# The Role of Entomopathogenic Fungi in the Management of Soybean Aphid

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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July 2011

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**Acknowledgements**

I would like to thank my adviser, Dr. David Ragsdale for his guidance and support. Without his encouragement, I would not have started down the path which led to this achievement. I would also like to thank my committee members, Dr. Tim Kurtti, Dr. Bill Hutchison, Dr. George Heimpel, and Dr. Jim Kurle, for sharing their time, insights, and knowledge.

I would especially like to thank Dr. Roger Moon whose generosity with his time, expertise, and statistical skills have helped me to become a better scientist. I would also like to acknowledge the help of Dr. David Andow. His expertise was indispensable for the completion of chapter 2.

My gratitude to my labmates Ms. Thelma Heidel and Ms. Diana Castillo for their advice and support. Thank you to Wally Rich, Alex Hacker, Andrea Dittmer, Barrett Srstka, Jason Zabeli, Nathan Drasler, Jimmy Ennen, Cammi Michaelson, and Dan Fischer who spent their summers counting aphids so that I could complete this research.

I would like to acknowledge the help of Kimon Karelis and everyone at the University of Minnesota Outreach, Research and Education Park and Ron Faber and Scott Garvin from the Sand Plains Research Farm for their valuable help with my field research.

I am grateful for the financial support of the NSF IGERT: Risk Analysis for Introduced Species and Genotypes (NSF DGE-0653827) and the University of Minnesota's Doctoral Dissertation Fellowship. I would also like to acknowledge the Minnesota Soybean Research and Promotion Council, and the North Central Soybean Research Program for funding my research.

Finally, my deepest gratitude to my husband, Eric, for his ceaseless interest in what I do and his unquestioning support of my academic endeavors. Thank you also to my parents, Bill and Dawne Koch, whose love, encouragement, and patience have made this acheivement possible.

## Dedication

This is dedicated to John P. Koch, whose final words to me are a constant reminder of what I am capable of and to Wayne Koch Jr. – I wish we had more time.

**Abstract**

Soybean aphid, *Aphis glycines* Matsumura, is an invasive arthropod pest of soybean which has been present in North America since 2000. Aphid outbreaks cause economic damage via increased insecticide use and reduced yield. Management of this pest has been achieved almost exclusively with foliar applications of broad spectrum insecticides. The purpose of this research is to determine how a fungal pathogen of soybean aphid, *Pandora neoaphidis*, can contribute to the natural regulation of aphid populations. *Pandora neoaphidis* is an endemic aphid pathogen and the most frequently reported pathogen infecting soybean aphid. Analysis and field validation of a mathematical model describing the soybean aphid-*P. neoaphidis* pathosystem has shown that the fungus can establish and persist when aphid densities are lower than the economic threshold of 250 per plant; thus confirming that *P. neoaphidis* is capable of contributing to aphid control before aphids reach damaging densities. Additionally, field experiments demonstrate that emerging aphid management tactics including aphid-resistant soybean plants and insecticide seed treatments have minimal impacts on aphid infection rates by *P. neoaphidis* and are likely compatible with this natural enemy. Cage studies demonstrate that the presence of *Harmonia axyridis* in soybean aphid colonies also harboring *P. neoaphidis* has no impact on aphid infection by the fungus. *Pandora neoaphidis* may also be an important regulator of aphid populations on the aphids' primary host, *Rhamnus cathartica*, or common buckthorn, as soybean aphid morphs which occur on this host are the most susceptible to infection. However, fungicide applications to soybean can have lasting impacts on the beneficial fungi infecting soybean aphid as foliar fungicide applications to soybean reduced the rate of aphid infection both before and after aphids moved to the primary host in the autumn. In summary, *P. neoaphidis* is likely an important member of the natural enemy community which provides a significant level of aphid biological control.

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# Chapter 1: A Review of Soybean Aphid Ecology and Management, with Particular Emphasis on Biological Control with Entomopathogenic Fungi

## Introduction

Cultivated soybean, *Glycine max* (L.) Merrill, is a major U.S. crop valued at nearly $32 billion with over 30 million ha harvested in 2009 (NASS 2010). Therefore, the introduction of soybean aphid dealt a devastating blow to soybean production, especially across the North Central region, where soybean production is concentrated. Soon after the aphid's introduction, it became apparent that growing soybean in North America would require more input of time and money in order to avoid economic losses to this pest. Researchers immediately began looking for alternatives to chemical control, with most of their effort focusing on biological control with arthropod natural enemies. While many studies have found that insect predators can be important regulators of soybean aphid populations (see section 4.2.4), only three papers to date have been published on soybean aphid's fungal natural enemies (Nielsen and Hajek 2005; Noma and Brewer 2007; Koch et al. 2010). The purpose of this literature review is to summarize the necessary information about soybean aphids, their fungal pathogens, and integrated pest management (IPM) to lay the groundwork for studies reported as part of this dissertation on the role of entomopathogenic fungi in the management of soybean aphid.

## Soybean Aphid

Soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), is an invasive, arthropod pest of cultivated soybean first discovered in North America in Wisconsin in 2000 (Ragsdale et al. 2004). In the year of its initial discovery, the aphid was found infesting soybean in 10 North Central states (Ragsdale et al. 2010). Since 2000, the aphid has invaded an additional 20 U.S. states and 3 Canadian provinces (Ragsdale et al. 2011). It is the most important arthropod pest of soybean in the North Central U.S. and has significantly altered soybean production in this region.

### Lifecycle

Soybean aphid is a heteroecious, holocyclic aphid, meaning it alternates between two hosts and it undergoes both asexual and sexual reproduction (Ragsdale et al. 2004). The primary host is the overwintering host and where sexual reproduction occurs. For soybean aphid, the most suitable primary hosts are *Rhamnus* species, which in North

America, includes *R. cathartica*, *R. alnifolia*, and *R. lanceolata* (Voegtlin et al. 2004, 2005; Yoo et al. 2005). A recent study indicates that a particular soybean aphid biotype, biotype 3, is able to utilize *Frangula alnus* (=*R.* frangula) as a primary host (Hill et al. 2010). The most commonly utilized primary host is *R. cathartica* or common buckthorn, which is itself an invasive species. The most important secondary host of soybean aphid is cultivated soybean (Ragsdale et al. 2004) because of its ubiquity and economic importance (NASS 2010). However, studies have shown that soybean aphid can utilize other species of Fabaceae and Solanaceae including red clover, *Trifolium pratense* (Ragsdale et al. 2004), and horsenettle, *Solanum carolinense* (Clark et al. 2006).

In the spring, overwintering soybean aphid eggs on the primary host hatch giving rise to a single fundatrix. The hatching usually corresponds with buckthorn bud break (Bahlai et al. 2007). This female is parthenogenic, meaning she reproduces asexually, depositing live nymphs. Several parthenogenic generations follow and with each subsequent generation, the proportion of alate, or winged, aphids increases. After three to four generations on buckthorn, winged spring migrants leave the primary host to colonize the secondary host, soybean. Early infestations on soybean are composed almost exclusively of wingless virginoparae which forfeit their migration ability (i.e. wings) in order to devote more energy to reproduction. Evidence of this is a soybean aphid doubling time of 1.5 days and total fecundity of 61 nymphs per female on soybean under ideal temperature conditions (25°C) (McCornack et al. 2004); thus leading to exponential population growth.

As aphid populations on soybean increase, summer migrants are produced. Summer migrants are winged virginoparae which can migrate to new soybean plants once their current host plants become over crowded (Lu and Chen 1993). Such migration can cause late season infestations in areas where the aphid cannot overwinter (Ragsdale et al. 2004). When the photoperiod shortens to below 14.5:9.5 (L:D), proportion of aphids becoming alatae increases (Hodgson et al. 2005). It is at this time that sexual migrants begin to be produced, most likely after a cool night (i.e. below 5°C) (D.W. Ragsdale, personal communication). Sexual migrants, or gynoparae, are winged females which leave soybean and return to buckthorn in the late summer and early fall (Dixon 1977). Once on buckthorn, the gynoparae produce oviparae, the egg-laying morph. Alate males

are produced on soybean approximately two weeks after the gynoparae, allowing time for the oviparae to mature. Oviparae mate with males and produce eggs, the overwintering stage of the aphid, which are deposited along the buckthorn buds (Dixon 1977).

**Ecology and Population Dynamics**

Soybean aphids interact closely with their hosts, natural enemies, other soybean pests and their environment. Each of these can become a factor which influences the aphids' population dynamics, which in turn, can impact management efforts. Since the aphid's introduction, much research has been performed in order to elucidate the role of soybean aphids in the ecology of the soybean agroecosystem and much has been uncovered through this research. Much less is known about the ecology of soybean aphid on its overwintering host, common buckthorn.

Soybean aphid populations in North America have been documented to have low genetic polymorphism, with all but one examined microsatellite locus having only two alleles per population (Michel et al. 2009). However, variations among aphid populations have been documented and three soybean aphid biotypes identified. The first soybean aphid biotypes were identified in 2008 when experiments by Kim et al. (2008) demonstrated that a soybean aphid population from Ohio was able to overcome aphid resistance caused by the *Rag1* gene, while a population from Illinois was susceptible to the resistance. The Illinois population became soybean aphid biotype 1 and the Ohio population became biotype 2. In 2010, biotype 3 was identified from a soybean aphid isolate collected from *Frangula alnus*, the apparent overwintering host, in Springfield Fen, IN (Hill et. al. 2010). Soybean aphids belonging to biotype 3 are distinguishable from biotypes 1 and 2 in that aphids belonging to biotype 3 are able to overcome aphid resistance conferred by the *Rag2* gene and can successfully overwinter on *F. alnus* (Hill et al. 2010). Biotype 3 aphids were also observed to be less susceptible to *Rag1* resistance (Hill et al. 2010). Identification of these biotypes will have important implications for the release of aphid resistant soybean cultivars. With the identification of aphid populations which can overcome two aphid resistance genes before the genes had been deployed on a large scale indicates that the utility of these resistance genes will be short-lived. Although stacking of multiple resistance genes has been an effective strategy in other crop plants, the ability of biotype 3 to overcome not only *Rag2*

resistance, but also, to some extent, *Rag1* resistance indicates that this strategy may not be effective. Thus, the search for more durable resistance genes must continue if host plant resistance is to remain a viable option for soybean aphid management.

Generally, aphids require symbiotic bacteria in order to obtain nine of the 20 essential amino acids, which they are unable to produce themselves (Douglas 1998). Soybean aphids have such a symbiotic relationship with *Buchnera aphidicola* and *Arsenophonus* sp. (Wille and Hartman 2009). While *B. aphidicola* is a common endosymbiont of aphids, soybean aphid is the first aphid species found to harbor an *Arsenophonus* species, which has only been identified in whiteflies (Wille and Hartman 2009). It is currently unclear how this unusual combination of endosymbionts might influence the ecology and management of soybean aphid.

Aphid morph and behavior can have an impact on soybean aphid population dynamics. A study by Zhang et al. (2009) indicated that there is a direct trade-off between flight and fecundity in soybean aphid. Their experiments demonstrated that alates engaging in long flights (>1.5 km) had a lower fecundity than alates engaging in flights of <0.5 km. This effect extended to the next generation with the offspring of alates experiencing long-distance flights depositing fewer nymphs than offspring of alates experiencing short flights (Zhang et al. 2009).

Soybean aphids follow a typical pattern of distribution and growth on soybean over the course of the growing season. Aphid colonization of a soybean field has been shown to be associated with the presence of overwintering hosts within 4 km of the field; however, this association was only observed in years when aphid densities were low (Bahlai et al. 2010). These initial infestations lack an edge effect because of the behavior of foundress alates as they move through a soybean patch (Ragsdale et al. 2004; Welsman et al. 2007). Spring migrants will locate a suitable host plant, alight, deposit nymphs and then move on to the next host plant where she will deposit more offspring. This behavior leads to a patchy distribution throughout an infested soybean field. Over time, this patchy distribution will coalesce leading to widespread infestation. Founding aphid colonies will remain at the new growth of the soybean plant while it remains in the vegetative stages (McCornack et al. 2008). As soybean moves into the reproductive stages, aphids tend to become more evenly distributed among plant nodes (McCornack et

al. 2008). At this time, aphids also change in their size and appearance. While soybean is in vegetative growth, aphids are typically large and bright green and have a high reproductive output. As soybean moves into reproductive growth, soybean aphids become smaller and paler in color and likely experience a reduced fecundity (Ragsdale et al. 2004). Causes of such changes in distribution and aphid appearance are unknown; however, changes in host plant quality, environmental factors such as rain events, action of natural enemies, or migration by alates have been suggested as possibilities (McCornack et al. 2008).

