

2015

# Beyond biotypes: *Aphis glycines* (Hemiptera: Aphididae) biology and the durability of aphid-resistant soybean

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**Beyond biotypes: *Aphis glycines* (Hemiptera: Aphididae) biology  
and the durability of aphid-resistant soybean**

by

**Adam Jerry Varenhorst**

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

DOCTOR OF PHILOSOPHY

Major: Entomology

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2015

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## TABLE OF CONTENTS

ABSTRACT	iv
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW	
Dissertation Organization	1
Literature Review	1
<i>Aphis glycines</i> biology and ecology	1
Natural enemies	3
Host plant resistance and insect resistance management	5
<i>Rag</i> genes	6
<i>Aphis glycines</i> biotypes	7
Fitness costs	8
Induced susceptibility	9
Aphid effector proteins	10
Author Contributions	12
References Cited	13
CHAPTER 2. AN INDUCED SUSCEPTIBILITY RESPONSE IN SOYBEAN PROMOTES AVIRULENT <i>APHIS GLYCINES</i> (HEMIPTERA: APHIDIDAE) POPULATIONS ON RESISTANT SOYBEAN	
Abstract	20
Introduction	21
Materials and Methods	24
Results	32
Discussion	36
Conclusions	40
Acknowledgements	41
References Cited	41
Figures	48
CHAPTER 3. DETERMINING THE DURATION OF <i>APHIS GLYCINES</i> (HEMIPTERA: APHIDIDAE) INDUCED SUSCEPTIBILITY EFFECTS IN SOYBEAN	
Abstract	53
Introduction	54
Materials and Methods	57
Results	60
Discussion	61
Acknowledgements	65
References Cited	65
Tables	70
Figure	72

CHAPTER 4. EXPLORING FACTORS THAT MAY INFLUENCE THE LONGEVITY OF *APHIS GLYCINES* (HEMIPTERA: APHIDIDAE) RESISTANCE GENES

Abstract	73
Introduction	74
Materials and Methods	77
Results	84
Discussion	89
Acknowledgements	94
References Cited	94
Table	100
Figures	101

CHAPTER 5. THE EFFECT OF INTERSPERSED REFUGES ON *APHIS GLYCINES* (HEMIPTERA: APHIDIDAE) AND NATURAL ENEMY POPULATIONS

Abstract	104
Introduction	105
Materials and Methods	109
Results	114
Discussion	120
Acknowledgements	124
References Cited	124
Tables	130
Figures	134

CHAPTER 6. GENERAL CONCLUSIONS 138

ACKNOWLEDGEMENTS 141

**ABSTRACT**

In North America, *Aphis glycines*, is capable of reducing soybean yields by as much as 40%. The management of *A. glycines* has relied heavily on the use of broad-spectrum insecticides that can be detrimental to both the pest and natural enemies that are present in soybean at the time of application. An alternative management strategy for *A. glycines* is the use of aphid-resistant soybean that contain *Rag* genes. The presence of three virulent *A. glycines* biotypes (*i.e.*, able to overcome aphid-resistance genes) in the US however raises the question about the durability and practicality of *Rag* genes. Here I examined the potential interactions that may be occurring between both virulent and avirulent *A. glycines* on soybean, and whether fitness costs exist for virulent biotypes. I also evaluated whether the use of an interspersed refuge strategy for resistant and susceptible soybean would manage *A. glycines* populations, and determined their impact on natural enemies present in soybean. Our results demonstrate that a virulent *A. glycines* biotype is capable of obviating the resistance gene in soybean; therefore, making the plant a suitable host for both an avirulent and virulent biotype. This effect occurs in the absence of the virulent biotype for up to a period of five days. Fitness costs were present for all virulent biotypes that have been discovered. An interspersed refuge strategy reduces *A. glycines* populations, and has minimal impacts on natural enemies present in soybean. Future research will need to investigate the mechanism responsible for the obviation of resistance effect. Work should also be conducted to determine the durability of *Rag* genes when a refuge in a bag approach is used.

## CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

### Dissertation Organization

The purpose of the research presented in this dissertation was to increase the understanding of the interactions that occur between *A. glycines* biotypes on susceptible and resistant soybean. This dissertation is organized into six chapters. In chapter one, we present a literature review on *Aphis glycines* Matsumura (Hemiptera: Aphididae), natural enemies, host plant resistance and insect resistance management, biotypes, *Rag* genes, induced susceptibility, and effector proteins. We report in chapter two on the ability of avirulent and virulent *A. glycines* biotypes to induce susceptibility of resistant soybean. In chapter three, we report on the duration that the induced susceptibility effect lasts in the absence of the initial population that induced the effect. We report in chapter four on fitness costs associated with virulent biotypes on susceptible soybean. In chapter five, we report on the effects of interspersed refuges on both *A. glycines* and their natural enemies. We present a brief summary in chapter six on the findings and conclusions reached based on the research presented here.

### Literature Review

#### *Aphis glycines* biology and ecology

*Aphis glycines* Matsumura (Hemiptera: Aphididae) has a typical heteroecious, holocyclic life cycle. The primary hosts of *A. glycines* are various species of buckthorn (Rhamnaceae: *Rhamnus spp.*) and the secondary host is soybean [*Glycine max* (L.) Merr.] (Ragsdale et al. 2011). The life cycle of *A. glycines* begins in the spring, when eggs hatch on *Rhamnus spp.* The emerging nymphs develop into wingless fundatrices. The

fundatrices remain on the primary host plant, where they produce a few generations of *A. glycines* (Ragsdale et al. 2004, Ragsdale et al. 2011).

Subsequent generations of *A. glycines* occur on *Rhamnus spp.* and develop into alates, or winged morphs. The alates emigrate from the *Rhamnus spp.* to soybean, and will deposit wingless nymphs to establish initial populations. Once on soybean, *A. glycines* will undergo several overlapping generations, during which apterous and alate morphs can be found. During the summer as many as fifteen generations of *A. glycines* can occur on the secondary host plant (Fox et al. 2004, Ragsdale et al. 2004, Fox et al. 2005, McCornack et al. 2005). It is also during the summer that *A. glycines* can double their populations approximately every 1.5 days when conditions are optimal (McCornack et al. 2004). In the fall, gynoparae alates are produced on soybean. The gynoparae emigrate from soybean in search of buckthorn. When buckthorn is located they feed and produce nymphs. The nymphs develop into apterous oviparae. Androparae, male alates, are also produced on soybean in the fall, and emigrate from soybean in search of the oviparae on buckthorn. The mating of the androparae and the oviparae represents the only sexual reproduction in the life cycle of *A. glycines* (Fox et al. 2004, Ragsdale et al. 2004, Fox et al. 2005, McCornack et al. 2005, Ragsdale et al. 2011). The oviparae lay eggs on buckthorn in late October to mid-November. These eggs hatch during the last week in March.

Since the discovery of *A. glycines* in Wisconsin in 2000 it has been a prevalent pest of soybean in North America (Venette and Ragsdale 2004, Ragsdale et al. 2011). Populations of *A. glycines* have the potential to reduce the yields of soybean by direct feeding, and are also capable of vectoring numerous plant viruses (Ragsdale et al. 2011).

Since 2000 *A. glycines* has dispersed to 23 states, and into areas of Canada (Venette and Ragsdale 2004, Ragsdale et al. 2011). Prior to the establishment of *A. glycines* in North America, soybean had not been challenged by many of its native pests. *Aphis glycines* originated from eastern Asia where it is also an economically important pest. *Aphis glycines* is also present in several other countries including Japan, The Philippines, South Korea, Indonesia, Malaysia, Thailand, Vietnam, and Russia (Wu et al. 2004).

### **Natural enemies**

Biological control refers to the management of a pest that is provided by natural enemies. The objective of biological control is the reduction of the pest's population to a density that is below a designated economic injury level or economic threshold (Smith and van den Bosch 1967, Debach and Rosen 1991). This form of management occurs when a natural enemy or a natural enemy community begin to suppress the pest's population before they have exceeded the economic threshold or economic injury level, or when the pest population density has exceeded those values (Wiedenmann and Smith 1997, Pedigo and Rice 2008).

Various attributes are used to determine how effective a natural enemy is at managing a pest population. Those attributes include the following: fitness and adaptability, searching capacity, power of increase, host specificity and preference, synchronization, density dependent performance, detection and responses to conditions of the host, and the natural enemies' competitive ability. The attributes for natural enemies differ greatly between each individual natural enemy species (Doutt and DeBach 1964, Beddington et al. 1978, Waage and Hassell 1982, Miller 1983, Waage 1990, Waage and Mills 1992).

A Biocontrol Services Index (BSI) is an equation used to determine the amount of control that is provided by the natural enemy community (Gardiner et al. 2009).

Calculating a BSI provides an estimation of the effectiveness of a natural enemy community by accounting for the number of prey that are removed. The BSI can be derived from data collected from cage studies. It compares the number of prey on a caged plant to the number of prey on an uncaged plant, and is able to determine the effectiveness of the natural enemy community based on the number of prey that were removed from the uncaged plant (Gardiner et al. 2009).

In North America there is a natural enemy community referred to as aphidophagous predators, which feed on aphids including *A. glycines*. Insects of this community include insects from the following orders and families: Coleoptera (Carabidae, Coccinellidae), Hemiptera (Anthocoridae, Pentatomidae), Neuroptera (Chrysopidae, Hemerobiidae), Hymenoptera (numerous parasitoids), Opiliones, and Araneae. Previous research has indicated that the two predators with the greatest impact on *A. glycines* populations are *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) and *Orius insidiosus* Say (Hemiptera: Anthocoridae) (Fox et al. 2004, Rutledge et al. 2004). The importance of carabids and spiders for controlling *A. glycines* has not been well documented. Nocturnal predators may also play an important role in the control of *A. glycines* (Fox et al. 2004, Rutledge et al. 2004). Overall, these aphidophagous predators are important for the management of *A. glycines* populations (Desneux et al. 2006, Schmidt et al. 2007, Schmidt et al. 2008, Ohnesorg et al. 2009, Varenhorst and O'Neal 2012).

## **Host plant resistance and insect resistance management**

Plant resistance to insects is the result of inherited traits in a plant that reduce the damage incurred by insects when compared to a known susceptible plant. There are three modalities of plant resistance to insects that can manifest. Those include antibiosis, antixenosis, and tolerance (Painter 1951, Smith 1989). Antibiosis negatively affects the biology of the pest insect, and results in pest populations that have reduced survival, growth, or reproduction. Antixenosis occurs when a plant serves as a poor host to the insect pest, which results in a reduction of feeding or oviposition by repelling the pest insect. Tolerance refers the ability of a plant to withstand or recover from damage caused by the pest insect. A pest can colonize tolerant plants, but significant yield loss will not occur (Painter 1951).

A type of resistance that can occur when susceptible plants are grown in association with resistant plants is associational resistance. This type of resistance occurs when the resistant plants that grow around susceptible plants reduce pest populations. A polyculture of plants (*i.e.*, multiple species) can also reduce pest populations at greater levels than monocultures. Associational resistance can slow the development of insect biotypes that are capable of overcoming the traits that confer resistance through diverting or delaying the insect pest populations (Smith 1989).

Resistance genes can be deployed in a field by use of horizontal or polygenic resistance strategies, or by vertical or monogenic resistance. Polygenic resistance strategies employ multiple sources of resistance that contain minor resistance genes. This strategy is not easily overcome by virulent biotypes. Monogenic resistance strategies employ a single major resistance gene.

Genes used for monogenic resistance strategies have higher levels (*i.e.*, major resistance genes) of resistance than those used in polygenic resistance strategies, but are also more readily overcome by virulent biotypes. One method used to increase the effectiveness of monogenic resistance strategies is the use of sequential cultivar release. The sequential cultivar release strategy results in a single major resistance gene being deployed at a time. Once virulent individuals in a pest population overcome the resistance conferred by that gene an additional major resistance gene is deployed (Gould 1986, Smith 1989). Another method used to improve the durability of major resistance genes is the pyramiding of two or more of these genes into a single cultivar. An additional method used to improve the durability of major resistance genes is the use of crop multilines. Crop multilines are comprised of numerous cultivars of a single species that contain different combinations of both major and minor resistance genes. Of the methods discussed, models have indicated that sequential deployment and crop multilines are more durable options than pyramiding genes. However, pyramided genes confer higher levels of resistance to pest populations, and planting a percentage of the total crop to a susceptible cultivar can enhance the durability of pyramided genes (Gould 1986, Smith 1989).

### ***Rag* genes**

Resistance to *A. glycines* was first discovered in Dowling, PI 71506, and Jackson cultivars (Hill et al. 2004). The genes conferring resistance are referred to as resistance to *Aphis glycines* (*Rag*) genes (Hill et al. 2006). The first *Rag* gene discovered was *Rag1* from the Dowling cultivar. The resistance that is provided by the *Rag1* gene is controlled by a single dominant gene (Hill et al. 2006), and if the *Rag1* gene is released on a large

scale it is assumed that *A. glycines* populations will overcome the gene in a short period of time (Gould 1986, Smith 1989, Mian et al. 2008a). Because of this concern, research was conducted to discover additional *Rag* genes. Additional *Rag* genes would present the opportunity of creating pyramids of resistance genes with different modes action, which is more likely to be effective at managing *A. glycines* populations and increase the durability of the genes that are deployed (Gould 1986, Smith 1989, Mian et al. 2008). A second resistance gene was discovered from the cultivar PI 234540, which was designated *Rag2* (Mian et al. 2008). A similar gene was also discovered in PI 200538, and has been determined to also be *Rag2* (Hill et al. 2009).

Before the release of commercially available varieties containing *Rag1* the use of insecticides represented the main form of aphid control (Hill et al. 2009). In 2012, there were eighteen soybean varieties with *A. glycines* resistance genes commercially available in Iowa (McCarville et al. 2012). The majority of these varieties contain only the *Rag1* gene (McCarville et al. 2012). Wiarda et al. (2012) conducted a study evaluating lines containing the *Rag1+Rag2* pyramided genes to lines that contained either the *Rag1* or *Rag2* genes alone. Their study determined that the lines with the pyramided genes were more effective at reducing *A. glycines* populations (Wiarda et al. 2012).

### ***Aphis glycines* biotypes**

A biotype is pseudo-taxonomic unit used to define populations of an insect species that vary in their virulence to cultivars of their host plant (Pedigo and Rice 2009). Therefore, a biotype is an intraspecies taxon that is classified based on a shared differentiated phenotype (Claridge and den Hollander 1983, Diehl and Bush 1984). In North America, there are currently four described *A. glycines* biotypes. Throughout North

America, the predominant biotype is the avirulent biotype-1 (Michel et al. 2011). The first virulent biotype, biotype-2, was observed in Ohio. Biotype-1 and biotype-2 were identified by their distinct abilities to form colonies on soybean containing different sources of resistance (Kim et al. 2008). Biotype-1 is the avirulent population, and has not been documented to survive well on soybean containing any of the known *Rag* genes. Biotype-2 is described as being virulent to the *Rag1* gene, and is capable of successfully colonizing *Rag1* soybean (Kim et al. 2008). Biotype-3 was discovered in Indiana, and is capable of surviving on *Rag2* soybean (Hill et al. 2010). The most recent biotype discovery was biotype-4, which is capable of surviving on soybean containing *Rag1*, *Rag2*, or *Rag1+Rag2* genes (Alt and Ryan-Mahmutagic 2013).

### **Fitness costs**

Fitness costs can be defined as trade-offs in which alleles that confer higher fitness in one environment reduce fitness in an alternative environment (Gassmann et al. 2009). The presence of fitness costs in a resistant (virulent) population can result in a reduction of the frequency of resistant alleles when refuges of susceptible plants are present (Gassmann et al. 2009). Fitness costs can be further subdivided into negative cross-resistance and negatively correlated resistance. Negative cross-resistance occurs when the allele(s) that confers virulence to one resistance gene also confers hypersensitivity to another resistance gene (Pittendrigh et al. 2008). An alternative to negative cross-resistance is negatively correlated resistance, which occurs when the gene responsible for virulence to one source of resistance is not the same gene that is responsible for hypersensitivity to another source of resistance (Pittendrigh et al. 2008).

### **Induced susceptibility**

Induced susceptibility (analogous to ameliorative effects described by Haukioja 1990a, 1990b, 1990c) is a term that describes positive interactions that can occur between insect herbivores and their host-plants, which result in increased host-suitability for subsequent herbivores (Price et al. 2011, Baluch et al. 2012, Robert et al. 2012). Induced susceptibility can occur between conspecifics on both susceptible (Rotem et al. 2003, Pitino and Hogenhout 2012, Robert et al. 2012, Atamian et al. 2013, Takemoto et al. 2013) and insect-resistant plants (Sauge et al. 2006, Baluch et al. 2012) regardless of the phenotype of the conspecific population (*i.e.*, both virulent and avirulent biotypes). The effect of induced susceptibility encompasses many different sub-categories of positive plant-insect interactions. The two subcategories that will be focused on in the following chapters are feeding facilitation (Price et al. 2011) and obviation of resistance (Baluch et al. 2012).

Feeding facilitation refers to increased host suitability or increased nutritional value of the plant caused by the initial herbivory (Price et al. 2011). This effect may be caused when the initial herbivore destroys defensive structures such as resin canals, or latex canals. Feeding facilitation may also occur when initial herbivore populations cause a nutrient sink in a plant due to their feeding (Price et al. 2011). Nutrient sinks are regions of a plant that contain enhanced levels of assimilates that occur due to the herbivory of phloem feeding insects (Price et al. 2011). Other examples of feeding facilitation occur when initial herbivores alter the plants nutrition simply by feeding on the plant. The mechanisms responsible for these cases of feeding facilitation are unresolved (Takemoto

et al. 2013), but are expected to be due to effector proteins (Pitino and Hogenhout 2012, Atamian et al. 2013).

The effect obviating of resistance occurs when an initial virulent population of herbivores removes the resistance present in a host plant, which allows subsequent avirulent conspecifics to then colonize and survive on an otherwise resistant plant (Baluch et al. 2012). This effect differs from feeding facilitation because the initial herbivore is specialized to feed on the resistant plant (*i.e.*, virulent population). The subsequent herbivores are otherwise incapable of successfully surviving on the plant (Baluch et al. 2012).

### **Aphid effector proteins**

Effectors are molecules that are secreted by plant pathogens and insects, which enable the colonization of the pests' host plant (Hogenhout et al. 2009, Hogenhout and Bos 2011). Effector molecules can range from proteins to small molecules that are capable of altering the function and structure of the host-cells (Hogenhout et al. 2009). The effector molecules may be delivered directly into host cells, or they may act in the apoplast of the host plant. In many instances, effector molecules suppress the immunity of the host plant and/or alter the plant's development (Hogenhout et al. 2009). Each interaction that occurs between an effector molecule and the host target can either result in either a positive, negative, or neutral effect. It is hypothesized that plants have decoy targets that when triggered by effector molecules result in an increased defense response by the host plant (van der Hoorn and Kamoun 2008).

Of the insect species that are herbivores, approximately 90% are specialists that feed on a single taxonomic family or a few closely related plant species (Schoonhoven et

al. 2005). One hypothesis is that this specialization is due to the presence of effector proteins in the saliva of these insects (Hogenhout and Bos 2011). Musser et al. (2002) were the first researchers to discover an herbivore effector protein that suppressed the defense response of the host plant. Although the first evidence of insect effector proteins was for a lepidopteran pest, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae), the same protein is now known to exist in many additional insects including aphids (Musser et al. 2002, Harmel et al. 2008). Additional research on aphid effector proteins has resulted in the identification of a salivary gland protein gene *C002*, which is present in both *Acyrtosiphon pisum* Harris (Hemiptera: Aphididae) and *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Mutti et al. 2006, Bos et al. 2010). Mutti et al. (2008) demonstrated that *C002* is necessary for *A. pisum* to feed on its host plant. Additionally, Bos et al. (2010) demonstrated that an overexpression of *C002* resulted in increased virulence of *M. persicae*. Their study also demonstrated that the protein *C002* is species specific (Bos et al. 2010).

Although the protein gene *C002* has been the focus of multiple studies (Mutti et al. 2006, Mutti et al. 2008, Bos et al. 2010) additional putative effector proteins have been identified in *Diuraphis noxia* Kurd. (Hemiptera: Aphididae) *A. pisum*, *M. persicae*, and *A. glycines* (Carolan et al. 2009, Nicholson et al. 2012, Atamian et al. 2013, Bansal et al. 2014). Many of these proteins have homologs that can be found in multiple species of aphids (Carolan et al. 2009, Atamian et al. 2013, Bansal et al. 2014). In *D. noxia*, there is evidence that the expression of effector proteins is biotype specific and may explain differences between avirulent and virulent individuals (Nicholson et al. 2012). It is not yet known if the genes that code for effector molecules in other aphids (e.g., *A. pisum*)

and are found in *A. glycines* (Bansal et al. 2014) are the basis of virulence to *Rag* genes in *A. glycines*.

### **Author Contributions**

#### **Chapter 2. An induced susceptibility response in soybean promotes avirulent *Aphis glycines* (Hemiptera: Aphididae) populations on resistant soybean**

Conceived and designed the experiments: AJV MTM MEO. Performed the experiments: AJV. Analyzed the data: AJV. Conceived and designed the model: MTM. Wrote the paper: AJV MTM MEO. Provided expertise and editing: AJV MTM MEO.

#### **Chapter 3. Determining the duration of *Aphis glycines* (Hemiptera: Aphididae) induced susceptibility effects in soybean**

Conceived and designed the experiment: AJV. Performed the experiment: AJV. Analyzed the data: AJV MTM. Wrote the paper: AJV MTM MEO. Provided expertise and editing: AJV MTM MEO.

#### **Chapter 4. Exploring factors that may promote the longevity of *Aphis glycines* (Hemiptera: Aphididae) resistance genes**

Conceived and designed the experiment: AJV. Performed the experiments: AJV. Analyzed the data: AJV. Conceived and designed the model: MTM. Wrote the paper: AJV MTM MEO. Provided expertise and editing: AJV MTM MEO.

#### **Chapter 5. The effect of seed mixtures on *Aphis glycines* (Hemiptera: Aphididae) and natural enemy populations**

Conceived and designed the experiment: AJV MEO. Performed the experiments: AJV. Analyzed the data: AJV. Wrote the paper: AJV MEO. Provided expertise and editing: AJV MEO.

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**Chapter 2**

**AN INDUCED SUSCEPTIBILITY RESPONSE IN SOYBEAN  
PROMOTES AVIRULENT *APHIS GLYCINES* (HEMIPTERA:  
APHIDIDAE) POPULATIONS ON RESISTANT SOYBEAN**

A paper accepted by Environmental Entomology

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**Abstract**

Observations of virulent *Aphis glycines* populations on resistant soybean in North America occurred prior to the commercial release of *Rag* genes. Laboratory assays confirmed the presence of four *A. glycines* biotypes in North America defined by their virulence to the *Rag1* and *Rag2* genes. Avirulent and virulent biotypes can co-occur and potentially interact on soybean, which may result in induced susceptibility. We conducted a series of experiments to determine if the survival of avirulent biotypes on susceptible and resistant soybean containing the *Rag1* or *Rag1+Rag2* genes was affected by the presence of either avirulent or virulent conspecifics. Regardless of virulence to *Rag* genes, initial feeding by conspecifics increased the survival of subsequent *A. glycines* populations on both susceptible and resistant soybean. Avirulent populations increased at the same rate as virulent populations if the resistant plants were initially colonized with virulent aphids. These results are the first to demonstrate that virulent *A. glycines* increase

the suitability of resistant soybean for avirulent conspecifics, thus explaining the lack of genetic differentiation observed in North America between *A. glycines* populations on resistant and susceptible soybean. These results suggest the occurrence of virulence towards *Rag* genes in North America may be overestimated. In addition this may alter the selection pressure for virulence genes to increase in a population. Therefore, insect resistance management models for *A. glycines* may need to incorporate induced susceptibility factors to determine the relative durability of resistance genes.

**Keywords:** host-plant resistance, *Rag* genes, feeding facilitation, obviation of resistance, soybean aphid

### Introduction

Plant resistance is an ecologically important trait that shapes ecosystems, plant-herbivore, and plant-pathogen interactions. Plant resistance is utilized in agricultural systems to provide an effective, economical method of pest control with limited environmental disruption (Smith 1989). Possibly the greatest challenge to deploying plant resistance on a large scale for pest control is the presence or evolution of pest genotypes capable of overcoming the plant resistance trait and causing crop injury. In the case of insect pests, genotypes that overcome resistant traits are often referred to as virulent biotypes. The term biotype in relation to resistant plant-insect interactions, encompasses groups of individuals categorized based on their response to a specific plant trait conferring resistance (*e.g.*, virulent to resistance gene/source 1, avirulent to resistance gene/source 2) (Day 1974, Smith 1989, Panda and Khush 1995).

