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An investigation of soybean aphid ecology and management in the context of agricultural sustainability

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An investigation of soybean aphid ecology and management in the context of agricultural sustainability

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ABSTRACT

Soybean aphid, *Aphis glycines* Matsumura, is a phytophagous insect capable of causing yield reduction of 40-50%. The introduction and spread of this invasive pest has caused dramatic changes in commercial soybean production management. Due to various economic factors, prophylactic application of insecticides and fungicides to soybean has become increasingly common for yield protection. Impacts of prophylactic pesticide use on soybean aphid ecology are not well characterized, but frequent or poorly timed pesticide use in other systems is associated with aphid population increases due to decreased aphid mortality attributable to decreased fungal disease outbreaks. The first paper of this study examines the impact of growth stage-based applications of fungicides, insecticides, and tank mixes on soybean aphid populations to see if similar population increases are observed. This study also examines the effect of growth-stage based pesticide applications on soybean yield, as research-to-date focuses on an integrated pest management approach.

The second portion of this study employs Bayesian statistical methods to calculate the probability of management tactics from the above study providing cost-effective soybean aphid management. Prior studies have examined the effectiveness of insecticidal seed treatments and pesticide application at soybean growth stage R1; most disease and insect pressure appear later in the season, thus, prophylactic pesticide use at growth stage R3 is more likely. Cost-effectiveness estimates for these situations were nonexistent. To determine the effectiveness of treatments, cost estimates based on pesticide costs and scouting and application fees were used to calculate gain thresholds for each treatment under potential

soybean market prices. The probabilities of each treatment reaching or exceeding estimated gain thresholds were calculated based upon collected yield data.

There is little literature available describing the community of entomopathogenic fungi utilizing soybean aphid as a host in North America. This may be due to the time-consuming bioassay and cultivation methods used to isolate and study these organisms. The third portion of this study endeavors to sequence the ITS region of a common entomopathogen, *Conidiobolus thromboides*, and to use this sequence information to develop a PCR-RFLP method to rapidly identify and distinguish *C. thromboides* in environmental samples.

CHAPTER 1.

GENERAL INTRODUCTION AND LITERATURE REVIEW

Thesis Organization

This thesis has been organized into 5 chapters. Chapter 1 contains a general introduction and literature review. The literature review will encompass a summary of damage caused by and the biology of soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), and biology and detection of common entomopathogenic fungi of the soybean aphid. Chapter 2 contains an article to be submitted to the *Journal of Economic Entomology* entitled "Investigating the Effects of Combined Insecticides and Fungicides on Soybean Aphid Management in Iowa". This article describes the impact of growth stage-based applications of fungicides, insecticides, and tank mixes on soybean aphid populations and soybean yield. Chapter 3 contains an article to be submitted to the *Journal of Economic Entomology* entitled "A Comparative Economic Analysis of Growth Stage-Based Strategies and Integrated Pest Management of Soybean Aphid". This article describes the break-even yield gain analysis of growth stage-based applications of pesticides. Chapter 4 is an article entitled "Development of a PCR-RFLP Method to Rapidly Identify Common Entomopathogenic Fungi Infecting Soybean Aphid in North America" and describes the development of a molecular method for the detecting and distinguishing between two soybean aphid entomopathogens. Finally, chapter 5 will present the general conclusions of this research study as well as acknowledgments.

Introduction and literature review

Impact of *Aphis glycines* on soybean yield and management

The phytophagous soybean aphid, *Aphis glycines* Matsumura, is an insect that feeds on soybean phloem. When large populations of aphids are present, feeding can cause significant damage to the plant. Injury typically presents as leaf curling, premature plant development, stunted growth, reduced pod set and fewer seeds per pod, reduced seed size, and increased protein/decreased oil content (Wang et al. 1994; Wang et al. 1996; Beckendorf et al. 2008). In addition, soybean aphids serve as vectors of several plant diseases, including soybean mosaic virus, bean yellow mosaic virus, alfalfa mosaic virus, tobacco etch virus, and tobacco vein mottling virus, which can impact seed quality and yield (Hill et al. 2001; Wang et al. 2006).

Untreated soybean aphid infestations can result in yield losses as great as 40-50% (Ragsdale et al. 2007; Johnson et al. 2009). Given this potential for economically significant damage, chemical control measures have been developed to protect soybean yields. Initially, the application of an organophosphate or pyrethroid insecticide when aphid populations exceeded 500 aphids per plant was found to provide consistent yield protection (Myers et al. 2005). A multi-state collaborative study over three years was later used to develop economic injury levels (EIL) and economic thresholds (ET) for soybean aphids (Ragsdale et al. 2007). The established ET is 250 aphids per plant; this robust estimate has remained accurate for a large range of control costs, yields, and fluctuating market values of soybean (Ragsdale et al. 2007; Ragsdale et al. 2011). This ET provides a seven day lead time before the established EIL of 674 aphids per plant is expected to be surpassed (Ragsdale et al.

2007). The establishment of an ET for soybean aphid led to the development of an integrated pest management (IPM) recommendation (Ragsdale et al. 2007). When aphid populations reach 250 aphids per plant and populations are increasing and plants are between growth stage R1 (flowering) and growth stage R5 (pod set) as determined by Fehr et al. (1971), a single foliar application of an organophosphate or pyrethroid insecticide is warranted (Ragsdale et al. 2007). ETs and EILs for late-season aphid populations (after growth stage R5.5) have not been developed at this time (Ragsdale et al. 2007).

Johnson et al. (2009) compared a prophylactic approach (the application of a combination of an insecticide and fungicide at plant growth stage R1), an insecticidal seed treatment, and the recommended IPM approach in an efficacy study based on break-even yield gain analysis. Despite the fact that seasonal aphid exposure for IPM treatment was at an intermediate level compared to the prophylactic approach and the untreated control, the IPM treatment resulted in yield gains high enough to offset the cost of treatment and provided the greatest probability of cost-effective soybean aphid management (Johnson et al. 2009). Additionally, an exclusion study by McCarville et al. (2011) examined the robustness of the IPM guidelines for soybean aphid management in the absence of predators and parasitoids and found the 250 aphid per plant threshold still provided yield protection.

Aphis glycines biology and ecology

The soybean aphid is native to Asia and is widely distributed throughout soybean-producing areas in China, Korea, Japan, the Philippines, Indonesia, Malaysia, Thailand, Vietnam, and Russia. Soybean aphid was first reported in North America in July 2000 and had spread to 30 states and 3 Canadian provinces by 2009 (Ragsdale et al. 2011).

The soybean aphid has a heteroecious holocyclic life cycle, alternating between sexual and parthenogenic reproduction and requiring two different host plants to successfully complete development (Ragsdale et al. 2004). Sexual reproduction occurs on the primary host, any one of several *Rhamnus* spp. (Voegtlin et al. 2005). In North America, the invasive common buckthorn, *R. cathartica* L., is the most commonly utilized primary host, although native *Rhamnus* spp. can also serve this purpose (Ragsdale et al. 2004; Voegtlin et al. 2005). Eggs laid the previous winter on *R. cathartica* hatch in the spring after bud break; the resulting fundatrices reproduce asexually, producing alate viviparous females at the third or fourth generation (Ragsdale et al. 2004). The females emigrate to the secondary host, *Glycine max*, where they deposit live apterous nymphs (Ragsdale et al. 2004).

Fifteen to eighteen overlapping generations can occur on soybean over a growing season with a population doubling time as short as 1.5 days under ideal conditions, leading to exponential increases in aphid numbers (McCornack et al. 2004). Optimal soybean aphid performance occurs between 22 and 25°C, with temperatures higher than 32°C causing a large decrease in reproductive rate and temperatures higher than 35°C resulting in death (Hirano et al. 1996; McCornack et al. 2004). Crowding and poor host quality can trigger the production of alatoid nymphs that will disperse to new secondary host plants (Dixon 1973). Environmental cues in late summer trigger the production of alates, both males and females (gynoparae). This generation migrates to *R. cathartica* and the gynoparae produce apterous female oviparae. The oviparae and alate males sexually reproduce and fertilized eggs are deposited near bud bases, where they will overwinter (Dixon 1973).

A well-developed suite of natural enemies comprised of predators, parasitoids, and pathogens normally holds soybean aphid populations below damaging levels in Asia (Liu et

al. 2004; Miao et al. 2007). In North America, predators are the most well studied of the natural enemy suite and provide occasional top-down management of soybean aphid populations (Fox et al. 2004; Rutledge et al. 2004; Fox et al. 2005; Rutledge and O'Neil 2005; Costamagna and Landis 2006; Schmidt et al. 2007; Costamagna and Landis 2011). Kaiser et al. (2007) found six species of hymenopteran parasitoids capable of using the soybean aphid as a host in the United States. However, the species described were all aphid generalists and this number is well below the diversity of parasitoids found in Asia (Kaiser et al. 2007). Introduction of the specialist parasitoid, *Binodoxys communis*, as a classical biological control agent began in 2007, but successful overwintering has not been documented (Wyckhuys et al. 2007).

Endemic fungal pathogens have been found utilizing the soybean aphid as a host in New York, Minnesota, and Michigan (Nielsen and Hajek 2005; Noma and Brewer 2007; Koch et al. 2010). Nielsen and Hajek (2005) conducted a survey of entomopathogenic fungi infecting soybean aphid on common buckthorn and soybean. On both buckthorn and soybean, *Pandora neoaphidis* was identified as the most common pathogen and high aphid density was associated with higher disease prevalence and epizootics (Nielsen and Hajek 2005). Additional species found on soybean were *Entomophthora chromaphidis, Conidiobolus thromboides* Drechsler, *Neozygites fresenii, Lecanicillium lecanii*, and an unidentified *Pandora sp.* that was distinct from *P. neoaphidis* (Nielsen and Hajek 2005). Koch (2010) found *P. neoaphidis* to be the most common pathogen of soybean aphids and also observed *C. thromboides* and an additional species, *Zoophthora radicans*, pathogenizing aphids on soybeans under field conditions. During 2005 and 2006, entomopathogens were endemic at two field locations in Minnesota but epizootics never occurred (Koch et al. 2010).

Taxonomy and biology of entomopathogenic fungi

Entomopathogenic fungi of the class Zygomycetes make up the majority of aphid-pathogenic fungi (Humber 1991). The seven species of fungi that have been identified pathogenizing soybean aphid in the United States all belong to the order Entomophthorales. This order consists of the following six families and most are obligate insect pathogens: Entomophthoraceae, Completoriaceae, Ancylistaceae, Meristacraceae, Neozygitaceae, and Basidiobolaceae (Humber 1989).

Fungi of the order Entomophthorales have substantial capacity for multiplication and expansion in host populations and therefore are well suited to cause epizootics (Latgé et al. 1983). Several distinguishing characteristics contribute to the relative success of Entomophthorales as insect pathogens. Most Entomophthorales exhibit high host specificity, often infecting only one or a few select host species (Latgé and Papierok 1988). Compared with other orders, Entomophthorales produce fewer conidia per host, but fewer conidia are required to cause successful infection (Pell et al. 2001).

Conidia are forcibly ejected from conidiophores, increasing their range of distribution and opportunities for contact with a host (Alexopoulos et al. 1996). These conidia are capable of sporulating and germinating within 2 – 4 hours if contact with a host occurs and conditions are suitable (Brobyn and Wilding 1977). Next, an appressorium will form at the end of the germ tube and penetrate the insect cuticle, although penetration of the cuticle by the germ tube without an appressorium is observed in some species (Brobyn and Wilding 1977; Hywel-Jones and Webster 1986; Butt et al. 1990).

Once inside the body, the fungus undergoes vegetative growth, producing mycelia

and hyphal bodies until host death. During this time, zygospores (thick-walled resting spores capable of surviving hostile conditions and persisting in the environment) are also produced inside the host (Alexopoulos et al. 1996; Papierok and Hajek 1997). Once the host dies, the fungus produces rhizoids to anchor the cadaver to substrate and switches to reproductive growth to produce external conidiophores and conidia (Alexopoulos et al. 1996). If conidia are ejected from the conidiophores and conditions are unsuitable for germination, secondary, tertiary, and quaternary conidia can be produced until appropriate germination conditions occur or energy is depleted (Alexopoulos et al. 1996).

Factors impacting successful infection by entomopathogenic fungi

Enzootic disease is the presence of a low, fairly constant number of infections in an insect population over a long period of time. Epizootics are sporadic and occur when the number of infections in the population increases greatly for a comparatively short period of time. Dredryver (1983) described enzootic and epizootic disease in aphid populations as the relation between the number of living aphids and the number of aphids killed by Entomophthorales. For an enzootic disease incident, the number of infected aphids remains approximately proportional to the number of live, healthy aphids throughout aphid population growth and decline (Dedryver 1983). In an epizootic, the;2 number of infected aphids within a population increases quickly and the proportion of infected aphids increases while the number of healthy aphids decreases, until there are more infected aphids than healthy aphids present (Dedryver 1983). For a successful epizootic to occur, four components must be in congruence: the host, the pathogen, disease transmission and the environment (Watanabe 1987; Tanada and Fuxa 1987; Andreadis 1987; Benz 1987).

A large number of abiotic environmental factors can affect various portions of the entomophthoralean life cycle. Humidity is considered to be the most important abiotic factor in entomopathogen success; conidia are extremely sensitive to humidity and most species require relative humidity of between 90 and 100% to sporulate and germinate (Millstein et al. 1983; Glare et al. 1986; Brobyn et al. 1987; Uziel and Kenneth 1991; Yu et al. 1995; Steinkraus 2006). Temperature is another key component of entomopathogens' ability to infect a host. Temperatures above the optimal range for a fungal species can result in decreased length of sporulation, decreased germination, conidial inactivation, and even inability to produce conidia (Glare et al. 1986; Oduor et al. 1996). For most Entomophthorales, the optimal temperature range is between ~10 – 20°C, a range that frequently occurs in temperate climates (Morgan et al. 1995; Yu et al. 1995; Shah et al. 2002). In a study of diurnal periodicity, Milner et al. (1984) found that all species studied exhibited a peak time of host death between 8 and 16 hours after dawn, which varied depending on species. Other abiotic factors that have been shown to impact Entomophthorales include photoperiod, leaf wetness, pH, and availability of specific nutrients (Callaghan 1978; Milner and Bourne 1983; Oduor et al. 1996).

Entomopathogenic fungi have varying pathogenicity, virulence, growth rate, latency, survival of infective propagules, and toxin production within and among species, all of which contribute to the ability of a species or strain of entomopathogenic fungi to cause an epizootic (Andreadis 1987; Tanada and Fuxa 1987). The importance of the ability for Entomophthorales to forcibly disperse conidia has already been discussed. Alate aphids can travel long distances after they have been infected, thereby providing a long distance mode of dispersal (Feng and Chen 2002; Feng et al. 2007). In addition, pathogen density is of

importance because densely distributed conidia are more likely to cause epizootic disease due to increased opportunities for a host to come in contact with the pathogen (Tanada and Fuxa 1987).

