

7-2016

# Soybean Tolerance to Soybean Aphid (*Aphis glycines* Matsumura) Herbivory

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SOYBEAN TOLERANCE TO SOYBEAN APHID (*APHIS GLYCINES*  
MATSUMURA) HERBIVORY

by

Lia Marchi Werle

A DISSERTATION

Presented to the Faculty of  
The Graduate College at the University of Nebraska  
In Partial Fulfillment of Requirements  
For the Degree of Doctor of Philosophy

Major: Entomology

Under the Supervision of Professors Thomas E. Hunt and Tiffany M. Heng-Moss

Lincoln, Nebraska

July, 2016

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University of Nebraska, 2016

Advisors: Thomas E. Hunt and Tiffany M. Heng-Moss

This research examined the soybean aphid tolerant soybean KS4202 and its possible role in soybean aphid management. Studies documented the stage at which the tolerance response was initially expressed, quantified the relationship between cumulative aphid-days (CADs) and yield loss, and determined economic injury levels (EILs) for the soybean aphid tolerant KS4202 soybean. At high aphid infestation levels, aphids negatively impacted yield for KS4202 and K03-4686 (susceptible) plants that were infested at V1 stage; however, KS4202 expressed tolerance to the soybean aphid at low aphid infestation levels. No significant differences in the yield parameters were detected for KS4202 when infested at any aphid infestation level; whereas K03-4686 was negatively impacted when CAD surpassed 10,000. The EILs calculated for KS4202 infested during reproductive stage soybean ranged from 826 to 3,415 aphids/plant, which were approximately 2.5-fold higher when compared to the EILs calculated for K03-4686.

Studies were also conducted to document the expression of constitutive and induced defense-related genes in tolerant and susceptible soybeans in response to soybean aphid feeding. Differences in the expression of several JA-associated transcripts were observed between genotypes, suggesting that the constitutive expression of JA-

associated transcripts may be important for KS4202 tolerance to soybean aphids, but not for K03-4686. The greater magnitude of PRX52, WRKY60 and PR1 induction in KS4202 relative to the susceptible genotype suggests that these transcripts may be contributing to the ability of KS4202 to tolerate high levels of oxidative stress.

The use of peroxidase activity and relative expression of peroxidase transcripts as potential assays to phenotype aphid-tolerant recombinant inbred lines (RILs) were also investigated. Peroxidase basal levels were similar among the genotypes for plants at V1 and V3 stages. Overall, there was an indication that KS4202 had greater abundance of peroxidase (PRX52) than the high-yielding and susceptible genotypes in response to aphid feeding. Despite the evidence of PRX52 involvement in KS4202 tolerance, no direct relationship between PRX2 and aphid feeding was detected.

The determination of EILs and identification of important mechanisms involved in plant tolerance is key for the development of successful breeding strategies and incorporation of this resistance into the IPM for soybean aphid.

*I dedicate this dissertation to my daughter Leila Annie, and my husband Rodrigo.*

*Family is forever, and it's everything.*

## Acknowledgments

The years of graduate school is one of the most memorable events of my life, and for that, I am very thankful to my advisors Drs. Tom Hunt and Tiffany Heng-Moss. You'll always be my role models and I cannot thank you enough for the time, patience, caring and friendship through both my Masters and PhD degrees. I hope that a day will come and I'll be able to reciprocate at least a little bit of what the two of you have provided me. I also thank Drs. George Graef and Blair Siegfried for serving in my committee and for proving their valuable knowledge to my research. I appreciated the opportunity of getting involved in some of the research activities of Dr. Graef's soybean-breeding group.

I have a lot of appreciation for the guidance, support and friendship from Dr. Teresa Donze-Reiner and Dr. Edson Baldin. Dr. Donze-Reiner patiently taught me many laboratory techniques and advised me on how to overcome challenges and become more confident in myself. Dr. Baldin, my former advisor at the time of college in Brazil, is a person whose character and knowledge has inspired me in many ways. I also want to recognize the hard work and friendship of current and past members our lab, including Crystal Ramm, Jenny Enchayan, Hillary Fischer, Kyle Koch, Katie Keller, Kait Chapman, Mitch Stamm and Travis Prochaska. You made graduate school much more pleasant. A special thank you to Hillary, who was present throughout these years and never measured efforts to help. You showed me how kindness and happiness can make the difference. I also thank the all Entomology graduate students, in special Camila Oliveira Hoffman, Debora Montenzano and Renata Pereira, for their friendship and

support. I'm also grateful to Renata Pereira, Luis Eduardo Pannuti, Jose Paulo da Silva and Jacqueline Barbosa for their assistance and contribution with multiple research projects. The completion of several research projects also relied on the assistance of Logan Dana, Lynn Junck and Bob Roselle. Thank you for your time and efforts in my research.

Finally, I want to express my deepest and sincere gratitude to my family. My parents, Otavio and Ana Maria, mean the world to me and words cannot explain it. You thought me that life is not easy, but it's fair for the honest and hard working people. Thank you being amazing parents and for every opportunity you provided me, none of this would have happened without your support. Noa and Betina, my sister and niece, thank you for making me a better and happier person. It's even harder to express how thankful I am to my beautiful daughter, Leila and husband, Rodrigo Werle. Your support, guidance, and love took me here. I love you both, very much.

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## CHAPTER I

### Literature Review

#### Soybean (*Glycine max* (L.) Merrill)

Soybean, *Glycine max* (L.) Merrill, is one of the most important domesticated crops for animal feed and human consumption. A member of the Fabaceae/Leguminosae and subfamily Papilionoideae, soybean is a self-pollinated and diploid plant ( $2n = 40$ ). Soybean seeds are well known for their rich amounts of oil, which contains unsaturated fatty acids, such as oleic, linoleic, and linolenic acids. Additionally, these seed have an average of 40% protein, including essential amino acids to provide quality nutrition for livestock and humans (Bilyeu et al. 2010).

The origin of cultivated soybean is linked to a domestication event in wild soybean (*Glycine soja* Seib et Zucc.) that occurred in ancient China approximately 5000 years ago (Hymowitz 2008). Most reports suggested that farmers domesticated soybean in northeast China, and that soybean was then disseminated to Indonesia, Japan, Malaysia, Myanmar, Nepal, India, Philippines, Thailand and Vietnam (Hymowitz 2008). Soybean was introduced to North America in the 1700s, where it was initially grown as a forage crop. Within the last 60 years, soybean has gained more attention and become the most prominent oilseed in world trade (Hymowitz 2008). The global soybean production for 2016/17 has been projected at 324 million tons (USDA/WASDE 2016), which corresponds to approximately 56% of the seed oil produced globally (USDA/WASDE 2016).

## **Distribution and Ecology of Soybean Aphids and Injury to Soybean**

The soybean aphid, *Aphids glycines* Matsumura (Hemiptera: Aphididae), is a hemipteran soybean pest native to eastern Asian. The soybean aphid's summer host is soybean (*Glycine max* L.). Although it does not always achieves pest status in its native habitat, soybean aphids are widely distributed in China, Japan, Korea, Indonesia, Malaysia, Taiwan, Thailand and the Philippines (Van den Berg et al. 1997).

In North America, soybean aphids were first observed in soybean fields in Illinois in 2000 (Hartman et al. 2001), although it is generally agreed that soybean aphids were present for some years prior to its first detection. In 2004, soybean aphids were distributed through at least 21 U.S. states and three Canadian provinces (Venette and Ragsdale 2004), expanding to 30 U.S. states in 2011 (Ragsdale et al. 2011).

The soybean aphid is a heteroecious and holocyclic species, with sexual reproduction on buckthorn (*Rhamnus* spp) and asexual reproduction on soybean (Ragsdale et al. 2004). The common buckthorn (*Rhamnus cathartica* L.) is considered the principal host of soybean aphids in North America (Ragsdale et al. 2004), although successful sexually reproduction and overwintering may also occur on *Rhamnus lanceolata* Pursh and *Rhamnus anifolia* L'Heritier (Voegtlin et al. 2005a). In the fall, oviparous females will deposit the eggs near buckthorn's buds, where overwintering will take place. Eggs are the most cold-hardy stage of soybean aphids, withstanding temperatures of up to -34°C (McCornack et al. 2005). In the spring, eggs hatch and aphids live on the buckthorn for three or four generations. Alate females produced on buckthorn then migrate to soybeans (Ragsdale et al. 2004). Soybean aphids arrive in soybean fields late spring to early summer. A temporal disjunction may occurs between

buckthorn and soybean since spring migrants can be found on buckthorn 2-3 weeks before colonies are detected on soybean, suggesting the existence of a transitional host (Ragsdale et al. 2004). In China, Sun et al. (2015) identified *Metaplexis japonica* (Thunb.) as a potential transitional host, however, research is still necessary to determine if this occurs in North America.

During the growing season, females feed and reproduce on soybeans in the absence of males (parthenogenic viviparae) (Ragsdale et al. 2004). In the early stages of colonization, aphids are mostly found on the underside of newly emerged trifoliolate leaves. The colonization pattern shifts as soybeans age and populations build, when insects are observed in the lower canopy and on petioles, stems and pods (Ragsdale et al. 2004). Under optimal environmental conditions and absence of natural enemies, soybean aphid populations can double in as little as 1.5-2 days (McCornack et al. 2004). Although under natural conditions (biological agents and abiotic factors), aphid density doubling time is increased to 6-7 days (Ragsdale et al. 2007).

Alate migrants are induced at the end of the season, permitting soybean aphid's returns to its primary host. The formation of migrants is triggered by a combination of signals resulting from soybean senescence, colder temperatures and shortening of photoperiod (Hodgson et al. 2005). It has also been suggested that decreases in nitrogen rate in the soybean's phloem at maturity may be related with migrant induction (Beckendorf et al. 2008). Sexual winged males and winged asexual females emigrate to buckthorn plants, where gynoparae females will give rise to wingless sexual females (oviparae). Males and oviparae mate, and overwintering eggs are deposited on buckthorn (Ragsdale et al. 2004).

Soybean aphids passively ingest soybean phloem using piercing-sucking mouthparts. Feeding can cause physical injury, such as soybean stunting and leaf chlorosis, which ultimately results in yield reduction. In Asia, infestations during soybean's early vegetative stages reduced yield by 50% (Wang et al. 1994). In North America, high levels of aphid infestation in 2001 were associated with a 20-25% yield reduction (Myers et al. 2005), although losses of 50% or more were also reported (Ragsdale et al. 2007, Beckendorf et al. 2008).

As aphid infestation increases, stronger negative correlations in the number of pods, seeds per pod and seed weight are observed (Beckendorf et al. 2008). Seed oil content also decreases as aphid numbers increased, whereas protein amount has a positive correlation with higher aphid densities (Beckendorf et al. 2008). Soybean aphids are also associated with indirect damage from virus transmission and sooty mold growth due to intense honeydew deposition (Clark and Perry 2002). Even at low densities, these insects have a significant impact on photosynthesis, substantially reducing gas exchange rates (Macedo et al. 2003).

### **Integrated Pest Management of Soybean Aphids**

The integrated pest management (IPM) concept was first presented by Stern et al. (1959), which at the time was referred as *integration of chemical and biological control*. The term was later renamed to IPM (Kogan 1998). Considered as “one the most robust constructs to arise in the agricultural science” (Kogan 1998), IPM seeks to suppress pest abundance while minimizing reliance on costly or environmentally hazardous

insecticides through a diversity approaches (e.g. biological control, host plant resistance (HPR), chemical and cultural control).

Stern and co-workers most significant contributions to IPM were the economic threshold (ET), and the economic injury level (EIL). Defined by Stern et al. (1959) as the “*lowest pest population that will cause economic damage*”, the EIL is expressed as a numerical value (e.g. number of herbivores/plant) where the economic yield loss caused by pest injury and management costs are equal. A supplemental tool for the EIL is the ET. The ET is a parameter with practical application in agricultural systems. Also expressed as a numerical value, ET is typically set to be lower than the EIL. In a practical use, the ET is an “alert” for growers, and represents the moment when control actions must be performed to avoid that pest populations from reaching the EIL (Stern et al. 1959). When available, the ET should be based on the pest population growth patterns and may also consider parameters such as pest mortality, survival rates and growth stage (Ostlie and Pedigo 1987).

The EIL is based on the pest management costs, commodity values, pest activity, plant response to pest injury, and efficiency of control strategies (Pedigo and Higley 1992), and is calculated based on the equation by Pedigo et al. (1986):  $EIL = C / (V \times I \times D \times K)$ , where C is the control cost (\$/ha), V is the crop value (\$/ha), I x D is yield loss per insect (in kg of soybean seed/insect), and K is the proportionate reduction in potential injury. EILs may change substantially with location, season and commodity prices. Determining and quantifying pest injury (I x D) is the most complex aspect of the EIL, which requires considerable data collection (Pedigo et al. 1986). The relationship of yield

loss x pest density typically follows a linear trend, or at least during the portion of the damage curve of most interest with respect to management.

IPM is the most cost effective management strategy for soybean aphid (Johnson et al. 2009). Major contributions to soybean aphid IPM was a multi-state study determining soybean aphid EILs and ETs (Ragsdale et al. 2007), and the development of a binomial sampling method that permits rapid and reliable assessment of soybean aphid numbers (Hodgson et al. 2007). The EILs for soybean aphids were developed using enumerative aphid counts (whole plant) using a common experimental strategy in six north-central states over three years (Ragsdale et al. 2007). Based on the average population doubling time (6.8 days), the average EIL for commercial soybean varieties was calculated as 674 aphids/plant. The average ET for soybean aphids was determined as 274 aphids per plant with 80% of the plants being aphid-infested. This ET is valid for soybeans in late vegetative through R5 (full size pods with developing seeds) (Ragsdale et al. 2007), and allows a lead-time of 5 -7 days to apply curative treatments. Different than other insect-pests, the soybean aphid ET is a comprehensive value calculated upon the population doubling time (Ragsdale et al. 2007). This means that the ET is held constant, even when commodity price is high. In the case a lower ETs, control actions would occur causing adverse effects on natural enemies which can keep soybean aphid populations in check at lower population densities, which provides significant advantage to growers (McCornack and Ragsdale 2006). The ET established for soybean aphids is highly adopted in North America, and is set high enough to permit maximum response by natural enemies.

## **Chemical Management Strategies for Soybean Aphid**

In the United States, soybean aphid management has relied heavily on foliar-applied insecticides. Before the arrival of soybean aphids in North America, less than 1% of the soybean fields were treated with insecticides. This scenario had drastically changed by 2006, when a sharp increase (estimated 130-fold) in insecticide applications occurred due to soybean aphid (Ragsdale et al. 2011). As a result, production costs were increased by \$16-33/ha (Ragsdale et al. 2007).

The current recommendation is to scout fields regularly and only spray foliar insecticides when the ET has been reached, and the aphid population is increasing (Ragsdale et al. 2007). The most common foliar insecticides used for soybean aphid management are the organophosphates chlorpyrifos and dimethoate, and pyrethroids (Chandrasena et al. 2011). A single application of chlorpyrifos during the early reproductive stages of soybean (R2 and R3) can significantly reduce yield loss (Myers et al. 2005).

Seed-treatment with neonicotinoids (imidacloprid and thiamethoxam) is another method used to manage soybean aphid. Although these insecticides can in part reduce yield loss caused by soybean aphids (Magalhaes et al. 2009), this practice as prophylactic and most University entomologists do not recommend this practice because it is inconsistent in regards to its return of investment (Hodgson and VanNostrand 2012, McCarville et al. 2014). The temporal mismatch between the typical timing of soybean aphid infestation and short residual activity (35-42 days) has raised questions in regards to its effectiveness in many soybean growing regions (McCornack and Ragsdale 2006).



## Biological Control of Soybean aphid

As part of integrated pest management (IPM), biological control is another management component that can reduce soybean aphid populations. In the absence of predators, soybean aphids numbers increased up to 7.7 fold, however the presence of predators restricted growth to a maximum of 2.9 fold (Desneux et al. 2006). The arthropod fauna that preys on soybean aphids is primarily composed of generalist predators, including lady beetles (Coleoptera: Coccinellidae, species *Harmonia axyridis* and *Coccinella septempunctata*), minute pirate bugs (Hemiptera: Anthocoridae, species *Orius insidiosus*), lacewings (Neuroptera: Chrysopidae) and parasitic wasps (Hymenoptera: Braconidae and Aphelinidae) (Rutledge et al. 2004). Studies conducted in the Midwest have identified *H. axyridis* and *O. indiosus* Say (Hemiptera: Anthocoridae) as the key predators of soybean aphids (e.g. Brosius et al. 2007). Together, these species may account for over 85% of all soybean aphid predators in some fields (Fox et al. 2004, Costamagna and Landis 2006).

Predators that occur early in the season and at high densities, such as *O. insidiosus*, are more efficient to prevent aphid outbreaks than those that appear later in the season, such as *H. axyridis* (Rutledge et al. 2004). *O. insidiosus* can successfully thrive on alternative prey (e.g. thrips) and establish vigorous populations before the arrival of soybean aphids (Brosius et al. 2007, Yoo and O'Neil 2009). However, its effects become limited once aphid densities reach high levels (Rutledge et al. 2005).

Other organisms, such as pathogens and parasitoids, may also assist with soybean aphid IPM. At least 7 species of aphid pathogenic fungi have been reported, of which *Pandora neoaphidis* is the most prevalent in the eastern and Midwestern U.S. (Nielsen

and Hajek 2005, Koch et al. 2010). Six hymenopteran species are known to parasitize soybean aphids, of which *Lysiphlebus testaceipes* is the most prevalent (Noma and Brewer 2008). Despite the occurrence of multiple species, parasitoids are considered a small portion of soybean aphid's predatory community in North America compared to Asia.

### **Host Plant Resistance (HPR)**

Host plant resistance (HPR) provides effective, economical and environmentally safe pest control. By definition, plant resistance to arthropods is the “*sum of the constitutive, genetically inherited qualities that result in a plant of one cultivar or species being less damaged than a susceptible plant lacking these qualities*” (Smith 2005). The degree of plant resistance is a relative characteristic measured in the presence of a susceptible plant, which is considerably damaged or killed under the same experimental conditions. In addition, plant resistance should be measured in the presence of resistant control plant, whose levels of resistance have been predetermined (Smith 2005).

HPR is arranged into three categories of resistance, as originally described by Painter (1951): antibiosis, antixenosis and tolerance. Antibiosis is characterized by certain host properties that adversely modify arthropod biology or life history (Smith 2005). Antibiosis may result from the biochemical synthesis, such as alkaloids, glucosinolates and other secondary metabolites, or even by the presence of trichomes (Panda and Khush 1995, Smith 2005). The effect of antibiosis on arthropods may range from mild to lethal. When individuals survive a plant's antibiotic responses, they frequently express a decline in size and weight that ultimately results in longer larval

stages and life cycles (Smith 2005). Antixenosis (non-preference) is a category of HPR that negatively affects insect behavior (Painter 1951, Smith 2005). The presence of physical barriers, including wax deposition on leaves, stems or fruits, thickened plant epidermal layers, or the presence of certain trichomes may induce arthropods to choose another host plant for feeding, ovipositing or mating (Smith 2005). Even when an individual comes in contact with antixenotic host, certain plant chemicals may prevent further colonization (Panda and Khush 1995). In addition, the presence of repellents and/or the absence of attractants substantially affect arthropod oviposition and feeding.

Tolerance to arthropod herbivory is the ability of a plant to withstand injury without the substantial damage or yield loss when compared to a given susceptible plant (Smith 2005). Different than antibiosis and antixenosis, plant tolerance is conferred by a collection plant characteristics and may not impose the same constraints in the arthropod biology or behavior. Although, in theory, it is possible that tolerance could affect herbivore performance (Stinchcombe 2002), researchers generally believe that arthropods on tolerant plants experience lower selection pressure than those on antibiotic or antixenotic plants. Hence, the likelihood of the emergence of virulent population (biotype) in response of a tolerant plant is minimized (Stinchcombe 2002, Smith 2005).

Plants possessing tolerance are particularly interesting for an IPM program. Plant tolerance is thought to exert no pressure on most natural enemies (Espinosa and Fornoni 2006), which enhances arthropod-pest management. Moreover, due to their ability to compensate for arthropod feeding, tolerant plants have a higher EIL than a susceptible plant. Collectively, enhanced biological control and greater EILs result in a reduction of insecticide applications (Panda and Khush 1995).

HPR has long served as an important management strategy, and an important component of IPM. Significant progress has been made in identifying soybeans with resistance to soybean aphid. Screenings of soybean selections has led to the discovery of several aphid resistant sources. Resistance was first identified in Dowling, Jackson and PI 71506 (Hill et al. 2004). Antibiosis was the major contributor for resistance in Dowling and Jackson (Hill et al. 2004), whereas resistance in PI 71506 was attributed to antixenosis (Hill et al. 2004, Mian et al. 2008). Other studies have identified antibiosis in PI 567541B, PI 567598B and PI 243540 (Mensah et al. 2005, Zhang et al. 2009). Antixenosis resistance to soybean aphids was later reported in Dowling, in PI 230977 (Hesler et al. 2007), and PI 567453C and PI 567597C (Mensah et al. 2005, Zhang et al. 2010).

Genetic approaches identified that resistance in Dowling and Jackson was due to a single dominant gene, both mapped to the same genomic region of chromosome 7 (Hill et al. 2006b, a). The resistance gene in Dowling was named *Rag1* (Resistance to *Aphis glycines*) whereas Jackson's was not named due to uncertainties in its genetic relationship with *Rag1* (Li et al. 2007). The *Rag2* gene was identified in PI 243540, however it mapped to chromosome 13 (Mian et al. 2008). Zhang et al. (2010) identified a resistance locus of PI 56743C on chromosome 16, which was named *Rag3*. In addition, *rag4* (under provision name) was found in PI 567541B (Zhang et al. 2009), and a *Rag5*, which was located near *Rag2*, has been proposed for PI 567301B (Jun et al. 2012). Oligogenic resistance to soybean aphid was reported in PI 567324 by Jun et al. (2013), who found two major quantitative trait loci (QTL) on chromosome 13 and a minor QTL on chromosome 6.

Soon after the identification of aphid resistance genes, soybean aphid virulent populations were noticed colonizing *Rag* soybeans. Referred as biotype 2 or Ohio biotype, this population can overcome the resistance imposed by *Rag1* (Kim et al. 2008). However, this gene still negatively affected Illinois' soybean aphids that infest *Rag1* soybean. For that reason, Kim et al. 2008 designated biotype 1 or Illinois biotype as susceptible to *Rag* genes. Soybean aphid biotype 3 was reported by Hill et al. (2010), and characterized by populations that survived on *Rag2*, but not on *Rag1* soybeans. To date, the latest biotype reported is biotype 4, which is virulent to both *Rag1* and *Rag2* genes (Alt and Ryan-Mahmutagic 2013b). Collectively, these findings indicate that single gene resistance is not a durable management strategy for the soybean aphid, and that polygenic sources of aphid resistance should be pursued.

Tolerance, a polygenic form of resistance, to soybean aphids has also been investigated, although in a lesser extent. In controlled environments, the soybean KS4202 infested with soybean aphids during the late vegetative (V3) and early reproductive stages (R1/R2) has showed moderate resistance based on the assessed aphid damage ratings (Pierson et al. 2010, Marchi 2012). Yield parameters of V3 and R1 stage control and infested KS4202 were also analyzed in Pierson et al. (2010) and Marchi (2012), and indicated no significant changes in the total number of seeds, seeds per pod and individual seed weight. Moreover, field trials conducted during three seasons also supported that KS4202 is tolerant to soybean aphid (Prochaska et al. 2013). In that study, aphid infestations on KS4202 reached approximately 53,000 CAD (cumulative aphid-days), which resulted in yield losses of only 13%. According to Ragsdale et al. (2007), at a similar CAD level growers would expect 24-36% yield loss.

Host plant tolerance to aphids, though understudied, is attractive because it raises the EIL, delays the need for insecticidal control and rarely induces insect resistance. The incorporation of soybean KS4202 into soybean aphid IPM programs is a promising idea, however, further a deeper understanding of the relationship between aphid feeding and KS4202 yield loss is necessary to incorporate tolerance into soybean breeding programs and provide proper recommendations for using soybean tolerance for soybean aphid IPM.

### **Hormone Role in Plant Resistance to Phloem Feeders**

Plants respond to herbivores using a variety of mechanisms that interact to activate defenses, and can be very specific to its stressor. It has been hypothesized that plants recognize phloem-feeding insects by detecting elicitors, possibly derived from the insect's saliva, such as  $\beta$ -glucosidases and peroxidases (Lapitan et al. 2007). Insect's elicitors are recognized by plant receptors, which in turn induce calcium- and reactive oxygen species (ROS)- related signaling. ROS, such as hydrogen peroxide, may be toxic to insects (Bi and Felton 1995). Moreover, ROS induces signaling pathways such jasmonic acid (JA), salicylic acid (SA) and ethylene (Eth) (Park et al. 2006).

A considerable number of studies have examined the role of plant hormones in the defense against phloem feeding insects (e.g. aphids and whiteflies). To date, most of these studies were conducted with plants that are reported "resistant", meaning that these plants possess traits that are either antibiotic or antixenotic to these insects. SA is regarded the most common hormonal pathway induced by aphids, and has been documented in several aphid-host interactions, including or Arabidopsis (*Arabidopsis thaliana*), tomato and soybean (Mewis et al. 2005, Li et al. 2006b, Li et al. 2008).

Arabidopsis PHYTOALEXIN DEFICIENT4 (PAD4) gene encodes for a nucleocytoplasmic protein, which is required for defense against pathogens (Weirmer et al. 2005). When examining the role of PAD4 in Arabidopsis defense against green peach aphid (*Myzus persicae*), Pegadaraju et al. (2005) found that PAD4 mutants had higher aphid densities and leaves that prematurely senesced. A normal functioning of PAD4 is required for antibiosis and antixenosis in Arabidopsis (Pegadaraju et al. 2005, Louis and Shah 2015). *Myzus persicae*-induced lipase (MPL1) was also required for green peach aphid antibiosis in Arabidopsis (Louis et al. 2010). Moreover, aphid feeding induces the expression of PR (PATHOGENESIS RELATED) genes and other transcripts associated with SA-signaling. Studies of PR proteins (e.g.  $\beta$ -glucanase, peroxidase and chitinase) found differential expression between resistant and susceptible plants in response to aphids. In wheat, activities of  $\beta$ -glucanase, peroxidase and chitinase were higher in resistant than in susceptible plants (van der Westhuizen et al. 1998b, a). Direct quantification of SA has demonstrated that Russian wheat aphid (RWA) (*Diuraphis noxia*) resistant wheat induced SA (Mohase and van derWesthuizen 2002). In tomato, research has been done with Mi-1.2, a resistance (R) gene that encodes a nucleotide binding site and leucine rich motifs and confers resistance to potato aphid (*Macrosiphum euphorbiae*) and silverleaf whitefly (*Bemisia tabaci*) (Rossi et al. 1998, Nombela et al. 2003). The P4 gene, homologous to PR1 in Arabidopsis, was highly expressed in Mi-1.2 plants infested with potato aphid (Li et al. 2006b). The participation of SA-signaling has also been noted in *Rag1* (aphid resistant) soybean. Transcriptome profiles revealed that PR1 was highly up regulated in *Rag1* plants infested with soybean aphids, when no

changes occurred in the susceptible genotype (Li et al. 2008, Studham and MacIntosh 2013).

Generally, most studies have associated increased biosynthesis of JA (octadecanoid pathway) and induction of JA-associated transcripts (e.g. lipoxygenases [LOX], coronatine-insensitive1 [COI1] and OPR [12-oxophytodienoate reductase]) with chewing insects (Mewis et al. 2005). Although phloem feeding insects can induce this pathway, this occurs in a lesser extent and it is likely a result of cell damage caused by stylet probing (Thompson and Goggin 2006). A variety of LOX-derived oxylipins (oxidized lipids) are known to function as defense modulating signaling molecules in plants. For example, LOX1-LOX5 genes and downstream JA-responsive genes (PI and VSP) were important for bluegreen aphid (*Acyrtosiphon kondoi* Shinji) resistance in *Medicago truncatula* (Gao et al. 2007). JA-responsive genes were also induced in *Arabidopsis* infested with green peach aphids (Moran et al. 2002). In addition, Ellis et al. (2002) found that COI1 mutants, which had a defective oxylipin signaling, harbored higher densities of green peach aphid than the wild type plant.

While SA and JA have important signaling functions in plant defense, the interaction between these hormones can be synergistic or antagonist. Gene expression studies in *Arabidopsis* indicated that green peach aphid induced PR1 and PDF.1, which are respectively correlated with SA and JA pathways ((Moran et al. 2002, Pegadaraju et al. 2005). An example of antagonist JA-SA relationship is the case of silverleaf whitefly (*Bemisia tabaci* type B) in *Arabidopsis*. Whitefly feeding induced SA as mechanism to repress JA in *Arabidopsis*, which was essential for the insect's optimal development on the host (Zarate et al. 2007).



Ethylene (Eth) is another plant hormone that has been indicated in plant defense against aphids. Aphid feeding altered protein composition in cucurbit phloem, where two ET biosynthesis enzymes increased and could be related with the synthesis of proteinase inhibitors and other defense proteins (Walz et al. 2004). It was also observed that potato aphids induced Eth-associated transcripts in Mi-1.2 tomato. Although silencing Eth did not compromise Mi-1.2 mediated resistance, it did decrease host susceptibility to potato aphid (Mantelin et al. 2009).

