered significant. A QTL was considered suggestive when the LOD value was larger than two and smaller than the genome-wide threshold value. In the CIM, molecular markers near a large-effect QTL, detected by SIM, were used as covariates, in order to reduce residual variation and to identify other QTL. This analysis uses forward selection to a fixed number of markers, followed by interval mapping, omitting any marker covariates within some fixed distance of the position under test. We used three marker covariates, and a window size on either side of the flanking loci was defined as 20 cM.

RESULTS

Phenotypic analysis

Significant differences (P<0.05) among lines for nymph density were observed in both populations. Given that the effects year and genotype x year interaction were not significant, we combined phenotypic data from 2003 and 2004. In addition, homogeneity of variance for nymph density across years was observed. Both populations showed segregation for the resistance trait (Figures 1-2). Transgressive segregation was observed for both populations. For Williams 79 x Corsoy 79, there were 17 transgressive segregants two standard deviations more resistant than the resistant cultivar, Corsoy 79, in 2004. For population Williams 79 x Cajeme, two transgressive segregants were two standard deviations more resistant than the resistant cultivar, Cajeme, in 2003.
In both populations, average nymph density of F$_{2:3}$ lines showed intermediate values of whitefly susceptibility (Table 1). For Williams 79 x Corsoy 79, the average nymph density value of the F$_{2:3}$ lines was not significantly different from Williams 79, in both years and in the combined analysis. Conversely, the average nymph density value of F$_{2:3}$ lines was significantly (P<0.05) different from Corsoy 79, in 2003 and in the combined analysis. For population Williams 79 x Cajeme, in both years, average nymph density across the F$_{2:3}$ lines was not significantly different from that of the resistant parent. However, the average of the F$_{2:3}$ lines was significantly (P<0.05) different from that of the susceptible parent, Williams 79. In both populations, significant (P<0.01) differences between parental lines were observed every year and in the combined analysis, except in 2004 where no significant differences were detected between Williams 79 and Corsoy 79 (Table 1).

**Genotypic analysis**

For population Williams 79 x Corsoy 79, 181 F$_2$ plants were genotyped with 101 polymorphic SSR markers. In the linkage analysis, 61 markers grouped into 15 linkage groups, while 50 remained unlinked. The length of the linkage map was 725 cM and the average distance between markers was 16.8 cM. For population Williams 79 x Cajeme, 90 polymorphic SSR markers were detected, from these, 60 mapped to 14 linkage groups and 30 markers remained unlinked. The length of the linkage map was 706 cM with an average distance between markers of 16.6 cM. The linkage groups detected in both populations corresponded to soybean chromosomes and the order of the majority of SSR markers coincided with the soybean consensus map (Song et al., 2004).
QTL analysis

Eight putative QTL were detected across the two populations (Table 2). QTL detected by SIM also were identified by CIM within the same significant marker interval. Phenotypic variance for whitefly resistance, explained by individual QTL detected in population Williams 79 x Corsoy 79, ranged from 6 to 13%. In population Williams 79 x Cajeme, QTL for whitefly resistance explained 5 to 18% of the phenotypic variation.

For population Williams 79 x Corsoy 79, the genome-wide 95% LOD thresholds were 3.16, 3.18, and 3.13 for 2003, 2004, and combined analysis, respectively. QTL were detected on chromosomes 10, 12, 16, 17, and 18 (formerly molecular linkage groups O, H, J, D2, and G respectively) and when combined explained 20.4, 20.2, and 19.9% of the phenotypic variation for 2003, 2004, and combined, respectively (Table 2). A major QTL for whitefly resistance was detected on chromosome 18 every year and in the combined analysis (Table 2; Figure 3). This QTL was highly significant in 2004 (LOD = 4.5) and in the combined analysis (LOD = 4.88). In 2003 the same QTL was detected as suggestive (LOD between 2 and the genome-wide threshold).

For population Williams 79 x Cajeme, the genome-wide 95% LOD thresholds for QTL detection were 3.07, 3.28, and 3.19 for 2003, 2004, and combined analysis, respectively. In 2003, QTL for whitefly resistance were detected on chromosomes 7, 12, 18, and 19 (formerly molecular linkage groups M, H, G, and L respectively), and in the combined analysis explained 52.6 % of the phenotypic variation (Table 2). Two significant QTL were detected on chromosomes 12 (LOD = 3.81) and 19 (LOD = 3.11), and two additional QTL on chromosomes 7 and 18 were declared suggestive (LOD between 2 and the...
genome-wide threshold) (Table 2; Figure 3). No significant QTL were detected in 2004, however, a suggestive QTL (LOD = 2.82) was detected on chromosome 1 (formerly molecular linkage group D1a), explaining 5% of the phenotypic variation. In the combined analysis, a suggestive QTL (LOD = 2.0) was detected on chromosome 12, explaining 4.9% of the phenotypic variation.

The two SSR markers flanking the most significant QTL region in the two populations were examined for allele-specific effects on phenotype. For population Williams 79 x Corsoy 79, the two SSR markers examined were Satt394 and Satt594. For population Williams 79 x Cajeme, Satt181 and Satt317 were examined for allele-specific effects on whitefly resistance. For the two populations, consistent with the phenotypes observed in the parental lines, the presence of Williams 79 alleles at the SSR markers correlated with an increase in nymph density (Figures 4-5). Conversely, the presence of alleles from the resistant parents represented a decrease in nymph density. For Williams 79 x Corsoy 79, intermediate phenotypes for whitefly resistance, were observed in individuals that were heterozygous for the two loci, consistent with additive effects of alleles at each locus. On the other hand, heterozygous individuals for the two SSR markers examined in population Williams 79 x Cajeme showed lower nymph density than the resistant parent.

**DISCUSSION**

Inheritance to whitefly resistance in soybean was studied in two F2 mapping populations evaluating nymph density on the lower leaf surface. We confirmed that whitefly
resistance was a polygenic trait, controlled by QTL on different chromosomes. Xu et al. (2009) reported that genetic resistance to whitefly was controlled by one major gene and polygenes in soybean. However, these authors did not report specific chromosome locations associated with the resistance trait. In our study, eight putative QTL for whitefly resistance were detected on different soybean chromosomes, across the two segregating populations. QTL detection varied between populations and years, although whitefly resistance QTL on chromosomes 12 and 18 were detected in more than one year and in both populations. For population Williams 79 x Corsoy 79, whitefly resistance was controlled by a major QTL on chromosome 18 and four minor QTL on chromosomes 10, 12, 16, and 17. These findings are in agreement with the major gene + polygenic mixed inheritance model suggested by Xu et al. (2009). Conversely, population Williams 79 x Cajeme had two major QTL on chromosomes 12 and 19, with three QTL of minor effect on chromosomes 1, 7, and 18.

Most of the significant and suggestive whitefly resistance QTL detected in our study where located on chromosomes that are known to contain several disease and insect resistance genes. Insect resistance in soybean is controlled by two mechanisms, antibiosis, in which feeding causes a disruption of growth and development of the insect; and antixenosis, where the insect is repelled by the host plant (Rector et al., 2000). In our study, we found major whitefly resistance QTL on chromosomes 12 and 18. These two chromosomes are known to have insect-resistance QTL associated with antibiosis and antixenosis (Komatsu et al., 2008; Narvel et al., 2001; Rector et al., 1998, 2000). We also observed one minor whitefly resistance QTL on chromosome 7. Insect resistance QTL associated with antibiosis and antixenosis previously have been mapped to this chromosome (Komatsu et al., 2004;
Narvel et al., 2001; Rector et al., 1998, 2000). Epistatic interaction between insect resistance QTL on chromosomes 7, 12, and 18 also has been documented. Zhu et al. (2006) reported epistatic interactions between a QTL on chromosome 7 controlling antixenosis and other resistance QTL on chromosomes 12 and 18. Similarly, Komatsu et al. (2008) observed interactions between common cutworm-resistance QTL on chromosomes 7 and 18. We detected another significant QTL associated with whitefly resistance on chromosome 19. Resistance QTL for sclerotinia (Arahana, 2001) and soybean cyst nematode (Guo et al., 2006) also have been mapped to this chromosome.

