Fall 11-26-2012

Investigating the tolerance response of early vegetative stage soybeans to *Aphis glycines* Matsumura

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Investigating the Tolerance Response of Early Vegetative Stage Soybeans to *Aphis glycines* Matsumura

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A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Entomology

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

Lincoln, Nebraska

December 2012

Investigating the Tolerance Response of Early Vegetative Stage Soybeans to *Aphis glycines* Matsumura

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University of Nebraska, 2012

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The objectives of this research were to evaluate the impact of soybean aphid (*Aphis glycines* Matsumura) feeding on the yield response of V1, V3, and R1 KS4202 soybean plants and examine the effect of soybean aphid feeding on peroxidase activity in V1 and V3 KS4202 plants. KS4202 plants infested during the early vegetative stages (VC, VE and V1) were identified as highly susceptible based upon plant damage and stunting of the plants. In contrast, V3, V4, and V5 stage KS4202 soybeans were classified as moderately resistant. In the yield response study, V1, V3 and R1 plants had aphid numbers that exceeded the average economic injury level threshold of 674 aphids per plant. Despite exceeding this level, V3 and R1 aphid-infested plants were not statistically different from their respective control plants for any of the yield parameters evaluated except average pod weight, which was statistically higher for plants exposed to the high aphid treatment than to the control treatment. Changes in protein content, peroxidase activity and isozyme profiles in response to aphid feeding were documented in V1 and V3 stages of tolerant (KS4202) and susceptible (SD76R) soybeans at 6, 16, and 22 days after aphid introduction. Protein content was similar between infested and control V1 and V3 stage plants for both KS4202 and SD76R at 6, 16, and 22 days after aphid

introduction. Enzyme kinetics studies documented that KS4202 V1 and SD76R V1 and V3 control and aphid-infested soybean had similar levels of peroxidase activity at the three time points evaluated. By contrast, KS4202 aphid-infested plants at the V3 stage had significantly higher peroxidase activity levels than control plants at 6 and 22 days after aphid introduction. The differences in peroxidase activity observed between infested and control V3 KS4202 plants throughout the course of the experiment suggest that peroxidases may be playing multiple roles in the tolerant plant. Gels stained for peroxidases identified differences in the isozyme profiles of aphid-infested and control plants for both KS4202 and SD76R. The results of this research provide insights to better understand the tolerance response in KS4202 and ultimately will result in improved management options for this important insect pest.

ACKNOWLEDGMENTS

I want to express my deepest gratitude to my advisors Dr. Thomas Hunt and Dr. Tiffany Heng-Moss, for their guidance, patience, support, caring, friendship, and also for providing me an excellent environment to perform my research. I also want to thank my supervisory committee, Dr. Z B Mayo and Dr. Lisa Baird, for sharing their valuable knowledge and inputs during the progress of my research. In addition, I want to thank Dr. Lisa Baird for her assistance with the electron microscopy and for teaching me the microscope techniques. I am also very grateful to my former advisor Dr. Edson Baldin, for his friendship, support and motivation during my undergraduate and master's program.

My research would not have been accomplished without the assistance of several past and present members of the Turfgrass Lab (Dr. Tiffany Heng-Moss, Mitch Stamm, Crystal Ramm, Travis Prochaska, Kyle Koch, Chelsea Piitz, Rachael Fithian, Jenny Freed, Courtney Spilker, Ginny Morgal, Kait Armitage and Natalie Goergen-Matz), for helping me in several aspects of my research, including the setup of experiments in the greenhouse through the analysis in the laboratory. Special thanks for all the assistance that I received from the staff members and professors of the Entomology Department at UNL. I'm also grateful to Travis Prochaska, Crystal Ramm, Rachael Fithian, Camila Oliveira, Jenny Freed and Karen da Silva, for their devotion and for being part of my life.

I want express my enormous gratitude to my family, because they are the ones that brought me to where I am. My parents, Otavio and Ana Maria, and my sister Noá, never measured efforts to help me to get through difficult moments and were always

present to celebrate my victories. I also thank my fiancée Rodrigo Werle, whose love, unselfishness, patience and encouraging words had help me to move forward with my graduate program. Finally, I thank God for all the amazing people he had put in life and the opportunities that he had offered me.

TABLE OF CONTENTS

Chapter 1. Introduction and Literature Review

Introduction and Literature Review…..……1

Thesis Objectives……...18

Chapter 2. Categorizing the Resistance of Vegetative and Reproductive Stages Soybeans to the Soybean Aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae) in the Greenhouse

Introduction……19

Materials and Methods...……22

Results and Discussion……..26

Tables and Figures…….29

Chapter 3. Physiological Responses of Resistant and Susceptible Soybean Genotypes to the Soybean Aphid (*Aphis glycines* Matsumura) Feeding

Introduction……36

Materials and Methods……...39

Results and Discussion……..43

Tables and Figures.……47

References Cited……...52

CHAPTER 1

Introduction and Literature Review

Soybeans

Soybean (*Glycine max* (L.) Merrill) is the dominant oil-seed in world trade and represents the majority of the oilseed produced in the world. Soybean production provides a variety of animal consumables and product goods from crayons to plastics (Endres 2001). Most soybeans are utilized for their oil and protein for livestock feed. Additionally, a small amount of soybean production is destined for human consumption, production of biofuels and industrial processing (Endres 2001).

In the United States, the cultivation of soybeans has contributed between $29.5 and $40 billion in cash receipts and grain production between 2008 and 2011. For the same period, an estimated area of 76 million acres was planted, resulting in a yield ranging from 3.0 to 3.06 billion bushels (USDA-ERS 2012). Over 80% of the acres planted with soybean are represented by 12 north-central states, including Nebraska. Nebraska is considered to be one of the largest producers in the country. From 2008 and 2011, approximately 5 million acres were harvested and 226 to 262 million bushels produced in Nebraska (USDA-ERS 2012).

Soybean aphid ecology in North America

The soybean aphid, *Aphis glycines* Matsumura, is an introduced pest in North America. Native to eastern Asia including China, Eastern Russia, Japan, Korea, Thailand, the Philippines and Vietnam, the soybean aphid was first detected in the United States in

2000 (Hartman et al. 2001). Although the soybean aphid is considered a sporadic pest in China (Wang et al. 1994), its impact in the Midwest United States has become extensive. Three years after its introduction, Venette & Ragsdale (2004) reported the presence of soybean aphid in 22 states in the United States and three provinces of Canada. The soybean aphid's distribution has expanded to 30 states in the United States as well as southeast Canada (Ragsdale et al. 2011).

The life cycle of soybean aphid in North America is similar to that in its native range, consisting of a heteroecious (alternation of hosts) and holocyclic (different physical forms) life cycle (Wu et al. 2004). Soybeans are considered a secondary host of the soybean aphid. *Rhamnus cathartica L.* (common buckthorn) is considered to be the primary host. This woody plant is widely distributed across North America and its presence is critical for soybean aphid winter survival (Ragsdale et al. 2004). During the spring and summer months, soybean aphids feed on soybeans and reproduce by parthenogenic viviparae (without mating), resulting in all offspring being female (Ragsdale et al. 2004). Asexual reproduction is a strategy that allows these aphids to have a high rate of reproduction. McCornack et al. (2004) found that soybean aphid population growth was greatest at 25^{o}C; at this temperature aphid numbers can double in 1.5 days. However, the presence of biotic and abiotic factors, such as natural enemies, diseases and extreme weather conditions decreases the rate of population doubling time to 5 – 6 days (Ragdale et al. 2007).

At the end of soybean growing season, both male and female aphids migrate back to *R. cathartica* (Ragsdale et al. 2007). The migration of soybean aphids to the buckthorn is by alate forms. The production of migrants usually occurs during the beginning of

soybean seed set, which is the period that coincides with decreasing temperatures and photoperiod. Hodgson et al. (2005) suggested that it is the combination of these signals that triggers the production of winged forms in preparation for overwintering. However, some authors speculate that aphid population dynamics and migration between soybean and buckthorn is influenced by the nitrogen concentration in the phloem, which dramatically drops once the soybean plant reaches R7 (Wu et al. 2004; Beckendorf et al. 2008).

The migrant males and females (gynoparae) feed and reproduce on buckthorn. The offspring produced through sexual reproduction results in females that produce overwintering eggs (Ragsdale et al. 2004). The overwintering eggs are very resistant to low temperatures. According to McCornack et al. (2005), soybean aphid eggs can survive temperatures as low as -34^{o}C. Once spring arrives, hatched eggs will begin a new generation of insects. Approximately three to four generations occur on buckthorn. Colonies on buckthorn produce alate females, which migrate to early vegetative soybeans in late spring/early summer (Bahlai et al. 2008). Probably due to the limited number of *R. cathartica* in Nebraska, the soybean aphid is generally detected here in late June to mid-July. The aphids are dispersed by jet-stream air currents and colonize reproductive stage soybeans (Brosius et al. 2007).

Feeding Injury on Soybean

The pattern of colonization on the soybean plant varies as the plant matures (McCornack et al. 2008). At the start of the growing season, soybean aphids target the newly emerged trifoliate leaves, specifically the undersides of these leaves. As the plant

develops and aphid numbers increase, individuals spread to the leaves, petioles, pods and stems of the lower canopy (Ragsdale et al. 2004). Initially, only a few aphids may occur per plant; however, favorable environmental conditions and the absence of management strategies (e.g., insecticides and natural enemies) lead to the formation of several hundred or even thousands of aphids per plant (Tilmon et al. 2011).

The insect feeds by piercing foliar and stem tissue in order to withdraw the phloem contents, which can result in the transmission of viral pathogens (Clark and Perry 2002), poor canopy development and significant reductions in photosynthesis (Macedo et al. 2003; Pierson et al. 2011). Heavy infestations are commonly associated with dark sooty mold (*Capnodium* sp.) development on the sugary excretions or "honeydew" that the aphids produce (Tilmon et al. 2011).

Ultimately, the injury caused by the soybean aphid manifests as a reduction in plant height, pod development and a lower number of seeds at maturity, consequently causing considerable yield loss (Ragsdale et al. 2007). Besides the effects on soybean yield, Beckendorf et al. (2008) also reported that soybean aphid injury decreases the amount of seed oil to an extent that can significantly affect the market value of soybean seeds.