The soybean aphid, *Aphis glycines* Matsumura, is an economically important pest of soybean in the United States (Ragsdale et al. 2011). Resistant soybean cultivars

carrying the *Rag1* and *Rag2* genes alone or combined can decrease *A. glycines* population growth by 34% and 49%, respectively (McCarville and O’Neal 2012). Commercial cultivars containing these genes were first sold in 2010, yet adoption has been slow despite their effectiveness for *A. glycines* management (McCarville et al. 2012). One reason for the slow adoption is the presence of *A. glycines* on resistant cultivars in the field, occasionally at economically damaging levels (Hesler et al. 2013, McCarville et al. 2014). The presence of economically damaging populations on resistant cultivars has been attributed to the occurrence of virulent biotypes in North America (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013). Initial estimates suggest biotype-2, which is virulent to *Rag1*, is not rare, comprising as much as 20% of the North American *A. glycines* population (Michel et al. 2011). The prevalence of virulent *A. glycines* in North America is unexpected given our understanding of the *A. glycines*-soybean system. The limited use of *Rag* genes in North America and the genetic bottleneck that *A. glycines* experienced coming from Asia would suggest virulence should be rare, due to low selection pressure and the pest population’s limited genetic diversity. Wenger and Michel (2013) suggest that a single gene-for-gene interaction does not fully explain the distribution and frequency of genetic biotypes of *A. glycines* in North America. Understanding how a non-genetic explanation for virulence to *Rag* genes occurs would contribute to estimating the frequency of virulence in *A. glycines* populations and the risk of virulence evolving to the extent that *Rag* genes lose their efficacy as a management tool.

The term ‘induced susceptibility’ encompasses the interactions that occur between insect herbivores and their host-plants that result in increased host-plant suitability for

subsequent herbivores (Price et al. 2011, Baluch et al. 2012, Robert et al. 2012). Induced susceptibility can occur between conspecifics on both susceptible (Rotem et al. 2003, Pitino and Hogenhout 2012, Robert et al. 2012, Atamian et al. 2013, Takemoto et al. 2013) and resistant plants (Sauge et al. 2006, Baluch et al. 2012) regardless of the phenotype of the conspecific population (both virulent and avirulent biotypes). For example, survival of avirulent *Myzus persicae* (Sulzer) is increased on resistant plants that were initially fed on by an avirulent *M. persicae* population (Sauge et al. 2006). Induced susceptibility can also occur among conspecifics with varying phenotypes. Survival of avirulent *Mayetiola destructor* (Say) on resistant wheat plants is dependent upon initial feeding by virulent individuals (Baluch et al. 2012). Induced susceptibility could therefore produce insect populations on resistant plants that appear phenotypically similar but are actually a diverse population comprised of both virulent and avirulent individuals (Claridge and Den Hollander 1983).

If induced susceptibility occurs among *A. glycines* on resistant soybean, it could complicate insect resistance management (IRM) plans. The aim of IRM is to limit the spread and increase of virulence alleles in a pest population. Production of insect resistant cultivars is a resource- and time-consuming endeavor; therefore, preservation of plant resistance is important and can be achieved through the implementation of an IRM plan.

Insect resistance management is the subject of a large body of literature, and is required for the deployment of transgenic insect-resistant cultivars in the United States (Hurley and Mitchell 2008). In general, virulence is initially considered rare and conferred by one or a few genes, and that without one (dominant or additive) or two (recessive) copies of the virulence allele an individual is incapable of surviving or

reproducing on the resistant cultivar (Claridge and Den Hollander 1983, Pan et al. 2011). This assumption may be correct for the majority of plant resistance developed from transgenic genes, which have not coevolved with herbivores. However, this assumption may not apply to the implementation of plant resistance derived from traditional breeding methods (*e.g.*, *Rag* genes) which ingress resistance genes from land races and wild relatives. Resistance obtained from these sources likely has an evolutionary history with the pest insect, and therefore virulence may occur prior to large-scale deployment of the resistance gene (Smith 1989, Panda and Khush 1995). If a non-genetic explanation like induced susceptibility can contribute to our understanding of virulence, it will require substantial changes in the models used to develop IRM plans.

Here we explore if induced susceptibility is a potential non-genetic explanation for the unexpectedly high prevalence of *A. glycines* on resistant soybean plants in North America. Furthermore, we explore the ability of virulent *A. glycines* populations to alter the induced susceptibility interaction. Finally, we modify a simple deterministic model (Crowder and Carrière 2009) to estimate the impact of this non-genetic explanation on the frequency of virulence alleles in an *A. glycines* population after the release of a plant resistance gene.

## **Materials and Methods**

### **Aphid colonies and soybean cultivars**

*Aphis glycines* populations used for this experiment were obtained from the Ohio State University. Two populations defined by their response to *Rag1* were utilized, an avirulent biotype (biotype-1) and a virulent biotype (biotype-2) (Kim et al. 2008). These populations were founded from individuals that were initially collected and identified in

Illinois (Kim et al. 2008). Their biotype identity (*i.e.*, virulent or avirulent to *Rag1*) was confirmed using detached leaf assays (Michel et al. 2010). After biotype identification, the two biotype populations used herein were derived from single clonal population. Efforts to measure the genetic variation between these two biotypes have revealed limited genetic diversity (Wenger and Michel 2013). For rearing, avirulent *A. glycines* were raised on susceptible soybean (IA3027), while virulent *A. glycines* were raised on a near-isogenic resistant soybean containing the *Rag1* gene (IA3027RA1).

For the experiments, we used three near-isogenic soybean cultivars containing no *Rag* genes (IA3027), *Rag1* (IA3027RA1), or *Rag1+Rag2* (IA3027RA12). These cultivars are near-isolines for the resistance genes *Rag1* and *Rag2*, and are approximately 93.25% genetically identical (Wiarda et al. 2012). Herein, we refer to cultivars with *Rag*-genes as being resistant, and the cultivar without *Rag* genes as susceptible.

### **Induced susceptibility experiment**

We hypothesized that initial feeding by avirulent *A. glycines* would increase host-plant suitability for conspecifics on both susceptible and resistant soybean, compared to *A. glycines* on plants that were not previously fed upon (*i.e.*, induced susceptibility). We tested for induced susceptibility by infesting plants with an initial population of *A. glycines*, termed an inducer population, and measuring the population density of a subsequent population of *A. glycines*, termed a response population, after 11 days of feeding.

We used six treatments to test our hypothesis in a growth chamber using individually potted plants. Each treatment was a combination of two factors, soybean cultivar and the density of the *A. glycines* inducer population. A resistant (IA3027RA12)

and a susceptible (IA3027) cultivar were used. Each cultivar was exposed to three densities of avirulent inducer populations: no inducer (none), 25 *A. glycines* (25 avirulent), or 50 *A. glycines* (50 avirulent). Inducer populations were added to plants 24 h prior to the addition of a response population. Therefore, the effect of inducer *A. glycines* feeding could be measured on performance of the response population of *A. glycines*. Induced susceptibility was confirmed if response populations were significantly larger in the presence of an inducer population (25 avirulent or 50 avirulent) than in its absence (none).

Avirulent inducer populations were applied to the first trifoliolate when plants reached the second trifoliolate growth stage. The entire first trifoliolate was then enclosed within a mesh net for the duration of the experiment. The effectiveness of the mesh nets at confining inducer populations to the first trifoliolate was confirmed in a preliminary experiment (supplementary data, S1). After 24 h the response population, consisting of five avirulent *A. glycines*, was added to the second trifoliolate of each plant. The response population was not enclosed within a mesh net, and allowed to move freely about the plant, with the exception of the first trifoliolate. Therefore, the inducer and response populations were spatially isolated and could be quantified separately. Individual potted plants were then enclosed within mesh nets to prevent plant-to-plant movement of response populations. We examined the response population after 24 h to confirm their successful establishment. The total number of *A. glycines* present in the response population on each plant was counted 11 days after the initial infestation. At this time the number of *A. glycines* in the inducer populations was also counted to confirm their persistence for the duration of the experiment.

This experiment was repeated three times using a randomized complete block design (RCBD) with four blocks per repetition (12 total experimental units per treatment). Individually potted soybean plants were grown in 8-cm diameter pots in a Percival E41L2C9 growth chamber (Percival Scientific, Incorporated, Perry, IA) using a 14:10 light dark cycle and a constant temperature of 27 °C with a relative humidity of 60%.

### **Induced susceptibility with virulent biotypes experiment**

We next hypothesized that the population density of *A. glycines* on a resistant soybean plant is affected by not only the presence of conspecifics, but also the conspecific's biotype. We tested our hypothesis using the same general procedure as outlined for the previous experiment. For this experiment, we measured the density of the response populations of *A. glycines* on *Rag1* (IA3027RA1) soybean, while altering the biotype of *A. glycines* in both the inducer and response populations.

We utilized six treatments, each a combination of an inducer population and a response population. Three inducer populations were used: no inducer (none), 50 avirulent *A. glycines* (50 avirulent), and 50 virulent *A. glycines* (50 virulent). Two response populations were used; five avirulent and five virulent *A. glycines*. Both inducer and response populations of *A. glycines* were added to plants and counted using the same methods as the previous experiment. The same planting procedure and growth chamber specifications as the previous experiment were used. This experiment was repeated three times using a RCBD with three blocks within each repetition (nine total experimental units per treatment).

### **Induced susceptibility in a semi-field setting experiment**

Our final hypothesis addressed the significance of the results from our first two experiments and their role in shaping *A. glycines* populations in a field setting. Specifically, we tested whether virulent *A. glycines* increase the host suitability of resistant soybean for avirulent *A. glycines* in the field. We hypothesized that the magnitude of the effects we observed in the lab were large enough to be observed in the field where abiotic factors such as rain, wind, and variation in temperature affect aphid populations (Dixon 1977, McCarville et al. 2011).

We conducted this experiment in a semi-field cage setting on the Iowa State University campus. We utilized three treatments to address our hypothesis. The first treatment consisted of an avirulent response population with no inducer (inducer: none; response: avirulent). The second treatment consisted of a virulent response population with no inducer (inducer: none; response: virulent). The third treatment consisted of a virulent inducer population of 50 *A. glycines* and an avirulent response population of five *A. glycines* (inducer: 50 virulent; response: avirulent). The third treatment was designed to measure the effect of a virulent inducer population on an avirulent response population, and to confirm that induced susceptibility observed in the laboratory could occur in the presence of abiotic factors. The first and third treatments were compared to test whether the virulent inducer population increased the performance of the avirulent response population (inducer: none; response: avirulent vs. inducer: 50 virulent; response: avirulent). The second and third treatments were compared to test whether induced susceptibility resulted in avirulent *A. glycines* presenting the same observed biotype as

virulent *A. glycines* (inducer: none; response: virulent vs. inducer: 50 virulent; response: avirulent).

We replicated each treatment ten times using a RCBD. Each plot for the experiment consisted of three plants grown in a 0.5-m row. Ten seeds of the assigned cultivar were planted in each plot on 15 May 2013, and thinned to 3 plants per plot on 25 June. Cages were used to prevent the movement of aphids among plots and to prevent predation from affecting aphid population growth (McCarville et al. 2011). Cages were constructed of mesh netting (Quest Outfitters, Sarasota, FL) wrapped over a PVC pipe frame with the dimensions of 1.1 m x 0.8 m x 0.8 m (h x l x w).

When plants reached the fifth trifoliate growth stage (10 July), all plants within plots assigned to the third treatment were infested with an inducer population of 50 virulent *A. glycines*, placed on the fourth trifoliate. The fourth trifoliate was then enclosed within a mesh net, as in the previous experiments. After 24 h, response populations were added to the fifth trifoliate of each plant within every plot according to the treatments outlined above. Response populations were allowed to move freely about the plant, except for the fourth trifoliate, which was confined within the mesh net. The total number of *A. glycines* present in the response population and inducer population was counted 11 days after the initial infestation. *Aphis glycines* were counted using the same protocol as the previous experiments.

### **Statistical analyses for experiments**

To address each of our hypotheses, we analyzed the number of *A. glycines* per plant in the response population at 11 days. To reduce heteroscedasticity the *A. glycines* per plant data were log transformed. All data were analyzed using the PROC MIXED

procedure with SAS statistical software version 9.3 (SAS Institute, Cary, NC). For all experiments, data were analyzed using an analysis of variance (ANOVA). Significant treatment effects were then separated using F-protected least-squares means test with a Tukey adjustment and a significance level of  $P < 0.05$ . Non-transformed data were used to construct figures and calculate percent differences between treatments.

The statistical model used to analyze data from the first growth chamber experiment (*i.e.*, induced susceptibility experiment) included the main effects of repetition, block, soybean cultivar, and inducer population. All two- and three-way interaction terms between the main effects were included in the model. The model for the second growth chamber experiment (*i.e.*, induced susceptibility with virulent biotypes experiment) utilized the same statistical model with the elimination of the main effect of soybean cultivar, as only one cultivar was used, and the addition of the main effects inducer population biotype and response population biotype (*i.e.*, avirulent vs. virulent). The statistical model to test for the occurrence of induced susceptibility within a semi-field cage experiment included the main effects of block and treatment.

### **Modeling the consequences of induced susceptibility**

We hypothesized that the induced susceptibility effects would affect the rate at which virulence alleles increase within a population of *A. glycines*. We utilized a simple deterministic, single-locus, two compartment genetic model developed for parthenogenic reproduction to track virulence alleles within a season (Crowder and Carrière 2009). *Aphis glycines* has a heteroecious, holocyclic lifecycle with 12 to 16 generations of asexual reproduction on soybean each year before sexual reproduction occurs on the primary host-plant, buckthorn. We therefore, tracked the change in the frequency of

virulence alleles across 14 generations of asexual reproduction (*i.e.*, a single season of soybean production).

We adjusted the parameters of the model according to published estimates available for *A. glycines*, including: fecundity (32 offspring, McCornack et al. 2004, McCarville et al. 2011), initial virulence allele frequency (0.2, Michel et al. 2011), fitness cost associated with virulence (0.0, Hill et al. 2010), relative fitness of avirulent individuals on resistant plants (0.59, McCarville and O’Neal 2012), and that virulence is complete (Hill et al. 2010). We assumed that a proportion of the landscape would be planted to susceptible plants (0.3) and that a relatively small initial herbivore population would colonize soybean randomly in the spring (10,000 *A. glycines*).

Induced susceptibility was modeled through the inclusion of a density-dependent increase in fitness. When the population density of *A. glycines* surpassed  $1.0 \times 10^{13}$  individuals per 1% of landscape, the fitness of individuals in that compartment of the landscape (*i.e.*, susceptible or resistant plants) was increased to 1.65. Additionally for the resistant landscape, if the population density of homozygous virulent individuals surpassed  $1.0 \times 10^{13}$  per 1% of landscape, the fitness of all individuals in the resistant landscape (both virulent and avirulent) was set to 1.0. We ran the model first without induced susceptibility effects and compared this to the model run with induced susceptibility effects included. We modeled virulence as a recessive, dominant, and additive trait.

## Results

### Induced susceptibility experiment

We confirmed our hypothesis that the population density of *A. glycines* on susceptible and resistant soybean is positively affected by the presence of conspecifics (*i.e.*, that induced susceptibility occurs). This was observed by analyzing data for the significance of the main effects of inducer population, soybean cultivar, and the interaction of inducer population by soybean cultivar. The main effects inducer population ( $F = 60.35$ ;  $df = 2, 12$ ;  $P < 0.0001$ ) and cultivar ( $F = 908.33$ ;  $df = 1, 12$ ;  $P < 0.0001$ ) significantly affected the response populations. We observed induced susceptibility occurring on both susceptible and resistant soybean, as evidenced by increased response population densities in the ‘50 avirulent’ inducer population treatments compared to the ‘none’ inducer population treatment (Fig. 1A and 1B). The presence of the inducer populations was confirmed at the end of the experiment for both the ‘25 avirulent’ and ‘50 avirulent’ inducer population treatments. The significant interaction of inducer population and soybean cultivar ( $F = 6.30$ ;  $df = 2, 12$ ;  $P < 0.0135$ ) indicated that induced susceptibility did not occur equally on the susceptible and resistant cultivars. This interaction is due to the decreased response population densities on resistant soybean, and also the density dependent response observed on resistant but not susceptible soybean. Therefore, we analyzed these data by soybean cultivar.

On susceptible soybean, the inducer population significantly affected response population density ( $F = 21.73$ ;  $df = 2, 12$ ;  $P < 0.0001$ ). Response populations for the ‘25 avirulent’ and ‘50 avirulent’ inducer population treatments were nearly equal ( $t = 0.28$ ;  $df = 2, 12$ ;  $P = 0.9578$ ), both approximately 279% greater than the response population that

received the ‘none inducer’ treatment ( $t = 5.84$ ;  $df = 2, 12$ ;  $P < 0.0002$  and  $t = 5.56$ ;  $df = 2, 12$ ;  $P < 0.0003$  respectively) (Fig. 1A). For the resistant cultivar, the main effect of inducer population was also significant ( $F = 13.28$ ;  $df = 2, 12$ ;  $P < 0.0009$ ). In contrast to the susceptible cultivar, response populations on the resistant cultivar were significantly different between plants that received the ‘25 avirulent’ and ‘50 avirulent’ inducer population treatments ( $t = 2.71$ ;  $df = 2, 12$ ;  $P < 0.0461$ ). Although not significantly different, the response population for the ‘25 avirulent’ *A. glycines* inducer treatment was 758% greater than the ‘none’ treatment ( $t = 2.44$ ;  $df = 2, 12$ ;  $P = 0.0746$ ). The ‘50 avirulent’ *A. glycines* treatment was 2400% greater when compared to the response population on plants that received the ‘none’ treatment ( $t = 5.15$ ;  $df = 2, 12$ ;  $P < 0.0007$ ) (Fig. 1B). The results for both the susceptible and resistant soybean indicated that induced susceptibility as previously defined occurred; however, the maximum effect of induced susceptibility was realized at a lower population density of *A. glycines* on the susceptible cultivar compared to the resistant cultivar. Therefore, to ensure that we observed an effect caused by the inducer population, regardless of plant genotype, we used 50 *A. glycines* for subsequent experiments.

### **Induced susceptibility with virulent biotypes experiment**

In our second experiment, we confirmed our hypothesis that the density of *A. glycines* on resistant soybean is positively affected not only by the presence of conspecifics, but also by the increased virulence of the conspecific (*i.e.*, virulent biotype). To test this hypothesis we analyzed these data for the significance of the main effects inducer population biotype, response population biotype, and their interaction. The main effects of inducer population biotype ( $F = 85.63$ ;  $df = 2, 41$ ;  $P < 0.0001$ ) and response

population biotype ( $F = 68.80$ ;  $df = 1, 41$ ;  $P < 0.0001$ ) both significantly affected the density of the response population. The interaction of the two main effects was also significant ( $F = 24.88$ ;  $df = 2, 41$ ;  $P < 0.0001$ ). In general, both the presence and the increased virulence of the inducer population positively affected the density of the response population (Fig. 2). The presences of the avirulent and virulent inducer populations were confirmed at the end of the experiment, indicating that the observed response population differences were attributable to the inducer populations.

In the absence of an inducer population, the virulent response population was 867% greater than the avirulent population ( $t = 7.23$ ;  $df = 2, 17$ ;  $P < 0.0001$ ). The virulent response population was 127% greater than the avirulent population in the presence of an avirulent inducer population ( $t = 3.41$ ;  $df = 2, 17$ ;  $P < 0.0333$ ). In the presence of a virulent inducer population, there was no difference between the population densities of virulent and avirulent response populations ( $t = 0.26$ ;  $df = 2, 17$ ;  $P = 0.9998$ ). In summary, the difference in the population density between the virulent and avirulent response populations diminished with the addition of an avirulent inducer population and completely disappeared in the presence of a virulent inducer population.

### **Induced susceptibility in a semi-field setting experiment**

In our third experiment, we confirmed our hypothesis that the results from the previous experiments were of a large enough magnitude to be observed in the presence of abiotic factors. We observed significant variation in the response populations among the three treatments tested in the semi-field setting ( $F = 570.85$ ;  $df = 2, 16$ ;  $P < 0.0001$ ). The presence of the virulent inducer population was confirmed at the end of the experiment. A means separation test confirmed that the presence of a virulent inducer population

increased the population density of the avirulent response population when compared to the treatment with no inducer population ( $t = 30.54$ ;  $df = 2, 16$ ;  $P < 0.0001$ ) (Fig. 3). This also resulted in equivalent population densities between the virulent response population and the avirulent response population in the presence of the virulent inducer population ( $t = 1.16$ ;  $df = 2, 16$ ;  $P = 0.4925$ ) (Fig. 3). Induced susceptibility, therefore, occurred in the field on resistant soybean and resulted in the avirulent response population growing to the same level as a virulent response population.

### **Modeling the consequences of induced susceptibility**

Induced susceptibility lowered the frequency of virulence alleles at the end of the season. In the absence of induced susceptibility, virulence alleles increased from the initial 0.20 to 0.40, 0.32, and 0.30 for dominant, additive, and recessive inheritance, respectively. With the inclusion of induced susceptibility the end of season virulence allele frequencies were 0.35, 0.28, and 0.26 for each mode of inheritance. Therefore, the end of season allele frequencies was reduced when induced susceptibility was present when compared to end of season allele frequencies where it was absent. Induced susceptibility, therefore, reduced directional selection for virulence by 25% to 40% within a single season.

The decreases in the rate of virulence evolution were due to two factors. First, between generations 10 and 11, the frequency of virulence alleles decreased due to the increased fecundity of individuals in the refuge. The fecundity of individuals in the refuge was temporarily higher because the density of individuals in that compartment of the landscape reached  $1.0 \times 10^{13}$  a generation prior to individuals on resistant soybean. This occurred because individuals in the refuge (both virulent and avirulent) had a

relative fitness of 1.0, whereas the population growth of avirulent individuals in the resistant compartment is slower due to their reduced fitness. Second, the selection pressure imposed by the resistance gene was alleviated by generation 11 because the density of homozygous resistant individuals exceeded  $1.0 \times 10^{13}$  in the resistant compartment of the landscape resulting in an induced susceptibility effect.

### **Discussion**

Our results indicate that feeding by avirulent and virulent *A. glycines* results in an induced susceptibility effect that increases host-plant suitability of both susceptible and resistant soybean. Furthermore, this effect is not an artifact of laboratory conditions, and occurs in a semi-field setting. Price et al. (2011) define induced susceptibility (analogous to ameliorative effects described by Haukioja 1990a, 1990b, 1990c) as an interaction whereby insect herbivory increases the host-plant's suitability for other herbivores. To more accurately describe our observations induced susceptibility can be divided into the two sub-categories 'feeding facilitation' (Price et al. 2011) and 'obviation of resistance' (Baluch et al. 2012). Feeding facilitation describes a sub-category of induced susceptibility where the presence of an herbivore, regardless of its genotype, increases the performance of conspecifics on either susceptible or resistant host-plants as observed in Figure 1. Obviation of resistance describes a sub-category of induced susceptibility, where the presence of a virulent herbivore increases the performance of avirulent conspecifics on a resistant host-plant as observed in Figures 2 and 3.

Although genetically distinct biotypes of *A. glycines* exist with clear phenotypic differences, the term 'biotype' is best described as a pseudo-taxonomic category that defines an intraspecies taxon by a shared differentiating phenotype (Diehl and Bush

1984). In addition to genetically distinct biotypes of *A. glycines* with clear phenotypic differences, there is evidence that a genetic model alone does not explain field observations of *A. glycines* on resistant and susceptible plants (Wenger and Michel 2013). Feeding facilitation resulted in avirulent *A. glycines* populations on resistant soybean resembling non-facilitated virulent populations (Figs. 2 and 3). Our results imply that feeding facilitation involving solely avirulent populations could explain instances of high populations of *A. glycines* on resistant soybean in the field. Obviation of resistance resulted in subsequent avirulent populations that were indistinguishable from virulent populations in respect to final population densities (Figs. 2 and 3). Therefore, the abundance of virulent *A. glycines* biotypes in North America may be overestimated, as these estimates are drawn largely from the relative field performance of *Rag* genes (*i.e.*, number of *A. glycines* on *Rag* containing plant compared to a susceptible plant) (Hesler et al. 2013). Our results indicate that in the presence of virulent *A. glycines*, avirulent populations can colonize resistant soybean and the two populations will be phenotypically indistinguishable in the field. Feeding facilitation and obviation of resistance may explain the lack of genetic differentiation observed between *A. glycines* populations on resistant and susceptible soybean (Wenger and Michel 2013).

These results suggest that population growth of *A. glycines* on soybean increases with an increasing density of the initial population (*i.e.*, the inducer population). Conversely, as the inducer population decreases, the likelihood of either feeding facilitation or obviation of *Rag*-resistance decreases. Such a relationship may produce an Allee effect in *A. glycines* populations, where the fitness of a given individual varies based on the population density on a given plant (Stephens et al. 1999). This suggests that

the ability of *A. glycines* to establish on a soybean plant (either resistant or susceptible) may require a ‘critical mass’ to result in a persistent population. To overcome reduced fitness at low populations, *A. glycines* would need to structure their populations in a way that resembles something other than a stochastic accumulation on soybean plants. We are not aware of any evidence that *A. glycines*’ initial colonization of soybean is not stochastic, though after initial colonization there is evidence that biotypes will disperse in a non-random manner (Whalen and Harmon 2012, Wenger et al. 2014). To what extent the colonization and subsequent dispersal within a soybean field by *A. glycines* leads to feeding facilitation or obviation of resistance requires additional study.