Aphids live and reproduce in close association with their host plants. It is not surprising, therefore, that bottom-up effects help explain soybean aphid dynamics (Costamagna et al. 2007). Soybean plants have a mutualistic relationship with nitrogen-fixing rhizobia in which rhizobia bacteria make nitrogen available to the plant while the plant provides organic acids to the bacteria. In a study examining how rhizobia may impact aphid abundance, Dean et al. (2009) inoculated soybean seed with commercially available rhizobia, allowed soybean to associate with naturally-occurring rhizobia, or fertilized soybean with nitrogen. They found that aphid densities were 50-54% lower on soybean associated with naturally-occurring rhizobia when compared to the inoculated or fertilized plants; however, other metrics were similar among the treatments (Dean et al. 2009). While the underlying causes of the differences in aphid densities are unknown, selection of rhizobia species or strain may be an important factor to consider if commercial inoculants are used and soybean aphid infestation is likely.

Soybean plant stage has been noted to impact soybean aphid populations across multiple studies (Ragsdale et al. 2004; McCornack et al. 2008), with younger plants in the vegetative stages leading to more rapid population expansion. Van den Berg et al. (1997) found that soybean aphid fecundity and survival were highest on 3 week-old plants and then declined linearly as plants aged. However, a study by Rutledge and O'Neil (2006) found that planting date and soybean growth stage had no impact on soybean aphid population dynamics or life history traits. In a related study, soybean maturity group has been found to have little impact on soybean aphid populations (Rhainds et al. 2010a). However, planting earlier maturing soybean could reduce

gynoparae populations if leaf abscission occurs before gynoparae are produced (Rhainds et al. 2010a).

Host plant nutrient composition is a likely cause of changes in soybean aphid dynamics. Potassium deficiency has been shown to increase soybean aphid densities via increased reproductive rate and survivorship in lab experiments; however, this effect was not observed in small plot field experiments with high, medium, and low potassium plots (Myers et al. 2005). The authors did observe that the study plots had much higher aphid populations than surrounding fields, supporting the hypothesis that potassium-deficient soybean may have caused increased aphid migration into the field leading to inflated aphid densities in all plots. Walter and DiFonzo (2007) were able to demonstrate impacts of potassium-deficient host plants in field studies, which confirmed the laboratory observations of Myers et al. (2005). When grown in potassium deficient soils, plants receiving potassium inputs had lower aphid densities than those without added potassium due to a quicker onset of reproduction and higher net fecundity (Walter and DiFonzo 2007). Asparagine was the only essential amino acid significantly impacted by potassium deficiency. Increased levels of asparagine in potassium deficient plants may have made nitrogen more available to aphids, leading to the observed increase in aphid populations (Walter and DiFonzo 2007). These results were confirmed in a larger scale study which found a negative association between aphid density and potassium levels in leaves and a positive association between aphid density and nitrogen levels in leaves (Noma et al. 2010). Avoiding potassium deficiency may aid in preventing economic losses due to soybean aphid infestations. In another study, soybean exposure to elevated carbon dioxide levels, expected as climate change proceeds, lead to an increase in soybean aphid densities (Dermody et al. 2008). However, when soybean plants were exposed to elevated ozone levels soybean aphid populations remained unchanged, suggesting changes in climate may increase the intensity of herbivore damage in the soybean system.

Temperature is likely one of the most important abiotic factors which influence the population dynamics of soybean aphid. The lower developmental threshold of soybean aphid has been calculated at 8.6°C and the upper developmental threshold is estimated to be 34.9°C (McCornack et al. 2004) under constant temperature in growth

chambers. Thus, temperatures must fall between these thresholds in order for soybean aphids to continue development. McCornack et al. (2004) studied soybean aphid demography at 20, 25, 30, and 35°C. Net fecundity was highest, 64 offspring per female, at 20°C, while the intrinsic rate of increase was highest, 0.474 $d^{-1}$, at 25°C (McCornack et al. 2004). Using this demographic data from various temperatures, McCornack et al. (2004) estimated that the optimal temperature for soybean aphid development is 27.8°C. Results of McCornack et al. (2004) are confirmed by observations made by Hirano et al. (1996) who found that the maximum fecundity of soybean aphid, 60 offspring per female, occurred at 22°C and maximum intrinsic rate of increase, 0.533 $d^{-1}$, occurred at 27°C. Thus, maximum soybean aphid population growth is likely to occur when temperatures are 20-28°C. Supercooling point (SCP) is the temperature at which an insect spontaneously freezes and is the lowest possible temperature an aphid egg could survive. The SCP of various soybean aphid morphs was determined and eggs were found to have the lowest SCP, -34°C, while the gynoparae and oviparae have the highest SCP, -15°C (McCornack et al. 2005). Thus, soybean aphids are most likely to successfully overwinter in areas where winter temperatures remain above -34°C; however, microclimatic conditions to which eggs are exposed maybe warmer than air temperatures. Therefore, successful overwintering may also be possible in areas where ambient temperatures below the SCP are likely.

Soybean aphids are not the only pests occurring on soybean. Management of or even the presence of other pest arthropods or pathogens can influence soybean aphid populations. Asian soybean rust is a fungal pathogen of soybean which requires preventive foliar fungicide applications in order to avoid substantial yield losses (Tenuta et al. 2008). Use of fungicides to manage soybean rust significantly lowers fungal disease levels in soybean aphid populations (Koch et al. 2010). Although no aphid population response was observed, the decrease in disease pressure has the potential to increase aphid densities. Co-occurrence of soybean aphids and viruses, including alfalfa mosaic virus, soybean mosaic virus, and bean pod mottle virus, has been shown to reduce aphid density by 50% compared to uninfected plants (Donaldson and Gratton 2007). When soybean plants were infested by cyst nematodes and soybean aphids, weight of soybeans from nematode-resistant plants were significantly reduced due to soybean aphid

damage and weight of soybeans from soybean aphid-resistant plants were significantly reduced by nematode damage (Avendano et al. 2007). Therefore, in places where both pests occur, care should be taken when choosing a soybean cultivar.

Natural enemies have been documented to significantly contribute to the natural regulation of soybean aphid populations. While biological control of soybean aphid will be most thoroughly discussed in section 4.2.4, some interesting relationships have been demonstrated and are pertinent to the discussion of aphid population dynamics. In some parts of the introduced range of the soybean aphid, researchers noticed a two year cycle in which even-numbered years, 2002, 2004, and 2006, were characterized by low aphid densities on soybean and odd numbered years, 2003, 2005, and 2007, were characterized by high densities on soybean (Rhainds et al. 2010b). Detailed observations of aphid densities and predator abundance through these two year cycles supported the hypothesis that the cycles were not simply caused by the seasonal dynamics of soybean aphid populations, but also by the activity of coccinellid predators (Rhainds et al. 2010b). In recent years, the cycle has not occurred in Minnesota (K.A.K., personal observation). This may be due to a disruption of the biological control provided by coccinellid predators resulting from increased insecticide use in soybean (Rhainds et. al. 2010b). Resident natural enemies have been shown to respond to soybean aphid populations in a density-dependent manner (Donaldson et al. 2007), meaning that such natural enemies have the potential to prevent aphid outbreaks. Costamagna and Landis (2011) found that generalist predators can exert significant pressure on soybean aphid populations partly because the aphids do not have a strong spatial refuge from such predators. However, they also found evidence for a weak refuge the lower canopy of soybean plants (Costamagna and Landis 2011). This observation was confirmed by another study which found that when aphid populations were exposed to natural enemies, more aphids were found lower in the canopy than if natural enemies had been excluded (Brosius et al. 2010). However, there is evidence that the pressure exerted by natural enemies on aphid populations may not always be large enough to prevent aphid damage. Noma et al. (2010) demonstrated that arthropod natural enemies are positively associated with soybean aphid densities. In spite of this aggregation response, the natural enemy to aphid ratio showed a negative relationship with aphid abundance, indicating that aphids can

reach a density at which natural enemies can no longer regulate aphid populations (Noma et al. 2010). Relationships between soybean aphids and their predators are constantly changing depending on population densities, abiotic conditions, and chemical usage. It is apparent, however, that arthropod predators play an important role in regulating soybean aphid populations.

The ecology and dynamics of soybean aphid populations on the primary host, *Rhamnus* spp. are still poorly understood. In China, Wang et al. (1962) observed that higher than normal densities of overwintering soybean aphid eggs led to higher aphid densities early in the growing season. Chen et al. (1984), also working in China, found that there was a significant correlation between the abundance of overwintering eggs and level of aphid infestation on 15 and 25 June of the following growing season; however, abnormal weather conditions and natural enemies may alter this relationship. The ratio of soybean (secondary hosts) to buckthorn (primary hosts) was also related to the abundance of overwintering eggs in Jilin Province, China (Chen et al. 1984). This observation was confirmed in Ontario, Canada, when researchers observed large densities of overwintering eggs in the fall of 2004 and then an aphid outbreak in the summer of 2005 (Welsman et al. 2007). In the same study, it was observed that egg populations declined 70% over the course of the winter of 2004-05, possibly due to severe abiotic conditions or predation (Welsman et al. 2007).

**Damage to Soybean**

Aphids are true bugs, meaning they have piercing-sucking mouthparts which are adapted to feed directly on the phloem of their host plants. Because feeding requires that aphids penetrate host plant tissues, aphids are also well known for their ability to transmit plant viruses. Specifically, soybean aphids can cause damage to soybean in three ways: direct feeding, virus transmission, and production of honeydew which encourages growth of sooty mold on soybean leaves. While relatively little is known about the impacts of sooty mold infection on soybean, it is generally assumed that extensive sooty mold growth reduces the ability of soybean plants to photosynthesize. Of most concern for soybean production, however, is the effect of direct feeding on soybean.

Aphid densities of greater than 20 individuals per leaflet have been found to significantly decrease the rate of photosynthesis in soybean (Macedo et al. 2003). While

this sounds like a low to moderate density of aphids, for a V7 plant, 20 aphids per leaflet translates to 420 aphids per plant, which is above the economic threshold (ET) (Ragsdale et al. 2007). Diaz-Montano et al. (2007) found that similar densities of aphids, 30 per leaf, were required to feed for 10 d before chlorophyll loss due to aphid feeding was detectable. Therefore, for the negative impacts of soybean aphid feeding to become economically relevant, large populations must occur. Similarly, Dai and Fan (1991) found that small aphid infestations (i.e. <100 aphids per plant) can actually lead to increased yields when soybean plants can compensate for the damage with increased growth. However, when aphid populations reach higher levels (i.e. >674 aphids per plant), economic damage is unavoidable (Ragsdale et al. 2007).

In China, large soybean aphid populations have been shown to cause curling of leaves, stunted growth, earlier development and fewer pods and seeds per plant in affected soybean (Wang et al. 1994; Wang et al. 1996). Confirming this, a US study found negative relationship between peak soybean aphid densities and pods per plant, seeds per pod, whole plant biomass, and yield (Beckendorf et al. 2008). Seed weight showed a weak negative relationship with peak aphid densities when plants were infested with aphids at the V5 stage, but a strong negative relationship when aphids were applied at R2 stage (full flower) (Beckendorf et al. 2008). Seed oil concentration also had a negative relationship with peak aphid densities, while seed protein concentration was positively associated with peak aphid densities (Beckendorf et al. 2008). Economic yield losses are most likely caused by plant stunting and the decreased number of pods and seeds per plant (Wang et al. 1996; Beckendorf et al. 2008).

Soybean aphids also cause damage to soybean and other crops via virus transmission. Soybean aphid has been documented to efficiently transmit *Soybean mosaic virus* (SMV) (Wang and Ghabrial 2002; Clark and Perry 2002; Burrows et al. 2005; Wang et al. 2006) and *Alfalfa mosaic virus* (AMV) (Clark and Perry 2002; Wang et al. 2006) from soybean to soybean. Studies indicate that soybean aphid is capable of transmitting *Bean yellow mosaic virus* (BYMV) (Wang et al. 2006) and *Tobacco ringspot virus* (TRSV) (Clark and Perry 2002) from soybean to soybean, but likely not as efficiently as other studies have been unable to document soybean aphid transmission of BYMV (Clark and Perry 2002) and TSRV (Wang et al. 2006). The efficiency of SMV

transmission by soybean aphids increased from < 1% to > 30% when aphids were allowed to feed on infected plants overnight versus being restricted to a 1 min probe, respectively (Wang and Ghabrial 2002). Burrows et al. (2005) found that increases in SMV incidence in soybean fields was temporally associated with increased aphid migration, implying that soybean aphids were primarily responsible for observed SMV epidemics.