Both host biology and behavior can impact transmission of entomopathogenic fungi. Fungal pathogens are transmitted horizontally among members of the same host population; in order for sufficient disease transmission to result in an epizootic, susceptible hosts must occur at high densities (Andreadis 1987). Epizootics of insect pathogens are considered density-dependent and high aphid population densities are critical to the development of epizootics (Feng et al. 1992). Host stress can also increase the probability of infection, particularly because aphids do not mount a strong immune response to Entomophthorales (Butt et al. 1990)

Human actions can impact the progression of epizootics in a number of ways. The most obvious and well-studied of these is the application of pesticides, which can affect a pathogens' ability to infect a host. Insecticides can indirectly affect entomopathogenic fungi by reducing host population density, which can prevent or delay epizootics (Steinkraus 2006). Direct impacts have not been observed; both Yendol (1968) and Vanninen and Hokkanen (1988) both found that insecticides had no impact on sporulation or conidial germination of Entomophthorales. Herbicides applied at the recommended field rate have been shown to inhibit and prevent the growth and germination of Entomophthorales in vitro (Vanninen and Hokkanen 1988; Poprawski and Majchrowicz 1995; Wei et al. 2004). However, fungicides have been the most closely examined for negative effects on entomopathogenic fungi.

In vitro, infectivity of *P. neoaphidis* conidia was reduced or completely inhibited by

16 of the 20 fungicides tested (Latteur and Jensen 2002). Exposure of *C. thromboides* to the fungicides thiophanate-methyl, iprodione, procymidone, mancozeb, prochloraz manganese chloride complex, and chlorothalonil at the recommended field application rate completely prevented germination (Wei et al. 2004). Detrimental effects of fungicides on growth and infectivity are more pronounced at 15°C than at 25°C (Majchrowicz and Poprawski 1993).

The impact of fungicides on entomopathogenic fungi has been examined in several field studies under production conditions. Naturally occurring entomopathogenic fungal control of green peach aphid, *Myzus persicae,* in potato is decreased by the application of fungicides used to manage late blight, *Phytophthora infestans*, such as macozeb, captafol, and metalaxyl (Nanne and Radcliffe 1971; Lagnaoui and Radcliffe 1998; Ruano-Rossil et al. 2004). In pecan orchards, the application of fungicide led to significantly fewer pathogenized black-margined aphids, *Monellia caryella*, than were present in untreated orchards (Pickering et al. 1990). Early season use of the fungicide carboxin in cotton plots resulted in greater populations of cotton aphid, *Aphis gossypii*, than in untreated control plots due to the suppression of the pathogen *N. fresenii* (Smith and Hardee 1996). Fungal epizootics by *N. fresenii* were delayed a week or more by the use of the fungicide chlorothalonil in cotton, allowing aphid densities to increase during that time (Wells et al. 2000). Finally, overall abundance of soybean aphids infected with entomopathogenic fungi was lower in soybean plots treated with soybean rust fungicides applied early in the season (growth stage R2) and late in the season (growth stage R5) (Koch et al. 2010).

Molecular detection of entomopathogenic fungi

Prior to the development of molecular detection methods, identification of entomopathogenic fungi was dependent on time-consuming bioassays and light microscopy. Since the early 1990s, the development and use of molecular techniques have become a source of information on fungal pathogens and have been used in ecological research of entomopathogenic fungi. The development of cultivation-independent molecular methods eliminates the times and resources needed for isolation and rearing of entomopathogens (Schwarzenbach et al. 2009). Species and isolates can be detected in insect cadavers, plant material, and soil samples. Moreover, species that are difficult to isolate or cultivate, fastidious, or morphologically indistinct can be identified more readily than via cultivation-based techniques (Fournier et al. 2008; Guzman-Franco et al. 2008).

Universal primers are available for different phyla, classes, species, and subspecies of entomopathogenic fungi (Borneman and Hartin 2000; Castrillo et al. 2003; Destefano et al. 2004; Tymon et al. 2004; Entz et al. 2005; Lynch and Thorn 2006; Castrillo et al. 2007; Fournier et al. 2008; Guzman-Franco et al. 2008). PCR length polymorphisms can be used to differentiate species and even isolates within a species (Rohel et al. 1997; Sierotzki et al. 2000; Nielsen et al. 2001; Hajek et al. 2003; Tymon et al. 2004). Restriction fragment length polymorphism (RFLP) analysis of the ITS rRNA gene cluster has been used to examine population structures, genotype variability of genera or species, and host-pathogen relationships of several entomopathogenic fungi, including *P. neoaphidis, Z. radicans,* and several *Conidiobolus* and *Pandora* spp. (Neuveglise et al. 1997; Coates et al. 2002; Tymon et al. 2004). At present, no species-specific molecular techniques exist for the detection of *C.*

thromboides and existing PCR length polymorphism and RFLP analyses have included other *Conidiobolus* spp., but not *C. thromboides.*

Research objectives

The objectives of each chapter of this study are listed below.

Chapter 2 objectives:

1) Characterize the effects of application of insecticides and fungicides compared alone or in combination on soybean aphid populations and yield.
2) Compare application effects of insecticides, fungicides, and insecticide-fungicide combinations made at plant growth stage R1 (beginning flowering) and plant growth stage R3 (beginning pod set) on soybean aphid populations and yield.
3) Assess the effectiveness of the recommended integrated pest management approach for soybean aphid as compared to the applications described above.

Chapter 3 objectives:

1) Examine the economic efficacy of preventative applications of soybean pesticides
2) Determine whether plant growth stage-based applications of pesticides at growth stage R1 (beginning flowering) or R3 (beginning pod set) are an economically sound practice in comparison with the recommended integrated pest management method.

Chapter 4 objectives:

1) Sequence the complete ITS region of the soybean aphid pathogen *Conidiobolus thromboides.*

2) Develop a cultivation-independent technique to distinguish between *Pandora neoaphidis* and *Conidiobolus thromboides* in environmental samples.

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CHAPTER 2.

COMPARISON OF COMBINED FOLIAR INSECTICIDES AND FUNGICIDES ON SOYBEAN APHID POPULATIONS AND SOYBEAN YIELD IN IOWA

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Abstract

Soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), is a major insect pest of soybean (*Glycine max* (L.) Merrill) in the United States. The introduction of soybean rust caused by *Phakopsora pachyrhizi* Sydow into the United States has led to increased availability of fungicides labeled for use in soybean. Increasing crop value and production costs have driven producers to explore previously uncommon management tactics, like prophylactic application of pesticides to protect soybean. We compared the impact of insecticides and fungicides, alone and combined, on soybean aphid management at several locations in Iowa over a 3-year period. Treatments included an untreated control, an integrated pest management (IPM) approach (i.e. insecticide applied based on weekly scouting and an economic threshold), and six treatments that applied insecticide and/or fungicide regardless of soybean aphid density. The IPM treatment was applied in 2008 and 2009, but not in 2010. Although all treatments that included an insecticide, regardless of the time of application, reduced aphid populations compared to the untreated control, we

observed significant variation in yield. Overall, insecticides applied alone or in combination with a fungicide resulted in the highest yield during 2008 and 2009 when applied at the R3 stage; no yield protection was observed in 2010. Our study confirms an IPM system prevents unnecessary application of an insecticide, and we discuss factors that resulted in greater protection when insecticides were applied based on calendar date and not an economic threshold.

Introduction

Soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), is a significant insect pest of soybean (*Glycine max* (L.) Merrill) in the United States. Since it was first detected in Wisconsin in 2000, this invasive insect has spread to 30 states and three Canadian provinces, causing yield losses of more than 40% when left untreated (Ragsdale et al. 2007; 2011). Before the discovery of soybean aphid in North America, foliar insecticides were rarely used in soybean; in 2000, < 0.1% of the total soybean acreage in the north central region of the United States was treated with insecticides (NASS/USDA 2001). During soybean aphid outbreaks on plants in reproductive growth stages, a single foliar application of a pyrethroid or organophosphate insecticide can provide yield protection (Myers et al. 2005). Ragsdale et al. (2007) recommended applying a foliar insecticide when soybean aphid populations exceed an economic threshold (ET) of 250 aphids per plant between flowering (growth stage R1, based on the developmental stages described by Fehr et al. [1971]) and early seed set (R5). Johnson et al. (2009) determined that insecticide

applications based on this recommendation were more cost effective than an application based only on plant growth stage.

Fungal pathogens can be another source of yield loss in soybean across the United States and Canada (Wrather and Koenning 2006). Fehr et al. (1971) established the crucial period for determination of soybean yield occurs between R1 and R5 – R6. Further research has indicated that fungicide application should be based on specific growth stages for optimal yield protection against some diseases. For example, Akem (1995) found that spraying benomyl at R1 and again at R3 provided the most effective control of frogeye leaf spot (*Cercospora sojina*) and application of fungicides based on a pre-determined schedule is a common strategy for the management of soybean rust (Levy 2005; Miles et al. 2007; Mueller et al. 2009). Several foliar fungal diseases of soybean are endemic in Iowa, including Septoria brown spot (*Septoria glycines*), Cercospora leaf blight (*Cercospora kikuchii*), and frogeye leaf spot. Economic thresholds for curative application of fungicides, which can inhibit further infection and disease development, are not available for these diseases. Nevertheless, foliar fungicides are applied to approximately 15% of soybean fields in Iowa each year (NASS 2013).

In some crops, foliar fungicides are applied based on developmental stages of the plant to promote physiological changes, even in the absence of disease. In spring wheat (*Triticum aestivum*), winter wheat (*Triticum hybernum*), and barley (*Hordeum vulgare*), application of strobilurin fungicides resulted in delayed senescence and increased grain yields (Grossmann and Retzlaff 1997; Mercer and Ruddock 1998; Glaab and Kaiser 1999; Ypema and Gold 1999; Wu and Tiedemann 2001; Ruske et al. 2003). However, in soybean these plant health effects may not result in greater yields, as Swoboda and Pedersen (2009)

found no increase in grain quality or yield when tebuconazole, pyraclostrobin, and tebuconazole and pyraclostrobin combined were applied to soybean at beginning flowering (R1) or beginning pod set (R3) in the absence of disease pressure. Despite the limited evidence for increased yield, fungicides are used on limited soybean acreage in Iowa (16% as of 2012), comparable to the amount of acres treated with insecticide (21%) (NASS 2013).

Fungal pathogens are a source of mortality for many species of aphids and a successful epizootic can result in an abrupt decline in aphid populations. Several species of entomopathogenic fungi have been confirmed as soybean aphid pathogens in North America. Nielsen and Hajek (2005) found *Pandora neoaphidis* to be the most prevalent entomopathogenic fungus in soybean fields in New York, with lower incidences of *Conidiobolus thromboides*, *Entomophthora chromaphidis*, *Neozygites fresenii*, and *Lecanicillium lecanii* also observed. Noma and Brewer (2007) and Koch et al. (2010) have also documented *P. neoaphidis* as a significant source of soybean aphid mortality in soybean fields in Michigan and Minnesota, respectively. These fungi may provide an important source of natural control of soybean aphid by contributing to aphid mortality. It is not clear what consequences, if any, the increased use of fungicides on soybean (NASS 2008) has had on the capacity for entomopathogenic fungi to contribute to natural control of the soybean aphid.

Several common fungicides have reduced or completely inhibited the infectivity of entomopathogenic fungal spores *in vitro*, including fungicides in the Fungicide Resistance Action Committee code 3 (triazole), 11 (strobilurin), and M classes (FRAC 2011) (Wilding and Brobyn 1980; Latteur and Jansen 2002). These fungicides include azoxystrobin (Quadris® and Quilt®, Syngenta), tebuconazole (Folicur®, Bayer CropSciences; several

generic products), propiconazole (Tilt® and Quilt®, Syngenta), and chlorothalonil (Bravo®, Syngenta; several other products). Fungicides containing these active ingredients are currently approved for use on soybean in the United States.

In field settings, a decrease in infectivity of entomopathogenic fungi due to the application of fungicides can translate to increased pest populations caused by the delay or prevention of epizootics. Aphid outbreaks due to reduced infectivity of entomopathogenic fungi after the use of fungicides have been observed in a variety of cropping systems. Fungicides, such as macozeb, captafol, and metalaxyl, applied to potato (*Solanum tuberosum*) for control of late blight, *Phytophthora infestans*, decreased the impact of entomopathogenic fungal control of green peach aphid, *Myzus persicae* (Nanne and Radcliffe 1971; Lagnaoui and Radcliffe 1998; Ruano-Rossil et al. 2004). Application of the fungicide carboxin in cotton limited the effectiveness of the fungal pathogen *N. fresenii* in the early season, resulting in higher aphid numbers in carboxin-treated plots as compared to untreated plots (Smith and Hardee 1996). Similarly, use of the fungicide chlorothalonil delayed fungal epizootics by *N. fresenii* for approximately one week, allowing aphid densities to increase during that time (Wells et al. 2000). Overall abundance of soybean aphids infected with entomopathogenic fungi was lower in soybean plots treated with fungicides targeting soybean rust (Koch et al. 2010). These differences in populations of infected aphids were observed for fungicide treatments applied early in the season (growth stage R2) and late in the season (growth stage R5) (Koch et al. 2010). The extent to which the use of fungicides in soybean production contributes to soybean aphid outbreaks is not clear.

Effective management of both soybean aphid and foliar fungal pathogens of soybean is crucial to profitable commercial soybean production. Therefore, it is necessary to

determine the impact of fungicides on soybean aphid populations under production conditions for the development of an ecologically and economically sound management plan for soybeans. Our objective was to characterize the effects of insecticides and fungicides applied alone or combined (i.e. a tank mix) at soybean growth stages R1 (beginning flowering) and R3 (beginning pod set) on soybean aphid populations and soybean yield. We compared these plant growth stage-based approaches to an integrated pest management (IPM) approach, in which an insecticide was applied when soybean aphids reached an ET (Ragsdale et al. 2007). We examined the effects of fungicides and tank mixes on soybean aphid populations and soybean yield. These trials were conducted in small- to mid-sized plots (24.5 to 58 m^2) with naturally occurring aphid infestations across the state of Iowa.

Materials and methods

Experimental design

Field trials were conducted in Iowa during 2008, 2009, and 2010. We established plots to test nine treatments at six locations in 2008, five locations in 2009, and three locations in 2010 (Table 2.1). Aphid-susceptible soybean varieties appropriate to each location were planted in mid- to late-May, depending on weather conditions. Plots at all locations were machine planted in rows spaced 76 cm apart and were managed using conventional practices, including glyphosate-based weed control. All soybeans were planted without a fungicide or insecticide applied to the seed. Soybean planted for the untreated (i.e. control) treatment were grown without pesticides, with the exception of glyphosate. Each plot was four rows wide in 2008 and six rows wide in 2009 and 2010. Plots were 10.7 to

15.3 m long, depending on location, and were arranged in a randomized block design, with five or six replications per site-year.