Economic Importance in North America

The soybean aphid is currently the primary pest of soybeans in the North-Central regions of the United States (Hodgson et al. 2012). Before the soybean aphid's introduction in 2000, there were only a few soybean pest issues in the Midwest. The USDA-NASS (2000) estimated that before 2000 less than 1% of the soybean fields were

treated with insecticides. During the years of 2003-2005, soybean aphid colonies reached numbers that exceeded 1,000 individuals per plant (O'Neal 2005), resulting in damage levels that forced growers to increase insecticide applications by approximately 130 times (Ragsdale et al. 2011). In 2003, farmers from Illinois invested between $9 -12 million to treat more than 0.75 million acres infested with soybean aphids (Steffey 2004). During the same period, Minnesota farmers treated over 3 million acres infested with soybean aphids, leading to yield reductions and management costs to be approximately $120 million (Ostlie 2004).

Plant Resistance

Several strategies have been adopted in an attempt to control the soybean aphid; including biological control, host plant resistance, and chemical control (Wu et al. 2004). Host plant resistance has been proposed as a viable alternative to pesticides. Plant resistance to arthropods is defined as 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 determination of the plant resistance level is crucial since the resistance is subject to the interference of environmental conditions. For that, the degree of resistance is estimated based on the comparison to the susceptible control plant, which is injured or killed under similar experimental conditions (Smith 2005).

The three categories of plant resistance are: antibiosis (1), antixenosis (2) and tolerance (3) (Painter 1951; Kogan and Ortman 1978). Resistance is called antibiosis when, upon feeding on a resistant plant, the arthropod biology is impacted by either

biophysical or biochemical plant defenses, which can be moderate or lethal (Painter 1951; Panda and Khush 1995; Smith 2005). Common effects of plant antibiosis on arthropods include death of early instars and reduction of adult fecundity. Young and adult individuals that survive the effects of antibiosis frequently express a decline in size and weight of larvae and nymphs, resulting in prolongation of the immature period and therefore their life cycle. Additionally, antibiotic effects can result in failure of larval pupation and also reduce the survival of overwintering insects (Panda and Khush 1995). Antibiosis can be referred to as vertical or a monogenic type of resistance, which relies on the effects of a single (or a couple of) major gene(s) (Smith 2005). Vertical resistance is hypothetically less stable than horizontal resistance (conferred by multiple genes) since it can be overcomed by the development of pest biotypes (Smith 2005).

Antixenosis, also known as non-preference, is defined as a resistance mechanism that causes adverse effects on insect behavior (Painter 1951; Kogan and Ortman 1978; Smith 2005). Biophysical, biochemical or both factors present in plants exhibiting antixenosis affect arthropod recognition of the plant as a suitable source of food, oviposition site, mating site, and/or shelter (Panda and Khush 1995). In certain occasions, even though the individual is able to contact the plant, the antixenotic features will not allow further colonization (Panda and Khush 1995). Antixenotic characteristics limit or prevent oviposition and feeding due to the presence of repellents, absence of attractants or by causing an unfavorable balance between both (Panda and Khush 1995). Other factors that contribute to plant antixenosis include thickened epidermal layers, waxy accumulation, and higher densities of trichomes on the leaves surface (Smith 2005).

The third kind of resistance described by Painter (1951) is tolerance. Tolerance is classified as horizontal or multigenic resistance and is likely more stable and durable than single gene or vertical resistance (Smith 2005). Tolerant plants do not impose those same levels of selection pressure as plants possessing antibiosis and antixenosis, where high selection pressure can result in biotypes (Horber 1972; Stinchcombe 2002; Smith 2005). Another positive aspect to tolerance is that in most situations it is compatible with biological control, which provides an additional management options to arthropod pests (Smith 2005). Furthermore, tolerant genotypes have a higher economic threshold than susceptible genotypes; hence fewer insecticide applications are necessary, which could enhance the effectiveness of the biological control (Panda and Khush 1995).

Since the soybean aphid introduction to North America in 2000, several screening studies have been conducted to identify resistant soybean genotypes. The first report of soybean resistance to soybean aphid in the United States was published by Hill et al. (2004), which identified genotypes expressing antibiosis and antixenosis. The genotypes 'Dowling', 'Jackson' and 'Sugao Zarai' were reported to negatively impact fecundity and increase mortality, suggesting that antibiosis-type resistance was responsible. On the other hand, the genotype PI 71506 also analyzed in this study was reported to express antixenosis due to the non-preference exhibited for this genotype during choice tests. Because Dowling and Jackson are not well adapted to the Midwest, several studies were conducted to identify the genes conferring antibiosis that could be bred into adapted cultivars. A single gene confers antibiosis in both Jackson and Dowling, which were respectively designated as *Rag* and *Rag1* (Hill et al. 2006a; Hill et al. 2006b). Although there is no knowledge about the genetic relationship between *Rag* and *Rag1*, it was found

that these genes are dominant and map to soybean linkage group M (Hill et al. 2006a; Hill et al. 2006b; Li et al. 2007). Another gene associated with antibiosis was identified in the genotype PI 24350, called *Rag2*, which is associated with linkage group F (Mian et al. 2008). Additional soybean lines expressing both antibiosis and antixenosis were also identified in other studies. A large screening study conducted over a 2-year period in Illinois evaluated 2147 Chinese soybeans entries from maturity groups 0 to 3. In non-choice tests, PI 567541B and PI 567598B had adverse effects on soybean aphid biology and increased mortality, and thus exhibited antibiosis (Mensah et al. 2005). PI 567543C and PI 567597C expressed resistance in the choice test but did not produce significant results in the no-choice test, indicating that antixenosis was the mechanism of resistance (Mensah et al. 2005). Diaz-Montano et al. (2006) screened 240 soybean cultivars and also identified sources of resistance to the soybean aphid. In their study, the cultivars K1639 and Pioneer 95B97 showed strong levels of antibiosis, and also antixenosis. Moreover, Diaz-Montano et al. (2006) also suggested the presence of antixenosis in addition to antibiosis already reported in Dowling and Jackson. Further analyses to assess aphid performance on some of the outstanding genotypes were also conducted by Diaz-Montano et al. (2007). The probing of aphids on resistant soybeans K1639, Pioneer 95B97, Jackson and Dowling required significantly longer time to reach the phloem, and ingested sap for a shorter period in comparison to the susceptible line (Diaz-Montano et al. 2007). In addition, Crompton and Ode (2010) evaluated soybean aphid feeding in Dowling and detected that the aphids were unable to ingest phloem at the sieve element, resulting in the aphids abandoning the host plant or dying of inadequate nutritional balance.

Unfortunately, as insect resistant plants possessing antibiosis and/or antixenosis become available, insect populations that are able to overcome these forms of resistance arise as well. Shortly after the identification of soybean plants with antibiosis in 2006, Kim et al. (2008) reported the first occurrence of soybean aphid biotypes. According to these authors, plants carrying the *Rag1* gene exposed to isolates from Ohio had aphid numbers that were comparable to the check Williams 82; however, isolates found in Illinois were still negatively affected by the *Rag1* gene. Subsequently, these Ohio isolates were designated as biotype 2. In Indiana, a study reported the existence of a third soybean aphid biotype (Biotype 3) (Hill et al. 2010). Soybean aphids collected from the overwintering host *Frangula alnus* Mill. were tested on plants containing either *Rag1* or *Rag2*. The aphids readily colonized plants with the Rag2 resistance gene, differentiating it from biotype 1 and biotype 2 and providing evidence for the third biotype (Hill et al. 2010).

Although considerable research has been devoted to identifying resistant sources in soybean, most of the studies have focused on plants exhibiting antibiosis. Because of the relatively quick development of soybean aphid biotypes following the release of single-gene soybean resistant cultivars expressing antibiosis, the use of antibiotic plants is not a sustainable strategy for managing soybean aphids (Ragsdale et al. 2011). Hence, the need for identifying tolerance sources of resistance is critical as growers are in search of effective strategies to manage the soybean aphid.

Tolerance

Plant tolerance has been identified in a number of plant species, with most of the work focusing on crops such as maize (*Zea may*s L.), sorghum (*Sorghum bicolor* (L.)), wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Havlickova (1997) proposed that tolerance played an important role in wheat resistance to bird cherry oat aphid (*Rhopalosiphum padi* L). Miller et al. (2003) also identified wheat genotypes conferring tolerance to the Russian wheat aphid, *Diuraphis noxia* (Mordvilko). In maize, genotypes were selected that had an overdeveloped root volume compared to susceptible genotypes because they were better able to tolerate feeding by the western corn rootworm (*Diabrotica virgifera virgifera* LeConte) (Rogers et al. 1976). Sources of tolerance in sorghum have also been identified for stalk borer, *Busseola fusca Fuller* (van den Berg et al. 1994), barley fly, *Delia flavibasis* (Macharia and Mueke 1986) and greenbug, *Schizaphis graminum* (Rondani) (Girma et al. 1998; Dogramaci et al. 2007).

Tolerance has also been the focus in other systems. Heng-Moss et al. (2003a) identified buffalograss genotypes (*Buchloe dactyloides* (Nuttall) Engelmann) expressing moderate to high level of tolerance to the chinch bug, *Blissus occiduus* Barber. McAuslane et al. (1996) proposed that resistance in zucchini squash (*Cucurbita spp.*) breeding lines to a physiological disorder caused by whitefly, *Bemisia argentifolii* Bellows and Perring, feeding was due to tolerance. The expression of tolerance to arthropod feeding also exists in legumes. A study conducted by Schaafsma et al. (1998) reported the tolerance of common beans (*Phaseolus vulgaris* L.) to the potato leafhopper, *Emposa fabae* (Harris). Kornegay and Cardona (1990) reported breeding lines of

common beans that expressed tolerance to another species of potato leafhopper, *Empoasca fabae* (Harris).

Unfortunately, a limited number of studies have focused on identifying tolerant soybean lines to soybean aphids. Greenhouse screening studies conducted by Pierson et al. (2010) first reported tolerance in soybeans to soybean aphids. The tolerant genotype KS4202 exhibited low levels of foliar damage under high aphid numbers when infested during reproductive stage plants, with yield parameters being similar to uninfested KS4202. Subsequent screening studies performed under field conditions added more evidence that KS4202 has moderate to high levels of tolerance to soybean aphid feeding (Prochaska et al. 2012).