Soybean aphid has also been found to transmit viruses to other important North American crops. Soybean aphid is capable of transmitting AMV, *Tobacco etch virus* (TEV), and *Tobacco vein mottling virus* (TVMV) from tobacco to tobacco (Wang et al. 2006). Interestingly, soybean aphid was more effective at transmitting AMV from tobacco to tobacco (11% transmission) than from soybean to soybean (2% transmission) (Wang et al. 2006). Soybean aphid has also been implicated as a competent vector of *Potato Virus Y* (PVY) (Davis et al. 2005) and *Potato leafroll virus* (PLRV) (Davis and Radcliffe 2008). When soybean aphids infest soybean borders around seed potato fields, an increase in PLRV incidence has been observed (Davis and Radcliffe 2008). While soybean aphid cannot survive and reproduce on tobacco or potato, the aphid can cause significant damage to these crops simply through quick probes of leaf surfaces and under laboratory conditions soybean aphid is capable of reaching potato phloem, which is necessary for PLRV transmission (Davis and Radcliffe 2008).

## Entomophthoran Fungi Infecting Soybean Aphid

Soybean aphids have been documented to be infected by eight species of fungi from two orders, Glomeromycota: Entomophthorales (Hibbett et al. 2007) and Ascomycota: Hypocreales (Nielsen and Hajek 2005; Koch et al. 2010; reviewed in Ragsdale et al. 2011). Of these eight species, seven are entomophthoralean fungi including *Pandora neoaphidis* (Remaudiere and Hennebert) Humber (=*Erynia neoaphidis* Remaudiere and Hennebert), *Conidiobolus thromboides* Drechsler, *Entomophthora chromaphidis* Burger and Swain, *Pandora* sp., *Zoophthora occidentalis* (Thaxter) Batko, *Z. radicans* (Brefeld) Batko, and *Neozygites fresenii* (Nowakowski) Remaudiere and Keller (Nielsen and Hajek 2005; Koch et al. 2010). A single species from the Hypocreales has been found to infect soybean aphid, *Lecanicillium lecanii*

(Zimmermann) Zare and W. Gams (Nielsen and Hajek 2005). Increased sampling of aphid fungal pathogens in the soybean system could uncover additional fungal species which infect soybean aphid. Consistently, the most common species infecting soybean aphid in the field is *P. neoaphidis* (Nielsen and Hajek 2005; Noma and Brewer 2007; Koch et al. 2010); thus *P. neoaphidis* will be the focal soybean aphid pathogen for this review and for the remainder of the dissertation.

**Life- and Infection-cycle**

Explicit details on the physiological processes of the life and infection cycle of *P. neoaphidis* is available in Butt et al. (1990). The following generalized description has been adapted from Glare and Milner (1991). *Pandora neoaphidis* begins the infection process as asexually formed infective spores called primary conidia. The primary conidium will germinate and will produce a germ tube or a secondary conidium. The structure produced as a result of conidial germination may depend on the availability of free water on the germination surface (Sierotzki et al. 2000). Successive germination into secondary and then tertiary conidia will continue either until a germ tube is formed or until the conidium's energy has been expended. Susceptible hosts include aphids (Hemiptera: Aphididae) and rarely other closely-related hemipterans (Wilding and Brady 1984). Hosts are critical to the fungus, as *P. neoaphidis* is an obligate pathogen and cannot survive saprophytically. Thus, *P. neoaphidis* requires live hosts for infection and proliferation.

Assuming contact with a susceptible host, the germ tube will penetrate the host's cuticle directly; thus highlighting an important characteristic of *P. neoaphidis* and other entomopathogenic fungi. The ability to directly penetrate the host cuticle is unique to fungi, as opposed to bacterial and viral pathogens which must be ingested by the host for infection to occur. Once the germ tube has penetrated the cuticle, the fungus begins to grow within the hemoceol as protoplasts. Protoplasts do not elicit an immune response from the aphid (Butt et al. 1990) and absorb nutrients directly from the host, causing host death (Boucias and Pendland 1998). Subsequent to host death, *P. neoaphidis* changes its within-host growth habit from protoplast to hyphal body production. Specialized hyphae called cystidia weaken the cuticle, allowing conidiophores to emerge from the cadaver. Rhizoids also emerge from the dead aphid, securely anchoring the body to the leaf

surface. At the conclusion of the infection process, the conidia, or infective spores, produced by the fungus are forcibly ejected from the conidiophores, aiding in the dispersal of infective propagules. This characteristic is extremely relevant in the case of soybean aphids because of their colonial lifestyle. An infected, apterous adult aphid will likely remain in close proximity of its offspring; thus, ample hosts will be available once the aphid is killed by the fungus and produces conidia.

The overwintering strategy of *P.neoaphidis* has not been definitively determined; however, potential overwintering structures have been described. Feng et al. (1992) described specialized hyphal bodies from infected pea aphids on alfalfa. These spherical hyphal bodies retained infectivity when exposed to above-ground environmental conditions in Bozeman, MT from November through April. However, cadavers lost infectivity quickly when stored below-ground under soil. Nielsen et al. (2003) found that thick-walled conidia called loriconidia may be a mechanism by which *P. neoaphidis* can survive adverse winter conditions. Such loriconidia were found on the outside of cadavers stored on soil at 5°C for one month. *Pandora neoaphidis* may be able to persist on the soil through the winter in lieu of the production of specialized infective spores. Grain aphids, *Sitobion avenae* F., became infected with *P. neoaphidis* when allowed to walk over soil collected in the late spring from aphid overwintering sites (Nielsen et al. 2003). Additionally, pea aphids became infected with *P. neoaphidis* after contacting soil exposed to typical winter conditions in the United Kingdom (Baverstock et al. 2008). Further supporting the overwintering ability of *P. neoaphidis*, Fournier et al. (2008) were able to detect *P. neoaphidis* in soil samples collected in November and March from a field which experienced a nettle aphid, *Microlophium carnosum* (Buckton), epizootic the previous fall. In contrast, another study found that although *P. neoaphidis* inoculum was present on the soil after an introduced strain caused an epizootic in pea aphids, infections of pea aphid the following spring were not caused by the introduced strain, but rather by inoculum from a strain originating outside of the experimental plots (Fournier et al. 2010).

Because *P. neoaphidis* forcibly ejects its conidia, it possesses an inherent ability to disperse short distances. If impacts of air currents and wind movement are factored in, fungal conidia can travel long distances without the aid of a biotic vector. *Pandora*

*neoaphidis* conidia have been documented to be abundant in air samples taken from above crop fields where *Metopolophium dirhodum*, the rose-grain aphid, occurred on winter wheat (Hemmati et al. 2001). No conidia were trapped in the first year of the study and no epizootic in the aphid populations was observed; however, in the second year of the study rainfall increased and conidia were trapped in abundance. Peak densities of conidia were trapped on days characterized by high RH, lower temperatures, and less sun exposure (Hemmati et al. 2001). Steinkraus et al. (1999) found that cotton aphids, *Aphis gossypii* Glover, became infected with *Neozygites fresenii* (Nowakowski) Remaudiere and Keller, a species related to *P. neoaphidis*, after being placed in a cotton field and downwind of the field, up to 100 m. Thus, wind transportation of conidia can play an important role in pathogen dispersal. Fungi can also disperse with their aphid hosts. Feng and Chen (2002) found that up to 68% of alate green peach aphids, *Myzus persicae* (Sulzer), trapped in flight were harboring fungal infections. A similar study found that up to 80% of infected green peach aphids trapped while flying were infected with *P. neoaphidis* (Feng et al. 2007). Winged grain aphids infected with *P. neoaphidis* were capable of flying long distances, an average of 2.10 km over 3.06 hr, and subsequently were able to deposit nymphs, an average of 4.64 nymphs per female, on new host plants prior to death (Chen and Feng 2004). Thus, aphid migration is likely a critical factor in the dispersal ability of *P. neoaphidis*.

*Influence of infection on host biology.* Fungal infection can impact the biology and behavior of aphid hosts prior to mycoses and death. Pea aphids exposed to *P. neoaphidis* showed reduced fecundity within 24 h of infection and this reduction continued until aphid death (Baverstock et al. 2006). Similarly, cotton aphids infected by *N. fresenii* also showed significantly lower daily fecundity for the 5 d post-infection when compared to uninfected controls (Kay and Steinkraus 2004). In the same experiment, the authors found that cotton aphids infected by *N. fresenii* produce significantly less honeydew, 12 droplets per aphid, when compared to uninfected aphids, 180 droplets per aphid (Kay and Steinkraus 2004). The presence of pea aphid cadavers sporulating after infection by *P. neoaphidis* did not affect aphid foraging on host plants (Baverstock et al. 2005a), suggesting that pea aphids cannot detect the presence of *P. neoaphidis*.

Roy et al. (1999) found that within 2-3 d of *P. neoaphidis* infection, pea aphids do not respond as readily to the production of alarm pheromone compared to uninfected aphids. However, these infected pea aphids do continue to produce increasing amounts of alarm pheromone up to death (Roy et al. 2005). Thus, manipulation of aphid response to and production of alarm pheromone may benefit the fungal pathogen by reducing the movement of infected aphids, while at the same time increasing movement of uninfected aphids. These behavioral changes can lead to infected individuals remaining on the plant to produce conidia, as opposed to moving or dropping off the plant in response to the alarm pheromone, while the uninfected aphids move in response to increased production of alarm pheromone leading to increased chance of disease transmission. Interestingly, pea aphids infected by the generalist fungal pathogen *Beauveria bassiana* produced less alarm pheromone than uninfected aphids and showed the same behavioral response to alarm pheromone as uninfected aphids. Such behavioral responses by host aphids may be related to pathogen species or biology (i.e. specialist vs. generalist pathogen).

Impacts of *P. neoaphidis* on subsequent aphid generations have also been studied. Baverstock et al. (2006) found that nymphs produced by *P. neoaphidis*-infected females had similar intrinsic rates of increase as nymphs produced by uninfected females (Baverstock et al. 2006). Thus there is no evidence that impacts of fungal infection extend to the progeny of infected individuals.

**Influence of Abiotic Conditions**

Entomophthoralean fungi are also characterized by their specific abiotic requirements. Effects of temperature and relative humidity are the most studied environmental factors influencing *P. neoaphidis* transmission. Milner and Bourne (1983) found that at 20°C, infected aphids died within 3-5 d of infection, while at 8°C aphids died 12-15 d after infection. These results suggest a lower developmental threshold for the fungus of 4°C, a threshold which is similar to that of the host aphid species, *Acyrthosiphon kondoi* Shinji, the bluegreen aphid. While time to aphid death had a negative relationship with temperature, infectivity was not consistently affected by temperature (Milner and Bourne 1983). Feng et al. (1999) demonstrated that temperature is more important for *P. neoaphidis* infectivity to pea aphid than photoperiod. The fungus was more virulent at a constant 20°C than at temperatures fluctuating between

5.4°C and 18.9°C. When infecting *Myzus nicotianae* (Sulzer), the red morph of the tobacco aphid, *P. neoaphidis* produced the most conidia when exposed to 10-25°C and 100% relative humidity (RH) or free water (Yu and Nordin 1995). No conidia were produced at 30°C or in less than 98% RH and optimal *in vitro* growth was obtained at 15°C. Brobyn et al. (1987) found that *P. neoaphidis* conidia held at 20°C retained their infectivity to pea aphids, *Acyrthosiphon pisum* Harris, longest at 40% RH when on glass coverslips and at 40% and 50% RH when on excised field bean (*Vicia faba* L.) leaves. Infectivity declined most rapidly when spores were kept at 70% RH on leaves and at 77% RH on coverslips. At relative humidity levels of 40-50%, conidia remained infective for up to 21 d.

Leaf wetness, rainfall, and solar radiation have also been explored for their impacts on the transmission and infectivity of *P. neoaphidis*. Infectivity of *P. neoaphidis* increased as the period of leaf wetness increased. At 20°C, at least 3 h of leaf wetness was required for infection of the bluegreen aphid and at 10°C at least 7 h was required (Milner and Bourne 1983). Rainfall significantly lowered the number of conidia on field bean plants, with more conidia being washed off the top of leaves than the underside of leaves. It took 1 h of simulated rainfall for a significant number of infected cadavers to be removed from plants, although those cadavers remaining after the 1 h simulation produced as many conidia as unexposed cadavers (Pell et. al. 1998). *Pandora neoaphidis* spores on field bean leaves grown outdoors remained infective to pea aphids for up to 14 d. Conidia remained infective longer on the bottom of the plant compared to the top and on the underside of leaves compared to the top surface of leaves. In the year with the coolest weather and least sun exposure, conidia also remained infective longer than the other years of the study. However, infectivity of conidia exposed to solar radiation did decline linearly with time (Brobyn et al. 1985).