WRKY transcription factors (TF) are known to modulate several plant responses against aphids and also pathogens (Boyko et al. 2006, Li et al. 2006a, Van Eck et al. 2010, Prochaska et al. 2015). WRKY proteins are a family of transcription factors classified by the presence of a 60 amino acid domain, which contain a conserved sequence WRKYGQK followed by a Zinc finger motif. In wheat and rice, WRKY53 represents an important role in leaf senescence and in the defense against RWA (Wu et al. 2008, Botha et al. 2010, Van Eck et al. 2010). When silenced, WRKY53 appears to reduce wheat resistance to RWA (Van Eck et al. 2010). Subsequent research on WRKY53 has proposed a network of genes involved in its pathway (Van Eck et al. 2014), suggesting WRKY53 regulates ROS release during hypersensitive responses (HR), which is initiated by biotic stressors such aphids and pathogens.

In soybeans, a transcriptome profile indicated that four WRKY TF were differentially expressed in soybean aphid infested tolerant plants (Prochaska et al. 2015). This study also indicated the involvement of the WRKY60. Other studies have suggested that aphids may manipulate certain WRKY TF to counteract a plant's defenses. Soybean aphids increased the expression of WRKY23 on susceptible soybeans, but not on *Ragl*

plants (Studham and MacIntosh 2013). The same TF was also induced at the feeding sites of soybean cyst nematode in soybean, whose biology was positively affected by the presence of soybean aphids (McCarville et al. 2012). A possible explanation for the role of WRKY23 comes from Grunewald et al. (2008), who found negative impacts on the performance of the cyst nematode (*Heterodera schachtii*) when reducing the levels of this WRKY TF in Arabidopsis. This suggests that WRKY23 in susceptible soybean may have a similar role, and could explain the effect on aphids and nematode populations.

### **Constitutive and Induced Resistance**

HPR to arthropods may be constitutively expressed or may be induced by the presence of arthropods. Constitutive resistance is regulated by preformed resistance traits, whereas induced resistance is triggered by herbivore attack that can be either localized or systemically expressed throughout the plant (Kessler and Baldwin 2002). An example of constitutive resistance is the Mi-1.2 gene in tomato (Rossi et al. 1998) and Vat NBS-LRR gene in melon that putatively encode a protein with NBS-LRR characteristics and controls resistance to cotton aphid (*Aphis gossypii*) (Boissot et al. 2010). It has been suggested that aphid resistance in lettuce (RGC2 gene), soybean (*Rag1* gene), and *Medicago truncatula* (AIN gene) could be potentially coordinated by NBS-LRR genes, as these genes map to chromosomal regions that surround NBS-LRR (Wroblewski et al. 2007, Klingler et al. 2009, Kim et al. 2010, Smith and Chuang 2014). Few studies have been done to document presence of constitutive defenses in aphid-tolerant plants. So far, it appears that aphid-tolerant plants may be predisposed to withstand damage

constitutively expressing certain hormonal pathways or oxidative enzymes at a greater level than a susceptible plant (Marimuthu and Smith 2012, Ramm et al. 2013).

Despite the presence of constitutive defenses, aphid-resistant sorghum and soybean also experienced substantial aphid-induced responses, and respond by overexpressing a large diversity of genes contributing to aphid resistance (Park et al. 2006, Li et al. 2008). These studies highlighted diversified transcriptomic changes in plants once aphids were introduced, which typically included homologs of constitutively expressed R genes, pathogenesis-related (PR) proteins, reactive oxygen species (ROS) elicitors, hormonal signaling pathway genes, and also genes necessary for the production of secondary metabolites and physical defenses. Conversely, the responses of a given susceptible plant resulted in the induction of a smaller and slower transcriptomic response. This has also been observed in aphid tolerant x susceptible soybean and barley, where aphid infestation resulted in a greater number of genes being induced in the tolerant plant (Gutsche et al. 2009a, Prochaska et al. 2015).

### **Specific Mechanisms of Tolerance to Aphid Herbivory**

Tolerance is described as a plant response independent of aphid presence, which functions using a network of compensatory mechanisms (Smith and Chuang 2014). The interactions within this network enable plants to withstand feeding injury and yield significantly more biomass than a susceptible plant under comparable conditions.

Tolerance to aphids has long been known in commonly grown crops such as wheat (Havlickova 1997, Miller et al. 2003), barley (Dogramaci et al. 2007), sorghum (Wilde and Tuinstra 2000), alfalfa (Nielson and Olson 1982), melon (Bohn et al. 1973) and

soybean (Pierson et al. 2010, Prochaska et al. 2013), and more recently in perennial grasses (Koch et al. 2014).

The mechanisms and genetics underlying plant tolerance to aphids are still poorly understood. However, consistent modifications in key aspects of plant metabolism such as photosynthesis, oxidative enzymes and also plant hormones have been reported. Increased photosynthetic activity and growth rates in RWA tolerance in wheat has been well documented (Haile et al. 1999, Heng-Moss et al. 2003). These changes occur as a result of a higher expression of genes that regulate photosynthesis and chlorophyll synthesis in aphid tolerant wheat (Boyko et al. 2006). By contrasting the response of wheat varieties expressing antibiosis, antixenosis and tolerance to RWA, Botha et al. (2014) reported that carbon flux and photosynthesis related genes (e.g. ferredoxin–thioredoxin reductase, fructose 1,6-bisphosphatase, chloroplast 50S ribosomal protein, ATP-dependent Clp protease proteolytic subunit) were up-regulated only in tolerant wheat, indicating that these plants can compensate for chlorophyll loss (Botha et al. 2014). Gutsche et al. (2009b) findings for RWA- tolerant barley corroborate the argument that tolerance to this aphid is associated with enhanced photosynthetic capacity. Tolerance in the soybean KS4202 to soybean aphids was also associated with enhanced photosynthetic capacity (Pierson et al. 2011). It was found that aphid tolerant soybeans had increased regeneration of rubilose-1,5-biphosphate (RuBP), a possible mechanism to compensate for aphid feeding.

Reactive oxygen species (ROS) have a fundamental role in the early signaling events that follow insect herbivory. Under normal cell conditions, the production and scavenging of ROS is under tight control (Apel and Hirt 2004, Mittler et al. 2004).

However, environmental and biotic stress (e.g. aphids) rapidly increases the synthesis of ROS, which may accumulate if not properly detoxified, causing permanent damage to proteins, DNA and lipids (Apel and Hirt 2004). The presence of oxidative enzymes, such as peroxidases, is required break down ROS into oxygen and water, keeping ROS under damaging levels (Apel and Hirt 2004). In addition to their role as ROS scavengers, class III peroxidases are involved in cell wall synthesis, auxin catabolism, wound healing and defense against stressors (Hiraga et al. 2001, Heng-Moss et al. 2004), and may also serve as ROS regenerators (Kawano 2003).

Several studies have documented increases in oxidative enzymes such as lipooxygenase, polyphenol oxidase, superoxide dismutase, catalase and peroxidase in response to arthropod feeding (Hildebrand et al. 1986, Felton et al. 1994a, Bi and Felton 1995, Hiraga et al. 2000). Further research has highlighted that increases in these enzymes may contribute to resistance (Felton et al. 1994b, van der Westhuizen et al. 1998b) and tolerance to aphids (Argandona et al. 2001, Gutsche et al. 2009a, Pierson et al. 2011, Marchi-Werle et al. 2014, Prochaska et al. 2015) and chinch bugs (Heng-Moss et al. 2004, Ramm et al. 2013). Specifically, peroxidases have been demonstrated to be important for tolerance to aphids in barley and soybean. Studies by Pierson et al. (2011) found that soybean aphid tolerance in soybean KS4202 was associated with higher levels of peroxidase when aphids were introduced in the reproductive stages. Moreover, Marchi-Werle et al. (2014) also correlated aphid infestation with elevated peroxidase activity during the vegetative stages of KS4202, stages at which KS4202 plants were showed to be tolerant to soybean aphids (Marchi 2012). In addition, these studies have reported that peroxidase profiling of KS4202 was different than aphid-susceptible

soybeans. Using high-throughput sequencing, Prochaska et al. (2015) compared and contrasted the transcriptome profile of KS4202 with a susceptible soybean, reporting that a few peroxidase transcripts were highly expressed in aphid-infested tolerant plants, when the levels of the same did not change in susceptible plants. Furthermore, a study performed to investigate the gene expression profiling of RWA tolerant barley x susceptible barley also detected two peroxidase transcripts (HvPRXA1 and HvPRXA2) that were up-regulated in tolerant barley in response to RWA feeding (Gutsche et al. 2009a). Collectively, these studies have proposed that aphid-stressed tolerant plants can maintain or elevate peroxidase activity to breakdown damaging ROS that accumulates as a result of stress, when the same mechanism is not as efficient in susceptible plants. While induced levels of peroxidase transcripts or peroxidase enzyme kinetics have showed to be important for the mechanism of plant tolerance to aphids, enhanced constitutive levels of peroxidases may also be important to buffalograss tolerance to chinch bugs (Ramm et al. 2013). Greater constitutive levels of peroxidases may allow a tolerant plant to utilize a greater portion of available resources for growth rather than initiating a defensive response.

As previously discussed, plant hormonal pathways are important for plant defense. However, only a few studies have specifically investigated the role of these pathways in plant tolerance to aphids. Wheat tolerance to RWA was associated with induced expression of JA, Eth and AUX-signaling genes (Smith et al. 2010). Conversely, barley tolerance to RWA feeding did not correlate with induction of defense related transcripts, but instead, it appears to be controlled by the constitutive expression of JA, Eth- and auxin-mediated defense (Marimuthu and Smith 2012).

The participation of Eth in tolerance to aphids appears to occur, but is sometimes controversial. Eth induction by bird cherry-oat aphid (*Rhopalophum padi*) and greenbug (*Schizaphis graminum*) was more expressive in aphid-tolerant barley than susceptible barley (Argandona et al. 2001). It was suggested that this hormone was correlated with oxidative responses of aphid-infested tolerant barley (Argandona et al. 2001). RWA feeding induced Eth responsive element binding protein (EREBP) in aphid-tolerant barley 'Sidney' (Gutsche et al. 2009a). However, a study by Miller et al. (1994) found that Eth synthesis was not modified in RWA infested PI 366450, a barley genotype tolerant to RWA.

### **Soybean breeding**

The majority of the traits desired for a breeding program are quantitatively inherited. The genes that control such traits are called quantitative trait loci (QTL). Molecular-based QTL analyses are used to characterize the associations between polymorphic DNA sites with the corresponding phenotypic variation of quantitative traits.

Studies with linkage map-based QTLs are performed by developing a mapping population. Recombinant inbred lines (RILs) are the result of successive inbreeding, and require considerable time. The construction of a RIL involves continuous selfing or sib mating the progeny of an F<sub>2</sub> population, and the process is repeated until populations become homozygous (Madhusudhana 2015). The construction of a RIL follows the single-seed descent method. A seed from each plant in an F<sub>2</sub> population is advanced to F<sub>3</sub>. Harvested F<sub>3</sub> seeds are then advanced to F<sub>4</sub>, and the process continues until the traits

are no longer segregating. RILs are a stable and permanent source for mapping that can be replicated in other locations and shared with other groups; once the alleles become fixed no changes occur upon inbreeding. Additionally, due to multiple meiosis cycles, RILs possess a higher degree of recombination than F<sub>2</sub> (Madhusudhana 2015).

Molecular markers, especially simple sequence repeats (SSR) and single nucleotide polymorphism (SNP), have been used extensively in soybean breeding programs for soybean aphid resistance. Identification of and recent expansion of soybean polymorphic SSR markers have facilitated the construction of linkage-maps, and allowed fine mapping and positional cloning of important genes (Song et al. 2004, Collard and Mackill 2008, Song et al. 2010). In the last decade, different QTLs governing soybean resistance gene (*Rag*) to soybean aphids were identified. The *Rag1* gene in Dowling and Jackson was mapped to chromosome 7 between the markers Satt435 and Satt463 (Li et al. 2007). Fine QTL mapping was later performed by Kim et al. (2010), which identified 2 SNP markers ( 46169.7 and 21A) using segregating plants from F<sub>2</sub> and F<sub>3</sub> generations of backcrossed populations (BC<sub>4</sub>). *Rag2* gene was also mapped in PI 243540 using SSR markers within F<sub>2</sub> and F<sub>2:3</sub> families from crosses between ‘Wyandot’ and PI 243540 (Mian et al. 2008). This gene is positioned between SSR markers Satt334 and Sct\_033 on chromosome 13. Moreover, *Rag3* was mapped in PI 567541B, in which two major QTLs explained 95.2% of the phenotypic variation (Zhang et al. 2010). RIL populations were used for genetic mapping in *Rag5* in PI 567301B, which revealed two loci associated with aphid resistance (Jun et al. 2012). These genetic maps have enabled marker-assisted selection (MAS) for soybean aphid resistance genes, which have been successfully incorporated into breeding lines adapted to the north-central regions of U.S.



Despite the success in mapping QTL in *Rag* soybean, to our knowledge, no studies have attempted to generate linkage maps for soybean with tolerance to soybean aphid. In fact, very few studies have identified QTLs associated with insect tolerance in other economic crops. This could be for various reasons, but most likely due to the complexity of the traits governing tolerance.

The use of RIL populations for QTL studies is more advantageous over F<sub>2</sub> or backcross populations for QTL studies (Burr and Burr 1991). A study by Nagaraj et al. (2005) developed RIL populations to map tolerance of sorghum 96-4121 to greenbug. A total of eight QTLs were found, of which three were related with greenbug biotype I, and five were related to biotype K feeding damage. Their study indicated that QTL for tolerance was biotype specific. Moreover, these QTLs only explained 9 to 19.6% of the phenotypic variation, and it has been speculated that these are clustered in small linkage groups on different chromosomes. RILs were also developed to map QTLs in GBIK sorghum, characterized as both resistant (i.e. antibiosis and/or antixenosis) and tolerant to greenbug (Agrama et al. 2002). In this study, 113 loci, including 38 SSR and 75 RAPD markers were mapped in 12 linkage groups (LG), covering a genomic region of 1,530 cM. In addition, nine putative QTLs were identified on LG A, B, C, D, and F, H, and J and were significantly related with resistance and tolerance of GBIK to greenbug biotype I and K. These results emphasize that tolerance is a polygenic resistance, and that MAS may be useful for breeding aphid-tolerant plants.

Increasing QTL density has implications for the development of more efficient breeding systems. A recurring complication with QTL data is that different parental combinations and/or experiments conducted in different locations frequently result in the

identification of partial or non-overlapping QTLs. These differences can be due to the significant genotype by environment interaction, sampling error due to population size and phenotypic evaluation (Rong et al. 2007). To date, the studies that conducted molecular QTL tolerance in sorghum have relied on phenotypical evaluations based on visual aphid feeding damage and readings with SPAD chlorophyll meter (Agrama et al. 2002, Nagaraj et al. 2005). The development of additional techniques to characterize aphid tolerant populations will improve selection criteria, and therefore increase the quality of QTL maps. Ultimately, an appropriate phenotypic evaluation may stimulate new breeding programs targeting development of crops with tolerance to insects.

## **Dissertation Objectives**

The deployment of plant tolerance is a valuable strategy that can mitigate the injury caused by soybean aphids. A detailed understanding on how tolerant soybeans respond to aphid will provide important information for incorporating these plants in the IPM programs for this insect. Under controlled environment, this research investigated the yield response of the tolerant soybean KS4202 at multiple developmental stages and infestation levels. Field trials were also conducted to quantify the relationship between CAD and yield loss in KS4202 and determine appropriate EILs for this genotype.

Additional knowledge at the molecular level is needed to understand key pathways that govern defense against soybean aphids in KS4202. The identification of these pathways will assist in the identification of phenotypic or genotypic characteristics of tolerant soybean, providing a baseline for the development of phenotypic assays to allow successful breeding of aphid-tolerant soybeans. Therefore, this research evaluated the differences in the expression of constitutive and induced defense-related transcripts (i.e. specific peroxidases, JA-associated transcripts and WRKY transcription factors) between KS4202 and a susceptible soybean in response to soybean aphid feeding. In addition, the use of total peroxidase activity and relative expression of specific peroxidase transcripts as potential assays to phenotypic assay for aphid-tolerant RIL were also investigated.

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## CHAPTER II

### **Yield Response of Tolerant and Susceptible Soybean to the Soybean Aphid (*Aphis glycines* Matsumura)**

\* This chapter represents a compilation of work done by Lia Marchi M.S thesis in 2012 and Lia Marchi Werle PhD dissertation in 2016. Sections from the M.S. thesis have been incorporated in this chapter.

#### **Introduction**

Soybean (*Glycine max* (L.) Merrill) is an important commodity in world trade and represents the majority of the oilseed produced in the United States (Bilyeu et al. 2010). The soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), native to Asia, was first reported in North America in July of 2000. Currently, this insect has been reported in 30 states in the United States as well as southeast Canada (Ragsdale et al. 2011). The insect feeds by removing foliar and stem phloem sap through piercing-sucking mouthparts, which negatively impacts photosynthesis (Macedo et al. 2003, Pierson et al. 2011). In addition, soybean aphids can also cause indirect injury to the plant, including virus transmission such as the soybean mosaic virus (SMV), and sooty mold development caused by honeydew accumulation (Clark and Perry 2002, Tilmon et al. 2011). High soybean aphid infestations can significantly reduce yield through reduced soybean growth, lower numbers of pods and seeds per pod, and lower individual seed weight (Beckendorf et al. 2008). A comprehensive, multi-state research project estimated a yield loss of 6.88% for every 10,000 CAD during the reproductive stages from first



flower (R1) to full pod (R5), and an average EIL of  $674 \pm 95$  aphids per plant (or  $\approx 5,563$  CAD) (Ragsdale et al. 2007).

In North America, soybean aphid management has relied heavily on foliar-applied insecticides. Before the arrival of soybean aphids in North America, less than 1% of the soybean fields were treated with insecticides. This scenario had drastically changed by 2006, when a sharp increase (estimated 130-fold) in insecticide applications was associated with the introduction of the soybean aphid to North America (Ragsdale et al. 2011). As a result, production costs were increased by \$16-33/ha (Ragsdale et al. 2007), which has stimulated the development of alternative pest management methods for this pest.

Host plant resistance (HPR) provides effective, economical and environmentally safe pest control and is considered an important component of integrated pest management (Smith 2005). Since soybean aphids were detected in North America, significant progress has been made in identifying soybeans with resistance to this insect. Resistance was first identified in Dowling, Jackson and PI 71506 (Hill et al. 2004). Antibiosis was the major contributor for resistance in Dowling and Jackson (Hill et al. 2004), whereas resistance in PI 71506 was attributed to antixenosis (Hill et al. 2004, Mian et al. 2008). Other studies have identified antibiosis in PI 567541B, PI 567598B and PI 243540 (Mensah et al. 2005, Zhang et al. 2009), and antixenosis resistance was also reported in Dowling, PI 230977 (Hesler et al. 2007), PI 567453C and PI 567597C (Mensah et al. 2005, Zhang et al. 2010). Resistance in those genotypes was attributed to a single dominant gene, named *Rag* (Resistance to *Aphis glycines*).

Not long after the identification of aphid resistance genes, soybean aphid virulent populations were observed colonizing *Rag* soybeans. Referred as biotype 2 or the Ohio biotype, this population has overcome the resistance imposed by *Rag1* (Kim et al. 2008). However, this gene still negatively affects Illinois' soybean aphids that infest *Rag1* soybean. For that reason, Kim et al. (2008) designated biotype 1 or the Illinois biotype as susceptible to *Rag* genes. Soybean aphid biotype 3 was reported by Hill et al. (2010), and characterized by populations that survived on *Rag2*, but not on *Rag1* soybeans. To date, the latest biotype reported is biotype 4, which is virulent to both *Rag1* and *Rag2* genes (Alt and Ryan-Mahmutagic 2013b). Collectively, these findings indicate that single gene resistance is not a durable management strategy for the soybean aphid, and that polygenic sources of aphid resistance should be pursued.

Tolerance is a polygenic form of resistance defined as the ability of a host plant to withstand arthropod feeding without suffering excessive injury (Smith 2005). This type of resistance has several advantages as a pest management strategy from an ecological viewpoint; however, a limited number of studies have focused on identifying soybean aphid-tolerant soybeans. In a controlled environment, studies by Pierson et al. (2010) first reported later vegetative and reproductive KS4202 soybeans to be tolerant to soybean aphid injury. Moreover, field trials conducted during three seasons also supported that KS4202 is tolerant to soybean aphid during the reproductive stages (Prochaska et al. 2013). In that study, aphid infestations on KS4202 reached approximately 53,000 CAD (cumulative aphid-days), which resulted in yield losses of only 13% (Prochaska et al. 2013). Although soybean aphids generally infest Nebraska's soybean fields when plants

are entering the reproductive stages (Prochaska et al. 2013), soybean aphids may impact other locations earlier in the season (Brosius et al. 2007)

A preliminary study based on visual plant damage documented that KS4202 is highly susceptible to the soybean aphid during the VE and VC; whereas, V1 is moderately susceptible, and V3, V4 and V5 stages are moderately resistant to this aphid (Marchi 2012). However, further studies are still needed to correlate this finding with yield. Therefore, the objective of this research was to investigate the yield response of the tolerant soybean KS4202 at multiple plant stages and aphid infestation levels.

## Materials and Methods

**Studies design.** Two greenhouse studies were performed to evaluate the impact of soybean aphids and plant stage on the yield response of KS4202. In the first study, soybean aphids were introduced at three vegetative stages: V1 (fully developed leaf at unifoliate node), V3 (fully developed leaf at third node) and R1 (reproductive stage from first flower) (Fehr and Caviness 1977). In addition, three levels of aphid infestation were implemented, control (uninfested), low (4,000 – 5,500 CAD) and high (7,500-8,500 CAD). The treatment design was a 3x3 factorial arranged in a completely randomized design with 10 replications. The low level, equivalent to 1,000-1,500 insects per plant, represented the high EILs for conventional soybean (i.e., non-tolerant) calculated by Ragsdale et al. (2007). Although these thresholds were determined for R1-R5 soybean, some level of loss could be expected for tolerant KS4202 soybean, but possibly not economic. The higher level represented a level where significant yield loss would be expected for KS4202.

A second study was performed to include the susceptible genotype, K03-4686 (Prochaska et al. 2015). The treatment design was a factorial with 2 genotypes (KS4202 and K03-4686) x 2 soybean stages (V1 and V3) x 3 infestation levels (control – uninfested, low and high CAD). Based on the results from the first study, low and high CAD treatments were increased to 9,000 – 12,000 and 18,000 - 25,000 CAD, respectively. The experimental design was a completely randomized design with 10 replications.

**Plant and insect source.** The seeds of each genotype (KS4202 and K03-4686) were planted in 15-cm diameter round plastic pots at a depth of approximately 3 cm. The

potting media was a mixture of 34% peat, 31% perlite, 31% vermiculite and 4% soil. Planting dates were staggered to ensure that plants in each study would reach the designated plant stage at the same time. Upon germination, plants were thinned to one plant per pot and placed in a plastic tray (35 cm x 50 cm) filled with water. Plants were fertilized every 2 weeks with a soluble (20:10:20 N/P/K) fertilizer. The greenhouse temperature was maintained at  $23 \pm 3^\circ\text{C}$ , with lighting supplemented by 400-watt high intensity lamps to produce a photoperiod of 16:8 (L: D) h.

Once the plants were at the desired stage, 10 (low CAD) or 20 (high CAD) soybean aphids (4<sup>th</sup> instars and adults) were placed on the youngest fully expanded leaf of the designated aphid-infested treatments. Aphids used in this study are progeny of a Nebraska isolate (biotype 1) initially collected during the 2011 growing season from commercial soybean near the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory, Concord, NE ( $42^\circ 23' 3'' \text{ N}$ ,  $96^\circ 59' 21'' \text{ W}$ ). The soybean aphid colony was maintained on KS4202 plants in a growth chamber at  $21 \pm 2^\circ\text{C}$  and a photoperiod of 16:8 (L: D) h. Upon aphid introduction, tubular polycarbonate plastic cages (15 cm of diameter and 61 cm of height, Makrolon Lexan) were placed on both infested and control (aphid-free) plants.

**Evaluations and yield parameters.** Plants were evaluated bi-weekly by recording aphid number and plant stage. After each evaluation, aphid number was used to calculate cumulative aphid-days, which provides an estimate of accumulated aphid pressure. CAD was calculated using the formula:  $\sum_{i=1}^n = [(x_i + x_{i-1})/2 + (t_i - t_{i-1})]$ , where  $n$  is the number of sample dates,  $x_i$  is the mean number of aphids per plant (i.e. average

per plot) on sample date  $i$ , and  $(t_i - t_{i-1})$  is the number of days between two consecutive sample dates (Hanafi et al. 1989, Ragsdale et al. 2007).

For both studies, aphids remained on the plants until the targeted infestation levels were reached after which plants were sprayed with the synthetic pyrethroid insecticide lambda-cyhalothrin (Warrior with Zeon technology<sup>®</sup>, Syngenta Crop Protection, Greensboro, NC). Plants were monitored closely within the next 24- 48 h after insecticide application, and cages were removed once aphid populations were completely eradicated. Plants were then tied to a bamboo stick (approximate length of 1 m) to ensure the main stem was properly supported.

Upon maturation (i.e. pods were completely yellow or brown), soybean pods were harvested and placed in a paper bag and oven dried. The yield parameters of each plant were individually calculated by evaluating the number of pods/plant, number of seeds/pod, average seed weight and average pod dry weight (Beckendorf et al. 2008, Pierson et al. 2010). An analysis of variance (ANOVA) was conducted for all plant stages and infestation levels to assess differences in yield parameters using PROC GLIMMIX in SAS 9.3 (SAS Institute, Cary, NC). Where appropriate ( $\alpha = 0.05$ ), means were separated using Fisher's least significant difference (LSD) test.

## Results and Discussion

**Study 1.** KS4202 plants exposed to the low cumulative aphid-days (LCAD) treatment during V1, V3 and R1 exceeded the average EIL of 674 aphids per plant reported by Ragsdale et al. (2007). No differences between V1 control (uninfested) and LCAD treated ( $3,710 \pm 304.7$  CAD) plants for any of the yield parameters evaluated (Table 2.1-2.6). In contrast, for V1 infestation at the high cumulative aphid-days (HCAD) treatment ( $7,790 \pm 769.1$  CAD), resulted in significant reduction in total seed weight, total pod weight, and seed number (33.8%, 21.74%, and 32.3% reduction, respectively) when compared to control plants (Table 2.2, 2.5, and 2.6). There was also a decrease in the number of pods (27.2%) between HCAD and control plants ( $P = 0.059$ ; Table 2.4)

Despite aphid numbers exceeding typical CAD EILs presented by Ragsdale et al. (2007), V3 and R1 plants at both the HCAD (resultant CAD =  $7,490 \pm 803.4$  and  $8,385 \pm 498.8$ , respectively) and LCAD treatments (resultant CAD =  $4,530 \pm 245.8$  and  $5,300 \pm 525.5$ , respectively) were not different from their respective control plants for any of the yield parameters evaluated (Tables 2.1-2.2, 2.4-2.6), except average pod weight for R1 plants, where R1 plants exposed to the LCAD treatment had an average pod weight that was higher than control soybeans ( $P = 0.02$ ; Table 2.3). Interestingly, HCAD treated V3 plants showed an increase of 58% in the single seed weight when compared to the control treatment ( $P = 0.06$ ; Table 2.1). Similarly to V3 plants, R1 plants exposed to different levels of aphid pressure also resulted in increases in some yield parameters. R1 plants exposed to LCAD had an increase of 44.4% in the total seed weight, 22.2% in the average pod weight and 28.6% in the number of seeds when compared to the control treatment (Tables 2.2, 2.3 and 2.6).

Comparisons between the yield parameters for LCAD and HCAD treatments were performed to obtain additional information on the impact of aphid infestation level. Although no statistical differences were detected in yield parameters for V1 aphid infested plants between HCAD and LCAD treatments, there was a trend for HCAD treatments to have lower means in total seed weight, number of pods, total pod weight, and number of seeds per plant (Tables 2.2, 2.4-2.6).

At the V3 stage, there were no significant differences in yield parameters between HCAD and LCAD (Tables 2.1-2.6). Although not significant at a  $P < 0.05$ , the difference for average seed weight neared significance, HCAD treatment had a greater average seed weight than the low aphid number treatment. Conversely, although yield parameters for R1 infested soybeans were not significantly different between LCAD and HCAD, there was a trend for the values to be lower in HCAD (Tables 2.1-2.6).

As an additional measure of the impact of soybean aphid infestation at different stages of KS4202 soybeans, comparisons between stages were performed (Table 2.7). Comparisons between V1 and V3 plants under LCAD did not indicate significant differences for any of the yield parameters, although there was a trend for total seed weight (22.5%), number of pods (17.4%) and total pod weight (17.5%) to be lower in plants infested at the V1 stage. In the HCAD treatment, significant differences were observed between V1 and V3 plants for all yield parameters except average pod weight. Mean yield parameter values for V1 plants were on average 30.1% lower than those for V3 plants under the same aphid treatment. A similar trend was observed between R1 and V3 yield parameters. In this case, the yield parameters were comparable between R1 and V3 plants under LCAD; however, significant reductions occurred for the yield parameters



analyzed at HCAD (exception for average pod weight in plants infested at the R1 stage). In this last comparison, significant reductions in the yield parameters for R1 were of 46.2% in average seed weight, 42.5% in total seed weight, and 28.7% in number of pods, 20.5% in total pod weight, and 34% in number of seeds. Finally, no statistical differences were observed between V1 and R1 yield parameters for either LCAD or HCAD, although V1 plants at the high aphid number treatment had lower total pod weight (24.6%) and number of pods (21.8%) than R1 plants.