Tolerance Mechanisms

The identification of mechanisms underlying plant tolerance is a crucial step to understanding how plants defend themselves from herbivores and identifying breeding strategies for incorporating tolerance traits into high yielding plants (Panda and Khush 1995). Plant tolerance is conferred by a number of compensatory mechanisms that enable fitness recovery from herbivore damage. Strauss and Agrawal (1999) provide an excellent review of several studies that have demonstrated the role of plant photosynthesis and plant physical structures in plant tolerance to arthropods. Plant hormones and oxidative enzymes have also demonstrated their role in plant tolerance (Gawronska and Kielkiewicz 1999; Heng-Moss et al. 2004; Pierson et al. 2011).

Enhanced levels of leaf photosynthesis followed by arthropod feeding have been one of the most cited mechanisms of tolerance. In wheat genotypes, the success in

compensating for the damage caused by *D. noxia* feeding was attributed to the plant's ability to maintain photosynthetic levels similar to uninfested plants because of limited damage to the photosystems (Haile et al. 1999; Heng-Moss et al. 2003b; Franzen et al. 2007). On the other hand, susceptible genotypes infested with *D. noxia* expressed significant reduction in total chlorophyll (chlorophyll A and B) and carotenoids (Haile et al. 1999; Heng-Moss et al. 2003b), including declines in carboxylation efficiency and RuBP generation (Franzen et al. 2007) when compared to control plants. Boyko et al. (2006) provided further evidence that photosynthetic compensation was involved in the tolerance response. DNA sequences encoding for chlorophyll and photosystem genes were overexpressed in wheat plants resistant to aphids. In buffalograss, chinch bug-tolerant and susceptible plants were analyzed for photosynthetic rates (Heng-Moss et al. 2006). Both control and chinch bug-infested tolerant plants had similar rates of photosynthesis, suggesting that compensatory photosynthesis was occurring. Additionally, tolerant plants were able to maintain the photochemical efficiency of photosystem II and the electron transport ratio; while, susceptible plants experienced significant reductions in these parameters (Heng-Moss et al. 2006). Similar results have also been reported for tolerant soybeans in response to soybean aphid feeding. Susceptible soybean plants infested with soybean aphids showed reduced photosynthetic capacity, specifically reductions in RUBISCO activity and carbon dioxide assimilation rates. On the other hand, photosynthetic levels in tolerant soybean were similar in both infested and uninfested plants (Pierson et al. 2011).

Modifications in plant architecture in response to arthropod feeding may also contribute to tolerance. An important mechanism by which plants are thought to

overcompensate for herbivory is through the release of apical dominance, which stimulates the growth of non-apical meristems. Rosenthal and Welter (1995) investigated the impact of stem borer herbivory, *Diatraea grandiosella* Dyar, in wild teosintes (maize wild relatives) and different species of maize (*Zea* spp). Typically, *D. grandiosella* feeding results in the destruction of the apical meristem; however, tolerant plants did not sustain significant damage to the apical meristem and were therefore able to produce a greater number of tillers and leaves to compensate for herbivory. In their study, wild teosintes were more tolerant than domesticated species. Cotton seedlings were shown to suppress the non-apical meristem vegetative development in response to feeding by cotton aphids, *Aphis gossypii* Glover as a tolerance mechanism to maintain yield (Rosenheim et al. 1997).

Plant hormones have also been shown to be involved in the tolerance response of plants to arthropod feeding. Gawronska and Kielkiewicz (1999) found that the levels of ABA measured from carmine spider mite, *Tetranychus cinnabarinus* Boisd, damaged and adjacent tomato tissue were higher in tolerant than in mite-susceptible tomato cultivars; however, the hormone levels were higher in susceptible cultivars under controlled conditions. Their study also demonstrated that ABA levels increased in mechanically wounded leaves of tolerant plants, but decreased in non-tolerant plants. Other plant hormones, such as jasmonic acid (JA) and salicylic acid (SA), are also known to be involved in the plant's defense to arthropods and pathogens, acting as key molecules in a complex signal network. However, to our knowledge no studies have specifically investigated the involvement of these hormones in plant tolerance to arthropods.

Oxidative enzymes have also been shown to play a role in plant tolerance. Studies have demonstrated that increased oxidative enzyme (e.g., peroxidase) activity is likely associated with the ability of a tolerant plant to compensate for insect feeding (Heng-Moss et al. 2004; Gutsche et al. 2009; Pierson et al. 2011). Peroxidases are enzymes that are found in plants, animals and microorganisms. Based on their primary structural and catalytic features, peroxidases are classified into three superfamilies: class I, II and III (Welinder 1992). Class I peroxidases are placed into three groups, known as microbial cytochrome c peroxidase, bacterial catalase-peroxidase, and ascorbate peroxidase. These molecules are intracellular enzymes present in almost all organisms except animals (Welinder 1992). Class II peroxidases are secretory peroxidases encoded solely by fungi, which include lignin peroxidase and manganese peroxidase (Welinder 1992; Ruiz-Duenas et al. 2001). Class III peroxidases are classical secretory plant peroxidases (Welinder 1992; Tognolli et al. 2002). Class III peroxidases are heme-containing glycoproteins that are widespread in the plant kingdom in several isoforms that are specific to plant development stage, tissue and environmental stimuli (Penel et al. 1992). Plant peroxidases are frequently found in a broad range of isoforms, suggesting that these enzymes have distinct physiological functions in the cell (Siegel 1993; Passardi et al. 2004). For example, the genome of *Arabidopsis thaliana* contains 73 different genes encoding class III peroxidases (Tognolli et al. 2002). Among the various forms of peroxidases, several forms are known to be induced by herbivores, pathogens and/or mechanical wounding. Allison and Schultz (2004) found that in response to gypsy moth, *Lymantria dispar* L., feeding and mechanical wounding, SA and JA along with six peroxidase isozymes were expressed in red oak (*Quercus ruba* L.) seedlings. This study

reported that at least three of the six isozymes observed responded differently to mechanical wounding and caterpillar feeding, suggesting that specific peroxidases are up and down regulated in response to insect feeding.

Plants produce signaling molecules, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), in response to biotic and/or abiotic stressors to trigger plant defense pathways. The term reactive oxygen species describes radicals and other non-radicals that are derived from oxygen, such as hydroxyl radical, hydrogen peroxide and superoxide anion (Apel and Hirt 2004); whereas, reactive nitrogen species primarily refers to nitric oxide derivatives (Leitner et al. 2009). In general, ROS are highly reactive and abundant levels of these molecules are frequently toxic to plant cells. In non-stressed plants, the cellular levels of ROS are low, but the presence of biotic stressors, such as pathogens or insects, induces cell oxidative stress by increasing production of ROS (Gill et al. 2010). A common characteristic shared by these molecules is their capacity to cause oxidative damage to proteins, DNA, and lipids (Imlay and Linn 1988; Apel and Hirt 2004). Most RNS are also important signaling molecules and are also produced in response to biotic and/or abiotic stressors (Durner and Klessig 1999). Studies have demonstrated that nitric oxide was induced in response to infection by bacteria and viruses (Durner et al. 1998). Similarly to ROS, the excessive accumulation of cellular RNS in response to these stressors has been implicated in host tissue injury, but low levels of these molecules may benefit the plant by activating plant defense pathways (Delledonne et al. 2001).

The presence of oxidative enzymes such as peroxidases is required to keep the amount of ROS under damaging levels. Peroxidases catalyze the conversion of hydrogen

peroxide to water in the presence of an electron acceptor. However, some forms of peroxidases can also serve as generators of hydrogen peroxide through the oxidation of NADH, which can serve as signaling molecules to trigger defense pathways (Apel and Hirt 2004; Sukalovic et al. 2005). Peroxidases have also been demonstrated to participate in a broad range of physiological responses. In the cell wall, peroxidases control the amount of hydrogen peroxide, which is a prerequisite for the cross-linking of phenol groups, such as polysaccharide linked ferulates, extensins and lignin monomers that eventually attach to the extracellular surface (Brisson et al. 1994). In addition, peroxidases found in the epidermis can generate cytotoxic compounds, and in combination to phenoloxidase these molecules can create polyphenolic barriers upon damage.

Peroxidases in plant defense to herbivory

Several studies have suggested that up-regulation or maintenance of peroxidase activity in resistant plants in response to insect feeding helps the plant compensate for insect feeding. It has been proposed that resistant plants are able to effectively detoxify the excessive accumulation of ROS through the higher expression of oxidative enzymes (e.g., peroxidases), while susceptible plants are not able to detoxify the ROS and as a result sustain more damage (Heng-Moss et al. 2004; Gutsche et al. 2009; Pierson et al. 2011). Furthermore, the up-regulation of peroxidases may lead to production of ROS, which can be an important signaling molecule (Apel and Hirt 2004).

Hildebrand et al. (1986) demonstrated that soybean resistance to *Tetranychus urticae* Koch (two-spotted spider mite) is strongly correlated to lipid peroxidation. In

their study, peroxidase activity increased with increasing lipid peroxidation, but increased less in the susceptible genotype 'Williams', which sustained greater levels of lipid peroxidation therefore higher damage levels. Felton et al. (1994) evaluated the response of soybean to three-cornered alfalfa leafhoppers and found increased levels of peroxidase activity, including the up-regulation of other oxidative enzymes, such as lipoxygenases, ascorbate oxidase, and polyphenol oxidase. A recent study performed by Pierson et al. (2010) is the first to report the effects of soybean aphid feeding on peroxidase activity in soybean plants. In their study, peroxidase activity increased in the tolerant soybean KS4202, suggesting that peroxidases may be involved in the tolerance response. In a subsequent study, Prochaska (2011) analyzed the transcriptional changes in infested and uninfested KS4202 and a susceptible genotype in order to gain insight into the genes involved in the tolerance response and mechanisms of the resistance. After 15 days of aphid feeding, two peroxidase genes, Glyma04g39860 and Glyma06g15030, were identified as being differentially expressed between KS4202 infested and uninfested plants. The same two peroxidases were not differentially expressed in the susceptible soybean in response to aphid feeding. Prochaska (2011) speculated that these two peroxidases might be serving to detoxify the ROS accumulated or be involved in triggering signaling molecules for specific plant defense pathways.