The following treatments were included at each location: an untreated control, a fungicide pre-mix (a triazole plus a strobilurin), a pyrethroid insecticide, an insecticide pre-mix (a neonicotinoid and a pyrethroid), and an insecticide-fungicide mix, with applications of each treatment at either plant growth stage R1 (beginning flowering) or R3 (beginning pod set) (Table 2.2). Because these pesticides were applied at the plant growth stage indicated, regardless of the level of insect or fungal disease pressure, these treatments will henceforth be referred to as prophylactic treatments. We also included a treatment referred to as the IPM treatment, in which an insecticide was applied based on an ET of 250 aphids per plant (Ragsdale et al. 2007); a fungicide was not included in the IPM treatment. Application rates for each pesticide varied depending on the product used (Table 2.2). Pesticides were applied using a CO_2 backpack sprayer with a handheld boom with flat fan nozzles calibrated to 187 L ha^{-1}. Application dates varied for all treatments depending on location and year (for details see Table 2.1).

Estimation of soybean aphid populations

We assessed soybean aphid populations once a week from mid-June to mid-September using nondestructive, whole plant counts of aphids (all growth stages of both apterae and alatae) on five to 20 consecutive plants within each plot. The number of plants counted was dependent on the proportion of plants infested throughout the field (Hodgson et al. 2004). Plants were arbitrarily selected from the center two rows of each plot in 2008 and from the second or fifth row of each plot in 2009 and 2010.

To estimate the total exposure of soybean plants to soybean aphids during the growing season, we calculated units of cumulative aphid-days (Hanafi et al. 1989). The calculation of cumulative aphid-days (CAD) is based on the number of aphids per plant counted on each sampling date. The exposure of the plants to aphids between two sampling dates is calculated using the following equation:

$$\sum_{n=1}^{\infty} = \left(\frac{x_{i-1} + x}{2} \right) \times t$$

where x is the mean number of aphids on the sample day i; x_{i-1} is the mean number of aphids on the previous sample day; and t is the number of days between samples i-1 and i.

Yield Determination

Each year, we harvested the center two rows of each plot using a plot combine. We measured total seed weight and seed moisture for each plot and estimated seed weight at 13% grain moisture.

Data Analysis

The PROC MIXED procedure in SAS statistical software version 9.2 (SAS Institute 2008) was used to compare soybean exposure to aphids (i.e. CAD) and yield across all treatments. Cumulative aphid-days for all treatments were natural log transformed to meet the assumptions of constant variance and normality. The statistical model used for both CAD and yield analysis defined overall treatment effects as fixed. Location, year, block, and

interaction effects (year×location, year×location×block, and year×location×treatment) were defined as random. Differences in treatment effects for each model (CAD and yield) were determined using least-squares means tests with a Tukey-Kramer correction for mean separation. For testing purposes, treatment effects were evaluated relative to the variance of the year×location×treatment interaction effects. In addition, select pairwise hypothesis tests were run to more closely analyze differences between the R1 and R3 applications of each treatment and to examine the performance of the IPM treatment compared to the prophylactic treatments.

In 2008, due to rapid aphid population growth and weather constraints, insecticide was not applied to the IPM treatments at O'Brien County until aphid populations had exceeded the ET and EIL (average CAD > 29,000). In addition, IPM plots at two locations (Boone and Floyd counties) experienced uncharacteristically high aphid exposure in excess of 23,000 and 21,000 CAD, respectively. These high populations occurred after the R5 growth stage, outside of the recommended period for applying insecticides for aphid management (Ragsdale et al. 2007). To determine whether these large aphid populations significantly affected the comparison of the IPM treatment to other treatments, we calculated an additional treatment, referred to as the 'adjusted IPM' treatment, which eliminated the outlier data from plots that received the IPM treatment and had CAD > 20,000. Four of six blocks in Boone County, four of six blocks in O'Brien County, and two of five blocks in Floyd County met these criteria and were eliminated. Both IPM and adjusted IPM were included in our comprehensive analysis.

Due to the high variability in aphid populations across the three years of the study, we also conducted least-squares means tests with a Tukey-Kramer correction for mean

separation for each individual year of the study (CAD and yield). Due to the minimal impact of using 'adjusted IPM' instead of the original IPM treatment in the multi-year analysis, 'adjusted IPM' was not included in the 2008 year-specific analysis.

Results

Soybean aphid populations

We observed considerable variation in the measurement of total soybean exposure to aphids (i.e. CAD) from year-to-year due to variation in aphid density (aphids per plant) across the three years of the study. Changes in the density of soybean aphids (mean aphids per plant) in untreated plots over the course of each growing season are presented in Figure 2.1. In 2008, aphid populations at most locations exceeded the ET of 250 aphids per plant, thus an insecticide application was made in the IPM treatment at all locations except Cass County (Table 2.1). In 2009, three locations (Story, Floyd, and O'Brien counties) had aphid populations exceeding the ET and received an IPM insecticide application. During 2009, aphids were present at Washington and Adair counties, but populations did not reach the ET. In 2010, the ET was not reached at any of the locations and the IPM treatment was not applied.

Despite the year-to-year variation in aphid populations, the covariance parameter estimate associated with the year×location×treatment random effect was small (0.2149), indicating consistency of overall treatment effects on CAD across all locations and years. Among the 11 treatments, we observed considerable differences in CAD ($F = 16.03$; df = 11, 103; $p < 0.0001$).

Across all site-years, the plants that experienced the greatest CAD were observed in plots treated at R1 with prothioconazole + trifloxystrobin and in the untreated control (Table 2.3). Overall, the abundance of aphids in plots treated at R1 with prothioconazole + trifloxystrobin was not greater than the abundance of aphids in the untreated plots. If fungicide application had an effect on aphid populations, we would have expected CAD for fungicide treatments to be significantly higher than the level observed in the untreated control. Across all site-years, treatments that contained a fungicide did not experience higher seasonal aphid exposure than the untreated control.

All management approaches that contained an insecticide reduced aphid exposure compared to the untreated control. For plots receiving an insecticide or the insecticide-fungicide tank mix, treatments applied at the R3 growth stage had lower aphid populations compared to the same insecticides or tank mixes applied at the R1 growth stage (Table 2.4). The lowest aphid populations were experienced in treatments receiving an insecticide regardless of the timing; no differences in CAD were observed among any of the treatments receiving only an insecticide.

The IPM treatment resulted in a peak population of aphids that was nearly double the ET (497 aphids per plant, Table 2.3) and resulted in plant exposure to aphids high enough to produce yield loss (7,220 CAD). As noted earlier, this abundance of aphids was due to late season outbreaks that occurred after the recommended period for managing the soybean aphid with a foliar insecticide in 2008. We identified these occurrences as outliers and conducted a second analysis that removed these observations (adjusted IPM). In this second analysis, the CAD for adjusted IPM was reduced to 2,341; a reduction of nearly 5,000 CAD from the original IPM treatment. With these adjustments, CAD differences were still

observed among treatments (F = 17.78; df = 9, 88; p < 0.0001). Changes in select pairwise hypothesis tests were minimal (Table 2.4). There was no difference in CAD between the IPM or adjusted IPM as compared to the R1 applications of imidacloprid + cyfluthrin, esfenvalerate, or tank mix. Both the IPM and the adjusted IPM treatments differed significantly from the R3 applications of a tank mix, imidacloprid + cyfluthrin, or esfenvalerate in their ability to provide protection from soybean aphids.

Results of the year-specific analyses were consistent with those of the comprehensive analysis (Table 2.6). In 2008 (F = 62.85; df = 9, 254; p < 0.0001), plants that experienced the greatest CAD were observed in the untreated control plots. CAD for plants in plots that were treated at R1 and R3 with prothioconazole + trifloxystrobin was not significantly different from the untreated plots. All management approaches that contained an insecticide reduced aphid exposure compared to the untreated control. As was previously mentioned, late application of the IPM treatment in O'Brien County and high late season aphid populations in Floyd and Story Counties contributed to the increased aphid exposure of IPM plots. As a result, R3 applications of imidacloprid + cyfluthrin, esfenvalerate, and a tank mix reduced CAD significantly more than the IPM treatment.

In both 2009 (F = 11.86; df = 9, 254; p < 0.0001) and 2010 (F = 4.5; df = 9, 129; p < 0.0001), the plants that experienced the greatest CAD were observed in plots treated at R1 with prothioconazole + trifloxystrobin. However, these plots did not experience significantly higher seasonal aphid exposure than the untreated control, R3 prothioconazole + trifloxystrobin, R1 imidacloprid + cyfluthrin, and R1 esfenvalerate.

Yield

Yield differences were observed among treatments receiving a pesticide (F = 5.22; df 11, 109; $p < 0.0001$). The greatest yield was observed in plots receiving the R3 tank mix, however this yield was not significantly different from treatments receiving only esfenvalerate at R3, imidacloprid + cyfluthrin at either R1 or R3, or a tank mix at R1 (Table 2.3). Only yields from plots receiving the R3 tank mix and the insecticides applied at R3 were significantly greater than the untreated plots.

We did not observe significant differences in yield among any of treatments receiving only an insecticide. This was the case regardless of timing (e.g. application at R1, R3, or based on an ET), insecticide mode of action, or number of active ingredients (esfenvalerate, imidacloprid + cyfluthrin). We did not observe a significant difference in yield between the IPM treatment and the adjusted IPM treatment, despite a difference of nearly 5,000 CAD.

In general, timing of pesticide application impacted soybean yield (Table 2.5). Overall, treatments receiving an application of insecticide at the R3 growth stage (either alone or in combination with a fungicide) had greater yields than treatments receiving R1 applications (Table 2.5). For treatments receiving only a fungicide, there was no difference in yield between the R1 and R3 prothioconazole + trifloxystrobin applications (Table 2.5). For the analysis with the adjusted IPM, yield differences were still observed among treatments (F = 6.67; df = 11, 103; $p < 0.0001$).

The year-specific analysis for 2008 (F = 17.72; df = 9, 200; $p < 0.0001$) shows greater yields for plots treated at R3 with a tank mix or insecticide alone. All treatments that contained an insecticide, regardless of timing, had greater yields than the untreated control. Treatments that received a fungicide alone at R1 or R3 had yields comparable to the

untreated control. In 2009, no yield differences were observed (F = 1.3; df = 9, 156; p = 0.2387). In 2010, R3 fungicide-treated plots out-yielded both the IPM and untreated control plots (F = 3.43; df = 9, 129; p = 0.0008).

Discussion

The purpose of this study was to assess the impact of foliar applications of insecticides and fungicides, applied at different soybean growth stages, on soybean aphid populations and soybean yield and to compare prophylactic approaches with existing IPM recommendations. At present, recommendations for soybean aphid management emphasize the importance of scouting and applying an insecticide only when populations exceed the established ET (Ragsdale et al. 2007). Our data show that an insecticide or insecticide-fungicide tank mix application based on plant growth stage can be effective when applied later in the season, at beginning pod set (R3).

Use of insecticides containing more than one active ingredient did not reduce aphid populations more than a single active ingredient or provide any additional yield protection, even though this combination included two modes of action. Previous studies have shown no benefit for soybean aphid management when two modes of action are combined. Johnson and O'Neal (2008) found no improvement in aphid control with the combination of two active ingredients (a pyrethroid and organophosphate), in pre-mixed insecticides. Ohnesorg et al. (2009) determined that the level of yield protection provided by foliar-applied imidacloprid was comparable to the protection provided by λ-cyhalothrin, a pyrethroid. Our study showed no observable difference in CAD or yield between the two insecticides

(imidacloprid + cyfluthrin and esfenvalerate) tested, despite different active ingredients. These results are similar to those observed in insecticide efficacy trials at Iowa State University across several years (Johnson and O'Neal 2007; Johnson and O'Neal 2008; O'Neal et al. 2010).

Analysis of fungicides and the insecticide-fungicide tank mix combining all locations and years did not show an increase in CAD (Table 2.3). Koch et al. (2010) did not observe an increase in soybean aphid populations in plots treated with fungicides when using CAD as a measure of seasonal aphid exposure, which is consistent with our results. Although the overall analysis from our three years' worth of data indicate that fungicides did not affect aphid populations, we did observe individual site-years when the CAD for treatments receiving a fungicide were greater than the untreated control (Figure 2.2). The R1 application of prothioconazole + trifloxystrobin at Floyd County in 2008 and the R1 application of a tank mix (prothioconazole + trifloxystrobin and imidacloprid + cyfluthrin) at Story County in 2009 both resulted in seasonal aphid exposure of 2X that of untreated controls. The average number of aphids per plant began to increase earlier in the season and reached higher populations overall than in the untreated control. This increase in the plants' exposure to aphids may be attributable to suppression of infectivity of endemic pathogenic fungal populations. Previous studies have shown that fungicides with more than one active ingredient cause greater suppression of entomopathogenic fungi than those with a single active ingredient (Lagnaoui and Radcliffe 1998; Ruano-Rossil et al. 2004; Koch et al. 2010). If the increase in soybean exposure to aphids at these locations was due to a reduction in mortality from entomopathogenic fungi, it is worth noting that increased aphid population

were only observed for 14.3% of all site-years. This may suggest that the conditions needed to produce epizootic outbreaks in soybean aphid populations are uncommon in Iowa.

Aphid populations in Floyd and Story counties experienced rapid growth late in the season and populations exceeded 250 aphids per plant when plants were at the R5 to R6 growth stages. Mean CAD for these two locations were 21,800 and 23,472 for Floyd and Story counties, respectively. Removing these outliers from our IPM treatment resulted in reduction of nearly 2,000 CAD for the Adjusted IPM treatment, and a reduction in the peak aphid per plant population to a level below the ET. However, we did not observe a difference in yield between the IPM and the Adjusted IPM treatments. The estimate CAD for the adjusted IPM was still quite high (4,985), and suggest that despite the removal of the outliers, yield loss still occurred due to insect damage. Because current IPM parameters recommend sprays only between R1 and R5.5 and do not specify whether to reapply if there is an aphid resurgence or late season population increase, growers face a difficult decision when confronted with heavy, late-season aphid populations. The similarity in soybean yield for the IPM and adjusted IPM treatments supports results from previous studies that indicate late season aphid populations (after growth stage R5) has negligible impacts on soybean yield (Ragsdale et al. 2007).

Plots assigned to the IPM treatment in this study only received an insecticide application at eight site-years (57%); IPM plots at the other six site-years did not receive any insecticide applications because the ET for soybean aphid was not reach. Since no foliar insecticides were applied to these plots at any time and seed treatments were not used, nearly half of the IPM plots were vulnerable to damage from other insect pests. Also, as noted above, applying the insecticide in a timely fashion based on the ET proved difficult in 2008.