**Study 2.** At V1 stage, KS4202 and K03-4686 (susceptible) plants under LCAD treatment accumulated  $11,623 \pm 464.9$  and  $10,392 \pm 461.1$  aphid-days, respectively. In the HCAD treatment, KS4202 plants had  $25,031 \pm 1,845.4$  CAD and K03-4686 had  $25,988 \pm 1402.7$  CAD. The LCAD treatment had a negative impact on the yield parameters (Tables 2.8-2.13) of both genotypes. However the impact was greater in K03-4686, where reductions of 29.7% in total pod number ( $P = 0.03$ ; Table 2.8), 39.9% in the total pod weight ( $P = 0.04$ ; Table 2.12) and 28.8% in the single pod weight ( $P = 0.02$ ; Table 2.13) were observed. In terms of proportions of reductions of LCAD in KS4202 relative to its respective control, the findings to Study 1 at 7,800 CAD (i.e. HCAD treatment of Study 1) are similar to Study 2. In contrast, HCAD treatment had a greater negative effect than LCAD for both soybean genotypes (Tables 2.8-2.13). KS4202 experienced reductions of 30% in the total pod weight ( $P = 0.01$ ; Table 2.12), 35.4% in the total seed weight ( $P = 0.01$ ; Table 2.9) and 29.9% in the seed number ( $P = 0.04$ ; Table 2.10), while K03-4686 experienced similar reductions in the same parameters, and also showed a significant reduction in total pod number ( $P = 0.04$ ; Table 2.8), seed number ( $P = 0.02$ ; Table 2.10), single seed weight ( $P = 0.03$ ; Table 2.11) and

single pod weight ( $P = 0.0001$ ; Table 2.13). It is noteworthy that the HCAD treatment applied in Study 2 ranged from 18,000 to 25,000, which is two to three-fold higher than the HCAD applied in Study 1, however, the proportion of yield reduction in KS4202 (relative to the control treatment) remained similar within these studies. Moreover, when contrasting LCAD x HCAD treatments within each genotype, no differences were identified (Tables 2.8-2.13). This indicates, independently of the genotype, that soybeans were susceptible to aphid infestation occurring at the V1 stage when CAD surpassed 10,000.

The mean LCAD for soybeans infested at the V3 stage in KS4202 was  $9,609 \pm 882.1$ , and  $11,537 \pm$  for K03-4686. At the HCAD treatment, KS4202 accumulated  $24,079 \pm 1,332.3$  aphid-days, while K03-4686 accumulated  $17,376 \pm 899.3$  aphid-days. In LCAD, both KS4202 and K03-4686 infested at V3 stage did not show differences in yield parameters (Tables 2.8-2.13), however, the HCAD treatment impacted all the yield parameters of K03-4686 (Tables 2.8-2.13). For this treatment, the yield parameters for K03-4686 were on average reduced by 27%, with the most noticeable impacts in total seed weight (Table 2.9) and total pod weight (Table 2.12). Conversely, KS4202 plants, which were exposed to  $\sim 24,000$  CAD, did not experience reductions in any of the yield parameters (Tables 2.8 – 2.13).

Different from infestations initiated during the V1 stage, the comparisons between infestation levels (i.e. LCAD x HCAD) for V3-infested plants indicated that higher aphid pressure did not affect the yield parameters in KS4202, but had a significant effect on K03-4686 (Tables 2.3 – 2.18). These data were also consistent with Study 1, indicating

that KS4202 tolerated various levels of aphid pressure during the V3 stage. In addition, the susceptible genotype showed yield loss when aphid pressure exceeded 10,000 CAD.

Despite the yield losses observed at the highest CAD treatment (25,000 CAD) for KS4202 and K03-4686 infested at V1 stage, lower CAD pressure (4,000 – 9,000 CAD) caused reductions in the yield parameters of KS4202 that varied from 0.93 to 17.7% relative to the control treatment (Table 2.1-2.13). The same parameters in the susceptible genotype ranged from 24 to 39%. Collectively, data from both studies demonstrate KS4202 is tolerant to soybean aphids within these lower levels of CAD during the V1 stage.

As the soybean's vegetative phase progressed, it was observed that plants infested at V3 stage were more resilient to aphid pressures above 10,000 CAD. Low aphid pressure (average of 10,000 CAD) had little or no impact on yield parameters of the susceptible genotype. This is consistent with previous studies, which found that at the early soybean stage, minor injury or severe injury quickly managed had no significant impact on soybean yields (He et al. 1991). Interestingly, some researchers found a positive relationship between low CAD and yield (Liere et al. 2015, Kucharik et al. 2016), suggesting some degree of overcompensation. We also noticed a slight increase in some yield parameters of the susceptible soybean at the V3 stage; however, these differences were not significant. Increased aphid pressure (>10,000 CAD) restricted yield on the susceptible genotype, where yield parameters were reduced by 17-40% when compared to healthy aphid-free plant of the same genotype. This finding was also corroborated by other studies, where continuous infestation caused a 20-30% yield reduction (Dai and Fan 1991, Rhainds et al. 2012). Most importantly, KS4202 tolerated

CAD pressure within the 17,000 to 25,000 CAD without a detrimental impact to the yield parameters evaluated.

This research also demonstrated that that under higher aphid pressure, KS4202 at the R1 was less tolerant than the same at V3. This could be a result of the physiological condition of KS4202 when aphids were introduced, and possibly the ability to compensate for early injury. Generally, soybeans are less sensitive to stress in the vegetative stages than in the reproductive stages. High soybean aphid densities at and before pod set have been shown to have a negative impact on seed weight and increased the proportion of shriveled pods (Lin et al. 1993). Soybeans were also able to compensate for bean leaf beetle, *Cerotoma trifurcata* (Forster), defoliation during the early vegetative stages (Hunt et al. 1994); however, stress during the reproductive period can cause a significant impact on yield, particularly in the later stages due to the reallocation of photosynthates from vegetative to reproductive structures (Ostlie 1984, Singer 2001).

From a pest management perspective, plant tolerance to insect herbivory has several advantages (Smith 2005). Different than antibiosis and antixenosis, plant tolerance is conferred by a collection plant characteristics and may not impose the same constraints in the arthropod biology or behavior. Although in theory it is possible that tolerance could affect herbivore performance (Stinchcombe 2002), researchers suggest that arthropods exposed to tolerant plants experience lower selection pressure than those on antibiotic or antixenotic plants. Hence, the likelihood of the emergence of virulent population (biotype) in response of a tolerant plant is minimal (Stinchcombe 2002, Smith 2005).

The cultivation of tolerant cultivars can favor the establishment of beneficial arthropods and raise the EIL, and possibly economic thresholds (ETs), thereby decreasing the need of early pest management intervention. The results from this research compare favorably with Pierson et al. (2010) and Prochaska et al. (2013), which found that KS4202 is tolerant to soybean aphids during the reproductive stages. In addition, this research documents that tolerance also occurs in the early vegetative stages (i.e. V3) of KS4202, although aphid infestation at the very early vegetative stages (i.e. V1) could result in economic loss. This research will contribute to the development of alternatives to mitigate the impacts of soybean aphid injury and will assist to establish EILs for the vegetative and reproductive stages of soybean aphid tolerant KS4202 soybeans.

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## Tables

**Table 2.1.** Means  $\pm$  SEM of single seed weight in soybean aphid-infested (low/high aphid number) and control KS4202 soybean (study 1).

Stage of infestation	Single Seed Weight (g)					
	C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i>		
				C x LCAD	C x HCAD	LCAD x HCAD
V1	0.1491 $\pm$ 0.017	0.1509 $\pm$ 0.005	0.1465 $\pm$ 0.007	0.97	0.97	0.93
V3	0.1703 $\pm$ 0.006	0.1593 $\pm$ 0.008	0.2694 $\pm$ 0.108	0.81	0.08	0.06
R1 <sup>e</sup>	0.1439 $\pm$ 0.004	0.1619 $\pm$ 0.006	0.1448 $\pm$ 0.008	0.78	0.99	0.79

**Table 2.2.** Means  $\pm$  SEM of total seed weight in soybean aphid-infested (low/high aphid number) and control KS4202 soybean (study 1).

Stage of infestation	Total Seed Weight (g)					
	C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i>		
				C x LCAD	C x HCAD	LCAD x HCAD
V1	9.92 $\pm$ 3.16	9.24 $\pm$ 3.59	6.57 $\pm$ 3.36	0.68	0.04	0.11
V3	14.70 $\pm$ 1.30	11.92 $\pm$ 1.19	12.94 $\pm$ 1.23	0.10	0.27	0.55
R1 <sup>d</sup>	7.11 $\pm$ 0.60	10.27 $\pm$ 1.24	7.44 $\pm$ 1.15	0.10	0.86	0.13

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

<sup>d</sup> R1: Tolerant control (Pierson et al. 2010)

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.3.** Means  $\pm$  SEM of single pod weight in soybean aphid-infested (low/high aphid number) and control KS4202 soybean (study 1).

Stage of Infestation	Single Pod Weight (g)					
	C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i>		
				C x LCAD	C x HCAD	LCAD x HCAD
V1	0.4477 $\pm$ 0.018	0.4387 $\pm$ 0.012	0.4400 $\pm$ 0.023	0.74	0.77	0.96
V3	0.4646 $\pm$ 0.016	0.4577 $\pm$ 0.017	0.4644 $\pm$ 0.013	0.81	0.99	0.82
R1 <sup>d</sup>	0.3751 $\pm$ 0.016	0.4758 $\pm$ 0.021	0.4167 $\pm$ 0.028	0.002	0.19	0.06

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

<sup>d</sup> R1: Tolerant control (Pierson et al. 2010)

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.4.** Means  $\pm$  SEM of pod number in soybean aphid-infested (low/high aphid number) and control KS4202 soybean (study 1).

Stage of Infestation	Pod Number					
	C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i>		
				C x LCAD	C x HCAD	LCAD x HCAD
V1	32.80 $\pm$ 2.94	32.11 $\pm$ 3.75	23.70 $\pm$ 4.27	0.88	0.06	0.09
V3	46.60 $\pm$ 3.77	38.87 $\pm$ 3.55	40.50 $\pm$ 2.89	0.13	0.20	0.74
R1 <sup>d</sup>	31.00 $\pm$ 2.29	32.14 $\pm$ 3.31	28.87 $\pm$ 2.57	0.84	0.70	0.55

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

<sup>d</sup> R1: Tolerant control (Pierson et al. 2010)

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.5.** Means  $\pm$  SE of total pod weight in soybean aphid-infested (low/high aphid number) and control KS4202 soybean (study 1).

Stage of Infestation	Total Pod Weight (g)					
	C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i>		
				C x LCAD	C x HCAD	LCAD x HCAD
V1	14.67 $\pm$ 1.33	21.51 $\pm$ 1.53	11.48 $\pm$ 1.59	0.73	0.04	0.10
V3	13.92 $\pm$ 1.70	17.75 $\pm$ 1.59	12.11 $\pm$ 1.53	0.10	0.21	0.61
R1 <sup>d</sup>	10.29 $\pm$ 0.86	18.88 $\pm$ 1.63	15.23 $\pm$ 1.44	0.14	0.79	0.21

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

<sup>d</sup> R1: Tolerant control (Pierson et al. 2010)

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.6.** Means  $\pm$  SEM of seed number in soybean aphid-infested (low/high aphid number) and control KS4202 soybean (study 1).

Stage of Infestation	Seed Number					
	C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i>		
				C x LCAD	C x HCAD	LCAD x HCAD
V1	66.60 $\pm$ 6.91	60.55 $\pm$ 6.50	45.10 $\pm$ 8.29	0.56	0.04	0.14
V3	86.60 $\pm$ 6.99	74.50 $\pm$ 6.35	74.62 $\pm$ 6.35	0.26	0.23	0.99
R1 <sup>d</sup>	49.43 $\pm$ 3.85	63.57 $\pm$ 7.88	49.25 $\pm$ 4.84	0.25	0.98	0.23

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

<sup>d</sup> R1: Tolerant control (Pierson et al. 2010)

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.7.** Effect of infestation level (low/high aphid number) and different plant stages (V1, V3, and R1) on yield parameters of KS4202 (study 1).

<b>Single seed Weight (g)</b>				<b>Total Seed Weight (g)</b>			
<b>Infestation level</b>	<b>V1 x V3</b>	<b>R1<sup>c</sup> x V1</b>	<b>R1<sup>c</sup> x V3</b>	<b>Infestation level</b>	<b>V1 x V3</b>	<b>R1<sup>c</sup> x V1</b>	<b>R1<sup>c</sup> x V3</b>
	<i>P-value</i>				<i>P-value</i>		
LCAD <sup>a</sup>	0.889	0.860	0.96	LCAD <sup>a</sup>	0.126	0.569	0.37
HCAD <sup>b</sup>	0.029	0.976	0.03	HCAD <sup>b</sup>	<0.001	0.609	0.002
<b>Single Pod Weight (g)</b>				<b>Total Pod Weight (g)</b>			
<b>Infestation level</b>	<b>V1 x V3</b>	<b>R1<sup>c</sup> x V1</b>	<b>R1<sup>c</sup> x V3</b>	<b>Infestation level</b>	<b>V1 x V3</b>	<b>R1<sup>c</sup> x V1</b>	<b>R1<sup>c</sup> x V3</b>
	<i>P-value</i>				<i>P-value</i>		
LCAD <sup>a</sup>	0.519	0.227	0.56	LCAD <sup>a</sup>	0.100	0.586	0.31
HCAD <sup>b</sup>	0.368	0.419	0.10	HCAD <sup>b</sup>	<0.01	0.412	0.003
<b>Pod Number</b>				<b>Seeds Number</b>			
<b>Infestation level</b>	<b>V1 x V3</b>	<b>R1<sup>c</sup> x V1</b>	<b>R1<sup>c</sup> x V3</b>	<b>Infestation level</b>	<b>V1 x V3</b>	<b>R1<sup>c</sup> x V1</b>	<b>R1<sup>c</sup> x V3</b>
	<i>P-value</i>				<i>P-value</i>		
LCAD <sup>a</sup>	0.194	0.995	0.22	LCAD <sup>a</sup>	0.211	0.793	0.35
HCAD <sup>b</sup>	<0.001	0.308	0.02	HCAD <sup>b</sup>	0.005	0.702	0.02

<sup>a</sup> LCAD: Low cumulative aphid-days

<sup>b</sup> HCAD: High cumulative aphid-days

<sup>c</sup> R1: Tolerant control (Pierson et al. 2010)

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.8.** Means  $\pm$  SEM of total pod number of KS4202 and K03-4686 infested and control at V1 and V3 stages (study 2).

Stage of Infestation	Genotype	Total Pod Number					
		C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i> <sup>a</sup>		
					C x LCAD	C x HCAD	LCAD x HCAD
V1	K03-4686	53.25 $\pm$ 4.39	37.42 $\pm$ 5.20	40.71 $\pm$ 5.69	0.03	0.04	0.29
	KS4202	62.10 $\pm$ 3.27	52.22 $\pm$ 5.39	47.14 $\pm$ 6.18	0.19	0.07	0.54
V3	K03-4686	67.62 $\pm$ 4.24	69.14 $\pm$ 4.13	54.11 $\pm$ 6.21	0.81	0.02	0.01
	KS4202	58.70 $\pm$ 3.10	61.40 $\pm$ 3.76	66.50 $\pm$ 2.24	0.62	0.15	0.35

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

Treatment means significantly different at  $P < 0.05$  by LSD test



**Table 2.9.** Means  $\pm$  SEM of total seed weight of KS4202 and K03-4686 infested and control at V1 and V3 stages (study 2).

Stage of Infestation	Genotype	Total Seed Weight (g)			<i>P-value</i> <sup>a</sup>		
		C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	C x LCAD	C x HCAD	LCAD x HCAD
V1	K03-4686	9.33 $\pm$ 1.14	6.03 $\pm$ 0.67	5.63 $\pm$ 2.11	0.12	0.08	0.85
	KS4202	17.18 $\pm$ 1.40	14.96 $\pm$ 1.46	12.15 $\pm$ 1.83	0.24	0.01	0.17
V3	K03-4686	12.38 $\pm$ 1.05	13.91 $\pm$ 1.08	7.37 $\pm$ 1.02	0.46	0.01	0.002
	KS4202	18.16 $\pm$ 1.23	16.83 $\pm$ 1.97	17.80 $\pm$ 1.09	0.45	0.83	0.58

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.10.** Means  $\pm$  SEM of seed number of KS4202 and K03-4686 infested and control at V1 and V3 stages (study 2).

Stage of Infestation	Genotype	Seed Number					
		C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i> <sup>a</sup>		
					C x LCAD	C x HCAD	LCAD x HCAD
V1	K03-4686	89.50 $\pm$ 8.38	62.71 $\pm$ 5.98	57.85 $\pm$ 6.16	0.07	0.02	0.78
	KS4202	128.41 $\pm$ 8.04	105.55 $\pm$ 11.55	94.28 $\pm$ 15.05	0.14	0.04	0.50
V3	K03-4686	118.35 $\pm$ 7.04	124.28 $\pm$ 7.17	84.33 $\pm$ 12.18	0.65	0.008	0.007
	KS4202	124.00 $\pm$ 8.13	125.10 $\pm$ 8.67	136.00 $\pm$ 5.66	0.92	0.29	0.34

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.11.** Means  $\pm$  SEM of single seed weight of KS4202 and K03-4686 infested and control at V1 and V3 stages (study 2).

Stage of Infestation	Genotype	Single Seed Weight (g)					
		C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i> <sup>a</sup>		
					C x LCAD	C x HCAD	LCAD x HCAD
V1	K03-4686	0.104 $\pm$ 0.006	0.098 $\pm$ 0.008	0.079 $\pm$ 0.011	0.57	0.03	0.12
	KS4202	0.136 $\pm$ 0.006	0.143 $\pm$ 0.005	0.130 $\pm$ 0.009	0.43	0.66	0.25
V3	K03-4686	0.105 $\pm$ 0.006	0.112 $\pm$ 0.006	0.092 $\pm$ 0.005	0.47	0.21	<0.0001
	KS4202	0.147 $\pm$ 0.005	0.130 $\pm$ 0.008	0.130 $\pm$ 0.006	0.07	0.06	0.97

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.12.** Means  $\pm$  SEM of total pod weight of KS4202 and K03-4686 infested and control at V1 and V3 stages (study 2).

Stage of Infestation	Genotype	Total Pod Weight (g)					
		C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i> <sup>a</sup>		
					C x LCAD	C x HCAD	LCAD x HCAD
V1	K03-4686	15.18 $\pm$ 1.67	9.12 $\pm$ 3.06	10.73 $\pm$ 0.86	0.04	0.05	0.61
	KS4202	25.70 $\pm$ 1.81	22.15 $\pm$ 2.57	17.97 $\pm$ 2.57	0.20	0.01	0.17
V3	K03-4686	19.89 $\pm$ 1.51	21.92 $\pm$ 1.51	12.65 $\pm$ 1.65	0.51	0.01	0.003
	KS4202	27.13 $\pm$ 1.94	25.28 $\pm$ 2.68	27.04 $\pm$ 1.72	0.48	0.97	0.50

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

Treatment means significantly different at  $P < 0.05$  by LSD test

**Table 2.13.** Means  $\pm$  SEM of single pod weight of KS4202 and K03-4686 infested and control at V1 and V3 stages (study 2).

Stage of Infestation	Genotype	Single Pod Weight (g)					
		C <sup>a</sup>	LCAD <sup>b</sup>	HCAD <sup>c</sup>	<i>P-value</i> <sup>a</sup>		
					C x LCAD	C x HCAD	LCAD x HCAD
V1	K03-4686	0.285 $\pm$ 0.02	0.203 $\pm$ 0.03	0.142 $\pm$ 0.02	0.02	0.0001	0.08
	KS4202	0.429 $\pm$ 0.02	0.425 $\pm$ 0.01	0.375 $\pm$ 0.02	0.83	0.17	0.13
V3	K03-4686	0.295 $\pm$ 0.01	0.321 $\pm$ 0.02	0.231 $\pm$ 0.01	0.47	0.06	0.01
	KS4202	0.462 $\pm$ 0.02	0.407 $\pm$ 0.02	0.407 $\pm$ 0.02	0.08	0.08	0.99

<sup>a</sup> C: Control (uninfested)

<sup>b</sup> LCAD: Low cumulative aphid-days

<sup>c</sup> HCAD: High cumulative aphid-days

Treatment means significantly different at  $P < 0.05$  by LSD test

## CHAPTER III

### **Economic Injury Levels for Soybean Aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), on the Soybean Aphid Tolerant KS4202 Soybean, *Glycine max* (L.) Merrill**

#### **Introduction**

The soybean aphid (*Aphis glycines* Matsumura), is an Asian pest that has become economically important for North American soybean (*Glycine max* (L.) Merrill) since its introduction in 2000 (Venette and Ragsdale 2004). At the time, there were no aphid species that colonized soybean in North America, so the niche was open and was quickly filled by the soybean aphid. Currently, the insect is present in the north central and eastern portion of the major North American soybean production region, including 30 states in the United States, and three Canadian provinces (Ragsdale et al. 2011). The severity of infestations fluctuates considerably within location and growing seasons (Johnson et al. 2008).

Soybean aphids have a complex life cycle, known as heteroecious holocyclic, where the insects alternate sexual reproduction on its primary and secondary hosts. In North America, common buckthorn (*Rhamnus spp.*) is considered the primary overwintering hosts of this aphid (Voegtlin et al. 2005b). Soybean is the alternative host, where females feed and reproduce in the absence of males (i.e. parthenogenic viviparae) during the most of the growing season (Ragsdale et al. 2004). Under favorable conditions, asexual reproduction allows population to grow dramatically. McCornack et al. (2004) reported that in controlled environments, soybean aphid population can double in as little as 1.5 d. However, climate, host plant quality, natural enemies and disease decrease doubling time to 5 – 6 days (Ragsdale et al. 2007).

The feeding damage caused by soybean aphids has a significant economic impact on soybean's yield. Reductions in plant height, pod development, number of seeds and oil content may cause yield reductions to exceed 40% (DiFonzo and Hines 2002, Ragsdale et al. 2007, Beckendorf et al. 2008). Prior to its introduction, insecticide treatment for soybean insect pests was uncommon in the north central U.S. soybean production region, but soon after its introduction, soybean aphid infestation resulted in a sharp increase in pesticide applications for soybeans, increasing production costs by \$16-33/ha and encouraging researchers to develop sustainable management alternatives (Ragsdale et al. 2007, NASS 2015).

Integrated pest management (IPM) is a well-established strategy for managing agronomic insect-pest populations (Pedigo et al. 1986), and has been identified as the most cost-efficient tool to reduce soybean aphid outbreaks (Johnson et al. 2009, Ragsdale et al. 2011). The economic threshold (ET) and economic injury level (EIL) are key concepts of IPM. ET and EIL for soybean aphids were determined based on the population growth rate of a 3- year multi-state project (Ragsdale et al. 2007), and considering control costs, market values and expected yield, the average estimated ET was 273 aphids per soybean plant. Once aphid densities reach the ET, and there is evidence that the population is increasing, treatment is recommended to avoid reaching the EIL (average of 674 aphids/plant). The treatment window based on the soybean aphid growth rate is approximately 7 days, requiring that growers remain alert and mobilize the necessary resources to avoid economic losses.

Host-plant resistance (HPR) is an important component of soybean aphid IPM. Currently, five soybean genes have been reported to provide some level of resistance to soybean aphids (Hill et al. 2006a, b, Zhang et al. 2010, Jun et al. 2013). Named *Rag* genes (Resistance to *Aphis glycines*), these genes negatively impact soybean aphid biology (antibiosis) and under certain

circumstances (e.g. genotype and gene type), may affect the insect's host preference (antixenosis). In the United States, varieties that contain *Rag1* and *Rag2* are available for growers, and can be commercialized either as single gene or pyramided (both genes). However, *Rag* soybeans have been threatened by the presence of three virulent soybean aphid populations (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013b), emphasizing the need of additional sources of HPR.

Tolerance is a category of HPR conferred by polygenic traits, which enables plants to withstand insect feeding without excessive yield losses (Smith 2005). The deployment of tolerant germplasm can benefit IPM programs in several ways. It results in a higher EIL and may allow the adoption of a higher ET, resulting in fewer insecticide applications and greater cost-effectiveness. Additionally, tolerant plants do not impose the same levels of selection pressure as antibiotic or antixenotic plants, minimizing the appearance of biotypes (Smith 2005). It's more compatible with biological agents, reducing soybean aphid outbreaks (Costamagna and Landis 2006, Schmidt et al. 2008) and maintaining populations below the ET.

Studies have reported that the soybean genotype KS4202 has moderate levels of tolerance to soybean aphids in both vegetative and reproductive stages (Pierson et al. 2010, Marchi 2012). Furthermore, field evaluations that included KS4202 have reported yield losses of 13% at a range of 35,000 - 50,000 cumulative aphid-days (CAD) (Prochaska et al. 2013), when, at that same level, Ragsdale et al. (2007) estimated approximate yield reductions of 24-36%. Considering these findings, deployment of tolerant soybeans to manage soybeans aphids requires refinement of current EILs. Thus, the objective of this research was to quantify the relationship between CAD and yield loss in the tolerant KS4202 and discuss the use of tolerance in soybean aphid IPM.



## Materials and Methods

**Agronomic practices.** The field studies were conducted in 2011 and 2013 at the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory, Concord, NE. In both growing seasons, soybeans were planted in a corn-soybean rotation in an Alcester-silt loam soil. Soil was disked prior to planting, following agronomic practices for northeastern Nebraska. Soybean seeds were planted at a density of 430,000 seeds/ha. Due to the wet conditions in May, and because soybean aphids are attracted to late planted soybeans, planting occurred on June 3, 2011 and June 11, 2013. In the first season, plots were not irrigated, as the irrigation system was inoperative, whereas plots in 2013 were irrigated via lateral irrigation system. Weeds were controlled with Flexstar GT® (Syngenta Crop Protection, Greensboro, NC), and Select Max™ (Valent U.S.A Corporation, Walnut Creek, CA) herbicides on May 3, in 2011. In 2013, Valor® (Valent U.S.A Corporation), 2,4-D ester and Durango® (DowAgroSciences LCC, Indianapolis, IN) were applied on 29 April, and Cadet® (FMC Corporation, Philadelphia, PA), Flexstar GT and Select max were applied on 18 June.

**Field plot design.** The experimental design for each year was a complete randomized block with four replications. Each plot consisted of four rows, measuring 15.24 m (50 ft) long and 3 m (10 ft) wide with 76.2-cm row spacing. There were five cumulative aphid-days (CAD) treatments designed for each season. Data collection was taken from the two center rows of each plot. Cumulative aphid-days provides a good estimation of aphid pressure over time, and is more informative than aphid number. CAD was calculated using the formula:  $\sum_{i=1}^n [(x_i + x_{i-1})/2 + (t_i - t_{i-1})]$  [equation1], where  $n$  is the number of sample dates,  $x_i$  is the mean number of aphids per plant (i.e. average per plot) on sample date  $i$ , and  $(t_i - t_{i-1})$  is the number of

days between two consecutive sample dates (Hanafi et al. 1989). In 2011, the CAD treatments were 0 CAD (control = aphid free), 3,000, 8,000, and 13,000 and untreated (=not treated with insecticide), whereas in 2013 they were 0 (control) 5,000, 13,000, and 22,000 and untreated. The untreated plots were designed to simulate soybean aphid population cycle. Once the desired treatment was achieved (average across the blocks), a foliar insecticide lambda-cyhalothrin at 28.0 g ([AI]/ha) (Warrior with Zeon Technology®, Syngenta Crop Protection) was applied using ground equipment. Although the complete eradication of aphids is not feasible, regular monitoring of the control plots (0 CAD) and CAD targeted plots was conducted to ensure that populations remained close to zero aphids per plant. Insecticide interventions were performed as needed.

**Aphid infestation, evaluations and harvest.** Soybean aphids naturally occurred and colonized soybeans in 2011. In 2013, populations of soybean aphids were low and intermittent in northeast Nebraska, so plots were artificially infested. As a precautionary measure due to low aphid population in 2012, two meshed cages measuring 2 x 2 x 2 m were installed in an adjacent soybean field in June. Aphid infested plants from a laboratory colony were introduced in the cages for population expansion and acclimatization prior to artificial infestation. The aphids used for the artificial infestation were from a colony maintained in a growth chamber ( $23 \pm 2^\circ\text{C}$  and 16:8 h [L: D]), and were progeny of a Nebraska isolate (biotype 1), collected in a nearby commercial field in 2011. The artificial infestation occurred on July 30 2013 to mimic a typical infestation of soybean aphids in northeast Nebraska. Leaf sections containing 10-50 aphid nymphs and adults were placed approximately 60 cm apart on the top trifoliolate of one soybean plant in the two center rows.

Evaluations were performed every five to seven days after the initial detection or artificial introduction of soybean aphids and were terminated once the number of insects per plant was close to zero. In each plot, five plants were destructively sampled for estimating aphid densities. Once the targeted CAD treatment levels were reached, plots were sprayed within 48 h.

At maturity, 10 plants from the treatment rows (two center rows) from each plot were manually cut at the base of the stem and stored in a cold walk-in chamber for further processing. The sampled material was oven-dried and the following yield parameters were determined: number of pods per plant, number of seeds per pod, average seed weight, average pod weight, and total biomass (Hill et al. 2004). The treatment rows were harvested on October 4, 2011 and October 29, 2013 with a small plot combine, and yield was adjusted to seed moisture of 13%.