The mechanisms responsible for feeding facilitation and obviation of resistance by *A. glycines* are currently unknown. Potential explanations for these two effects could be aphid endosymbiotic bacteria (Oliver et al. 2010), plant viruses (Mauck et al. 2012, Casteel et al. 2013), or aphid effector proteins (Rodriguez and Bos 2013). Although we cannot definitively determine the cause of these effects from the experimental designs used in this manuscript, we hypothesize that the simplest explanation for both of these phenomenon is the presence of effector proteins present in the saliva of aphids (reviewed by Rodriguez and Bos 2013). Bansal et al. (2014) found 47 effector protein transcripts in the saliva glands of *A. glycines* that matched *Acyrtosiphum pisum* effectors with known function. In *A. pisum*, these proteins alter plant physiology to improve host quality. Our results suggest that feeding facilitation is due to an increased population of conspecifics on soybean, and that obviation of resistance is due to the virulence of biotype-2. This conclusion for obviation of resistance would suggest that different *A. glycines* biotypes have different effector proteins, populations of either endosymbiotic bacteria, or transmit

different plant viruses. The spatial separation of *A. glycines* inducer and response populations would limit the opportunities for the horizontal transfer of endosymbiotic bacteria from the virulent to the avirulent populations. We are unaware of any research that supports the suggestion that biotypes of *A. glycines* in North America have different endosymbionts. For obviation of resistance to be due to a plant virus infection *A. glycines* would need to vector a persistently transmitted plant virus to the experimental soybean plants. In contrast to non-persistently transmitted viruses, persistently transmitted plant viruses have the potential to make the host plant more suitable for aphid vectors, and promote long term feeding (Mauck et al. 2012). Such a plant virus is unlikely to cause the obviation of *Rag*-resistance as the only persistently transmitted soybean virus in North America is *Soybean dwarf virus* (SbDV), and transmission of SbDV by *A. glycines* is rare (Harrison et al. 2005, Wang et al. 2006, Damsteegt et al. 2011). Furthermore, we did not observe symptoms of virus infection among the 426 soybean plants used in these experiments.

Results of the model indicated the induced susceptibility effects of feeding facilitation and obviation of resistance function to reduce the within-season directional selection for virulence by 25-40%. This reduction in selection pressure would be magnified across multiple years potentially creating large delays in the evolution of virulence. Induced susceptibility effects, specifically obviation of resistance, may be a mechanism plants have evolved to maximize the trade-offs between resisting herbivore attack and selecting for virulence. Virulence is theorized to be rare in herbivore populations; therefore, an individual plant or crop field is more likely to be colonized by avirulent individuals first, assuming colonization is a stochastic process (Wilhoit 1991).

Therefore, the majority of early season herbivore populations would be avirulent and resistant plants would gain a fitness advantage over susceptible plants. Upon arrival of virulent herbivores, resistant plants could then shut down defense responses (*i.e.*, obviate resistance), eliminating selection for virulence and preserving the efficacy of the resistance genes to be realized in future years by their progeny. Managing this trade-off between resisting herbivore attack and selecting for virulence with induced susceptibility effects would likely be most advantageous for systems where (1) the herbivore or pathogen has multiple generations with asexual reproduction, (2) plant-plant dispersal of the herbivore or pathogen population occurs multiple times during the season and is less than 100%, and (3) the plant is less susceptible to reductions in fitness late in the season. All three of these conditions are met in the *A. glycines*-soybean system. We hypothesize that induced susceptibility would also occur between other plants and herbivores that share these life-history traits.

### **Conclusions**

The results of our study indicate that interactions between two different biotypes of *A. glycines* on susceptible and resistant soybean can increase the survival of an avirulent biotype. This suggests that *A. glycines* virulence may not be as frequent as perceived in field trials due to the induction of susceptibility allowing avirulent populations to proliferate on resistant soybean. Furthermore, our initial attempt to model the impact of this interaction suggests that the consequences of these interactions on *Rag*-soybean can function to slow the rate at which virulence increases in *A. glycines* populations. If confirmed, this may help alleviate concerns that virulent biotypes already identified in North America would quickly dominate the populations of *A. glycines*. If

additional empirical and modeling studies support these results, the perception of risk associated with virulent biotypes could be positively affected leading to greater commercial adoption of resistant soybean. Beyond the *A. glycines*-soybean system, the general phenomenon of induced susceptibility is not considered in IRM models. Understanding the consequences of induced susceptibility and the mechanisms underlying these interactions could also lead to novel IRM strategies. Therefore, future research should explore both the possible mechanisms for feeding facilitation and obviating of resistance and their consequences for the evolution of virulence.

### **Acknowledgements**

This study was funded in part by the Soybean Checkoff through a grant from the North Central Soybean Research Program. We thank Erin Hodgson for suggestions on earlier versions of this manuscript. We thank Andy Michel for the initial populations of *A. glycines*, and also for comments on earlier versions of the manuscript. We also thank Patrick Wagner for assisting with data collection.

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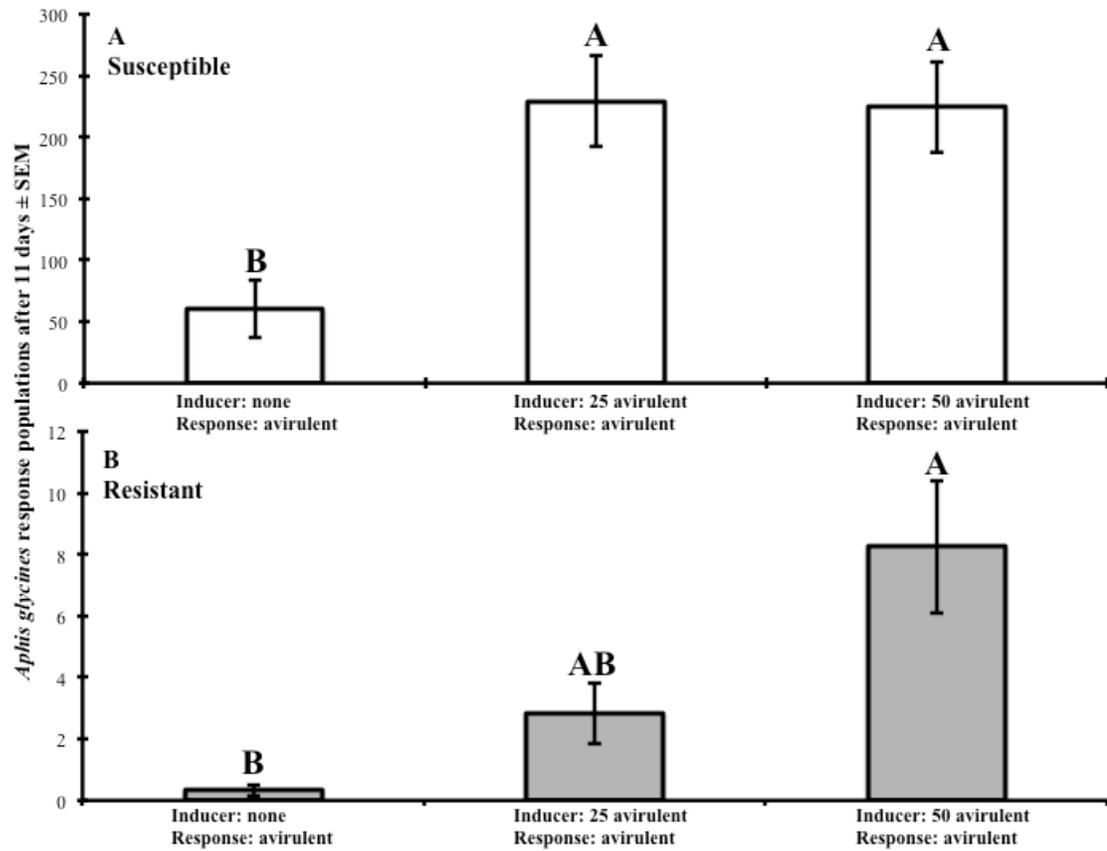
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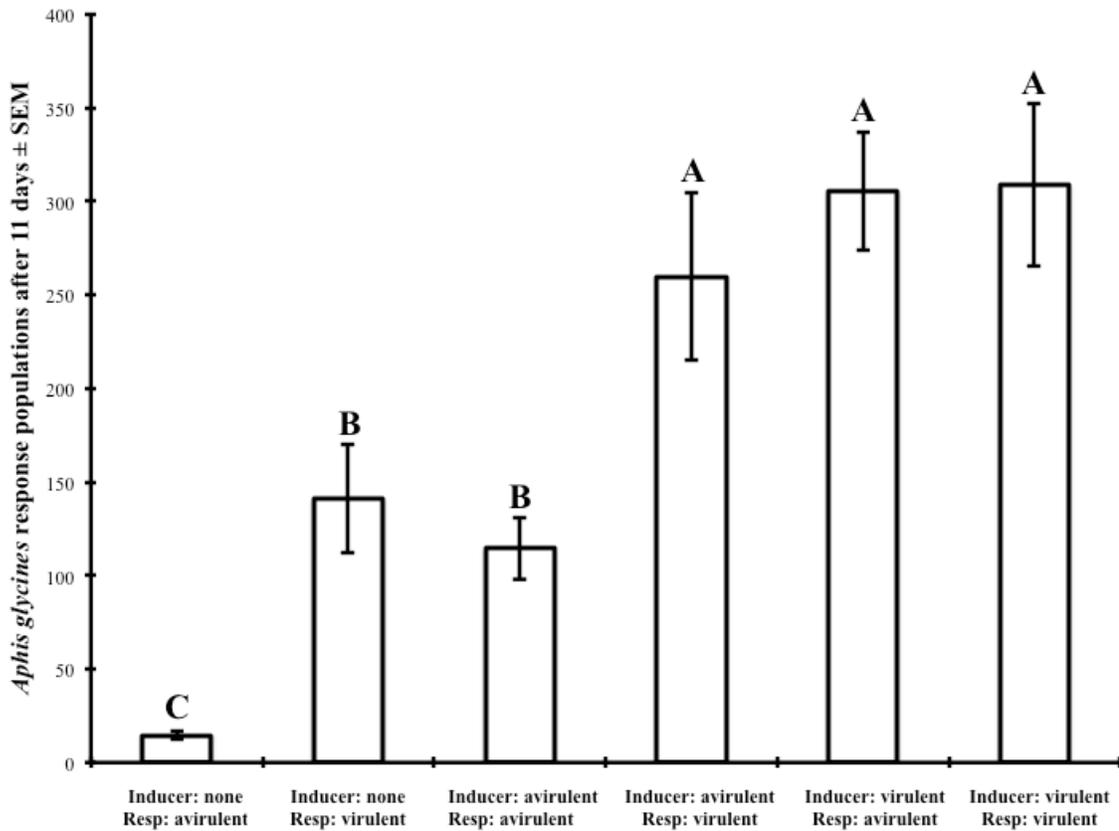
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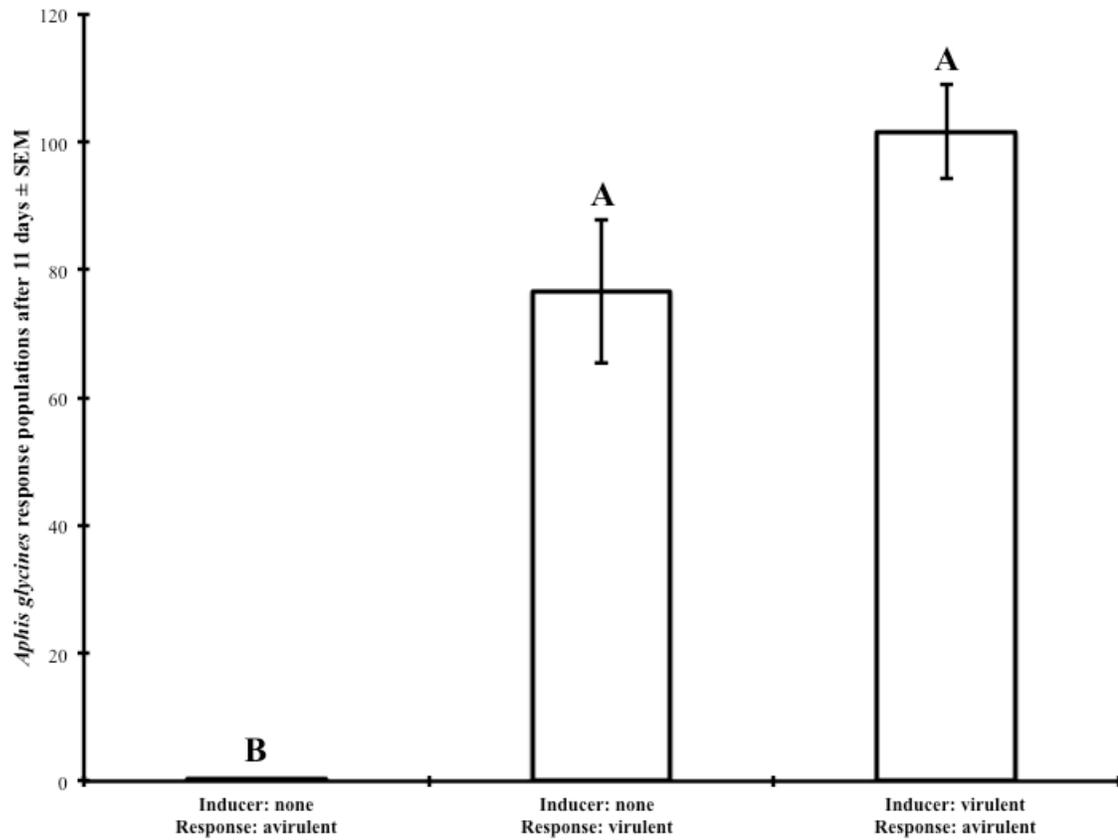
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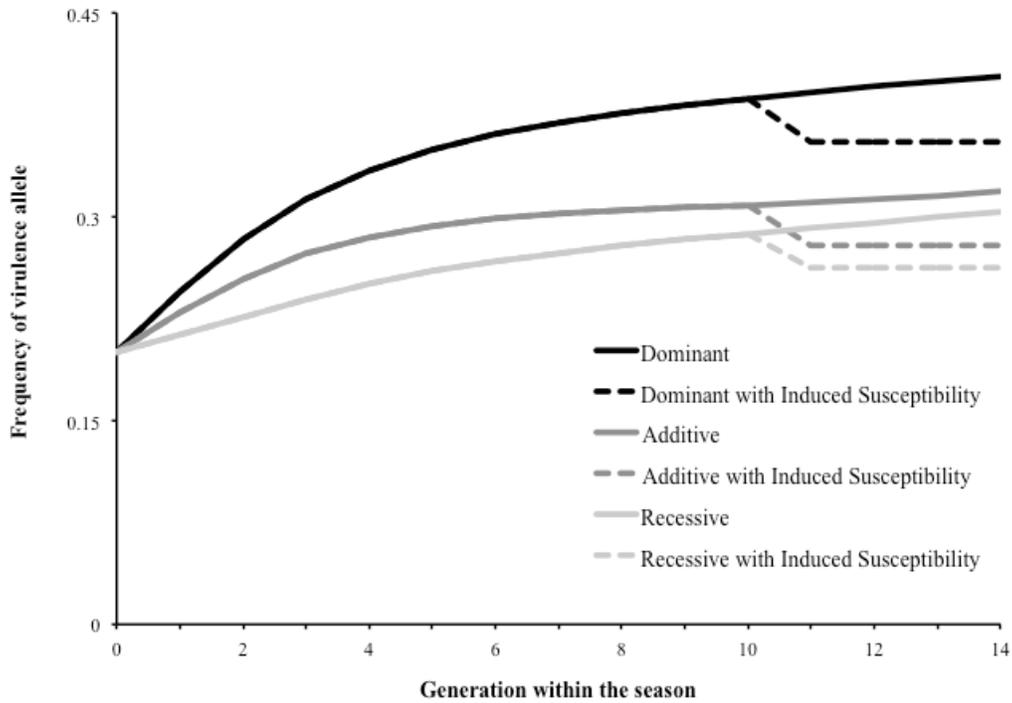
**Figure 1.** A comparison of the effect produced by varying densities of avirulent inducer populations on avirulent response populations on both susceptible (A) and resistant (B) soybean. For this experiment the susceptible soybean cultivar was IA3027 and the resistant cultivar was IA3027RA12. Capital letters indicate significance among treatments ( $P < 0.05$ ). Data were transformed for analysis. Plotted values represent data prior to log-transformation.



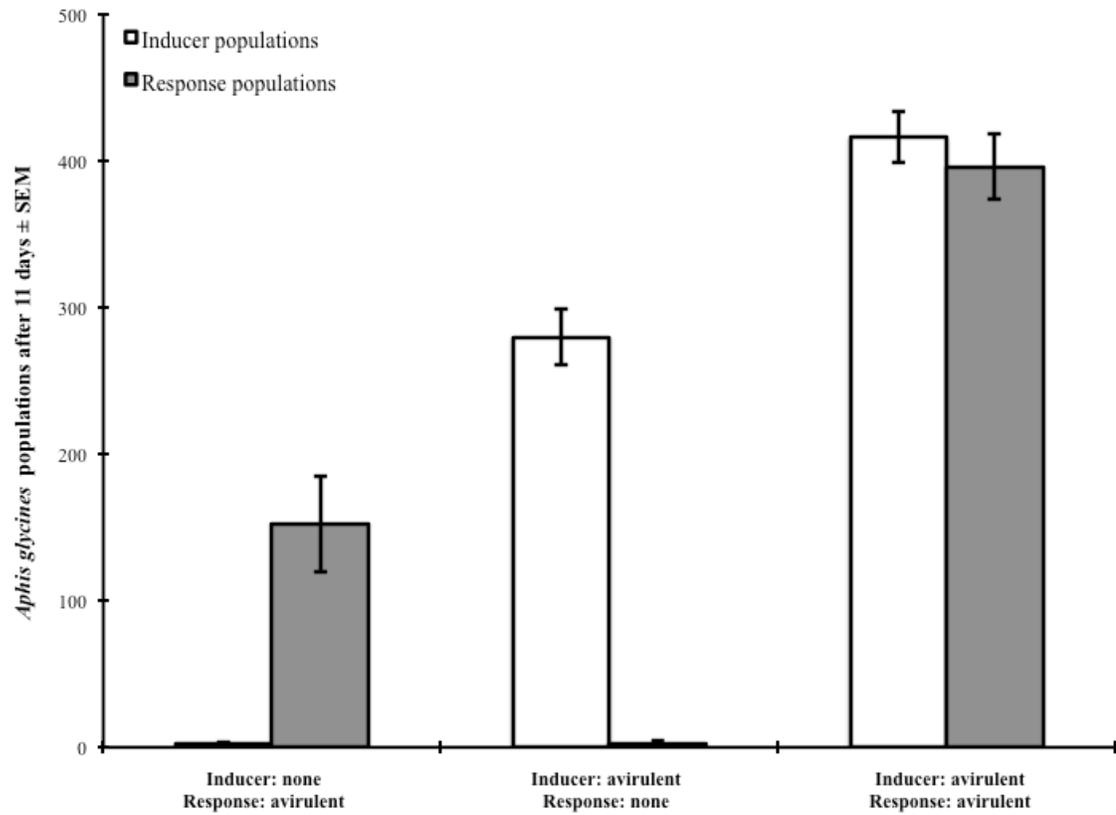
**Figure 2.** A comparison of the effects produced by varying inducer population phenotypes (avirulent or virulent) on varying response population phenotypes (avirulent or virulent). For this experiment the resistant soybean cultivar IA3027RA1 was used. Capital letters indicate significance among treatments ( $P < 0.05$ ). Data were log-transformed for analysis. Plotted values represent data prior to transformation.



**Figure 3.** A comparison of the effect produced by the presence of a virulent inducer population on an avirulent response population to avirulent and virulent response populations without inducer populations in a semi-field setting. For this experiment the resistant cultivar was IA3027R1. Capital letters indicate significance among treatments ( $P < 0.05$ ). Data were transformed for analysis. Plotted values represent data prior to transformation.



**Figure 4.** The frequency of an allele in an *Aphis glycines* population that confers virulence to a host-plant resistance gene (*Rag1*) was tracked throughout a single growing season. The initial allele frequency was assumed to be 0.2 and the rate of change was tracked assuming dominant, additive, or recessive inheritance of resistance. We compared the increase in virulence alleles for a model including induced susceptibility to the same model without induced susceptibility included.



**Supplemental Figure 1.** Containment confirmation for single trifoliolate mesh cages. For this experiment avirulent *A. glycines* and the susceptible soybean cultivar IA3027 were used. Data were transformed for analysis. Plotted values represent data prior to transformation.

**Chapter 3**  
**DETERMINING THE DURATION OF *APHIS GLYCINES***  
**(HEMIPTERA: APHIDIDAE) INDUCED SUSCEPTIBILITY**  
**EFFECTS IN SOYBEAN**

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**Abstract**

*Aphis glycines* feeding modifies soybean defense pathways and primary metabolism that favor the performance of *A. glycines*. The phenomenon whereby herbivory increases the suitability of the host plant for conspecifics is termed induced susceptibility. Induced susceptibility effects can be separated into feeding facilitation, whereby *A. glycines* feeding increases the performance of conspecifics regardless of the genotype of *A. glycines* and soybean, and obviation of resistance, where feeding of virulent *A. glycines* increases the performance of avirulent conspecifics on resistant plants. In natural and agricultural settings, aphid populations can colonize plants for brief periods before leaving or being removed due to emigration, predation, or insecticide application. However, induced susceptibility may last beyond the period when aphids are present on the plant. We tested the duration of induced susceptibility in the *A. glycines*-soybean system by measuring the duration of these effects in a growth chamber experiment. *Aphis glycines* resistant soybean were infested with an inducer population of either virulent, avirulent, or no aphids. Inducer populations were removed after 24 h and

response populations were added at three post-infestation times (24 h, 120 h, 216 h). Densities of all response populations were measured 11 d after infestation. Feeding facilitation was lost within 24 h of the removal of avirulent inducer populations. Obviation of resistance diminished over time and was completely lost within 216 h of the removal of the virulent inducer populations. We discuss how the legacy of induced susceptibility may impact the durability of *A. glycines* resistance in soybean.

**Keywords:** soybean aphid, induced susceptibility, feeding facilitation, obviation of resistance, legacy effects

### **Introduction**

Insect herbivores can directly or indirectly alter the suitability of a host plant for themselves and for subsequent herbivores (Karban and Myers 1989). Such alterations of host-plants can be categorized as either negative, such as induced resistance, or positive, such as induced susceptibility, for subsequent herbivores (Karban and Myers 1989, Price et al. 2011). These herbivore-induced effects in plants affect an herbivore's survival, fecundity or preference for the host plant (Karban and Baldwin 1997, Price et al. 2011). The duration of either induced resistance or susceptibility can vary, and are classified as being either short or long term effects, depending upon the plant-insect system studied (Karban and Myers 1989, Karban and Baldwin 1997). In general, short-term responses are elicited by and affect the initial herbivore, while long-term responses are elicited by an initial herbivore and affect subsequent herbivores (Karban and Myers 1989, Karban and Baldwin 1997). Some potential causes of induced effects include physical contact, chemical cues, plant viruses, insect endosymbionts, or insect proteins (Schoonhoven et al. 2005, Oliver et al. 2010, Casteel and Jander 2013, Pitino and Hogenhout 2013).

Takemoto et al. (2013) demonstrated that initial *Acyrtosiphum pisum* Harris (Hemiptera: Aphididae) feeding led to improved plant suitability for subsequent *A. pisum* populations. Similar results have been observed regarding *Myzus persicae* (Hemiptera: Aphididae) on *Prunus persica* (Rosales: Rosaceae) (Sauge et al. 2006). Karban and Baldwin (1997) report a total of six cases where aphids are recognized for inducing susceptibility of their host plants. Of these cases, all are reported as affecting the same generation of aphids, but there are also two for which the induced susceptibility effect also altered the plant for the next generation of aphids (Karbon and Baldwin 1997).

*Aphis glycines* is an invasive pest of soybean that can greatly reduce yield in North America (Ragsdale et al. 2011). Soon after the discovery of *A. glycines* in the US, soybean breeders discovered several genes that confer resistance to *A. glycines* (*Rag*-genes) in the soybean germplasm (reviewed in Ragsdale et al. 2011). When tested in the field, *Rag*-containing plants consistently have fewer *A. glycines* than aphid-susceptible lines (*i.e.*, no *Rag* genes) but the resistant lines are rarely free of aphids and sometimes can support large populations (Hesler et al. 2013) that exceed an economic threshold (McCarville et al. 2014). Despite the genetic bottleneck associated with *A. glycines* arrival in North America (Michel et al. 2011) and the limited commercial use of *Rag*-genes in North America (Hesler et al. 2013), several virulent biotypes have been found in the US (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013). These virulent biotypes are defined by the specific *Rag*-genes on which they can survive. To date, for every *Rag* gene that has been incorporated into a soybean line either alone or in a combination, a virulent biotype has been found in the US (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013).