Johnson et al. (2009) observed that an insecticide applied based on the ET of 250 aphids per plant kept populations below the EIL proposed by Ragsdale et al. (2007) and resulted in yields equal to that of an insecticide and fungicide combination applied when plants began flowering (i.e. the R1 growth stage). We observed higher yields for soybeans treated at R3 compared to those treated based on an ET. Similarly, Myers et al. (2005) found that a foliar application of insecticide at or near soybean growth stages R2 to R3 coincided with peak aphid population and prevented yield losses during years with high aphid populations.

While the prophylactic treatments with insecticides and insecticide-fungicide tank mixes kept aphid populations below the ET used in this study, they should be used with caution and only in situations when high aphid population densities have been confirmed. Prophylactic application of insecticides and fungicides on a yearly basis to control soybean aphid and foliar diseases could result in many detrimental side effects. Unwarranted use of insecticides and fungicides would speed the development of resistance in both soybean aphid and foliar diseases, respectively. Strains of the fungus responsible for frogeye leaf spot (*Cercospora sojina*), a common foliar disease of soybean, have already developed resistance to strobilurin fungicides and can tolerate fungicide concentrations 200 to 7,000 times greater than required to inhibit spore germination in baseline isolates (Bradley 2010). Additionally, broad-spectrum insecticides negatively impact natural enemy populations, including many insect predators and parasitoids that use the soybean aphid as food or host. The absence of these natural enemies in the field can lead to significant increases in soybean aphid populations (Liu et al. 2004; Fox et al. 2004; Costamagna and Landis 2006).

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Table 2.1. Locations, dates of planting and treatment applications of fungicides and insecticides for experimental trials in Iowa

Year and location (county)	Soybean variety	Planting date	R1 application[a]	R3 application[b]	IPM application[c]
2008					
Boone	PB2636[d]	May 19	July 11	August 4	August 19
Cass	DSR[e] 3155RR	May 12	July 2	July 30	NA
Floyd	AG2107[f]	May 17	July 13	August 4	August 29
Hancock	AG2107	May 19	July 14	August 6	August 15
O'Brien	AG2107	May 13	July 9	July 31	July 31
Washington	DSR 3155RR	May 22	July 7	August 5	September 5
2009					
Floyd	Navaho 720RR	May 20	July 16	July 29	August 22
O'Brien	Navaho 720RR	May 14	July 13	July 28	August 14
Story	Navaho 720RR	May 22	July 15	July 27	August 13
Washington	Cherokee 1029RR2Y	May 21	July 17	July 30	NA
Adair	Cherokee 1029RR2Y	May 19	July 15	July 31	NA
2010					
Floyd	AG2430	May 19	July 9	July 21	NA
O'Brien	AG2430	May 17	July 6	July 28	NA
Story	AG2430	May 19	July 6	July 28	NA

[a] The growth stage R1 (beginning bloom) treatment was applied when 50% of plant had one flower at any node

[b] The growth stage R3 (beginning pod set) treatment was applied when 50% of plants had a ½ cm long pod at one of the four uppermost nodes with a completely unrolled leaf

[c] The IPM treatment was applied when soybean aphid populations reached an average of 250 aphids per plant; NA, not applied

[d] Prairie Brand Seed

[e] Dairyland Seed Research

[f] Asgrow

Table 2.2. Active ingredients and application rates of fungicides and insecticides, applied alone or in combination, for experimental trials in Iowa, 2008 to 2010

Timing	Active ingredient(s)	Rate per hectare (ml)
---	Untreated control	---
Fungicides		
R1[a]	Prothioconazole + trifloxystrobin[b]	292
R3[c]	Prothioconazole + trifloxystrobin	292
Insecticides		
R1	Imidacloprid + cyfluthrin[d]	275
R3	Imidacloprid + cyfluthrin	275
R1	Esfenvalerate[e, f]	702
R3	Esfenvalerate	702
IPM	Esfenvalerate	702
Fungicide + insecticide		
R1	Prothioconazole + trifloxystrobin; imidacloprid + cyfluthrin[g]	292; 275
R3	Prothioconazole + trifloxystrobin; imidacloprid + cyfluthrin	292; 275

[a] The growth stage R1 (beginning bloom) treatment was applied when 50% of plant had one flower at any node
[b] Stratego® YLD (Bayer CropScience, Leverkusen, Germany)
[c] The growth stage R3 (beginning pod set) treatment was applied when 50% of plants had a ½ cm long pod at one of the four uppermost nodes with a completely unrolled leaf [d] Leverage 2.7SE™ (Bayer CropScience, Leverkusen, Germany)
[e] Asana® (DuPont, Wilmington, DE)
[f] In 2010, the R1 and R3 esfenvalerate treatments were only applied at the Story County location
[g] Tank mix of Stratego® YLD and Leverage 2.7SE

Table 2.3. Comparison of plant exposure to aphids (CAD) and yield for soybeans grown with fungicides and insecticides, applied alone or in combination, in Iowa from 2008 to 2010

Application time and active ingredient	Cumulative aphid-days ± SEM	Peak aphids per plant ± SEM (date)	Yield (kg ha^{-1}) ± SEM
Untreated control	15,809 ± 401 a[a]	1,040 ± 163 (23 Aug)	3,699 ± 139 c
R1[b] prothioconazole + trifloxystrobin	15,519 ± 455 a	896 ± 152 (26 Aug)	3,766 ± 142 c
R3[c] prothioconazole + trifloxystrobin	14,049 ± 455 ab	854 ± 141 (25 Aug)	3,904 ± 142 bc
R1 imidacloprid + cyfluthrin	6,390 ± 455 c	562 ± 90 (26 Aug)	3,950 ± 142 bc
R3 imidacloprid + cyfluthrin	1,987 ± 455 d	292 ± 97 (27 Aug)	4,129 ± 142 ab
R1 esfenvalerate	4,593 ± 696 bc	717 ± 101 (23 Aug)	3,889 ± 155 bc
R3 esfenvalerate	443 ± 696 d	498 ± 139 (16 Aug)	4,209 ± 156 ab
R1 tank mix[d]	5,931 ± 455 c	484 ± 70 (28 Aug)	3,985 ± 142 abc
R3 tank mix	1,116 ± 394 d	204 ± 163 (25 Aug)	4,268 ± 142 a
IPM[e] (all data)	7,220 ± 401 bc	497 ± 85 (16 Aug)	3,899 ± 16 bcd
Adjusted IPM[f]	2,341 ± 408 c	153 ± 27 (16 Aug)	3,850 ± 143 bc

[a] Treatment means within a column followed by the same lowercase letter are not different at $P \leq 0.05$ according to least-square means tests with Tukey-Kramer adjustment

[b] The growth stage R1 (beginning bloom) treatment was applied when 50% of plant had one flower at any node

[c] The growth stage R3 (beginning pod set) treatment was applied when 50% of plants had a ½ cm long pod at one of the four uppermost nodes with a completely unrolled leaf

[d] Tank mix consisted of prothioconazole + trifloxystrobin and imidacloprid + cyfluthrin

[e] IPM applications of insecticides were made when soybean aphid populations exceeded the ET of 250 aphids per plant. In 2010, aphid populations did not reach the ET, so the IPM treatment was not applied

[f] The adjusted IPM calculation eliminated data for site-years (O'Brien, Story, and Floyd Co., 2008) with atypically high cumulative aphid-days due to high late season aphid population

Table 2.4. Select pairwise hypothesis testing results for equal mean log cumulative aphid-days by treatment for all years and locations

Treatment 1	Treatment 2	Estimate[a]	*P* – value
R1 prothioconazole + trifloxystrobin	R3 prothioconazole + trifloxystrobin	0.1974	0.3626
R1 esfenvalerate	R3 esfenvalerate	1.0265	0.0005
R1 imidacloprid + cyfluthrin	R3 imidacloprid + cyfluthrin	0.9655	<0.0001
R1 tank mix[b]	R3 tank mix	1.1257	<0.0001
IPM	R1 esfenvalerate	0.1877	0.2163
Adjusted IPM[c]	R1 esfenvalerate	0.0423	0.8746
IPM	R3 esfenvalerate	1.2141	<0.0001
Adjusted IPM	R3 esfenvalerate	1.0687	0.0001
IPM	R1 imidacloprid + cyfluthrin	0.1527	0.4819
Adjusted IPM	R1 imidacloprid + cyfluthrin	0.0204	0.9282
IPM	R3 imidacloprid + cyfluthrin	1.1183	<0.0001
Adjusted IPM	R3 imidacloprid + cyfluthrin	0.9858	<0.0001
IPM	R1 tank mix	0.2163	0.4951
Adjusted IPM	R1 tank mix	0.0158	0.9444
IPM	R3 tank mix	1.2738	<0.0001
Adjusted IPM	R3 tank mix	1.1413	<0.0001
IPM	Untreated control	-0.4465	0.0309
Adjusted IPM	Untreated control	-0.5688	0.0088

[a] A positive estimate indicates treatment 1 experienced greater aphid exposure than treatment 2. A negative estimate indicates treatment 2 experienced greater aphid exposure than treatment 1

[b] Tank mix consisted of prothioconazole + trifloxystrobin and imidacloprid + cyfluthrin

[c] The adjusted IPM calculation eliminated data for site-years (O'Brien, Story, and Floyd Co., 2008) with atypically high cumulative aphid-days due to high late season aphid populations

Table 2.5. Select pairwise hypothesis testing results for equal yield (kg ha^{-1}) by treatment for all years and locations

Treatment 1	Treatment 2	Estimate[a]	*P* – value
R1 prothioconazole + trifloxystrobin	R3 prothioconazole + trifloxystrobin	-138	0.1559
R1 esfenvalerate	R3 esfenvalerate	-318	0.0158
R1 imidacloprid + cyfluthrin	R3 imidacloprid + cyfluthrin	-179	0.0671
R1 tank mix[b]	R3 tank mix	-283	0.0044
IPM	R1 esfenvalerate	-38	0.7400
Adjusted IPM[c]	R1 esfenvalerate	-41	0.7346
IPM	R3 esfenvalerate	-358	0.0027
Adjusted IPM	R3 esfenvalerate	-359	0.0040
IPM	R1 imidacloprid + cyfluthrin	-99	0.3050
Adjusted IPM	R1 imidacloprid + cyfluthrin	-101	0.3225
IPM	R3 imidacloprid + cyfluthrin	-278	0.0047
Adjusted IPM	R3 imidacloprid + cyfluthrin	-279	0.0071
IPM	R1 tank mix	-134	0.1667
Adjusted IPM	R1 tank mix	-135	0.1860
IPM	R3 tank mix	-417	<0.0001
Adjusted IPM	R3 tank mix	-418	<0.0001
IPM	Untreated control	153	0.0940
Adjusted IPM	Untreated control	151	0.1160

[a] A positive estimate indicates treatment 1 produced greater yields than treatment 2. A negative estimate indicates treatment 2 produced greater yields than treatment 1

[b] Tank mix consisted of prothioconazole + trifloxystrobin and imidacloprid + cyfluthrin

[c] The adjusted IPM treatment eliminated data for site-years (O'Brien, Story, and Floyd Co., 2008) with atypically high cumulative aphid-days due to high late season aphid populations

Table 2.6. Yearly comparison of plant exposure to aphids (CAD) and yield for soybeans grown with fungicides and insecticides, applied alone or in combination, in Iowa from 2008 to 2010

Application time and active ingredient	Cumulative aphid-days ± SEM 2008	2009	2010	Yield (kg ha^{-1}) ± SEM 2008	2009	2010
Untreated control	26,635 ± 5,840 a	10,854±1,975 ab	212 ± 43 ab	3,293 ± 249 f	4,056 ± 143 a	3,827 ± 335 b
R1[b] prothioconazole + trifloxystrobin	22,885 ± 4,079 ab	17,398 ± 2,114 a	243 ± 51 a	3,402 ± 249 ef	3,931 ± 162 a	3,990± 335 ab
R3[c] prothioconazole +trifloxystrobin	18,995 ± 4,117 abc	19,110 ± 2,289 a	202 ± 38 abc	3,539 ± 249 def	4,039 ± 162 a	4,133 ± 335 a
R1 imidacloprid + cyfluthrin	10,229 ±1,798 cd	9,342 ± 1,216 abc	101 ± 14 abc	3,612 ± 249 de	4,297 ± 162 a	3,902 ± 335 ab
R3 imidacloprid + cyfluthrin	4,206 ±1,416 e	4,222 ± 483 d	106 ± 25 c	3,931 ± 249 abc	4,152 ± 162 a	4,069 ± 335 ab
R1 esfenvalerate	12,766 ± 1,218 d	13,261 ±1,923 abc	75 ± 14 abc	3,705 ± 264 bcde	3,947 ± 162 a	3,781 ± 348 ab
R3 esfenvalerate	5,805 ± 1,698 e	10,710 ± 2,277 cd	37 ± 9 c	4,163 ± 264 ab	4,119 ± 162 a	4,031 ± 348 ab
R1 tank mix[d]	9,751 ± 1,625 cd	8,585 ±1,114 bcd	137 ± 25 abc	3,781 ± 249 bcd	4,042 ± 162 a	3,994 ± 335 ab
R3 tank mix	2,118 ± 559 e	4,996 ± 787 d	115 ± 38 bc	4,154 ± 249 a	4,239 ± 162 a	4,131 ± 335 a
IPM[e]	13,143 ± 2,463 bcd	3,749 ±774 cd	211 ± 44 ab	3,668 ± 249 cde	4,100 ± 143 a	3,800 ± 335 b

[a] Treatment means within a column followed by the same lowercase letter are not different at $P \leq 0.05$ according to least-square means tests with Tukey-Kramer adjustment

[b] The growth stage R1 (beginning bloom) treatment was applied when 50% of plant had one flower at any node

[c] The growth stage R3 (beginning pod set) treatment was applied when 50% of plants had a ½ cm long pod at one of the four uppermost nodes with a completely unrolled leaf

[d] Tank mix consisted of prothioconazole + trifloxystrobin and imidacloprid + cyfluthrin

[e] IPM applications of insecticides were made when soybean aphid populations exceeded the ET of 250 aphids per plant. In 2010, aphid populations did not reach the ET, so the IPM treatment was not applied