Aphid population growth and EIL calculation. Soybean aphid population growth rates ( $r$ ) across 2011 and 2013 were calculated within the time interval of when 80% of the plants were infested and populations reached peak densities (Ragsdale et al. 2007). Aphid densities were natural log transformed and graphed against time to determine the growth rate (i.e. slope of the linear regression). In addition, discrete daily growth rate ( $\lambda$ ) was calculated based on the average of both growing seasons, using the expression:  $\lambda = e^r$  [equation 2]

The EIL was calculated using the slope obtained from the regression curve built with the targeted CAD treatments for both years and the corresponding proportion maximum yield. The equation [3] for EIL was:

$$\frac{C}{VxDxIxK}$$

where,  $C$  is the treatment cost (\$/ha) of soybean aphid infested fields, which includes insecticide market price and associated costs with equipment and labor;  $V$  is the crop value (\$/ton);  $K$  is the expected rate of control (proportion);  $D$  is measure of loss of value (\$) per unit on a per insect

basis; and  $I$  is the yield reduction (ton/ha) per pest injury. The control costs used in this study were based on the survey conducted by Ragsdale et al. (2007). The crop value was determined based on the current US soybean prices by the National Agricultural Statistical Services (NASS 2016). For comparisons purposes, soybean prices reported in Ragsdale et al. (2007) were also included in this calculation. The approximate rate of insecticide control ( $K$ ) for soybean aphids is considered high. For convenience, this parameter was set to 1 (100% control).

The EIL expressed in CAD was calculated based on the equation provided by a linear regression of percentage of maximum yield (relative to control plots) and CAD. Yield loss per CAD injury ( $D \times I$ ) was obtained from the slope of the linear regression. The conversion of EIL in CAD to aphids per plant ( $I$ ) proceeded as outlined by Ragsdale et al. (2007) with the expression  $I = \frac{s(\lambda-1)+1}{\lambda}$  [equation 4], where  $s$  is the EIL in CAD (per plant) and  $\lambda$  is the discrete daily population growth rate. The time (in days) that a given population feeding on KS4202 would require to reach the EIL once the ET (average of 273 aphids per plant from Ragsdale et al. 2007) was calculated with the population growth model:  $N_t = N_0 e^{rt}$  or  $\ln(N_t) = \ln(N_0) + rt$  [equation 5], where  $N_0$  is the initial aphid density,  $r$  is the population growth rate, and  $t$  is time expressed in days.

**Statistical Analysis.** An analysis of variance (ANOVA) was used to analyze yield parameters and plot yield in PROC GLIMMIX in SAS 9.3 (SAS Institute, Cary, NC). Experimental treatments for both seasons were treated as fixed factors, whereas replication blocks nested within experimental runs were treated as random factors. Means were separated when the interaction or main effect was significant ( $P < 0.05$ ). The results presented for each growing season were originated from the same mixed model analysis.

To evaluate the treatment effect (CAD) and percentage of maximum yield for KS4202, an “F-test” was performed in R version 2.15.1 (R Foundation for Statistical Computing, Wien, Austria), according to Ritz and Streibig (2008). This statistical analysis computes the difference between residual sum of squares (RSS) for two considered models. The models need to be fitted to the data: a full model (FULL) and a sub-model (SUB) of the full model. In the full model, the CAD treatments for each year were estimated separately, whereas the sub-model estimated the parameters for a single model fit to the data of all treatments combined. Models were fitted to the data and parameters estimated using the nls function of R (version 2.15.1, R Foundation). The following equation represents the F-test performed:

$$\frac{(RSS_{sub} - RSS_{full}) / (df_{sub} - df_{full})}{RSS_{full} / df_{full}}$$

where  $RSS_{sub}$  and  $RSS_{full}$  indicate the minimized RSS for the CAD and yield estimates of the sub-model and full model, respectively;  $DF_{sub}$  and  $DF_{full}$  represent the degrees of freedom for the sub-model and full model, respectively. A large F-value indicates that two nested models are different, whereas a small F-value indicates that both models provide similar fit to the data. Next, the F-value was converted to a P-value from the F-distribution ( $df_{SUB} - df_{FULL}$ ,  $df_{FULL}$ ). A significant analysis ( $P < 0.05$ ) indicates that models are statistically different. Under this circumstance, full model can be used along with the parameters for each treatment level, whereas a non-significant test ( $P > 0.05$ ) indicates that nested models are not different and that a sub-model may be used.

## Results and Discussion

**Soybean aphid population density and cumulative aphid-days (CAD).** The economic threshold established for soybean aphids of 273 insects per plant (Ragsdale et al. 2007) was surpassed in all treatments in 2011 and 2013, with the exception of 0 CAD (control = aphid-free) treatment (Fig. 3.1 and 3.2). Infestation began in late July when plants were in the early reproductive stage (R1/R2) (Fehr and Caviness 1977). In the untreated plots, where soybean aphids were allowed to colonize soybeans throughout the season, the mean peak aphid number for both seasons was  $2,513 \pm 594$ , and ranged from 1,918 to 3,108 aphids per plant. Aphid peak density occurred on August 18, 2011 and September 6, 2013 when KS4202 plants were within R4 stage (full size pods) and R5 stage (beginning seed).

Soybean aphid growth rate and discrete daily increase rate were consistent for the two seasons (Table 3.1), resulting in population doubling time of 9.64 days and 9.13 days, in 2011 and 2013, respectively. Peak aphid numbers in 2013 (Fig. 3.1b) were generally lower than 2011 (Fig. 3.1a), however, aphid infestation was prolonged in 2013 (Fig. 3.2a and 3.2b). In 2011, the targeted CAD treatments of 0, 3,000, 8,000 and 13,000 had an actual CAD mean of  $163 \pm 13$ ;  $4,354 \pm 405$ ;  $8,313 \pm 506$  and  $13,776 \pm 1,044$ , respectively. The actual CAD mean in 2013 for the treatments of 0, 5,000, 13,000 and 22,000 CAD were  $542 \pm 62$ ;  $5,458 \pm 330$ ;  $12,138 \pm 234$  and  $22,303 \pm 2779$ . In the untreated treatment, where soybean aphids were allowed to colonize KS4202 throughout the season, CAD reached  $44,959 \pm 4,148$  in 2011 and  $38,174 \pm 4,790$  in 2013.

**KS4202 yield response to soybean aphids.** There were no differences in total yield among 0 (control), 3,000 8,000 and 13,000 CAD treatments in 2011. However, untreated plots had a yield reduction of 13.33%, which was statistically different than the remaining treatments

(Table 3.2a). A similar pattern occurred in 2013, although there was not a significant difference in total yield among the treatments, even when soybean aphids were allowed to colonize field throughout the season (Table 3.2b). In the untreated plots, yield was reduced by 12.60% when compared to 0 CAD treatments ( $P = 0.06$ ), which is also consistent with the data from the previous season.

Yield parameters were also evaluated to investigate how KS4202 compensates for aphid feeding injury. In 2011 (Table 3.3), total pod weight and total seed weight, total plant biomass for CAD treatments of 3,000, 8,000 and 13,000 were not statistically different than 0 CAD (control) treatment. However, there was a significant reduction in those parameters when compared to untreated plots, at which cumulative levels were near 45,000 AD. There were no significant differences in number of pods per plant between the 8,000 and 13,000 CAD and 0 CAD plots, although plants from 3,000 CAD and untreated treatments had significantly fewer pods than the 0 CAD treatments (Table 3.3). Untreated ( $P = 0.01$ ) and 3,000 CAD ( $P = 0.08$ ) treatments also had significantly fewer seeds than 0 CAD treatment. Furthermore, the single seed weight for the 8,000 and 13,000 CAD treatments did not differ from 0 CAD treatment, but untreated treatment produced smaller seeds than 0 CAD treatment ( $P = 0.01$ ). Seeds from 3,000 CAD plots were approximately 8% heavier than seeds from control plot (Table 3.3), indicating that plants exposed to this treatment may be compensating for a reduction in seed number by producing heavier seeds and thus no differences were observed in total yield (Table 3.2).

Total biomass, number of pods, pod weight, number of seeds and total seed weight were not significantly different among any of the treatments in 2013 (Table 3.4), although single seed weight for untreated plots (CAD ~38,000) was significantly lower than 0, 5,000, 13,000 and 22,000 CAD treatments.

KS4202 tolerance to soybean aphids was initially documented in greenhouse studies (Pierson et al. 2010, Marchi 2012). Pierson et al. (2010) examined tolerance in the reproductive stages of KS4202, and found no impact on the average seed weight or number of seeds per pod in the presence of soybean aphids. Marchi (2012) also reported KS4202 tolerance in the early vegetative and reproductive stages, where most of the yield parameters for plants infested during the V3 and R1 stages were unaffected at 1,000 or 2,000 aphids per plant (corresponding range of 4,500 - 8,000 CAD). In field trials, Prochaska et al. (2013) corroborated the presence of tolerance in KS4202. Their research included multiple field seasons, and also found that KS4202 tolerated soybean aphid feeding without the expected severe impact on yield.

To standardize the yield data from both years and permit a direct statistical comparison, the proportion of maximum yield (relatively to 0 CAD treatment) was calculated (Fig. 3.3). An F-test indicated there was no significant difference in the proportion of maximum yield by CAD across seasons ( $P = 0.39$ ), so 2011 and 2013 were included in one model. An inverse relationship between CAD and yield was detected (Fig. 3.3;  $F = 23.91$ ;  $df = 1, 38$ ;  $R^2 = 0.37$ ;  $P < 0.0001$ ). The intercept of the equation  $y = -3.102E^{-6}x + 1.001$  passes through 100% of the proportion maximum yield (Fig. 3.3); this indicates that linear regression was adequate to explain the relationship between yield loss-CAD. No evidence of feeding by bean leaf beetle, *Cerotoma trifurcata* (Forster), or injury caused by other pests or diseases was observed, indicating that yield losses observed were caused by soybean aphid feeding.

The CAD treatment over two growing seasons in this study varied from 3,000 to 44,000. A visual comparison between CAD and proportion of maximum yield from Ragsdale et al. (2007) multi-state study and this research is provided on Fig. 3.4. Ragsdale et al. (2007) calculated that soybean yield is reduced by 6.88% for every 10,000 aphid-days accumulated for



soybean aphid susceptible soybeans. In contrast, the slope of the regression obtained for KS4202 was  $-3.102 \times 10^{-6}$ , indicating that yield was reduced by 3.10% (95% CI of 1.82 - 4.38%) for every 10,000 aphid-days accumulated (Fig. 3.4), so yield loss in KS4202 is approximately 45% of the yield loss of the susceptible soybean varieties used in the Ragsdale et al. (2007) multi-state study.

**Economic injury levels (EILs).** The EILs calculated for KS4202 ranged from 826 to 3,415 aphids per plant (CAD = 12,696 to 52,545), averaging 2,118.4 aphids per plant (CAD = 29,724.5) (Table 3.5). Considering to the current commodity prices of \$202.09 used by Ragsdale et al. (2007) and the control cost of \$16.41/ha, the EIL for KS4202 is 1,702 per plant (Table 3.5), when under the same conditions is at most 684 aphids per plant in the Ragsdale et al. (2007) multi-state study.

The establishment of an ET prevents pest populations from reaching the EIL (Pedigo et al. 1986). The ETs presented in the Ragsdale et al. (2007) multi-state study are based on the mean rate of soybean aphid population growth ( $r = 0.127$ ), and provide a lead-time of 3-7 days to arrange curative action (i.e. apply insecticide). Lead-time is particularly important with respect to soybean aphid because of the soybean aphid rapid population growth potential. Soybean aphid populations cannot only reach the EIL in a relatively short time, but also increase well beyond the EIL to levels that frequently result in yield losses >20%. However, even with a recommended lead-time of 7 days (Ragsdale et al. 2007, Hodgson et al. 2012), this can pose significant problems for farmers, where weather and scheduling delays, or even late decision-making (i.e. initiating scouting after populations reach the ET) can result in treatment well after populations reach and exceed the EIL.

The higher EILs of soybean aphid tolerant varieties, such as KS4202, can help mitigate treatment delay problems by lengthening the treatment lead-time. For example, the mean ET for soybean aphid from Ragsdale et al. (2007) is 273 aphids per plant with a corresponding mean EIL of 674 aphids per plant. The lead-time for aphid populations to increase from 273 aphids per plant to 674 aphids per plant is 7 days. For the soybean aphid tolerant KS4202, a corresponding lead-time would be on average 16 days (in this study, soybean aphid growth rate,  $r = 0.074$ , was lower than the multi-state average ( $r = 0.127$ ) but within the range reported by Ragsdale et al. (2007), so calculations used  $r = 0.127$ ), which substantially increases the lead-time designed for susceptible soybeans. The time interval between scouting and employment of control strategies is of importance especially when dealing with pests of rapid growth rates and high economic impact. While most soybean aphid management tactics are employed within 7 d of determining the need, difficulties such as inclement weather, equipment malfunction, or scheduling difficulties can delay insecticide application and result in economic loss. In this case, the advantage of using tolerant plants is the flexibility to schedule chemical control.

Although a case can be made for keeping the practical and widely adopted soybean aphid ET (250 aphids per plant) and benefiting from the more flexible insecticide application lead-time associated with a soybean aphid tolerant soybean, increasing the ET could also be argued. As a basic component of decision making in pest management, the ET is set to guide growers on when to take control action. Redefining (i.e. increasing) the ET for tolerant soybeans would result in delayed control applications and possibly fewer applications and associated costs. Although insecticide resistance has not been reported in the United States, it's crucial to consider the impacts of repeated exposure of these chemicals as aphids have a high capacity of reproduction and dispersion (McCornack et al. 2004, Zhang et al. 2008). In that sense, the use of

tolerance in general may result in reduced insecticide application. This has long-term benefits, as minimizing chemical control enhances the conservation of natural enemies. The establishment of a strong predator and parasite community enhances soybean aphid IPM, extending soybean aphid biological control even after winged forms return to the overwintering host (Yoo et al. 2005).

Future research should focus on the implementation of KS4202 as a platform to backcross antibiotic/antixenotic (single or pyramided) genes. The combination of tolerance with traits that are biologically detrimental or affect soybean aphid's host preference may provide a more stable management by keeping its population under economic damaging levels. Moreover, tolerant plants require less antibiosis or antixenosis than non-tolerant plants when considering the total effect of the resistant plant on the insect, and may be more durable (Smith 2005). Even if virulent aphid populations emerge in response of the higher pressure imposed by antibiotic and antixenotic traits, the aphid tolerant background in these plants is likely to prevent substantial yield losses.

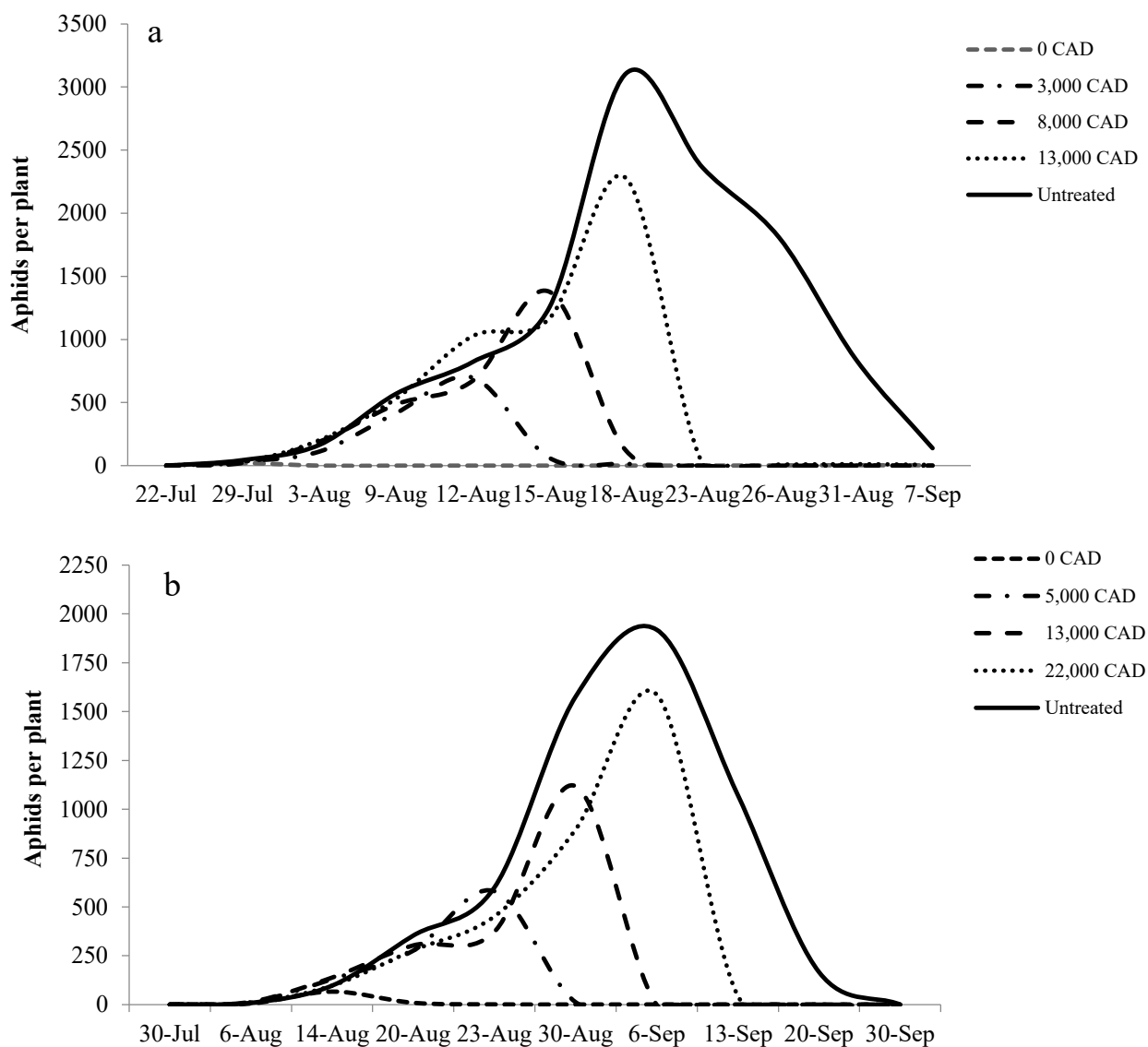
The integration of tolerant plants into IPM programs is a valuable tactic that remains underexplored. Difficulties in identifying tolerance mechanisms for incorporation in breeding programs or perhaps the ability of harboring large insect populations may have caused tolerance to receive little attention. This work represents the first attempt to develop an adequate EIL for aphid-tolerant soybeans and provides support for the proper use of KS4202 in field conditions.

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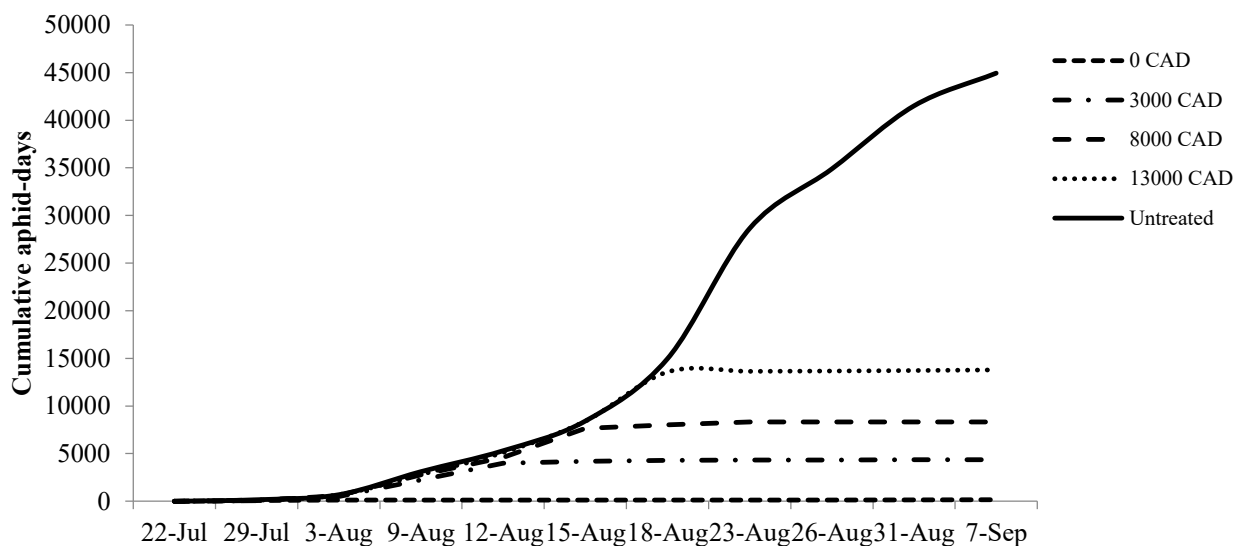
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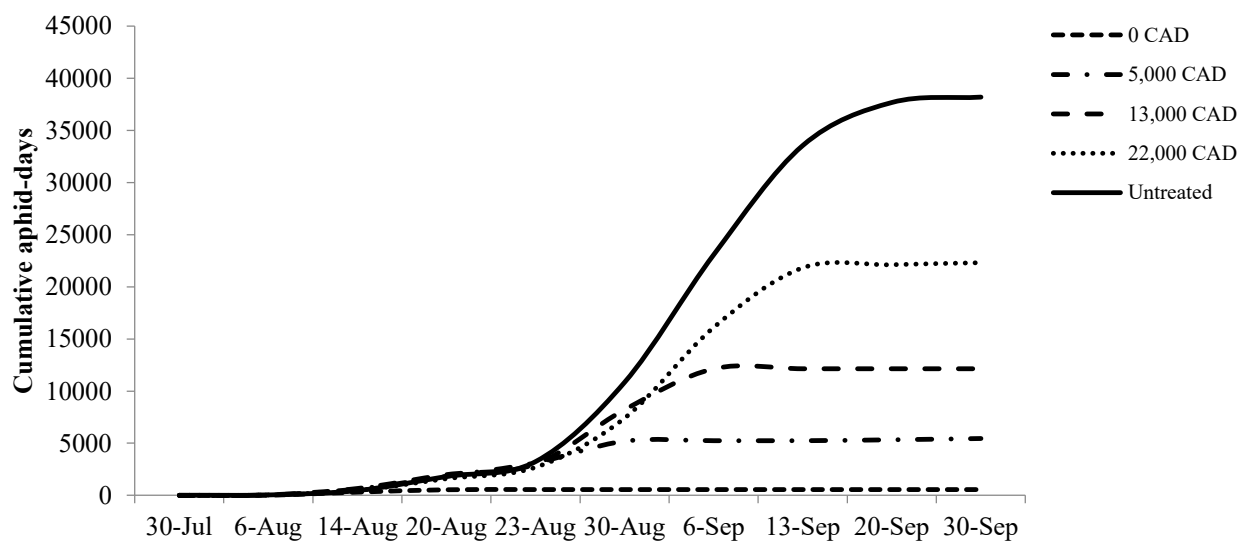
### Figures and Tables



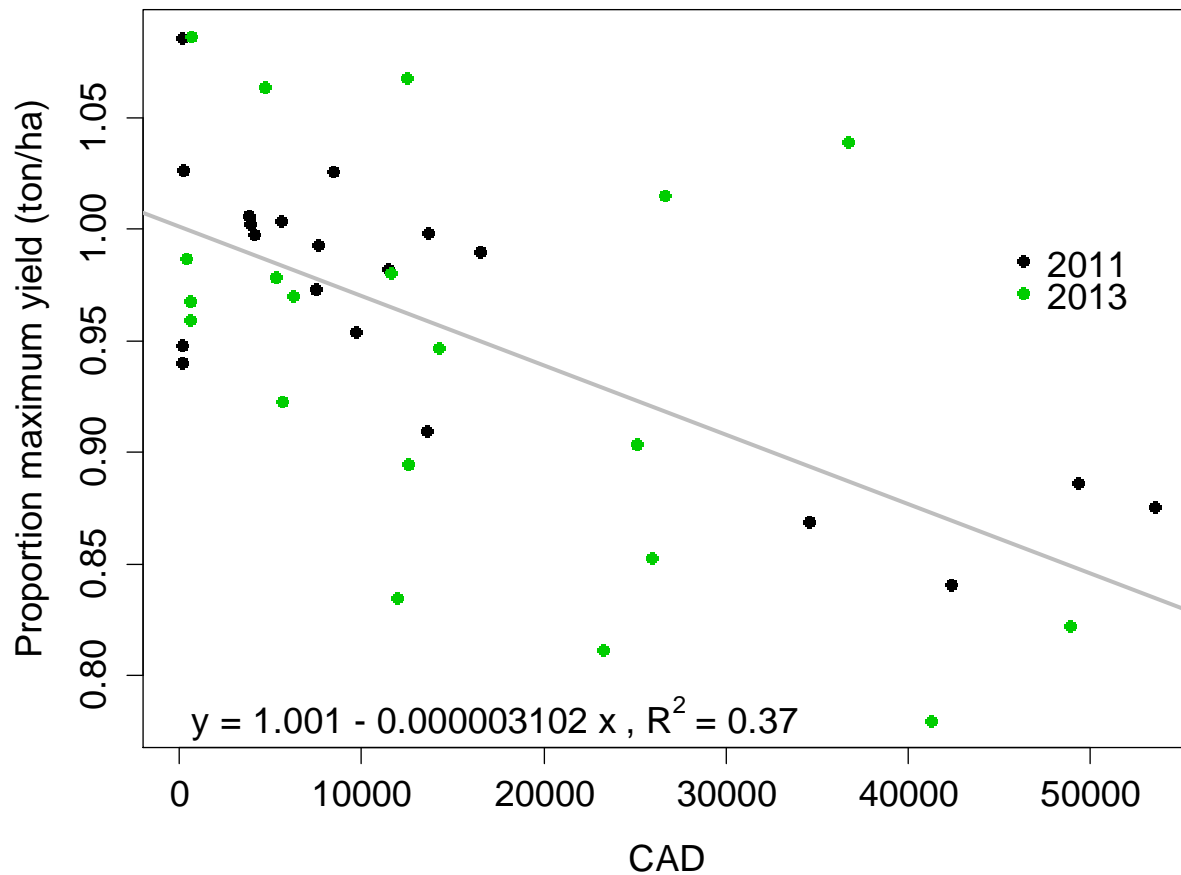
**Fig. 3.1.** Mean aphid number for KS4202 during the weekly evaluations in the growing season of 2011 (a) and 2013 (b) in each respective target CAD treatments.



**Fig. 3.2a.** Cumulative aphid-days in the target CAD treatments in 2011. Actual CAD treatment means were: 0 CAD: 163 CAD; 3,000: CAD 4,354 CAD; 8,000 CAD: 8,313 CAD; 13,000 CAD: 13,776 CAD; untreated plots: 44,958 CAD.

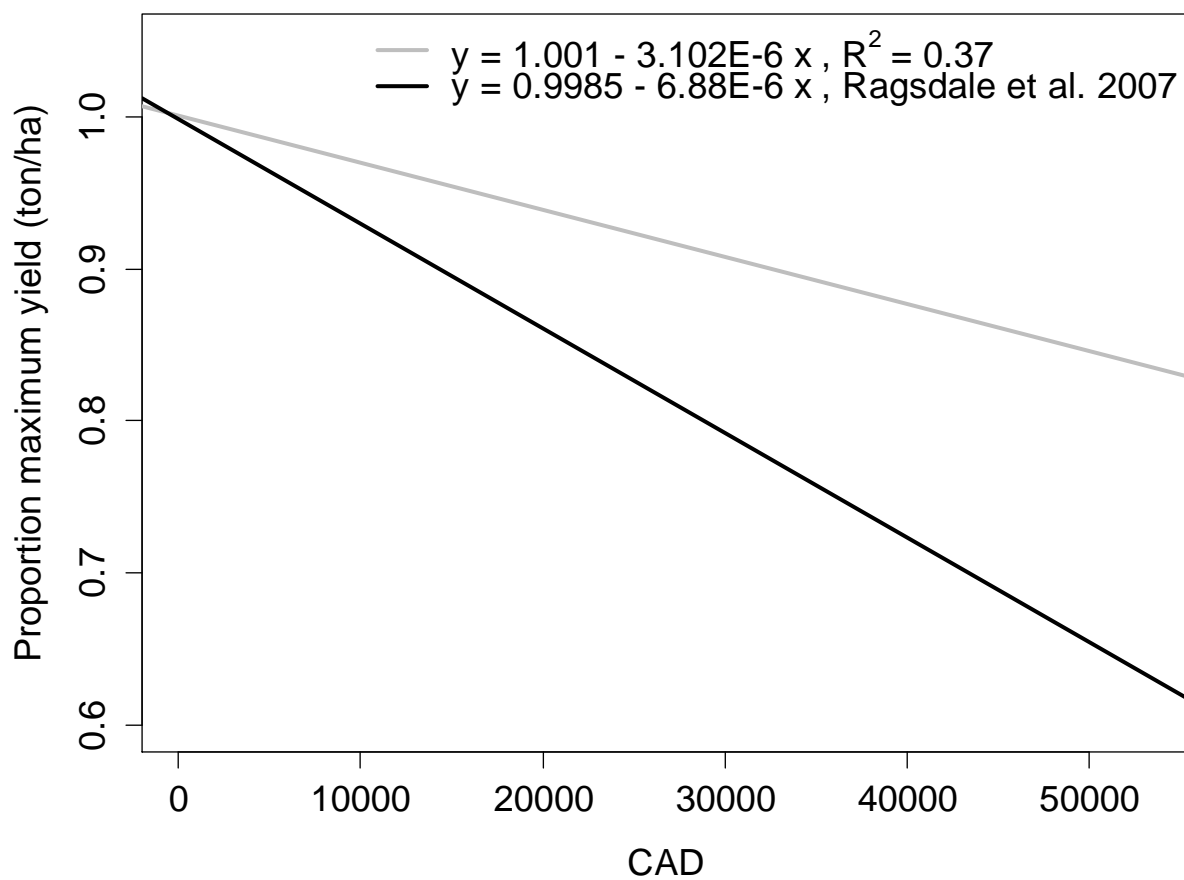


**Fig. 3.2b.** Cumulative aphid-days in the target CAD treatments in 2013. Actual CAD treatment means were: 0 CAD: 542 CAD; 5,000 CAD: 5,458 CAD; 13,000 CAD: 12,138 CAD; 22,000 CAD: 22,303 CAD; untreated plots: 38,174 CAD.