Thesis Objectives:

Additional research is needed to better understand the tolerance response in KS4202. Therefore, the objectives of my thesis were to:

- Document the presence of tolerance in the early vegetative stages of KS4202 soybean plants.
- Evaluate the impact of soybean aphid feeding on the yield response of V1, V3, and R1 KS4202 soybean plants.
- Examine the effect of soybean aphid feeding on peroxidase activity in V1 and V3 KS4202 plants.

Chapter 2

Categorizing the Resistance of Soybean Vegetative and Reproductive Stages to the Soybean Aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae) in the Greenhouse

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 (Endres 2001). The soybean aphid, *Aphis glycines* Matsumura, native to Asia, was first reported in North America in July of 2000. Currently, the soybean aphid has expanded its distribution to 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 eventually results in poor canopy development and 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 (*Capnodium* spp.) due to honeydew accumulation (Clark and Perry 2002; Tilmon et al. 2011). High infestations by soybean aphid 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% for every 10,000 cumulative aphid-days (CAD) during the early vegetative stages to pod set (R4), and the average economic injury level (EIL) was 674 ± 95 aphids per plant (Ragsdale et al. 2007).

Several management strategies have been employed to control soybean aphids, including chemical, cultural and biological control methods (Wu et al. 2004). Among

these, chemical control still represents the primary pest management tool used to manage soybean aphids in the United States (Hodgson et al. 2010). However, considering the high costs and potential negative effects on the environment and human health, alternative methods to chemical control should be considered. Host plant resistance has become a viable alternative to reduce the sole reliance on pesticides.

Several screening studies have been conducted to identify possible sources of soybean resistance to soybean aphid. In the United States, Hill et al. (2004) reported the first resistant soybean cultivars that expressed antibiotic and/or antixenotic effects to soybean aphid. To date, most resistance research has focused on antibiosis (Mensah et al. 2005; Diaz-Montano et al. 2006; Hill et al. 2006a; Hill et al. 2006b; Diaz-Montano et al. 2007; Kim et al. 2008; Mian et al. 2008; Crompton and Ode 2010). However, these antibiotic sources of resistance have not been sustainable given the development of biotypes (Ragsdale et al. 2011; Hesler et al. 2012). Hence, sustainable alternatives for managing soybean aphids are needed.

Plant tolerance is classified as horizontal or multigenic resistance; therefore, it is assumed to be more stable and durable than antibiotic and antixenotic traits, which are normally controlled by single genes (Smith 2005). Unfortunately, a limited number of studies have focused on identifying soybean aphid injury tolerant soybeans. Greenhouse screening studies conducted by Pierson et al. (2010) first reported later vegetative/reproductive KS4202 soybeans to be tolerant to soybean aphid injury. Subsequent screening studies performed under field conditions added more evidence that KS4202 possesses moderate to high levels of tolerance to soybean aphid injury (Pierson et al. 2010; Prochaska et al. 2012). However, further research is necessary to evaluate the

tolerance response of KS4202 in the early vegetative stages. Therefore, the objectives of this research were to document the presence of resistance (e.g., tolerance) in the early vegetative stages of KS4202 soybean and evaluate the impact of soybean aphid injury on the yield response of V1, V3, and R1 KS4202 soybean.

Materials and Methods

Documenting the presence of resistance in the vegetative stages of KS4202

Two greenhouse studies were conducted to evaluate the presence of tolerance in the VE to V5 vegetative stages. For each study, four seeds were planted in 15-cm diameter round plastic pots at a depth of approximately 3 cm. The potting media was a 34% peat, 31% perlite, 31% vermiculite and 4% soil mix. Planting dates were staggered to ensure that plants 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 maintained under 400-watt high intensity lamps with a 16:8 (L: D) h photoperiod at a temperature of 23 ± 3^{o}C.

For the first study, soybean plants were infested in the early vegetative stages VE (cotyledons above the soil), VC (unrolled unifoliolate leaves) and V1 (fully developed leaf at unifoliate node). For the second study, aphids were introduced at the V3 (fully developed leaf at third node), V4 (fully developed leaf at fourth node) and V5 (fully developed leaf at fifth node) stages. The experimental design used for both studies was a completely randomized design with 10 replications per treatment. Adult apterous soybean aphid females used in the studies were progeny of a Nebraska isolate (Biotype 1) initially collected in the growing season of 2011 from commercial soybean near the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory, Concord, NE (42° 23′ 3″ N, 96° 59′ 21″ W). The soybean aphid colony was maintained on KS4202 plants in a growth chamber at 21 ± 2^{o}C and a photoperiod of 16:8 (L: D) h. At the start of each study, 20 soybean aphids (4th instars and adults) were placed on the youngest fully expanded leaf using a camel hair paintbrush. Following infestation, plants

were individually caged with tubular 0.05 cm clear polycarbonate plastic (15 cm diameter x 61 cm height) covered with organdy fabric to prevent aphid escape. Plants were evaluated within 48 hours of aphid infestation to assess aphid survival and reinfested if lower than 20 aphids.

Soybean plants were evaluated for aphid numbers, plant development stage, and plant damage semi-weekly. Damage ratings were performed using a 1-5 scale, 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% or more of leaf area with yellowing discoloration or dead tissue (Heng-Moss et al. 2002; Pierson et al. 2010; Prochaska et al. 2012). Studies were concluded when the designated plant stages had reached 10,000 CAD.

At the conclusion of the studies, the level of resistance was established for each vegetative stage using the following categories: HS = highly susceptible (damage rating $\geq$ 4), MS = moderately susceptible (damage rating ≥ 3 but < 4), MR = moderately resistant (damage rating > 1 but < 3), and HR = highly resistant (damage rating = 1) (Heng-Moss et al. 2002; Pierson et al. 2010).

Cumulative aphid-days (CAD) were calculated to provide an estimate of accumulated aphid pressure. Cumulative aphid-days = ((N1+N2)/2)*T, where N1 is the number of aphid per plant on the previous evaluation, N2 is the number of aphids per plant in the subsequent evaluation, and T is the period (days) in between the two sampling dates (Hanafi et al. 1989, Ragsdale et al. 2007). Damage ratings and cumulative aphid-days were analyzed by generalized mixed model analysis (PROC GLIMMIX, SAS

Institute) with a 5% level of significance. When appropriate, means were separated using Fisher's least significant difference (LSD) test.

Effect of soybean aphid on the yield response of V1, V3, and R1 KS4202 plants

Soybean establishment and maintenance were similar to the methods previously described. Plants were again maintained in a greenhouse under 400-watt high intensity lamps with a 16:8 (L: D) h photoperiod at a temperature of 23 ± 3^{o}C.

The experimental design was a completely randomized with a 3x3 factorial treatment design that included three soybean growth stages (V1, V3, and R1) and three aphid infestation levels (0, 10 and 20 soybean aphids per plant) with 10 replications. The R1 stage plants served as the reference control for this study (Pierson et al. 2010). Once the plants were at the desired vegetative stage (i.e., V1, V3, and R1), 10 or 20 soybean aphids (4th instars and adults) were placed on the youngest fully expanded leaf of the designated aphid-infested treatments. Aphids were obtained from the same colony previously described. Following aphid introduction, plants were individually caged to prevent aphid escape. Soybeans plants designated as the control (i.e. 0 aphids) were also caged. Aphids remained on the plants until numbers reached levels of 1,000 – 1,500 (low level) or 2,000 – 2,500 (high level) insects per plant, after which plants were sprayed with the synthetic pyrethroid insecticide lambda-cyhalothrin (Warrior ®) and allowed to mature. Upon maturation, soybean pods were harvested and placed in a paper bag and dried to adjust seed moisture level to 13% prior processing. 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 dry weight of pod (Beckendorf et al. 2008; Pierson et al. 2010). Yield parameters were analyzed by generalized mixed models

(PROC GLIMMIX, SAS Institute), and once appropriate (5% level of significance), means were separated using Fisher's least significant difference (LSD) test.

Results and Discussion

Documenting the presence of resistance in the vegetative stages of KS4202

VE, VC, and V1 Stages. Significant differences in aphid damage ratings were detected among the KS4202 plants infested in the VE, VC and V1 stages (F = 5.38; df = 2, 27; P = 0.0108) (Table 1). At approximately 10,000 CAD, the three vegetative stages were highly susceptible and had an average damage rating of ≥3. At approximately 20,000 or more CAD, the average damage rating was >4 (Table 1).

Plants infested at the VE stage had damage ratings (4.9 ± 0.10) that were significantly higher than those of V1 stage plants (4.1 ± 0.23) (Table 1). On the other hand, damage exhibited by plants infested at the VC stage was not statistically different from either VE or V1 stages. By visual analysis, plants infested at the VE and VC stages were stunted and had delayed plant development when compared to the V1 stage plants (Figure 1). Based on plant damage ratings, the VE, VC and V1 stages were classified as highly susceptible (Table 1).

V3, V4, and V5 Stages. Plant damage ratings were not significantly different among the three plant stages (F = 1.82; df = 2, 27; P = 0.1808) (Table 2), and V3-V5 stages were classified as moderately resistant. At 21 days after soybean aphid introduction, the V3, V4 and V5 stages had similar CAD values, ranging from to 8,739 to 12,486 (Table 2) (F = 2.44; df = 2, 27; P = 0.1066).

The level of resistance found in this study for the V3, V4 and V5 stages is comparable with Pierson et al. (2010), which first documented KS4202 resistance to the soybean aphid during the reproductive stages (Figure 2). Conversely, the early vegetative

stages VC, VE and V1 were identified as highly susceptible due to higher plant damage and stunting of the plants (Figure 1).