In addition to the QTL previously associated with insect resistance, we also detected QTL on chromosomes 1, which is known to have QTL for pubescence density (Komatsu et al., 2007). Pubescence type and density are associated with whitefly resistance in soybean. Cultivars with dense pubescence have higher whitefly infestation than glabrous cultivars. On the other hand, cultivars that have trichomes laying flat on the leaves were very resistant to whitefly infestation (Arioglu et al., 1989; Kilen and Lambert, 1993; Lambert et al., 1995; McAuslane, 1996; McAuslane et al., 1995). Future research will help to elucidate the relationship between the whitefly-resistance QTL on chromosome 1 and the previously reported pubescence density QTL on the same chromosome. Furthermore, it is necessary to clarify the relationship between whitefly resistance of cultivars Corsoy 79 and Cajeme and pubescence density, for a better identification of the resistance mechanisms of whitefly resistance in soybean.

We concluded that the presence of major-effect QTL and several small-effect QTL indicates complex inheritance of whitefly resistance in soybean. Development of insect-
resistance cultivars is difficult because plant resistance to insects is most often a quantitatively inherited trait (Kogan and Turnipseed, 1987; McAuslane, 1996). However, identification of molecular markers linked to whitefly-resistance QTL could be used in marker-assisted selection (MAS) programs to develop whitefly-resistance soybean cultivars. To implement MAS in a breeding program, however, a few QTL with consistent large effects across breeding populations have to be identified (Holland, 2004).

Location of whitefly-resistance QTL in soybean were detected in this study. Knowledge of the approximate locations of QTL is a starting point for fine mapping or for studying candidate genes that are close to the identified QTL (Bernardo, 2008). Fine-mapping of the large-effect whitefly-resistance QTL detected in this study would be helpful to identify tightly linked markers for MAS programs for cultivar development of whitefly-resistant soybean cultivars.

**Acknowledgements**

We thank Dr. John Tamulonis for facilitating the genotyping performed by the Monsanto molecular laboratory in Huxley, IA. This is a joint contribution of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project No. 4403 and the USDA-ARS, Corn Insects and Crop Genetics Research Unit, and supported by Hatch Act and State of Iowa. The research was in part financed by funds provided by the Iowa Soybean Association (ISA), and the North Central Soybean Research Program (NCSRP).
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Table 1. Population mean, standard error, and tests of significance of the difference between genotypic classes, for whitefly infestation in populations Williams 79 x Corsoy 79, and Williams 79 x Cajeme, in 2003, 2004, and the combined analysis of both years.

<table>
<thead>
<tr>
<th></th>
<th>Mean nymph density†</th>
<th>t test (P-value) of the difference between populations</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$F_{2:3}$ population</td>
<td>Williams 79 (susceptible)</td>
<td>Resistant†</td>
</tr>
<tr>
<td>Williams 79 X Corsoy 79</td>
<td>2003</td>
<td>24.06 ± 0.8</td>
<td>30.93 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>2004</td>
<td>24.54 ± 1.1</td>
<td>27.83 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>24.29 ± 0.6</td>
<td>29.38 ± 3.8</td>
</tr>
<tr>
<td>Williams 79 X Cajeme</td>
<td>2003</td>
<td>24.93 ± 1.1</td>
<td>39.87 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>2004</td>
<td>21.39 ± 1.2</td>
<td>35.22 ± 6.2</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>23.16 ± 0.8</td>
<td>37.54 ± 4.3</td>
</tr>
</tbody>
</table>

†Nymph density is the number of nymphs in an area of 6.5 cm$^2$
Table 2. Summary of significant† and suggestive‡ whitefly resistance quantitative trait loci (QTL) for populations Williams 79 x Corsoy 79, and Williams 79 x Cajeme, in 2003, 2004, and in the combined analysis across years.

<table>
<thead>
<tr>
<th>Population</th>
<th>Chromosome</th>
<th>Position (cM)</th>
<th>LOD‡</th>
<th>P-value</th>
<th>R² (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Williams 79 x Corsoy 79</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2003</td>
<td>18</td>
<td>22.0</td>
<td>2.03</td>
<td>0.441</td>
<td>5.9</td>
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<tr>
<td></td>
<td>16</td>
<td>4.0</td>
<td>2.33</td>
<td>0.276</td>
<td>6.9</td>
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<tr>
<td></td>
<td>10</td>
<td>17.8</td>
<td>2.33</td>
<td>0.276</td>
<td>7.6</td>
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<tr>
<td>2004</td>
<td>18</td>
<td>23.0</td>
<td>4.50</td>
<td>0.001</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>17.0</td>
<td>2.42</td>
<td>0.222</td>
<td>7.5</td>
</tr>
<tr>
<td>Combined</td>
<td>18</td>
<td>22.0</td>
<td>4.88</td>
<td>0.002</td>
<td>13.3</td>
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<tr>
<td></td>
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<td>0.457</td>
<td>6.6</td>
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<td>Williams 79 x Cajeme</td>
<td></td>
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<tr>
<td>2003</td>
<td>12</td>
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<td>3.81</td>
<td>0.009</td>
<td>18.0</td>
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<td>74</td>
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<tr>
<td>Combined</td>
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<td>10</td>
<td>2.00</td>
<td>0.123</td>
<td>4.92</td>
</tr>
</tbody>
</table>

† A QTL was declared significant when the Logarithm of Odds (LOD) value was higher than the genome-wide LOD threshold for significance level of 5%.

‡ A QTL was declared suggestive if LOD was higher than 2 but lower than genome-wide LOD threshold for a significance level of 5%.

§ For Williams 79 x Corsoy 79, genome-wide LOD thresholds were 3.16, 3.18, and 3.13 for 2003, 2004, and combined analysis, respectively. For Williams 79 x Cajeme, genome-wide LOD thresholds were 3.07, 3.28, and 3.19 for 2003, 2004, and combined analysis, respectively.
**Figure 1.** Distribution of whitefly infestation among 150 F$_{2:3}$ lines from the population Williams 79 x Corsoy 79. Histograms show distribution of mean nymph density (number of nymphs in an area of 6.5 cm$^2$) in 2003, 2004, and in the combined analysis of both years. Arrows indicate parental mean values (continuous arrows for Williams 79 and dashed arrows for Corsoy 79).
Figure 2. Distribution of whitefly infestation among 90 F$_{2:3}$ lines from the population Williams 79 x Cajeme. Histograms show distribution of mean nymph density (number of nymphs in an area of 6.5 cm$^2$) in 2003, 2004, and in the combined analysis of both years. Arrows indicate parental mean values (continuous arrows for Williams 79 and dashed arrows for Cajeme).
Figure 3. Logarithm of Odds (LOD) curves from interval mapping analysis in the combined analysis of two years for Williams 79 x Corsoy 79, and in 2003 for Williams 79 x Cajeme. The dashed horizontal lines indicate the genome-wide 95% LOD threshold.
Figure 4. Nymph density (number of nymphs in an area of 6.5 cm$^2$) for different genotypes at the two SSR markers flanking a QTL on chromosome 18 (formerly molecular linkage group G) in the combined analysis. Error bars represent 95% confidence intervals. A: alleles from Williams 79, and B: alleles from Corsoy 79.
Figure 5. Nymph density (number of nymphs in an area of 6.5 cm$^2$) for different genotypes at the two SSR markers flanking a QTL on chromosome 12 (formerly molecular linkage group H) in 2003. Error bars represent 95% confidence intervals. A: alleles from Williams 79 and B: alleles from Cajeme.
CHAPTER 4: NECTAR COMPOSITION AND FLOWER CHARACTERISTICS OF
WILD PERENNIAL GLYCINE SPECIES

A paper to be submitted to *American Journal of Botany*

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ABSTRACT

The genus *Glycine* contains the cultivated annual soybean *G. max*, the wild annual soybean *G. soja*, and about 25 wild perennial *Glycine* species. Outcrossing by insects occurs at a very low frequency (<1%) in the cultivated species, between <1% to 20% in the wild annual species, and can exceed 50% in certain wild perennial species. Unfortunately, there is a paucity of data on the plant traits that contribute to outcrossing in the wild perennial species.

The objective was to determine nectar composition (sucrose, glucose, fructose, and total carbohydrates) and flower size from greenhouse-grown wild perennial plants. Nectar samples were collected from 70 accessions representing 19 perennial species. Sugars were analyzed by spectrophotometric enzyme assays. Sugar ratios were the most meaningful for comparative purposes. We found variation for nectar sugar composition, and flower size across all the species. Sugar proportions varied across species and on average, the nectar was dominated by sucrose (62.5%), followed by fructose (25.1%) and glucose (12.4%).