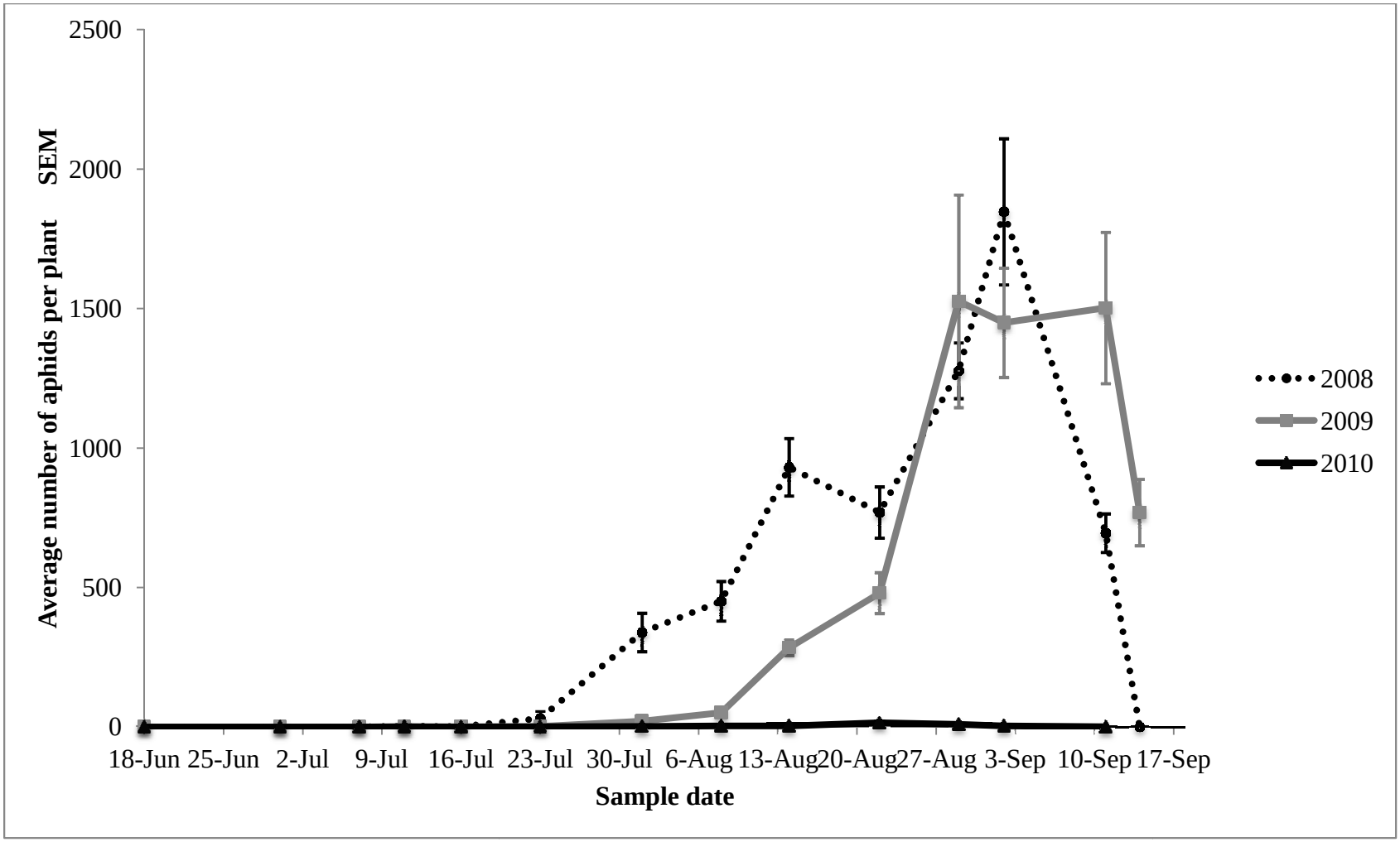

Figure. 2.1. Abundance (mean ± SEM) of soybean aphids in soybean plots that did not receive a pesticide application (i.e. the untreated controls) in 2008, 2009, and 2010. Means for each date were calculated by averaging across replications and locations

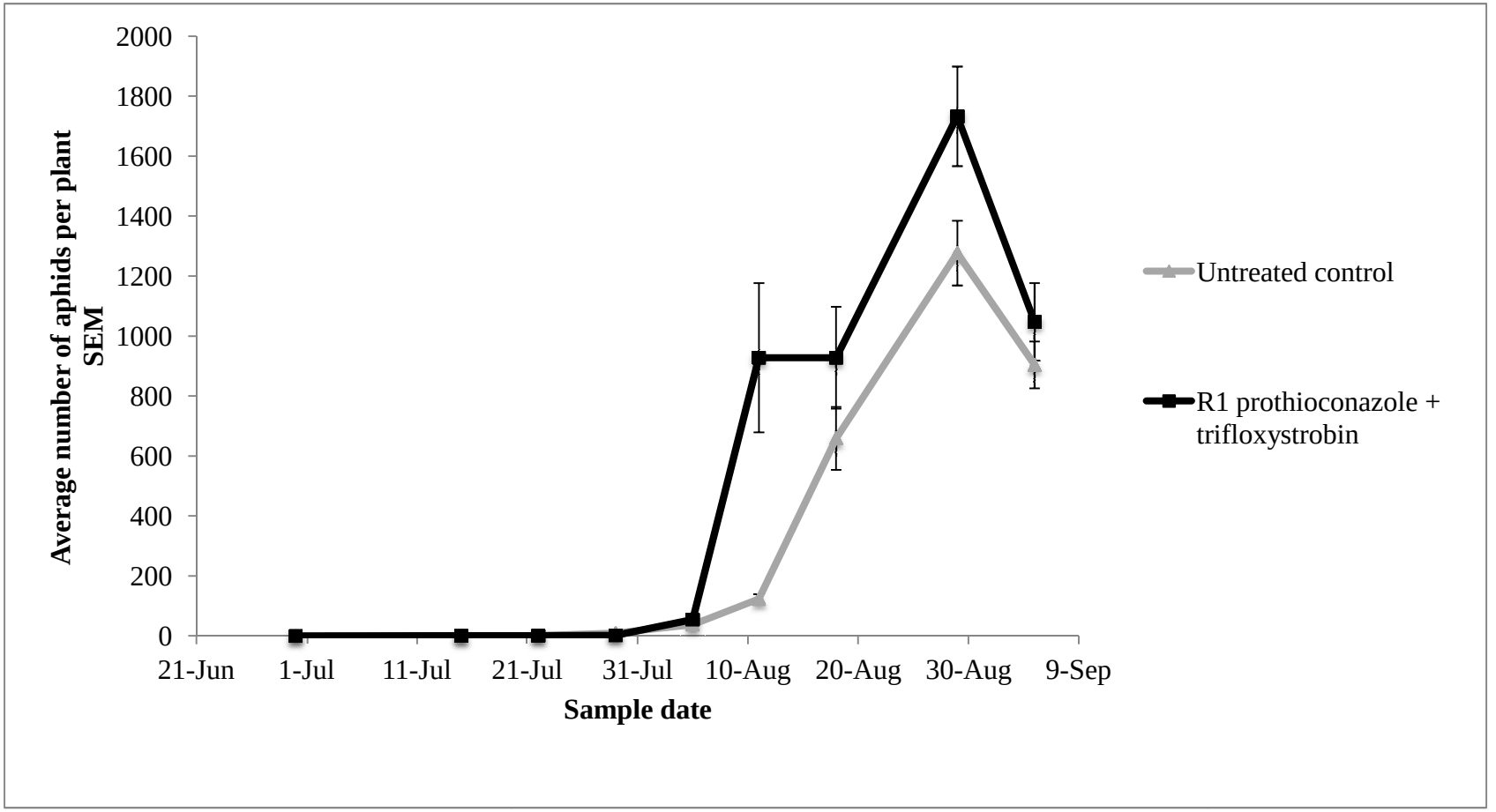

Figure. 2.2. Abundance (mean ± SEM) of soybean aphids in untreated control plots and plots treated with prothioconazole and trifloxystrobin at growth stage R1 in Floyd County, 2008

CHAPTER 3.

A COMPARATIVE ECONOMIC ANALYSIS OF INTEGRATED PEST MANAGEMENT AND GROWTH STAGE-BASED STRATEGIES FOR MANAGEMENT OF SOYBEAN APHID

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Abstract

Although soybean (*Glycine max* (L.) Merrill) production in the United States has traditionally been low input, the introduction the soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae) and soybean rust, *Phakopsora pachyrhizi* Sydow, has resulted in increased use of both insecticides and fungicides, including prophylactic pesticide applications to provide yield protection. Given the high costs of pesticides and application fees, the economic viability of these prophylactic methods lacks evidence. We established small plots to test eight growth stage-based treatments and an integrated pest management approach across several locations in Iowa over a three-year period (2008 to 2010) and collected data on soybean aphid populations and soybean yield. A break-even yield gain analysis was performed to assess each management plan's probability of a positive economic return at three soybean market prices. All insecticide applications, regardless of timing, reduced aphid populations as compared to the untreated control. Our study confirms that a

single application of an insecticide provides yield protection in a cost effective manner. Additionally, the application of an insecticide-fungicide tank mix can provide the same benefit. Application of the IPM treatment at an estimated cost of $43.54/ha provided a moderate to high probability (73 – 97%) of surpassing the GT. Due to differences in overall costs and yields, application of an insecticide or an insecticide-fungicide tank mix at soybean growth stage R3 provided the highest probabilities of recouping treatment costs.

Introduction

Soybean (*Glycine max* (L.) Merrill) is a major product of the United States agricultural industry. In 2010, 77.4 million acres of soybean were planted and ~3,329 million bushels were produced, with more than 50% produced in the North Central region (ERS 2011). Traditionally, soybean production has been relatively low input, with few insect and pathogen problems and low usage of fungicides and insecticides (NASS 1999). However, in the past decade, soybean producers have faced increasing pest pressure.

In 2000, the soybean aphid (*Aphis glycines* Matsumura) was found in Wisconsin. Since then, it has spread throughout the United States and Canada (Ragsdale et al. 2011). Soybean aphid feeding results in reduced plant height, pod number, seed size, seed quality, and yield (Ostlie 2001). Soybean aphid infestations can lead to yield losses of more than 40% if left untreated (Ragsdale et al. 2007). At this time, foliar insecticide application is the main management strategy for soybean aphid.

In 2004, soybean rust caused by *Phakopsora pachyrhizi* Sydow was reported for the first time in the continental United States (Schneider et al. 2005). Introduction of this

pathogen to other continents has resulted in devastating yield loss. Kawuki et al. (2003) reported that yields losses in excess of 45% were observed in untreated plots in Uganda. In South America, yield losses of 60% and 30-75% have been reported in Paraguay and Brazil, respectively (Yorinori et al. 2005). Soybean rust is now endemic in kudzu (*Pueraria lobata*) throughout the southern United States; given favorable weather conditions, soybean rust has the potential to significantly impact soybean production (Sikora and Hershman 2008).

Although soybean rust has not caused yield loss thus far in the North Central region, several other foliar fungal pathogens are endemic to the area. Septoria brown spot (*Septoria glycines*), Cercospora leaf blight (*Cercospora kikuchii*), and frogeye leaf spot (*Cercospora sojina*) are often present in low levels in soybean fields (Wrather and Koenning 2006). Because disease estimates for these pathogens are typically low, disease thresholds for the application of fungicides have not been developed.

The cost of soybean production on a per bushel basis has increased dramatically in the past decade. Between 2000 and 2011, the estimated cost of production in Iowa increased by 57% (ADM 2011). Thirty-three percent of this increase was attributed to seed, fertilizer, and pesticides (ADM 2011). Simultaneously, the market price for soybean has been on the rise, from $6.43 per bushel in 2006 to $10.60 per bushel in 2010 (ERS 2011). With crop value and production costs soaring and invasive pests threatening to diminish yields, producers are looking for new management tactics to protect their crop.

One management tactic that has gained appeal in the past few years is the prophylactic application of both fungicides and insecticides. Organophosphate and pyrethroid insecticides can be used to manage soybean aphid infestations that occur during the plants' reproductive growth stages (Myers et al. 2005). A multi-state, three year study

conducted by Ragsdale et al. (2007) established an economic threshold (ET) for soybean aphid (250 aphids per plant). However, prophylactic application of an insecticide based on a calendar date or plant growth stage eliminates the need for scouting.

Due to the threat of soybean rust, several fungicides have been labeled for use in soybean. In southern soybean producing states, prophylactic fungicide use has been a successful management tactic to limit yield loss (Wrather and Koenning 2006). Strobilurin fungicides in particular are popular choice for prophylactic application as they are associated with numerous non-fungicidal plant health effects. For example, in tobacco (*Nicotiana tabacum*), use of pyraclostrobin was associated with increased resistance to both tobacco mosaic virus and the wildfire pathogen, *Pseudomonas syringae* pv. *tabaci* (Herms et al. 2002). Physiological and developmental changes triggered by strobilurin application to spring wheat (*Triticum aestivum*), winter wheat (*Triticum hybernum*), and barley (*Hordeum vulgare*) cause a greening effect, with delayed senescence and increased grain yields (Grossmann and Retzlaff 1997; Mercer and Ruddock 1998; Glaab and Kaiser 1999; Ypema and Gold 1999; Wu and Tiedemann 2001; Ruske et al. 2003).

Field research on the impact of strobilurin fungicides on soybean yield is limited. Swoboda and Pedersen (2009) found that the application of fungicides (tebuconazole, pyraclostrobin, or tebuconazole and pyraclostrobin combined) in the absence of disease pressure was not associated with an increase in grain quality or yield.

In order to ensure the continued use of the recommended integrated pest management strategy for soybean aphid despite the increasing popularity and perceived benefits of prophylactic pesticide application, it is necessary to establish an economic justification. Johnson et al. (2009) examined the cost-effectiveness of several soybean aphid management

tactics and found that application of an insecticide when aphid populations reached the ET (250 aphids per plant) provided the highest probability of a positive economic return, as compared to an insecticide seed treatment and a prophylactic foliar application of an insecticide-fungicide tank mix applied at growth stage R1 (beginning flowering). However, this study did not examine prophylactic application of pesticides later in the reproductive growth stages of the plant when aphid populations are typically higher.

The objective of our study was to examine the economic efficacy of preventative applications of soybean pesticides and to determine whether plant growth stage based applications of pesticides at growth stage R1 (beginning flowering) or R3 (beginning pod set), as described by Fehr et al. (1971), are an economically sound practice in comparison with the recommended integrated pest management method. We conducted this experiment over three years at multiple locations in the state of Iowa.

Materials and methods

Experimental design

In 2008, 2009, and 2010, small plots were established throughout the state of Iowa to examine early- and mid-season applications of pesticides based on plant growth stage. Plots were 24.5 to 58 m^2 with 76.2 cm row spacing and were arranged in a randomized block design. Aphid-susceptible soybean varieties appropriate to each location were machine planted in mid- to late-May, depending on weather conditions, and treatment application dates varied for all treatments depending on location and year (Table 2.1). An untreated control was compared with a total of four pesticide treatments (one fungicide, two

insecticides, one insecticide-fungicide tank mix) and an IPM treatment based on weekly scouting and a 250 aphid per plant threshold (Table 2.2). For all treatments except the IPM treatment, pesticide application was made at one of two plant growth stages: R1 (beginning flowering) or R3 (beginning pod set); application dates varied for all treatments depending on location and year (Table 2.1). These treatments were tested at six locations in 2008, five locations in 2009, and three locations in 2010. Pesticides were applied using a CO_2 backpack sprayer with a handheld boom with flat fan nozzles calibrated to 187 L ha^{-1} and application rates varied depending on the product used (Table 2.2).