Effect of soybean aphid on the yield response of V1, V3, and R1 KS4202 plants

In this study, V1, V3 and R1 plants had aphid numbers that exceeded the average economic injury level threshold of 674 aphids per plant reported by Ragsdale et al. (2007). Despite exceeding this level, V3 and R1 plants at both the high (CAD = 7,490 ± 803.42 and 8,385 ± 498.8, respectively) and low aphid number treatments (CAD = 4,530 ± 245.8 and 5,300 ± 525.5, respectively) were not statistically different from their respective control plants for any of the yield parameters evaluated except average pod weight (Tables 3, 4, 5, 6, 7, and 8). Interestingly, the R1 plants designated as the high aphid number treatment (2,000 – 2,500 aphids plant^{-1}) had significantly higher pod weights than control plants (Table 5) (t = -2.00; df = 70; *P*= 0.0497).

V1 plants at the low aphid number treatment (1,000 – 1,500 aphids plant^{-1} or 3,710 ± 304.7 CAD) were not significantly different from control plants for any of the yield parameters evaluated (Table 3, 5, 6 and 7). At the high aphid number treatment (2,000 – 2,500 aphids plant^{-1} or 7,790 ± 769.1 CAD), aphid free plants had significantly higher total seed weights and number of seeds compared to the infested treatment. Plant exposed to the high infestation level experienced a 33.8% reduction in total seed weight (Table 4) and 32.3% fewer seeds (Table 8) when compared to control plants. All other yield parameters were not significantly different between the high aphid number treatment and the control plants.

Despite yield loss at the high infestation level for V1 plants, the same stage plants exposed to the lower infestation level were not significantly different when compared to

control plants for any of the yield parameters evaluated, demonstrating the presence of tolerance at this infestation level. The low aphid infestation level applied in this study represents aphid numbers approximately twice the average economic injury level calculated by Ragsdale et al. (2007).

Pierson et al. (2010) also evaluated the effect of aphid feeding on the yield parameters of reproductive stage KS4202 plants under greenhouse conditions. They reported infested KS4202 to have similar average seed weight and number of seeds per pod when compared to control plants. However, significant decreases in total seed weight, number of pods per plant and number of seeds per plant in comparison to control plants were detected. The significant differences in these yield parameters for KS4202 were likely due to the large number of aphids on this genotype. KS4202 consistently had up to 10-fold the aphid numbers compared to the other genotypes evaluated.

The results from this research compare favorably with Pierson et al. (2010) and Prochaska et al. (2012) and document the presence of varying levels of tolerance in the V1 through the reproductive stages of KS4202. Although Diaz-Montano et al. (2006) categorizing KS4202 as susceptible during the seedling stages, their study focused on the identification of soybean genotypes exhibiting antibiosis and/or antixenosis and did not account for levels of foliar damage and yield parameters. Further research is needed to develop economic injury levels for the various vegetative and reproductive stages of KS4202.

Table 1. Mean ± SE damage ratings and cumulative aphid-days for the genotype KS4202 after initial infestation of 20 aphids per plant.

Plant Infestation Stage	Mean of Damage Level [a]	Cumulative Aphid Days [b]	Resistance Level [c]
VE	4.9 ± 0.10 a	21,299 ± 3,158.7 a	HS
VC	4.6 ± 0.16 ab	27,029 ± 2,167.5 a	HS
V1	4.1 ± 0.23 b	27,342 ± 2,675.5 a	HS

Means within columns followed by the same letter are not significantly different (P > 0.05; LSD test).
[a] F value = 5.38; df =2, 27 ; P-value = 0.0108; damage level at 30 days after initial infestation.
[b] F value = 1.59; df = 2, 27; P-value = 0.2223.
[c] HS = Highly Susceptible

Table 2. Mean ± SE damage ratings and cumulative aphids-days for the genotype KS4202 after initial infestation of 20 aphids per plant.

Plant Infestation Stage	Mean of Damage Level [a]	Cumulative Aphid Days [b]	Resistance Level [c]
V3	2.3 ± 0.30 a	12,486 ± 1,539.9 a	MR
V4	2.8 ± 0.13 a	11,817 ± 946.3 a	MR
V5	2.2 ± 0.25 a	8,738.2 ± 1,285.5 a	MR

Mean within columns followed by the same letter are not significantly different (P >0.05; LSD test).

[a] F value = 1.82; df= 2, 27; P-value = 0.1808; damage level at 21 days after initial infestation.

[b] F value = 2.44; df = 2, 27; P-value = 0.1066.

[c] MR = Moderately resistant.

Figure 1. Damage caused by *Aphis glycines* to the VE, VC and V1 stages of KS4202 at 30 days after aphid introduction.

Figure 2. Damage caused by *Aphis glycines* to the V3, V4 and V5 stages of KS4202 at 21 days after aphid introduction.

Table 3. Means ± SE of average seed weights in soybean aphid-infested (low/high aphid number) and non-infested KS4202 soybean plants

	Average Seed Weight (g)								
Stage of infestation	**No-aphids**	**Low aphid number**	**P-value[1]**	**No-aphids**	**High aphid number**	**P-value[1]**	**Low aphid number**	**High aphid number**	**P-value[1]**
V1	0.1491 ± 0.017	0.1509 ± 0.005	0.975	0.1491 ± 0.017	0.1465 ± 0.007	0.9749	0.1509 ± 0.005	0.1465 ± 0.007	0.939
V3	0.1703 ± 0.006	0.1593 ± 0.008	0.851	0.1703 ± 0.006	0.2694 ± 0.108	0.0766	0.1593 ± 0.008	0.2694 ± 0.108	0.064
R1	0.1439 ± 0.004	0.1619 ± 0.006	0.786	0.1439 ± 0.004	0.1448 ± 0.008	0.9893	0.1619 ± 0.006	0.1448 ± 0.008	0.790

[1] Means significantly different at $P \leq 0.05$ by least significant difference.

Table 4. Means ± SE of total seed weight in soybean aphid-infested (low/high aphid number) and non-infested KS4202 soybean plants

	Total Seed Weight (g)								
Stage of infestation	**No-aphids**	**Low aphid number**	**P-value[1]**	**No-aphids**	**High aphid number**	**P-value[1]**	**Low aphid number**	**High aphid number**	**P-value[1]**
V1	9.92 ± 3.16	9.24 ± 3.59	0.681	9.92 ± 3.16	6.57 ± 3.36	0.0391	9.24 ± 3.59	6.57 ± 3.36	0.107
V3	14.70 ± 1.30	11.92 ± 1.19	0.105	14.70 ± 1.30	12.94 ± 1.23	0.273	11.92 ± 1.19	12.94 ± 1.23	0.550
R1	7.11 ± 0.60	10.27 ± 1.24	0.102	7.11 ± 0.60	7.44 ± 1.15	0.8613	10.27 ± 1.24	7.44 ± 1.15	0.129

[1] Means significantly different at $P \leq 0.05$ by least significant difference.

Table 5. Means ± SE of average pod weigh in soybean aphid-infested (low/high aphid number) and non-infested KS4202 soybean plants

	Average Pod Weight (g)								
Stage of infestation	**No-aphids**	**Low aphid number**	**P-value[1]**	**No-aphids**	**High aphid number**	**P-value[1]**	**Low aphid number**	**High aphid number**	**P-value[1]**
V1	0.1473 ± 0.005	0.1513 ± 0.006	0.694	0.1473 ± 0.005	0.1612 ± 0.007	0.1659	0.1513 ± 0.006	0.1612 ± 0.007	0.337
V3	0.1484 ± 0.005	0.1518 ± 0.006	0.751	0.1484 ± 0.005	0.1491 ± 0.006	0.944	0.1518 ± 0.006	0.1491 ± 0.006	0.802
R1	0.1424 ± 0.004	0.1584 ± 0.008	0.182	0.1424 ± 0.004	0.1654 ± 0.013	0.0497	0.1584 ± 0.008	0.1654 ± 0.013	0.547

[1] Means significantly different at $P \leq 0.05$ by least significant difference.

Table 6. Means ± SE of number of pods in soybean aphid-infested (low/high aphid number) and non-infested KS4202 soybean plants

	Number of Pods								
Stage of infestation	**No-aphids**	**Low aphid number**	**P-value[1]**	**No-aphids**	**High aphid number**	**P-value[1]**	**Low aphid number**	**High aphid number**	**P-value[1]**
V1	32.80 ± 2.94	32.11 ± 3.75	0.888	32.80 ± 2.94	23.70 ± 4.27	0.0594	32.11 ± 3.75	23.70 ± 4.27	0.089
V3	46.60 ± 3.77	38.87 ± 3.55	0.130	46.60 ± 3.77	40.50 ± 2.89	0.2032	38.87 ± 3.55	40.50 ± 2.89	0.748
R1	31.00 ± 2.29	32.14 ± 3.31	0.841	31.00 ± 2.29	28.87 ± 2.57	0.7002	32.14 ± 3.31	28.87 ± 2.57	0.554

[1] Means significantly different at $P \leq 0.05$ by least significant difference.

Table 7. Means ± SE of total pod weight in soybean aphid-infested (low/high aphid number) and non-infested KS4202 soybean plants

	Total Pod Weight (g)								
Stage of infestation	**No-aphids**	**Low aphid number**	**P-value[1]**	**No-aphids**	**High aphid number**	**P-value[1]**	**Low aphid number**	**High aphid number**	**P-value[1]**
V1	4.75 ± 0.35	4.67 ± 0.42	0.906	4.75 ± 0.35	3.720 ± 0.55	0.0854	4.67 ± 0.42	3.720 ± 0.55	0.119
V3	6.81 ± 0.45	5.82 ± 0.44	0.120	6.81 ± 0.45	5.940 ± 0.35	0.145	5.82 ± 0.44	5.940 ± 0.35	0.855
R1	4.37 ± 0.30	4.95 ± 0.39	0.409	4.37 ± 0.30	4.675 ± 0.42	0.6582	4.95 ± 0.39	4.675 ± 0.42	0.681

[1] Means significantly different at $P \leq 0.05$ by least significant difference.