Multivariate analysis was used to detect groups of species with similar nectar sugar characteristics. Two groups were detected, one group was formed by the two species *G. falcata* and *G. canescens*, which were similar in nectar sugar concentration and composition. The other 17 species conformed the second group. The information gained from the floral nectar composition of the wild perennial species will guide research for nectar composition-outcrossing associations in the cultivated species. The overall goal is to produce large quantities of F₁ hybrid seed for plant breeding studies, and for commercial hybrid seed production.

**Key words:** flower size; *Glycine*; nectar sugar composition; soybean wild relatives.
INTRODUCTION

The soybean [Glycine max (L.) Merr.] is an economically important crop rich in seed protein (about 40%) and oil (about 20%), and is ranked number one in world oil production in the international trade markets among the major oil seed crops (Wilson, 2004). Given the importance of this crop, breeding efforts have been made to enhance soybean as a food, feed, medicinal, and industrial crop (Palmer, 1999). The gene pools of the soybean, or groups of genes useful for genetic improvement, comprise species of the genus Glycine. This genus is divided into two subgenera Glycine (perennials) and Soja (Moench) F.J. Herm. (annuals). The progenitor of the soybean is the wild annual species Glycine soja (Sieb. and Zucc.) (Hymowitz, 2004). Despite the rich diversity in the soybean gene pools, the genetic base of soybean cultivars is extremely narrow. Gizlice et al. (1993) found that fewer than 15 progenitors constituted the major portion of the genetic base for cultivars in U.S soybean production. Soybean breeding programs could exploit the genetic diversity present in exotic germplasm as G. soja and the wild perennial Glycine species. Limited numbers of interspecific crosses between G. max and G. soja have been made in attempts to broaden the genetic base of the cultivated soybean, however, the G. soja accessions harbor many undesirable genetic traits as lodging, vining growth habit, susceptibility to biotic and abiotic stresses, lack of complete leaf abscission, pod shattering, and black seed coat (Palmer and Hymowitz, 2004). Nevertheless, a successful example of the introgression of G. soja germplasm into the cultivated soybean is the development of small-seeded cultivars, where G. soja has been used as the donor parent (Fehr et al., 1990a, 1990b; Carter et al., 1995; Singh and Hymowitz, 1999).
The unexploited valuable pool of genetic diversity of the wild perennial species is largely unexplored by soybean breeders for improvement of the cultivated soybean (Singh and Hymowitz, 1999). These species offer a potential source of new germplasm, but embryo rescue techniques are necessary to obtain viable hybrids (Singh et al., 1990, 1993; Hymowitz, 2004; Palmer and Hymowitz, 2004). The wild perennial *Glycine* species have genes that could contribute to the improvement of the cultivated soybean.

Hybrids have proven to be a practical method of crop improvement. According to Palmer et al. (2001), five components are crucial for the successful development of commercial hybrid soybean. The first is that parent combinations must produce heterosis levels superior to the best pure-line cultivars. The second is that a stable male-sterile, female-fertile sterility system is needed. The third is that a selection system to obtain 100% female (pod parent) plants that set seed normally and can be harvested mechanically is necessary. The fourth is that the pollen transfer mechanism has to be efficient in transfer of pollen from the male to pod parent. The fifth requires that hybrid seed production process be profitable for the seed industry and for the farmers. Pollen transfer is the most limiting factor for hybrid soybean production because natural cross-pollination in the cultivated soybean is generally less than 1% (Carlson and Lersten, 2004, Palmer et al., 2009), and manual cross-pollination to produce large quantities of hybrid seed is difficult, time consuming, and expensive. The small size of the soybean flowers, the low success rate and the few seeds obtained per hybrid pod contribute to the difficulty of manually producing large quantities of hybrid seed (Fehr, 1991). Insect cross-pollination of male-sterile soybean plants facilitates the production of hybrid seed (Nelson and Bernard, 1984; Lewers et al., 1996; Ortiz-Perez et
al., 2007, Perez et al., 2009a, 2009b). The cultivated soybean attracts relatively few bees (Blickenstaff and Huggans, 1962); however, bees do visit the flowers for both pollen and nectar.

*Glycine* species are predominantly self-pollinated, although they possess chasmogamous flowers capable of outcrossing. An example of this phenomenon, two species of the wild perennial *Glycine, G. argyrea* and *G. clandestina*, have a dual flowering strategy of both self-fertilized cleistogamous flowers and chasmogamous flowers on the same plant (Brown et al., 1986; Schoen and Brown, 1991). In a study of selfing in these two species, Schoen and Brown (1991) showed differences in the levels of selfing due to the contrast in the variability of environmental conditions for insect-mediated pollination between the habitats evaluated. In a study of seed set of chasmogamous and cleistogamous flowers of *G. clandestina*, Hempel (2004) found that pollinator limitation in chasmogamous flowers is an important factor limiting seed production.

The identification and characterization of sources of insect attraction among the wild perennial species of the genus *Glycine* could be useful in soybean breeding programs for hybrid production, because in contrast with the low natural out-crossing in the cultivated soybean, out-crossing in the wild perennial soybean species can exceed 50%. We believe that flowers of the perennial *Glycine* have characteristics that attract and reward insect-pollinators. Insect pollinator attraction is determined by flower color, flower accommodation, flower anthesis and pollen dehiscence, volatile production, and nectary structures and secretion. Nectar is a rich source of sugars and amino acids and provides reward to pollinators, which in turn help to increase the fecundity of those plants that provide nectar.
(Perret et al., 2001; Carter et al., 2006). The nectar of soybean is a complex mixture of many compounds, consisting of sugars, amino acids, proteins, lipids, and other compounds that provide nutritional and protective functions (Horner et al., 2003). In soybean, honeybees visit the flowers mainly for nectar collection, which has sugar content between 37 and 45% (Erickson, 1975; Chiari et al., 2005). The quantification and qualification of some of these characteristics, such as the content of glucose, fructose, sucrose (and their ratios), and total carbohydrates would give us an insight into the factors that may affect cross-pollination in the wild perennial soybean.

Thus far, no studies have been done in the search for useful genes for insect attraction in the wild perennial soybean. We performed a comprehensive study of a sample of the wild perennial soybean species to elucidate their variations in nectar composition and in flower characteristics that could be determinants for pollinator-insect attraction and reward in the cultivated soybean. The objective of this study was to determine nectar composition (sucrose, glucose, fructose, and total carbohydrates), and floral morphology from 19 greenhouse-grown wild perennial Glycine species.

**MATERIALS AND METHODS**

**Study plants**

Species belonging to subgenus Glycine are perennial and largely indigenous to Australia and the South Pacific Islands and have been a focus of attention for over two decades because of their relationship to the annual cultivated soybean (Hymowitz, 2004). The perennial Glycine species have intricate and overlapping combinations of genomes,
multiple origins, and ecological variation. In this study, 70 accessions, representing 19 species of the wild perennial soybean were surveyed for nectar composition and flower size (Table 1).

**Study site and methods**

Representative accessions of 19 perennial *Glycine* species were grown in a greenhouse at the Commonwealth Scientific and Industrial Research Organization (CSIRO) in Canberra, Australia from April 2000 to April 2001. The 19 species were accessions from Australia, Papua New Guinea, Indonesia, the Philippines, and Taiwan (Table 1).

Flowers were collected daily from November 2000 to April 2001, between 9:00 am (after watering) until 12:00 pm (noon). The anthers were removed before collecting nectar to minimize pollen contamination of the nectar samples. A 1 µL micropipette was inserted at the base of the standard petal and nectar was taken up by capillary action, approximately 0.50 µL of nectar were collected per flower and nectar samples were stored at -20°C until assayed.

**Nectar analysis and flower evaluation**

Quantification of glucose, sucrose, fructose, and total carbohydrates were done with Kits for Carbohydrate Analysis (Sigma-Aldrich). Standard curves for glucose, fructose, sucrose, and total carbohydrates were prepared and used to calculate the concentration of each sample; each sample was measured three times. Assay results were obtained using a 96-well plate reader and absorbance was read using a Schimadzu spectrophotometer.
Soybean has typical papilionaceous flowers with a standard petal, two lateral wing petals, and an anterior keel petal (Carlson and Lersten, 2004) (Figure 1). In order to estimate floral structure of the perennial soybean, we recorded length and width of standard, wing, and keel petals. Petal perimeter was estimated as the sum of petal length and width (Dafni and Neal, 1997). In order to simplify the analysis of complete set of petal variables, a principal component analysis (PCA) was performed to reduce the number of variables and summarize the sources of variation. We found most of the variation was explained by the first component, and each petal size had a similar contribution to the variance. For this reason, we concluded it was appropriate to add standard perimeter, wings perimeter, and keel perimeter, to create a new variable called flower size.