Recently, we (Varenhorst et al. in press) observed that *A. glycines* feeding results in induced susceptibility for subsequent *A. glycines* on resistant soybean. This induced susceptibility was observed on both resistant and susceptible soybean and with virulent and avirulent aphids. The general phenomenon of induced susceptibility was observed and could be divided into two different mechanisms: feeding facilitation and obviation of resistance. Feeding facilitation was defined by significant increases in avirulent *A. glycines* populations on resistant soybean after initial herbivory by an inducer population of avirulent *A. glycines*, or by significant increases in virulent *A. glycines* populations on resistant soybean after initial herbivory by an inducer population of virulent *A. glycines*. Obviation of resistance was defined by significant increases in avirulent *A. glycines* populations on resistant soybean after initial herbivory by an inducer population of virulent *A. glycines*, to the extent that the performance of avirulent populations was equivalent with virulent populations (Varenhorst et al. in press). Wenger et al. (2014) observed an improvement in aphid fitness for both virulent and avirulent *A. glycines* on a shared host. They described this as an inter-biotype interactive effect that occurred when both biotype-1 (avirulent) and biotype 3 (virulent) were present on an aphid-susceptible and aphid-resistant (*i.e.*, *Rag2*) soybean. This effect is similar to obviation of resistance observed by Varenhorst et al. (in press), and provides further evidence for the ability of *A. glycines* biotypes to interact on a shared host.

Previous experiments were conducted with plants that were co-infested with both an inducer population and a subsequent response population. In natural and agricultural settings, aphid populations can colonize plants for brief periods before leaving due to emigration, predation, or insecticide application. It is not known if the impact of an

inducer population on the soybean plant will persist if it is removed from the plant. In this paper, we measured the duration of induced susceptibility using the methods developed by Varenhorst et al. (in press). We amended the design using both a virulent and avirulent biotype of *A. glycines* and a *Rag*-containing soybean line to explore the duration of both feeding facilitation and obviation of resistance.

### **Materials and Methods**

**Aphid colonies and soybean cultivars.** Two populations of *A. glycines* from The Ohio State University were used for this experiment. The populations are defined by their response to *Rag1*, an avirulent population (biotype-1) and a virulent population (biotype-2) (Kim et al. 2008). Individuals used to create these populations were initially collected and identified in Illinois (Kim et al. 2008). The avirulent population was raised on aphid-susceptible soybean (IA3027), while the virulent population was raised on a near-isogenic, aphid-resistant soybean containing the *Rag1* gene (IA3027RA1). These two cultivars are near isogenic, sharing approximately 93.25% genetic identity (Wiarda et al. 2012).

**Duration of induced susceptibility effects.** We hypothesized that both feeding facilitation and obviation of resistance would persist in soybean after the removal of the initial *A. glycines* populations (*i.e.*, a legacy effect). We measured the duration of these effects by infesting *Rag1* containing soybean (IA3027RA1) with an initial population of *A. glycines*, termed an inducer population, and allowing them to feed for a period of 24 h. After 24 h, the inducer population was removed using a fine tip paintbrush, and a subsequent population of *A. glycines*, termed response populations, was infested. The response populations were defined by the time between the removal of the inducer

population and their infestation (post infestation interval, or PII). The response population densities were measured 11 d after being added to plants, a time span that allows for the production of two generations of *A. glycines* (McCornack et al. 2004). Table 1 outlines the timing of these events. During the 11 d period alates of *A. glycines* were not observed.

To test our hypothesis we used nine treatments. Each treatment was a combination of two factors, inducer populations and response population infestation time. The three inducer-populations used were: no inducer (none), 50 avirulent *A. glycines* (avirulent), and 50 virulent *A. glycines* (virulent). Three response infestation times used: 24 h PII, 120 h PII, and 216 h PII. Inducer populations were applied to the first full trifoliolate of individual potted plants when the plants reached the second trifoliolate growth stage. Each individual potted plant was enclosed within a mesh net to prevent plant-to-plant movement of either the inducer or the subsequent response population. After 24 h the inducer populations were removed from all of the previously infested plants using a fine tip paintbrush. Varenhorst et al. (in press) determined that the maximum effect of induced susceptibility occurred with an inducer population of 50 *A. glycines*. Therefore, to determine the duration of induced susceptibility, inducer populations of 50 avirulent and 50 virulent *A. glycines* were used. Both inducer population and response population were compromised of *A. glycines* nymphs.

Infestations of the response population were applied at three intervals, defined by the time between the removal of the inducer population and the infestation of the response population. These treatments occurred at 24 h PII, 120 h PII, and 216 h PII. Response populations were added to the second full trifoliolate of each plant, and consisted

of five avirulent *A. glycines* that were allowed to move freely about the plant. The total number of *A. glycines* present in each response population was counted 11 d after the response population was infested. We measured both the presence and length of induced susceptibility by adding response populations at various times after the removal of the inducer population (Table 1).

Each experimental unit (*i.e.*, potted plant) was grown in 16-cm diameter pots in a Percival E41L2C9 growth chamber (Percival Scientific, Incorporated, Perry, IA) using a 14: 10 light: dark cycle and a constant temperature of 27 °C with a relative humidity of 60%. Each of the experimental units received one of the nine treatment combinations. This experiment was repeated twice in a growth chamber using a randomized complete block design with three blocks per repetition (six total experimental units per treatment).

**Statistical Analysis.** To address our *a priori* hypotheses, we analyzed the number of *A. glycines* per plant in the response population at 11 days after plants were infested with response populations. Data were analyzed separately for each PII time point. To reduce heteroscedasticity, the *A. glycines* per plant data were log transformed. All data were analyzed using the PROC MIXED procedure with SAS statistical software version 9.3 (SAS Institute, Cary, NC). The impact of each treatment factor was determined using an analysis of variance (ANOVA). The statistical model used to analyze data for each of the PII included the fixed effect of inducer population treatment. The random effects included repetition, inducer population treatment\*repetition, and block(repetition). We tested for the significance of all random effects using a log-likelihood ratio statistic (-2RES Log Likelihood). The log-likelihood statistic follows an approximate  $\chi^2$  distribution with one degree of freedom (Littell et al. 2002).

The duration of the induced susceptibility effects were determined by comparing the effect of the inducer population treatment factor on the response population abundance at each PII. If we determined that the effect was present, we next tested for whether it was the effect of feeding facilitation, obviation of resistance or both. We tested for these effects using contrast statements within PROC MIXED using the same model as previously described with a significance level of  $P < 0.05$ . We compared the avirulent inducer population to the no inducer control to test for feeding facilitation. We tested for obviation of resistance by comparing the virulent inducer population to the avirulent inducer population.

## Results

**Duration of induced susceptibility.** We confirmed our hypothesis that induced susceptibility (*i.e.*, feeding facilitation and obviation of resistance) persist in soybean after the removal of the initial *A. glycines* populations. This was observed by analyzing data for the significance of the fixed effect of inducer population for each of the PII levels. The inducer population treatment significantly affected the response populations at 24 h PII and 120 h PII, but not at 216 h PII (Table 2).

Because the inducer population significantly affected the response populations at the 24 h PII and 120 h PII, we compared the impact of the various inducer population treatments at each PII. The response population on plants receiving an avirulent inducer population (*i.e.*, avirulent treatment) was significantly greater than on plants that did not have an inducer population (*i.e.*, none treatment) at 24 h PII ( $F = 128.63$ ;  $df = 1, 2$ ;  $P < 0.0077$ ) (Fig. 1; 24 h PII), but not at 120 h PII or 216 h PII. The response population for the virulent treatment was significantly greater than that of the response population for

the avirulent treatment at 24 h PII ( $F = 843.04$ ;  $df = 1, 2$ ;  $P < 0.0012$ ) (Fig. 1; 24 h PII) and 120 h PII ( $F = 42.92$ ;  $df = 1, 2$ ;  $P < 0.0225$ ) (Fig. 1; 120 h PII). At 216 h PII there was no significant differences among the treatments. Therefore, induced susceptibility effects were observed for the avirulent treatment at 24 h PII, and for the virulent treatment at 24 h PII and 120 h PII.

### Discussion

Our results demonstrate that *A. glycines* feeding alters resistant soybean such that it is more susceptible to future infestations of conspecifics. The length of time this effect lasts after the inducer population of *A. glycines* is removed varies by aphid phenotype (*i.e.*, virulence). Increases in *A. glycines* populations due to an inducer population (*i.e.*, induced susceptibility) occurred in two ways, by feeding facilitation (Price et al. 2011) or obviation or resistance (Baluch et al. 2012). Feeding facilitation was observed when the response population increased when the plant received an avirulent inducer treatment compared to the none inducer treatment. Feeding facilitation was only observed at 24 h PII. Obviation of resistance was observed when the response population increased on the virulent inducer treatment compared to the avirulent inducer treatment, which occurred at 24 h PII and at 120 h PII but with diminished impact. There was no evidence of either feeding facilitation or obviation of resistance at 216 h PII for any of the treatments. Therefore, we conclude that in the absence of the inducer population the effect of feeding facilitation persists for 24 h and the effect of obviation resistance persists for at least 120 h.

An indirect effect that persists for an extended period of time in the absence of the causal species (*i.e.*, inducer) of the effect, or when the activity of the causal species has

ceased has been described as a legacy effect (Cuddington 2011). While both feeding facilitation and obviation of resistance produced short-term effects in the absence of the inducer population, the effect of feeding facilitation did not persist beyond 120 h and therefore should not be considered a legacy effect. The obviation of resistance by the virulent *A. glycines* did not persist beyond 216 h but did produce an indirect effect that persisted after the removal of the inducer population. Based on these results we conclude that obviation of resistance produces a legacy effect.

Based on the duration of the obviation of resistance we hypothesize a mechanism responsible for this effect. There are several factors that can explain how the physiology of the plant was altered by *A. glycines*, including endosymbionts (Oliver et al. 2010), viruses (Mauck et al. 2012, Casteel et al. 2013), and effector proteins found in salivary excretions (Rodriguez and Bos 2013). These factors may help explain how an avirulent aphid could survive on a resistant plant that is co-infested with a virulent biotype. For example, virulence could be delivered by endosymbionts (Oliver et al. 2010). We did not observe evidence of the horizontal transmission of endosymbionts between the virulent and avirulent populations. Endosymbiotic bacteria are unlikely to be the cause of obviation of resistance because horizontal transmission of bacteria is rare. Also our inducer and response populations were temporally and spatially separated on the soybean plant making horizontal transmission even less probable (Oliver et al. 2010).

In a review, Mauck et al. (2012) describe the potential for plant viruses to enhance vector attraction to a host plant, and then also affect vector settling and feeding preferences. In contrast to non-persistently transmitted viruses, persistently transmitted plant viruses have the potential to make the host plant more suitable, and promote long

term feeding. The persistently transmitted viruses generally are acquired through extended feeding bouts and benefit from vector settling. Plant virus infection is unlikely to be the cause of the observed obviation of resistance as the only persistently transmitted soybean virus in North America is *Soybean dwarf virus* (Hartman et al. 1999), which is rarely vectored by *A. glycines* (Harrison et al. 2005, Wang et al. 2006, Damsteegt et al. 2011). Additional evidence that a plant virus is unlikely responsible for the obviation of resistance is the reduction in the response populations at 120 h, and 216 h for the virulent inducer population treatment. This observation is not consistent with results from other studies analyzing the effects of plant virus infection on aphid populations where the virus infection improved aphid populations for up to one week post-infection (Casteel et al. 2014). Due to the asymptomatic nature of our plants, and the reduction of the effect over time we conclude that a plant virus was not the cause of the obviation of resistance.

We suggest that effector proteins are the most probable explanation of obviation of resistance. Previous research has indicated that aphid effector proteins are capable of suppressing host plant defense pathways and modulating a range of host cell processes (Hogenhout and Bos 2011, Pitino and Hogenhout 2013, Rodriguez and Bos 2013). Pitino and Hogenhout (2013) demonstrated that the impact of aphid effector proteins vary by aphid species. On *Arabidopsis* (Brassicaceae), homologs of effector proteins from *Acyrtosiphum pisum*, a specialist of plants in the Fabaceae family, did not improve reproduction of *Myzus persicae*, a generalist capable of utilizing plants from multiple families. In contrast, expression of *M. persicae* effector homologs did result in increased *M. persicae* reproduction. Both enzymes and binding proteins are present in the saliva of aphids and are potential explanations for how aphids influence the host plant's defense

response to herbivory (Will et al. 2007, Harmel et al. 2008, Hogenhout and Bos 2011). In addition to explaining speciation, effector proteins may also help explain variation within an aphid species in the form of virulence to aphid-resistant traits (Rodriguez and Bos 2013). Our hypothesis is further validated by Bansal et al. (2014)'s discovery of 47 effector protein transcripts present in *A. glycines* that matched effectors present in *A. pisum* with known functions.

The short duration and apparent degradation of the effect between 24 h PII and 120 h PII for obviation of resistance further support the role of effector proteins in this aphid-plant system. The decline of obviation of resistance that was observed over time in our experiment may be attributable to the degradation of an aphid enzyme or protein present in the host-plant. Therefore, we hypothesize that the effect of obviation of resistance is strongest when the inducer and response populations are present on the plant simultaneously, but the effect persists until the effector proteins are degraded. This is likely a function of the density of the aphids that are injecting effector proteins and the capacity of the plant to recognize and/or degrade them.

The results from this paper provide a framework for future research on the mechanism of *A. glycines* virulence. Future work should investigate effector protein candidates, and determine the mechanism of these effector proteins as potential targets for novel pest control technologies. We predict that if effector proteins are the cause of the biotypic variation in virulence towards *Rag* genes, then variation within the effector proteins among these biotypes should also be present. This variation may not only be responsible for the virulence of a biotype towards a resistance gene, but may also affect

the duration of the obviation of resistance effect (*i.e.*, the legacy of effector proteins may differ by biotype).

### Acknowledgements

This study was funded in part by the Soybean Checkoff through a grant from the North Central Soybean Research Program. We thank Andy Michel for the initial *A. glycines* populations, and also for comments on earlier versions of this manuscript.

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**Table 1. Sequence of events for legacy effect experiment**

Event	24 h	120 h	216 h
Planting	Day 1	Day 1	Day 1
Infestation of inducer <sup>a</sup>	Day 17	Day 17	Day 17
Removal of inducer	Day 18	Day 18	Day 18
Infestation of response <sup>b</sup>	Day 18	Day 22	Day 26
Counting of response	Day 29	Day 33	Day 37

<sup>a</sup> Inducer populations consisted of 50 avirulent, 50 virulent, or no *A. glycines*.

<sup>b</sup> Response populations consisted of 5 avirulent *A. glycines*.

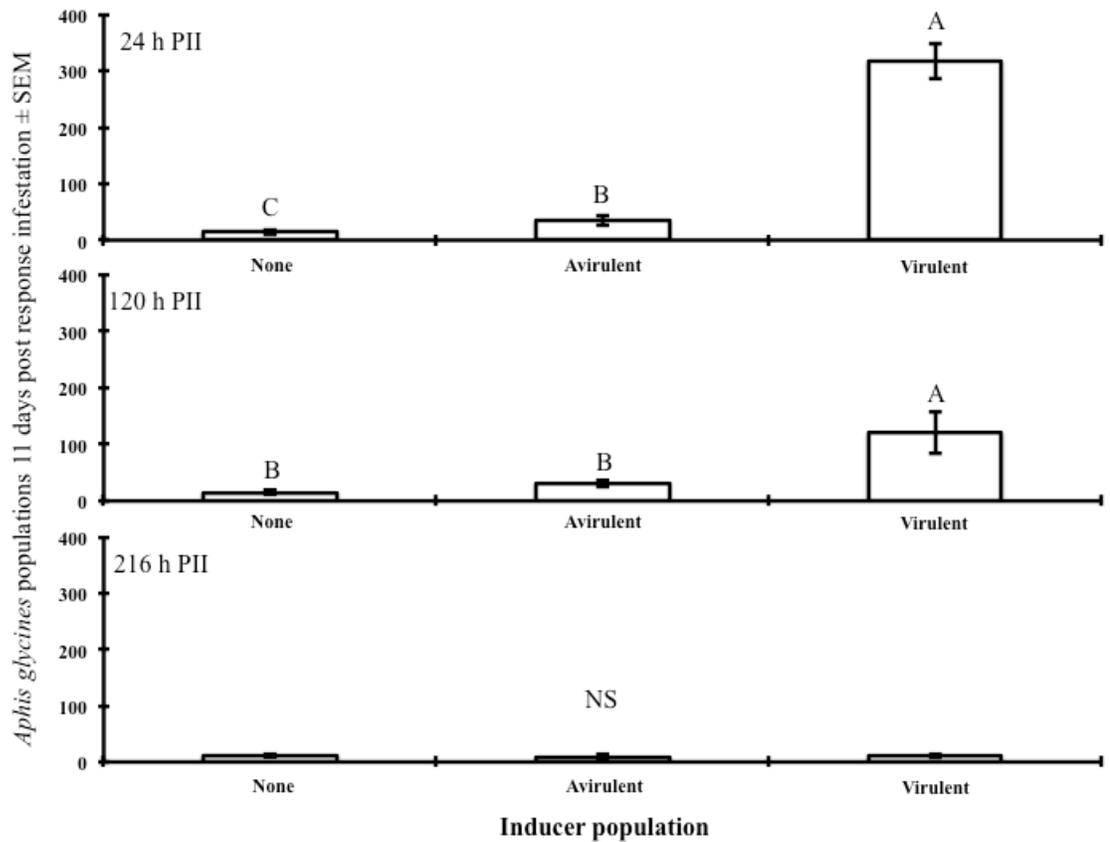
**Table 2. Analysis of variance tables of treatment effects.**

Effect	Fixed/Random	df	F statistic/ $\chi^2$ <sup>a</sup>
24 h PII <sup>b</sup>			
repetition	R	1	0.20
block(repetition)	R	1	1.00
inducer population	F	2, 2	867.31**
repetition*inducer population	R	1	4.30*
120 h PII			
repetition	R	1	0.40
block(repetition)	R	1	5.10*
inducer population	F	2, 2	50.65*
repetition*inducer population	R	1	1.20
216 h PII			
repetition	R	1	0.20
block(repetition)	R	1	0.00
inducer population	F	2, 2	4.83
repetition*inducer population	R	1	0.50

\*Significant effect at  $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$

<sup>a</sup> An F statistic was used to test for the significance of fixed effects, while a  $\chi^2$  test was used for random effects.

<sup>b</sup> Post-inducer population infestation.



**Figure 1.** The duration of induced susceptibility effects was measured in a growth chamber experiment. Aphid-resistant plants were infested with an inducer population of either virulent, avirulent, or no aphids for 24 h and then removed from plants. Response populations were then added at three post-inducer infestation times (24 h PII, 120 h PII, and 216 h PII). Capital letters indicate significance among treatments ( $P < 0.05$ ).

**Chapter 4**  
**EXPLORING FACTORS THAT MAY INFLUENCE THE**  
**LONGEVITY OF *APHIS GLYCINES* (HEMIPTERA: APHIDIDAE)**  
**RESISTANCE GENES**

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**Abstract**

To date four virulent biotypes of *Aphis glycines* have been found in North America with laboratory bioassays. These biotypes are defined by their capacity to survive on aphid-resistant cultivars of soybeans (e.g., biotype-2 survives on *Rag1* soybean). Although fitness costs have been reported for biotype-3 on aphid susceptible and *Rag1* soybean, it is not clear if the general phenomenon of virulence to aphid resistance is associated with a decrease in fitness on aphid susceptible cultivars. We determined if there are fitness costs for all currently identified virulent biotypes found in North America on susceptible soybean. Our experiments included avirulent *A. glycines* (biotype-1) and also virulent biotype-2, biotype-3, and biotype-4. Our results indicate that fitness costs exist for biotype-2, biotype-3, and biotype-4 on an aphid-susceptible soybean cultivar. In addition, we also observed negative cross-resistance for biotype-2 on *Rag3*, and biotype-3 on *Rag1* soybean. We also determined that biotype-1 is capable of obviating the fitness costs experienced by biotype-2 and biotype-3 on the aphid susceptible cultivar. These results are the first to document fitness costs for biotype-2 and

biotype-4 on susceptible soybean, and also the first to document negative cross-resistance of virulent biotypes toward *Rag* genes that they are not virulent towards. Future insect resistance management models for *A. glycines* should incorporate fitness costs, negative cross-resistance, and also biotype-1 obviation of fitness costs to determine the relative durability of the currently employed *Rag* genes.

**Keywords:** soybean aphid, host plant resistance, *Rag1*, *Rag2*, *Rag1+Rag2*, fitness costs, negative cross-resistance, virulence, biotypes

### Introduction

In 2000, *Aphis glycines* Matsumura was first observed in the US. Prior to 2000, insecticide use in north central US soybean was infrequent (NASS/USDA 1999); however, after the establishment of *A. glycines* insecticide use on soybean in north central US dramatically increased (NASS/USDA 2005). The reason for the increase in insecticide use is attributed to soybean yield reductions of up to 40% caused by *A. glycines* feeding (Ragsdale et al. 2011). Insecticides are effective at reducing *A. glycines* populations and preventing associated yield loss while also being cost effective (Johnson et al. 2009, Ragsdale et al. 2011). The insecticides commonly used to manage *A. glycines* populations are broad-spectrum and reduce populations of natural enemies present in soybean during application (Olson et al. 2008, Ohnesorg et al. 2009, Varenhorst and O'Neal 2012). However, concerns for the future development of insecticide-resistant *A. glycines* populations if insecticides are consistently used suggest a need for additional management tools. An alternative management strategy that is potentially more cost effective with negligible effects on natural enemies is soybean that contain one or more resistant to *A. glycines* genes (*Rag* genes) (Ragsdale et al. 2011,

Hesler et al. 2013, McCarville et al. 2014). Although there is evidence that *Rag* genes are effective, a limited number of varieties containing *Rag1*, *Rag2*, or *Rag1+Rag2* are commercially available and their adoption has been limited (McCarville et al. 2012, Hesler et al. 2013, McCarville et al. 2014).

Initially the low adoption of *Rag* soybean was attributed to the concern for reduced yields associated with soybean varieties containing *Rag* genes. However, no yield drag is associated with the presence of *Rag1*, *Rag2*, or both genes (Kim and Diers 2009, Mardorf et al. 2010, Brace and Fehr 2012, Kim and Diers 2013). A second factor limiting the production and adoption of *A. glycines*- resistant soybean is the discovery of multiple virulent (*i.e.*, able to feed on soybean containing aphid-resistance genes) *A. glycines* biotypes (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013). To date four biotypes have been confirmed in North America (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013). Biotype-1 is avirulent to all of the resistance to *Rag* genes currently known. Biotype-2 is virulent towards the *Rag1* gene (Kim et al. 2008). Biotype-3 is virulent towards the *Rag2* gene (Hill et al. 2010), and biotype-4 is virulent towards both the *Rag1* and *Rag2* genes, as well as pyramids constructed from *Rag1* and *Rag2* (Alt and Ryan-Mahmutagic 2013).

A majority of the research conducted on *A. glycines* and *Rag* genes has been focused on the discovery of virulent biotypes (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013), identifying new *Rag* genes (Hesler et al. 2013), and the efficacy of *Rag* genes towards biotype-1 (McCarville et al. 2012). Less research has focused on the relative importance of these biotypes for the continued use of *Rag* genes, specifically the relative fitness of virulent *A. glycines* biotypes. Only two studies have

evaluated the fitness of a single virulent biotype, biotype-3. Wenger et al. (2014) evaluated the fitness costs associated with virulence to *Rag2* (*i.e.*, biotype-3's fitness on susceptible soybean), while Enders et al. (2014) examined biotype-3 for any negative cross-resistance to *Rag1*. Wenger et al. (2014) observed fitness costs for biotype-3 on susceptible soybean, and also concluded that virulence is not complete. Enders et al. (2014) observed negative cross-resistance for biotype-3 on *Rag1* soybean.

Gassmann et al. (2009) define fitness costs as trade-offs in which alleles that confer higher fitness in one environment (*e.g.*, on *Rag* soybean) reduce fitness in an alternative environment (*e.g.*, on an aphid-susceptible soybean). Fitness costs in a virulent population can result in a reduction of the frequency of virulent alleles when refuges of susceptible plants are present (Gassmann et al. 2009). In addition to fitness costs, negative cross-resistance can also reduce the frequency of virulence in a population. Negative cross-resistance occurs when the allele(s) that confer virulence to one resistance gene also confer hypersensitivity to another resistance gene (Pittendrigh et al. 2008). An alternative to negative cross-resistance is negatively correlated resistance, which occurs when the gene responsible for virulence to one source of resistance is not the same gene that is responsible for hypersensitivity to another source of resistance (Pittendrigh et al. 2008). Crowder and Carrière (2009) determined that fitness costs for parthenogenic insects would only be effective for a short period of time (*i.e.*, 20 generations) if they were experienced on both crop and refuge. However, it is possible that the presence of these factors in virulent *A. glycines* biotypes could affect the rate at which virulence alleles increase in the environment, and reduce their perceived importance as a hindrance to the successful and sustainable adoption of *A. glycines*-resistant soybean varieties.

The objective of this study was to determine if fitness costs or negative cross-resistance are associated with virulence to *Rag1* or *Rag2* for *A. glycines* biotype-1, biotype-2, biotype-3, and biotype-4 populations on near-isogenic resistant and susceptible soybean cultivars. In addition to this evaluation, we sought to determine if *A. glycines* biotype-1 could obviate any fitness cost associated with virulence to *Rag1* or *Rag2* (Varenhorst et al. in press). Finally we used a deterministic genetic model to predict the relative frequency of virulent *A. glycines* in light of fitness costs and negative cross-resistance observed herein.