**Interactions with Host**

Aphid characteristics, including clone, morph, endosymbiont presence, and host plant can also influence the infectivity and transmission of *P. neoaphidis*. Milner (1982) first reported differences in susceptibility between aphid clones. Milner (1982) identified two pea aphid biotypes, one susceptible to *P. neoaphidis* infection and one resistant. He found that both biotypes were present in field-collected populations and that at least two

*P. neoaphidis* strains were capable of overcoming the defenses of the resistant clone (Milner 1982). Similarly, Ferrari et al. (2001) found that 20 pea aphid clones exhibited a spectrum of susceptibility to *P. neoaphidis*. In another study, four pea aphid clones were studied for their susceptibility to *P. neoaphidis* under different temperatures (Stacey et al. 2003). Susceptibility to the fungus was related to both clone and temperature, with susceptibility of two clones at experimental temperatures being positively associated with the growth rate of *P. neoaphidis in vitro* at those same temperatures (Stacey et al. 2003). However, the other two aphid clones show changes in susceptibility to *P. neoaphidis* over the range of experimental temperatures which are distinct from both the other two clones and the growth of *P. neoaphidis* at the same temperatures (Stacey et al. 2003). Thus aphid clone or biotype may be an important factor in *P. neoaphidis* transmission.

Aphids generally exhibit phenotypic plasticity which allows them to develop into either a migratory, winged morph or a sedentary, wingless morph, depending on conditions before or shortly after birth. The morph of these aphids is related to dispersal ability and fecundity as well as susceptibility to fungal pathogens. Alatoid nymphs of the tobacco aphid were more susceptible than apterous nymphs to *P. neoaphidis* infections, with $76.3 \pm 3.4\%$ and $25.0 \pm 4.9\%$ becoming infected, respectively, after challenge with the fungus (Yu and Nordin 1995). Conidia density required for mortality of 50% of exposed grain aphids was significantly lower for winged compared to wingless aphids for both the green and brown color morph (Dromph et al. 2002); thus implying an increased susceptibility of winged aphids to *P. neoaphidis* infection.

Endosymbionts have also been identified as a cause of differential susceptibility of aphids to fungal pathogens. Pea aphids possessing a γ-proteo-bacteria informally referred to as PAUS (U-type) exhibited increased resistance to *P. neoaphidis* infection; however, the effect of the symbiont could not be disentangled from the impact of host plant (Ferrari et al. 2004). Further study indicated that pea aphid infection by a PAUS endosymbiont, *Regiella insecticola*, improved aphid resistance to *P. neoaphidis* (Scarborough et al. 2005). This study separated the effect of host plant as *R. insecticola* -infected and uninfected pea aphids were reared on the same host plant prior to exposure to *P. neoaphidis* (Scarborough et al. 2005). This does not exclude impacts of aphid host plant on *P. neoaphidis* transmission and infection. Experiments by Tkaczuk et al. (2007)

indicate that pea aphids are most susceptible to *P. neoaphidis* infection when continuously kept on dwarf bean. However, when pea aphids were moved from dwarf bean to an alternate host plant, pea, after exposure to *P. neoaphidis*, susceptibility decreased. The authors hypothesized that the decreased virulence when aphids were moved to alternate host plants may be caused by aphid physiological stress due to host-switching (Tkaczuk et al. 2007). The bird cherry-oat aphid, *Rhopalosiphum padi* L., is a host-alternating aphid and is infected by different species of entomophthoran fungi on its primary host, *Prunus padi*, than on its secondary host, small grains (Nielsen and Steenberg 2004). Pea aphids collected from red clover, *Trifolium pratense*, and *Lotus uliginosus* differed in their susceptibility to *P. neoaphidis* (Ferrari and Godfray 2003). After all aphid clones were reared on broad bean, *Vicia faba*, for several generations, none of the aphids tested which were originally collected from red clover became infected by *P. neoaphidis*, while at least 60% of aphids tested which were originally collected from *L. uliginosus* became infected (Ferrari and Godfray 2003). Thus host plant appears to play an important role in resistance to entomophthoran pathogens, at least in aphid species which exhibit host races.

*Establishment density.* The study of insect epizootiology is rooted in general concepts of epidemiology originally developed for vertebrates (see Kermack and McKendrick 1927). However, differences in the immune response of insects compared to vertebrates, mainly the lack of acquired immunity, makes modeling and subsequent empirical study of insect epizootiology simpler than vertebrate epidemiology (Anderson and May 1981). Modeling dynamics of the soybean aphid-*P. neoaphidis* pathosystem can provide important insights into the role of *P. neoaphidis* in soybean aphid population regulation. In other words, existing biological information on the transmission and infection of aphids by *P. neoaphidis* can be utilized in a model to explore requirements for successful aphid population regulation by *P. neoaphidis*, the most basic of which related to host (i.e. soybean aphid) and pathogen (i.e. *P. neoaphidis*) population densities (Anderson and May 1981). Estimates of these requirements are critical in understanding the role *P. neoaphidis* can play in soybean aphid management.

Kermack and McKendrick (1927) introduced the concept of a threshold density of hosts required for the initiation of an epidemic. At host densities below this threshold, no

epidemics would be observed. At host populations close to the threshold density, small epidemics should occur; however, if the threshold density is exceeded by a large margin, much larger epidemics are possible. Termination of such epidemics was thought to come about from either the elimination of susceptible hosts from the population or from a decline in the virulence of the pathogen (Kermack and McKendrick 1927). Kermack and McKendrick (1927) theorized that instead, based on the threshold density concept, the termination of epidemics was due to relationships between host density, host recovery and death rates, and infectivity of the pathogen. While their theory referred to human epidemiology, the importance of the threshold density concept in the context of invertebrate epizootiology was confirmed by Anderson and May (1981) at which time they introduced several generalized models describing invertebrate epizootiology under differing assumptions.

Infection of aphids by entomophthoran fungi have some unique properties which must be accounted for in any model describing dynamics of the system. Fungal transmission is exclusively horizontal and does not require ingestion by the host. Once infected, aphids do not recover. Infected aphids do not become infectious (i.e. they cannot cause additional cases of disease) until after death when sporulation occurs; thus infections can be considered latent. Conidia can remain infective for at least 14 d in the environment (Brobyn et al. 1985) and resting spores or other overwintering structures can infect aphids months after they are produced (Nielsen et al. 2003). Horizontal transmission and lack of recovery simplify disease dynamics; however, latent infections and free-living conidia complicate the system.

Soybean aphid infection by *P. neoaphidis* can be most accurately described by adding two concepts, latent infection and free-living infectious stages, to the basic dynamical model developed by Anderson and May (1981). The basic model includes two state variables, susceptible and infected hosts, and five parameters, host birth rate, host death rate, host mortality rate (due to infection), host recovery rate, and a transmission constant. Because aphids cannot recover, recovery rate can be removed from the model. Fecundity is reduced in infected aphids, so a new parameter describing the birth rate of infected individuals must be included. The latent infection model introduces a state variable which designates those individuals who are infected but not

yet infectious. These infecteds are assumed to become infectious at a constant rate which is the death rate of infected individuals. Infectious individuals, or sporulating cadavers, are assumed to decay (i.e. exhaust their sporulation capacity) at a constant rate, introducing another parameter, decay rate of infectious individuals. Because conidia can survive outside host aphids, a state variable describing the density of these conidia must be added to the model. Conidia are assumed to be produced, lose infectivity, and germinate at constant rates, thereby introducing three more parameters to the model. Thus, the dynamics of soybean aphid infection by *P. neoaphidis* can be described with four state variables, susceptible, infected, and infectious aphids and free-living conidia, and eight parameters, birth rate for susceptible and infected aphids, aphid death rate, aphid mortality rate due to infection, transmission constant, rate of conidia production, rate of loss of infectivity of conidia, and decay rate of infectious aphids.

The establishment density of soybean aphid for maintenance of *P. neoaphidis* infection is the aphid density when the reproductive rate, $R$, of *P. neoaphidis* is equal to 1, meaning that each soybean aphid infection gives rise to exactly one other infection. *Pandora neoaphidis* can be maintained in the population when $R=1$, but epizootics occur when $R>1$. Therefore, epizootics can only occur when the establishment density has been exceeded and will cease once the host population falls below this density. Thus estimating the establishment density of soybean aphid for maintenance of *P. neoaphidis* has value when attempting to determine the magnitude of the role that *P. neoaphidis* can play in aphid management.

**Interactions with Other Organisms**

Research exploring the interactions of *P. neoaphidis* and non-aphid arthropods has traditionally focused on the predators and parasitoids which compete with the fungus for the aphid resource. Understanding how these organisms interact can be key in understanding the importance of *P. neoaphidis* in aphid pest management. There are many potential interactions between arthropod and fungal natural enemies of aphids in the context of pest management. When co-occurring, the two groups of natural enemies can be additive, in which aphid control in the presence of both groups is equal to the sum of the control provided by each group. If arthropod predators decrease aphid densities by 10% and fungal pathogens decrease aphid densities by 15%, an additive relationship

would occur if the aphid population was reduced by 25% when both predators and pathogens are present. If the resulting control is larger than the sum of control provided by both natural enemy groups, their interaction is synergistic. If the resulting control is less than the sum of control provided by both groups, their interaction is considered antagonistic (see Ferguson and Stiling 1996). In terms of pest management, additive or synergistic interactions are preferred, as they can maximize the benefit of natural enemies.

Potentially antagonistic and synergistic interactions have been demonstrated in the relationship between ladybird beetles and *P. neoaphidis*. In laboratory studies, the coccinellid *Coccinella septempunctata* L. has been documented to consume pea aphids killed by *P. neoaphidis* infection when kept in petri dishes (Pell et al. 1997). Consumption of the infected cadavers has the potential to reduce the inoculum available for infection of susceptible aphids, thereby leading to an antagonistic relationship between the pathogen and the predator. However, *C. septempunctata* preferred uninfected aphids over the dead, infected aphids and consumption of the infected cadavers was most likely to occur when beetles had been starved (Pell et al. 1997). In a similar experiment using whole plants, adult and larval *C. septempunctata* consumed infected pea aphid cadavers, with starved individuals consuming the infected prey more frequently (Roy et al. 2003). However, the coccinellids also moved to new plants more frequently when presented with infected prey; thus, demonstrating that infected prey are less desirable than healthy prey.

Intraguild predation of *P. neoaphidis* has also been demonstrated in the problematic species *Harmonia axyridis* Pallas, which has become a widespread invasive pest outside of its native Asia (Roy et al. 2008). In the UK, the tendency of *H. axyridis* to consume *P. neoaphidis*-infected aphid cadavers was compared to that of a native species, *C. septempunctata* (Roy et al. 2008). *H. axyridis* was more likely to engage in intraguild predation of *P. neoaphidis* than native *C. septempunctata* individuals. Furthermore, results of choice experiments indicated that *H. axyridis* originating from invasive populations, as opposed to those originating from native populations in Japan, did not discriminate between infected and uninfected aphids (Roy et al. 2008). Thus,

coccinellids, particularly individuals from invasive populations, have the potential to engage in antagonistic interactions with *P. neoaphidis*.

Antagonistic relationships between coccinellids and *P. neoaphidis* are not necessarily one-way. When *C. septempunctata* larvae were reared on *N. fresenii*-infected, but living cotton aphids, time for second, third, and fourth instar larvae to reach the next stadium was significantly longer when compared to larvae reared on uninfected aphids (Simelane et al. 2008). In addition, mortality of larvae raised on infected aphids was higher than for larvae raised on healthy aphids and egg production by females reared on infected aphids was significantly lower than those reared on uninfected aphids (Simelane et al. 2008). Thus, *C. septempunctata* can experience fitness costs when engaging in intraguild predation of entompathogenic fungi.

On the other hand, coccinellids have the potential to enter into synergistic relationships with *P. neoaphidis*. Adult *C. septempunctata*, when allowed to forage on plants with sporulating pea aphid cadavers prior to being introduced to healthy populations, initiated infection with up to 10% of aphids becoming infected (Pell et al. 1997). However, when the time between coccinellid inoculation and coccinellid introduction to healthy aphid populations increased to 4 h or more, no aphids became infected (Roy et al. 2001). Thus, the synergistic impacts of coccinellid foraging may be minimal.

Co-occurrence of predators and pathogens may also impact aphid behavior, possibly having significant impacts on disease transmission and aphid population dynamics. Adult *C. septempunctata* were allowed to forage on plants with both uninfected aphids and individuals infected by *P. neoaphidis* in order to determine if there was a relationship between aphid infection status and presence of the predator on the location of aphids on the plant (Roy et al. 2002). In the case of pea aphids on bean plants, the presence of adult coccinellids caused aphids to move to lower parts of the plant, regardless of aphid infection status (Roy et al. 2002). Location of *Sitobion avenae* on wheat plants was not significantly impacted by the presence of a foraging coccinellid, but infected *S. avenae* were found higher on the plant than healthy individuals (Roy et al. 2002).