Data analysis

All the statistical analyses were done with the general statistical software R (The R Project for Statistical Computing, 2005). Analyses were performed for three sets of variables. The first set included sugar (sucrose, glucose, and fructose) and carbohydrates content. The second set was formed by sugar proportions (sucrose, glucose, and fructose percentages) and the variables sugar ratio (sucrose/hexoses). The third set of variables included flower size measurements (petal perimeters and total flower size). Initially, summary statistics were obtained for each of the 19 species, and Pearson correlation coefficients were determined for each pair of variables in a given set. Subsequently, we focused the analyses on species with a sample size equal or larger than ten. Multivariate analysis of variance (MANOVA) for each pair of species was done using nectar information as response variables. Given that we had fewer measurements of flower size per species, and that MANOVA requires more degrees of
freedom, we performed a single factor analysis of variance (ANOVA) for flower size. Since the ANOVA was significant for this variable, t-tests were done for each pair of species.

**RESULTS**

**Summary statistics**

Sugar contents were estimated for each accession in each species by averaging the results from three measurements per flower. Across the 19 species, the mean value of sucrose was 196 g L\(^{-1}\) (Table 2). Mean hexose sugars values were 44.9 g L\(^{-1}\) and 82.3 g L\(^{-1}\) for glucose and fructose, respectively. Carbohydrates had a mean value of 117.2 g L\(^{-1}\). Pearson correlation coefficients were obtained for each pair of variables (Table 2). A high positive association was observed for fructose and glucose (r=0.9); sucrose content had a similar correlation with glucose and fructose (r=0.6); and carbohydrate content was not associated with sugar contents.

Sugar ratio (sucrose/hexoses) was estimated for each species and the average value across species was 2.2 (Table 3). This result suggested that the nectar composition of the perennial *Glycine* species was dominated by sucrose. Sugar proportions varied across species and on average, the nectar was dominated by sucrose (62.5%), followed by fructose (25.1%) and then glucose (12.4%). Negative correlations between sucrose and hexoses percentages were observed (Table 3).

Petal perimeter of the perennial *Glycine* species was estimated as the sum of petal length and width (Dafni and Neal, 1997). Linear measurements of the floral display could
give an accurate estimate of the true area; however, these measurements could be bias depending on the shape and size differences of the flowers under study (Dafni and Neal, 1997). In our study, we used linear measurement estimates only for comparative purposes among the perennial species. On average, the standard petal was larger than the keel and wing petals, although, the perimeter of the two wings combined was larger than the standard petal (Table 4). Total flower size was estimated by adding the measurements of the petals. Flower size had a high positive correlation with standard and wing petal perimeters (Table 4).

**Nectar and flower characteristics of perennial *Glycine* species**

Sample size per species varied from 2 to 134 flowers due to differences in availability of accessions and flowers per species. Sugar and carbohydrates contents showed a large variation among species (Figure 2). Sucrose, glucose, and fructose content varied from 45.0 to 281.3 g L$^{-1}$, from 4.5 to 76.6 g L$^{-1}$, and from 22.3 to 149.2 g L$^{-1}$, respectively (Table 5). Total carbohydrates per flower varied from 94.5 to 170.9 g L$^{-1}$. The hybrid *G. max x G. tomentella* showed the highest content of all sugars, although, only two plants were evaluated. On the other hand, *G. pescadrensis* had the highest carbohydrate content, with four plants evaluated (Table 5).

Sucrose/hexose ratio showed that the nectar of the perennial *Glycine* species evaluated was sucrose-dominant, ranging from 1.2 to 8.9 (Table 6, Figure 3). The lowest sucrose/hexoses ratios were observed in *G. pullenii, G. falcata, G. pindanica, and G. canescens*. Sucrose percentages ranged from 44.7 to 85.5 %, while the ranges of the hexoses were from 4.1 to 32 %, and from 7.7 to 33.4 %, for glucose and fructose, respectively.
Considering hexoses only, the proportion of fructose was always higher than that of glucose, except in *G. pindanica* and *G. albicans*.

Flower size varied across species (Figure 4). The number of flowers evaluated for this variable was lower than the number of flowers evaluated for nectar composition, and for many species only one accession per species was evaluated. The hybrid *G. max x G. tomentella* had the largest flowers, however, only two flowers were evaluated (Table 7). *G. tabacina* and *G. falcata* also exhibited large flowers and the number of flowers evaluated was 8 and 16, respectively. The smallest flower perimeter was observed in *G. aphynota*, where only two flowers were evaluated. With a larger sample size (n=38), *G. tomentella* (diploid) also exhibited small flower size.

**Analysis of variance**

A multivariate analysis of variance (MANOVA) was performed using information from species where more than 10 flowers were evaluated. Pair-wise comparisons between species were performed using different sets of data. The first multivariate analysis included the response variables: sucrose, glucose, fructose, and carbohydrates content. Wilks lambda test and p-values were reported for each pair of species. Species that were not significantly different were grouped together. Using this approach, two groups were detected; one group was formed by *G. falcata* and *G. canescens*, and the other species formed a second group (Table 8). Another multivariate analysis was performed using the variables sucrose/hexoses ratio, and sucrose, glucose, and fructose percentages. The same group patterns were detected using this approach (Table 9). After the multivariate analysis was done, a single factor analysis of variance (ANOVA) was performed for each sugar nectar variable. The variables
that contributed the most to the differences among wild perennial *Glycine* species were sugar ratio ($F_{9:247}= 29.23, P<0.0001$), sucrose percentage ($F_{9:247}= 28.7, P<0.0001$), glucose percentage ($F_{9:247}= 14.34, P<0.0001$), fructose content ($F_{9:256}= 9.7, P<0.0001$), sucrose content ($F_{9:247}= 8.6, P<0.0001$), and total carbohydrates content ($F_{9:256}= 2.4, P=0.013$).

An ANOVA for flower perimeter was performed with species with more than six flowers evaluated. Species were significantly ($F_{9:69} = 8.05, P < 0.0001$) different for flower size. Pair-wise comparisons between species, using t-test, were done. *Glycine tomentella* (diploid) had significantly smaller flower size than that of the other species, except for *G. lactovirens*, where no significant differences were observed (Table 10). *Glycine tabacina* flowers were larger than those of the other perennial *Glycine* species. However, these differences were significant only for *G. canescens*, and *G. tomentella* (diploid). The second largest flowers were observed for *G. falcata*. Its flowers were significantly larger than those of *G. canescens*, and *G. tomentella* (diploid and tetraploid).

**DISCUSSION**

Nectar composition and flower size of 19 wild perennial *Glycine* species were assessed. Variation was found for nectar sugar composition and flower size across all species. Perennial *Glycine* species have predominantly sucrose-rich nectars. Species with sucrose-rich nectar usually are pollinated by hummingbirds, butterflies, moths, and long-tongued bees; conversely, flowers with hexose-rich nectar are pollinated by short-tongued bees, flies, perching birds, and bats (Baker and Baker, 1983; Freeman et al., 1985; Lammers
and Freeman, 1986; Baker et al., 1998; Perret et al., 2001; Dupont et al., 2004; Krömer et al., 2008). To date, no studies on pollinator syndrome of the perennial *Glycine* have been reported. The cultivated soybean is pollinated by short-tongued bees from the families Apidae, Megachilidae, Halictidae, Anthophoridae, and Adrenidae (Erickson, 1975; Chang and Kiang, 1987; Ortiz-Perez et al., 2007; Perez et al., 2009). Pollinators from the Lepidoptera family also have been observed on soybean (Chiari et al., 2005).