**Fig. 3.3.** Percentage of maximum yield comparing aphid-free (control) plots with the target CAD treatments in 2011 and 2013 seasons.  
 $F = 23.91$ ;  $df = 1, 38$   $P < 0.0001$





**Fig. 3.4.** Comparisons of simple regressions of proportion of maximum yield (ton/ha) and cumulative aphid-days (CAD) of soybean KS4202 and multi-state study by Ragsdale et al. (2007).

**Table 3.1.** Soybean aphid growth rate, discrete daily increase and population doubling time on KS4202 during 2011 and 2013 growing seasons.

Season	Y Intercept $\pm$ SEM	R <sup>2</sup>	Growth rate (r) $\pm$ SEM <sup>a</sup>	P	Discrete daily increase rate ( $\lambda$ ) <sup>b</sup>	DT (days) <sup>c</sup>
2011	1.742 $\pm$ 0.104	0.96	0.0719 $\pm$ 0.006	0.0004	1.0745	9.64
2013	1.188 $\pm$ 0.230	0.91	0.0759 $\pm$ 0.001	0.0003	1.0788	9.13

<sup>a</sup> Aphid population growth rate in untreated plots using the equation  $N_t = N_0 e^{rt}$ , where  $N_0$  = initial population density,  $r$  = population growth rate (linear regression slope), and  $t$  (in days) is based on the interval when 80% of the plants were infested until aphid densities reached a peak.

<sup>b</sup> Discrete daily increase rate =  $e^r$ .

<sup>c</sup> DT = Population doubling time (days);  $DT = \ln(2)/r$ .

**Table 3.2a.** Estimated yield (ton/ha) for KS4202 under different cumulative aphid-days (CAD) treatments in 2011.

Target Treatment	Mean CAD $\pm$ SE	Yield $\pm$ SE	Yield reduction (%) <sup>a</sup>
0 CAD	63 $\pm$ 13	2.85 $\pm$ 0.10 a	-
3,000 CAD	4,354 $\pm$ 405.2	2.85 $\pm$ 0.00 a	0
8,000 CAD	8,313 $\pm$ 506.9	2.81 $\pm$ 0.04 a	1.40
13,000 CAD	13,776 $\pm$ 1,044	2.76 $\pm$ 0.06 a	3.15
Untreated	44,958 $\pm$ 4,148	2.47 $\pm$ 0.03 b	13.33

Means within the same column followed by the same letter are not statistically different ( $P > 0.05$ ), LSD test.

<sup>a</sup>Yield reduction (%) relative to aphid-free (control) plots for each growing season.

**Table 3.2b.** Estimated yield (ton/ha) for KS4202 under different cumulative aphid-days (CAD) treatments in 2013.

Target Treatment	Mean CAD $\pm$ SE	Yield $\pm$ SE	Yield reduction (%) <sup>a</sup>
0 CAD	542 $\pm$ 62	3.49 $\pm$ 0.09 a	-
5,000 CAD	5,458 $\pm$ 330	3.43 $\pm$ 0.10 a	1.72
13,000 CAD	12,138 $\pm$ 234	3.29 $\pm$ 0.18 a	5.73
22,000 CAD	22,303 $\pm$ 2,779	3.21 $\pm$ 0.15 a	8.02
Untreated	38,174 $\pm$ 4,790	3.05 $\pm$ 0.20 a	12.60

Means within the same column followed by the same letter are not statistically different ( $P > 0.05$ ), LSD test.

<sup>a</sup>Yield reduction (%) relative to aphid-free (control) plots for each growing season.

**Table 3.3.** Yield parameters of KS4202 under different CAD treatments harvested in 2011.

<b>Treatment <sup>a</sup></b>	<b>Total biomass/plant (g)</b>	<b>No. of pods/plant</b>	<b>Total pod weight/plant (g)</b>
0 CAD	18.87 ± 1.52 a	38.10 ± 3.95 a	11.95 ± 0.97 a
3,000 CAD	17.40 ± 0.95 a	32.78 ± 1.55 bc	11.21 ± 0.70 a
8,000 CAD	18.84 ± 0.80 a	37.48 ± 2.01 a	11.92 ± 0.56 a
13,000 CAD	17.62 ± 0.52 a	35.70 ± 1.91 ab	11.37 ± 0.39 a
Untreated	14.76 ± 0.51 b	30.98 ± 0.94 c	9.11 ± 0.31 b

<b>Treatment <sup>a</sup></b>	<b>No. of seeds/plant</b>	<b>Total seed weight/plant (g)</b>	<b>Single seed weight (g)</b>
0 CAD	74.38 ± 6.59 a	8.34 ± 0.60 a	0.113 ± 0.002 b
3,000 CAD	65.93 ± 3.97 ab	7.95 ± 0.51 a	0.121 ± 0.001 a
8,000 CAD	74.28 ± 3.63 a	8.38 ± 0.35 a	0.113 ± 0.003 b
13,000 CAD	71.18 ± 3.58 a	7.98 ± 0.26 a	0.113 ± 0.003 b
Untreated	58.68 ± 0.84 b	6.29 ± 0.25 b	0.107 ± 0.003 c

Means within the same column followed by the same letter are not statistically different ( $P > 0.05$ ), LSD test

<sup>a</sup> Treatment column indicates target treatments; Actual CAD treatment means were: 0 CAD: 163 CAD; 3,000: CAD 4,354 CAD; 8,000 CAD: 8,313 CAD; 13,000 CAD: 13,776 CAD; untreated plots: 44,958 CAD.

**Table 3.4.** Yield parameters of KS4202 under different CAD treatments harvested in 2013.

<b>Treatment <sup>a</sup></b>	<b>Total biomass/plant (g)</b>	<b>No. of pods/plant</b>	<b>Total pod weight/plant (g)</b>
0 CAD	31.52 ± 3.41 a	53.50 ± 5.31 a	25.18 ± 2.88 a
5,000 CAD	33.46 ± 2.90 a	56.70 ± 5.15 a	27.15 ± 2.52 a
13,000 CAD	32.79 ± 3.46 a	56.25 ± 6.10 a	26.37 ± 3.04 a
22,000 CAD	34.78 ± 4.43 a	59.08 ± 7.34 a	28.07 ± 3.91 a
Untreated	32.87 ± 3.63 a	61.93 ± 6.53 a	26.61 ± 3.23 a

<b>Treatment <sup>a</sup></b>	<b>No. of seeds/plant</b>	<b>Total seed weight/plant (g)</b>	<b>Single seed weight (g)</b>
0 CAD	111.69 ± 11.75 a	19.54 ± 2.23 a	0.173 ± 0.005 a
5,000 CAD	121.03 ± 11.49 a	21.05 ± 1.93 a	0.175 ± 0.005 a
13,000 CAD	118.90 ± 13.30 a	20.46 ± 2.33 a	0.173 ± 0.004 a
22,000 CAD	126.13 ± 16.61 a	21.79 ± 2.98 a	0.173 ± 0.005 a
Untreated	127.95 ± 14.55 a	20.28 ± 2.47 a	0.158 ± 0.005 b

Means within the same column followed by the same letter are not statistically different ( $P > 0.05$ ), LSD test.

<sup>a</sup> Treatment column indicates target treatments; Actual CAD treatment means were: 0 CAD: 542 CAD; 5,000 CAD: 5,458 CAD; 13,000 CAD: 12,138 CAD; 22,000 CAD: 22,303 CAD; untreated plots: 38,174 CAD.

**Table 3.5.** Economic injury levels (EILs) for soybean aphids on tolerant KS4202 soybean.

<b>Soybean price (\$/ton)</b>	<b>Control cost (\$/ha)</b>	<b>EIL Cumulative aphid- days</b>	<b>EIL Aphids per plant</b>
202.09	16.41	26,177	1,702
	24.51	39,098	2,541
	32.94	52,545	3,415
220.46	16.41	23,995	1,560
	24.51	35,840	2,330
	32.94	48,167	3,131
238.83	16.41	22,150	1,440
	24.51	33,083	2,150
	32.94	44,462	2,890
376.66	16.41	14,044	913
	24.51	20,977	1,364
	32.94	28,192	1,833
416.66	16.41	12,696	826
	24.51	18,963	1,233
	32.94	25,485	1,657

## CHAPTER IV

### Expression Profiling of Constitutive and Induced Defense-Related Transcripts in Soybean Aphid Tolerant and Susceptible Soybean

#### Introduction

The soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae) became the most economically important pest of soybean [*Glycine max* (L.) Merrill] in the North Central region of the United States (Ragsdale et al. 2011). It has been estimated that US\$2.36 to 3.7 billion may be lost due to soybean aphid from 2003 to 2017 (Song and Swinton 2009). While chemical control is still the most common method to manage the soybean aphid, host plant resistance (HPR) has received considerable attention (Ragsdale et al. 2011).

Three functional categories of HPR were proposed by Painter (1951): antibiosis, antixenosis and tolerance. Antibiosis may result from physical or biochemical properties that adversely affect pest biology (Panda and Khush 1995, Smith 2005). Antixenosis resistance (non-preference) negatively affects pest behavior through physical barriers and/or repellents. The effect of antixenosis reduces host colonization by a pest, which ultimately chooses another host plant for feeding, ovipositing or mating (Painter 1951, Smith 2005). Most soybean genotypes expressing antibiosis and antixenosis resistance to the soybean aphid were linked to a single dominant gene, named *Rag* (Hill et al. 2004, Mensah et al. 2005, Hesler et al. 2007, Mian et al. 2008, Zhang et al. 2009, 2010). Currently, soybean expressing *Rag1* and *Rag1+Rag2* (pyramid) genes are commercially

available; however, the sustainable deployment of these antibiotic traits faces considerable limitations due to the quick emergence of soybean aphid biotypes (Kim et al. 2008, Hill et al. 2010, McCarville and O'Neal 2012, Alt and Ryan-Mahmutagic 2013b).

Tolerance is a result of a network of compensatory features, which allows a host plant to withstand arthropod feeding and still yield significantly more biomass than a given susceptible host (Smith 2005). This category of resistance is particularly interesting for integrated pest management (IPM), as it exerts minimal negative impacts on the targeted arthropod pest as well as most natural enemies (Espinosa and Fornoni 2006). Due to their compensatory mechanism, tolerant plants have a higher economic injury level, which may delay or reduce the need of insecticide treatments (Panda and Khush 1995). Moreover, pests that feed on a tolerant plant likely experience lower selection pressure than those on an antibiotic or antixenotic plant; therefore, the likelihood of a biotype emergence is minimized (Smith 2005). Despite these advantages, the absence of detailed knowledge in the mechanisms and genetics underlying plant tolerance limits its incorporation in breeding programs and IPM.

The current literature on compatible and incompatible interactions between different plants and several aphid species has uncovered that plants defend against these insects using a variety of defense signaling pathways. These pathways may be dependent on several hormonal pathways, including but not limited to salicylic acid (SA), jasmonic acid (JA) and ethylene (Eth) signaling molecules (Howe and Jander 2008, Kerchev et al. 2012, Smith and Chuang 2014). Plants receptors recognize aphid-feeding probe via elicitors, which may be derived from an aphid's saliva or even products of endosymbiotic



bacteria (Lapitan et al. 2007). Upon aphid probing, a cascade of defense reactions occurs via the recognition of aphid elicitors, where calcium- and reactive oxygen species (ROS)-related signaling play important roles in trigger the activation of defense pathways such as JA, SA and Eth (Park et al. 2006, Kerchev et al. 2012).

SA mediates localized plant tissue hypersensitive and systemic acquired responses, and induces the expression of defense responsive transcripts, including *pathogenesis-related* (PR) genes and PR proteins. Studies found that aphid-resistant wheat had synthesized a higher amount of PR proteins comparatively to susceptible wheat (van der Westhuizen et al. 1998b, a). In soybean, PR1 was highly expressed in aphid resistant *Rag1* plants infested with soybean aphids, when no changes occurred in the susceptible genotype (Li et al. 2008, Studham and MacIntosh 2013). Studies have also shown that phloem feeding insects may induce JA-associated transcripts as well, although this pathway is better characterized in plants stressed by chewing insects (Mewis et al. 2005). Aphid-infested wheat and barley have also induced JA-associated transcripts including lipoxygenases (LOX), coronatine-insensitive1 (COI1), OPR (12-oxophytodienoate reductase) and cytochrome P450 (Boyko et al. 2006, Smith et al. 2010).

The orchestration of defense pathways of plant tolerance to aphids has not been well explored. So far, the limited amount of studies available indicated that constitutive and aphid induced hormonal pathways as well as oxidative enzymes play an important role in host plant tolerance. In wheat, tolerance to Russian wheat aphid (*Diuraphis noxia*) was associated with induced expression of JA, ethylene (ET) and auxin-signaling genes (Smith et al. 2010); whereas tolerance to the same insect in barley appeared to be

controlled by the constitutive expression of JA, ET- and auxin-mediated defense (Marimuthu and Smith 2012).

In previous studies, plant tolerance (reduced yield loss relative to a susceptible plant) to the soybean aphid was observed in the soybean genotype KS4202 (Pierson et al. 2010, Prochaska et al. 2013). Defensive mechanisms in this genotype may be composed of metabolic changes that involve oxidative enzymes and a primed photosynthetic system (Pierson et al. 2011, Marchi-Werle et al. 2014). Functional transcriptomic approaches revealed a wide variety of responses induced by soybean aphids in KS4202, including the overexpression of peroxidases, cytochrome P450, and WRKY transcription factors in the tolerant soybean (Prochaska et al. 2015).

A deeper understanding of the transcriptional changes that occur in KS4202 in response to soybean aphid feeding may provide more insights into the mechanisms governing plant tolerance. In addition, this knowledge will help to identify phenotypic characteristics linked to tolerance that will assist breeding of tolerant plants. Thus, the objectives of this study were to evaluate the differences in constitutive and induced responses between tolerant KS4202 and susceptible K03-4686 soybeans by monitoring the expression of selected defense-related transcripts, including specific peroxidases, JA-associated transcripts and WRKY transcription factors.

## Materials and Methods

**Plant and insect sources.** Seeds of the tolerant soybean KS4202 (Pierson et al. 2011, Marchi-Werle et al. 2014) and susceptible K03-4686 (Prochaska et al. 2015) were pre-germinated to ensure homogeneity of the seedlings. Seeds were maintained in wet paper rolls inside plastic bags and placed in a dark cooler at room temperature ( $24 \pm 2^\circ\text{C}$ ) for 3 days to allow radical emergence. Two seedlings of each genotype were planted in each pot (15 cm diameter) at a depth of 3 cm containing Fafard Growing Media (Mix No. 3B; Conrad Fafard, Awawam, MA), and later thinned to one plant per pot. Soybeans were grown and maintained in a growth chamber ( $24 \pm 2^\circ\text{C}$  and 15: 9 h light: dark) until the completion of the studies.

Soybean aphids used in this research were progeny of a Nebraska isolate (biotype 1), collected in a commercial field near the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory, Concord, NE ( $42^\circ 23' 3''$  N,  $96^\circ 59' 21''$  W) during the summer of 2011. The colony was maintained on V1-V5 stage KS4202 soybean in a growth chamber at  $24 \pm 2^\circ\text{C}$  and a photoperiod of 16: 8 h (light: dark).

**Plant infestation.** Soybean plants at the V3 stage (fully developed leaves at the second trifoliate, third trifoliate leaf unrolled) (Fehr and Caviness 1977) were used for all experiments. A preliminary study (study 1) was conducted to compare the overall changes in expression of defense related transcripts between tolerant and susceptible soybeans in the presence and absence of soybean aphids. Plants were organized in a completely randomized design with a factorial design that included two soybean genotypes (KS4202 and K03-4686), two soybean aphid infestation levels (0 [control] and

15 aphids per plant) and two plant evaluation/harvesting times (0 and 7 days post infestation [D0 and D7]). A second study (study 2) was conducted with the aforementioned conditions; however, evaluation and harvesting times occurred at 0, 1, 3, and 7 days after aphid introduction (D0, D1, D3 and D7). In both studies, apterous soybean aphid females (4<sup>th</sup> instar and adults) were placed on a single trifoliolate of each plant (first emerged trifoliolate). The infested trifoliolate was caged to prevent insect escape using a custom-built plastic petri dish cage (8.9 by 2.5 cm). Each cage had two mesh windows (7 cm diameter) and a small hole on the side of the cage was made to fit the stem. A metal clip was placed on each side of the cage to secure the petri dishes. To provide cage support and prevent leaves from bending, a bamboo stick was cut to the appropriate height and placed in the potting soil.

At each evaluation time, soybean aphid numbers were recorded and insects were gently removed with a soft paintbrush. In addition, feeding injury was addressed according a 1-5 scale developed by Heng-Moss et al. (2002) and Pierson et al. (2010). Trifoliate from both control and infested treatments were then excised and flash frozen with liquid nitrogen. Samples were stored at -80°C for RNA extraction and qRT-PCR analysis.

**RNA isolation and cDNA synthesis.** In preparation for RNA isolation, approximately 300 mg of leaf tissue per sample was ground with liquid nitrogen and transferred into a 2 ml plastic tube. The total RNA was extracted from cells using the TRIzol® reagent, according to manufacturer's protocol (Invitrogen, Grand Island, New York). Samples were treated with RNase-free DNase I for 10 min at room temperature and sequentially purified using RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA),

following manufacturer's instructions. The integrity of RNA bands was confirmed by agarose gel electrophoresis, and quantification and purity of RNA was determined with a spectrophotometer (NanoDrop 1000, Wilmington, DE). cDNA first strand was synthesized using 2.5 µg of total RNA with ThermoScript RT-PCR system (Life Technologies) according to manufacturer's protocol.

**Quantitative real-time PCR (qRT-PCR).** qRT-PCR reads were performed on a 7500 Fast Real-time PCR (Applied Biosystems). Each qRT-PCR reaction contained 20 µL, which was composed of 10 µL of SsoAdvanced SYBR Green (Bio-Rad Laboratories, California, USA), 9 µL of cDNA (1:50 water dilution) and 1 µL of forward and reverse primers (10 µM). Each PCR plate included non-infested controls for comparisons and calculations with several transcripts at different time points. There were three technical replicates of each cDNA biological replicate per plate with cyclophilin (CYP) (Hu et al. 2009) was used as the UCE internal control gene. At least four biological replications were used per treatment in each study. Primers were designed using NCBI platform and sequences are provided in Table 1.

The amplification protocol was 95°C for 30 sec for polymerase activation and DNA denaturation, then 40 amplification cycles at 95°C for 5 sec (denaturation) and 60°C for 30 sec (annealing/extension). After the amplification step, the instrument also performed the melt curve analysis to ensure that the readings obtained come from the amplification of a single product. In this case, a single peak indicated that only the gene of interest was amplified. PCR conditions for the melt curve analysis were: 95°C for 15 secs min, 60°C for 1 min and increase in set point temperature after cycle 2 by 1% for each cycle at every 15 sec.

**Fold-change calculations and statistical analysis.** Calculations of  $\Delta\Delta CT$  were performed with the values of cycle threshold (CT) for each primer and cyclophilin (CYP) as an endogenous control (Hu et al. 2009), according to Schmittgen and Livak (2008). Changes in transcript abundance were determined for each control and infested soybean genotype. In addition, comparisons between control treatments in KS4202 and K03-4686 at each time point were also calculated. To determine the impact of aphid feeding on the selected transcripts in studies 1 and 2, the statistical significance of CT values was determined through generalized mixed model analysis (PROC GLIMMIX, SAS Institute 2008). Means were separated using Fisher protected least significant difference (LSD) procedure when appropriate ( $P < 0.05$ ).

## Results and Discussion

**Aphid number and visual damage.** In study 1, soybean aphids developed similarly in tolerant KS4202 ( $150.8 \pm 18.9$ ) and susceptible K03-4686 ( $138.6 \pm 16.5$ ) at D7. The growth pattern of soybean aphids in study 2 was similar to study 1. At D1, KS4202 and K03-4686 trifoliates had on average  $24.9 \pm 2.9$  and  $19.8 \pm 3.4$  aphids, respectively. At D3,  $48.6 \pm 7.24$  aphids were recorded on KS4202, whereas K03-4686 had  $46 \pm 15.29$  aphids. At D7, the number of aphids in the infested trifoliolate in KS4202 and K03-4686 was  $91.8 \pm 20.2$  and  $127.8 \pm 27.89$ , respectively. Consistent with previous studies (Marchi-Werle et al. 2014, Prochaska et al. 2015), the brief soybean aphid infestation in both studies did not cause visual damage for either soybean genotype (data not shown).

### **Constitutive levels (control) of defense-associated transcripts.**

**Study 1.** No differences were found for the constitutive levels of PRX52, PRX2, LOX1, LOX2, OPR3, PR1 and WRKY60 transcripts between KS4202 and K03-4686 at D0 (Table 4.2). However, the constitutive expression of LOX10 was markedly greater in KS4202 than in K03-4686 (fold-change = 12.64;  $P = 0.02$ , Table 4.2). At D7, the constitutive expression of the selected transcripts was similar for both tolerant and susceptible genotypes (Table 4.2).

**Study 2.** The constitutive expression of the selected transcripts at D0 and D1 was similar to study 1, except for LOX10 at D0 (Table 4.3). Evaluations performed at D1 showed that constitutive levels of all transcripts were similar between tolerant and susceptible soybean (Table 4.3). At D3, the constitutive levels of PRX52 and PR1 were lower in KS4202 than in K03-4686 ( $P = 0.001$ ), although the remaining transcripts were statistically similar (Table 4.3). At D7, the constitutive expression of LOX1, LOX2,

LOX10 and OPR3 transcripts was greater in KS4202 than in K03-4686 (Table. 4.3). The most remarkable changes occurred in LOX1 and LOX10, with a fold-change of 28.55 and 108.85, respectively.

### **Soybean aphid induced levels of defense-associated transcripts.**

**Study 1.** At D7 post soybean aphid introduction there was a 10.95 fold-increase in the levels of PRX52 in KS4202 comparatively to the control plants ( $P = 0.002$ ); whereas, no changes were observed in K03-4686 (Fig. 4.1). This is consistent with previous studies by Prochaska et al. (2015). Soybean aphid feeding did not statistically impact the abundance levels of PRX2 and WRKY60 in either KS4202 or K03-4686, although the abundance of WRKY60 in infested KS4202 was 3 fold higher than in control KS4202. The SA-related transcript, PR1, was induced in KS4202 ( $P = 0.02$ ), but not in K03-4686. Conversely, there were no differences in the expression of LOX1, LOX2, LOX10 and OPR3 transcripts when aphids were present in either KS4202 or K03-4686 (Fig. 4.2).

**Study 2.** Soybean aphids did not impact the expression of PRX52 at D1 in KS4202 or K03-4686, but at D3, aphid feeding resulted in a lower PRX52 expression (fold-change = 0.09;  $P = 0.005$ ) in K03-4686 relative to its respective control treatment (Fig. 4.3). Consistent with study 1, intensified aphid infestation at D7 resulted in greater expression of PRX52 in KS4202 ( $P = 0.04$ ); whereas no changes occurred in K03-4686 (Fig. 4.3). The aphid induced levels of PRX2 remained steady across D1, D3 and D7 in KS4202 (Fig. 4.4), although a transient induction of this transcript occurred at D3 in K03-4686, (Fig. 4.4).



The induced expression of WRKY60 in KS4202 was significantly higher at D1 (3.89 fold-change;  $P = 0.02$ ) and D7 (13.94 fold-change;  $P < 0.0001$ ), but did not change in K03-4686 for those same evaluation times (Fig. 4.5). The fold-change for WRKY60 at D7 was higher in study 2 than study 1, but the trend between both studies is comparable. Aphid feeding did not significantly induce LOX1, LOX2 and LOX10 transcripts in KS4202 at either harvest time in study 2 (Fig. 4.6, 4.7 and 4.8). In the susceptible genotype, LOX1 was induced at D7 ( $P = 0.04$ ) while the expression of LOX2 and LOX10 remained similar across D1, D3 and D7 (Fig. 4.6, 4.7 and 4.8). Moreover, soybean aphid feeding at D1 and D3 did not influence the expression of OPR3 in KS4202 and K03-4686, but a slight increase (2 fold-increase) was observed at D7 for both genotypes (Fig. 4.9). The SA-related transcript, PR1, was induced by soybean aphids in KS4202 at D7 ( $P = 0.008$ ), but not at D1 and D3 (Fig. 4.10). Conversely, soybean aphids induced the expression of PR1 in K03-4686 at D1 ( $P = 0.02$ ; Fig. 4.10).

Plants have developed complex systems for defense against insects. The interactions between plants and insects can be classified as antibiosis, antixenosis, tolerance, and or a combination of multiple categories of resistance (Smith 2005). Many biochemical processes orchestrating these resistance categories may overlap with pathways needed for plant development and metabolism (Smith and Chuang 2014). Plant tolerance differentiates itself from the remaining resistance categories by expressing certain characteristics (e.g. regrowth and enhance metabolic activities) that enable these plants to endure greater insect injury (Smith 2005). This research monitored the expression of selected defense-related transcripts in the absence (constitutive) and

presence (induced) of soybean aphids via qRT-PCR to identify important components of soybean tolerance to soybean aphids.

Constitutive resistance is regulated by preformed resistance traits while induced resistance is triggered by insect injury that can be either localized or systemically propagated through the host plant (Kessler and Baldwin 2002). The constitutive levels of the peroxidases (PRX52 and PRX2) transcripts evaluated in this research were similar for tolerant and susceptible soybean; whereas soybean aphid feeding strongly induced PRX52 transcript in the tolerant KS4202. This finding corroborates with transcriptomic analysis by Prochaska et al. (2015), who found induction of PRX52 in KS4202 after 15 days of aphid feeding. The orthologous PRX52 in *Arabidopsis thaliana* (AtPRX52) encodes an apoplastic peroxidase, which was strongly induced by pathogen infection (Floerl et al. 2012). The role of PRX52 in KS4202 may be similar to *Arabidopsis* where the product of this transcript acts in the general defense system against pathogens. Moreover, the greater magnitude of PRX52 induction in KS4202 relative to the susceptible genotype suggests that this transcript may be contributing to the ability of this genotype to tolerate high levels of oxidative stress by aphid feeding. Conversely, no correlations could be made with PRX2 in KS4202's tolerance.

WRKY proteins are a family of transcription factors identified by the presence of a 60 amino acid domain with a conserved sequence WRKYGQK followed by a Zinc finger motif (Pandey and Somssich 2009). Studies on *Arabidopsis*, wheat and rice have indicated that WRKY53 is conserved across these plant families (Miao et al. 2004, Van Eck et al. 2014). Wheat TaWRKY53 and rice OsWRKY53 have two conserved domains (Van Eck et al. 2014), and assume a similar function to AtWRKY53 in leaf senescence,

where hydrogen peroxide might be involved in the signal transduction (Miao et al. 2004). Further, this transcription factor was also linked with the defense against Russian wheat aphid (Wu et al. 2008, Van Eck et al. 2010). Van Eck et al. (2014) proposed that WRKY53 transcriptional network regulates oxidative burst caused by biotic stress, where the interaction between membrane-bound glutathione S-transferases (GST) and WRKY53 induce detoxifying gene products (peroxidases) that protect the photosynthetic machinery from damage by reactive oxygen species (ROS). WRKY60 (Glyma16g026400) appears to be orthologous to AtWRKY53 (Prochaska et al. 2015), and could presumably play a role similar to AtWRKY53 in soybean.

Accumulating evidence on the increased expression of peroxidase transcripts and activity in tolerant soybean suggest that these enzymes effectively protect them from excessive accumulation of ROS, when the same mechanism does not provide sufficient protection in susceptible plants (Pierson et al. 2011, Marchi-Werle et al. 2014, Prochaska et al. 2015). This study shows that WRKY60 and PRX52 were induced simultaneously. Based on the previous role of its analogous in rice and wheat, we speculate that WRKY60 is involved in the mechanism of tolerance in KS4202 by supporting the action of ROS-fighting enzymes.

Previous studies have indicated that plants make use of hormonal signaling pathways to mobilize defense strategies against aphids by the inducing transcripts associated with these pathways (Moran et al. 2002, Mewis et al. 2005, Pegadaraju et al. 2005, Thompson and Goggin 2006, Gutsche et al. 2009a, Botha et al. 2010). Our data indicate that JA-associated transcripts were constitutively expressed in tolerant soybean. Conversely, soybean aphids induced LOX1 and OPR3 in susceptible soybean at D7.

Research has shown that the induction of LOX transcripts in response to aphids occurs rapidly (12 -36 hours) in antixenotic plants, in the meantime, no changes occurred in the susceptible plants (Gao et al. 2007). The greater induction of JA-related transcripts in the susceptible genotype could be related with lower constitutive expression of these transcripts while this action in the tolerant soybean was mostly dismissed in tolerant soybean. Marimuthu and Smith (2012) observed that JA-related genes were induced to greater levels in susceptible plants than in barley tolerant to Russian wheat aphid, when most of these genes were constitutively expressed at greater levels in tolerant plants. In combination, these studies support the hypothesis that induction JA-transcripts do not condition tolerance, but instead the constitutive expression of JA-transcripts appear to be an important component of plant tolerance to aphids.