Both parasitoids and fungal pathogens can concurrently colonize the same aphid individual, leading to interesting interactions. The nature of these interactions depends on the relative timing of fungal infection to parasitism. In the case of *P. neoaphidis* and *Aphidius rhopalosiphi* (De Stefani Perez) utilizing the rose-grain aphid, if *P. neoaphidis* infects the aphid prior to or within four days of parasitization by *A. rhopalosiphi*, the pathogen will successfully out-compete the parasitoid (Powell et al. 1986). However, if *A. rhopalosiphi* is able to parasitize the aphid 4 or more days before *P. neoaphidis* infects the aphid, *A. rhopalosiphi* will be the successful competitor. Thus, in this case, the pathogen has the competitive advantage due to its shorter generation time. However, if the pathogen in question has a wide enough host range, the pathogen could become an intraguild 'predator' of susceptible parasitoid larvae (Brodeur and Rosenheim 2000). While it may be advantageous for these parasitoids to be able to detect and avoid infected aphids, there is no evidence they can do so. Baverstock et al. (2005b) found that foraging *Aphidius ervi* (Haliday) did not avoid attacking infected pea aphids until they were sporulating, even when given the choice between healthy and infected, living aphids.

Due to its narrow host range, which is restricted to aphids (Hemiptera: Aphididae), *P. neoaphidis* does not infect co-occurring parasitoids. However, other interactions between the parasitoid and fungus are possible, leading to impacts on aphid management. Increased nettle aphid infection was observed when *P. neoaphidis* and *Aphidius microlophii* (Pennachio and Tremblay) occur together compared to *P. neoaphidis* alone (Baverstock et al. 2008). Interestingly, in the same system, when a lepidopteran herbivore, *Inchis io*, was added, nettle aphid infection with *P. neoaphidis* also increased (Baverstock et al. 2008). Similarly, Fuentes-Contreras and Niemeyer (2000) found that densities of *S. avenae* were lowest when *P. neoaphidis* and *A. rhopalosiphi* occurred together. However, adding the effect of an aphid-resistant wheat variety did not increase aphid control in this situation. Thus, it is unlikely that co-occurrence of parasitoids and fungal pathogens leads to antagonistic interactions that would decrease aphid control. Although, these interactions may have negative impacts on parasitoid or pathogen populations, depending on the species involved and abiotic conditions.

Ants (Hymenoptera: Formicidae) are known to interfere with the ability of parasitoids to parasitize aphid hosts (Wyckuys et al. 2007). This mutualism benefits both the ant, which receives honeydew from aphids, and the aphid, which receives protection from natural enemies. Such an arrangement can be less than desirable if ant-tending increases the density of pest aphids. This may the case for soybean aphids that are tended by *Lasius neoniger*. Schwartzberg et al. (2010) have documented a significant increase the biomass of soybean aphid populations when tended by ants. If ants can also detect fungal natural enemies, then ant-tending may also interfere with transmission of fungal pathogens within aphid populations. Interestingly, a recent study indicates that ants, *Formica podzolica*, tending milkweed aphids, *Aphis asclepiadis*, not only detect and remove infected aphid cadavers, but also clean infective conidia from healthy aphids, thereby preventing infection (Nielsen et al. 2010). Thus it is possible for ants tending soybean aphids to prevent the establishment and transmission of pathogenic fungi.

**Tritrophic Interactions**

The third trophic level, in this case the aphid host plant, has the potential to impact not only the aphids which utilize it as a resource, but also aphid- pathogenic fungi. Changes in the quality, physiology, or genotype of host plants and even changes in aphid location on the plant have the potential to alter the susceptibility or behavior of the aphid which in turn may impact pathogenic fungi (Cory and Hoover 2006). If changes in the third trophic level increase aphid vigor, pathogenic fungi may be unable to overcome aphid defenses, thus leading to decreased levels of disease. However, if such changes increase aphid stress or susceptibility, pathogens may infect aphids more easily, increasing levels of disease.

Effects of plant quality on entomopathogenic fungi have been studied via the effects of plant volatiles produced in response to host plant damage. When *P. neoaphidis* conidia were exposed to leaf volatiles of tobacco, *Nicotiana tabacum* (L.), from aphid-damaged or undamaged in-tact leaves, significantly more conidia germinated when exposed to undamaged leaves compared to aphid-damaged leaves (Brown et al. 1995). However, exposure of *P. neoaphidis* conidia to host plant volatiles did not significantly impact the infection rate of *Myzus nictianae* (Blackman) (Brown et al. 1995). In a similar study, when *P. neoaphidis* conida were exposed to volatiles from undamaged broad-bean,

*V. faba*, significantly more conidia germinated when compared to conidia exposed to volatiles from bean plants damaged via pea aphid feeding (Baverstock et al. 2005c). However, this difference in germination did not lead to similar differences in the infectivity of *P. neoaphidis* towards pea aphid. Therefore, host plant condition has the potential to impact *P. neoaphidis* transmission.

Dara and Semtner (2006) showed that in August, a significantly larger proportion of *M. persicae* was infected by *P. neoaphidis* in pre-flowering tobacco plants when compared to flowering tobacco plants, indicating that host plant physiology may also impact aphid pathogens.

Genetically-based resistance to aphids is often a desired character for crops consistently impacted by aphid damage; however, this resistance has the potential to impact aphid pathogens. Fuentes-Contreras et al. (1998) found that aphid resistance in wheat did not significantly impact $LC_{50}$ or $LC_{90}$ values for *P. neoaphidis* infecting *S. avenae*. However, another study by Fuentes-Contreras and Niemeyer (2000) indicated that wheat resistance may reduce the aphid population control incurred by *P. neoaphidis*. When comparing a variety of pea plants, *Pisum sativum*, with reduced leaf surface wax to its near isoline with regular wax levels, Duetting et al. (2003) found that significantly more pea aphids became infected with *P. neoaphidis* on the plants with reduced wax compared to aphids on plants with normal wax levels, when aphids and plants were exposed to a similar level of inoculum. Interestingly, infection initiation was similar on both types of plants; however, the proportion of aphids infected on plants with reduced wax increased nearly two times faster than on plants with normal wax levels (Duetting et al. 2003).

The location of an aphid on its host plant may also impact the likelihood that the aphid succumbs to fungal infection. In the case of cotton aphid on cotton, when aphids are equally distributed between the top and bottom of the plant, significantly more aphids located on the bottom of the plant became infected with *N. fresenii* when compared to aphids located near the top of the plant (O'Brien et al. 1993). In the case of *M. persicae* on tobacco, at the peak of the epizootic, aphids residing on the top of the plant were more often infected by *P. neoaphidis* than those located on the bottom half of the plant (Dara and Semtner 2006). Thus the impacts of aphid location on the host plant likely depends

on the architecture of the host plant canopy, with the observed differences in infection between the bottom and top of host plants most likely attributable to microclimatic conditions.

**Impacts of Agricultural Chemicals**

Agricultural chemicals can cause drastic changes in the flora and fauna present in a treated area. Understanding how the use of chemicals can impact resident organisms, especially those which provide a valuable service, can improve the efficiency and efficacy of pest management programs. Entomopathogenic fungi in agricultural systems are most likely impacted, either directly or indirectly, by the use of insecticides and fungicides (Steinkraus 2006).

*Insecticides*. Insecticides indirectly impact aphid pathogens by altering the density of aphids. If insecticides reduce aphid populations to a level that cannot sustain pathogen transmission, insecticide use can eliminate the pathogen from the host aphid population (Steinkraus 2006). However, this was not the case in a study examining the effects of imidacloprid on *N. fresenii* epizootics in cotton aphid populations. When imidacloprid was applied to cotton, *N. fresenii* was able to persist in cotton aphid populations and the fungus was likely responsible for early-season reductions in aphid density (Wells et al. 2000). On the other hand, if insecticides do not effectively kill aphids, but kill their arthropod predators, aphid densities can increase in the aftermath of an application; thus increasing the host availability, and possibly the prevalence of the pathogen (Steinkraus 2006).

Insecticides may also directly impact the pathogen if the insecticide has some fungistatic or fungicidal activity. This aspect of the interaction between insecticides and entomopathogenic fungi has been studied in systems including non-entomophthoralean fungi, likely because these fungi, including *Beauveria bassiana* and *Metarhizium anisopliae*, can also survive saprophytically outside of their hosts and would be more likely to come in direct contact with insecticides in an agricultural setting. Three neonicotinoid insecticides, imidacloprid, thiamethoxam, and acetamiprid, at three rates, field rate, 0.7 times the field rate, and 1.3 times the field rate, were studied for their effects *in vitro* on condia germination, mycelial growth, and conidia production of *B. bassiana, M. anisopliae*, and *Paecilomyces* sp. (Neves et al. 2001). Results were mixed

in that some insecticide/rate combinations increased pathogen growth and productivity, while other caused a reduction, which also varied by species (Neves et al. 2001). In light of the somewhat conflicting results, the authors concluded that in most instances, these insecticides would be compatible with these species of fungi in an integrated pest management program (Neves et al. 2001). A similar study, based on both *in vitro* and field tests of fungal growth and conidial production, found that thiamethoxam was compatible with *B. bassiana*, *M. anisopliae*, *Hirsutella thompsonii*, *Nomuraea rileyi*, *Paecilomyces farinosus*, and *Verticillium lecanii* (Filho et al. 2001). Consistent with these findings, Anwar et al. (2007) found that the prevalence of *N. fresenii* in cotton aphids was not impacted by thiamthoxam treatment; however, at peak disease prevalence plots treated with acetamiprid, dicrotophos, and imidacloprid had significantly lower levels of disease compared to untreated plots and those treated with thiamethoxam (Anwar et al. 2007). This effect was observed in spite of the thiamethoxam treatment having the lowest average aphid density (Anwar et al. 2007). Thus, it is likely that the use of thiamethoxam is compatible with entomopathogenic fungi.

*Fungicides.* Synthetic fungicides are chemicals that have been developed to specifically kill or inhibit the growth of pest fungi in order to protect valuable crop or horticultural plants. Therefore, broad-spectrum fungicides have the potential to negatively impact beneficial entomopathogenic fungi. This phenomenon has been documented in many agricultural systems. In a three year study of triphenyltin hydroxide impacts on entomophthoralean fungi infecting the blackmargined aphid, *Monellia caryella* (Fitch), observed aphid mortality due to fungal infection was consistently lower in fungicide-treated plots when compared to untreated plots (Pickering et al. 1990). In potatoes, it has been consistently observed that frequent fungicide applications lead to green peach aphid outbreaks coupled with decreased aphid fungal disease prevalence in fungicide-treated plots when compared to untreated plots (Nanne and Radcliffe 1971; Lagnaoui and Radcliffe 1998; Ruano-Rossil et al. 2004). In cotton, when fungicides were applied as granules at planting, early season fungal disease levels in cotton aphids were significantly lower, compared to untreated plots; however this difference disappeared as the growing season progressed (Smith and Hardee 1996). When chlorothalonil was applied to cotton, aphid densities were consistently higher in

chlorothalonil-treated plots, though not always significantly higher, and *N. fresenii* epizootic development in cotton aphid populations was delayed one week when compared to an untreated control (Wells et al. 2000). Fungicides applied to soybean for soybean rust infection and plant health effects, namely triazole, strobilurin, and chloronitrile fungicides, were found to significantly reduce soybean aphid fungal disease under field conditions; however, no aphid population response was observed (Koch et al. 2010).

Negative impacts of fungicides on aphid fungal disease prevalence in the field are supported by *in vitro* assessments of the impacts of fungicides on entomopathogenic fungi. Majchrowicz and Poprawski (1993) found that among the many species of fungi assessed, the entomophthoralean species, *Conidiobolus thromboides* and *C. coronatus*, were more severely impacted by fungicide exposure than non-entomophthoran species. When chlorothalonil was applied to excised broad bean leaves at the field rate, bioassays confirmed that *P. neoaphidis* lost all of its infectivity toward pea aphids (Latteur and Jansen 2002). Under identical procedures, the soybean rust fungicides propiconazole, azoxystrobin, and tebuconazole reduced *P. neoaphidis* infectivity by 37%, 68% and 100%, respectively (Latteur and Jansen 2002). Thus, widespread use of fungicides in systems in which fungal pathogens are important regulators of aphid populations could lead to increased aphid densities and host plant damage, particularly in the case of entomophthoralean fungi.