In the present study, none of the species evaluated had sucrose/hexoses ratios lower than one. The perennial *Glycine* species *G. falcata* had one of the lowest sugar ratios of all the species studied. This species also had larger flowers than those of most of the other species. This could be indicative of a different pollinator syndrome. Based on our current knowledge about phylogenetic relationships in perennial *Glycine* species, *G. falcata* belongs to a unique taxonomic/phylogenetic group (Brown et al., 2002; Doyle et al., 2002, 2003, 2004). This species, along with *G. canescens*, was different in nectar composition from the other *Glycine* species. Another similarity between these two species is their geographic distribution pattern, both collected from dry interior areas, in contrast to the other species, which were collected mainly in the east coast of Australia (Figure 5). Brown et al. (1990) reported that *G. canescens* is found in zones of high genetic diversity, which are associated with valleys in the lower slopes of mountain ranges. On the other hand, these two species differed in flower size, *G. falcata* had significantly larger flowers than those of *G. canescens*.

The *G. tomentella* complex was of particular interest given that it is the most widely distributed and diverse of the perennial *Glycine* species. The *G. tomentella* complex has overlapping combinations of genomes (Brown et al., 2002; Doyle et al., 2002). We found
that both *G. tomentella* ploidy levels grouped with most of the species under study for nectar composition. However, diploid *G. tomentella* had significantly smaller flowers than those of the other *Glycine* species and its tetraploid counterpart.

Several approaches are being explored by us to assess nectar output and composition, nectary structure and development, and increased seed set in the wild perennial *Glycine* and wild annual *Glycine soja* species to determine feasibility to introduce them into the cultivated soybean. We report a wide range of variation for nectar composition and flower size across the perennial *Glycine* species. This variation could be used to improve pollinator-insect attraction in the cultivated soybean. Although, embryo rescue techniques are necessary to obtain viable interspecific hybrids (Singh et al., 1990, 1993; Hymowitz, 2004; Palmer and Hymowitz, 2004). In the present study, one interspecific hybrid, *G. tomentella* x *G. max*, was evaluated. Interestingly, this hybrid had very high sugar contents and large flowers. We could speculate that interspecific hybrids exhibit beneficial insect-pollinator attraction traits. However, only two plants from this hybrid were evaluated.

Commercial hybrid soybean production could be feasible if insect-pollinator attraction is increased. Perennial *Glycine* species have a great range of genetic variability for nectar and flower characteristics and eventually, desirable insect-pollinator attraction traits could be introduced into the cultivated soybean. Also, the information gained from the floral nectar composition of the wild perennial species will guide research for nectar composition-outcrossing associations in the cultivated soybean. The overall goal is to produce large quantities of F₁ hybrid seed for plant breeding studies, and for commercial hybrid seed
production. Unfortunately, there is a paucity of data on the native insect-pollinators of the wild perennial *Glycine* species.

**Acknowledgements**

We are most grateful to Dr. Anthony H.D. Brown and to CSIRO Plant Industry, Canberra, Australia for maintenance of the *Glycine* species and for their hospitality during Dr. Reid G. Palmer's study visit.

**REFERENCES**


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Table 1. Species evaluated of the genus *Glycine* Willd., subgenus *Glycine*, and the interspecific hybrid *G. max x G. tomentella*, ploidy level, genome type, accession number, and geographic distribution*.

<table>
<thead>
<tr>
<th>Subgenus <em>Glycine</em></th>
<th>2n</th>
<th>Genome</th>
<th>Accession number**</th>
<th>Geographic distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. albicans</em> Tind. and Craven</td>
<td>40</td>
<td>II</td>
<td>G 2049, G 2937</td>
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<tr>
<td><em>G. aphyonota</em> B.E. Pfeil</td>
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<td>?</td>
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<td>G 3136</td>
<td>Australia</td>
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<td>G 1232, G 1301, G 1302, G 1485, G 3004, G 3014, G 3088, G 3094</td>
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<tr>
<td><em>G. canescens</em> F.J. Herman</td>
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<td>AA</td>
<td>G 1126, G 1171, G 2347, G 2860, G 3114</td>
<td>Australia</td>
</tr>
<tr>
<td><em>G. clandestina</em> Wendl.</td>
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<td>A1A1</td>
<td>G 1396</td>
<td>Australia</td>
</tr>
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<tr>
<td><em>G. falcata</em> Benth.</td>
<td>40</td>
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<td>Australia</td>
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<tr>
<td><em>G. lactovirens</em> Tind. and Craven</td>
<td>40</td>
<td>I1I1</td>
<td>G 1142, G 1298, G 3123</td>
<td>Australia</td>
</tr>
<tr>
<td><em>G. latifolia</em> (Benth.) Newell and Hymowitz</td>
<td>40</td>
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<td>G 2951</td>
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<td><em>G. max x G. tomentella</em></td>
<td>40</td>
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<tr>
<td><em>G. pindanica</em> Tind. and Craven</td>
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<td><em>G. pullenii</em> B.E Pfeil, Tind. and Craven</td>
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<tr>
<td></td>
<td>78 - 80</td>
<td>Complex</td>
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<td>Australia, Papua New Guinea, Indonesia, Philippines, Taiwan</td>
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* Adapted from Hymowitz (2004)
** Accession number, CSIRO Plant Industry, Canberra, Australia.
Table 2. Nectar sugar content and correlations across perennial *Glycine* species and the interspecific hybrid *G. max x G. tomentella*.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>s.d</th>
<th>Correlation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sucrose</td>
</tr>
<tr>
<td>Sucrose (g L⁻¹)</td>
<td>196</td>
<td>76.7</td>
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<tr>
<td>Glucose (g L⁻¹)</td>
<td>44.9</td>
<td>30.3</td>
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<tr>
<td>Fructose (g L⁻¹)</td>
<td>82.3</td>
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<td>1</td>
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<td>Carbohydrates (g L⁻¹)</td>
<td>117.2</td>
<td>48.1</td>
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Table 3. Sugar ratio, sugar percentages, and correlations across perennial *Glycine* species and the interspecific hybrid *G. max x G. tomentella*,