## **Materials and Methods**

### **Aphid colonies and soybean cultivars**

*Aphis glycines* populations used for this experiment were obtained from The Ohio State University and the University of Wisconsin. Four populations that were defined by their response to *Rag1* and *Rag2* genes were utilized. A biotype avirulent to *Rag1* and *Rag2* (biotype-1; The Ohio State University), a biotype virulent to *Rag1* but not *Rag2* (biotype-2; The Ohio State University), a biotype virulent towards *Rag2* but not *Rag1* (biotype-3; The Ohio State University), and a biotype virulent towards *Rag1* and *Rag2* (biotype-4; University of Wisconsin) (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013). These populations were initially collected and identified in Ohio and Wisconsin using detached leaf assays described by Michel et al. (2010). Biotype-1 *A. glycines* were reared and maintained on susceptible soybean, biotype-2 *A. glycines* reared and maintained on *Rag1* soybean, biotype-3 *A. glycines* reared and maintained on *Rag2* soybean, and biotype-4 *A. glycines* reared and maintained on *Rag1+Rag2* soybean. The cultivars used for rearing and maintaining the aphids are near-isogenic ( $\geq 75\%$  of

genes from the recurrent parent IA3027). The soybean plants used containing no *Rag* genes (IA3027), *Rag1* (IA3027RA1), or *Rag1+Rag2* (IA3027RA12) are near-isolines for the resistance genes *Rag1* and *Rag2* (approximately 93.25% genetically identical). (Mardorf et al. 2010, Brace and Fehr 2012). The near-isogenic line containing only the *Rag2* gene is an experimental soybean line with 75% of its genes derived from the recurrent parent line IA3027 (Wiarda et al. 2012).

### **Fitness costs associated with virulence of biotype-2 and biotype-3 on susceptible soybean**

We hypothesized that the fitness of biotype-2 and biotype-3 *A. glycines* would be lower on susceptible soybean, when compared to biotype-1 (*i.e.*, fitness costs would be associated with virulence to *Rag1* or *Rag2*). We estimated the fitness of each biotype on *Rag1*, *Rag2*, and susceptible soybean by infesting individual plants with five *A. glycines* of either biotype-1, biotype-2, or biotype-3, and measuring the population density of *A. glycines* present on the plant 11 d after infestation. The population densities were compared to determine if fitness varied with virulence. Fitness costs were identified if the population densities of the virulent biotype-2 and biotype-3 *A. glycines* were significantly lower than that of biotype-1. In addition, negative cross resistance was observed if the population density of a virulent biotype (*i.e.*, biotype-3) was significantly lower than that of biotype-1 on soybean containing a resistance gene that it is not virulent towards (*i.e.*, *Rag1*). In total, we used nine treatments to test our hypothesis in a growth chamber using individually potted plants. Each treatment was a combination of two factors, soybean cultivar (3 levels) and *A. glycines* biotype (3 levels).

Five adult *A. glycines* were transferred from colonies maintained at Iowa State University to the first full trifoliolate of individually potted plants at the second trifoliolate growth stage (V2 according to Fehr et al. 1971). Plants were enclosed within mesh nets to prevent plant-to-plant movement of aphid populations. After 24 h we examined *A. glycines* populations to confirm their successful establishment. The total number of *A. glycines* (both nymphs and adults) present on each plant was counted 11 d after initial infestation.

This experiment was repeated twice using a randomized complete block design (RCBD) with five blocks per repetition (10 total experimental units per treatment). Individually potted soybean plants were grown in 16-cm diameter pots in a Percival E41L2C9 growth chamber (Percival Scientific, Incorporated, Perry, IA) using a 14:10 light:dark cycle and a constant temperature of 27° C with a relative humidity of 60%.

#### **Fitness cost associated with virulence of biotype-4 on susceptible soybean**

Biotype-4 was not included in the previous experiment because a colony had not been established at the onset of that experiment. We next hypothesized that *A. glycines* biotype-4 fitness would be lower on susceptible soybean when compared to biotype-1 (*i.e.*, fitness costs would be present). We tested for fitness costs using the same experimental procedures as described previously. Plants were infested with biotype-1, or biotype-4, and the population density was measured 11 d after initial infestation.

We utilized a two factor experimental design, with eight total treatments. Each treatment was a combination of four soybean cultivars and two *A. glycines* biotypes. Three of the soybean cultivars used were the same as described in the previous experiment, with the addition of a resistant cultivar containing *Rag1+Rag2*

(IA3027RA12). Each cultivar was infested with five biotype-1 or five biotype-4 *A. glycines*. The fitness of these two biotypes on each soybean cultivar was measured in population density and compared to determine the presence of fitness costs. The methods for infesting *A. glycines* populations from the previous experiment were used. The same planting procedure and growth chamber specifications as the previous experiment were used. This experiment was repeated twice using a RCBD with five blocks within each repetition (10 total experimental units per treatment).

### **Impact of induced susceptibility on fitness costs of biotype-2 and biotype-3 on susceptible soybean**

Varenhorst et al. (in press) demonstrated that feeding by biotype-2 aphids improves the suitability of soybean containing the *Rag1* resistance gene for avirulent biotype-1 *A. glycines* (*i.e.*, obviating of resistance). Our third hypothesis was that the herbivory by biotype-1 *A. glycines* would improve the quality of susceptible soybean for biotype-2 and biotype-3. We tested this hypothesis using the same experimental design as outlined by Varenhorst et al. (in press). This design involved infesting soybean plants with an initial inducer population for 24 h prior to infesting plants with a second response population. The population density of the response population 11 d after infestation is used as a measurement of the effect of the inducer population. The effect of the inducer population on the performance of the response population can then be assessed in comparison to plants receiving a response population but not an initial inducer population. If response populations are greater in the presence of a biotype-1 inducer population than in their absence then induced susceptibility alleviates fitness costs.

For this experiment, we used a three factor experimental design with ten total treatments. The three factors included plant cultivar, inducer population biotype, and response population biotype. We utilized three soybean cultivars: susceptible, *Rag1*, and *Rag2*. Four inducer populations were used: no inducer (none), 50 biotype-1 *A. glycines* (B1), 50 biotype-2 *A. glycines* (B2), or 50 biotype-3 *A. glycines* (B3). Three response populations were used: five biotype-1, five biotype-2, or five biotype-3 *A. glycines*. Inducer populations were applied to the first full trifoliolate when the plants reached the second trifoliolate growth stage. The entire first trifoliolate was then enclosed within a mesh net for the duration of the experiment. After 24 h, the response populations were added to the second full trifoliolate and allowed to move freely about the plant, with the exception of the first trifoliolate, which was enclosed in a net and contained the inducer population. Varenhorst et al. (in press) confirmed the effectiveness of the mesh nets for separating the inducer and response populations. Individually potted plants were enclosed within mesh nets to prevent plant-to-plant movement. Response populations were examined after 24 h to confirm successful establishment. The total number of *A. glycines* present in the response population on each plant was counted 11 d after the initial infestation. The experiment was repeated twice using a RCBD with three blocks per repetition (six total experimental units per treatment). The same planting procedure and growth chamber specifications as the previous experiments were used.

### **Statistical analysis**

To address each hypothesis, we analyzed the number of *A. glycines* per plant after 11 d. To reduce heteroscedasticity the *A. glycines* per plant data were log transformed. All data for the first two experiments were analyzed using the PROC MIXED procedure with

SAS statistical software version 9.3 (SAS Institute, Cary, NC). For both experiments, data were analyzed using an analysis of variance (ANOVA). Significant treatment effects were then separated using F-protected least-squares means with a significance level of  $P < 0.05$ .

The statistical model used to analyze data from the first two experiments (*i.e.*, fitness costs of biotype-2 and biotype-3, and fitness costs of biotype-4) included the main effects of repetition, block, soybean cultivar, and *A. glycines* biotype. All two and three-way interactions of the main effects were included in the model.

All data from the third experiment (*i.e.*, biotype-1 obviation of fitness costs on susceptible soybean) were analyzed using the PROC GLM procedure. Data were analyzed using an analysis of variance (ANOVA) with significant treatment effects separated using Student-Newman-Keuls (SNK) grouping with a significance level of  $P < 0.05$ . The statistical model included the main effects of repetition, block, inducer population biotype, and response population biotype. All two- and three-way interaction terms between the main effects were included in the model.

### **Modeling the consequences of fitness costs and negative cross-resistance**

We hypothesized that the fitness cost and negative cross-resistance effects we observed would affect the rate at which virulence alleles increased within *A. glycines* populations. We utilized a simple deterministic, single-locus, four compartment genetic model developed for *A. glycines* to track changes in virulence alleles (Varenhorst et al. in press). The model was adapted from one created for parthenogenic reproducing insects similar to *A. glycines* (Crowder and Carrière 2009). We tracked the change in the frequency of virulence alleles across 25 years with 14 generations of asexual

reproduction and one generation of sexual reproduction occurring within each year. We assumed virulence to the *Rag1* and *Rag2* genes to be conferred by two independently segregating genes. Each virulence gene was assumed to have two alleles, with one allele conferring virulence and another conferring avirulence. Mating was assumed to be completely random with alleles returning to Hardy-Weinberg equilibrium after each year's generation of sexual reproduction. We tracked the frequency of virulence alleles to *Rag1* for 25 years beginning at the initial deployment of resistant cultivars. We report the number of years for the frequency of the *Rag1* virulence allele to surpass 50% in the population. We report the allele frequency after 25 years for the *Rag1* virulence allele in cases where the frequency fails to surpass 50% in 25 years.

Our goal was to evaluate the relative potential importance of fitness costs and negative cross-resistance for the development of virulence, not to evaluate all possible scenarios for the development of virulence to *Rag* genes. Therefore, we assessed a small proportion of possible scenarios for the development of virulence in *A. glycines*. We used values from empirical data for specific parameters in the *A. glycines*-soybean system whenever possible, including the field-to-field movement rate of *A. glycines* (5% static rate, Donaldson et al. 2007), the efficacy of the *Rag1* and *Rag2* genes (41% reduction in *A. glycines* fitness, McCarville and O'Neal 2012), the efficacy of a *Rag1*+*Rag2* pyramid (59% reduction in *A. glycines* fitness, McCarville and O'Neal 2012).

Fitness costs and negative cross-resistance were included as reductions in the fitness of virulent individuals on susceptible and resistant plants, respectively. The exact values used for the reduction in fitness due to fitness costs and negative cross-resistance were based on the results of experiments one and two. We included induced susceptibility

effects in all of our models (Varenhorst et al. in press) as these can affect the rate at which virulence alleles increase in the population. The inclusion of a density-dependent increase in fitness was used to model induced susceptibility. A starting population density of  $1.0 \times 10^2$  individuals per 1% of the landscape was used for each year of the model. Fitness of individuals in a compartment increased to 1.65 if the population density surpassed  $1.0 \times 10^{13}$  individuals per 1% of landscape. Obviation of resistance was modeled by setting the fitness of all individuals in a resistant compartment to 1.0 when the population density of homozygous virulent individuals surpassed  $1.0 \times 10^{13}$  per 1% of landscape. Obviation of fitness costs was modeled using the same method as obviation of resistance, except the obviation of fitness costs was based on the population density of homozygous avirulent individuals in the susceptible compartment of the landscape.

We ran the model for a range of values for unknown parameters, including the dominance of virulence (recessive, additive, and dominant), and initial virulence allele frequency (0.02 and 0.2, Michel et al. 2011). We assumed a single static 25% refuge size of susceptible plants in the landscape for each run of the model. We investigated two resistance gene deployment strategies by partitioning the remaining 75% of the landscape to either plants containing only the *Rag1* gene or plants containing the *Rag1+Rag2* genes. In total, we ran the model 24 times once with fitness costs and negative cross-resistance included and once without for each combination of dominance of virulence (three levels), initial allele frequency (two levels), and gene deployment strategy (two levels).

## Results

### **Fitness costs associated with virulence of biotype-2 and biotype-3 on susceptible soybean**

We confirmed our hypothesis that the population densities of *A. glycines* biotype-2 and biotype-3 would be lower on susceptible soybean, when compared to biotype-1 (*i.e.*, that fitness costs for these biotypes exist). This was observed by analyzing data for the significance of the main effects of soybean cultivar, *A. glycines* biotype, and the interaction of soybean cultivar by *A. glycines* biotype. The population densities of the biotypes of *A. glycines* varied significantly by the main effects of soybean cultivar ( $F = 14.41$ ;  $df = 2, 76$ ;  $P < 0.0001$ ), *A. glycines* biotype ( $F = 5.83$ ;  $df = 2, 76$ ;  $P < 0.0044$ ), and their interaction ( $F = 58.00$ ;  $df = 4, 76$ ;  $P < 0.0001$ ). We observed differences in the population density of the biotypes that were not equal among the soybean cultivars. Therefore, we analyzed these data by soybean cultivar.

On susceptible soybean, the population density of *A. glycines* biotype-1 was significantly greater than those of biotype-2 ( $t = 4.13$ ;  $df = 2, 22$ ;  $P < 0.0004$ ) or biotype-3 ( $t = 6.80$ ;  $df = 2, 22$ ;  $P < 0.001$ ) (Fig. 1). The population density of biotype-2 was also significantly greater than that of biotype-3 ( $t = 2.68$ ;  $df = 2, 22$ ;  $P < 0.0138$ ) on susceptible soybean (Fig. 1). For the *Rag1* soybean cultivar, the population density of biotype-2 was significantly greater than those for biotype-1 ( $t = 6.72$ ;  $df = 2, 22$ ;  $P < 0.0001$ ) or biotype-3 ( $t = 11.76$ ;  $df = 2, 22$ ;  $P < 0.0001$ ) (Fig. 1). The population density of biotype-1 was also significantly greater than that of biotype-3 ( $t = 5.04$ ;  $df = 2, 22$ ;  $P < 0.0001$ ) on *Rag1* soybean. On *Rag2* soybean, the population density of biotype-3 was significantly greater than those of biotype-1 ( $t = 5.15$ ;  $df = 2, 22$ ;  $P < 0.0001$ ) or biotype-2 ( $t = 8.68$ ;  $df = 2, 22$ ;  $P < 0.0001$ ). The population density of biotype-1 was also significantly greater than that of biotype-2 ( $t = 3.53$ ;  $df = 2, 22$ ;  $P < 0.0019$ ) on *Rag2* soybean (Fig. 1).

These results indicate that for the susceptible soybean cultivar that was tested, the population densities of both biotype-2 and biotype-3 were lower than those of biotype-1. In addition, these results indicate that population densities of biotype-2 and biotype-3 were lower on *Rag2* and *Rag1* respectively when compared to biotype-1.

#### **Fitness cost associated with virulence of biotype-4 on susceptible soybean**

In our second experiment, we confirmed our hypothesis that the population density of *A. glycines* biotype-4 would be lower on susceptible soybean, when compared to biotype-1 (*i.e.*, that fitness costs for this biotype exist on susceptible soybean) (Fig. 2). As in the first experiment, data were analyzed for the significance of the main effects of soybean cultivar, *A. glycines* biotype, and the interaction of soybean cultivar by *A. glycines* biotype. The population densities of the biotypes of *A. glycines* varied significantly by the main effects of soybean cultivar ( $F = 50.31$ ;  $df = 3, 67$ ;  $P < 0.0001$ ) and *A. glycines* biotype ( $F = 138.00$ ;  $df = 1, 67$ ;  $P < 0.0001$ ). Similar to the previous experiment we observed reductions in the populations of the *A. glycines* biotypes that were not equal among the soybean cultivars. The interaction soybean cultivar by *A. glycines* biotype was significant ( $F = 98.36$ ;  $df = 3, 67$ ;  $P < 0.0001$ ), and indicated that the *A. glycines* biotypes did not respond similarly to the tested soybean cultivars. Therefore, data were analyzed by soybean cultivar.

On susceptible soybean, the population density of *A. glycines* biotype-1 was significantly greater than that of biotype-4 ( $t = 6.91$ ;  $df = 1, 13$ ;  $P < 0.0001$ ) (Fig. 2). For the *Rag1* ( $t = 4.19$ ;  $df = 1, 13$ ;  $P < 0.0011$ ), *Rag2* ( $t = 9.29$ ;  $df = 1, 13$ ;  $P < 0.0001$ ), and the *Rag1+Rag2* ( $t = 18.74$ ;  $df = 1, 13$ ;  $P < 0.0001$ ) soybean cultivars, the population density of biotype-4 was significantly greater than that of biotype-1 (Fig. 2). These

results indicate that for the susceptible soybean cultivar that was tested the population density of biotype-4 was lower on susceptible soybean when compared to biotype-1.

### **Impact of induced susceptibility on fitness costs of biotype-2 and biotype-3 on susceptible soybean**

For our third experiment, we confirmed that *A. glycines* biotype-1 is capable of alleviating the fitness costs observed for biotype-2 and biotype-3 on the susceptible soybean tested. To test this hypothesis, we analyzed these data for the significance of the main effects of inducer population biotype, response population biotype, and the interaction of inducer population biotype by response population biotype. The main effects of inducer population biotype ( $F = 31.05$ ;  $df = 1, 41$ ;  $P < 0.0001$ ) and response population biotype ( $F = 28.83$ ;  $df = 2, 41$ ;  $P < 0.0001$ ) both significantly affected the density of the response population. The interaction of inducer population biotype by response population biotype was also significant ( $F = 5.06$ ;  $df = 2, 41$ ;  $P < 0.0209$ ). In agreement with results from Varenhorst et al. (in press), both the presence and herbivory of biotype-1 positively affected the density of the biotype-2 and biotype-3 response populations on susceptible soybean (Fig. 3).

When inducer populations were absent the biotype-1 response population was 184% greater than biotype-2 and 196% greater than biotype-3. The biotype-1 response population without an inducer population was 21% greater than biotype-2 and 19% greater than biotype-3 when both had their respective biotype inducer populations present. In the presence of the biotype-1 inducer population, there were no significant differences among the population densities of biotype-1, biotype-2, or biotype-3 response populations (Fig. 3). In summary, the differences between the population densities of

avirulent biotype-1 and virulent biotype-2 and biotype-3 *A. glycines* on susceptible soybean were diminished with the addition of an inducer population with the same shared biotype on susceptible soybean. The impact of an inducer population was greatest when all three biotypes response populations were on soybean that were induced by biotype-1 *A. glycines*.

### **Modeling the consequences of virulent biotype fitness costs and induced susceptibility**

Based on the results of our first three experiments we modeled fitness costs and negative cross-resistance as providing equivalent selection pressure as *Rag* genes (*i.e.*, single *Rag* genes, fitness costs and negative cross-resistance all reduce fitness by 41%). In general, fitness costs and negative cross-resistance delayed the development of virulence. This delay was observed when virulence was rare (*i.e.*, 0.02), regardless of the mode of inheritance. When virulence was common (*i.e.*, 0.2) the delay was only observed with recessive or additive modes of inheritance (Table 1). Fitness costs resulted in the frequency of additively inherited virulence alleles to actually decrease over the course of 25 years. Dominantly inherited virulence alleles were only slightly delayed by the presence of fitness costs.

The decreases in the rate of virulence evolution (*i.e.*, reduced virulent allele frequency) were due to two factors. First, between generations 10 and 11, the frequency of virulence alleles decreased due to the increased fecundity of individuals in the refuge (*e.g.*, a result of fitness costs for virulent biotypes on susceptible soybean). The fecundity of individuals in the refuge was temporarily higher because the density of individuals in that landscape reached  $1.0 \times 10^{13}$  a generation prior to individuals on resistant plants (*e.g.*,

a result of obviation of fitness costs). Second, the selection pressure imposed by the resistance gene was alleviated by generation 11 because the density of homozygous resistant individuals exceeded  $1.0 \times 10^{13}$  in the resistant landscape (*e.g.*, a result of induced susceptibility).

### Discussion

Our results indicate that fitness costs exist for biotype-2, biotype-3 and biotype-4 *A. glycines* on the susceptible soybean cultivar (IA3027) that was used for these experiments when compared to cultivars on which they are virulent. We determined that the populations of biotype-2 were reduced by 84% (Fig. 1), biotype-3 by 88% (Fig. 1), and biotype-4 by 73% (Fig. 2) compared to biotype-1 on the susceptible soybean. The difference in reproduction between the virulent biotypes and our avirulent biotype on the susceptible soybean can be explained by two general hypotheses. Both hypotheses would require a change of the language used to describe the *A. glycines*-soybean system.

Our first hypothesis is that the susceptible cultivar used for these experiments possesses some heretofore-unknown form of *Rag* resistance that provides resistance towards the biotypes that are virulent to known *Rag* genes. If this hypothesis is correct, it would require us to discover the gene providing the resistance. Our second hypothesis is that biotypes that are virulent towards the known *Rag* genes contain specialized effector proteins that prevent them from effectively reproducing on susceptible soybean. This hypothesis, if correct, would require us to refer to susceptible soybean as “wild type” as the innate resistance prevents the establishment of virulent biotypes.

Evidence that our second hypothesis is correct was observed by the presence of either negative cross-resistance or negatively correlated resistance of biotype-2 to *Rag2*,

and biotype-3 to the *Rag1* soybean cultivars that were used. These results indicate that the specialization of biotype-2 to feed on *Rag1* and biotype-3 to feed on *Rag2* prevent it from successfully feeding on the alternative *Rag* gene.

We further hypothesize that the observed effect is due to effector proteins, which are likely the source of virulence for biotype-2 and biotype-3. Varenhorst et al. (in review) hypothesized that virulence in biotype-2 is due to the presence or expression of effector proteins in the saliva. Although there is no preliminary evidence that effector proteins are responsible for virulent aphids in the *A. glycines*-soybean system, there is evidence from other aphid-plant systems (Nicholson et al. 2012). These salivary secretions may alter the plants' physiology such that it becomes a better host for the aphid (Mutti et al. 2008, Bos et al. 2010). Although the function has not been determined, genes coding for effector proteins in other aphids have been found in *A. glycines* (Bansal et al. 2014).

Assuming virulence is due to effector proteins, it is possible that the gene or genes responsible for producing these promote virulent biotypes (*e.g.*, biotype-2) to feed on resistant soybean (*e.g.*, *Rag1*) are also responsible for the hypersensitivity to the other resistance genes (*e.g.*, *Rag2*). A similar effect is observed when effector molecules associated with pathogens elicit either positive or negative responses in their host plant (Hogenhout et al. 2009). We propose the following functioning model as an explanation for our hypothesis. The expression of effector proteins is likely unique to each biotype (Nicholson et al. 2012). Based on this assumption, biotype-1 (avirulent to both *Rag1* and *Rag2*) would have standard effector protein expression with no modifications for virulence to *Rag1* or *Rag2* soybean. Therefore, biotype-1 effectors would be described as

*Rag1* negative and *Rag2* negative (B1: *Rag1*-, *Rag2*-). Because biotype-2 is virulent to *Rag1* the expression of its effector proteins are likely modified for virulence on *Rag1* and are best described as *Rag1* positive, but is avirulent for *Rag2* soybean making it *Rag2* negative (B2: *Rag1*+, *Rag2*-). Biotype-3 is virulent to *Rag2* and the expression of its effector proteins would likely vary from that of biotype-1 or biotype-2 and provide virulence to *Rag2* but is avirulent to *Rag1* (B3: *Rag1*-, *Rag2*+). Biotype-4 has effector proteins that are modified for virulence to both *Rag1* and *Rag2* genes (B4: *Rag1*+, *Rag2*+).

This model further supports our second hypothesis that the specialization of virulent biotypes results in fitness costs on susceptible (wild type) soybean and *Rag* genes that they are not virulent to. The modified expression of the effector proteins present in the virulent biotypes may not be effective at altering the physiology of a susceptible plant or may actually elicit an increased defense response (Hogenhout et al. 2009). Because virulent *A. glycines* feeding on susceptible plants have effector proteins that are expressed in a manner that allows them to effectively manipulate specific defense pathways associated with the presence of *Rag* genes they are not able to manipulate the defense pathways present in a soybean cultivar lacking these genes (*i.e.*, the susceptible cultivar). A similar case is present for virulent biotypes feeding on soybean containing resistant genes that they are not specialized for, which results in negative cross-resistance.

As previously mentioned, only two of the many studies on *A. glycines* biotypes have addressed fitness costs, and both of those manuscripts were addressing fitness costs associated with biotype-3. We note that the fitness cost observed by Enders et al. 2014 is in agreement with our results that biotype-3 populations decrease significantly on *Rag1*

soybean. Our results were also in agreement with those from Wenger et al. (2014), that a fitness cost for biotype-3 was observed on susceptible soybean. Although a different soybean cultivar was used by Wenger et al. (2014) for their susceptible plant (SD-01 76R), it performed similarly to the one used in our current study (IA3027).

The studies by Kim et al. (2008), Hill et al. (2010), and Alt and Ryan-Mahmutagic (2013) that reported the presence of each virulent biotype did not directly assess the presence of fitness costs for each biotype, and these studies found no evidence of fitness costs for biotype-2, biotype-3, or biotype-4 on the susceptible varieties that were used in their experiments. Differences in experimental methodology between our study and those may provide a possible explanation for why fitness costs were not previously observed. Previous studies used different: susceptible soybean lines (some near-isogenic, others not genetically related) from one another and also from our study, artificially infested plants with different densities of *A. glycines*, varied in temperature at which infested plants were maintained, and the duration of each experiment. All of these factors can influence the reproduction of *A. glycines* (Ragsdale et al. 2011).