**Role of Entomophthorales in Aphid Biological Control**

Aphid biological control with entomophthorales has focused on classical, inoculative, and conservation biological control strategies. Classical biological control involves the introduction of a natural enemy, here an entomophthoran pathogen, which originated in the same geographic area as the pest of concern, with the expectation that the agent will establish, reproduce, and persist in the introduced range. Inoculative biological control is the introduction of a natural enemy with the expectation that the agent will establish and reproduce within a particular time-frame, such as a single field season. The agent is not necessarily imported from the native range of the target pest and is not expected to persist in the environment, but would need to be re-released in the future. Conservation biological control does not involve the release of natural enemies,

instead it relies on the preservation and encouragement of existing natural enemies. In the case of entomophthorales, this may involve irrigating crops to make the environment more conducive for disease transmission (Wilding et al. 1986) or restricting the use of fungicides which may inhibit fungal development (Koch et al. 2010).

Fungi are the most common microbes released as classical biological control agents against arthropods; however, less than one third of releases actually result in establishment of the agent (Hajek and Delalibera Jr. 2010). While *P. neoaphidis* has not been a classical biological control agent, seven other species of entomophthoran fungi have been released as part of classical biological control programs (Hajek and Delalibera Jr. 2010). A strain of *N. fresenii* originating from Arkansas has been released against cotton aphid in the San Joaquin Valley of California as part of a classical biological control program (Steinkraus et al. 2002). The release of dried, infected cadavers and inoculated, live aphids both resulted in transmission and establishment of the fungus through the end of the growing season. However, long-term persistence of *N. fresenii* in the cotton aphid populations in the region of release is doubtful, as it has not been detected there since the end of the field season of release (Steinkraus et al. 2002).

Inoculative biological control releases of *P. neoaphidis* have been attempted against aphid pests in crop plants on several occasions. Several species of entomophthoran fungi were released against *A. fabae* populations on bean over several field seasons in order to initiate epizootics earlier in the season to prevent the build-up of damaging aphid populations (Wilding 1981). Results of the inoculations showed that when the weather was dry and warm, establishment of the pathogens occurred, but they were unable to spread throughout the aphid population. However, cool, wet years saw rapid fungal spread and quicker aphid population reductions in plots receiving the inoculum (Wilding 1981). Wilding (1981) concluded that the inoculative release of entomophthoran fungi did increase aphid mortality; however, mortality observed was not adequate to prevent yield loss. In the same *A. fabae*-bean system, Wilding et al. (1986) explored the impact of inoculum type and irrigation on the success of inoculative releases of *P. neoaphidis* (=*Erynia neoaphidis*). The authors found that release of triturated infected cadavers was as effective as releasing living laboratory-infected aphids. Also, irrigation during dry periods in July also increased the proportion of aphids infected.

However, in spite of these successes, the inoculative release did not result in adequate control of the aphid (Wilding et al. 1986). *Pandora neoaphidis* successfully established in *M. persicae* populations on tobacco when it was released as infected, living aphids plus sporulating cadavers, infected, living aphids alone, or triturated infected cadavers; however, aphid disease prevalence was significantly lower in plots where triturated cadavers were released compared to the other two release methods (Dara and Semtner 2005). In spite of the successful establishment of *P. neoaphidis*, aphid densities were not significantly affected by the release of the pathogen (Dara and Semtner 2005). These studies suggest that entomophthoran pathogens can establish as inoculative biological control agents; however, they may not be able to control aphid densities sufficiently to prevent crop damage.

Few examples of conservation biological control programs involving entomophthoran pathogens are available. This may be due to our limited knowledge of the ecology of these pathogens and their interactions with their hosts in the field. However, entomophthoran fungi have commonly been described as important mortality factors in many agricultural systems. Thus, there is potential for conservation biological control to become an important component of aphid management. Naturally occurring populations of *P. neoaphidis* have been noted as causing aphid mortality in the *M. persicae*-spinach (Elkassabany et al. 1992; Mcleod et al. 1998), *A. pisum*-alfalfa (Hutchison and Hogg 1985), *A. glycines*-soybean (Nielsen and Hajek 2005), *M. persicae*-tobacco (Dara and Semtner 2006), cereal aphid (*M. dirhodum, S. avenae, and D. noxia*)-spring wheat (Feng et al. 1991), *Brevicoryne brassicae*-cabbage (Gopalakrishnan and Mohan 1993), and *M. persicae*-potato (Ruano-Rossil et al. 2004) systems.

## Integrated Pest Management

Pest management has been described by Pedigo (1999) as

> *"…the application of technology, in the context of biological knowledge, to achieve a satisfactory reduction of pest numbers or effects."*(p. 285)

Therefore, integrated pest management, or IPM, is a pest management strategy which integrates multiple, compatible pest management tactics, potentially including biological, chemical, and/or cultural control measures. Integration of multiple types of management

tactics can increase effectiveness of pest management programs by preventing or slowing pest resistance to a particular tactic, increasing pest suppression, and lowering management costs.

**Soybean Aphid IPM**

Soybean aphid is the most damaging insect pest of cultivated soybean in the North Central region of the US. After its discovery in 2000, researchers and growers rapidly responded by developing an IPM program for this pest in order to stem economic losses. Soybean aphid management has been focused on the use of insecticides; however, research continues on alternative management strategies, including the release of a parasitoid wasp as part of a classical biological control program. In spite of the relatively recent introduction of soybean aphid into North America, continuous, high quality research has lead to a rich source of information on which management decisions can be made.

*Sampling and Thresholds.* Thresholds are important components of IPM, and, in the case of soybean aphid, an economic injury level (EIL) and economic threshold (ET) have been developed for inclusion in a comprehensive soybean aphid IPM program. The EIL is the aphid density at which economic losses are sustained and is determined using information including cost of the management tactic, value of the crop, injury inflicted per aphid, damage in relation to injury, and reduction in aphid density achieved with the management tactic (Pedigo 1999). The ET is the aphid density at which some action to reduce aphid injury should be completed in order to avoid economic loss. If aphid densities have already reached the EIL, economic damage has already occurred, degrading the value of any management action. The goal is to balance the risk of an unnecessary management action if aphid densities reach the ET but not the EIL and the risk of failing to act before the EIL is reached (Pedigo 1999).

Initially, a working ET of 250 aphids per plant was introduced shortly after soybean aphid was found in North America. Because of the immediate need for treatment guidance, this threshold was not based on thorough field trials to assess injury and damage levels exerted by soybean aphid. Instead this threshold was a nominal threshold- essentially an experience-based rather than research-based threshold (Pedigo

1999).  While this was not ideal, it did provide some level of protection against unnecessary treatments until a more rigorous economic threshold could be developed.

In 2007, a research-based ET and EIL were published (Ragsdale et al. 2007). Data was collected across six North Central states, where 19 experiments were performed over three years to assess soybean aphid caused yield losses.  Results indicated that the EIL was 674 ± 95 soybean aphids per plant (Ragsdale et al. 2007).  The ET was calculated to be 273 ± 38 soybean aphids per plant, which allowed for a 7 d window in which treatment could be applied without aphid densities reaching the EIL.  Because the nominal threshold was within the 95% confidence interval of the calculated ET, the familiar ET of 250 aphids per plant was confirmed and retained (Ragsdale et al. 2007). These ET and EIL values are only applicable to soybean plants up to the full pod reproductive stage (R5).  It is unknown how exceeding the EIL in later reproductive stages affects yield.

Another initial problem encountered in soybean aphid IPM was the rather large sampling effort required to estimate whole-plant aphid densities, a measure on which treatment decisions were made.  In response, 'speed scouting' was developed by Hodgson et al. (2004).  'Speed scouting' is a binomial sequential sampling plan for soybean aphid.  If aphid densities are very large or very small, a 'treat' or 'do not treat' decision can be made quickly, reducing the sampling effort required (Hodgson et al. 2004).  Once plants are found to harbor at least 40 aphids, the plant is considered 'infested' and once 84% of the plants are found to be 'infested', a 'treat' decision is made (Hodgson et al. 2004).  The >40 tally threshold combined with the 84% infestation level corresponded to the 250 aphid per plant ET.  This sampling method is most accurate in minimizing incorrect treatment decisions when aphid densities are at extremes in relation to the ET.  For those instances when aphid densities are close to the ET, additional binomial samples are required and if after additional samples are taken a decision still cannot be made, sampling must be repeated within one week (Hodgson et al. 2004).

To further minimize sampling effort, McCornack et al. (2008) developed a reduced sampling unit based on the vertical distribution of soybean aphids among nodes. In instances when whole-plant aphid counts are still required to make treatment decisions, this sampling plan can limit the time and effort required to make such

decisions. Sampling the node with the most aphids ($N_{MAX}$) can lead to an accurate estimate of whole-plant aphid density; however, this requires the sampler to accurately determine which node is the most infested by visual inspection, which can lead to incorrect identification of the node of interest and inaccurate density estimates (McCornack et al. 2008). However, a three node sampling unit, including the top node (terminal growth, $N_1$), the third node from the top ($N_3$), and the middle node of those remaining on the plant ($N_{MR}$) was also able to accurately estimate whole-plant aphid density while limiting incorrect node identifications (McCornack et al. 2008). This reduced sampling unit can be used to estimate soybean aphid densities in both the open field and in cages which exclude natural enemies for experimental purposes; however when attempting to estimate whole-plant densities on caged plants alternate parameter values must be utilized in order to compensate for differences in aphid distribution on caged plants (Costamagna et al. 2010).

Across the North Central US, suction traps have been installed which sample winged, migratory aphids in the air fauna (Rhainds et al. 2010c). Knowing the density of migratory soybean aphids in the spring may allow managers to predict aphid densities for the coming field season, if a significant correlation with local soybean aphid densities is established. Rhainds et al. (2010c) attempted to find this relationship. They found that cumulative numbers of soybean aphids in suction traps did correlate with local aphid densities (Rhainds et al. 2010c). However, high variation in aphid densities among fields within the same county prevents this sampling technique from being useful in field-based management decisions (Rhainds et al. 2010c).

*Insecticides.* When using insecticides to manage pest populations, an active ingredient, application method, and time of application must be selected. In the context of soybean aphid IPM, these decisions must remain flexible and must consider the broader impact of such applications. An important part of soybean aphid IPM is the use of thresholds. If the ET and EIL are considered in treatment decisions, insecticides will be used therapeutically rather than preventatively. However, with the emergence of insecticide seed treatments, an inherently preventative tactic, a growing proportion of insecticide usage in soybean is no longer in response to aphid densities surpassing the

ET. The value of such seed treatments is still under debate while use of the ET as the trigger for foliar insecticide applications is gaining popularity.

Foliar insecticide applications to manage soybean aphid have generally utilized broad-spectrum chemistries including pyrethroids and organophosphates. However, 'softer' chemicals have been studied and show promise, especially in the context of IPM. Myers et al. (2005) showed that if a single, scheduled broad-spectrum insecticide application were used, yield was protected most effectively when applications were made at the soybean plant reproductive stages of R2 or R3. However, such timed applications which do not utilize the ET, have been found to be less economical for soybean aphid management (Johnson et al. 2009). Instead, relying on the IPM strategy of weekly sampling and treatment only after the ET is surpassed was found to lead to the highest return on investment, even when sampling costs are considered (Johnson et al. 2009). Foliar applications of 'reduced-risk' insecticides imidacloprid and pymetrozine were found to be as effective in preventing soybean aphid yield damage as the broad-spectrum insecticide lambda-cyhalothrin (Ohnesorg et al. 2009). Products used in organic agriculture, including pyrethrins, insecticidal soap, and narrow-range mineral oil were also found to cause high levels of soybean aphid mortality in lab studies and because these exposure to these products are less harmful to an important aphid predator, *H. axyridis*, they are likely compatible for organic and IPM systems (Kraiss and Cullen 2008).

Soybean seeds are most commonly treated with the neonicotinoid insecticides thiamethoxam or imidacloprid in addition to up to three different fungicides which prevent fungal diseases of soybean seed and seedlings. Benefits of such treatments likely include increased safety for the applicator, decreased cost of applications, and decreased non-target impacts to natural enemies (Ohnesorg et al. 2009) and the environment due to the elimination of drift and the targeted application method. However, the efficacy of such seed treatments in soybean aphid management is still being debated. Seed treatments are only active against soybean aphids for the first 30 to 40 days after planting (McCornack and Ragsdale 2006). Therefore, if treatments are going to be cost effective, they must exert enough control on aphid populations within that 30 to 40 day window which would protect yield that would otherwise have been lost. This is not always the

case, as even if aphid colonization is delayed for those initial 30 days, after this time colonization and population build-up can occur and cause yield damage in spite of the treatment. Alternatively, if aphid densities remain below the ET throughout the growing season, but seed treatments were utilized anyway, there would be an economic loss for the unnecessary treatment. Because aphid densities vary greatly among years, it is difficult to predict if preventative seed treatments will be effective.