<table>
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<th>s.d.</th>
<th>Correlation</th>
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<td>Sucrose (%)</td>
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<td>12.1</td>
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<td>Glucose (%)</td>
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<td>5.9</td>
<td>1 0.44</td>
</tr>
<tr>
<td>Fructose (%)</td>
<td>25.1</td>
<td>8.2</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4. Flower size across species and correlations across perennial *Glycine* species and the interspecific hybrid *G. max* x *G. tomentella*.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (mm)</th>
<th>s.d.</th>
<th>Correlation</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Flower size</td>
<td>Standard</td>
<td>Wings</td>
<td>Keel</td>
<td></td>
</tr>
<tr>
<td>Flower size</td>
<td>3.9</td>
<td>0.6</td>
<td>1</td>
<td>0.94</td>
<td>0.96</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Standard perimeter</td>
<td>1.5</td>
<td>0.2</td>
<td>1</td>
<td>0.85</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wings perimeter</td>
<td>1.7</td>
<td>0.3</td>
<td>1</td>
<td></td>
<td>0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keel perimeter</td>
<td>0.7</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Nectar sugar content [mean (s.d.]) of perennial *Glycine* species and the interspecific hybrid *G. max x G. tomentella*.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Sucrose (g L⁻¹)</th>
<th>Glucose (g L⁻¹)</th>
<th>Fructose (g L⁻¹)</th>
<th>Carbohydrates (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. max x G. tomentella</em></td>
<td>2</td>
<td>281.3 (65)</td>
<td>76.6 (33.7)</td>
<td>149.2 (67.7)</td>
<td>146.1 (38.6)</td>
</tr>
<tr>
<td><em>G. arenaria</em></td>
<td>2</td>
<td>252.1 (11.9)</td>
<td>53.8 (3.0)</td>
<td>104.4 (6.9)</td>
<td>122.4 (18.2)</td>
</tr>
<tr>
<td><em>G. aphyonota</em></td>
<td>4</td>
<td>242.2 (31.7)</td>
<td>66.3 (19.7)</td>
<td>126.0 (27.1)</td>
<td>95.1 (18.4)</td>
</tr>
<tr>
<td><em>G. curvata</em></td>
<td>4</td>
<td>231.5 (48.7)</td>
<td>27.0 (19.0)</td>
<td>50.1 (18.8)</td>
<td>137.9 (13.9)</td>
</tr>
<tr>
<td><em>G. clandestina</em></td>
<td>15</td>
<td>223.6 (55.4)</td>
<td>41.3 (26.4)</td>
<td>69.8 (33.4)</td>
<td>120.4 (44.3)</td>
</tr>
<tr>
<td><em>G. albicans</em></td>
<td>7</td>
<td>219.8 (75.6)</td>
<td>27.5 (39.4)</td>
<td>22.9 (16.9)</td>
<td>129.4 (29.3)</td>
</tr>
<tr>
<td><em>G. cyrtoloba</em></td>
<td>12</td>
<td>215.2 (91.1)</td>
<td>19.1 (15.1)</td>
<td>37.0 (11.4)</td>
<td>140.4 (74.1)</td>
</tr>
<tr>
<td><em>G. canescens</em></td>
<td>67</td>
<td>204.6 (75.9)</td>
<td>63.7 (32.1)</td>
<td>116.6 (51.4)</td>
<td>119.2 (29.9)</td>
</tr>
<tr>
<td><em>G. latifolia</em></td>
<td>33</td>
<td>196.6 (77.5)</td>
<td>28.0 (20.4)</td>
<td>62.0 (44.7)</td>
<td>120.4 (33.6)</td>
</tr>
<tr>
<td><em>G. lactovirens</em></td>
<td>9</td>
<td>195.3 (62.3)</td>
<td>9.3 (4.3)</td>
<td>22.3 (11.0)</td>
<td>121.2 (35.2)</td>
</tr>
<tr>
<td><em>G. tomentella (diploid)</em></td>
<td>55</td>
<td>193.1 (78.8)</td>
<td>50.5 (25.7)</td>
<td>92.1 (47.1)</td>
<td>118.6 (31.8)</td>
</tr>
<tr>
<td><em>G. argyrea</em></td>
<td>8</td>
<td>192.7 (40.1)</td>
<td>39.3 (15.8)</td>
<td>73.1 (18.8)</td>
<td>102.3 (36.1)</td>
</tr>
<tr>
<td><em>G. pescadrensis</em></td>
<td>4</td>
<td>181.7 (148.6)</td>
<td>40.7 (40.5)</td>
<td>54.5 (36.3)</td>
<td>170.9 (75.6)</td>
</tr>
<tr>
<td><em>G. microphylla</em></td>
<td>12</td>
<td>177.5 (57.9)</td>
<td>31.9 (35.6)</td>
<td>62.6 (41.7)</td>
<td>131.4 (31.3)</td>
</tr>
<tr>
<td><em>G. tomentella (tetraploid)</em></td>
<td>31</td>
<td>170.5 (68.9)</td>
<td>39.3 (25.4)</td>
<td>71.3 (39.3)</td>
<td>121.9 (31.6)</td>
</tr>
<tr>
<td><em>G. tabacina</em></td>
<td>15</td>
<td>169.8 (78.1)</td>
<td>34.3 (28.3)</td>
<td>62.0 (46.2)</td>
<td>122.8 (35.8)</td>
</tr>
<tr>
<td><em>G. pullenii</em></td>
<td>5</td>
<td>163.8 (34.7)</td>
<td>47.9 (9.7)</td>
<td>88.1 (16.2)</td>
<td>106.1 (39.4)</td>
</tr>
<tr>
<td><em>G. falcata</em></td>
<td>17</td>
<td>161.5 (86.1)</td>
<td>48.3 (29.8)</td>
<td>93.8 (47.5)</td>
<td>116.2 (36.5)</td>
</tr>
<tr>
<td><em>G. stenophita</em></td>
<td>1</td>
<td>71.6</td>
<td>4.5</td>
<td>27.7</td>
<td>94.5</td>
</tr>
<tr>
<td><em>G. pindanica</em></td>
<td>2</td>
<td>45.0 (35.3)</td>
<td>29.0 (30.8)</td>
<td>22.6 (6.8)</td>
<td>117.4 (23)</td>
</tr>
</tbody>
</table>
Table 6. Sugar ratios and sugar percentages [mean (s.d.)] of perennial *Glycine* species and the interspecific hybrid *G. max x G. tomentella*.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>S/H*</th>
<th>Sucrose (%)</th>
<th>Glucose (%)</th>
<th>Fructose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. pullenii</em></td>
<td>5</td>
<td>1.2 (0.3)</td>
<td>54.5 (6.1)</td>
<td>16.0 (2.4)</td>
<td>29.5 (3.8)</td>
</tr>
<tr>
<td><em>G. falcata</em></td>
<td>17</td>
<td>1.2 (0.4)</td>
<td>52.8 (6.3)</td>
<td>13.8 (5.6)</td>
<td>33.4 (8.2)</td>
</tr>
<tr>
<td><em>G. pindanica</em></td>
<td>2</td>
<td>1.2 (1.2)</td>
<td>44.7 (31.3)</td>
<td>32.1 (35.6)</td>
<td>23.1 (4.4)</td>
</tr>
<tr>
<td><em>G. canescens</em></td>
<td>67</td>
<td>1.24 (0.5)</td>
<td>53.67 (7.6)</td>
<td>15.79 (3.9)</td>
<td>30.54 (6.0)</td>
</tr>
<tr>
<td><em>G. aphyonota</em></td>
<td>4</td>
<td>1.3 (0.2)</td>
<td>56.1 (2.9)</td>
<td>15.0 (1.9)</td>
<td>28.9 (2.0)</td>
</tr>
<tr>
<td><em>G. max x G. tomentella</em></td>
<td>2</td>
<td>1.3 (0.3)</td>
<td>56.4 (5.7)</td>
<td>14.8 (1.8)</td>
<td>28.8 (3.9)</td>
</tr>
<tr>
<td><em>G. arenaria</em></td>
<td>2</td>
<td>1.6 (0.2)</td>
<td>61.5 (2.6)</td>
<td>13.1 (0.8)</td>
<td>25.4 (1.8)</td>
</tr>
<tr>
<td><em>G. tomentella (diploid)</em></td>
<td>51</td>
<td>1.6 (0.9)</td>
<td>58.8 (8.2)</td>
<td>13.4 (3.9)</td>
<td>27.9 (5.5)</td>
</tr>
<tr>
<td><em>G. argyrea</em></td>
<td>8</td>
<td>1.8 (0.4)</td>
<td>63.6 (5.3)</td>
<td>12.5 (2.9)</td>
<td>23.9 (3.0)</td>
</tr>
<tr>
<td><em>G. tomentella</em> (tetraploid)</td>
<td>29</td>
<td>1.8 (0.6)</td>
<td>62.0 (8.8)</td>
<td>12.3 (5.3)</td>
<td>25.6 (6.9)</td>
</tr>
<tr>
<td><em>G. stenophita</em></td>
<td>1</td>
<td>2.2</td>
<td>68.9</td>
<td>4.4</td>
<td>26.7</td>
</tr>
<tr>
<td><em>G. tabacina</em></td>
<td>13</td>
<td>2.2 (0.7)</td>
<td>67.3 (8.4)</td>
<td>10.2 (4.0)</td>
<td>22.5 (4.7)</td>
</tr>
<tr>
<td><em>G. pescadrensis</em></td>
<td>4</td>
<td>2.3 (1.0)</td>
<td>67.4 (8.3)</td>
<td>11.6 (6.4)</td>
<td>21.1 (4.8)</td>
</tr>
<tr>
<td><em>G. clandestina</em></td>
<td>15</td>
<td>2.4 (1.0)</td>
<td>68.0 (9.2)</td>
<td>11.4 (4.5)</td>
<td>20.6 (6.1)</td>
</tr>
<tr>
<td><em>G. latifolia</em></td>
<td>33</td>
<td>2.8 (1.3)</td>
<td>71.1 (71.1)</td>
<td>8.6 (3.9)</td>
<td>20.3 (5.9)</td>
</tr>
<tr>
<td><em>G. microphylla</em></td>
<td>12</td>
<td>3.2 (2.4)</td>
<td>69.2 (14.7)</td>
<td>9.2 (7.6)</td>
<td>21.6 (9.4)</td>
</tr>
<tr>
<td><em>G. curvata</em></td>
<td>4</td>
<td>3.4 (1.3)</td>
<td>75.5 (9.1)</td>
<td>8.4 (4.9)</td>
<td>16.1 (4.2)</td>
</tr>
<tr>
<td><em>G. cyrtoloba</em></td>
<td>12</td>
<td>4.2 (2.2)</td>
<td>78.1 (8.0)</td>
<td>6.7 (2.5)</td>
<td>15.2 (6.8)</td>
</tr>
<tr>
<td><em>G. lactovirens</em></td>
<td>8</td>
<td>6.7 (2.9)</td>
<td>85.4 (5.3)</td>
<td>4.1 (1.6)</td>
<td>10.6 (4.1)</td>
</tr>
<tr>
<td><em>G. albicans</em></td>
<td>7</td>
<td>8.9 (6.3)</td>
<td>83.6 (13.9)</td>
<td>8.6 (11.4)</td>
<td>7.7 (4.5)</td>
</tr>
</tbody>
</table>