For example, Varenhorst et al. (in press) observed that the reproduction of *A. glycines* is positively influenced by population density of the initial population of aphids. Sufficiently large initial populations can induce susceptibility such that the fitness of subsequent colonizers is improved. Therefore, initial populations that were used in previous studies may affect the outcome of the experiments (*i.e.*, no fitness costs were observed). McCornack et al. (2004) estimated that optimal *A. glycines* development occurred at 27.8 °C. The temperatures that were used for the biotype determination experiments varied from 20-27°C. The variation among temperatures used for these

experiments could be a possible explanation for the differences in results. Furthermore, there may be interactions between abiotic factors, like temperature, and the impact of *Rag* genes (Whalen and Harmon 2015). Also, the duration that the biotype determination experiments were conducted varied from 7-14 d. According to results by Varenhorst et al. (in review) this could also contribute to variation due to density of aphids that would be present on a plant at 7 d compared to a density at 14 d. Finally, Michel et al. (2010) determined that the as much as 50% of the genotypic diversity of an *A. glycines* colony can be lost within 10 generations of laboratory establishment. Therefore, the genetic diversity of the *A. glycines* colonies that were used for these experiments was likely different.

Given these sources of variation in methodology, it is difficult to compare across studies when significant methodological variation exists. In the future, we propose a standard for *A. glycines* laboratory and greenhouse bioassays in which biotype identity is characterized. We propose that a temperature regime of  $27.8 \pm 1$  °C be used for future laboratory experiments with *A. glycines*. In addition, we suggest that initial infestations of *A. glycines* should be limited to five individuals (adults and nymphs) per plant, on early vegetative stage soybean. Finally, we propose that experiments be conducted for 10 to 12 days. The proposed method would potentially reduce the variation observed among these studies.

Contrary to the results from Crowder and Carrière (2009) for parthenogenic insects our genetic model indicate the presence of fitness costs and negative cross-resistance could result in large delays or even the prevention of the evolution of virulence. However, the relative inheritance or expression of virulence (recessive, additive or

dominant) had large impacts on both the rate at which virulence evolved and the relative importance of fitness costs. These results indicate that the durability of *Rag* genes is likely to be much greater than traditional resistance management models would predict. Our genetic model highlights three important conclusions regarding the *A. glycines*-resistant soybean system. First, the discovery that virulent *A. glycines* biotypes are not rare in the US is not an insurmountable obstacle to the sustainable deployment of *Rag* genes. Second, the relative inheritance or expression of virulence in each biotype must be determined before an accurate assessment can be made of the durability of current *Rag* genes. Finally, if virulence is a dominant trait a resistance pyramid containing three or more *Rag* genes may be needed to significantly delay an increase in the occurrence of virulence.

In conclusion, our results indicate that *Rag* genes are a suitable management option for *A. glycines*. Although virulent biotypes of *A. glycines* are present in the United States, they should not be considered an insurmountable obstacle to the widespread adoption of *Rag* genes. The durability of *Rag* genes will rely on the utilization of refuges, and as Wenger et al. (2014) proposed, the use of an interspersed refuge would likely be most effective. Development of pyramids with additional resistance genes could be a necessary step in the development of a sustainable gene deployment strategy for *Rag* genes.

### **Acknowledgements**

We thank Danaisa Green, Tyler Stallman, and Patrick Wagner for assistance with data collection. This study was funded in part by the Soybean Checkoff through a grant from the North Central Soybean Research Program and the Iowa Soybean Association.

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**Table 1. Simulated effect of induced susceptibility and fitness costs on virulence development.**

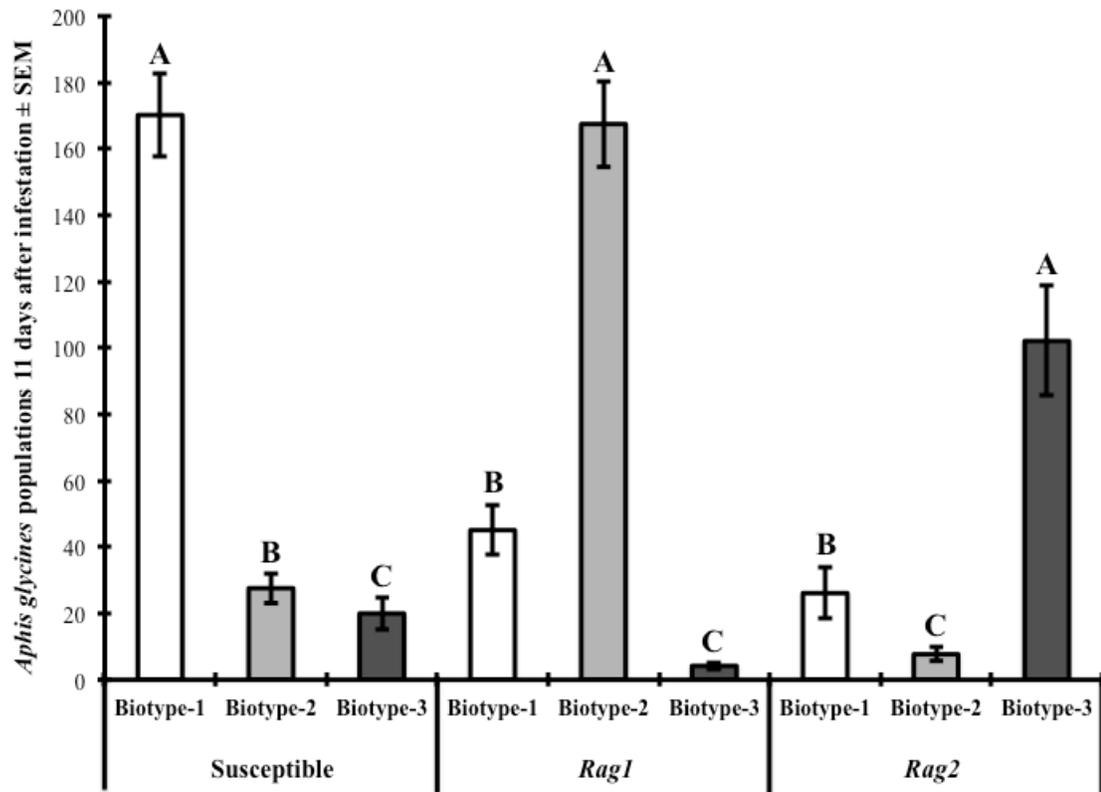
Model Factors Included	Default <sup>a</sup>		Default Including Fitness Costs	
Initial Vir. Allele Frequency	0.02	0.2	0.02	0.2
Recessive <sup>b</sup>				
<i>Rag1</i> Alone	>25 <sup>c</sup> (0.02) <sup>d</sup>	4	>25 (0.02)	18
<i>Rag1+Rag2</i> Pyramid	>25 (0.02)	25	>25 (0.02)	>25 (0.03)
Additive				
<i>Rag1</i> Alone	>25 (0.03)	4	>25 (<0.01)	>25 (<0.01)
<i>Rag1+Rag2</i> Pyramid	>25 (0.02)	14	>25 (<0.01)	>25 (<0.01)
Dominant				
<i>Rag1</i> Alone	>25 (0.06)	3	>25 (0.03)	3
<i>Rag1+Rag2</i> Pyramid	>25 (0.02)	4	>25 (<0.01)	4

<sup>a</sup> Default simulations were run with the inclusion of induced susceptibility effects but not fitness costs or negative cross-resistance effects.

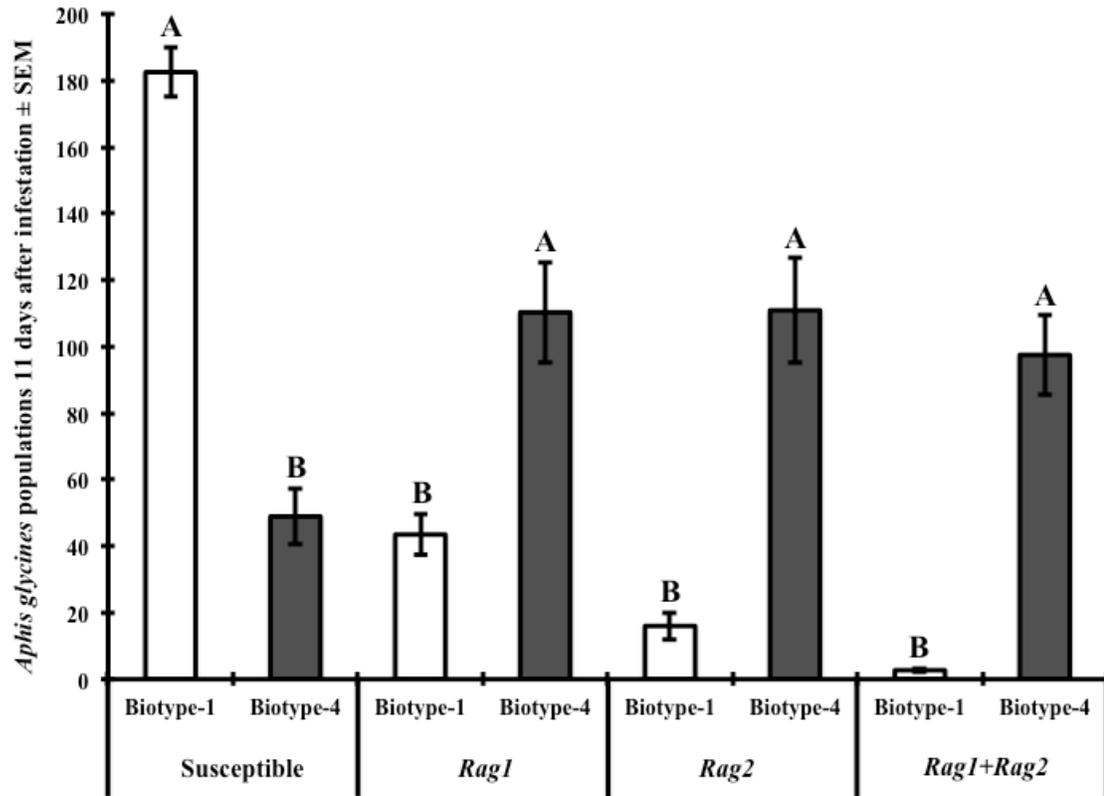
<sup>b</sup> Inheritance of virulence and fitness costs.

<sup>c</sup> Years until the frequency of the allele conferring virulence to *Rag1* exceeded 0.50.

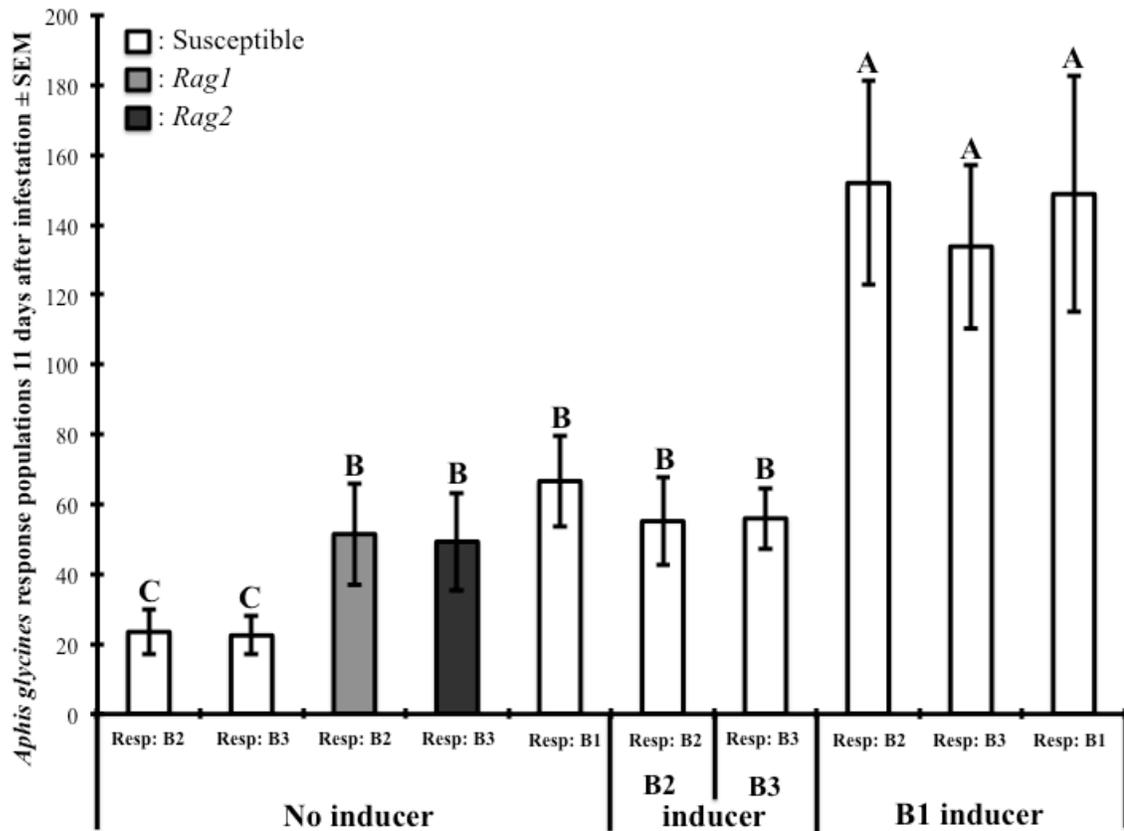
<sup>d</sup> If the frequency of the virulence allele failed to surpass 0.50 within 25 years, its frequency after 25 years is provided in parentheses.



**Figure 1.** Determining if a fitness cost exists for biotype-2 and/or biotype-3 *A. glycines* on susceptible soybean. For this experiment the susceptible soybean cultivar was IA3027, the *Rag1* cultivar was IA3027RA1, and the *Rag2* cultivar was IA3027RA2. Data were analyzed by soybean cultivar, and capital letters indicate significance among biotype populations ( $P < 0.05$ ).



**Figure 2.** Determining if a fitness cost exists for biotype-4 *A. glycines* on susceptible soybean. For this experiment the susceptible soybean cultivar was IA3027, the *Rag1* cultivar was IA3027RA1, the *Rag2* cultivar was IA3027RA2, and the *Rag1+Rag2* cultivar was IA3027RA12. Data were analyzed by soybean cultivar, and capital letters indicate significance between biotype populations ( $P < 0.05$ ).



**Figure 3.** A comparison of the effects produced by varying inducer population biotypes on varying response population biotypes on susceptible soybean. For this experiment the susceptible cultivar IA3027, *Rag1* cultivar IA3027RA1, *Rag2* cultivar IA3027RA2, and *Rag1+Rag2* cultivar IA3027RA12 were used. Capital letters indicate significance among treatments ( $P < 0.05$ ).

**Chapter 5**  
**THE EFFECT OF AN INTERSPERSED REFUGE ON *APHIS***  
***GLYCINES* (HEMIPTERA: APHIDIDAE) AND NATURAL ENEMY**  
**POPULATIONS**

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**Abstract**

Soybean production in the north central United States has relied heavily on the use of foliar and seed applied insecticides to manage populations of *Aphis glycines* (Hemiptera: Aphididae). An additional management strategy is the use soybean cultivars containing *A. glycines* resistance genes (*Rag*). Previous research has demonstrated that *Rag* cultivars are capable of preventing yield loss equivalent to the use of foliar and seed applied insecticides. However, the presence of three virulent biotypes in North America has raised concern for the durability of *Rag* genes. A resistance management program that includes a refuge for avirulent biotypes could limit the rate at which virulent biotypes occur within a population. To what extent such a refuge reduces the effectiveness of aphid resistant soybean is not clear. We conducted an experiment to determine if a susceptible refuge mixed into resistant soybean (*i.e.*, interspersed refuge) affects the seasonal exposure of aphids, their biological control, and yield protection provided by aphid resistance. We compared three ratios of interspersed refuges (resistant: susceptible; 95:5, 90:10, 75:25) to plots grown with 100% susceptible or resistant soybean. We

observed no significant difference in seasonal exposure to aphids between plots grown with 100% resistant soybean and a 95:5 mixture. Interspersed refuges had negligible effects on yield, the natural enemy community, and the amount of biological control occurring did not differ among treatments. We discuss the compatibility of interspersed refuges for *A. glycines* management and whether resistance management can prolong the durability of *Rag* genes.

**Keywords:** host-plant resistance, soybean aphid, refuge in a bag

### Introduction

Since 2000 the soybean aphid, *Aphis glycines* Matsumura, has been an economically important pest of soybean in North America (Alleman et al. 2002, Ragsdale et al. 2007, Ragsdale et al. 2011). The current management strategy for *A. glycines* is the use of broad-spectrum insecticides (*e.g.*, organophosphates, pyrethroids) (Olson et al. 2008, Ragsdale et al. 2011). One concern for the prolonged use of insecticides to manage *A. glycines* is the potential for insecticide resistant populations to evolve, an effect observed in other aphid species (Furk and Hines 1993, Devonshire et al. 1998). Although additional classes of insecticides (*e.g.*, neonicotinoids, ketoenoles) have proven effective against *A. glycines*, these products are not marketed for use on soybean (Ohnesorg et al. 2009, Varenhorst et al. 2012). An additional alternative to broad-spectrum insecticides is the implementation of resistant soybean cultivars. Resistance to aphids in soybean may be produced through the introduction of toxins (*i.e.*, *Bt* proteins) into the plant genome (*e.g.*, plant incorporated protectants, PIP) (Chougule et al. 2013), or the incorporation of *A. glycines* resistance genes (*Rag*) from the soybean germplasm (McCarville et al. 2014). Regardless of the mechanism, an insect resistance management (IRM) programs can

extend the length of time that the resistance trait can be used (Tabashnik et al. 2013).

Aspects of the biology and ecology of *A. glycines* in North America suggest that an IRM plan is needed, initially for *Rag*-genes and possibly for future PIPs.

Previous research has demonstrated that soybean cultivars containing either the single *Rag1* gene or a pyramid of *Rag1+Rag2* genes are effective at reducing *A. glycines* populations without an associated yield drag (Brace and Fehr 2012, McCarville et al. 2014). However, their availability and adoption is limited, and occasionally economically damaging populations of *A. glycines* can be observed on cultivars containing *Rag1* (Michel et al. 2011, McCarville et al. 2012). Although *A. glycines* populations are not reported to reach economically damaging levels on *Rag1+Rag2* cultivars, a potential limit to the adoption of *Rag* genes is the presence of multiple virulent *A. glycines* biotypes. In North America, there are currently four recognized *A. glycines* biotypes. Biotype-1 is described as being avirulent towards both *Rag1* and *Rag2* genes (Kim et al. 2008). Biotype-2 is virulent towards *Rag1* but not *Rag2* (Kim et al. 2008). Biotype-3 is virulent towards *Rag2* but not *Rag1* (Hill et al. 2010). Biotype-4 is virulent towards *Rag1*, *Rag2*, and the pyramid containing both genes (Alt and Ryan-Mahmutagic 2013). Although their geographic distribution is largely unknown, it appears that these biotypes are widespread across North America (Michel et al. 2011). Furthermore, the limited genetic diversity among *A. glycines* biotypes may indicate that a simple gene-for-gene explanation is not appropriate to explain this aphid-plant system (Wenger et al. 2013).

Previous studies have shown that a refuge of susceptible plants can reduce the frequency of virulent alleles in a population. A refuge is defined as a habitat in which the target pest (*i.e.*, *A. glycines*) is not under selection pressure due to the presence of a

toxin/resistance gene (Bourguet et al. 2005). A refuge can be considered both wild host plants as well as crop hosts that do not contain the source for selection pressure (*i.e.*, *Rag* genes). The underlying principle of a refuge is that resistant individuals emerging from the resistant host plants will mate with susceptible individuals emerging from the susceptible refuges (Bourguet et al. 2005). Although *A. glycines* do not mate on soybean (Ragsdale et al. 2011), it is possible that a refuge strategy may contribute to the aphids returning to *Rhamnus* spp., where sexual reproduction takes place. By increasing the amount of avirulent individuals on *Rhamnus* spp., the frequency of virulence for *Rag* genes could be reduced. Furthermore, a fitness cost for virulent biotypes on susceptible soybean could also contribute to reduce selection pressure when both susceptible and resistant plants are grown together (Enders et al. 2014, Wenger et al. 2014).

Based on the biology of *A. glycines* and the acceptance of farmers, it is possible that an interspersed refuge would be ideal for an *A. glycines* IRM program. Gray (2011) determined that farmer willingness to incorporate a refuge was greatest when a 5% refuge was recommended. The refuge-in-a-bag (RIB) approach incorporated a 5% refuge of susceptible seed into a bag of 95% resistant seed. This method ensures planting compliance of a refuge. Wenger et al. (2014) provide evidence that a refuge strategy may be appropriate for the management of virulent biotypes. In a laboratory assay, an interspersed refuge with either 75% or 25% susceptible seed was evaluated for the production of virulent and avirulent biotypes. The inclusion of a refuge of susceptible seed increased the fitness of biotype-1 *A. glycines* while it decreased the fitness advantage of biotype-3, resulting in the persistent population of aphids even when a resistant cultivar was the most dominant plant genotype used. To what extent an

interspersed refuge that combines aphid-resistant and susceptible soybean in a field setting can allow for *A. glycines* to persist throughout the season is not known.

A possible concern when considering the implementation of a refuge is the impact on the natural enemy community of the pest. In soybean, the most abundant natural enemies are *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) and *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) and both are sources of mortality for *A. glycines* in North America (Fox et al. 2004, Rutledge et al. 2004, Fox et al. 2005, Schmidt et al. 2008, Varenhorst and O'Neal 2012). *Orius insidiosus* is generally a source of early season mortality for *A. glycines* while *H. axyridis* arrives later in the season (Fox et al. 2004, Rutledge et al. 2004, Desneux et al. 2006, Schmidt et al. 2008). In addition to these two species, soybean in Iowa are also inhabited by members of Aphelinidae, Anthocoridae, Braconidae, Carabidae, Cecidomyiidae, Chamaeyiidae, Chrysopidae, Coccinellidae, Forficulidae, Hemerobiidae, Opiliones, Staphylinidae, and Syrphidae (Schmidt et al. 2008), all of which feed on *A. glycines* (Rutledge et al. 2004). To what extent these predators will respond to *A. glycines* within soybean fields that combine resistant and susceptible cultivars is unknown. As noted by McCarville and O'Neal (2012), natural enemies added to the mortality that *A. glycines* experienced on soybean plants containing *Rag* genes. In a field setting, these two mortality forces may reduce the likelihood that *A. glycines* persist until the end of the growing season and return to the overwintering host. Such a scenario would be inconsistent with the goals of a refuge.

To our knowledge no research has been conducted to evaluate the effect of interspersed refuges of resistant and susceptible soybean in a field on *A. glycines* and their natural enemies. We conducted an experiment to determine an optimum interspersed

refuge ratio in soybean that both prevents yield loss and conserves the natural enemy community in soybean. The objectives of this paper were to examine the effect of interspersed refuges on 1) *A. glycines* performance, 2) natural enemy abundance, and 3) natural enemy performance.

## **Materials and Methods**

### **Field Site**

The experiment was conducted at Iowa State University's Johnson Research Farm in 2012 and 2013, and at Iowa State University's Curtiss Research Farm and Northwest Research Farm in 2014. Both the Johnson and Curtiss Research Farms are in Story County, IA, and the Northwest Research Farm is in O'Brien County, IA. Conventional tillage practices were used at all locations during each year. Weed management was performed by hand, and at the Johnson and Curtiss Research Farms herbicides were not applied to the experiment. At the Northwest Research Farm, a pre-emergent conventional herbicide was applied to soil before soybean was planted, and a foliar conventional herbicide was applied when soybean reached the V5 growth stage (Fehr et al. 1971). Soybean were planted on 12 May 2012, and 18 June 2013 at the Johnson Research Farm. In 2014, soybean was planted at the Northwest Research Farm on 19 May and at the Curtiss Research Farm on 12 June.

### **Experimental design**

For this experiment we used a randomized complete block design with six blocks. In 2012, soybean were planted in six 23 by 15m blocks. For 2013, soybean was planted in six 23 by 9-m blocks. Each block contained 5 adjacent plots that were 5 by 15 m in 2012, and 5 by 9 m in 2013. In 2014, soybean were planted at the Curtiss Research Farm

in six 23 by 15-m blocks, while at the Northwest Research Farm they were planted in six 23 by 3-m blocks. At all locations and years plots were planted with 76 cm row spacing and a seed population of 370,000 seeds per ha. At the Johnson and Curtiss Research Farms 9 m alleys separated each block, while at the Northwest Research Farm 6 m alleys separated blocks. All block and alley size variations were due to space constraints at the previously indicated research farms.

### **Interspersed refuge treatments**

For this experiment we used two near-isogenic cultivars containing no *Rag* genes (IA3027, herein referred to as susceptible) or *Rag1+Rag2* (IA3027RA12, referred to as resistant). These two soybean cultivars are near isogenic, and are approximately 93.25% genetically identical (Wiarda et al. 2012). These cultivars were planted in the following treatments: 100% susceptible, 100% resistant, 25%:75% susceptible:resistant, 10%:90% susceptible:resistant, and 5%:95% susceptible:resistant where the ratios were determined by seed weight. All seeds were weighed and mixed before being placed into cloth bags to ensure accurate ratios and even distribution of susceptible and resistant seed.