Much research has focused on the efficacy, utility, and economics of insecticide seed treatments in soybean aphid management. In Minnesota, McCornack and Ragsdale (2006) found that soybean plants which were seed treated with thiamethoxam caused the direct mortality of soybean aphids up to 35 days after planting and that toxicity did not persist past the V4 vegetative growth stage. While the cumulative aphid days (CAD), a measure of season-long aphid pressure, was significantly lower in three of the four location-years, a significant yield gain was observed in only one location-year which experienced very high aphid densities (McCornack and Ragsdale 2006). Thus, in this study, the seed treatment was unnecessary 75% of the time. Similarly, a study in Nebraska found that in a low aphid year, 2005, seed treatments of imidacloprid and thiamethoxam did not result in yield improvement compared to the untreated control (Magalhaes et al. 2009). However, in 2006, high aphid densities were observed and yield was significantly higher in imidacloprid and thiamethoxam treated plots compared to untreated plots (Magalhaes et al. 2009). Studies of preventative soybean seed-treatments in Iowa also demonstrated that such management tactics are not effective against soybean aphid, as seed treatments did not lead to yield increases over the untreated controls in any of the five location-years studies (Johnson et al. 2008). Trials performed in Kansas found that soybean seed treatments of insecticide + fungicide and of fungicide alone equally improved plant density and yield; thus, it may be the fungicide component of the seed treatment rather than the insecticide which leads to yield improvement (Gordon 2009). While occasionally seed treatments do improve soybean yields compared to untreated controls (McCornack and Ragsdale 2006; Magalhaes et al. 2009), it does not improve yields compared to the more conservative IPM approach (Johnson et al. 2009). The IPM approach rejects preventative treatments and instead using scouting of aphid densities and the ET and EIL to make therapeutic treatment decisions. This IPM approach was found

to be not only as effective as preventative insecticide applications, including seed treatment, but also more cost effective (Johnson et al. 2009). Thus the majority of evidence suggests that the year to year variability in soybean aphid populations makes preventative insecticide treatments, including seed treatments, either unnecessary or ineffective.

*Host Plant Resistance.* With the recent release of the first soybean-aphid resistant soybean varieties, understanding the efficacy and potential impacts of such varieties has become important. While it is expected that utilization of aphid-resistant varieties will lower aphid populations, it is unknown how these changes in aphid population dynamics may impact the aphids' natural enemies—an important component of IPM. Potential impacts of host plant resistance (HPR) on natural enemies can be indirect, via changes in the physiology or behavior of soybean aphid, or direct if the resistant plant itself affects the natural enemy.

Host plant resistance can be characterized by the type of resistance exhibited, including antibiosis, antixenosis, or tolerance (Pedigo 1999). Antibiosis is characterized by physiological or behavioral changes in the aphid resulting from feeding on the resistant plant. These changes often lead to reduced growth rates, fecundity, lifespan and/or adult size, increased mortality, and morphological and/or behavioral abnormalities (Pedigo 1999) all of which lead to reduced aphid densities on the resistant plants. Antixenosis, or nonpreference, refers to resistance which results from aphid avoidance of the resistant host plant. Such avoidance can result from morphological changes in the host plant leading to physical barriers which prevent normal aphid behavior or from changes in plant chemistry, commonly the production of secondary metabolites, which cause the aphid to avoid the resistant plant (Pedigo 1999). Tolerant host plants do not impact the aphid, instead the tolerant plant is able to withstand and compensate for aphid damage, leading to higher yields than would be expected from a non-tolerant plant under the same level of aphid pressure (Pedigo 1999).

Currently three aphid resistance genes have been identified in soybean, *Rag1* (Hill et al. 2006), *Rag2* (Rouf Mian et al. 2008), and *Rag3* (Zhang et al. 2010). Because *Rag1* was the first gene identified, it is the most studied and was recently made commercially available. The *Rag1* gene is a single, dominant gene identified in the varieties 'Dowling'

and 'Jackson' (Hill et al. 2006) which exhibits both antibiosis and antixenosis to soybean aphid (Diaz-Montano et al. 2006). Soybean aphids were found to exhibit decreased fecundity and survival on when feeding on *Rag1* resistant plants, compared to susceptible soybean (Li et al. 2004). Diaz-Montano et al. (2007) found that it took 4 h longer for aphids to reach sieve elements in *Rag1* resistant soybean than in non-resistant plants. Confirming this finding, Crompton and Ode (2010) found that soybean aphids had increased difficulty in reaching phloem cells when feeding on *Rag1* resistant soybean compared to non-resistant varieties. In field trials, soybean plants with the *Rag1* gene were found to be nutritionally inferior for soybean aphid compared to non-resistant varieties (Chiozza et al. 2010). The *Rag2* gene was identified from plant introduction (PI) 243540 as a novel soybean gene conferring resistance to soybean aphid (Rouf Mian et al. 2008). Zhang et al. (2009) identified the *Rag3* gene from PI 567543C. The identification of the resistance genes *Rag2* and *Rag3* was done so recently that relatively little is known about their mechanism of resistance.

One of the most important components of HPR research is developing strategies to avoid aphid tolerance of the resistant host plant, which would eliminate HPR as a management tactic until an alternative aphid-resistant variety was developed. Aphid populations able to overcome *Rag1* and *Rag2* resistance have already been identified in North America (Kim et al. 2008; Hill et al. 2010). These populations are called biotypes, or races, because they can be differentiated from other soybean aphid populations by the soybean varieties they can colonize (Pedigo 1999). Three such soybean aphid biotypes have been identified in North America. Biotype 1 is simply the wild-type and is susceptible to all three resistance genes identified. Biotype 2 is the soybean aphid population identified in Ohio which can overcome aphid-resistance attributed to the *Rag1* gene (Kim et al. 2008). Biotype 3 is a soybean aphid population capable of overcoming resistance caused by the *Rag2* gene which was identified on an alternate primary host, *Frangula alnus*, in Illinois (Hill et al. 2010). The emergence of biotypes 2 and 3 is disconcerting as the genetic background required by soybean aphids to overcome two of the three resistance genes are already present in wild populations in North America. Thus it is likely that the development of aphid-resistant soybean varieties will continue.

In the single published study of the effect of aphid-resistant soybean on soybean aphid natural enemies, aphid-resistance was found to significantly reduce lifespan and survival of *H. axyridis* adults (Lundgren et al. 2009). These impacts were direct, as the *H. axyridis* individuals were only exposed to the different soybean genotypes through physical contact with the plant and not via consumption of aphids colonizing the plants (Lundgren et al. 2009). More research is require to determine any indirect effects of aphid-resistance on natural enemies via prey consumption and to determine how these impacts might affect soybean aphid biological control on aphid-resistant soybean.

*Biological Control.* As mentioned in section 3.7, biological control can take on several forms including classical, augmentation (inoculative or inundative), and conservation biological control. Classical, or importation, biological control involves the importation of a non-native biocontrol agent to control a non-native pest. Augmentation biological control can be inoculative, in which a small number of biocontrol agents are released under the assumption that they will reproduce and control the pest on a short-term basis, or inundative, in which large numbers of the biocontrol agent are released to control the pest, without the expectation of reproduction after release. In both types of augmentation biological control, establishment of the biocontrol agent is not expected. Conservation biological control does not involve the introduction of a biocontrol agent, instead it occurs when the environment is made more conducive for existing natural enemies which can control the target pest. Both classical and conservation biological control have been studied for their use in soybean aphid management.

Shortly after the discovery of soybean aphid in North America researchers initiated a classical biological control program. Observations of soybean aphid in its native range showed that parasitoids were a common and effective natural enemy guild utilizing soybean aphid in Asia but generally lacking in North America (Liu et al. 2004; Miao et al. 2007). These observations led to the importation of several species of parasitoids from China, with the hope for classical biological control releases against soybean aphid in North America (Heimpel et al. 2004; Wyckhuys et al. 2007). In 2007, one of the imported parasitoids, *Binodoxys communis*, was approved for release against soybean aphid after intensive host-range testing (Wyckhuys et al. 2009). Currently, it is not known whether *B. communis* has successfully established, though releases continue.

Because so little is known about the overwintering behavior of the parasitoid, it is unclear if or how *B. communis* can overwinter in North America.

Conservation biological control of soybean aphid has also been explored as a way to reduce economic losses due to aphid damage. Adding a refuge to experimental soybean plots did not increase the density of ground- and foliar-dwelling generalist aphid predators (Fox and Landis 2003). However, when these generalist predators were present, they significantly reduced the survival of soybean aphids (Fox and Landis 2003). Another study found that increasing landscape diversity within 1.5 km of soybean fields increased the level of soybean aphid biological control observed (Gardiner et al. 2009). This result suggests that enhancing or conserving landscape diversity in areas surrounding soybean fields can enhance the natural control of soybean aphid and might lead to a reduction in insecticide use.

Resident natural enemies have responded quickly to the arrival of soybean aphid; thus providing aphid control without human intervention. Such biological control is ideal as it does not require a grower to put in any additional effort or financial means to take advantage. The vast majority of published research on the impact of natural enemies on soybean aphid populations indicates that resident natural enemies are important regulators of aphid populations which can prevent aphid outbreaks. Much of this research has focused on generalist arthropod natural enemies including coccinellids and anthocorids. Cage studies performed shortly after soybean aphid's arrival in North America demonstrated that the resident natural enemies, including *H. axyridis* and *Orius insidiosus*, were responding to the new resource and were capable of suppressing aphid populations (Fox et al. 2004; Desneux et al. 2006). In Michigan, coccinellids, including *H. axyridis*, were found to be the dominant predator of soybean aphids (Costamagna et al. 2008). While in Indiana, the anthocorid *O. insidiosus*, the insidious flower bug, was found to be the key soybean aphid predator (Rutledge and O'Neil 2005; Desneux et al. 2006). Such generalist predators have been found to prevent or delay aphid establishment (Fox et al. 2005) and reduce the densities of already established aphid populations (Donaldson et al. 2007), leading to increased soybean yield (Costamagna and Landis 2006). While *H. axyridis* and *O. insidiosus* are likely the key predators of soybean aphid in the North Central region (Rutledge et al. 2004), other resident predators

and parasitoids have been observed utilizing soybean aphids. In 2003 and 2004, fifteen parasitoid and predator y fly species were found consuming soybean aphids in Michigan, with the predatory fly *Aphidoletes aphidimyza* (Rondani) being the most commonly encountered species (Kaiser et al. 2007). Also in Michigan, Noma and Brewer (2008) observed six parasitoid and nine predatory fly species responding to soybean aphids. Throughout the four-year study, parasitism rates of soybean aphids remained below 10%, further demonstrating the general lack of this guild in natural aphid control (Noma and Brewer 2008). In addition, a study in New York State found 60 species of ground-dwelling carabid beetles in pitfall traps located in soybean fields (Hajek et al. 2007). The most commonly found species, *Agonum muelleri* (Herbst), was found to consume soybean aphid in a no-choice assay (Hajek et al. 2007).

While extensive research has demonstrated the impacts of arthropod natural enemies on soybean aphid populations, relatively little is known about such impacts of fungal natural enemies. In 2005, Nielsen and Hajek (2005) demonstrated that fungal pathogens already present in North America were infecting soybean aphids on both its primary and secondary host in New York State. Among the pathogens found, *P. neoaphidis* was the most common (Nielsen and Hajek 2005). Similarly, in Michigan, fungal infection of soybean aphid was confirmed, with all infections observed being caused by *P. neoaphidis* (Noma and Brewer 2007). Koch et al. (2010) confirmed that fungal pathogens were utilizing soybean aphid in Minnesota and that the use of soybean rust fungicides significantly lowered aphid disease prevalence. In this study, *P. neoaphidis* was again the most common pathogen species, causing more than 90% of the infections (Koch et al. 2010). Thus, it is clear that aphid fungal pathogens play a role in the natural regulation of soybean aphid populations; however, the extent of that role is not known.

## Conclusion

The purpose of the remaining chapters of this dissertation is to elucidate the role of fungal entomopathogens in soybean aphid IPM. While these pathogens are already present and readily utilizing soybean aphid, understanding when they occur and how their presence influences aphid population dynamics will be an important first step in

recognizing the importance of these pathogens not only in soybean aphid management, but also in the management of other aphid pests. Furthermore, determining how such pathogenic fungi interact not only with the target aphid, but also with other aphid natural enemies and additional management tactics will inform growers, allowing them to get the most out of the natural service that these pathogens can provide.