* S/H = [%Sucrose]/[%Glucose + %Fructose]
Table 7. Flower size and petal perimeter [mean (s.d.)] of perennial *Glycine* species and the interspecific hybrid *G. max x G. tomentella*.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Flower size (mm)</th>
<th>Standard perimeter (mm)</th>
<th>Wings perimeter (mm)</th>
<th>Keel perimeter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. max x G. tomentella</em></td>
<td>2</td>
<td>5.0</td>
<td>1.8</td>
<td>2.4</td>
<td>0.8</td>
</tr>
<tr>
<td><em>G. tabacina</em></td>
<td>8</td>
<td>4.7 (0.5)</td>
<td>1.8 (0.2)</td>
<td>2.2 (0.3)</td>
<td>0.7 (0.0)</td>
</tr>
<tr>
<td><em>G. pescadrensis</em></td>
<td>2</td>
<td>4.7</td>
<td>1.8</td>
<td>2.2</td>
<td>0.7</td>
</tr>
<tr>
<td><em>G. stenophita</em></td>
<td>2</td>
<td>4.6</td>
<td>1.9</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td><em>G. falcata</em></td>
<td>16</td>
<td>4.4 (0.4)</td>
<td>1.6 (0.2)</td>
<td>2.0 (0.2)</td>
<td>0.7 (0.1)</td>
</tr>
<tr>
<td><em>G. cyrtoloba</em></td>
<td>8</td>
<td>4.3 (0.5)</td>
<td>1.6 (0.2)</td>
<td>2.0 (0.3)</td>
<td>0.7 (0.0)</td>
</tr>
<tr>
<td><em>G. clandestina</em></td>
<td>8</td>
<td>4.1 (0.6)</td>
<td>1.5 (0.3)</td>
<td>1.9 (0.2)</td>
<td>0.7 (0.1)</td>
</tr>
<tr>
<td><em>G. microphylla</em></td>
<td>10</td>
<td>4.1 (0.2)</td>
<td>1.6 (0.0)</td>
<td>1.8 (0.2)</td>
<td>0.7 (0.1)</td>
</tr>
<tr>
<td><em>G. tomentella</em> (tetraploid)*</td>
<td>24</td>
<td>4.0 (0.5)</td>
<td>1.5 (0.2)</td>
<td>1.8 (0.2)</td>
<td>0.7 (0.1)</td>
</tr>
<tr>
<td><em>G. argyrea</em></td>
<td>4</td>
<td>4.0 (0.4)</td>
<td>1.7 (0.2)</td>
<td>1.6 (0.2)</td>
<td>0.7 (0.0)</td>
</tr>
<tr>
<td><em>G. latifolia</em></td>
<td>24</td>
<td>4.0 (0.3)</td>
<td>1.5 (0.2)</td>
<td>1.8 (0.2)</td>
<td>0.7 (0.1)</td>
</tr>
<tr>
<td><em>G. albicans</em></td>
<td>4</td>
<td>3.8 (0.5)</td>
<td>1.3 (0.1)</td>
<td>1.6 (0.3)</td>
<td>0.8 (0.1)</td>
</tr>
<tr>
<td><em>G. canescens</em></td>
<td>16</td>
<td>3.8 (0.2)</td>
<td>1.5 (0.1)</td>
<td>1.7 (0.2)</td>
<td>0.6 (0.0)</td>
</tr>
<tr>
<td><em>G. lactovirens</em></td>
<td>6</td>
<td>3.7 (0.6)</td>
<td>1.3 (0.3)</td>
<td>1.6 (0.2)</td>
<td>0.8 (0.1)</td>
</tr>
<tr>
<td><em>G. curvata</em></td>
<td>2</td>
<td>3.6</td>
<td>1.4</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td><em>G. pullenii</em></td>
<td>4</td>
<td>3.5 (0.1)</td>
<td>1.4 (0.0)</td>
<td>1.6 (0.1)</td>
<td>0.6 (0.1)</td>
</tr>
<tr>
<td><em>G. tomentella</em> (diploid)*</td>
<td>38</td>
<td>3.2 (3.2)</td>
<td>1.2 (1.2)</td>
<td>1.4 (1.4)</td>
<td>0.6 (0.6)</td>
</tr>
<tr>
<td><em>G. pindanica</em></td>
<td>2</td>
<td>3.1</td>
<td>1.2</td>
<td>1.4</td>
<td>0.5</td>
</tr>
<tr>
<td><em>G. arenaria</em></td>
<td>2</td>
<td>3.1</td>
<td>1.3</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td><em>G. aphyonota</em></td>
<td>2</td>
<td>2.7</td>
<td>1.0</td>
<td>1.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Table 8. Multivariate analysis of variance (MANOVA) for nectar sugar content, using species with a sample size larger than ten. Wilk's lambda test and p-value (in parentheses) are reported. Wilk's lambda test and p-value of a pair of species that are non-significantly different in nectar sugar content are reported in bold.

<table>
<thead>
<tr>
<th>Glycine</th>
<th>tomentella (diploid)</th>
<th>tomentella (tetraploid)</th>
<th>falcata</th>
<th>latifolia</th>
<th>clandestina</th>
<th>tabacina</th>
<th>microphylla</th>
<th>cyrtoloba</th>
<th>lactovirens</th>
</tr>
</thead>
<tbody>
<tr>
<td>canescens</td>
<td>0.85 (0.00)</td>
<td>0.82 (0.00)</td>
<td>0.95 (0.40)</td>
<td>0.55 (0.00)</td>
<td>0.65 (0.00)</td>
<td>0.78 (0.00)</td>
<td>0.74 (0.00)</td>
<td>0.53 (0.00)</td>
<td>0.52 (0.00)</td>
</tr>
<tr>
<td>tomentella (diploid)</td>
<td>0.92 (0.17)</td>
<td>0.83 (0.09)</td>
<td>0.70 (0.00)</td>
<td>0.75 (0.00)</td>
<td>0.91 (0.23)</td>
<td>0.77 (0.00)</td>
<td>0.54 (0.00)</td>
<td>0.56 (0.00)</td>
<td></td>
</tr>
<tr>
<td>tomentella (tetraploid)</td>
<td>0.81 (0.07)</td>
<td>0.81 (0.01)</td>
<td>0.78 (0.04)</td>
<td>0.94 (0.70)</td>
<td>0.81 (0.09)</td>
<td>0.55 (0.00)</td>
<td>0.55 (0.00)</td>
<td>0.52 (0.00)</td>
<td></td>
</tr>
<tr>
<td>falcata</td>
<td>0.44 (0.00)</td>
<td>0.52 (0.00)</td>
<td>0.55 (0.00)</td>
<td>0.67 (0.04)</td>
<td>0.27 (0.00)</td>
<td>0.24 (0.00)</td>
<td>0.24 (0.00)</td>
<td>0.24 (0.00)</td>
<td></td>
</tr>
<tr>
<td>latifolia</td>
<td>0.89 (0.27)</td>
<td>0.94 (0.63)</td>
<td>0.91 (0.41)</td>
<td>0.75 (0.02)</td>
<td>0.66 (0.00)</td>
<td>0.66 (0.00)</td>
<td>0.66 (0.00)</td>
<td>0.66 (0.00)</td>
<td></td>
</tr>
<tr>
<td>clandestina</td>
<td>0.86 (0.46)</td>
<td>0.84 (0.40)</td>
<td>0.62 (0.03)</td>
<td>0.58 (0.04)</td>
<td>0.58 (0.04)</td>
<td>0.58 (0.04)</td>
<td>0.58 (0.04)</td>
<td>0.58 (0.04)</td>
<td></td>
</tr>
<tr>
<td>tabacina</td>
<td>0.85 (0.51)</td>
<td>0.63 (0.05)</td>
<td>0.44 (0.01)</td>
<td>0.44 (0.01)</td>
<td>0.44 (0.01)</td>
<td>0.44 (0.01)</td>
<td>0.44 (0.01)</td>
<td>0.44 (0.01)</td>
<td></td>
</tr>
<tr>
<td>microphylla</td>
<td>0.73 (0.18)</td>
<td>0.62 (0.10)</td>
<td>0.71 (0.25)</td>
<td>0.71 (0.25)</td>
<td>0.71 (0.25)</td>
<td>0.71 (0.25)</td>
<td>0.71 (0.25)</td>
<td>0.71 (0.25)</td>
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</table>
Table 9. Multivariate analysis of variance (MANOVA) for nectar sugar ratio and percentages, using species with a sample size larger than ten. Wilk's lambda test and p-value (in parentheses) are reported. Wilk's lambda test and p-value of a pair of species with non-significant differences in nectar sugar proportions are reported in bold.