### **Effect of interspersed refuges on *A. glycines* populations and soybean yield**

We hypothesized that increased amounts of the susceptible cultivar present in the interspersed refuges would positively affect *A. glycines* populations. Sampling for *A. glycines* populations was conducted on a weekly basis throughout each summer. Ten plants in the middle four rows of each plot were scouted for the presence of *A. glycines* populations at the Johnson and Curtiss Research Farms. The middle two rows of each plot at the Northwest Research farm were scouted. All *A. glycines* (immatures and adults) present on those soybean plants were counted. When 100% of the ten plants in the

susceptible plot were infested five plants per plot were then scouted. Soybean exposure to *A. glycines* populations throughout a growing season was measured by calculating cumulative aphid days (CAD), an estimate of plant exposure to aphids throughout a growing season (Hanafi et al. 1989).

In conjunction with our previous hypothesis, we also hypothesized that increased amounts of resistant seed would prevent yield loss. Yields for 2012 and 2013 were measured by harvesting all six rows of the plots. In 2014, at the Curtiss Research Farm the yields were measured by harvesting the middle four rows of each plot. In 2014, at the Northwest Research Farm yields were measured by harvesting the middle two rows of each plot. All yields were adjusted to 13% moisture.

#### **Effect of interspersed refuges on natural enemy abundance and performance**

We next hypothesized that the abundance of natural enemies would vary based on the amount of susceptible cultivar within each treatment. We monitored plots for the presence of predators once a week using a sweep-net (BioQuip Products, Rancho Dominguez, CA). Sweep-nets were chosen due to previous research in Iowa that demonstrated their effectiveness at collecting aphidophagous natural enemies in soybean (Schmidt et al. 2008, Ohnesorg et al. 2009, Varenhorst and O'Neal 2012). The middle two rows of each plot were sampled using 10 pendulum swings per row for a total of 20 pendulum swings per plot. One sweep-net sample consisted of 20 pendulum swings, and as described by Varenhorst and O'Neal (2012). the contents of each sweep-net sample were emptied into a one-gallon plastic bag and stored at -20 °C until the specimens were identified and counted. All insects collected were identified to at least the family level, and Coccinellidae and *O. insidiosus* were identified to species. Voucher specimens were

deposited into the Iowa State University Insect Collection at Iowa State University, Ames, IA.

Our final hypothesis addressed the impact of natural enemies on *A. glycines* in each treatment. Specifically we used sentinel plants that were artificially infested with *A. glycines* to measure the mortality from natural enemies. To do this at the Johnson and Curtiss Research Farms we planted four susceptible and resistant plants into the 25%:75% susceptible:resistant, 10%:90% susceptible:resistant, and 5%:95% susceptible:resistant treatments. In the 100% susceptible plots, four susceptible seeds were planted, and in the 100% resistant plots four resistant seeds were planted. The soybean seeds were arbitrarily planted into the middle two rows of each plot. When the soybean plants reached the V4 growth stage in the treatments containing interspersed refuges one susceptible and resistant soybean plant were caged according to methods described by McCarville et al. (2012), and the uncaged resistant and susceptible plants were marked with flags. In 100% susceptible and resistant plots, one plant was caged and one uncaged plant was marked with a flag in each plot. All caged and uncaged flagged plants were infested with pieces of soybean leaves containing approximately 50 mixed age *A. glycines*. Infested leaves were paper clipped to the top full trifoliolate of each plant. The success of the infestation was checked after 24 h. The caged and uncaged plants were then counted 12 d after their initial infestation. This was repeated when the second set of soybean plants in each plot reached the V6 growth stage.

To determine if natural enemies reduced the *A. glycines* that were infested on the plants a Biocontrol Services Index (BSI) was calculated for each treatment. A BSI is calculated by using the following equation from Gardiner et al. (2009):

$$BSI = \frac{\sum_{p=1}^4 (A_{c,p} - A_{o,p})}{n}$$

where  $A_c$  is the number of aphids on the caged plant at each sampling date,  $A_o$  is the number of aphids on the open plant at each date,  $p$  is the plot, and  $n$  is the number of replicates. The upper limit of the values calculated from this equation is 1 representing the complete biological control of *A. glycines*. When negative BSI values were measured they were reported as zeroes; such values occurred when the uncaged plant had more *A. glycines* than the caged plant. These could be due to the arrival of a large amount of immigrating alate *A. glycines*. Data collected to determine the presence of a biological control were used to calculate a BSI for each treatment.

### **Statistical analyses for experiments**

To address each of our hypotheses data were analyzed using the PROC MIXED procedure with SAS statistical software version 9.3 (SAS Institute, Cary, NC). Data were analyzed using an analysis of variance (ANOVA). Significant treatment effects were separated using F-protected least-squares means tests with a Tukey adjustment and a significance level of  $P < 0.05$ .

The statistical model used to analyze the CAD data included the main effects of location-year, block, and interspersed refuge. All two- and three-way interaction terms between the main effects were included in the model. The model for determining the impact of interspersed refuges on yield included the main effects of location-year, block, and interspersed refuge. Again, all two- and three-way interaction terms between the main effects were included in the model.

To determine if the abundance of natural enemies varied between 2012, 2013, and 2014 we compared the total populations of all the natural enemies present in each treatment for all location years. To determine if interspersed refuges had an effect on the most abundant and important aphidophagous natural enemies, we individually compared the abundance of Araneae, Chrysopidae, *H. axyridis*, Nabidae, and *O. insidiosus* among treatments using repeated measures analyses. For these analyses, the model included the main effects location-year, date, block, interspersed refuge ratio, and the interaction interspersed refuge ratio by date(location-year). All two- and three-way interactions terms between the main effects were included in the model.

The impact of interspersed refuges on biological control was determined by comparing the BSI values for resistant and susceptible soybean for the 25%:75% susceptible:resistant, 10%:90% susceptible:resistant, and 5%:95% susceptible:resistant treatments. For this analysis the model included the main effects location-year, repetition, block, and interspersed refuge ratio. All two- and three-way interactions terms between the main effects were included in the model.

## **Results**

### **Effect of interspersed refuges on *A. glycines* populations and soybean yield**

We confirmed our hypothesis that higher percentages of susceptible seed present in the interspersed refuges lead to greater *A. glycines* populations. To determine if the varying ratios of susceptible and resistant soybean seed present in the different interspersed refuge treatments had an impact on the seasonal exposure of soybean to *A. glycines*, we analyzed the CAD data for the significance of the main effects location-year, block, and interspersed refuge ratio. The main effect of location-year was

significant ( $F = 474.40$ ;  $df = 3, 747$ ;  $P < 0.0001$ ) so data were analyzed by location-year. The main effect interspersed refuge ratio was significant for Johnson Research Farm in 2012 ( $F = 8.29$ ;  $df = 4, 40$ ;  $P < 0.0001$ ), Johnson Research Farm in 2013 ( $F = 19.49$ ;  $df = 4, 36$ ;  $P < 0.0001$ ), Northwest Research Farm in 2014 ( $F = 6.19$ ;  $df = 4, 24$ ;  $P < 0.0014$ ), and Curtiss Research Farm 2014 ( $F = 27.23$ ;  $df = 4, 28$ ;  $P < 0.0001$ ).

At each location we observed significant differences among the treatments with the 100% susceptible experiencing the greatest exposure to *A. glycines*. At the Johnson Research Farm in 2012, the CAD for the 100% susceptible treatment was significantly greater than the CAD for the 10%:90% susceptible:resistant ( $t = 2.90$ ;  $df = 4, 40$ ;  $P < 0.0455$ ), 5%:95% susceptible:resistant ( $t = 3.43$ ;  $df = 4, 40$ ;  $P < 0.0117$ ), and the 100% resistant ( $t = 3.90$ ;  $df = 4, 40$ ;  $P < 0.0031$ ) treatments (Fig. 1A). There was no significant difference between the 100% susceptible and 25%:75% susceptible:resistant treatments. The CAD for the 25%:75% susceptible: resistant treatment was significantly greater than that of the 10%: 90% susceptible:resistant ( $t = 3.37$ ;  $df = 4, 40$ ;  $P < 0.0136$ ), 5%:95% susceptible:resistant ( $t = 3.90$ ;  $df = 4, 40$ ;  $P < 0.0031$ ), and the 100% resistant ( $t = 4.38$ ;  $df = 4, 40$ ;  $P < 0.0008$ ) treatments (Fig. 1A). There were no significant differences in CAD among the 10%:90%, 5%:95% susceptible:resistant, or 100% resistant treatments.

For the Johnson Research Farm in 2013, the CAD for the 100% susceptible treatment was significantly greater than the 25%:75% susceptible:resistant ( $t = 3.78$ ;  $df = 4, 36$ ;  $P < 0.0049$ ), 10%:90% susceptible:resistant ( $t = 4.73$ ;  $df = 4, 36$ ;  $P < 0.0003$ ), 5%: 95% susceptible:resistant ( $t = 5.82$ ;  $df = 4, 36$ ;  $P < 0.0001$ ), and 100% resistant ( $t = 8.56$ ;  $df = 4, 36$ ;  $P < 0.0001$ ) treatments (Fig. 1B). The CAD for the 100% resistant treatment were significantly lower than the 25%:75% susceptible:resistant ( $t = 4.78$ ;  $df =$

4, 36;  $P < 0.0003$ ), and 10%: 90% susceptible: resistant ( $t = 3.82$ ;  $df = 4, 36$ ;  $P < 0.0043$ ) treatments (Fig. 1B). There was no significant difference between the 5%:95% susceptible:resistant and 100% resistant treatments.

The CAD for the 100% susceptible treatment at the Northwest Research Farm in 2014, were significantly greater than the 10%: 90% susceptible: resistant ( $t = 3.72$ ;  $df = 4, 24$ ;  $P < 0.0085$ ), 5%:95% susceptible:resistant ( $t = 3.92$ ;  $df = 4, 24$ ;  $P < 0.0053$ ), and 100% resistant ( $t = 4.31$ ;  $df = 4, 24$ ;  $P < 0.0020$ ) treatments (Fig. 1C). There was no significant difference between the 100% susceptible and 25%:75% susceptible:resistant treatments. There were also no significant differences among the other treatments. For the Curtiss Research Farm in 2014, the CAD for the 100% susceptible treatment were significantly greater than the 25%:75% susceptible:resistant ( $t = 3.64$ ;  $df = 4, 28$ ;  $P < 0.0090$ ), 10%:90% susceptible:resistant ( $t = 6.60$ ;  $df = 4, 28$ ;  $P < 0.0001$ ), 5%:95% susceptible:resistant ( $t = 8.21$ ;  $df = 4, 28$ ;  $P < 0.0001$ ), and 100% resistant ( $t = 8.99$ ;  $df = 4, 28$ ;  $P < 0.0001$ ) treatments (Fig. 1D). The CAD for the 25%:75% susceptible:resistant treatment were significantly greater than the 10%:90% susceptible:resistant ( $t = 2.96$ ;  $df = 4, 28$ ;  $P < 0.0452$ ), 5%:95% susceptible:resistant ( $t = 4.57$ ;  $df = 4, 28$ ;  $P < 0.0008$ ), and 100% resistant ( $t = 5.35$ ;  $df = 4, 28$ ;  $P < 0.0001$ ) treatments (Fig. 1D). There were no significant differences among the other treatments. From the four location-years we did not observe a significant difference in CAD between plots grown with 100% resistant cultivar compared to the 5%:95% susceptible:resistant mix.

We did not confirm our second hypothesis that increased amount of susceptible seed in interspersed refuge treatments would lead to decreased yields. On the contrary, we observed either no differences or greater yields with higher percentages of susceptible

seed present. The main effects of location-year ( $F = 2.88$ ;  $df = 3, 107$ ;  $P < 0.0392$ ) and block ( $F = 2.70$ ;  $df = 5, 107$ ;  $P < 0.0245$ ) were significant. Therefore, we analyzed yield data by location-year. For Johnson Research Farm in 2012 (Fig. 2A), Johnson Research Farm in 2013 (Fig. 2B), and Curtiss Research Farm in 2014 (Fig. 2C), there were no significant differences among treatments. At the Northwest Research Farm in 2014, the main effect interspersed refuge was significant ( $F = 5.75$ ;  $df = 4, 20$ ;  $P < 0.0030$ ). The 100% susceptible treatment had a significantly greater yield than the 10%:90% susceptible:resistant ( $t = 3.15$ ;  $df = 4, 20$ ;  $P < 0.0357$ ), 5%:95% susceptible:resistant ( $t = 4.49$ ;  $df = 4, 20$ ;  $P < 0.0019$ ), and 100% resistant ( $t = 3.01$ ;  $df = 4, 20$ ;  $P < 0.0482$ ) treatments (Fig. 2D). There were no significant differences among the yields of the other treatments.

### **Effect of interspersed refuges on natural enemy population density and performance**

We confirmed our third hypothesis that varying interspersed refuges would affect the total abundance of natural enemies. In all four location-years, the 100% susceptible plots had more natural enemies than any of the other treatments, however this was only significant for two of the four location-years. This difference was observed by analyzing data for the significance of the main effects location-year, date, block, interspersed refuge ratio, and the interaction of date by interspersed refuge ratio(location-year). The total abundance of natural enemies varied significantly by the main effects location-year ( $F = 88.81$ ;  $df = 3, 906$ ;  $P < 0.0001$ ) and interspersed refuge ratio ( $F = 34.31$ ;  $df = 4, 906$ ;  $P < 0.0001$ ). The significance of the main effects indicates that the total abundance of natural enemies varied by the location-years, and that the total abundance of natural enemies was

not equal among the interspersed refuge treatments. This difference was further illustrated by the significant interaction of location-year by interspersed refuge ratio, which indicated that the natural enemy abundances were not equal among treatments across the four location-years of the study. The significant interaction of date by interspersed refuge ratio (location-year) ( $F = 7.49$ ;  $df = 136, 906$ ;  $P < 0.0001$ ) indicated that within a year the total abundance of natural enemies was affected by sampling date. Therefore, all natural enemy abundance analyses were conducted by location-year as a repeated measures analysis. Analyses were not conducted by date, as there were limited observations for individual dates.

During two location-years we observed significant differences among the treatments (Table 2A). For the Johnson 2013 location-year, the 100% susceptible treatment had significantly more natural enemies present than the 5%:95% susceptible:resistant treatment ( $t = 2.85$ ;  $df = 4, 232$ ;  $P < 0.0378$ ) and the 100% resistant treatment ( $t = 4.29$ ;  $df = 4, 232$ ;  $P < 0.0002$ ). During the Curtiss 2014 location-year the 100% susceptible treatment had significantly more natural enemies than the 25%:75% susceptible:resistant ( $t = 4.46$ ;  $df = 4, 199$ ;  $P < 0.0001$ ), 10%:90% susceptible:resistant ( $t = 5.14$ ;  $df = 4, 199$ ;  $P < 0.0001$ ), 5%:95% susceptible:resistant ( $t = 5.20$ ;  $df = 4, 199$ ;  $P < 0.0001$ ), and 100% resistant ( $t = 5.42$ ;  $df = 4, 199$ ;  $P < 0.0001$ ) treatments. There were no differences among the other treatments for Curtiss 2014.

To further evaluate the effect that the varying interspersed refuge ratios had on specific aphidophagous natural enemies we evaluated *H. axyridis* and *O. insidiosus* due to the previous research that demonstrated their importance as predators of *A. glycines*. We also evaluated the natural enemies that were most abundant across each of the four

location-years; Araneae, Chrysopidae, and Nabidae (Table 1). Only at the Curtiss 2014 location year did the abundance of *H. axyridis* vary among the treatments, with more in the 100% susceptible treatment than the 25%:75% susceptible:resistant ( $t = 4.40$ ;  $df = 4$ , 194;  $P < 0.0002$ ), 10%:90% susceptible:resistant ( $t = 4.80$ ;  $df = 4$ , 194;  $P < 0.0001$ ), 5%:95% susceptible:resistant ( $t = 5.02$ ;  $df = 4$ , 194;  $P < 0.0001$ ), and 100% resistant ( $t = 5.08$ ;  $df = 4$ , 194;  $P < 0.0001$ ) treatments (Table 2B). There were no significant differences in *H. axyridis* abundance among the other treatments. For *O. insidiosus* (Table 2C) and Araneae (Table 2D) there were no differences in abundance during any of the four location-years among any of the treatments. We observed significant differences in Chrysopidae abundance among the treatments at Johnson 2013 and Curtiss 2014. For Johnson 2013, the abundance of Chrysopidae was significantly greater in the 10%:90% susceptible:resistant treatment than the 5%:95% susceptible:resistant ( $t = 2.92$ ;  $df = 4$ , 232;  $P < 0.0310$ ) resistant and 100% resistant ( $t = 3.19$ ;  $df = 4$ , 232;  $P < 0.138$ ) treatments (Table 2E). There were no other differences in Chrysopidae abundance for Johnson 2013. For Curtiss 2014, there were significantly more Chrysopidae in the 100% susceptible treatment than the 25%:75% susceptible:resistant ( $t = 4.47$ ;  $df = 4$ , 203;  $P < 0.0001$ ), 10%:90% susceptible:resistant ( $t = 4.99$ ;  $df = 4$ , 203;  $P < 0.0001$ ), 5%:95% susceptible:resistant ( $t = 5.46$ ;  $df = 4$ , 203;  $P < 0.0001$ ), and 100% resistant ( $t = 5.34$ ;  $df = 4$ , 203;  $P < 0.0001$ ) treatments (Table 2E). During the Johnson 2013 location-year there were significantly more Nabidae in the 100% susceptible treatment than the 5%:95% susceptible:resistant ( $t = 2.98$ ;  $df = 4$ , 232;  $P < 0.0266$ ) and 100% resistant ( $t = 3.25$ ;  $df = 4$ , 232;  $P < 0.0114$ ) treatments (Table 2F). There were no significant differences among the other treatments. For the individual taxa that were evaluated, in all instances of

significant differences there were more of the individuals in the 100% susceptible treatment than the 5%:95% susceptible:resistant and 100% resistant treatments.

We rejected our fourth hypothesis that varying interspersed refuge ratios would affect predation of *A. glycines* by natural enemies. This was observed by analyzing the BSI calculated for the main effects location-year, replication, interspersed refuge ratio, and the interaction of location-year by interspersed refuge ratio. The main effect location-year ( $F = 64.28$ ;  $df = 2, 201$ ;  $P < 0.0001$ ) was significant. We observed differences in the BSI values that were attributed to the location-year. This indicates that BSI was not equal across all location-years in all treatments. Therefore, we analyzed these data by location-year. For each of the location-years, we did not observe any significant differences in BSI among the interspersed refuge treatments (Fig. 3). Indicating that biological control occurred equally among the treatments.

### Discussion

Results from these experiments indicate that an interspersed refuges composed of aphid-susceptible and aphid-resistant soybean is capable of reducing the exposure of *A. glycines* populations similar to what is observed in plots grown with only a resistant cultivar (Fig. 1). Although we observed reduced *A. glycines* exposure on the interspersed refuge and 100% resistant treatments, we did not observe yield differences when compared to the 100% susceptible treatment which experienced the greatest exposure. For the Johnson 2012 and Johnson 2013 location-years we attribute this to limited exposure to *A. glycines* populations, as the CAD accumulated were too low to affect yield, (Fig. 1A & B). For the Curtiss 2014 location-years we attribute the absence of yield loss to an epizootic fungal outbreak that greatly reduced *A. glycines* populations within a two-

week period (Supplemental Fig. 1). So although the average number of aphids per plant resulted in significantly greater exposure than what was observed in 2013, the length of this exposure was likely too short to negatively effect yield. At the Northwest 2014 location-year we observed a significantly greater yield in the 100% susceptible treatment. This is remarkable because the CAD experienced on the 100% susceptible treatment was great enough over several weeks such that we anticipated yield loss compared to the other treatments. It is unclear to us why the yield was significantly lower in the three of the interspersed refuge treatments (Fig. 2C). Although this difference was significant, it accounts for only a 14% difference between the 100% susceptible and 100% resistant treatments.

Our results also indicate that interspersed refuges have a minimal impact on the abundance of natural enemies (Table 1A). Although there were significantly more natural enemies present in the 100% susceptible treatment during the Johnson 2013 and Curtiss 2014 location-years this increase can be directly related to increased *A. glycines* populations present in those plots (Table 2A) (Fig. 1B and D). The same trend was observed when the abundance of individual taxa was evaluated for the interspersed refuge treatments (Table 2). These results also indicate that although some differences were observed in the total abundance of natural enemies among the treatments the biological control provided in each plot was similar (Fig. 3).

The presence of virulent biotypes in North America may suggest that sustainable use of *Rag* genes is unlikely. However, there is increasing evidence (Wenger et al. (2014), Varenhorst et al. (in press), and Varenhorst et al. (in prep)) that widespread use of *Rag* genes may not lead to an increase in the frequency of virulent biotypes such that *Rag*

genes are no longer useful. Wenger et al. (2014) observed an increase in an avirulent biotype population on *Rag2* soybean when simultaneously co-infested with a virulent biotype. Varenhorst et al. (in press) observed a similar effect with *Rag1* and determined that a virulent biotype can obviate the *Rag*-resistance allowing for survival of avirulent biotypes. Using a simple, single-gene deterministic model we determined that virulence to *Rag*-genes, the frequency of virulent genes within *A. glycines* populations, was negatively affected by this obviation of resistance, prolonging the durability of *Rag* genes. Furthermore, Varenhorst et al. (in prep) observed fitness costs associated with virulent biotypes on susceptible soybean (*i.e.*, soybean without *Rag* genes). When a model combining induced susceptibility and fitness costs was constructed the results indicated that *Rag1* is expected to be effective for 18 years, and *Rag1+2* for more than 25 years when 25% of the landscape was composed of susceptible soybean. However, this model did not specify if the susceptible soybean was grown in a block or interspersed refuge.

The challenge for the sustainable use of *Rag*-genes is the production of a refuge that is compatible with soybean production. Farmer adoption of a refuge will determine to a large extent how successful this approach to IRM will be. We suggest that an interspersed refuge would be ideal for an *A. glycines* IRM program, as this method ensures greater compliance by farmers to plant a refuge. The combined results of our study indicate that interspersed refuges composed of susceptible and resistant soybean may provide a viable method for the management of *A. glycines*. Our results indicate that an interspersed refuge allows for the production of aphids throughout the growing season without interfering with yield or biological control of *A. glycines*. Many questions regarding the use of a refuge in soybean still exist. For a refuge to be successful, avirulent

*A. glycines* populations must exist in soybean fields, albeit at populations that would not cause economic loss. Based on the results from this study, a refuge size of 25% or more susceptible soybean would be required to produce avirulent individuals. The CAD for the treatments containing 10% or 5% susceptible soybean were often not significantly different from that of the 100% resistant treatment (Fig. 1).

While our study evaluated the effectiveness of interspersed refuges for managing *A. glycines* in a single season, we did not evaluate the proportion of avirulent:virulent individuals produced within these refuges. To determine if interspersed refuges are appropriate for soybean production future research should expand to include investigations of the biotype composition of *A. glycines* in interspersed refuge plots for virulent individuals, determine the abundance of *A. glycines* successfully immigrating to *Rhamnus spp.* in the fall, and model the impact of both natural enemies and interspersed refuges on the success of *A. glycines* populations. However, our results do not indicate that a virulent biotype was present, as the CAD on the 100% resistant treatment were very low in every location year of the experiment.

Based on how *A. glycines* responds to conditions at the landscape scale (Gardiner et al. 2009, Schmidt et al. 2011) it is possible that in the future area-wide management may be possible. Area-wide suppression of an insect pest through the adoption of pest-resistant cultivars has been observed for other field crops (Carrière et al. 2003, Wu et al. 2008, Hutchison et al. 2011). For example the use of *Bt*-containing maize cultivars on only 63% of corn acres in the U.S. has resulted in area-wide suppression of the *Ostrinia nubilalis*, providing yield and quality benefits to non-Bt maize (Hutchison et al. 2011). Similar results have been observed for cotton pests in the North America (Carrière et al.

2003) and China (Wu et al. 2008). Large-scale adoption of *Rag* soybean would potentially also greatly reduce *A. glycines* populations over a large geographic area. However, the success of *Rag* soybean would require an effective IRM plan to combat the evolution of virulent biotypes and the ineffectiveness of released *Rag* genes.

### **Acknowledgements**

We thank Michael McCarville, Shelby Pritchard, Tyler Stallman, and Patrick Wagner for assistance with data collection. We also thank Kent Burns and Josh Sievers for assistance with preparation and management of soybean plots. This study was funded in part by the Soybean Checkoff through a grant from the North Central Soybean Research Program.