In addition to the role of JA in plant tolerance, Marimuthu and Smith (2012) have proposed that the constitutive expression of ET-responsive transcripts is important for barley's tolerance to Russian wheat aphid. Further, resistance to green peach aphid (*Myzus persicae*) in Arabidopsis has also relied on constitutive expression of JA or ET (Ellis et al. 2002). JA and ET have a synergistic relationship, and inhibition of ET biosynthesis may lead to a reduced accumulation of JA (Penninckx et al. 1998, Wang et al. 2002).

In the soybean aphid-soybean interaction, studies that have compared the responses of *Rag* x susceptible soybeans observed the induction of JA biosynthesis in susceptible soybean at D1 and D7 after infestation, however induction of JA-responsive transcripts only occurred at D1 (Studham and MacIntosh 2013). The lack of JA-

biosynthesis at D7 led to the hypothesis that soybean aphids may be suppressing JA to overcome effective defense responses.

PR genes such as PR1, PR2 and PR5 have been associated with and used as a marker for SA-mediated pathway (Fu and Dong 2013). SA accumulation in response to stressors (e.g. pathogens or insects) triggers the release of NPR1 (non-expressor of PR genes 1) protein, which is required for the activation of *PR* genes (Fu and Dong 2013). PR genes encode small proteins that may have antimicrobial or antifungal properties (Van Loon and Van Strien 1999). Many WRKY proteins, including AtWRKY53, act in the upstream of NPR1 and positive regulate the transcription of NPR1 (Yu et al. 2001, Wang et al. 2006). Interestingly, overexpression of WRKY53 in wheat induced PR proteins and reduced symptoms of pathogen infection in rice (Marcel et al. 2010). Studies have indicated that WRKYs can interact with both negative and positive regulators of SA (Wang et al. 2006, Pandey and Somssich 2009, Fu and Dong 2013). Induced SA pathway has been proposed as a general mechanism of antibiosis or antixenosis to aphids with a weak response in susceptible plants (Gao et al. 2007). The PR2 ( $\beta$ -1,3-glucanase) and PR3 (chitinase) transcripts and peroxidases have been associated with wheat antibiosis resistance to Russian wheat aphid (van der Westhuizen et al. 1998b, a). Our data show that induction of PR1 in tolerant soybean was stronger but delayed in regards to the susceptible reference. Interestingly, PR1 was induced simultaneously to PRX52 and WRKY60 in the tolerant soybean, suggesting that SA may be also be involved in this resistance.

Overall, this research shows that constitutive expression of JA-associated transcripts is important for soybean tolerance to soybean aphids. Differently than the

usual rapid transcriptional response observed in soybeans expressing antibiosis and or antixenosis (*Rag* genes) to soybean aphids (Li et al. 2008, Studham and MacIntosh 2013), induced transcriptional responses in tolerant soybean were slower and only detected later (D7). Nevertheless, primed induction of PRX52, WRKY60 and PR1 transcripts may still be contributing to tolerance. Pending future validation, the expression profile of constitutive JA transcripts may provide a baseline for screening soybean for aphid tolerance.

Additional research using soybean with impaired hormonal pathways will provide more clues on the involvement of SA and JA in tolerance to soybean aphids, and may also help to understand whether soybean aphids can manipulate plant defenses through these hormones. Furthermore, studies should investigate the role of ET-associated transcripts and the possible interaction with JA as these hormonal pathways have shown their importance in host plant tolerance response in other plant-aphid systems.

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## Figures and Tables

**Table 4.1.** Primers designed for studies 1 (preliminary) and 2.

Primer	Accession Number	Description	Sequences
PRX52	NM_001254985.2	Peroxidase 52-like	F: CCGCCATGATCAAGATGGGA R: AACCCACCACGGAATCCAAA
PRX2	XM_014766488.1	Peroxidase-like	F: TTGCTGTAGGCTTTGGTCCT R: TTTGTTGGCCCAAGGTA CT
LOX1	XM_003531548.3	Linoleate 9S-lipoxygenase-3	F: TGCCGTGATTGAGCCATTG R: AGTGATTGTCGAGCAAGTGC
LOX2	XM_003537901.3	Linoleate 13S-lipoxygenase 2	F: ATGGAAATCAACGCGCTTGC R: TGCAGGTCGAATTGCCATTG
LOX10	NM_001250409	Lipoxygenase-10	F: TCTGCATCTCAAAATGTGATACCTC R: CATCCATCCAGACAGATTCACTTG
OPR3	XM_003542310.3	12-oxophytodienoate reductase 3	F: GTGTATCAGCCTGGTGGG R: GCACGAGGCTCTGGATAG
WRKY60	KT031239.1	WRKY transcription factor	F: ATGGCAGCATGATGGATTCC R: TTCTGTGCACGTTGACATGG
PR1	XM_003545723.3	Pathogenesis-related protein 1-like	F: AACTATGCTCCCCCTGGCAACTATATTG R: TCTGAAGTGGTAGCTTCTACATCGAAACAA
CYP	XM_014764426.1	Protein folding	F: ACGACGAAGACGAGTGG R: CGACGACGACAGGCTTGG

**Table 4.2.** Constitutive transcript abundance of peroxidases (PRX52 and PRX2), lipoxygenases 1, 2 and 10 (LOX1, LOX2, LOX10), 12-oxophytodienoate reductase 3 (OPR3), WRKY53 transcription factor and pathogenesis-related 1 (PR1) when comparing KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D0 and D7 (study 1). A fold change >1 represents higher constitutive transcript abundance in KS4202. A fold change <1 indicates higher constitutive transcript abundance in K03-4686. A fold change equal to 1 indicates no difference between the constitutive transcript abundance plants of either genotype. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference.

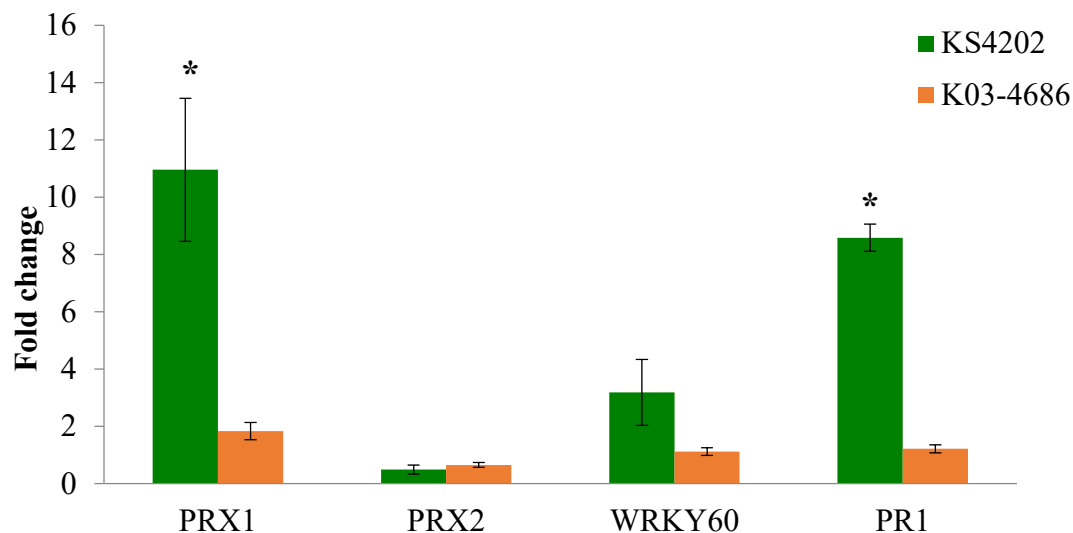
<b>Transcript</b>	<b>D0</b>	<b><i>P</i>-value<sup>a</sup></b>	<b>D7</b>	<b><i>P</i>-value<sup>a</sup></b>
<b>PRX52</b>	0.68 ± 0.22	0.70	0.83 ± 0.19	0.76
<b>PRX2</b>	0.30 ± 0.10	0.22	1.76 ± 0.57	0.72
<b>LOX1</b>	1.95 ± 0.76	0.58	0.77 ± 0.24	0.67
<b>LOX2</b>	1.65 ± 0.67	0.59	1.12 ± 0.26	0.66
<b>LOX10</b>	12.64 ± 2.59	0.02	1.89 ± 0.64	0.26
<b>OPR3</b>	0.42 ± 0.07	0.36	0.67 ± 0.03	0.62
<b>PR1</b>	1.85 ± 0.27	0.55	1.52 ± 0.09	0.40
<b>WRKY60</b>	1.03 ± 0.43	0.98	1.61 ± 0.58	0.80

<sup>a</sup> Statistical significance at  $P < 0.05$

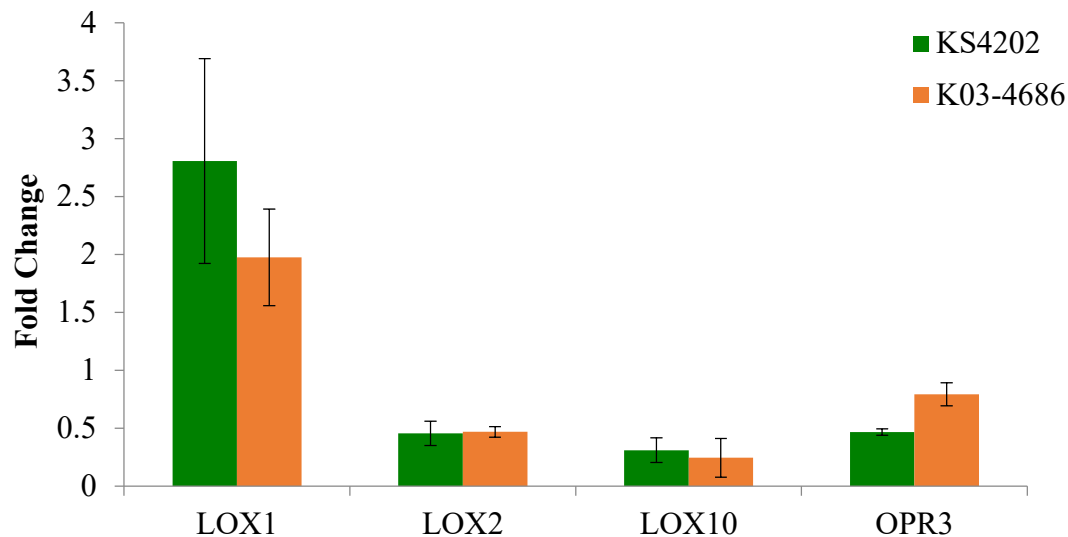
**Table 4.3.** Constitutive transcript abundance of peroxidases (PRX52 and PRX2), lipoxygenases 1, 2 and 10 (LOX1, LOX2, LOX10) and 12-oxophytodienoate reductase 3 (OPR3), WRKY53 transcription factor and pathogenesis-related 1 (PR1) when comparing KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D0, D1, D3 and D7 (study 2). A fold change >1 represents higher constitutive transcript abundance in KS4202. A fold change <1 indicates higher constitutive transcript abundance in K03-4686. A fold change equal to 1 indicates no difference between the constitutive transcript abundance plants of either genotype. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference.

<b>Transcript</b>	<b>D0</b>	<b><i>P</i>-value<sup>a</sup></b>	<b>D1</b>	<b><i>P</i>-value<sup>a</sup></b>	<b>D3</b>	<b><i>P</i>-value<sup>a</sup></b>	<b>D7</b>	<b><i>P</i>-value<sup>a</sup></b>
<b>PRX52</b>	0.73 ± 0.10	0.63	0.39 ± 0.04	0.14	0.22 ± 0.03	0.001	1.16 ± 0.13	0.83
<b>PRX2</b>	2.46 ± 0.11	0.14	0.73 ± 0.04	0.39	0.76 ± 0.10	0.95	0.33 ± 0.03	0.09
<b>LOX1</b>	1.65 ± 0.31	0.50	1.00 ± 0.19	0.99	0.48 ± 0.09	0.32	28.55 ± 4.18	<0.0001
<b>LOX2</b>	0.45 ± 0.04	0.11	0.95 ± 0.05	0.93	1.90 ± 0.20	0.21	2.79 ± 0.21	0.048
<b>LOX10</b>	0.49 ± 0.07	0.47	1.69 ± 0.32	0.57	2.33 ± 0.81	0.30	108.85 ± 9.16	<0.0001
<b>OPR3</b>	1.17 ± 0.05	0.67	0.51 ± 0.05	0.15	0.49 ± 0.08	0.10	3.40 ± 0.24	0.0004
<b>PR1</b>	1.28 ± 0.08	0.71	0.83 ± 0.04	0.22	0.26 ± 0.01	0.008	2.46 ± 0.30	0.86
<b>WRKY60</b>	0.79 ± 0.05	0.67	0.57 ± 0.05	0.12	1.29 ± 0.13	0.64	2.82 ± 0.29	0.06

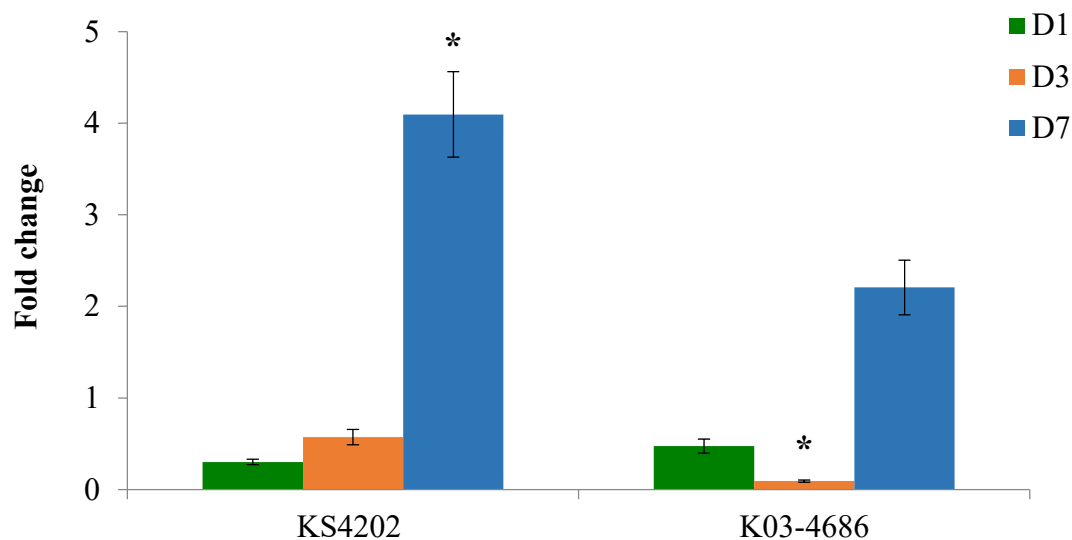
<sup>a</sup> Statistical significance at  $P < 0.05$



**Fig. 4.1.** Mean  $\pm$  SEM of transcript abundance of peroxidases (PRX52 and PRX2), WRKY53 transcription factor and pathogenesis-related 1 (PR1) induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D7 (study 1). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).

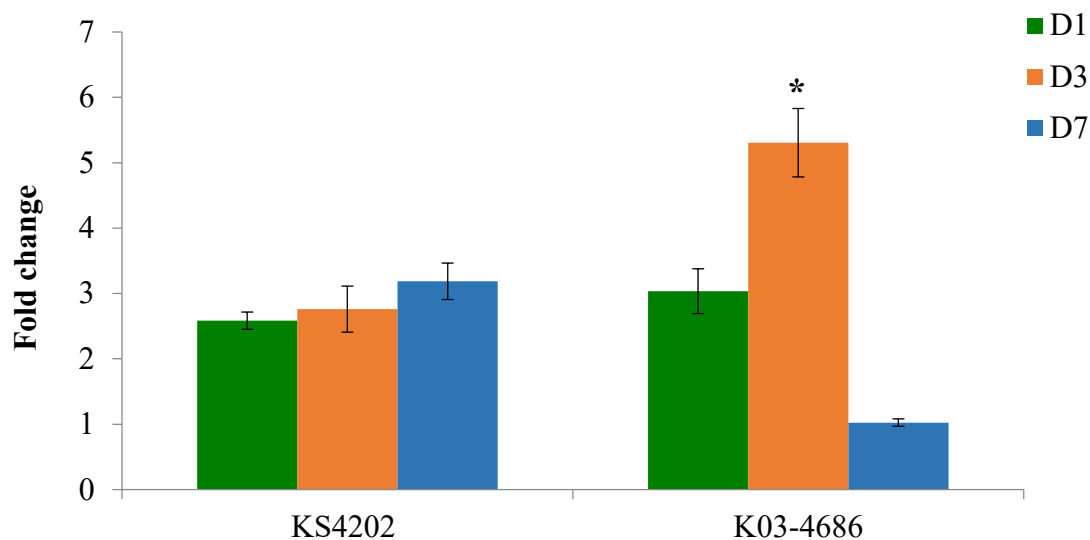


**Fig. 4.2.** Mean  $\pm$  SEM of transcript abundance of lipoxygenase 1 (LOX1), lipoxygenase 2 (LOX2), lipoxygenase 10 (LOX10) and 12-oxophytodienoate reductase 3 (OPR3) induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D7 (study 1). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Fold change equal to 1 represents no difference between infested and control treatments. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).

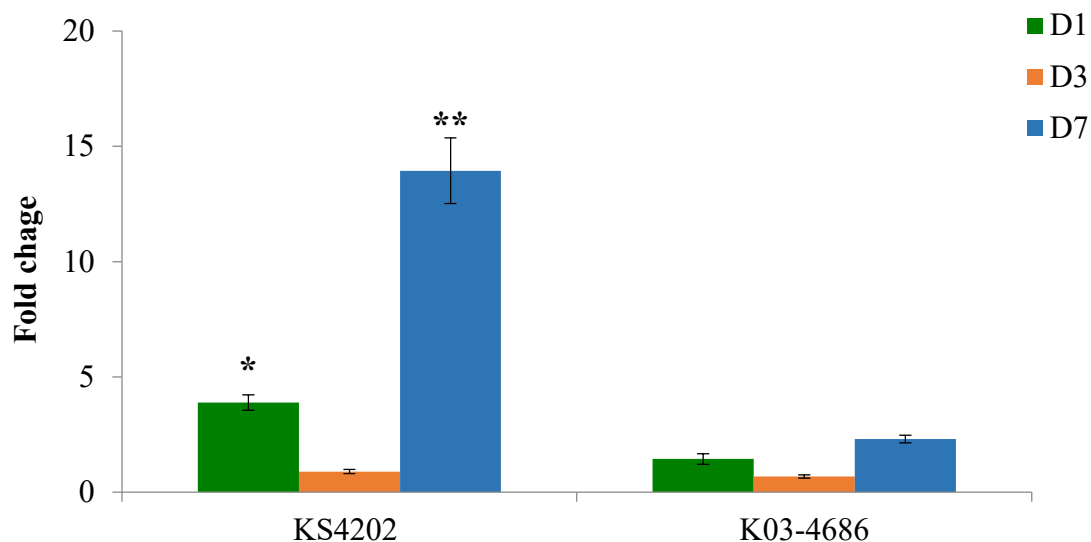


**Fig. 4.3.** Mean  $\pm$  SEM of transcript abundance of peroxidase (PRX52) induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D1, D3 and D7 (study 2). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).

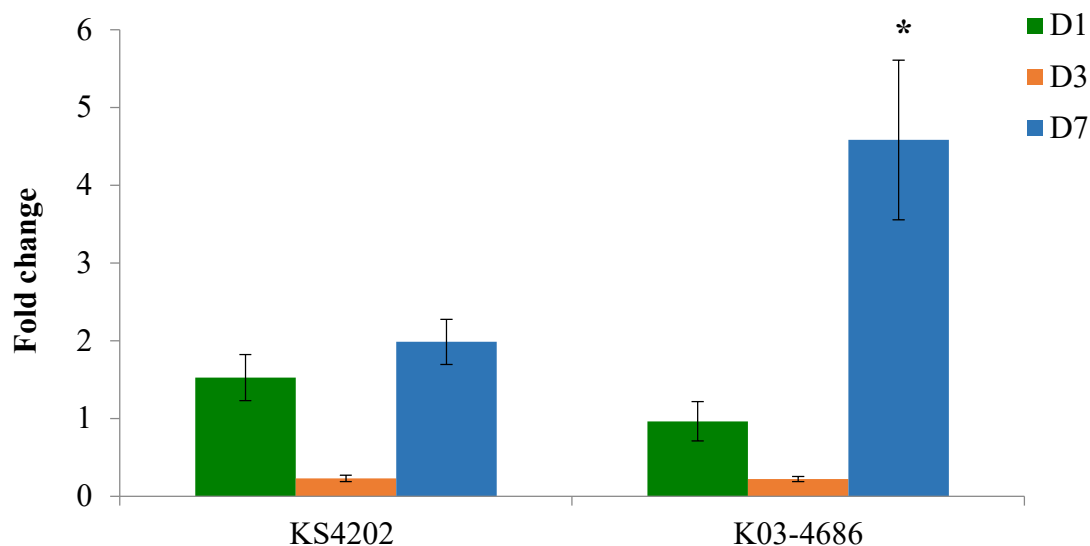




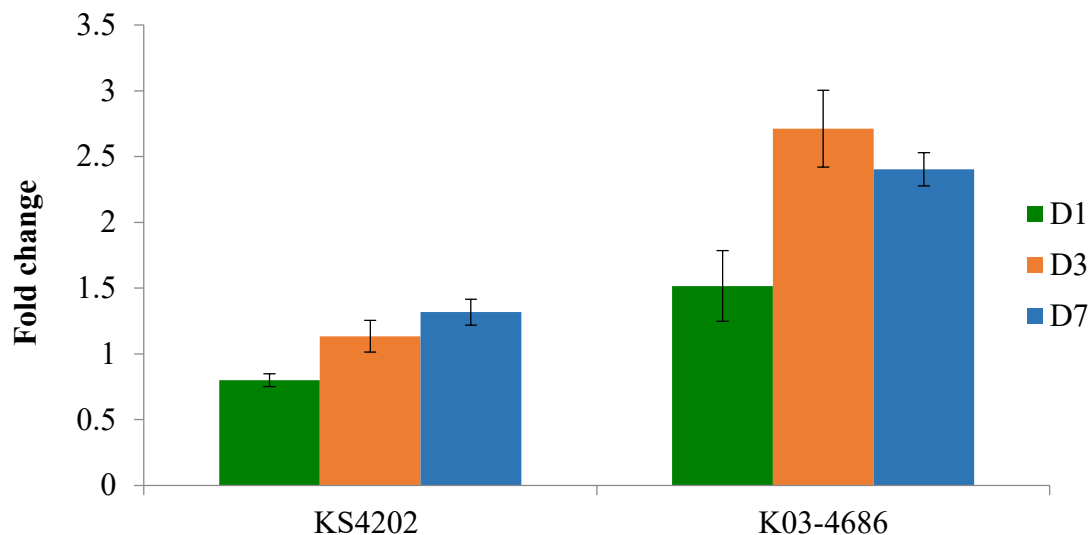
**Fig. 4.4.** Mean  $\pm$  SEM of transcript abundance of peroxidase (PRX2) induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D1, D3 and D7 (study 2). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).



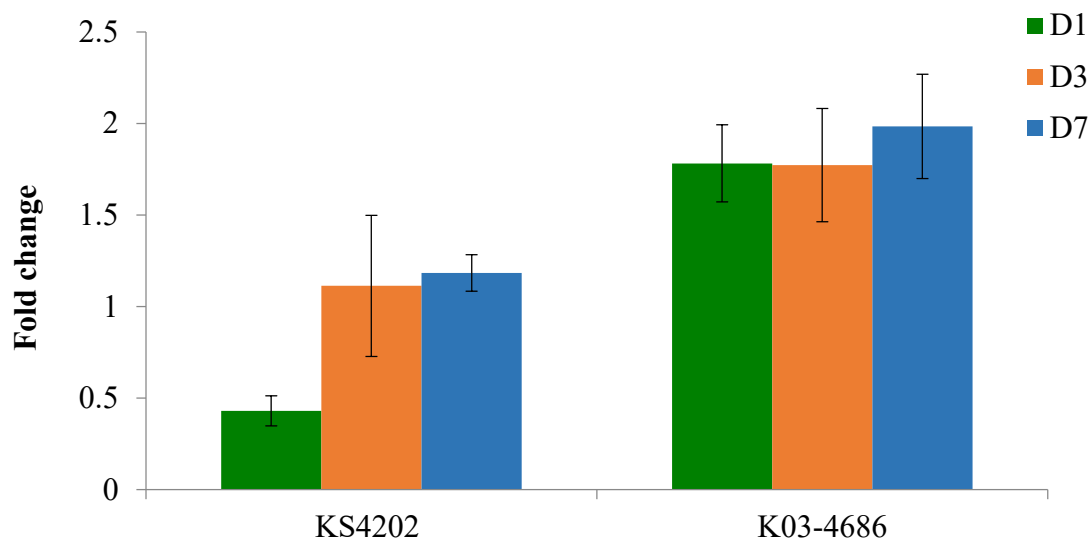
**Fig. 4.5.** Mean  $\pm$  SEM of transcript abundance of WRKY60 induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D1, D3 and D7 (study 2). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).



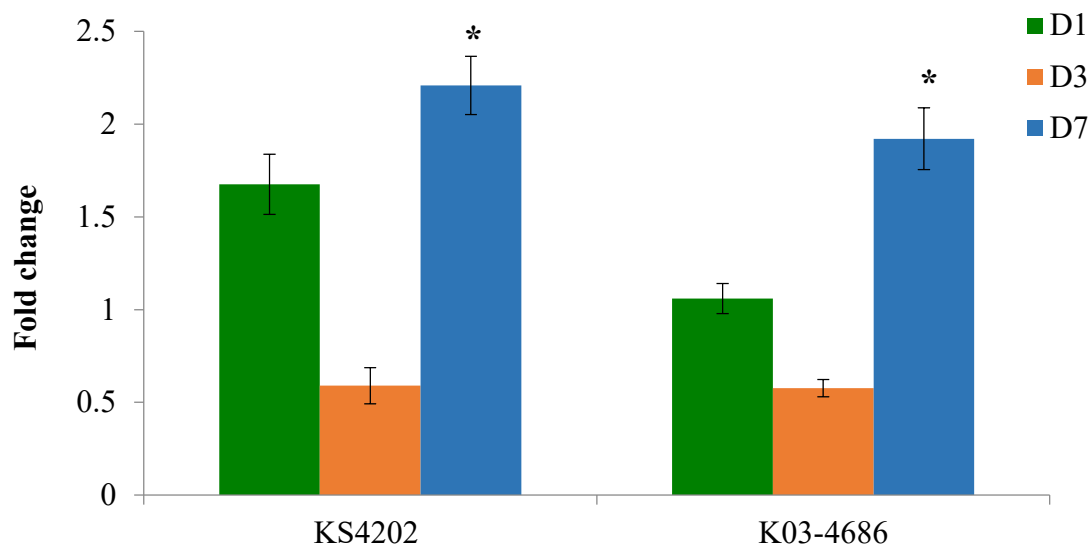
**Fig. 4.6.** Mean  $\pm$  SEM of transcript abundance of lipoxigenase 1 (LOX1) induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D1, D3 and D7 (study 2). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).



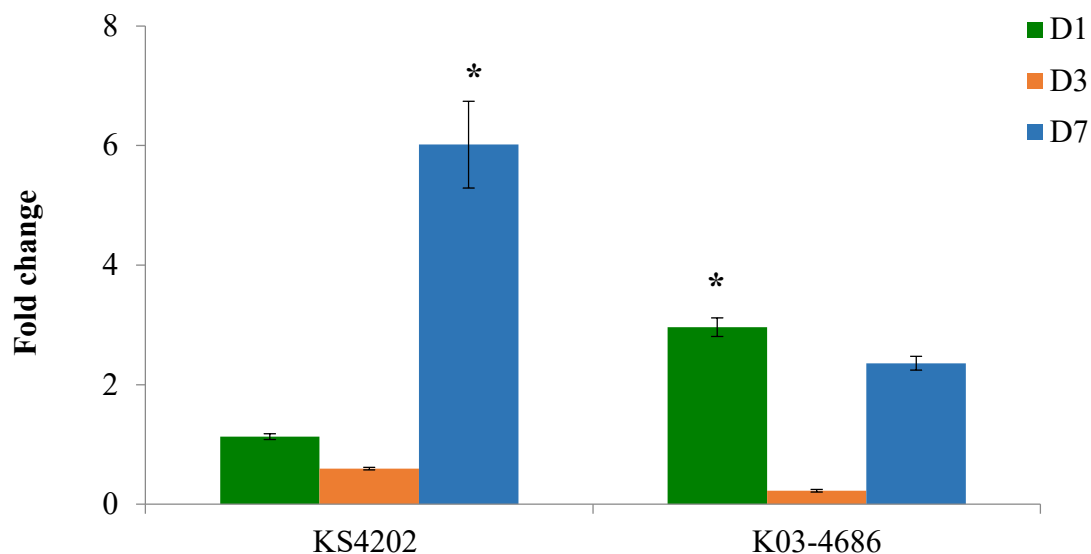
**Fig. 4.7.** Mean  $\pm$  SEM of transcript abundance of lipoxigenase 2 (LOX2) induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D1, D3 and D7 (study 2). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).



**Fig. 4.8.** Mean  $\pm$  SEM of transcript abundance of lipoxygenase 10 (LOX10) induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D1, D3 and D7 (study 2). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).



**Fig. 4.9.** Mean  $\pm$  SEM of transcript abundance of 12-oxophytodienoate reductase 3 (OPR3) induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 at D1, D3 and D7 (study 2). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).