Table 8. Means ± SE of number of seeds in soybean aphid-infested (low/high aphid number) and non-infested KS4202 soybean plants

	Number of Seeds								
Stage of infestation	**No-aphids**	**Low aphid number**	**P-value[1]**	**No-aphids**	**High aphid number**	**P-value[1]**	**Low aphid number**	**High aphid number**	**P-value[1]**
V1	66.60 ± 6.91	60.55 ± 6.50	0.565	66.60 ± 6.91	45.10 ± 8.29	0.0382	60.55 ± 6.50	45.10 ± 8.29	0.144
V3	86.60 ± 6.99	74.50 ± 6.35	0.266	86.60 ± 6.99	74.62 ± 6.35	0.2432	74.50 ± 6.35	74.62 ± 6.35	0.991
R1	49.43 ± 3.85	63.57 ± 7.88	0.249	49.43 ± 3.85	49.25 ± 4.84	0.9879	63.57 ± 7.88	49.25 ± 4.84	0.228

[1] Means significantly different at $P \leq 0.05$ by least significant difference.

Chapter 3

Physiological Responses of Resistant and Susceptible Soybean Genotypes to the Soybean Aphid (*Aphis glycines* Matsumura) Feeding

Introduction

Since its detection in July of 2000, the soybean aphid (*Aphis glycines* Matsumura) has become a serious pest of soybeans in the United States and southeast Canada (Ragsdale et al. 2011). The insect pierces foliar and stem tissue in order to withdraw the phloem contents, which can result in the transmission of viral pathogens (Clark and Perry 2002), poor canopy development and reductions in photosynthesis (Macedo et al. 2003; Pierson et al. 2011). An indirect effect of heavy soybean aphid infestation is commonly associated with the development of dark sooty mold (*Capnodium* spp.) on the sugary excretions produced by these insects (Tilmon et al. 2011). Ultimately, the injuries caused by the soybean aphid manifests as a reduction in plant height, pod development, a lower number of seeds at maturity, and ultimately yield loss (Ragsdale et al. 2007). Furthermore, soybean aphids can also decrease the amount of seed oil, which can affect the market value of soybean seeds (Beckendorf et al. 2008).

Several management strategies have been employed in an attempt to control the soybean aphid, including biological control, plant resistance, and chemical control (Wu et al. 2004). Nevertheless, chemical control still represents the primary pest management tool used for soybean aphids in the United States (Hodgson et al. 2010). As an alternative to chemicals, research has been conducted to identify possible sources of soybean resistance to the soybean aphid. Most of this research has focused on cultivars possessing antibiosis and/or antixenosis (Hill et al. 2004; Mensah et al. 2005; Diaz-Montano et al.

2006; Hill et al. 2006a; Hill et al. 2006b; Diaz-Montano et al. 2007; Kim et al. 2008; Mian et al. 2008; Crompton and Ode 2010), and only a few studies have investigated soybeans that are tolerant to the soybean aphid (Pierson et al. 2010; Pierson et al. 2011; Prochaska et al. 2012). Despite the progress achieved in identifying soybean aphid resistant sources, limited research has been done to understand the mechanisms that are underlying plant resistance, specifically tolerance in soybeans.

The identification of mechanisms underlying plant tolerance is a crucial step to understanding how plants defend themselves from herbivores and identifying breeding strategies for incorporating tolerance traits into high yielding plants (Panda and Khush 1995). Plant tolerance is conferred by a number of compensatory mechanisms that enable plants to compensate for herbivore feeding and injury. Photosynthetic compensation, plant morphology and architecture, plant hormones and oxidative enzymes have all been shown to be involved in the plant's defense response to insect herbivory (Gawronska and Kielkiewicz 1999; Haile et al. 1999; Heng-Moss et al. 2004; Boyko et al. 2006; Heng-Moss et al. 2006; Franzen et al. 2007; Gutsche et al. 2009; Pierson et al. 2011).

Biotic stressors (e.g. arthropods and pathogens) are known to trigger the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2). Hydrogen peroxide acts as signaling molecule to activate plant defense; however, excessive accumulation of ROS can result in toxicity and cellular damage (Klessig et al. 2000; Delledonne et al. 2001). Therefore, the presence of ROS-scavenging enzymes, such as peroxidases and other oxidative enzymes, function to degrade the H_2O_2 synthesized in response to stress (Apel and Hirt 2004). Peroxidases also participate in several processes of plant metabolism, such as lignification, suberization, wound healing,

and other forms of defense to arthropods and pathogens (Brisson et al. 1994; Kawano 2003; Almagro et al. 2009). Several studies have demonstrated a direct correlation between resistance and increased levels of oxidative enzymes, such as superoxide dismutase, catalase, and peroxidase (Hiraga et al. 2000; Chaman et al. 2001; Ni et al. 2001; Heng-Moss et al. 2004; Park et al. 2006; Gutsche et al. 2009). A limited number of studies have examined the role of oxidative enzymes in the tolerance response of soybean to arthropod feeding (Hildebrand et al. 1986; Felton et al. 1994; Pierson et al. 2011). Pierson et al. (2011) reported reproductive stage KS4202 (tolerant) soybeans to have higher peroxidase activity in response to aphid feeding; whereas, aphid-infested and control soybean plants of the susceptible cultivar had similar levels of peroxidase activity. Additional studies are needed to further assess the role of oxidative enzymes in the defense response of soybeans to the soybean aphid. Therefore, the aim of the present study was to investigate the effect of soybean aphid feeding on peroxidase activity in V1 and V3 KS4202 plants.

Materials and Methods

Soybeans and soybean aphids. Seeds of two soybean genotypes, a susceptible line SD76R (Chiozza et al. 2010) and a tolerant line KS4202 (Pierson et al. 2010; Prochaska et al. 2012) were selected for this study. Four seeds of each genotype were planted in potting media (34% peat, 31% perlite, 31% vermiculite, and 4% soil mix) in 15 cm diameter round plastic pots at a depth of approximately 3 cm. Planting dates were staggered to ensure that plants 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. The plants were maintained in a greenhouse under 400-watt high intensity lamps with a 16:8 (L: D) h photoperiod at a temperature of 23 ± 3^{o}C.

The experimental design was a completely randomized design, with a 2 x 2 x 2 x 3 factorial treatment design that included 2 soybean genotypes, 2 aphid infestation levels (control and 20 soybean aphids per plant), 2 vegetative stages (V1 and V3) and 3 harvest dates (6, 16 and 22 days after aphid introduction). Each treatment combination was replicated six times. Adult apterous soybean aphid females used in this study were progeny of a Nebraska isolate (Biotype 1) collected from commercial soybean near the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory in Concord, NE (42° 23′ 3″ N, 96° 59′ 21″ W). The soybean aphid colony was maintained on KS4202 plants in a growth chamber at 21 ± 2^{o}C and a photoperiod of 16:8 (L: D) h. Once soybeans were at the desired vegetative stage, 20 soybean aphids (4th instars and adults) were placed on the youngest fully expanded tissue using a small paintbrush. Following aphid introduction, both infested and non-infested plants were

individually caged to prevent aphids from escaping. Cages were built with a tubular 0.05 cm clear Makrolon Tuffak Lexan polycarbonate plastic (15 cm diameter x 61 cm height) covered with organdy fabric at the top. Plants were evaluated within 48 hours of aphid infestation to assess aphid survival and reinfested if lower than 20 aphids. At each harvest day, damage ratings were performed using a 1-5 scale, 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% or more of leaf area with yellowing discoloration or dead tissue (Heng-Moss et al. 2002; Pierson et al. 2010; Prochaska et al. 2012). At the time of harvest, the total number of soybean aphids on infested plants was determined, cumulative aphid-days (CAD) were calculated, and plant stage was recorded. Aphids were carefully removed with a paintbrush. The youngest fully developed trifoliate was harvested and flash frozen with liquid nitrogen, sequentially stored at -80^{o}C for later processing.

Preparation of Soybean Samples. Soybean tissue was prepared for protein analysis through modified protocols from Hildebrand et al. (1986) and Heng-Moss et al. (2004). Using a mortar and pestle, soluble proteins were extracted by grinding soybean tissue with 3.0 mL of 20mM HEPES buffer (pH 7.2), 1% polyvinylpolypyrrolidone (PVP) and a plant protease cocktail inhibitor (0.3ml/1g of tissue). The extracted homogenate was centrifuged at 10,000 rpm for 10 minutes at a temperature of 4^{o}C. The supernatants were collected and stored at 4^{o}C (less than 2 hours) for protein and peroxidase analysis.

Protein and Peroxidase Assays. Total protein content was measured using a commercially available bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Bovine serum albumin was used as the standard for protein concentration. Procedures were carried out according to Pierce's protein assay instructions. Five replications of each treatment combination were analyzed in triplicates.

Peroxidase activity was analyzed using a modified protocol from Hildebrand et al. (1986) and Heng-Moss et al. (2004). The activity was determined by monitoring the increase in absorbance at 470 nm for 2 minutes. The reaction was started by adding 75 µL of 18 mM guaiacol and 2.5 µL of 30% hydrogen peroxide to microplate wells containing 5 µL of plant extract, 25 µL of 200 mM HEPES buffer pH 6.0, 71.3 µL of distilled water. Peroxidase specific activity was calculated using the molar absorptivity of guaiacol at 470 nm ($26.6 \times 10^3\ M^{-1}cm^{-1}$). Five replications of each treatment combination were analyzed in triplicates.

Peroxidase profiles. Native gel electrophoresis was performed to profile peroxidase patterns from extracted soybean proteins. Equal amounts of protein (60 µg) were diluted 1:1 with a gel-loading buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 40% glycerol, and 0.01% bromphenol blue prior to loading. The samples were loaded in pre-cast 12-well 10-20% polyacrylamide gels (BioRad Criterion gel, BioRad, Richmond, CA) and electrophoresed at 120V for 90 - 100 minutes at 4^oC. Gels were stained for peroxidase activity using a modified protocol from Vallejos (1983) and Heng-Moss et al. (2004). Gels were soaked for 10 minutes in a 50 mM sodium acetate solution at room temperature. Band development was initiated by adding 0.01 g of 4-chloronapthol dissolved in 0.5 mL of methanol with 20 µl of 30% hydrogen peroxide to the buffer solution.

Statistical Analysis. Accumulated aphid-days, protein content and peroxidase activity were analyzed using generalized mixed model (PROC GLIMMIX, SAS Institute) to detect differences between infested x control of soybeans KS4202 (tolerant) and SD76R (susceptible) at different vegetative stages. When appropriate, means were separated using Fisher's least significant differences (LSD) procedures ($\alpha = 0.05$).