<table>
<thead>
<tr>
<th>Glycine</th>
<th>tomentella (diploid)</th>
<th>tomentella (tetraploid)</th>
<th>falcata</th>
<th>latifolia</th>
<th>clandestina</th>
<th>tabacina</th>
<th>microphylla</th>
<th>cyrtoloba</th>
<th>lactovirens</th>
</tr>
</thead>
<tbody>
<tr>
<td>canescens</td>
<td>0.88</td>
<td>0.79</td>
<td>0.94</td>
<td>0.46</td>
<td>0.69</td>
<td>0.68</td>
<td>0.39</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.15)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
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<tr>
<td>tomentella</td>
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<td>0.80</td>
<td>0.82</td>
<td>0.53</td>
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<td>(0.00)</td>
<td>(0.00)</td>
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<tr>
<td>tomentella</td>
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<td>0.77</td>
<td>0.82</td>
<td>0.88</td>
<td>0.81</td>
<td>0.53</td>
<td>0.30</td>
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</tr>
<tr>
<td>(tetraploid)</td>
<td>(0.01)</td>
<td>(0.00)</td>
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<td>(0.16)</td>
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<tr>
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<td>0.12</td>
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</tr>
<tr>
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<td>(0.00)</td>
<td>(0.00)</td>
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<td>(0.00)</td>
<td>(0.00)</td>
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<td>0.84</td>
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</tr>
<tr>
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<td>0.70</td>
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Table 10. Pair wise comparisons of flower size, using species with sample size three or larger. Student t-test and p-values (in parentheses) are reported. t-test and p-value of a pair of species with non-significant differences in flower size are reported in bold.

<table>
<thead>
<tr>
<th>Glycine</th>
<th>tomentella (diploid)</th>
<th>tomentella (tetraploid)</th>
<th>falcata</th>
<th>latifolia</th>
<th>clandestina</th>
<th>tabacina</th>
<th>microphylla</th>
<th>cyrtoloba</th>
<th>lactovirens</th>
</tr>
</thead>
<tbody>
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<td>canescens</td>
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<td>-2.11</td>
<td>-1.23</td>
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<td>-2.23</td>
<td>-1.67</td>
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<td>(0.10)</td>
<td>(0.31)</td>
<td>(0.00)</td>
<td>(0.14)</td>
<td>(0.20)</td>
<td>(0.90)</td>
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<td>tomentella</td>
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<td>-4.89</td>
<td>-5.51</td>
<td>-3.56</td>
<td>-1.31</td>
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<td></td>
</tr>
<tr>
<td>(diploid)</td>
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<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
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<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.32)</td>
</tr>
<tr>
<td>tomentella</td>
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<td>-0.51</td>
<td>-0.56</td>
<td>-0.74</td>
<td>-1.01</td>
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<tr>
<td>(tetraploid)</td>
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<td>(0.61)</td>
<td>(0.62)</td>
<td>(0.11)</td>
<td>(0.46)</td>
<td>(0.29)</td>
<td>(0.56)</td>
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<tr>
<td>falcata</td>
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<td>0.89</td>
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<td>(0.45)</td>
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<td>(0.45)</td>
<td>(0.52)</td>
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<td>(0.44)</td>
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<td>tabacina</td>
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<td>1.02</td>
<td>2.01</td>
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<td>microphylla</td>
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<td>(0.52)</td>
<td>(0.45)</td>
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</tr>
<tr>
<td>cyrtoloba</td>
<td>1.20</td>
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<td></td>
<td></td>
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</tr>
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<td>(0.33)</td>
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</tr>
</tbody>
</table>
**Figure 1.** Typical soybean flower. A) Standard petal. B) Two wing petals. C) Fused keel petals.
Figure 2. Distribution of nectar sugar content of perennial *Glycine* species* with a sample size larger than ten. Mean value represented by X symbol.

*G. tomentella_D* represents diploid species of *Glycine tomentella* and *G. tomentella_T* represents tetraploid species of *Glycine tomentella*.
Figure 3. Distribution of nectar sugar ratio and sugar percentages of perennial *Glycine* species* with a sample size larger than ten. Mean value represented by X symbol.

*G. tomentella_D* represents diploid species of *Glycine tomentella* and *G. tomentella_T* represents tetraploid species of *Glycine tomentella*
Figure 4. Distribution of petal perimeter and flower size of perennial *Glycine* species* with a sample size larger than ten. Mean value represented by X symbol.

*G. tomentella_D* represents diploid species of *Glycine tomentella* and *G. tomentella_T* represents tetraploid species of *Glycine tomentella*.
Figure 5. Geographic distribution of *G. clandestina*, *G. cyrtoloba*, *G. latifolia*, *G. microphylla*, *G. tomentella*, *G. tabacina*, *G. canescence*, *G. falcata*, and *G. lactovirens*. 
CHAPTER 5: GENERAL CONCLUSIONS

The purpose of this research project was to investigate different strategies for soybean improvement, from genetic resistance to biotic stresses, to surveying perennial wild relatives for insect-pollinator attraction characteristics that could be useful in hybrid seed production in the cultivated species.

The first study evaluated four plant introductions from south-central China to identify new sources of resistance to BSR. Four F₂ segregating populations were developed and BSR resistance/susceptibility was evaluated under growth chamber conditions. Using single marker regression with SSR markers linked to previously identified BSR resistance genes, high association was found between the SSR markers used and BSR resistance in populations PI 594638B X Century 84, PI 594650A X Century 84, and PI 594658B X Century 84. It was concluded that the BSR resistance genes present in these PIs could be allelic to previously identified BSR resistance genes on chromosome 16. Population PI 594637 X Century 84 showed non-association with SSR Satt547. This could be indicative of the presence of a new BSR resistance gene in PI 594637. In the same population, highly resistant F₂:₃ individuals were present. This highly resistant progeny can be used for development of BSR resistant cultivars. In order to test the hypothesis of a different BSR resistance gene present in PI 594637, allelism tests between PI 594637 and genotypes possessing Rsb1, Rbs2, and Rbs3 genes need to be performed.

The objective of the second study was to investigate the genetic factors associated with whitefly resistance in soybean. Two F₂ segregating populations for whitefly resistance
were developed for QTL analysis. It was found that whitefly resistance was a polygenic trait, controlled by QTL on different chromosomes. Eight putative whitefly-resistance QTL were detected on different soybean chromosomes across the two populations. QTL detection varied between populations and years, although whitefly-resistance QTL on chromosomes 12 and 18 were detected in more than one year and in both populations. Most of the whitefly-resistance QTL detected in the study were located on chromosomes that are known to contain several disease and insect resistance genes. The presence of major-effect QTL and several small-effect QTL indicated complex inheritance of whitefly resistance in soybean. Future research should focus on fine-mapping of the large-effect whitefly-resistance QTL detected in this study. This will help to identify tightly linked markers to whitefly-resistance QTL, which can be used for marker-assisted selection.

The third study surveyed the floral characteristics of wild perennial *Glycine* species. The nectar sugar composition and flower size varied among the 19 species evaluated. The study found that perennial *Glycine* species have predominantly sucrose-rich nectars. These nectars are usually attractive to pollinators such as hummingbirds, butterflies, moths, and long-tongued bees. Studies of pollinator syndrome of wild perennial Glycine species are needed. In this study, multivariate analysis of nectar sugar characteristics identified one group consisting of *G. falcata* and *G. canescens*, which had different nectar sugar characteristics in comparison with the other perennial species evaluated. These two species have similar geographic distribution, both collected from dry interior areas. The information gained from the floral nectar composition and flower morphology of the wild perennial species will guide research for nectar composition-outcrossing associations in the cultivated
soybean. Future research should focus on the native insect-pollinators of the wild perennial *Glycine* species.