**Fig. 4.10.** Mean  $\pm$  SEM of transcript abundance of pathogenesis-related (PR1) induced by soybean aphids relative to control treatment in KS4202 (aphid tolerant) and K03-4686 (aphid susceptible) at D1, D3 and D7 (study 2). A fold change  $>1$  represents higher transcript abundance in the infested treatment, while fold changes  $<1$  indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance at  $P < 0.05$  indicated by (\*), and  $P < 0.0001$  indicated by (\*\*).

## CHAPTER V

### **Integrating Plant Tolerance into Breeding Programs for Soybean Aphid (*Aphis glycines* Matsumura) Management**

#### **Introduction**

The soybean aphid, *Aphis glycines* Matsumura, has become the most notorious pest of soybean (*Glycine max* (L.) Merrill) in North America since its first detection during the growing season of 2000 (Ragsdale et al. 2004). Methods for minimizing the damage caused by these aphids have relied heavily on chemical control (Hodgson et al. 2010), which has caused a 130-fold increase in insecticide applications in less than a decade (Ragsdale et al. 2011, Hodgson et al. 2012). At the same time, host plant resistance (HPR) has gained considerable attention as an effective and sustainable management strategy to reduce soybean aphid populations.

Several studies have identified resistant soybean that impact the biology (antibiosis) and behavior (antixenosis) of soybeans aphids. These have been associated with a single dominant gene named *Rag* (**R**esistance to *Aphis glycines*). Currently, five different *Rag* genes (*Rag1*-*Rag5*) are known to provide HPR against soybean aphids in several soybean genotypes (Hill et al. 2006b, a, Li et al. 2007, Mian et al. 2008, Zhang et al. 2010, Jun et al. 2013). However, the implementation of *Rag* soybeans has been challenged by the presence of four soybean aphid biotypes (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013a). The precise distribution of the biotypes,



frequency patterns, and migration is still the subject of future research; however, studies from Cooper et al. (2015) found a high degree of virulence diversity in North America. When associated with high dispersion capacity of aphids, large virulence diversity could render *Rag* genes ineffective as resistant plants become more prevalent in the field.

Tolerance is another category of plant resistance, defined by Smith (2005) as the “ability of a plant to withstand or recover from damage caused by arthropod populations equal to those on susceptible cultivars”. Plant tolerance does not impose the same levels of selection pressure as antibiosis and antixenosis, therefore virulent populations are less likely to evolve. It is considered a multigenic resistance, which is directly correlated to a complex of plant characteristics (Smith 2005). The soybean genotype KS4202 has been identified as tolerant to soybean aphids in both vegetative and reproductive stages (Pierson et al. 2010, Marchi 2012), capable of withstanding high aphid populations with minimal yield loss (Prochaska et al. 2013, Marchi-Werle unpublished data). Studies performed to identify the mechanisms of KS4202 tolerance to soybean aphids have suggested that photosynthetic compensation plays an important role (Pierson et al. 2011). In addition, several studies, using both transcriptomic and enzyme kinetic techniques, have investigated the role of peroxidases in insect-tolerant plants, including soybeans (Heng-Moss et al. 2004, Gutsche et al. 2009a, Pierson et al. 2011, Ramm et al. 2013, Marchi-Werle et al. 2014, Prochaska et al. 2015). It has been proposed that insect-stressed tolerant plants can maintain or elevate peroxidase activity to breakdown damaging ROS that accumulated as a result of stress, when the same mechanism is not as efficient in susceptible plants.

The appropriate breeding schemes for developing plants with resistance to insects are associated with the complexity of the resistance traits (i.e. monogenic vs polygenic). In the last decade, hundreds of plant introductions (PI) were screened for soybean aphid resistance and many were confirmed to possess antibiosis and/or antixenosis (via *Rag* genes) (Hill et al. 2004, Li et al. 2004, Mensah et al. 2005). Breeding schemes for these categories of resistance in soybean have become more efficient and precise with the construction of fine quantitative trait loci (QTL) maps. To date, the *Rag1* gene was mapped in Dowling and Jackson (Li et al. 2007, Kim et al. 2010), and the *Rag2* gene was mapped in PI 243540 (Mian et al. 2008). Moreover, *Rag3* and *Rag5* were also mapped in PI 567541B and PI 567301B, respectively (Zhang et al. 2010, Jun et al. 2012). QTL maps have enabled marker-assisted selection (MAS) for aphid resistance genes in soybeans adapted to the North-Central regions of U.S.

Studies with linkage map-based QTL are performed by developing a mapping population. In this case,  $F_2$  can be developed via backcross, doubled haploids, near-isogenic lines or recombinant inbred lines (RILs). Recombinant inbred lines (RILs) are the result of successive inbreeding, and require considerable time. The construction of RIL involves continuous selfing (self-pollinated crops) or sib mating (cross-pollinated crops) the progeny of an  $F_2$  population, and then repeating the process until populations become homozygous, following the single-seed descent method (Madhusudhana 2015). Once the desired cycles of inbreeding are completed, RIL become a permanent resource for trait mapping and analysis.

Despite the success in mapping QTL in *Rag* soybean, to our knowledge, no studies have attempted to generate linkage maps for soybean with tolerance to soybean

aphid. In fact, very few studies have identified QTLs associated with insect tolerance in other economic crops. RIL populations were used to map tolerance to greenbug in '96-4121' sorghum, which was partially associated with eight QTLs (Nagaraj et al. 2005). RIL were also developed to map QTLs in 'GBIK' sorghum, characterized as both resistant (i.e. antibiosis and/or antixenosis) and tolerant to greenbug (Agrama et al. 2002). In this study, 113 loci, including 38 single sequence repeats (SSR) and 75 random amplified polymorphic DNA (RAPD) markers were mapped in linkage group 12. These studies indicate that tolerance is a polygenic resistance, and that MAS may be useful for breeding aphid-tolerant plants.

A recurring complication with QTL data is that different parental combinations and/or experiments conducted in different locations frequently result in the identification of partial or non-overlapping QTLs. These differences can be due to the significant genotype by environment interaction, sampling error due to population size and phenotypic evaluation (Rong et al. 2007). To date, the studies that conducted molecular QTL for tolerance in sorghum have relied on phenotypical evaluations based on visual aphid feeding damage and readings with SPAD chlorophyll meter (Agrama et al. 2002, Nagaraj et al. 2005). Additional techniques to characterize aphid tolerant populations will improve selection criteria, and therefore increase the quality of QTL maps. Ultimately, an appropriate phenotypic evaluation may stimulate new breeding programs targeting development of crops with tolerance to insects. Thus, the objectives of this research were: (i) determine the susceptibility of two high yield soybean genotypes involved in the breeding platform to develop aphid tolerant RIL; (ii) characterize the peroxidase activity

and relative expression of peroxidase transcripts in the parents of RIL and (iii) identify an assay to phenotype aphid-tolerant RIL.

## Materials And Methods

**Study 1. Susceptibility determination of two high yield soybean genotypes involved in the breeding platform to develop aphid tolerant RIL.** Two evaluations (Screening A and B) were performed to investigate the level of susceptibility of two high yield soybeans in two vegetative stages. The genotype KS4202, previously categorized as tolerant (Pierson et al. 2010, Prochaska et al. 2013, Marchi-Werle et al. 2014), and a soybean aphid susceptible genotype, SD76R (Marchi-Werle et al. 2014), served as references to evaluate two high yield soybeans, U09-105007 and U11-919011. The high-yielding soybeans in this study were developed by University of Nebraska-Lincoln soybean breeding program and are involved in a project to develop recombinant inbred lines (RIL) along with KS4202. U09-105007 (MG I) is a line with resistance to phytophthora root rot, and U11-919011 (MG II) is heterogeneous for resistance to phytophthora root rot. None of the high-yielding soybean in this study possesses a soybean aphid resistance gene (*Rag*), however determining the presence of soybean aphid tolerance was one of the objectives of this study.

Prior to planting, soybean seeds for both experiments were pre-germinated to ensure homogeneity of the seedlings. Seeds were maintained in a wet paper roll inside plastic bags at room temperature for 3 days to allow radical emergence. Two seedlings of each genotype were planted in Fafard Growing Media (Mix No. 3B; Conrad Fafard, Awawam, MA) in 15 cm diameter pots at a depth of 3 cm. After the emergence of unifoliate leaves, plants were thinned to one plant per pot and maintained in the greenhouse until the completion of the studies. Greenhouse conditions were set at  $25\pm 7$

°C with the supplemented LED lights (Pro 325, Lumigrow, Novato, CA) to produce a photoperiod of 16:8 (L:D).

For Screening A, plants were infested in the V1 stage (fully developed leaves at unifoliate node, first trifoliate leaf unrolled), while plants in Screening B were infested in the V3 stage (fully developed leaves at the second trifoliate, third trifoliate leaf unrolled) (Fehr and Caviness 1977). The two screening evaluations were synchronized. Soybean plants were infested with 20 apterous adult females of soybean aphids, which were placed on the youngest trifoliate of each plant. The insects used in this study were progenies of a Nebraska isolate (biotype 1), collected in a commercial field near the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory, Concord, NE (42° 23' 3" N, 96° 59' 21" W) during the summer of 2011. The insect colony was maintained on V1-V5 stage KS4202 plants in a growth chamber at 23±2°C and 16:8 (L:D) h. Following aphid introduction, plants were individually caged to prevent insect escape. Cages were constructed with clear Makrolon Lexan polycarbonate plastic (15 cm of diameter and 61 cm of height) with vents covered with organdy fabric.

Soybean aphid damage, aphid number and plant growth stage were recorded biweekly for each genotype. Damage ratings were assigned according to 1-5 scale developed by Heng-Moss et al. (2002) and Pierson et al. (2010), where 1 = 10% or less of leaf area with yellowish discoloration; 2 = 11-30% of leaf area with yellowish discoloration; 3 = 31-50% of leaf area with yellowish discoloration; 4 = 51-75% of leaf area with yellowish discoloration; and 5 = ≥75% of leaf area with yellowing discoloration or dead tissue (Fig. 5.1). To better estimate aphid pressure on the genotypes the over time, the cumulative aphid-days (CAD) was calculated using aphid population

for each evaluation. Cumulative aphid-days is calculated by  $= ((N1+N2)/2)*T$ , where N1 is the aphids count per plant on the previous evaluation, N2 is the aphid count per plant in the subsequent evaluation, and T is the time (days) between evaluations (Hanafi et al. 1989). Studies were terminated when at least one of the susceptible references genotypes had reached damage rating of four or greater. Subsequently, plants were categorized in response to aphid damage using the following the scale by Pierson et al. (2010): HS = highly susceptible (mean damage ratings  $\geq 4$ ); MS = moderately susceptible (mean damage ratings  $\geq 3$  but  $< 4$ ); MR = moderately resistant (mean damage ratings  $\geq 2$  but  $< 3$ ); and HR = highly resistant (mean damage ratings  $< 2$ ).

The experimental design for screenings A and B was organized in completely randomized design, with four genotypes. Each treatment combination consisted of 10 replications, and each plant represented one experimental unit. Prior to statistical analysis, data were verified with the univariate procedure (PROC UNIVARIATE, SAS 9.3, SAS Institute, Cary, NC) for Gaussian assumptions of normality and homogeneity of variance. The data was analyzed using a generalized mixed model analysis (PROC GLIMMIX, SAS) for all the CAD values to measure aphid pressure. Means were separated by Fisher least significant difference (LSD) procedure when the interaction or main effect was significant ( $P < 0.05$ ). Means damage ratings were analyzed through a nonparametric analysis using a rank based procedure (PROC RANK, SAS), generating relative damage ranking. For clarification purposes, results and discussion section report mean damage ratings rather than relative damage ranking.

**Study 2. Characterization of peroxidase activity and relative expression of peroxidase transcripts in the parents of RIL.** To evaluate the potential of peroxidases

to phenotype aphid-tolerant RIL, a second study was conducted to evaluate gene expression of two peroxidase genes PRX52 and PRX2 (Table 5.1). This study was performed with the potential soybean parental genotypes, KS4202, U09-105007 and U11-919011, which were infested during V1 and V3 vegetative stages as described in Study 1. Seeds were pre-germinated as described in Study 1. One seedling was sowed in a SC-10 Super Cell Single Cell cone-tainer (3.8 cm in diameter by 21 cm in depth; Stuewe & Sons, Inc., Corvallis, OR) containing a Fafard Growing Media (Mix No. 3B; Conrad Fafard, Awawam, MA). Cone-tainers were placed in 96-well cone-tainer trays filled with water and maintained in a greenhouse at  $25 \pm 7$  °C with the lighting supplemented by LED lights (Pro 325, Lumigrow, Novato, CA) to generate a 16:8 (L:D) h photoperiod.

Once soybeans were at the desired vegetative stage, plants were randomly assigned to an infestation level of 0 (uninfested control) or 20 soybean aphids (4<sup>th</sup> instars and adults). Aphids were placed on the youngest fully expanded trifoliolate leaf using a small paintbrush. Following aphid introduction, all plants were caged with tubular plastic cages (4 cm diameter by 46 cm height) with vents covered by organdy fabric to prevent aphid scape. Evaluations and tissue harvestings were conducted at three sampling days (D4, D6 and D8 after aphid introduction). The experiment was organized in a completely randomized with a 4 x 2 x 3 (four soybean genotypes, two levels of aphid infestation and three evaluations) and five replications. At each evaluation time, aphid number was determined and soybeans were examined for developmental stage and damage ratings, as described previously. Following evaluations, insects were removed from plants with a soft paintbrush and the youngest fully expanded trifoliolate was excised and immediately



frozen in liquid nitrogen. The harvested trifoliates were stored in  $-80^{\circ}\text{C}$  freezer, and later prepared for protein and RNA extraction.

**Total Peroxidase Activity.** Approximately 100 mg/sample of frozen soybean leaf tissue was weighed for soluble protein extraction. Protein was extracted with Minute Total Protein Extraction for Plant Tissues (Invent Biotechnologies, Eden Prairie, MN) according to manufacturer recommendations. During the extraction process, a protease inhibitor cocktail for plant tissue (Sigma-Aldrich, Saint Louis, MO) was added to each sample to prevent protein degradation (300  $\mu\text{L/g}$  of tissue).

A total of 20  $\mu\text{L}$  plant extract was transferred in to 1000  $\mu\text{L}$  of 95% acetone and incubated at  $-20^{\circ}\text{C}$  for 1 hour. Samples were centrifuged at 13,000 rpm for 10 minutes at  $4^{\circ}\text{C}$ . The supernatant was discarded and pellet allowed to air dry at room temperature. The pellet was dissolved in 120  $\mu\text{L}$  of 25 mM of sodium hydroxide and the resulting solution was diluted in water at a 1:11 ratio. The commercially available bicinchoninic acid (BCA) protein assay (Pierce, Rockford) was used to measure soluble protein, where bovine serum albumin served as the standard for protein quantification. In a 96-well microplate, 25  $\mu\text{L}$  of the diluted protein extract was loaded per well along with manufacturer recommended rate of the reagents A and B (provided in the protein assay kit). Samples were incubated at  $37^{\circ}\text{C}$  for 30 min before absorbance was measured with a spectrophotometer (Biotek PowerWave; Winooski, VT) at a wavelength of 562 nm.

The enzymatic activity for peroxidases was determined by a modified protocol from Hildebrand et al (1986) and Pierson et al. (2011). Each well of a microplate (96 wells) was loaded with 5  $\mu\text{L}$  of plant extract. The reaction was started by adding 2.5  $\mu\text{L}$  of 30% hydrogen peroxide, 75  $\mu\text{L}$  of 18 mM guaiacol, 25  $\mu\text{L}$  of 200 mM HEPES buffer

(pH 6.0) and 71.3  $\mu\text{L}$  of distilled water in the well containing the undiluted plant extract. Peroxidase activity measured in spectrophotometer (Biotek PowerWave) by monitoring the increase in absorbance at 470 nm for 2 min. The specific activity of total peroxidase was calculated using the molar absorptivity of guaiacol at 470 nm ( $26.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ). Each treatment consisted of five replications, where each replication was measured in a triplicate (3 assays per sample). Data analysis for total peroxidase activity was performed in PROC GLIMMIX, SAS, at a significance level of 5%. When necessary, means were separated by Fisher LSD test.

**RNA extraction and qRT-PCR assays.** In preparation for RNA isolation, approximately 200-300 mg of leaf tissue per sample was ground with liquid nitrogen and transferred into a 2 ml plastic tube. The total RNA was extracted from cells using the TRIzol® reagent, according to manufacturer's protocol (Invitrogen, Carlsbad, CA). Samples were treated with RNase-free DNase I for 10 min at room temperature and sequentially purified using RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA), following manufacturer's instructions. The integrity of total RNA bands was confirmed by agarose gel, and RNA quantification was determined with a spectrophotometer (NanoDrop 1000, Wilmington, DE).

cDNA first strand was synthesized using 2.5  $\mu\text{g}$  of total RNA with ThermoScript RT-PCR system (Life Technologies) according to manufacturer's protocol. There were at least 4 replications per treatment, measured in a triplicate (3 reactions per sample). qPCR reaction was performed in 20  $\mu\text{l}$ , which contained 10  $\mu\text{l}$  of SsoAdvanced SYBR Green (Bio-Rad Laboratories, California, USA), 9  $\mu\text{l}$  of cDNA (1:50 water dilution) and 1  $\mu\text{l}$  of 10  $\mu\text{M}$  primer mix. Primers were designed using NCBI platform and sequences are

provided in Table 5.1. Reads were performed on a 7500 Fast Real-time PCR (Applied Biosystems) with the following cycling parameters: 95°C for 30 sec for polymerase activation and DNA denaturation, then 40 amplification cycles at 95°C for 5 sec (denaturation) and 60°C for 30 sec (annealing/extension).

Calculations of  $\Delta\Delta CT$  were performed with the values of cycle threshold (CT) for each primer, and cyclophilin (CYP) as an endogenous control (Hu et al. 2009). Changes in transcript abundance were determined for each control and infested soybean genotype. In addition, differences among genotypes for the control treatment at each time point within the same vegetative stage (V1 or V3) were also calculated. Differences in relative expression were calculated using the equation  $2^{-\Delta\Delta CT}$ , and the statistical significance of CT values was determined through generalized mixed model analysis (PROC GLIMMIX, SAS) at significance of 5% as described in Study 1.

## Results and Discussion

**Study 1.** The overall statistical analysis for cumulative aphid-days (CAD) was not significant among the genotypes infested at the V1 stage (screening A) indicating that the high-yielding genotypes, U09-105007 and U11-919011, sustained soybean aphid populations that were comparable to the susceptible (SD76R) and tolerant (KS4202) genotypes (Table 5.2,  $F = 0.83$ ;  $df = 3, 36$ ;  $P = 0.48$ ). Conversely, there was a statistical significance for relative damage ranking at V1 stage (Table 5.2;  $F = 17.63$ ;  $df = 3, 36$ ;  $P < 0.0001$ ). KS4202 had the lowest damage rate among the genotypes evaluated, while U11-919011 was similar to the susceptible genotype. Further, U09-105007 sustained more damage ( $4.5 \pm 0.22$ ) than U11-919011 and SD76R, which resulted in a significantly higher damage ranking (Table 5.2).

No significant differences were detected in the CAD general statistical analysis in screening B, where plants were infested in the V3 stage (Table 5.3,  $F = 2.38$ ;  $df = 3, 36$ ;  $P = 0.086$ ). However, the accumulated aphid-days for U11-919011 was significantly lower than SD76R ( $13187.3 \pm 2552.5$  and  $17863 \pm 1496.82$ , respectively). The statistical analysis for relative damage ranking in the V3 stage was also significant (Table 5.3,  $F = 15.65$ ;  $df = 3, 33$ ;  $P < 0.0001$ ). Similarly to screening A (V1 stage), KS4202 had the lowest damage rating, when the performance of the high-yielding genotypes was similar to the susceptible reference (SD76R).

Based on the damage ratings, KS4202 was categorized as moderately resistant during both stages of infestation (Table 5.4). In the V1 stage, U09-105007 was highly susceptible to soybean aphids, when U11-919011 was found moderately susceptible.

When soybean aphids were introduced during the V3 stage, both high-yielding genotypes behaved similarly to SD76R, and therefore were categorized as moderately susceptible.

The levels of resistance found in KS4202 during the V3 stage is consistent with Marchi (2012), although V1 stage was reported as highly susceptible in the aforementioned study. The total CAD reported in Marchi (2012) is approximately 2-fold higher than this study, which indicates the observed divergence in the resistance category. Meanwhile, the resistance categories of U09-105007 and U11-919011 were comparable to SD76R (Table 5.4), indicating that these genotypes are susceptible to soybean aphids.

**Study 2.** When infested at V1 stage, the genotypes harbored a similar number of soybean aphids at D4 and D6 (Table 5.5). At D8, aphid population maintained a similar development trend as the previous evaluations; however, U09-105007 had significantly less aphids ( $142.0 \pm 32.3$  aphids/plant) than KS4202 ( $251.6 \pm 26.5$  aphids/plant). In the V3 stage, aphid number was similar among the genotypes at D4, but U09-105007 harbored fewer aphids than KS4202. At D6, both high-yielding soybeans had aphid populations that were significantly lower than KS4202, and at D8 U11-919011 and the susceptible SD76R had aphid populations that were statistically lower than KS4202 and U09-919011 (Table 5.5). Due to the briefness of the study, no visible aphid injury was observed on the four genotypes at D4, D6 and D8 after soybean aphids were introduced (data not shown).

**Total peroxidase activity basal levels.** The basal levels (i.e. uninfested or control plants) of peroxidase activity were statistically similar for V1-plants at D4 and D6; however, U11-919011 had significantly higher basal levels when compared to the other genotypes at D8 (Table 5.6). No apparent trends were observed in the basal peroxidase

levels at the V3 stage within the time points investigated in this study (Table 5.6).

Although the peroxidase specific activity is consistently increasing in both stages as the genotypes are aging, no evidence was found to support that there are differences among the genotypes. Therefore, the use of peroxidase basal levels does not appear to be a suitable indicator of soybean aphid tolerance under these experimental conditions.

**Total peroxidase activity induced by soybean aphids.** A small increase in total peroxidase was detected on V1 stage infested soybean relatively to the control, but no statistical differences were found for either time points (Table 5.7). Conversely, plants infested during the V3 stage responded differently. KS4202 had a significant increase in peroxidase activity when contrasted to the respective control at both D4 and D6 (Table 5.8; D4:  $P < 0.0001$ ; D6:  $P < 0.0001$ ); whereas, only a slight increase was observed for SD76R at those time points (Table 5.8; D4:  $P = 0.25$ ; D6:  $P = 0.11$ ). In addition, no differences were found between control and infested plants for the high yielding genotypes, except for U11-919011 at D4 (Table 5.8;  $P < 0.0001$ ). Lastly, there were no significant changes between control and infested plants sampled at day 8 (Table 5.8). Our findings compare favorably with Marchi-Werle et al. (2014), who found no differences in peroxidase activity between control and infested V1- KS4202 and V1 and V3 -SD76R at D6. In addition, the elevated enzymatic activity for infested V3- KS4202 reported in this study also aligns with Marchi-Werle et al. (2014), compiling more evidence of the role of peroxidases in KS4202 tolerance to aphids in later vegetative stages.

**Abundance of peroxidase transcripts in control (non-infested) plants.** The basal levels of PRX52 during V1 and V3 stages were statistically similar SD76R, U09-105007 and U11-919011 when compared to KS4202 for time points evaluated (Fig. 5.2

and 5.3). However, there was a tendency for higher abundance of this transcript in the high-yielding soybeans at both stages. No consistent trends were detected in the PRX2 basal levels of SD76R, U09-105007 and U11-919011 relatively to KS4202. Although SD76R had a higher abundance of PRX2 than KS4202 at both D6 (Fig. 5.4;  $P = 0.01$ ) and D8 (Fig. 5.4;  $P = 0.04$ ), no differences were found for the high-yielding soybeans (Fig. 5.4). Moreover, there was a lower transcript abundance of PRX2 at D6 and D8 for V3-U09-105007 (Fig. 5.5; D6:  $P = 0.0003$ ; D8:  $P = 0.0006$ ) and V3- U11-919011 at day 6 (Fig. 5.5;  $P = 0.0016$ ).

**Abundance of peroxidase transcripts in soybean aphid infested plants.** For the soybean aphid infested V1 plants (Fig. 5.6), no changes occurred at D4 for the genotypes analyzed. In the later time points, KS4202 infested plants had a fold change of 6.4 (Fig. 5.6;  $P = 0.16$ ) and a 9.3 (Fig. 5.6;  $P = 0.002$ ) at D6 and D8, respectively, when compared to aphid free plants. No changes were observed for U09-105007 at all times; however, infested U11-919011 had increased PRX52 transcript abundance at D8 ( $P = 0.002$ ). In V3 stage, the comparisons between controls and infested plants for PRX52 transcript abundance did not result in statistical differences (Fig. 5.7). Although the trends reveal that at D8, infested KS4202 had the highest fold change (4.6) among the genotypes in this study.

V1-infested SD76R showed lower PRX2 transcript expression in infested plants at D4 (Fig. 5.8;  $P = 0.01$ ) and D6 (Fig. 5.8;  $P = 0.001$ ). There was also a reduction in the abundance of this transcript for KS4202 ( $P < 0.0001$ ) and high yielding genotypes (U09-105007:  $P = 0.01$ ; U11-919011;  $P = 0.01$ ) at D8 after aphid introduction. In the V3 stage, there were generally no alterations in the expression of PRX2 transcript when contrasting

aphid infested and control soybeans (Fig. 5.9), although infested KS202 at D6 (Fig. 5.9,  $P = 0.02$ ) had a slight reduction in the expression of PRX2 in regards to its control. Overall these analyses revealed that in the presence of soybean aphids, the tolerant genotype, KS4202, has an elevated activity of peroxidases and a higher transcript abundance of PRX52 than the high-yielding and susceptible soybean genotypes.

Breeding programs targeting insect-tolerant plants still remain largely underexplored. Several factors have likely contributed to this, including limited knowledge of the mechanisms of tolerance and the genes controlling the tolerance response. Considered as polygenic resistance, tolerance is a category of HPR that reduces the detrimental effects of herbivore damage on plant fitness (Smith 2005). Different than antibiosis and antixenosis, tolerance is a plant's response that can only be quantified in relation to other genotypes or species (Tiffin 2000).

As a preliminary screening method, several studies have successfully used biophysical characters like damage ratings scores to identify tolerance in buffalograss, soybean and switchgrass to phloem-feeding insects (Heng-Moss et al. 2002, Pierson et al. 2010, Koch et al. 2014). However, in a breeding program, the use of damage scores when making selections may not always be the most appropriate criterion. CIAT (1985) made selections based on low damage scores in the early generations ( $F_2$ ) and tested yield performance in  $F_5$  generations in common beans (*Phaseolus vulgaris* L.) tolerant to leafhopper (*Empoasca kraemeri* Ross & Moore). After five cycles of recurrent selection, they found no significant progress in the program. In that case, feeding damage scores were found moderately associated with unprotected yield, which lead the group to



implement yield response as the primary criteria for selection (Kornegay and Cardona 1990).

Identifying reliable and fast methods for phenotypically classify populations of RIL are of great importance to ensure breeding objectives are achieved. Based on previous indications of the role of peroxidases in the tolerance of KS4202 to soybean aphids (Pierson et al. 2011, Marchi-Werle et al. 2014, Prochaska et al. 2015), this study investigated the potential of these enzymes as markers to categorize RIL for a breeding program. The enzyme kinetics assays indicated no differences or consistencies when contrasting the basal levels of peroxidase activity of tolerant, high-yielding and susceptible genotypes in both the V1 and V3 stages. Therefore, the use of peroxidase basal levels is not a feasible parameter for phenotyping RIL. In the presence of soybean aphids, a trend for higher peroxidase activity in KS4202 was observed at the V3 stage. In contrast, the high-yielding (U09-105007 and U11-919011) and susceptible (SD76R) genotypes were not able to produce a similar response. Despite the potential of this assay to evaluate the parental lines (pending validation with RIL populations), this technique may not be ideal. There is a definite limitation in the quantity of plants that can be tested simultaneously since aphid infestation is required.

Transcriptomic analysis performed to contrast the basal levels (from uninfested plants) of PRX52 and PRX2 was also not adequate to create a phenotypic profile of the genotypes tested in this study. Our results indicated that constitutive levels of these transcripts were not different among aphid-tolerant, susceptible and high-yielding genotypes. Future research should verify the potential of other transcripts involved in soybean's defense to soybean aphids. A study from Marchi-Werle (unpublished) found

that aphid-tolerant KS4202 had greater transcript abundance of basal levels of LOX1 (lipoxygenase 1), LOX2 (lipoxygenase 2), LOX10 (lipoxygenase 10) and OPR3 (12-oxophytodienoate reductase 3) than aphid-susceptible plants, indicating a potential candidate for further work. In addition, it would be of interest to pair such experiments with proteomic studies (e.g. lipoxygenase assays), and later validate these findings with the populations derived from the phenotyped parents (i.e. KS4202 and high-yielding lines).

Overall, there was an indication that KS4202 had greater abundance of PRX52 than the high-yielding and susceptible genotypes when soybean aphids were introduced at stages analyzed. In functional transcriptomic approaches, Prochaska et al. (2015) also detected peroxidases to be up-regulated in infested KS4202. In their study, PRX52 (Glyma06g15030) to be up regulated in late vegetative KS4202 at 15 days post soybean aphid introduction, when no changes occurred in susceptible plants. Despite the evidence of PRX52 involvement in KS4202 tolerance, no direct relationship in the expression of PRX2 and soybean aphid feeding, in both V1 and V3 stages, was detected in this study. This could be due to the versatility of plant peroxidases. These enzymes are known to be present in several isoforms, and many are specific to plant development stage, tissue and certain environmental stimuli (Lagrimini 1992).