Soybean aphid population and yield determination

Throughout the experiment, naturally occurring soybean aphid populations were sampled weekly using in situ whole-plant counts, based on the method described in Hodgson et al. (2004). The number of aphids per plant between two sampling was then used to calculate cumulative aphid-days (Hanafi et al. 1989), an estimate of the summative seasonal exposure of the soybean plant to soybean aphids. To determine yield, the center two rows of each plot were harvested using a small combine. Seed moisture was adjusted to 13% and yield was converted to kg per ha.

Data analysis

Seasonal soybean exposure to aphids (i.e. CAD) and yield were compared using the PROC MIXED procedure in SAS statistical software version 9.2 (SAS Institute 2008). To meet assumptions of constant variance and normality, cumulative aphid-days for all treatments were natural log transformed. The statistical model defined overall treatment

effects as fixed, while location, year, block, and interaction effects (year×location, year×location×block, and year×location×treatment) were defined as random. Least-square means tests with a Tukey-Kramer correction were used to determine differences in treatment effects and mean separation for each model (CAD and yield) (Table 2.3).

To assess the effectiveness of each management plan, a break-even yield gain analysis was performed to determine whether a treatment resulted in the increased yield volume necessary to recoup the costs of that treatment. Costs of pesticides, application, and scouting services, expected crop price, and expected yield were used to calculate a gain threshold (GT) in kg per ha (Table 3.1). Scouting and application service cost estimates were provided by Iowa State University field crop extension agronomist Clarke McGrath (personal communication) and costs of pesticides were based on 2010 market prices. To account for variability in costs, two GTs were calculated for each management plan. The 'high cost' calculation assumes all pesticides were purchased at retail value and scouting and application each cost $19.77 per ha. The 'low cost' calculation assumes fungicides were purchased at a rebated price and that scouting and application costs were $12.36 per ha and $14.83 per ha, respectively. Based on recent futures prices, three soybean prices ($8, $12, and $16 / 27.2 kg (1 US bushel) were used in this analysis.

The yield impacts of insecticide-fungicide combinations are still poorly defined, therefore, Bayesian statistical methods were used to calculate the probability of each management strategy being cost-effective. These calculations were based on approaches developed by Johnson et al. (2009) and Munkvold et al. (2001). Unlike traditional frequentist methods, Bayesian methods model the uncertainty about parameters using probability distributions, so that prior knowledge (prior distribution) is informed by the

observed data and translated into updated knowledge (posterior distribution). For this Bayesian analysis, a prior distribution is proposed for the treatment means and a non-informative prior distribution is used. These data are combined with the prior distribution to obtain the posterior distribution for the treatment means and any functions thereof, such as the pairwise differences. With non-informative prior distributions, the posterior distributions are scaled t-distributions that are functions of the least-square means and their standard errors. Using these distributions, the probability that the difference in yield between a treatment and the untreated control will exceed the GT at each soybean price can be calculated. This probability was calculated based upon *t(GT)*, a re-centered t-quantile (Equation 1), and derived as the one-tailed probability of a random variable with a t-distribution exceeding *t(GT)* (Equation 2). Calculations were performed using SAS software.

$$t(GT) = \frac{GT - (\bar{y}_t - \bar{y}_c)}{s\sqrt{1/n_t + 1/n_c}} \qquad [1]$$

where *GT* is the gain threshold, $\bar{y}_t$ is the observed mean treatment yield, $\bar{y}_c$ is the observed mean control yield, $\bar{n}_t$ is the number of treatment observations, $\bar{n}_c$ is the number of control observations, and *s* is the pooled standard deviation.

$$P_{net} = 1 - PROBT[t(GT), df_e] \qquad [2]$$

where *t(GT)* is the re-centered t-quantile (Equation 1) and df_e is the error degrees of freedom associated with the pooled standard deviation, *s*.

Results

Aphid exposure and yield

Significant differences in CAD were observed among management tactics across all location-years ($F = 17.98$; $df = 9, 91$; $p = < 0.0001$). Highest levels of aphid exposure occurred in the untreated plots and in plots that received an R1 application of prothioconazole + trifloxystrobin. All management options that included an insecticide reduced seasonal aphid exposure as compared to the untreated control, regardless of when applied (growth stage R1 or R3) or whether a fungicide was included (Table 2.3). Significant differences in soybean yield were also observed among treatments ($F = 14.83$; $df = 9, 633$; $P < 0.0001$). Lowest yields were observed in untreated plots and in plots receiving an R1 application of prothioconazole + trifloxystrobin. Highest yields were observed in plots that received an R3 tank mix application of prothioconazole + trifloxystrobin in combination with imidacloprid + cyfluthrin. There was no evidence of difference in soybean yield among the other treatments.

Break-Even Yield Gain and Cost-Effectiveness Analysis

Increases in crop price resulted in greater probability of recovering incurred treatment costs (Table 3.1). Although the differences in yield among many of the treatments were not statistically significant, the differences were large enough to result in highly variable probabilities of recovering treatment costs. The likelihood of recouping treatment costs was lower for the high cost estimates of all treatments as a greater GT is necessary to recover higher costs. Insecticide (esfenvalerate or imidacloprid + trifloxystrobin) and tank mix

treatments applied at growth stage R3 had the highest probability of earning back the associated treatment costs for both high and low cost estimates (Table 3.2). The prothioconazole + trifloxystrobin applied alone at growth stage R1 had the lowest probability of recovering incurred treatment costs, with between 18% probability (at $8 per 27.2 kg) and 66% probability (at $16 per 27.2 kg) of increasing yields enough to break even at the low cost estimate and only 4% to 49% probability of recovering costs at the high cost estimate.

Discussion

This study sought to characterize the economic viability of a variety of prophylactic management tactics for soybean aphid, comparing them to IPM methods. As Kennedy (2000) said, "IPM technologies and tactics must be cost competitive with, or otherwise offer a clear advantage over, alternative technologies and tactics already in use if they are to be adopted." Soybean aphid IPM has been widely adopted (Olson et al. 2008); however, as row crop agriculture grows more dependent on pesticide use due to pest pressure from invasive species and profit margins fluctuate, it is necessary to reassess the economic viability of pest management recommendations.

We found the IPM method at low estimated cost ($43.54/ha) to have a high probability of resulting in soybean yield gain increases great enough to break even (73% - 97% for $8 to $16 per 27.2 kg soybeans), although the probability of recouping treatment costs was less favorable (45 – 84% for $8 to $12 per 27.2 kg soybeans) for the IPM method at high estimated cost ($55.90/ha). However, at the highest calculated market price for soybean ($16 per 27.2 kg), there was still a high probability (94%) of IPM treatment costs

being recovered. Treatments containing an insecticide applied at the R3 growth stage (esfenvalerate, imidacloprid + cyfluthrin, and tank mix) had probabilities of > 99.9% regardless of high or low estimated treatment cost and soybean market price.

These results were inconsistent with the findings of Johnson et al. (2009), who determined that an IPM approach emphasizing scouting followed by the application of an insecticide only when soybean aphid populations reach an ET, as recommended by Ragsdale et al. (2007), was the most cost-effective method of soybean aphid management. However Johnson et al. (2009) did not examine preventative application of pesticides later in the growing season (after growth stage R1) as we did. Additionally, Johnson et al. (2009) found a much lower probability (63% at $8 per 27.2 kg and 74% at $12 per 27.2 kg) of a preventative application of a tank mix of lambda-cyhalothrin and pyraclostrobin ($58.06/ha) increasing yields enough to recoup costs than probabilities for a similar treatment in our study.

This discrepancy might be attributable in part to insect pressure from other pests (such as Japanese beetle, bean leaf beetle, stinkbug, and grasshopper). These pests were not closely monitored in this study, but were observed in several fields during weekly soybean aphid scouting, particularly in 2010. All of these insects are occasional pests of soybean but rarely cause economic damage. Despite low aphid pressure in 2010, application of an insecticide alone at R3 resulted in yield increases of 204 and 242 kg per hectare for esfenvalerate and imidacloprid + cyfluthrin, respectively (data not shown). These yields were not significantly greater than the untreated control; however, as was observed in this study, even statistically insignificant yield differences can result in sizeable differences in probability of recovering treatment costs.

As previously mentioned, our study was a simplified analysis. It accounted for easily calculable pesticide costs and assumed that application and scouting would be performed by a cooperative for a fixed price. However, it did not account for additional services typically provided by full-service scouting agencies, such as soil nutrient analysis, monitoring weeds, monitoring other insect pests, assessing diseases pressure, and providing consultation services. It did not take into account pest management costs for producers that conduct scouting and application themselves, including time, labor, machinery, and fuel. This trial also used small plots and a backpack sprayer, so yield loss and soil compaction associated with ground application of pesticides were not a concern. In Indiana, Hanna et al. (2007) estimated yield loss ranging from 1.3 – 4.9% (depending on sprayer boom width) due to wheel tracks when pesticide application occurred after growth stage R1. Ground application to fields with hillsides, which are common in Iowa, or application in wet conditions would result in even greater yield loss (D. Mueller, personal communication).

Aside from these tangible omissions, there are difficult to quantify impacts of pesticide overuse, which are a risk when using preventative pesticide applications. Conditions that could lead to ecological backlash are well documented in soybean. After application of insecticides, soybean aphid may be replaced by the two-spotted spider mite (*Tetranychus urticae*), typically a secondary pest. Exposure to imidacloprid, a commonly used insecticide in soybean, can increase the fecundity and longevity of two-spotted spider mites (James and Price 2002). Predators are the most well studied natural enemies of soybean aphid and, under the right conditions, predation can decrease aphid populations and aphid density (Fox and Landis 2003; Fox et al. 2004; Rutledge and O'Neil 2005; Donaldson et al. 2007; Schmidt et al. 2007). However, broad-spectrum insecticides that are typically

used for soybean aphid management are toxic to these natural enemies (Galvan et al. 2005; Ohnesorg et al. 2009). Myers et al. (2005) attributed the increase of soybean population density after the application of a broad-spectrum insecticide to the aphids' rapid reproductive rate and changes in the pest-to-natural enemy ration. Finally, there is the issue of insecticide resistance. At present, soybean aphid has not exhibited any resistance to insecticides, but other major aphid pests, such as *Myzus persicae* and *Aphis gossypii* have developed a variety of modes of resistance to pyrethroid and organophosphate insecticides (Kerns and Gaylor 1992; Moores et al. 1994; Martinez-Torrez et al. 1999).

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Table 3.1. Yield gain thresholds for three soybean prices at high and low estimated treatment costs

Management tactic	Low estimated cost (US$/ha)[b]	Gain threshold (kg ha^{-1}) by soybean price[a] $8	$12	$16	High estimated cost (US$/ha)[c]	Gain threshold (kg ha^{-1}) by soybean price $8	$12	$16
Prothioconazole + trifloxystrobin	44.58	152	101	76	59.31	202	134	101
Esfenvalerate	31.18	106	71	53	36.13	123	82	61
Imidacloprid + cyfluthrin	32.37	110	73	55	37.31	127	85	63
Tank mix[d]	62.12	211	141	106	76.85	261	174	131
IPM	43.54	148	99	74	55.90	190	127	95

[a] Soybean prices in US$/27.2 kg (1 US bushel)

[b] Low estimated costs assumed market price insecticides, rebated fungicides, $14.83/ha for pesticide application, and $12.36/ha for scouting services

[c] High estimated costs assumed market price pesticides, $19.77/ha for pesticide application, and $19.77 for scouting services

[d] Tank mix consisted of prothioconazole + trifloxystrobin and imidacloprid + cyfluthrin

Table 3.2. Probability of yield gain from pesticide treatments exceeding the gain threshold for low and high estimate treatment costs at three soybean prices

	Probability by soybean price per 27.2 kg[a]					
	Low cost estimate			High cost estimate		
Management tactic	$8	$12	$16	$8	$12	$16
R1 prothioconazole + trifloxystrobin	0.18	0.49	0.66	0.04	0.27	0.49
R3 prothioconazole + trifloxystrobin	0.88	0.98	0.99	0.62	0.93	0.98
R1 esfenvalerate	0.84	0.94	0.96	0.77	0.91	0.95
R3 esfenvalerate	1[b]	1	1	1	1	1
R1 imidacloprid + cyfluthrin	0.99	1	1	0.97	1	1
R3 imidacloprid + cyfluthrin	1	1	1	1	1	1
R1 tank mix[c]	0.93	1	1	0.72	0.98	1
R3 tank mix	1	1	1	1	1	1
IPM	0.73	0.93	0.97	0.45	0.84	0.94

[a] 27.2 kg = 1 US bushel

[b] Probabilities of > 0.999 are expressed as 1

[c] Tank mix consisted of prothioconazole + trifloxystrobin and imidacloprid + cyfluthrin

CHAPTER 4.

DEVELOPMENT OF A PCR-RFLP METHOD TO RAPIDLY DISTINGUISH *PANDORA NEOAPHIDIS* AND *CONIDIOBOLUS THROMBOIDES* INFECTING SOYBEAN APHID IN NORTH AMERICA

Rebekah M. Ritson, Matthew E. O'Neal, and Alison E. Robertson

Abstract

Entomopathogenic fungi are considered a promising means for biological control of many insect pests, particularly aphids. Several fungal pathogens of soybean aphid, *Aphis glycines* Matsumura, have been identified in North America, including *P. neoaphidis* and *C. thromboides*. In this study, a PCR-RFLP diagnostic tool was developed to distinguish between the two pathogens. Lab-reared soybean aphids were inoculated with each pathogen and genomic DNA was isolated from resultant cadavers. Amplification and digestion with *Hinf*I of the ITS region of DNA extracted from *C. thromboides*-infected aphids resulted in two fragments of ca. 375 bp and ca. 300 bp. The same process conducted on *P. neoaphidis*-infected aphids resulted in two fragments of ca. 500 bp and ca. 400 bp. This technique could be used to monitor the presence of *P. neoaphidis* and *C. thromboides* in the environment to gain a more complete understanding of entomopathogen ecology.

Introduction

The soybean aphid, *Aphis glycines* Matsumura, is rarely an economically important pest in Asia, its native range. However, since 2000, *A. glycines* has spread throughout soybean producing regions of the Unites States and Canada, establishing itself as a major pest and causing yield losses of > 40% when infestations are left untreated (Ragsdale et al. 2007).

Epizootics caused by fungi frequently occur, consequently, fungal pathogens are considered a promising means of biological control for aphids, particularly in cotton (Steinkraus et al. 1995; Pell et al. 2001). The majority of aphid pathogenic fungi are classified in the order Entomophthorales (Zygomycota) and include *Pandora*, *Zoophthora*, *Entomophaga*, and *Entomophthora* species. Several species of entomopathogens have documented incidences of soybean aphid infection in the United States. Three studies have attempted to quantify the impact of entomopathogenic fungi on soybean aphid populations.