Results

Aphid numbers. At day 6, CADs were similar between KS4202 and SD76R for both the V1 and V3 stages. Cumulative aphid-days were 441.5 ± 39.7 and 425.5 ± 67.1 for V1 stage KS4202 and SDR76R plants, respectively. Cumulative aphid-days were lower for the V3 stage with KS4202 and SD76R plants having 321 ± 31 and 234.5 ± 36.5 aphid-days, respectively.

By 16 days after aphid introduction, V1 stage KS4202 and SD76R had accumulated 5,080 ± 1,018 and 3,235.8 ± 662.8 aphid-days, respectively ($t = 1.40$; $df = 20$; $P = 0.17$). In a similar pattern, KS4202 and SD76R plants infested at the V3 stage accumulated 3,633 ± 790 and 3,796.6 ± 1,174.9 aphid-days ($t = -0.12$; $df = 20$; $P = 0.90$).

Both SD76R and KS4202 V1 stage plants exceeded the economic threshold of 10,000 CAD (Ragsdale et al. 2007) by day 22. KS4202 had 21,342 ± 2,122 aphid-days, which was significantly higher than SD76R plants (15,440 ± 2,360 aphid-days) ($t = 2.2$; $df = 20$; $P = 0.04$). For treatments that had aphids introduced at the V3 stage, no differences in CAD were detected between the two genotypes ($t = 0.29$; $df = 20$; $P = 0.77$). KS4202 and SD76R plants had CAD values of 9,998 ± 968 and 9,196 ± 1,950, respectively.

Protein and Peroxidase Assays. Protein content was not significantly different between infested and control V1 and V3 stage plants for both KS4202 and SD76R (Table 1). At 6 days after soybean aphid introduction, similar levels of peroxidase activity were detected between control and infested plants for both KS4202 and SD76R V1 stage plants (Table 2). For V3 plants, infested KS4202 had significantly higher peroxidase activity than KS4202 control plants ($t = -2.7$; $df = 4, 24$; $P = 0.01$) (Table 2). Although not

significantly different (t = -1.34; df = 4, 24; P = 0.19), SD76R infested plants had slightly higher peroxidase activity than control plants at 6 days after aphid introduction.

At 16 days after aphid introduction, KS4202 and SD76R V1 and V3 plants had similar levels of peroxidase activity when compared to their respective control plants (Table 2). By day 22, KS4202 infested V3 stage plants had significantly higher peroxidase activity levels than control plants (Table 2) (t= -2.4; df = 32; P = 0.03); whereas, V1 infested and control KS4202 had similar activity levels (Table 2). Although not statistically different, V1 stage SD76R infested plants had slightly higher levels of peroxidase activity when compared to their control plants (t = -1.54; df = 32; P = 0.13) (Table 2). Peroxidase activity levels were similar between SD76R infested and control V3 plants.

Peroxidase profiles. At day 6, no specific banding patterns were observed between control and infested plant for both genotypes at the V1 stage (Figure 1). However, differences in band intensity between control and infested plants were evident in the isozyme profiles for KS4202 and SD76R plants at the V3 stage, which is consistent with the higher peroxidase specific activity detected in the peroxidase kinetics study. SD76R control plants had slightly darker banding patterns than their respective control plants for the V1 and V3 stage plants at 16 days after aphid introduction (Figure 2). KS4202 infested plants, on the other hand, had darker banding patterns than KS4202 control plants at day 16. At day 22, visual differences in the banding intensity were observed between control and infested SD76R V1 and KS4202 V3 stage plants (Figure 3).

Discussion

Peroxidases are a large class of oxidative enzymes involved in a broad range of physiological responses in plants. These molecules play a key role in the oxidation of reactive oxygen species (ROS), such as hydrogen peroxide. Peroxidases can also serve as generators of hydrogen peroxide through the oxidation of NADH (Apel and Hirt 2004; Koutaniemi et al. 2005). Peroxidases can also be involved in cell wall lignification, suberization, wound healing and defense against pathogen infection (Brisson et al. 1994; Kawano 2003; Almagro et al. 2009). Several studies have also suggested that increased peroxidase activity in the plant may be associated with resistance (Hildebrand et al. 1986; Heng-Moss 2004; Hemmat 2007; Gutsche et al. 2009). Pierson et al. (2011) reported reproductive stage KS4202 (tolerant) soybeans to have higher peroxidase activity in response to aphid feeding; whereas, aphid-infested and control soybean plants of the susceptible cultivar had similar levels of peroxidase activity. Through transcriptional profiling, Prochaska (2011) identified two peroxidases that were significantly up regulated in response to aphid feeding in the tolerant soybean KS4202.

In this study, KS4202 V1 and SD76R V1 and V3 control and aphid-infested had statistically similar levels of peroxidase activity throughout the sampling periods of the experiment. KS4202 infested plants at the V3 stage had significantly higher peroxidase activity levels than control plants at 6 and 22 days after aphid introduction. The differences in peroxidase activity observed between infested and control V3 KS4202 plants throughout the course of the experiment suggest that peroxidases are playing multiple roles in the tolerant plant. The increase peroxidase activity at day 6 may be resulting in the production of ROS, specifically H_2O_2, which can serve as a signaling

molecule for triggering several plant defense pathways. On the other hand, the increases in peroxidase activity at day 22 are likely involved in the detoxification of the reactive oxygen species that accumulate as a result of aphid feeding. Although not significant, slight increases in peroxidase activity were observed between control and infested SD76R V1 and V3 stage plants. However, the level of activity was insufficient in preventing accumulation of ROS and as a result, these plants experienced visible plant damage.

The results from this study compare favorably to Pierson et al. (2011), which also found higher levels of peroxidase activity in KS4202 exposed to soybean aphids during the reproductive stages. The results provided here add more evidence that oxidative enzymes, such as peroxidases, are likely involved in the soybean's tolerance mechanism to soybean aphid. Additional gene expression studies are needed to understand the roles of specific peroxidases in the defense response of KS4202.

Table 1. Total protein concentration for KS4202 (tolerant) and SD76R (susceptible) at 6, 16 and 22 days after soybean aphid introduction.

		Total Protein (μg/μl)		
	Stage of Infestation	**Control**	**Infested**	***P*-value[1]**
		Day 6		
SD76R	V1	6.55 ± 0.70	6.61 ± 0.86	0.96
KS4202	V1	6.37 ± 0.31	7.20 ± 1.07	0.46
SD76R	V3	8.02 ± 1.10	8.00 ± 0.71	0.98
KS4202	V3	9.34 ± 0.71	8.59 ± 0.54	0.51
		Day 16		
SD76R	V1	6.40 ± 0.46	6.11 ± 0.61	0.72
KS4202	V1	7.81 ± 0.59	6.90 ± 0.80	0.27
SD76R	V3	5.27 ± 0.29	6.21 ± 0.39	0.25
KS4202	V3	7.12 ± 0.50	7.27 ± 0.72	0.85
		Day 22		
SD76R	V1	7.16 ± 0.78	6.31 ± 0.98	0.34
KS4202	V1	7.36 ± 0.14	6.05 ± 0.59	0.17
SD76R	V3	6.77 ± 0.79	6.22 ± 0.38	0.53
KS4202	V3	7.72 ± 0.43	8.00 ± 0.34	0.75

[1] Means significantly different at $P \leq 0.05$ by least significant difference.

Table 2. Peroxidase specific activity (μmol/min x mg protein) for KS4202 (tolerant) and SD76R (susceptible) at 6, 16 and 22 days after soybean aphid introduction.

	Peroxidase Activity (μmol/min x mg)			
	Stage of Infestation	**Control**	**Infested**	***P*-value[1]**
		Day 6		
SD76R	V1	1.43 ± 0.44	1.25 ± 0.35	0.79
KS4202	V1	1.37 ± 0.18	1.16 ± 0.27	0.75
SD76R	V3	1.64 ± 0.39	2.53 ± 0.47	0.19
KS4202	V3	2.67 ± 0.62	4.44 ± 0.74	0.01
		Day 16		
SD76R	V1	1.84 ± 0.33	1.69 ± 0.36	0.86
KS4202	V1	2.34 ± 0.60	2.60 ± 0.64	0.76
SD76R	V3	2.27 ± 0.24	3.19 ± 0.71	0.16
KS4202	V3	3.70 ± 0.97	4.35 ± 0.75	0.47
		Day 22		
SD76R	V1	2.65 ± 0.32	4.04 ± 1.18	0.13
KS4202	V1	2.68 ± 0.19	2.43 ± 0.30	0.78
SD76R	V3	3.83 ± 0.83	4.39 ± 0.60	0.54
KS4202	V3	5.10 ± 0.39	7.33 ± 0.69	0.02

[1] Means significantly different at $P \leq 0.05$ by least significant difference.