To our knowledge, this research represents the first attempt to use peroxidases as a marker to phenotype parental lines of RIL populations for breeding soybean aphid tolerance in soybeans. Additional research is still needed to investigate other defense-related transcripts including those involved in JA-signaling (LOX1, LOX2, LOX10 and OPR3) as a basal level (or constitutive) marker for soybean tolerance. From breeding

perspective, measuring basal levels is more appealing than aphid-induced changes as it maximizes time and resources to select large populations of RIL. There is also a need to address the efficiency of these assays for proper phenotyping of the RIL populations. The data obtained from phenotyping are valuable to conduct quality QTL analysis (SSR and SNP markers) and thus map plant tolerance traits. Proper phenotypic followed by genotypic analysis will open new niches for exploring plant tolerance to insects, facilitating its incorporation into breeding programs.

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**Figures and Tables****Table 5.1.** Primer sequences and NCBI accession number.

<b>NCBI Accession Number</b>	<b>Symbol</b>	<b>Forward sequence</b>	<b>Reverse sequence</b>
NM_001254985.2	PRX52	CCGCCATGATCAAGATGGGA	AACCCACCACGGAATCCAAA
XM_014766488.1	PRX2	TTGCTGTAGGCTTTGGTCCT	TTGTTGGCCCAAGGTACTC
XM_014764426.1	CYP	ACGACGAAGACGAGTGG	CGACGACGACAGGCTTGG



**Table 5.2.** Mean  $\pm$  SE cumulative aphid-days (CAD) and damage ratings for soybean genotypes infested with soybean aphids in the V1 stage (study 1).

<b>Genotype</b>	<b>CAD<sup>1</sup></b>	<b>Damage rating<sup>2</sup></b>	<b>Relative damage ranking</b>
KS4202	14339.3 $\pm$ 1239.10 a	2.35 $\pm$ 0.10	10.25 $\pm$ 1.81 c
SD76R	16973.0 $\pm$ 864.71 a	3.10 $\pm$ 0.18	24.05 $\pm$ 3.45 b
U09-105007	15201.3 $\pm$ 1713.9 a	4.50 $\pm$ 0.22	42.70 $\pm$ 2.72 a
U11-919011	16010.0 $\pm$ 947.36 a	3.05 $\pm$ 0.24	24.90 $\pm$ 4.21 b

Treatment means within the same column followed by the same letter indicate no significant differences ( $P > 0.05$ ), LSD test.

<sup>1</sup>CAD and damage levels measured 16 days after aphid introduction

<sup>2</sup>Damage ratings based on 1-5 scale, where 1 = 10% or less of the leaf area damaged; 2 = 11-30% of the leaf area damaged; 3 = 31-50% of the leaf area damaged; 4 = 51-70% of the leaf area damaged; and 5 = 71% or more of the leaf area damaged and the plant near death.

<sup>3</sup>Damage ratings analyzed as nonparametric data, using a rank-based procedure.

**Table 5.3.** Mean  $\pm$  SE cumulative aphid-days (CAD) and damage ratings for soybean genotypes infested with soybean aphids in the V3 stage (study 1).

<b>Genotype</b>	<b>CAD<sup>1</sup></b>	<b>Damage rating<sup>2</sup></b>	<b>Relative damage ranking<sup>3</sup></b>
KS4202	15400.0 $\pm$ 1389.09 ab	2.00 $\pm$ 0.21	7.05 $\pm$ 1.79 c
SD76R	17863.0 $\pm$ 1496.82 a	3.45 $\pm$ 0.17	26.05 $\pm$ 3.29 ab
U09-105007	15487.9 $\pm$ 1173.80 ab	3.15 $\pm$ 0.11	20.05 $\pm$ 2.10 a
U11-919011	13187.3 $\pm$ 2522.50 b	3.60 $\pm$ 0.14	28.85 $\pm$ 2.37 ab

Treatment means within the same column followed by the same letter indicate no significant differences ( $P > 0.05$ ), LSD test.

<sup>1</sup>CAD and damage levels measured 16 days after aphid introduction

<sup>2</sup>Damage ratings based on 1-5 scale, where 1 = 10% or less of the leaf area damaged; 2 = 11-30% of the leaf area damaged; 3 = 31-50% of the leaf area damaged; 4 = 51-70% of the leaf area damaged; and 5 = 71% or more of the leaf area damaged and the plant near death.

<sup>3</sup>Damage ratings analyzed as nonparametric data, using a rank-based procedure.

**Table 5.4.** Resistance categories based on damage ratings of soybean genotypes infested with soybean aphids at V1 and V3 stages (study 1).

<b>Genotype</b>	<b>V1 stage</b>	<b>V3 stage</b>
KS4202	MR	MR
SD76R	MS	MS
U09-105007	HS	MS
U11-919011	MS	MS

Highly susceptible (HS, mean damage ratings  $\geq 4$ ); moderately susceptible (MS, mean damage ratings  $\geq 3$  but  $< 4$ ); moderately resistant (MR, mean damage ratings  $\geq 2$  but  $< 3$ ); and highly resistant (HR, mean damage ratings  $< 2$ ).

**Table 5.5.** Mean  $\pm$  SE for soybean genotypes infested with soybean aphids during V1 and V3 stages at D4, D6 and D8 (study 2).

	<b>D4</b>	<b>D6</b>	<b>D8</b>
<b>Genotype</b>	<b>V1 stage</b>		
KS4202	76.2 $\pm$ 17.3 a	67.0 $\pm$ 4.4 a	251.6 $\pm$ 26.5 a
SD76R	78.4 $\pm$ 12.1 a	45.0 $\pm$ 9.1 a	206.2 $\pm$ 36.6 ab
U09-105007	76.0 $\pm$ 17.5 a	46.8 $\pm$ 7.6 a	142.0 $\pm$ 32.3 b
U11-919011	65.6 $\pm$ 9.70 a	58.2 $\pm$ 7.4 a	178.8 $\pm$ 36.7 ab
<b>Genotype</b>	<b>V3 stage</b>		
KS4202	102.4 $\pm$ 19.2 a	102.4 $\pm$ 19.2 a	222.6 $\pm$ 36.3 a
SD76R	73.8 $\pm$ 16.7 ab	73.8 $\pm$ 16.7 ab	131.2 $\pm$ 31.8 b
U09-105007	58.0 $\pm$ 5.70 b	42.0 $\pm$ 11.9 b	190.2 $\pm$ 57.7 a
U11-919011	64.2 $\pm$ 13.0 ab	40.4 $\pm$ 7.40 b	128.2 $\pm$ 6.60 b

Treatment means within the same column followed by the same letter indicate no significant differences ( $P > 0.05$ ), LSD test.

**Table 5.6.** Basal levels (uninfested plants) of peroxidase activity ( $\mu\text{mol}/\text{min} \times \text{mg}$  of protein) for V1 and V3 stage soybeans at D4, D6 and D8 (study 2).

	<b>D4</b>	<b>D6</b>	<b>D8</b>
<b>Genotype</b>	<b>V1 stage</b>		
KS4202	1.73 $\pm$ 0.25 a	1.52 $\pm$ 0.07 ab	2.50 $\pm$ 0.44 b
SD-0176R	1.43 $\pm$ 0.35 a	1.65 $\pm$ 0.35 ab	2.40 $\pm$ 0.54 b
U09-105007	1.62 $\pm$ 0.22 a	1.19 $\pm$ 0.13 b	2.41 $\pm$ 0.31 b
U11-919011	1.56 $\pm$ 0.33 a	1.98 $\pm$ 0.13 a	3.89 $\pm$ 0.47 a
<b>Genotype</b>	<b>V3 stage</b>		
KS4202	2.65 $\pm$ 0.35 a	2.02 $\pm$ 0.32 a	3.78 $\pm$ 0.52 a
SD-0176R	1.32 $\pm$ 0.52 b	1.50 $\pm$ 0.29 a	2.36 $\pm$ 0.92 a
U09-105007	1.73 $\pm$ 0.23 ab	1.85 $\pm$ 0.16 a	3.51 $\pm$ 0.44 a
U11-919011	2.56 $\pm$ 0.31 a	2.09 $\pm$ 0.42 a	3.60 $\pm$ 0.92 a

Treatment means within the same column followed by the same letter indicate no significant differences ( $P > 0.05$ ), LSD test.

**Table 5.7.** Peroxidase specific activity ( $\mu\text{mol}/\text{min} \times \text{mg}$  of protein) for soybean genotypes infested during the V1 stage at D4, D6 and D8 after aphid introduction (study 2).

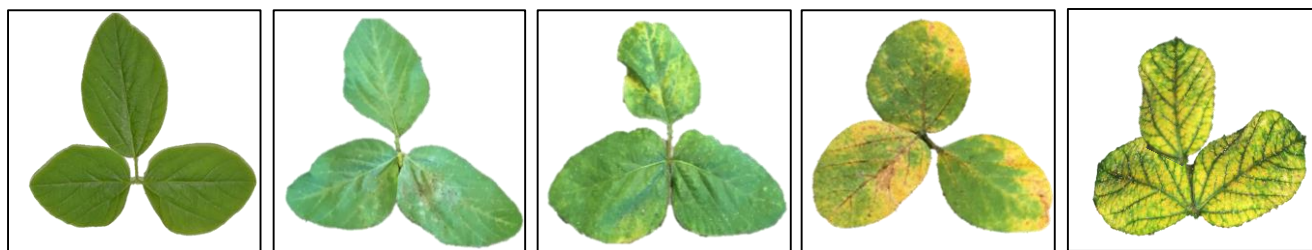
Genotype	D4			D6			D8		
	Control	Infested	<i>P-value</i>	Control	Infested	<i>P-value</i>	Control	Infested	<i>P-value</i>
KS4202	1.73 $\pm$ 0.25	2.79 $\pm$ 0.60	0.14	1.52 $\pm$ 0.07	2.62 $\pm$ 0.33	0.15	2.50 $\pm$ 0.44	2.32 $\pm$ 0.17	0.80
SD76R	1.43 $\pm$ 0.35	2.55 $\pm$ 0.68	0.14	1.65 $\pm$ 0.35	2.54 $\pm$ 0.05	0.29	2.40 $\pm$ 0.54	3.13 $\pm$ 0.50	0.33
U09-105007	1.62 $\pm$ 0.22	2.29 $\pm$ 0.43	0.41	1.19 $\pm$ 0.13	1.93 $\pm$ 0.17	0.33	2.41 $\pm$ 0.31	2.56 $\pm$ 0.37	0.83
U11-919011	1.56 $\pm$ 0.33	2.09 $\pm$ 0.29	0.46	1.98 $\pm$ 0.13	2.71 $\pm$ 0.20	0.31	3.89 $\pm$ 0.47	4.22 $\pm$ 0.94	0.64

Statistical significance at  $P < 0.05$

**Table 5.8.** Total peroxidase activity ( $\mu\text{mol}/\text{min} \times \text{mg}$  of protein) for soybean genotypes infested during the V3 stage at D4, D6 and D8 after aphid introduction (study 2).

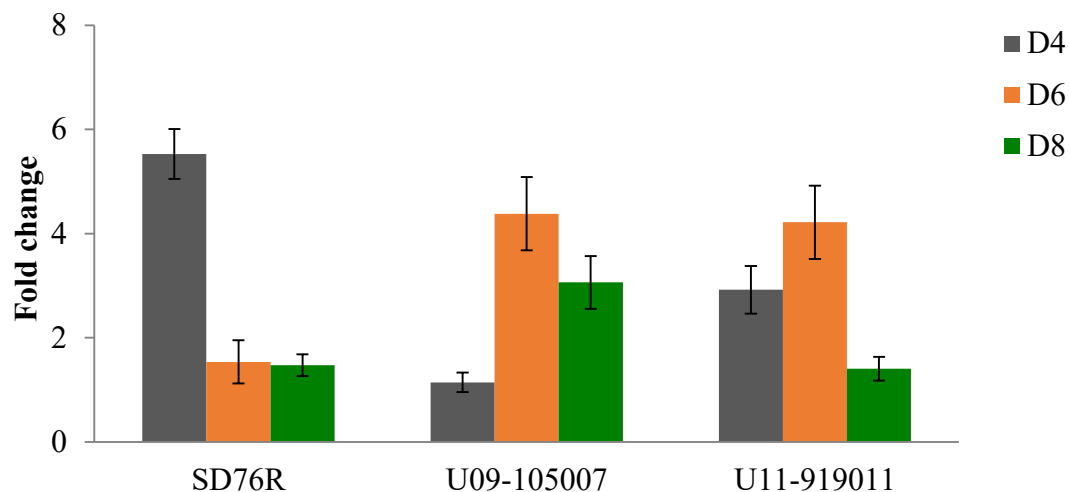
Genotype	D4			D6			D8		
	Control	Infested	<i>P-value</i>	Control	Infested	<i>P-value</i>	Control	Infested	<i>P-value</i>
KS4202	2.65 $\pm$ 0.35	5.36 $\pm$ 1.49	<0.0001	2.02 $\pm$ 0.32	5.18 $\pm$ 0.64	<0.0001	3.78 $\pm$ 0.52	3.11 $\pm$ 0.61	0.38
SD76R	1.32 $\pm$ 0.52	2.15 $\pm$ 0.27	0.25	1.50 $\pm$ 0.29	2.81 $\pm$ 0.37	0.11	2.36 $\pm$ 0.92	2.12 $\pm$ 0.22	0.99
U09-105007	1.73 $\pm$ 0.23	2.42 $\pm$ 0.37	0.33	1.85 $\pm$ 0.16	1.95 $\pm$ 0.13	0.88	3.51 $\pm$ 0.44	3.42 $\pm$ 0.66	0.90
U11-919011	2.56 $\pm$ 0.31	5.40 $\pm$ 1.21	<0.0001	2.09 $\pm$ 0.42	2.58 $\pm$ 0.23	0.54	3.60 $\pm$ 0.92	3.83 $\pm$ 0.52	0.75

Statistical significance at  $P < 0.05$

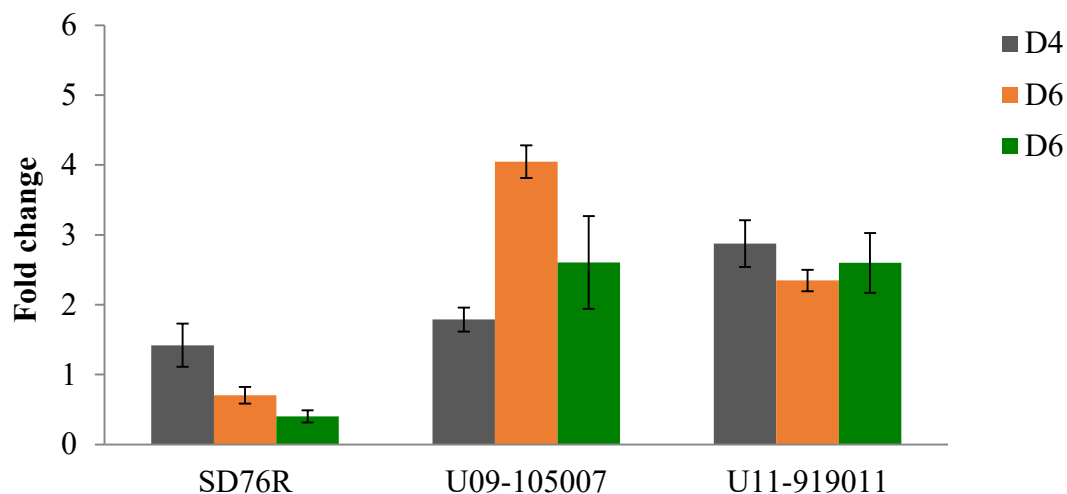


**Fig. 5.1.** Damage ratings were assigned according to 1-5 scale; 1 = 10% or less of leaf area discolored; 2 = 11-30% of leaf area discolored; 3 = 31-50% of leaf area discolored; 4 = 51-75% of leaf area discolored; and 5 =  $\geq 75\%$  of leaf area discolored or dead tissue.

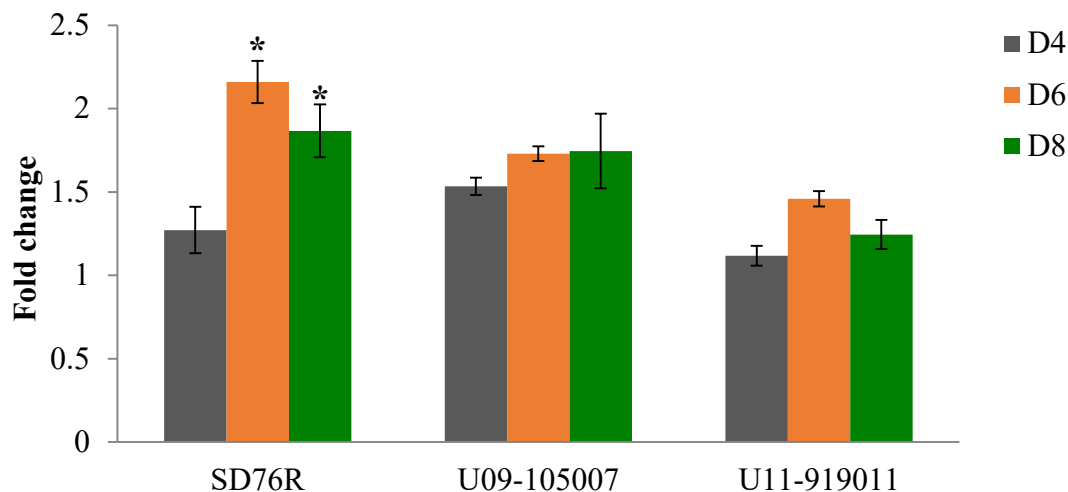




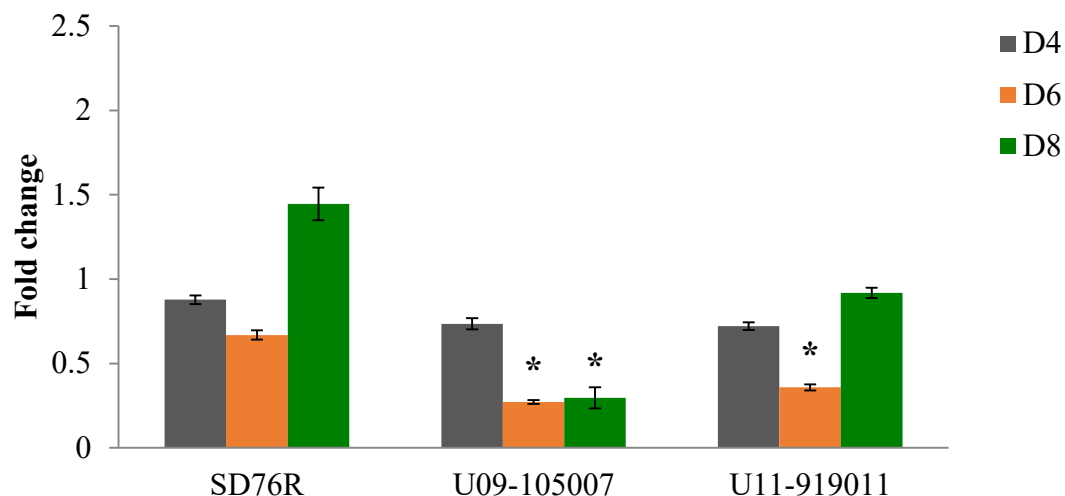
**Fig. 5.2.** PRX52 transcript abundance of SD76R, U09-105007 and U11-919011 relative to KS4202 at D4, D6 and D8 after plants reached V1 stage (study 2). A fold change >1 indicates greater transcript abundance in the given genotype compared to KS4202, while a fold change <1 represents a higher transcript abundance in KS4202 when compared to the given genotype. A fold change of 1 indicates no differences between KS4202 and the compared genotype. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI}) - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance ( $P < 0.05$ ) indicated by (\*).



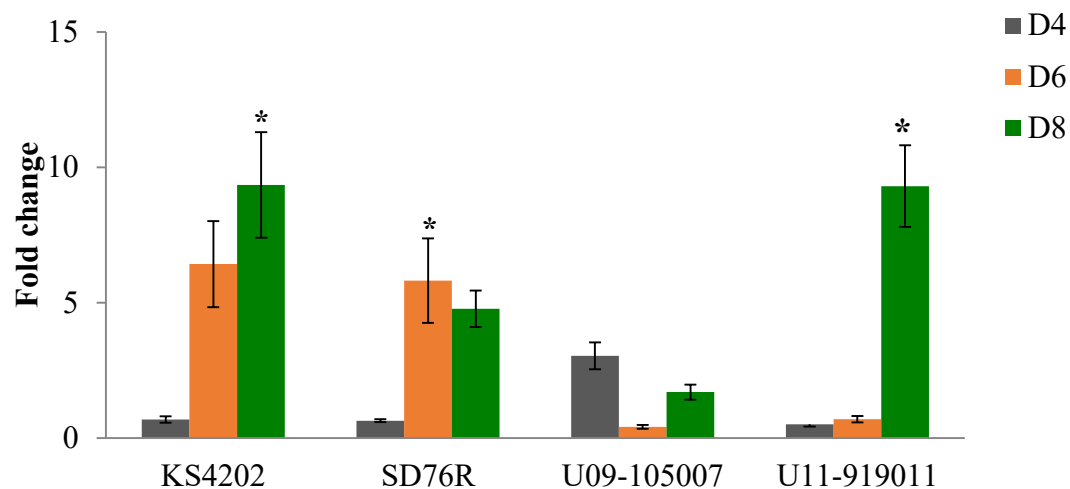
**Fig. 5.3.** PRX52 transcript abundance of SD76R, U09-105007 and U11-919011 relative to KS4202 at D4, D6 and D8 after plants reached V3 stage (study 2). A fold change >1 indicates greater transcript abundance in the given genotype compared to KS4202, while a fold change <1 represents a higher transcript abundance in KS4202 when compared to the given genotype. A fold change of 1 indicates no differences between KS4202 and the compared genotype. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI}) - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance ( $P < 0.05$ ) indicated by (\*).



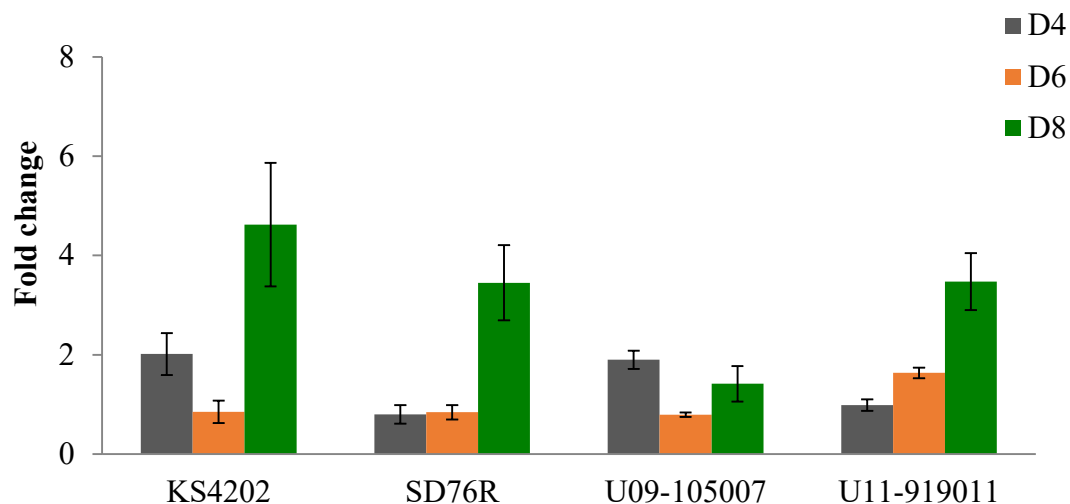
**Fig. 5.4.** PRX2 transcript abundance of different soybean genotypes relative to KS4202 at D4, D6 and D8 after plants reached V1 stage (study 2). A fold change >1 indicates greater transcript abundance in the given genotype compared to KS4202, while a fold change <1 represents a higher transcript abundance in KS4202 when compared to the given genotype. A fold change of 1 indicates no differences between KS4202 and the compared genotype. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI}) - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance ( $P < 0.05$ ) indicated by (\*)



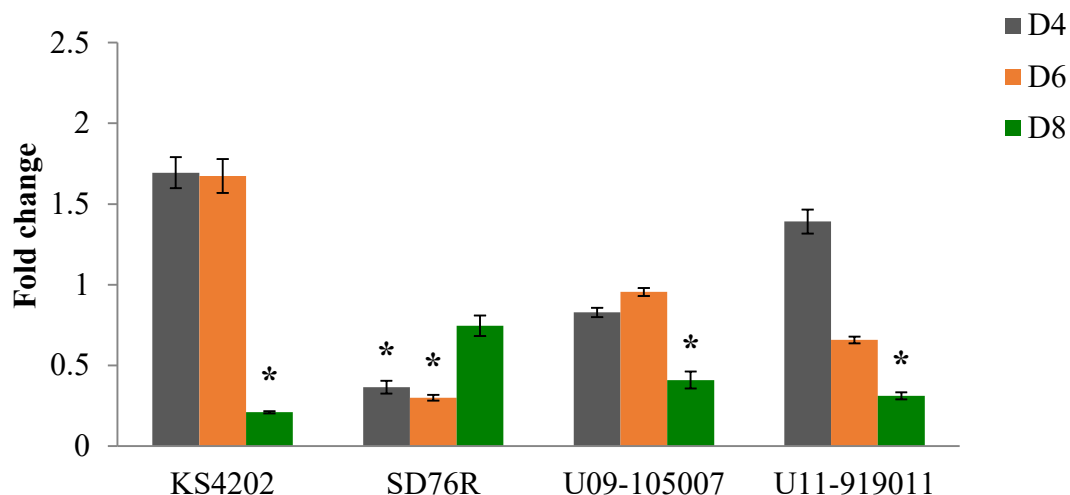
**Fig. 5.5.** PRX2 transcript abundance of different soybean genotypes relative to KS4202 at D4, D6 and D8 after plants reached V3 stage (study 2). A fold change >1 indicates greater transcript abundance in the given genotype compared to KS4202, while a fold change <1 represents a higher transcript abundance in KS4202 when compared to the given genotype. A fold change of 1 indicates no differences between KS4202 and the compared genotype. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI}) - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance ( $P < 0.05$ ) indicated by (\*).



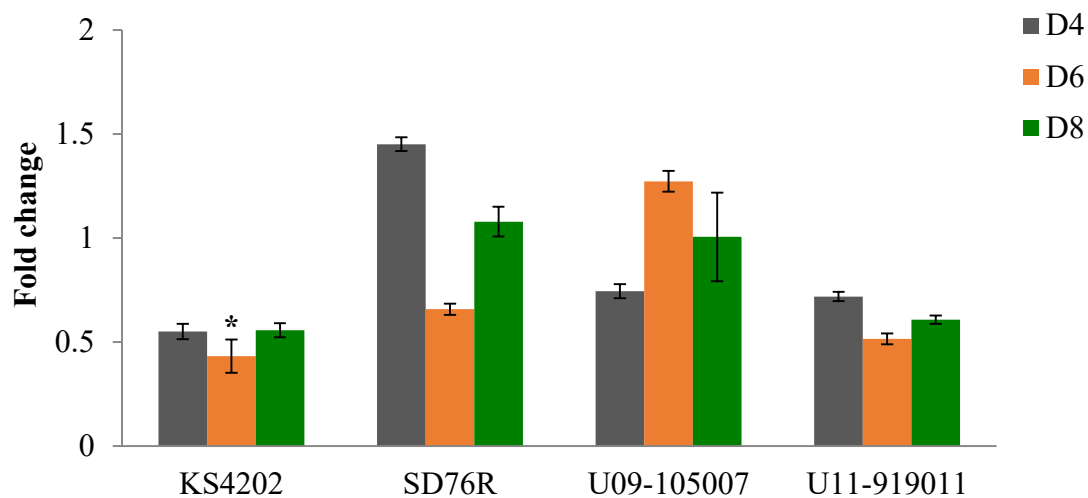
**Fig. 5.6.** Relative expression of PRX52 in the presence of soybean aphids introduced at the V1 stage at D4, D6 and D8 (study 2). A fold change >1 represents higher transcript abundance in the infested treatment, while fold changes <1 indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance ( $P < 0.05$ ) indicated by (\*).



**Fig. 5.7.** Relative expression of PRX52 in the presence of soybean aphids introduced at the V3 stage at D4, D6 and D8 (study 2). A fold change >1 represents higher transcript abundance in the infested treatment, while fold changes <1 indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance ( $P < 0.05$ ) indicated by (\*).



**Fig. 5.8.** Relative expression of PRX2 in the presence of soybean aphids introduced at the V1 stage at D4, D6 and D8 (study 2). A fold change >1 represents higher transcript abundance in the infested treatment, while fold changes <1 indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance ( $P < 0.05$ ) indicated by (\*).



**Fig. 5.9.** Relative expression of PRX2 in the presence of soybean aphids introduced at the V3 stage at D4, D6 and D8 (study 2). A fold change >1 represents higher transcript abundance in the infested treatment, while fold changes <1 indicates higher transcript abundance in the control treatment of the given genotype. Fold change equal to 1 represents no difference between infested and control treatments. Fold change calculated as  $\Delta\Delta Ct = [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{GI})] - [(Ct \text{ for sample cDNA} - Ct \text{ for control cDNA}_{CYP})]$ ; GI = gene of interest; CYP = UCE internal control reference. Statistical significance ( $P < 0.05$ ) indicated by (\*).