Epizootics in soybean aphid have been reported in several states and provinces, including Georgia, Minnesota, New York, Wisconsin, and Ontario, Canada. Two studies examined and quantified commonly occurring fungal pathogens of soybean aphid in the field (Nielsen and Hajek 2005; Koch 2010). Nielsen and Hajek (2005) identified six species of entomopathogenic fungi infecting *A. glycines* in soybean fields in New York. In order of prevalence, these species were *Pandora neoaphidis* (90.1% of total infections), *Neozygites fresenii* (4.6%), *Entomophthora chromaphidis* (3.8%), *Conidiobolus thromboides* (1.1%), an unidentified *Pandora sp.* (0.4%), and *Lecanicillium lecanii* (0.05%), and *Zoophthora occidentalis* (0.05%) (Nielsen and Hajek 2005). Koch (2010) also found *P. neoaphidis*

(90.1% of total infections) and *C. thromboides* (9.0% of total infections) infecting *A. glycines* in soybean fields and identified *Zoophthora radicans* as an occasional *A. glycines* pathogen (0.9% of total infections). Both studies used bioassays and light microscopy for isolation and identification of the entomopathogenic fungi.

Molecular tools present an exciting opportunity for rapid identification of entomopathogenic fungi in soil and plant samples and from insect cadavers. Such tools have already been used to characterize fungal communities in different ecosystems, determine geographic distribution of fungal isolates, and detect entomopathogenic fungi on insect cadavers or other environmental samples (Schwarzenbach et al. 2007). A few species of entomopathogenic fungi have been extensively studied for interspecific variation; these studies have utilized amplification of the highly conserved ITS region and some have used RFLP and RAPD analyses to detect polymorphisms between species (Hodge et al. 1995; Hajek et al. 1996; Rohel et al. 1997; Sierotzki et al. 2000; Jensen and Eilenberg 2001; Nielsen et al. 2001). Jensen and Eilenberg (2001) developed Entomophthorales-specific primers for the detection of fungi in insect cadavers. Tymon et al. (2004) developed a method of distinguishing *P. neoaphidis* from related entomopathogenic fungi using species-specific diagnostic primers.

In addition to these efforts, molecular tools for the isolation of entomopathogenic fungi from environmental samples have recently been developed for a few frequently occurring fungal species. Detection and quantification of the pathogen *Entomophaga maimaiga* in soil via PCR assay was first attempted by Castrillo et al. (2007). Fournier et al. (2008) developed a diagnostic tool used to detect *P. neoaphidis* spores in the environment, particularly in soil and on plant leaves. Molecular tools that target environmental samples

can be extremely useful for studying entomopathogen life cycles, including overwintering strategies and persistence in the environment, in addition to being used to quantify pathogen prevalence in insect populations. This information could provide valuable insight for the further development of entomopathogenic fungi as biological control agents, particularly in row crop production.

The objectives of this study were to develop a cultivation-independent technique to detect and distinguish between *P. neoaphidis* and *C. thromboides* infections of soybean aphid in Iowa and use the method to monitor the incidence of each pathogen in soybean aphid populations in Iowa in 2009 and 2010.

Materials and methods

Fungal strains and cultivation techniques

Three strains of *Conidiobolus thromboides* (ARSEF 7209, 7210, and 7211) and one strain of *Pandora* neoaphidis (obtained from the University of Minnesota) were grown in 95mm × 15mm Petri dishes (Fisherbrand®, Mediamiser) containing SDAY/4 that were placed in closed plastic boxes at 20°C, ~100% humidity, 24 hr dark. To obtain mycelial mass for DNA extraction, 3-mm plugs were removed from 2-3 week old SDAY/4 cultures and ten plugs were placed in each flask containing 150 ml liquid Sabouraud dextrose media with yeast extract prepared as detailed by Gardner and Pillai (1987). Liquid cultures were placed on a shaker table and incubated for 5 to10 days at room temperature (22°C, 100 rpm, 24 hr dark). Mycelia from all liquid cultures was harvested using vacuum filtration through Whatman filter paper and stored at -20°C.

Aphid inoculation with *P. neoaphidis* and *C. thromboides*

Lab-reared soybean aphids, initially obtained from naturally infested plants in Iowa, were transferred onto excised soybean leaves. All leaves were obtained from aphid-susceptible cultivars and were surface sterilized using 70% ethanol prior to aphid transfer. The stem of the leaf was inserted into florists' foam (Smithers-Oasis Company, Cuyahoga Falls, OH), which was hydrated with sterile distilled water. The leaf was placed in a Petri dish and aphids were monitored for three days for evidence of contamination (disease and presence of thrips or whiteflies). If no contaminants were present, a Petri dish containing an actively sporulating culture of *C. thromboides* or *P. neoaphidis* was inverted over an open Petri dish of soybean aphids and the aphids exposed to sporulating cultures for 0.5, 1, 4, 8, and 12 h. The culture was then removed and the leaf with aphids was sealed inside a petri dish using Parafilm (Pechiney Plastic Packaging, Chicago, IL) to ensure high relative humidity levels. Aphids were monitored for five days post-exposure for mortality or signs of infection. Infected aphid cadavers were removed from the leaf for DNA extraction, in batches of 10 to 15 aphids.

Establishing sentinel aphid colonies and screening for entomopathogens

Sentinel colonies of *A. glycines* were established in Iowa at five locations in 2009 and three locations in 2010. To ensure establishment of aphid colonies and eliminate predation by aphidophagous natural enemies, a single exclusion cage was placed in a center row of each untreated plot. Three soybean plants in each plot were caged using a tomato cage (0.4 diameter, 1 m height) with two metal garden stakes zip-tied to the tomato cage for support.

Cage covers, made of fine mesh no-see-um netting (Balson-Hercules, New York, NY) were sewn to fit the tomato cage, placed over the cages and buried at a depth of 4 – 5 cm. Any insects inside the cage were removed before plants were infested with fifteen *A. glycines* from the established laboratory colony in late June. If aphid colonies did not establish, cages were re-infested one to two additional times. From 13 July 2009 to 21 August 2009 and from 12 July 2010 to 20 August 2010, one to two leaves containing *A. glycines* from the middle or upper canopy were arbitrarily removed from each cage at weekly intervals. Depending on aphid density within the cage, 10 to 50 live aphids from each plot were desiccated, then stored in 95% ethanol/ 2% glycerol at -20°C until DNA extraction.

DNA extraction

Genomic DNA from inoculated aphids and mycelia of *P. neoaphidis* and *C. thromboides* was extracted using the DNeasy Plant Mini Kit (Qiagen, Basel, Switzerland)**.** Samples of DNA from these sources were processed differently prior to extraction. To extract DNA from mycelia, 0.1-0.5 g of frozen mycelial mass was placed in liquid nitrogen and ground to a fine powder with a mortar and pestle. To extract DNA from aphid cadavers, 10 to 35 lyophilized aphids were suspended in 10 µl of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen Carlsbad, CA, USA) and macerated with an Eppendorf® micropestle (Eppendorf, Hamburg Germany), vortexed for 10 s, and remacerated. DNA was also extracted from uninfected, lab-reared aphid cadavers for use as a control.

PCR and ITS region sequencing

The ITS 5/4 primer pair developed by White et al. (1990) was synthesized by the Iowa State University DNA Facility using a MerMade-192 synthesizer (BioAutomation, Plano, TX, USA). This primer pair amplified the 3' end of the small sub-unit (SSU), the ITS1, 5.8S, and ITS2 regions and the 5' end of the large sub-unit (LSU) of the ribosomal gene. PCR amplifications were performed in thin wall PCR tubes with attached caps (BrandTech Scientific, Essex, CT, USA). Each 50 μl reaction contained 200 mM of each dNTP, 0.2 mM of each primer, 0.5 U Taq DNA polymerase (Bioline, Tauton, MA) in 1× reaction buffer with 2 μl of template DNA extracted from mycelia and 5 μl of template DNA extracted from aphids (concentration between 25-50 ng/μl). Negative controls containing sterile water were also included. Reactions were run in an Endurance TC-412 thermal cycler (Techne, Cambridge, UK). The thermal cycling conditions were as follows: one cycle of denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 1.5 min, with a final extension at 72°C for 5 min. For DNA concentrations below 40 ng/ μl, 5 additional cycles were run.

Eight µl aliquots of PCR products were separated by agarose gel electrophoresis (1.5% wt/vol) in 1× TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA; pH 8) with 1 Kb and 100 bp (Invitrogen) size markers. Gels were stained with ethidium bromide (0.1 g ml^{-1}) and photographed using the GelDoc EQ (Bio-Rad Laboratories, Hercules, CA, USA). When the correct product was detected, products were purified with the Qiaquick PCR product purification kit (Qiagen). Sequencing reactions were analyzed by Iowa State University's DNA Facility with the ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

ITS-RFLP analysis *in silico*

Complete ITS region sequences from *P. neoaphidis* and *C. thromboides* were tested *in silico* using EnzymeX (Mek & Tosj, Aalsmeer, The Netherlands) for restriction analysis with eight restriction enzymes. Due to the lack of availability of ITS sequences for most soybean aphid entomopathogens in the United States, available ITS sequences of closely related entomophthoralean species obtained from GenBank were also used (Table 4.1). Based on the results of the *in silico* simulations, two restriction enzymes, *Hinf*I and *Swa*I (New England Biolabs, Ipswich, MA, USA), were selected for testing *in vivo*.

ITS-RFLP analysis *in vivo*

Aliquots of 15 µl of ITS-region DNA amplified from mycelial extraction were used for restriction digestion in 30 µl reactions. Reactions of *Hinf*I contained 5 or 10 U of enzyme in 1 × reaction buffer. Reactions of *SwaI* contained 5, 10, and 15 U of enzyme in 1 × reaction buffer were also used. Digests of *Hinf*I were incubated at 37°C for 4 hours and digests of *Swa*I were incubated at 25°C for 8 hours. RFLP digests were separated by gel

electrophoresis (2% wt/vol) in 1 × TBE.

Results

Aphid inoculation with *P. neoaphidis* and *C. thromboides*

The infection rate of aphids with either pathogen for lower exposure times was poor (Table 4.2). Regardless of exposure time, aphids exposed to *C. thromboides* exhibited a slightly higher infection rate than aphids exposed to *P. neoaphidis*. *C. thromboides* is a faster-growing fungus than *P. neoaphidis*, therefore, the total diameter of *C. thromboides* cultures was greater when cultures began sporulating, allow ejection of spores onto a greater area of the excised soybean leaf and aphids below the culture. Infected aphids that were exposed for 12 hours were used as positive controls in the molecular work.

PCR and ITS region sequencing

The ITS regions of both *P. neoaphidis* and *C. thromboides* were successfully amplified using the ITS 5/4 primers developed in Tymon et al. (2004) (Figure 4.1). The presence of aphids in the PCR reactions did not inhibit formation of a PCR product or restriction enzyme activity (Figure 4.1). No size polymorphisms were detected between the three strains of *C. thromboides*. Amplification of the complete ITS region gave sizes of ca 900 bp for *C. thromboides* ARSEF strains 7209, 7210, and 7211 and ca 1100 bp for the *P. neoaphidis* isolate from Minnesota. . Eight hundred bp segments of the ITS regions of the three *C. thromboides* strains were sequenced.

ITS-RFLP analysis *in silico*

Results of *in silico* testing revealed interspecific variation for many of the entomophthoralean species tested (Table 4.3). *In silico* testing for species with multiple ITS region sequences available revealed no variability within species. Based on these results and enzyme cost, two enzymes, *Hinf*I and *Swa*I, were selected for *in vivo* analysis.

ITS-RFLP analysis *in vivo*

Using *Hinf*I, restriction analysis revealed interspecific variation between *P. neoaphidis* and *C. thromboides* as anticipated by simulation *in silico* (Figure 4.2). Digests of *C. thrombroides* using the enzyme *Hinf*I produced two fragments of ca. 375 bp and ca. 300 bp (Figure 4.2). No variability was detected among the *C. thromboides* isolates (data not shown). Digests of *P. neoaphidis* resulted in two fragments of ca 500 bp and ca 400 bp (Fig. 2). *Swa*I did not successfully cut ITS amplicons, despite the use of a range of concentrations of enzyme (data not shown).

Detection of *C. thromboides* and *P. neoaphidis* in aphids collected from sentinel plots

Amplification of the ITS region from aphids collected from the sentinel plots was not successful. Consequently restriction analysis with *Hinf*I was not possible.

Discussion

In this study, we developed a PCR-RFLP based approach for distinguishing between two of the four most commonly occurring soybean aphid pathogens in the United States, *C.*

thromboides and *P. neoaphidis.* Although some species-specific primers exist, these are mostly limited to very common entomopathogenic fungi and no species-specific primers have been developed for *C. thromboides.* By using a universal primer pair, testing of environmental samples can be accomplished with only one amplification and one digestion per sample. *In silico* restriction analyses allowed identification of the most appropriate and cost effective restriction enzymes to differentiate between the species of interest.

Although digests with *Swa*I were unsuccessful, restriction analysis of *Hinf*I products was able to distinguish between *C. thromboides* and *P. neoaphidis*. However, complete ITS region sequence information for all *A. glycines* entomopathogens as determined by Nielsen and Hajek (2005) and Koch et al. (2010) is unavailable, particularly *E. chromaphidis*, therefore this analysis needs to be substantiated to determine if digestion of the ITS region with *Hinf*I can distinguish *C. thromboides* and *P. neoaphidis* from other reported entomopathogens. Furthermore, in order for the tool developed in this study to be used to detect the presence of *Zoophthora* spp., a different primer for the ITS region would need to be developed. Tymon et al (2004) reported the ITS 5/4 primer set used in this study was not effective on two *Zoophthora* species, *Z. phalloides* and *Z. occidentalis,* due to base dissimilarity at one or both primer sites when amplification was attempted. Previous studies have reported *Zoophthora* spp. account for <1% of total infections of soybean aphid in the United States (Nielsen and Hajek 2005; Koch et al. 2010).

Finally, extraction of DNA from aphid cadavers inoculated with *P. neoaphidis* and *C. thromboides* in the lab using the DNeasy Plant Mini Kit was successful, typically yielding 25-60 ng/ µl of DNA. Amplification of DNA extracted from aphids collected from sentinel plots did not yield detectable quantities of target DNA using gel electrophoresis. Every

effort was made to optimize reaction conditions, including altering reaction concentrations of Mg^{2+}, dNTPs, primers, and *Taq* polymerase, and an additional 10 cycles of amplification. There are several possible causes of the unsuccessful amplification. One possibility is that PCR inhibiting factors were co-extracted from environmental samples, as reported by Wilson (1997). However, when target DNA was spiked with either DNA extracted from lab-reared aphids that had been exposed to fungal pathogens or DNA from harvested mycelia and amplification of the ITS region was successful, indicating no presence of PCR inhibiting factors in the field-collected aphid samples. Another possible explanation is that the target DNA from entomopathogens was not present or was present at such low levels that it was undetectable. Collection of soybean aphids from an irrigated field in Minnesota during 2005 and 2006 found 4.6% and 0.7% of the population to be infected with entomopathogenic fungi (Koch et al. 2010). Aphids collected from another location in Minnesota during the same years exhibited an infection rate of <1% (Koch et al. 2010). Fournier et al. (2008) reported successful extraction and amplification of *P. neoaphidis* DNA from single aphid cadavers using species-specific primer pairs. Over all the years of this study and sample locations, 5,560 aphids were tested using the molecular technique. If a rate of infection similar to that seen by Koch et al. (2010) had occurred, between 39 and 262 aphids should have been infected with entomopathogenic fungi. This leads us to conclude that the presence of *P. neoaphidis* and *C. thromboides* in soybean aphid populations in Iowa was extremely low during the period of this study.