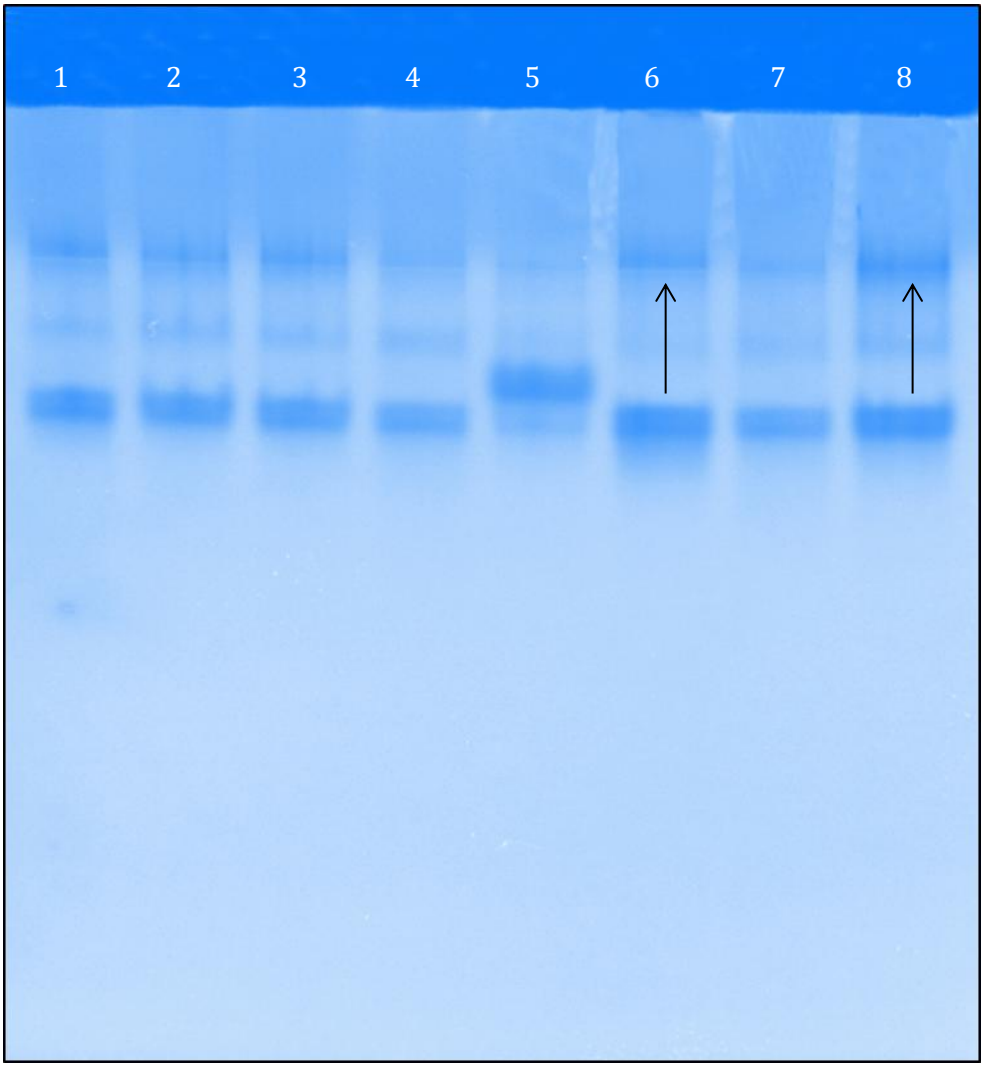

Figure 1. Native gel (10-20%) stained for peroxidase activity 6 days after soybean aphid introduction. Lane 1: V1 stage SD76R control; Lane 2: V1 stage SD76R infested; Lane 3: V1 stage KS4202 control; Lane 4: V1 stage KS4202 infested; Lane 5: V3 stage SD76R control; Lane 6: V3 stage SD76R infested; Lane 7: V3 stage KS4202 control; Lane 8: V3 stage KS4202 infested.

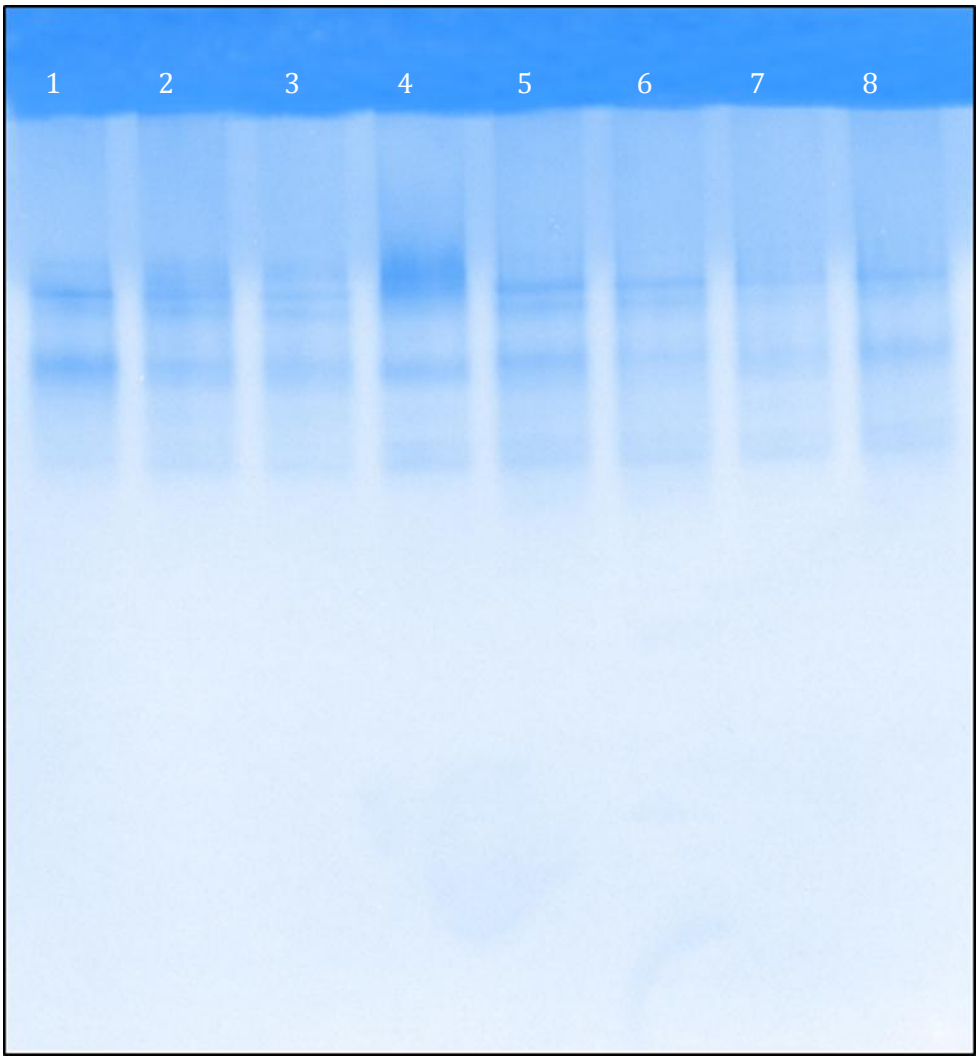

Figure 2. Native gel (10-20%) stained for peroxidase activity 16 days after soybean aphid introduction. Lane 1: V1 stage SD76R control; Lane 2: V1 stage SD76R infested; Lane 3: V1 stage KS4202 control; Lane 4: V1 stage KS4202 infested; Lane 5: V3 stage SD76R control; Lane 6: V3 stage SD76R infested; Lane 7: V3 stage KS4202 control; Lane 8: V3 stage KS4202 infested.

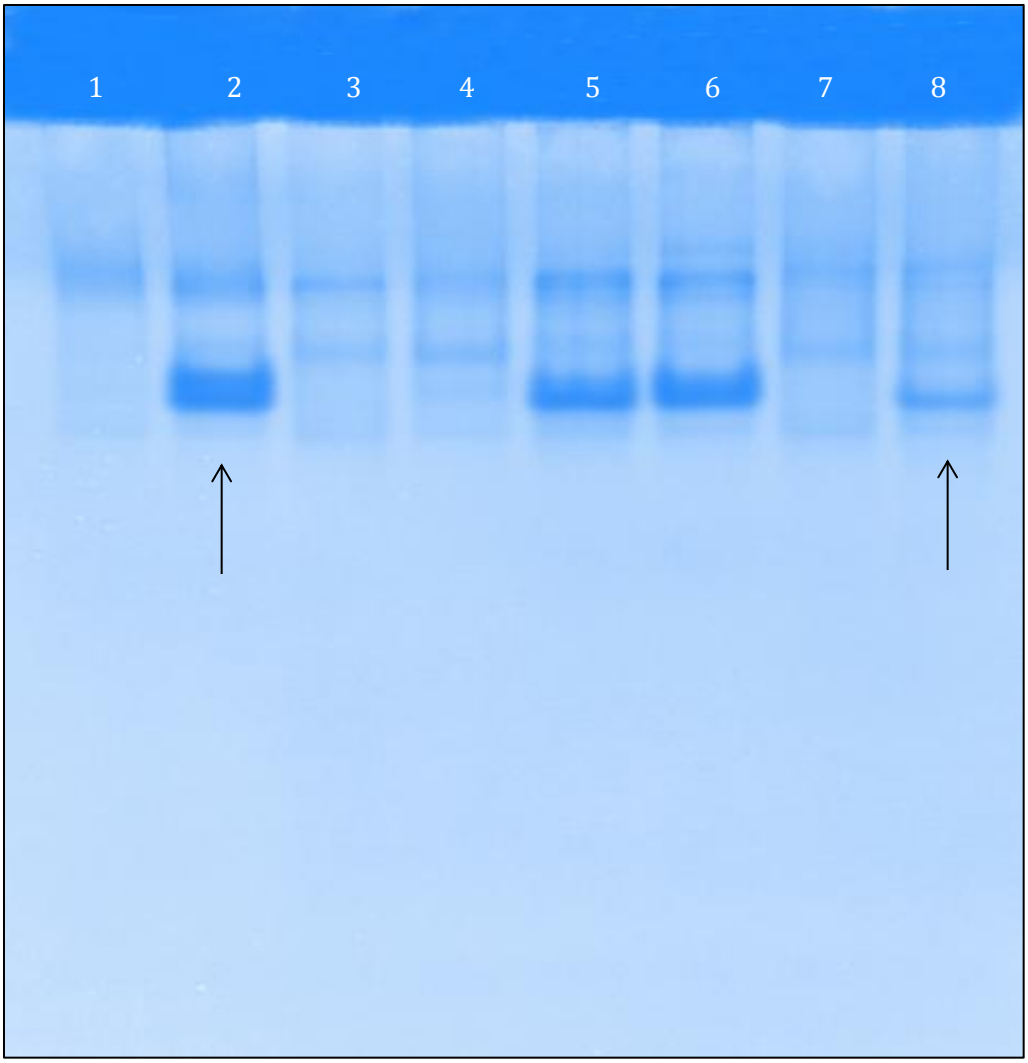

Figure 3. Native gel (10-20%) stained for peroxidase activity 22 days after soybean aphid introduction. Lane 1: V1 stage SD76R control; Lane 2: V1 stage SD76R infested; Lane 3: V1 stage KS4202 control; Lane 4: V1 stage KS4202 infested; Lane 5: V3 stage SD76R control; Lane 6: V3 stage SD76R infested; Lane 7: V3 stage KS4202 control; Lane 8: V3 stage KS4202 infested.

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