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**Table 1. Natural enemy community present in Iowa soybean.**

		<b>Aphidophagous natural enemies</b>				
Order	Family	Species	% of total abundance			
			Johnson 2012	Johnson 2013	Northwest 2014	Curtiss
2014						
Araneae			40.8	7.20	38.8	3.67
Coleoptera	Coccinellidae	<i>Coccinella septumpunctata</i>	0.09	0.00	0.00	2.23
		<i>Coleomegilla maculata</i>	0.09	0.04	0.00	0.40
		<i>Cycloneda munda</i>	0.15	1.90	0.00	2.23
		<i>Harmonia axyridis</i>	0.26	7.82	0.00	22.3
		<i>Hippodamia convergens</i>	0.13	1.51	0.00	15.9
		<i>Hippodamia parenthesis</i>	0.04	0.12	0.00	0.69
Diptera	Dolichopodidae		16.9	1.04	10.4	7.29
	Syrphidae		0.74	3.52	15.4	4.96
Hymenoptera	Aphelinidae		0.01	0.00	0.00	0.00
	Braconidae		2.38	1.35	0.00	0.10
Hemiptera	Anthocoridae	<i>Orius insidiosus</i>	11.1	7.04	17.7	9.03
	Nabidae		9.6	34.1	5.89	4.91
	Pentatomidae		0.32	0.31	0.00	0.00
Neuroptera	Chrysopidae		16.1	17.4	12.9	22.4
	Hemerobiidae		0.00	0.35	0.00	3.57
Opiliones			0.00	0.00	0.00	0.12
		<b>Non-aphidophagous natural enemies</b>				
Order	Family	Species	% of total abundance			
			Johnson 2012	Johnson 2013	Northwest 2014	Curtiss
2014						
Diptera	Asilidae		0.15	0.00	0.00	0.00
	Tachinidae		0.00	0.12	0.00	0.00
Hemiptera	Reduviidae		0.31	0.70	0.00	0.00

Hymenoptera	Chalcididae	0.00	0.00	0.00	0.00
	Ichneumonidae	0.68	6.23	0.00	0.20
	Pteromalidae	0.37	9.25	0.00	0.00

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Natural enemies were collected with a sweep-net.

**Table 2. Comparison of natural enemy populations for 2012, 2013, and 2014.**

	Johnson 2012	Johnson 2013	Northwest 2014	Curtiss 2014
<b>A. Mean total all natural enemies</b>				
100% susceptible	8.17 ± 1.76	12.28 ± 1.87a <sup>1</sup>	1.07 ± 0.57	21.3 ± 6.04a
25% susceptible: 75% resistant	6.63 ± 1.44	10.02 ± 1.32ab	0.40 ± 0.30	7.04 ± 1.70b
10% susceptible: 90% resistant	6.70 ± 1.53	10.11 ± 1.40ab	0.43 ± 0.33	4.96 ± 1.38b
5% susceptible: 95% resistant	7.90 ± 1.62	9.09 ± 1.22b	0.57 ± 0.62	4.71 ± 1.05b
100% resistant	6.61 ± 1.35	7.48 ± 1.05b	0.37 ± 0.43	4.04 ± 1.11b
<b>B. <i>H. axyridis</i></b>				
100% susceptible	0.01 ± 0.01	0.87 ± 0.20	0	6.96 ± 2.16a
25% susceptible: 75% resistant	0	0.70 ± 0.13	0	1.24 ± 0.44b
10% susceptible: 90% resistant	0.02 ± 0.01	0.69 ± 0.13	0	0.72 ± 0.32b
5% susceptible: 95% resistant	0.03 ± 0.02	0.85 ± 0.17	0	0.39 ± 0.16b
100% resistant	0.02 ± 0.01	0.63 ± 0.15	0	0.30 ± 0.16b
<b>C. <i>O. insidiosus</i></b>				
100% susceptible	0.74 ± 0.18	0.63 ± 0.16	0.17 ± 0.12	0.92 ± 0.33
25% susceptible: 75% resistant	0.80 ± 0.17	0.83 ± 0.16	0.03 ± 0.03	0.87 ± 0.30
10% susceptible: 90% resistant	0.86 ± 0.16	0.63 ± 0.17	0.03 ± 0.03	0.68 ± 0.25
5% susceptible: 95% resistant	0.66 ± 0.15	0.63 ± 0.14	0.13 ± 0.13	0.62 ± 0.23
100% resistant	0.67 ± 0.14	0.65 ± 0.14	0.10 ± 0.10	0.77 ± 0.25
<b>D. Araneae</b>				
100% susceptible	3.08 ± 0.35	0.63 ± 0.07	0.43 ± 0.18	0.26 ± 0.08
25% susceptible: 75% resistant	2.52 ± 0.26	0.70 ± 0.09	0.23 ± 0.11	0.26 ± 0.08
10% susceptible: 90% resistant	2.58 ± 0.32	0.74 ± 0.09	0.10 ± 0.06	0.22 ± 0.09
5% susceptible: 95% resistant	3.08 ± 0.36	0.80 ± 0.10	0.20 ± 0.09	0.57 ± 0.22
100% resistant	2.53 ± 0.29	0.57 ± 0.10	0.13 ± 0.10	0.30 ± 0.10

**E. Chrysopidae**

100% susceptible	1.32 ± 0.24	1.66 ± 0.26ab	0.07 ± 0.05	4.77 ± 1.24a
25% susceptible: 75% resistant	1.02 ± 0.21	1.70 ± 0.25ab	0.07 ± 0.05	1.65 ± 0.42b
10% susceptible: 90% resistant	1.02 ± 0.20	2.35 ± 0.35a	0.17 ± 0.08	1.48 ± 0.40b
5% susceptible: 95% resistant	2.2 ± 0.25	1.35 ± 0.22b	0.07 ± 0.05	1.69 ± 0.26b
100% resistant	1.74 ± 0.22	1.26 ± 0.28b	0	2.56 ± 0.39b

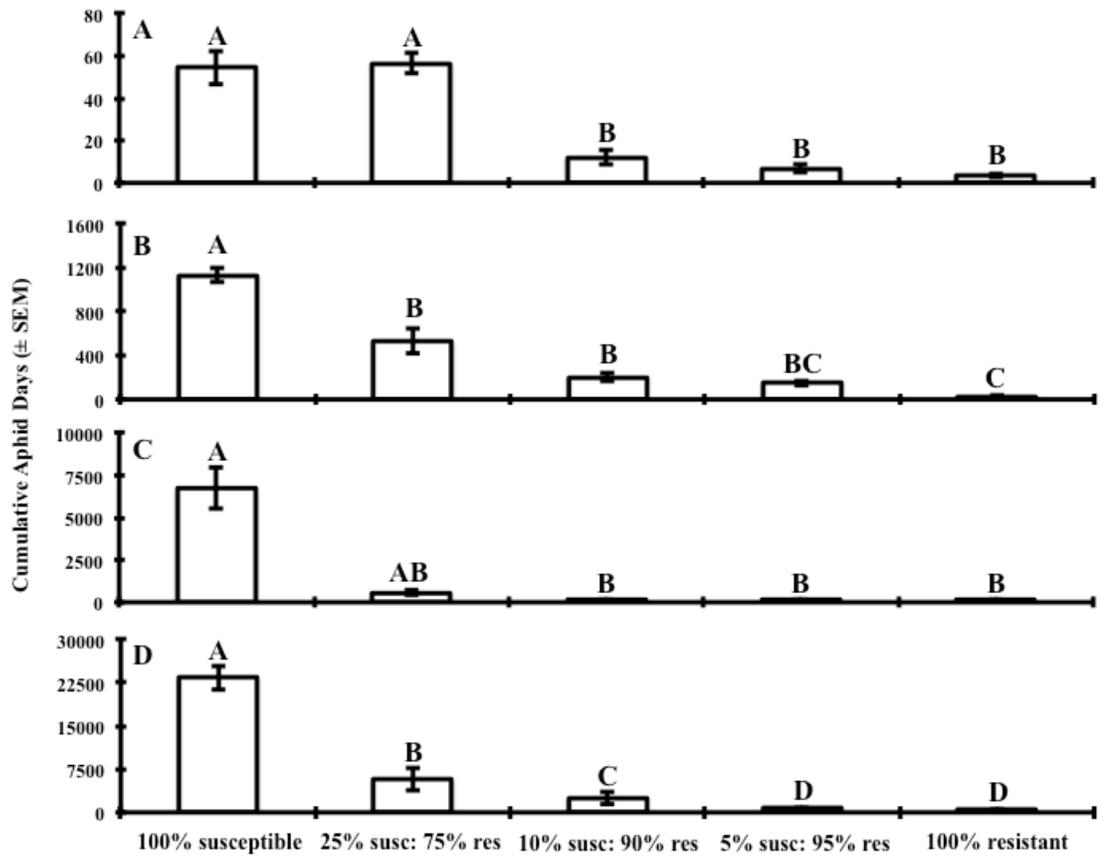
**F. Nabidae**

100% susceptible	0.58 ± 0.10	4.35 ± 0.59a	0.03 ± 0.03	0.64 ± 0.19
25% susceptible: 75% resistant	0.59 ± 0.12	3.39 ± 0.42ab	0.03 ± 0.03	0.43 ± 0.16
10% susceptible: 90% resistant	0.57 ± 0.10	3.20 ± 0.45ab	0	0.35 ± 0.11
5% susceptible: 95% resistant	0.77 ± 0.13	2.76 ± 0.40b	0	0.39 ± 0.13
100% resistant	0.74 ± 0.13	2.61 ± 0.36b	0.07 ± 0.05	0.33 ± 0.11

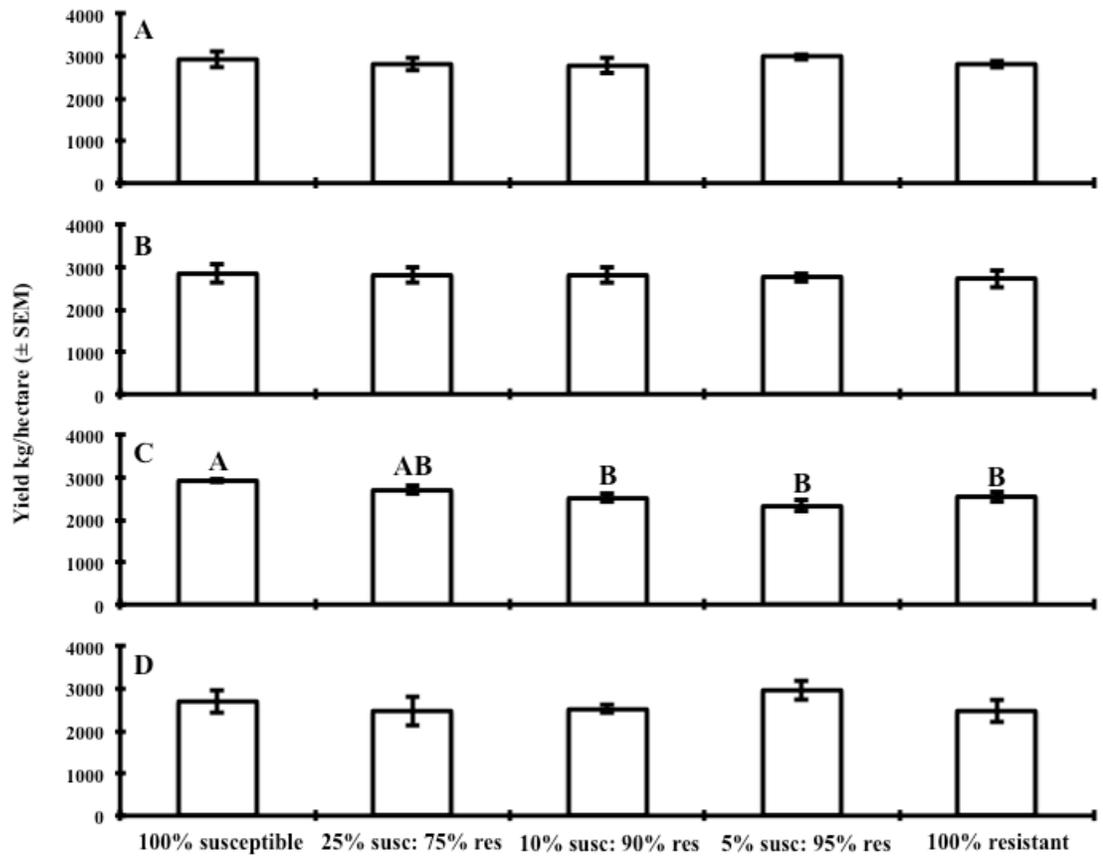
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Natural enemies were collected from sweep-net sampling

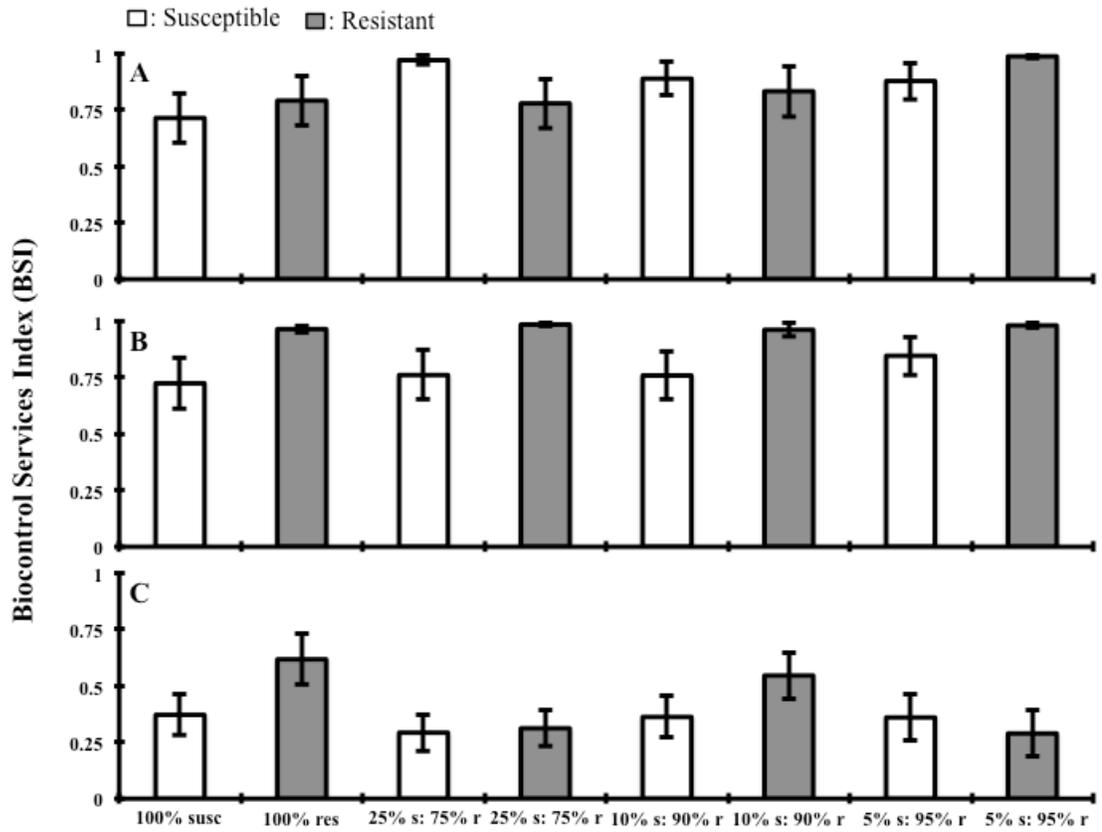
<sup>1</sup> Letters indicate significance among treatments for a given location-year ( $P < 0.05$ ).



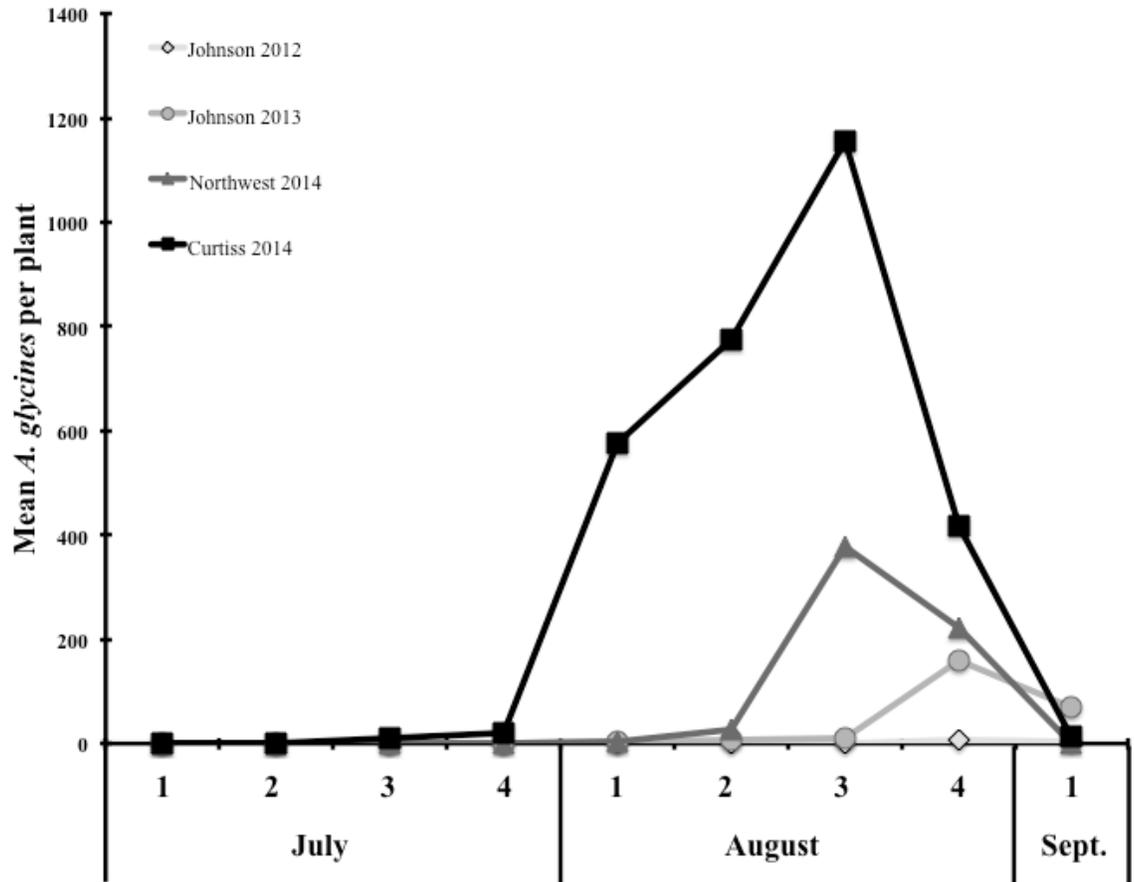
**Figure 1.** A comparison of the impact that varied ratios of susceptible and resistant soybean had on the exposure of soybean to *A. glycines* populations. Exposure was measured in cumulative aphid days (CAD) for the Johnson Research Farm in 2012 (A), Johnson Research Farm in 2013 (B), Northwest Research Farm in 2014 (C), and Curtiss Research Farm in 2014 (D). The susceptible soybean cultivar used for this experiment was IA3027, and the resistant cultivar was IA3027RA12. Capital letters indicate significance among treatments ( $P < 0.05$ ).



**Figure 2.** A comparison of the impact that varying ratios of susceptible and resistant soybean seed mixtures had on yield (average kg/ha) for Johnson Research Farm in 2012 (A), Johnson Research Farm in 2013 (B), Northwest Research Farm in 2014 (C), and the Curtiss Research Farm in 2014 (D). The susceptible soybean cultivar used for this experiment was IA3027, and the resistant cultivar was IA3027RA12. Capital letters indicate significance among treatments ( $P < 0.05$ ).



**Figure 3.** A comparison of the Biocontrol Services Index (BSI) ratings for the varying ratios of susceptible and resistant soybean seed mixtures for Johnson Research Farm in 2012 (A), Johnson Research Farm in 2013 (B), the Curtiss Research Farm in 2014 (C). A value of one indicates a level of 100% biological control, where a value of zero indicates the absence of biological control. The susceptible soybean cultivar used for this experiment was IA3027, and the resistant cultivar was IA3027RA12.



**Supplemental Figure 1.** A comparison of the *A. glycines* populations in the 100% susceptible soybean plots for Johnson 2012 (light gray), Johnson 2013 (gray), Northwest 2014 (dark gray), and Curtiss 2014 (black) location-years. The susceptible soybean cultivar was IA3027.

## Chapter 6

### GENERAL CONCLUSIONS

Shortly after its discovery in 2000, *Aphis glycines* became an economically important pest of soybean in North America, and is still capable of causing economic losses. The current management strategy for *A. glycines* is the use of synthetic broad-spectrum insecticides. While these insecticides are effective at reducing *A. glycines* populations they have the potential to also reduce the populations of natural enemies present in soybean during application. An alternative management strategy is the use of host-plant resistance, mediated by resistance found in the soybean germplasm or through plant-incorporated protectants (*e.g.*, *Bacillus thuringiensis*). The research presented in this manuscript focused on the use of resistance genes that were discovered in the soybean germplasm.

Although *A. glycines* resistant soybean cultivars are commercially available, farmer adoption of resistant cultivars has been slow. The slow adoption of resistant soybean has been attributed to performance issues of the initial cultivars that were released, and the occurrence of three virulent biotypes in North America. The recognized biotypes of *A. glycines* in North America are biotype-1 (avirulent to all tested *Rag* genes), biotype-2 (virulent to *Rag1*), biotype-3 (virulent to *Rag2*), and biotype-4 (virulent to *Rag1*, *Rag2*, and *Rag1+Rag2*). The results from the second chapter of the manuscript demonstrate that initial populations of biotype-2 on *Rag1* soybean are capable of obviating *Rag1*-resistance for biotype-1 (*i.e.*, induced susceptibility). A model of this interaction determined that induced susceptibility results in a decrease in the virulent allele frequency of *A. glycines* over the course of a single growing season. In the third

fourth chapter, we observed fitness costs for biotype-2, biotype-3, and biotype-4 on susceptible soybean. When both induced susceptibility and fitness costs were modeled the results indicated that the combination of these two factors leads to a large reduction of virulent alleles. Together these results indicate that virulent biotypes in North America may not pose a hurdle for the use of *Rag* resistance in soybean. Assuming that the models are correct the durability of *Rag* genes could be prolonged due to the presence of these effects.

The mechanism responsible for induced susceptibility as observed in the soybean-*A. glycines* interaction is unknown. However, the results from the third chapter assist in the determination. While a definitive answer cannot be determined, we hypothesize that induced susceptibility effects are due to the presence of effector proteins in the saliva of *A. glycines*. We observed differences in the duration of feeding facilitation and obviation of resistance (*i.e.*, sub-categories of induced susceptibility), with the obviation of resistance effect lasting longer in the absence of the initial inducer population. This difference indicates that initial virulent biotype-2 inducers are capable of altering the resistance of *Rag1* soybean in a way that benefits avirulent biotype-1 response populations. This difference in virulence between biotype-1 and biotype-2 is best explained by the hypothesis of effector proteins.

Previous researchers have demonstrated that the use of resistant cultivars may lead to a reduction of natural enemies present due to reduced prey availability. The use of interspersed refuges of both resistant and susceptible soybean could theoretically provide an adequate supply of prey while also reducing the selection pressure for virulent biotypes. Our results indicate that interspersed refuges do not reduce the abundance of

natural enemies, and that they are also capable of effectively managing *A. glycines* populations. We also observed no difference among the treatments in the biocontrol provided by natural enemies indicating that interspersed refuges do not negatively affect aphidophagous predators.

The implementation of *Rag* mediated host-plant resistance into the soybean system could lead to an integrated management approach. The results from these studies indicate that our knowledge of the soybean-*A. glycines* system is incomplete. Future research should be focused on further exploring the mechanisms responsible for induced susceptibility. Additional research should also be conducted to determine the impact of interspersed refuges on the virulent allele frequency of *A. glycines*.

## ACKNOWLEDGEMENTS

I thank God for the perseverance and intellect He has given me. Without His guiding grace there were many times that I would have lost my way. I would also like to thank my parents, Jerry and Donna Varenhorst. Without their constant encouragement for advancing my education I wouldn't have considered pursuing a Ph.D. They have also encouraged me to finish anything I start, and without that encouragement I wouldn't have reached this point in my life. I also thank my parents for the initial financial support that facilitated my undergraduate studies, and allowed me to discover my love for learning. For these reasons I also thank my paternal, Norman and Lois Varenhorst, and maternal, Donald and Mabel Held, grandparents. My family's pride in my academic progress has given me the determination to continue pushing forward to reach new goals, and I strive everyday to make them proud. I thank all of my extended family for their support, and encouragement. My thanks also go to my advisor Dr. Matthew E. O'Neal for his guidance and support throughout my graduate education. I also thank my committee members Drs. Bryony Bonning, John Hill, Erin Hodgson, and Forrest Nutter, Jr. for their advice and the time commitments associated with my Ph.D. program of study. I would also like to thank Kelly Kyle. Without her assistance my graduate career would not have been as enjoyable. I thank the past and current graduate students Michael McCarville, Kelly Gill, Cody Kuntz, Joe Wheelock, Mike Dunbar, Shelby Pritchard, Eric Clifton, Ryan Keweshan, Mike Dunbar, Nick Schmidt, Kevin Johnson, and Missy Rynerson. Their friendship and willingness to help made graduate school much more enjoyable. My thanks also go to Greg VanNostrand and Dr. Thelma Heidel-Baker for their assistance and advice on research projects. I must thank the numerous hourly workers who helped

me collect data. I would also like to thank Dr. Ted Wilson who reminded me of my interest in insects when I took an entomology course taught by him at Briar Cliff University. I would be remiss if I did not thank my friends Dr. Drew Dula, Bill Engle, Dr. Aaron Kunz, Patrick Wagner, and Dan Meier for the moral support over the years.