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Table 4.1. List of ITS sequences retrieved from GenBank and used to generate sequence alignments

Species	**Strain**	**Accession number**
Basidiobolus ranarum	MCCL W6	AY211271
Conidiobolus coronatus	P1	AJ345094
Conidiobolus thromboides	ARSEF 7209	Sequenced as part of this research
Conidiobolus thromboides	ARSEF 7210	Sequenced as part of this research
Conidiobolus thromboides	ARSEF 7211	Sequenced as part of this research
Entomophaga aulicae	FPMI 646	U35394
Pandora kondoiensis	ARSEF 828	AF543199
Pandora kondoiensis	ARSEF 5707	AF543200
Pandora kondoiensis	ARSEF 5708	AF543201
Pandora neoaphidis	NW 343	AF543202
Pandora neoaphidis	NW 356	AF543203
Pandora neoaphidis	NW 195	AF543204
Pandora neoaphidis	NW 283	AF543205
Pandora neoaphidis	NW 316	AF543206
Pandora neoaphidis	NW 327	AF543207
Pandora neoaphidis	NW 415	AF543208
Pandora neoaphidis	ARSEF 835	AF543209
Pandora neoaphidis	ARSEF 1609	AF543210
Pandora neoaphidis	ARSEF 5374	AF543211

Table 4.2. Percent of infected aphids by length of exposure to sporulating fungal cultures

	Percentage of infected aphids by length of exposure				
Species	0.5 h	1 h	4 h	8 h	12 h
P. neoaphidis	<1%	1.3%	3.0%	5.4%	9.1%
C. thromboides	<1%	1.5%	3.4%	6.1%	9.9%

Table 4.3. Results of *in silico* restriction enzyme analyses using EnzymeX on entomophthoralean ITS sequences with the number and size of fragments generated

	Restriction enzymes tested Number of fragments(size of fragments in bp)						
Species	*Cla*I	*Dpn*I	*Hinf*I	*Mae*I	*Mbo*II	*Sau3*AI	*Swa*I
B.[a] *ranarum*	2(351; 199)	5[b]	3(290; 252; 8)	2(469; 81)	2(334; 216)	5	4(292; 117; 82; 59)
C.[c] *coronatus*	2(347; 341)	2(391; 324)	4(237; 167; 156; 155)	0	3 (357; 184; 174)	2(393; 322)	3(374; 204; 137)
C. thromboides	2(482; 364)	2(499; 347)	3(416; 371; 59)	3 (411; 364; 71)	2 (465; 381)	2(501; 345)	2 (482; 364)
E.[d] *aulicae*[e]	4	11	17	17	10	11	9
P.[f] *kondoiensis*	2(999; 475)	3(982; 430; 62)	3(529; 522; 422)	5	5	3(980; 432; 62)	4 (895; 417; 104; 58)
P. neoaphidis	2(660; 440)	3(457; 365; 278)	3(504; 388; 208)	5	2(677; 423)	3(459; 363; 278)	5

[a] *Basidiobolus*

[b] Due to the large number of fragments generated for these digests, fragment sizes for digests with ≥5 are not listed

[c] *Conidiobolus*

[d] *Entomophaga*

[e] Due to the large number of fragments generated for these digests, fragment sizes for *E. aulicae* are not listed

[f] *Pandora*

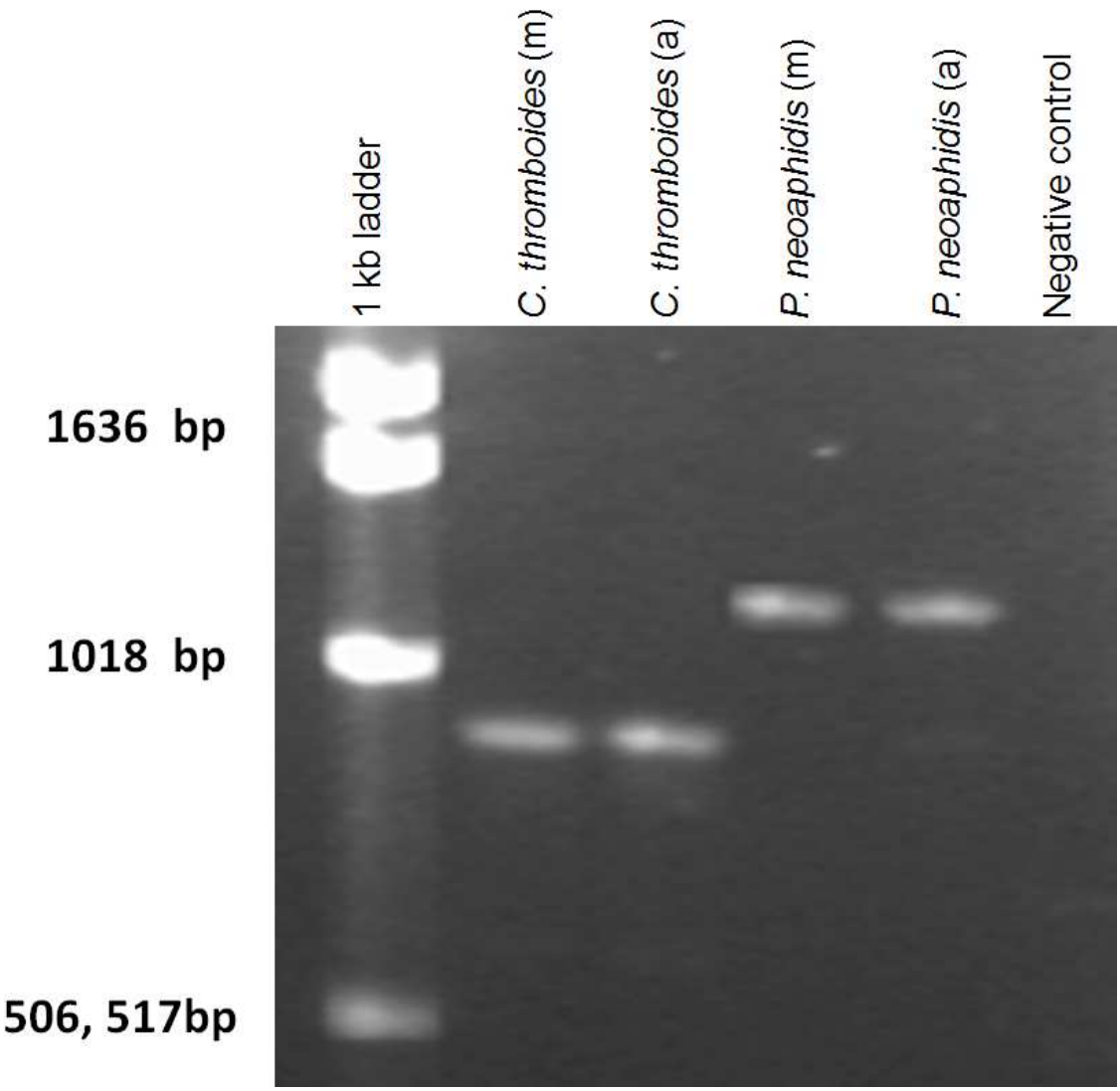

Figure. 4.1. Amplification of ITS regions of *P. neoaphidis* and *C. thromboides* from mycelia (m) and infected aphids (a) using universal primer set ITS 5/4. Size marker = 1 kb.

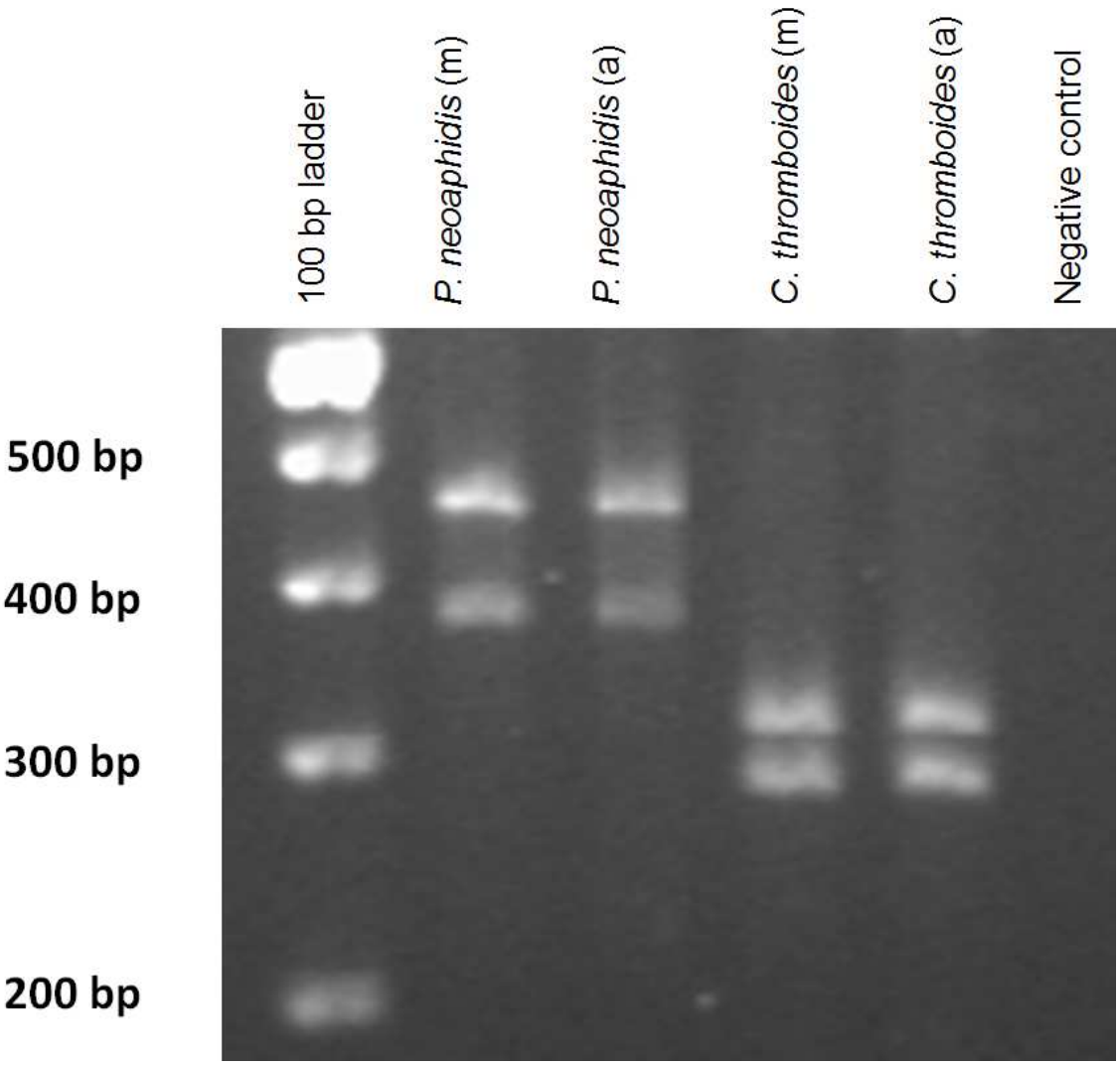

Figure. 4.2. RFLP analysis of ITS regions of *P. neoaphidis* and *C. thromboides* digested with *Hinf*I. Size marker = 100 bp.

CHAPTER 5.

SUMMARY

General conclusions

Based on the findings of this study and previous studies conducted at the University of Minnesota, a single application of a fungicide or insecticide-fungicide tank mix to soybean does not appear to cause increases in aphid population. However, given the high cost of fungicides and application, return on investment for such prophylactic fungicide application is rare. This research confirmed the efficacy of application of an insecticide based on IPM recommendations. At present, few studies have examined a growth stage-based application of insecticides or insecticide-fungicide tank mixes. Based on this research, growth stage-based application of insecticides or insecticide-fungicide tank mixes at R3 can provide soybean aphid population suppression and yield protection on par with, or superior to, applications according to IPM guidelines. Yield gains associated with these treatments are large enough to pay off treatment costs.

In terms of detecting entomopathogenic fungi of soybean aphid, molecular methods using Entomophthorales-specific primers are a promising possibility. PCR-LP and RFLP methodologies have been developed for the detection of the most common soybean aphid pathogen, *P. neoaphidis.* The method developed in this study was useful in distinguishing between *P. neoaphidis* and another pathogen *C. thromboides.* However, this method is still cultivation-dependent, as detection of low levels of *C. thromboides* in environmental samples proved difficult.

Future research

To more fully understand the overall impacts of prophylactic pesticide use, a number of experiments could be performed. Development of *A. glycines* resistance to a variety of commonly used insecticides could be pursued. Soybean aphid natural enemy population presence and absence, specifically during and after pesticide applications at soybean growth stage R3 –R4, could be used to investigate the possibility of late season aphid resurgence in the absence of natural enemies. Field trials to determine yield loss associated with ground application of pesticides in various conditions across the state of Iowa could be examined to calculate more specific loss estimates, which could be used to calculate more precise economic analyses.

Additional trials with IPM applications that successfully suppress aphid populations below the ~5,500 CAD threshold at which yield damage is observed Further investigation into the effects of extremely high aphid populations (>1,000 aphids per plant) at plant stages R5-R7 on soybean yield could help fine tune existing IPM treatment recommendations.

Given the previous success of similar detection methods for a variety of related entomopathogenic fungi and the success of restriction analysis of positive controls (*P. neoaphidis* and *C. thromboides*), use of *Hinf*I as a tool to detect and differentiate these entomopathogens. For continued development of a PCR-RFLP tool to detect common North American soybean aphid pathogens, DNA isolation and amplification from environmental samples must be improved. Further attempts at amplification of target DNA from environmental samples could make use of enzymes or master mixes (e.g. DreamTaq™ DNA Polymerase or High Fidelity PCR Enzyme mix) with higher sensitivity than the *Taq* DNA

polymerase used in this study. A larger quantity of field-collected aphids could be used for each DNA extraction in order to potentially yield greater quantities of the target DNA. A different DNA extraction method, such as a modified CTAB method could be used to although trace amounts of some of the reagents for this protocol, such as phenol, are known to inhibit thermostable DNA polymerases.