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# Chapter 2: Determining the Soybean Aphid Density Required for the Establishment of *Pandora neoaphidis* in Aphid Populations on Soybean

## Introduction

The soybean aphid, *Aphis glycines* Matsumura, is an invasive pest of cultivated soybean that was first discovered in North America in 2000 (Ragsdale et al. 2004). In Asia, its native range, this aphid rarely reaches pest status, likely because of the action of natural enemies. In North America, however, the soybean aphid undergoes population outbreaks which cause economic damage via increased insecticide use and decreased yield (Ragsdale et al. 2007). In order to prevent the economic and ecological costs of such insecticide applications, a significant amount of research exploring the impacts of biological control on soybean aphid populations has been performed (Ragsdale et al. 2011). To date, most research has focused on arthropod natural enemies and has demonstrated that predatory arthropods can lead to significant aphid control under certain conditions (Desneux et al. 2006; Fox et al. 2005; Costamagna et al. 2006, 2007; Costamagna and Landis 2011). However, field observations have indicated that pathogenic fungi, particularly *Pandora neoaphidis* (Remaudière and Hennebert) Humber, may play an important role in aphid population regulation (Nielsen and Hajek 2005; Noma and Brewer 2007; Koch et al. 2010). Despite these observations research into the impacts of such pathogens is lacking.

The soybean aphid exhibits a heteroecious, holocyclic life cycle in which sexual reproduction occurs on the primary host, *Rhamnus cathartica* or common buckthorn, and asexual reproduction via parthenogenesis occurs on the secondary host, *Glycine max* or cultivated soybean (Dixon 1973; Ragsdale et al. 2004; Voegtlin et al. 2005). In spring, nymphs hatch from eggs on common buckthorn. The first generation is composed of wingless females called fundatrices that remain on *Rhamnus.* In subsequent generations, an increasing proportion of parthenogenetic females are winged and these winged aphids are spring migrants which leave *Rhamnus* in search of soybean (Ragsdale et al. 2004). Foundress alates locate soybean and deposit nymphs which will develop into apterous, parthenogenetic females and many such generations of parthenogenetic females follow. As fall approaches and photoperiod shortens, sexual females (gynoparae) and males, both of which are alatae, are produced. The gynoparae and males return to buckthorn where gynoparae produce oviparae, apterous females which mate with males, and deposit eggs, the overwintering stage, near buckthorn buds (Dixon 1973).

Fungal pathogens belonging to the fungal order Entomophthorales are important regulators of aphid populations in a variety of cropping systems including potato (Lagnaoui and Radcliffe 1998; Ruano-Rossil et al. 2004), alfalfa (Hutchison and Hogg 1985), and cotton (Steinkraus et al. 1995). The most common entomophthoralean fungus infecting soybean aphid in Minnesota is *P. neoaphidis* (Koch et al. 2010). The host range of *P. neoaphidis* is restricted to aphids (Hemiptera: Aphididae; Keller 1991). The infection process of *P. neoaphidis* infecting the pea aphid, *Acyrthosiphon pisum*, has been documented by Butt et al. (1990). The fungus penetrates the aphid cuticle directly and upon entering the aphid's hemocoel, the pathogen grows vegetatively, absorbing nutrients from aphid tissues. Once infected, aphid fecundity drops by up to 45% (Baverstock et al. 2006) and as the infection process proceeds aphids die of starvation. Some entomophthoralean fungi produce toxins which kill the host; however, there is no evidence of toxin production by *P. neoaphidis* (Boucias and Pendland 1998). Aphids become infectious only after death, when *P. neoaphidis* produces conidia, the infective spore, on the end of conidiophores which emerge from the aphid cadaver. The conidia are then forcibly ejected from the ends of the conidiophores, which is the main method of fungal dispersal. The entire infection process, from penetration of the aphid cuticle through fungal spore dissemination, takes three to five days. An immune response by the aphid was not observed in this study and new data suggests that aphids infected by entomophthoralean fungi produce a relatively small amount defense compounds, implying that aphid immune response to fungal infection is limited (Grell et al. 2011). so recovery after becoming infected is unlikely (Butt et al. 1990).

*Pandora neoaphidis* has no definitive overwintering stage, although two have been proposed (Feng et al. 1992; Nielsen et al. 2003). Because no overwintering stage has been confirmed, it is believed that winged aphids redistribute the fungus each season. Infected alates are capable of flying long distances, locating a host plant, and producing nymphs before death, at which time the infected aphid is capable of causing secondary infection in her offspring (Feng and Chen 2002; Feng et al. 2004; Feng et al. 2007). Therefore, it is most likely that infected alates will bring infection into a soybean field where a population of soybean aphid already exists.

While *P. neoaphidis* has the potential to contribute to the population regulation of soybean aphids on soybean, there are some practical issues which may hinder the ability of fungal pathogens to consistently establish in aphid populations. Entomophthoralean fungi require specific abiotic conditions in order to successfully infect aphid hosts- mainly high relative humidity of 96-100% (Steinkraus 2006). In addition, these fungal pathogens are density-dependent, meaning they require a minimum host aphid density in order to establish and persist in an aphid population (Anderson and May 1981).

Determining the threshold density necessary to establish fungal infection in a soybean aphid population is the first step in determining the potential for these pathogens to contribute to meaningful aphid suppression in the field. We will refer to this threshold aphid density as the establishment density (ED) throughout the remainder of the paper to avoid confusion with the soybean aphid *economic threshold* (ET) used in aphid management. Soybean aphids cause significant economic damage when they reach densities of 674 aphids per plant, the economic injury level (EIL) (Ragsdale et al. 2007). When considering the ability of aphid populations to expand quickly, an ET of 250 aphids per plant has also been confirmed (Ragsdale et al. 2007). The ET is the aphid density at which a grower would have one week to treat his or her field to prevent economic damage (Ragsdale et al. 2007). If *P. neoaphidis* is unable to establish and maintain itself in a soybean aphid population below 674 aphids per plant, there is little chance it will be able to contribute to natural control of the pest below damaging levels. However, if the opposite is true, and *P. neoaphidis* is capable of being transmitted at lower aphid densities, its potential as a natural control agent should be considered and further research into its importance in aphid population dynamics would be warranted. In addition, conservation of this group of natural enemies would become critical, especially considering that the level of natural control exerted by this fungus is unknown and any disruption of its abilities could lead to increased frequency of aphid outbreaks.

Following the model of Anderson and May (1981), we predict that there is a minimum soybean aphid density at which *P. neoaphidis* can establish, we call this the establishment density or *ED*. In order to predict the *ED*, we adapted dynamic models presented in Anderson and May (1981) to the soybean aphid-*P. neoaphidis* pathosystem. Furthermore, to determine the validity of the model, we performed microplot cage studies

in 2009 to develop an independent field estimate of the *ED* which can be compared to and confirm model results.

We do not attempt to describe the complicated ecological processes which occur within this pathosystem, rather we aim to quickly and economically estimate the *ED* relative to the soybean aphid ET and EIL in order to determine if this pathogen has the potential to establish in sub-economic aphid densities and contribute meaningfully to aphid management. Thus, these methods will not only inform soybean aphid management, but may also be applied to pests in other agricultural and natural systems. We hypothesize that these methods may be useful as a screening tool for classical or augmentative biological control agents for their potential effectiveness prior to release as model parameters may be estimated in laboratory or quarantine situations or from the published literature.

## Materials and Methods

### Model Development and Analysis

*Model Development.* Because the soybean aphid is a pest on cultivated soybean, we focused on determining the *ED* for soybean aphid on the soybean host, rather than on its primary host, common buckthorn. Parameters are estimated from the literature or, if necessary, from unpublished data obtained via personal communications. Parameters relating to time are on per day time scale. The spatial scale for any parameters relating to density will be on a per $mm^2$ scale; thus model results will be in aphids per $mm^2$. To convert this to a more meaningful density, the *ED* will be multiplied by a constant based on O'Neal et al. (2002) which estimates the leaf area of soybean plants at differing growth stages. Multiplying by this constant will convert the *ED* to aphids per soybean plant. Because soybean leaf area changes throughout the season, the constant will standardize aphid densities to those on a typical vegetative stage 7 soybean plant (V7; Fehr and Caviness 1977). Therefore, the *ED* can be considered dynamic in that it will change as the leaf area changes throughout the season. Relatively speaking, *ED* will be smaller for younger soybeans plants and larger for older soybean plants. The per plant spatial scale was used because current scouting procedures for soybean aphid monitoring utilize per plant counts and the current ET and EIL are also on a per plant basis.

Anderson and May (1981) developed several dynamic models that describe the host-pathogen interaction in arthropod disease systems. The relationship between the soybean aphid and *P. neoaphidis* is a complicated one and required the integration of three models proposed by Anderson and May (1981): the parasite-induced reduction of host reproduction model (model B), the latent period of infection model (model D), and the free-living infective stages model (model G). Major underlying assumptions of these models are that the pathogen reproduces within the host and is directly and horizontally transmitted. Production of large numbers of infective spores by a single aphid provides ample evidence of pathogen reproduction within the host (Sierotzki et al. 2000). Additionally, there is no evidence that *P. neoaphidis* requires an intermediate host for transmission; however, it has been shown that aphid predators can increase transmission (Roy et al. 2001). Barta and Cagáň (2003) demonstrated that *P. neoaphidis* is capable of horizontal transmission among aphid species and Baverstock et al. (2005) showed horizontal transmission within species. Strictly speaking, vertical transmission is also possible; however, this transmission occurs after birth when infected mothers sporulate near their daughters (Chen and Feng 2004). Therefore, assumptions of within host reproduction and direct, horizontal transmission are satisfied and these models can be adapted to describe the dynamics of *P. neoaphidis* infection of soybean aphid.

Aphids become infected with *P. neoaphidis* via the direct penetration of pathogen conidia through aphid cuticle (Butt et al. 1990). However, once infected with the pathogen, the aphid does not become infectious to other aphids until after death, which occurs three to five days after inoculation (Butt et al. 1990), thereby making the disease latent. Once the aphid dies and becomes infectious, it produces thousands of conidia that are forcibly ejected into the surrounding environment (Butt et al. 1990). Conidia can cause infections in aphids for up to 28 d after their release into the environment (Brobyn et al. 1985); thus there is a group of free-living infectious stages that can lead to aphid infection.

Using a schematic depiction of interactions, based on what we know about the soybean aphid-*P. neoaphidis* pathosystem (Fig. 1), a system of equations can be developed to describe changes in aphid and pathogen populations as infection occurs. To begin, we describe the susceptible aphid population:

$$\frac{dX}{dt} = (a_1 - b)X + a_2 Z - vXW \quad (1)$$

where $X$ is the population of susceptible aphids (susceptibles), $Z$ is the population of infected aphids (infecteds), $W$ is the population of infective conidia (conidia), $a_1$ is the per capita birth rate of susceptible aphids, $b$ is the natural death rate of aphids (due to causes other than *P. neoaphidis* infection), $a_2$ is the per capita birth rate of infected aphids, and $v$ is a transmission constant. Susceptibles are produced via parthenogenetic reproduction by susceptibles, $a_1X$, and infecteds, $a_2Z$, and are lost to natural death, $bX$, and infection of susceptibles, $vXW$, at which time susceptibles become infecteds. While recovery from infection is another potential cause of changes in susceptible population, the histological study of *P. neoaphidis* by Butt et al. (1990) provides no evidence of aphid recovery after *P. neoaphidis* has successfully infected a host.

Changes in the population of infecteds can be described as:

$$\frac{dZ}{dt} = vWX - (\alpha + b)Z \quad (2)$$

where $\alpha$ is the rate at which infected hosts succumb to the infection and become infectious hosts. Infected hosts result from the contact of susceptibles with infectious conidia, $vWX$,

while infecteds leave the population via death due to infection, $\alpha Z$, or natural death, $bZ$ (due to factors other than infection).

Changes in the population of infectious aphids can be described as:

$$\frac{dY}{dt} = \alpha Z - \beta Y \quad (3)$$

where $Y$ is the population of infectious aphids and $\beta$ is the rate at which infectious aphids decay—or stop actively producing infective conidia. We termed this phenomenon 'decay' because aphids have already succumbed to infection, but also cease to produce infective conidia after several days, depending on abiotic conditions. Infectious aphids are produced as the result of infecteds succumbing to *P. neoaphidis* infection, $\alpha Z$. As infectious aphids decay, $\beta Y$, they leave the population of infectious individuals.

Finally, changes in the *P. neoaphidis* population can be described as:

$$\frac{dW}{dt} = \lambda Y - \mu W - vW(X + Z) \quad (4)$$

where $\lambda$ is the production of *P. neoaphidis* in number of conidia produced per unit area by an infectious aphid and $\mu$ is the rate of death experienced by infective conidia in the environment. Conidia are produced as a function of the population of infectious aphids and the density of conidia, number per $mm^2$, they produced, $\lambda Y$, and spores are lost via natural spore death, $\mu Y$, and spore 'consumption' by susceptibles, $vWX$, and infecteds, $vWZ$. In this case, when a spore contacts a susceptible or infected aphid and germinates, the spore leaves the pathogen population (i.e. can no longer cause additional infection); thus aphids which are already infected by the pathogen can be considered 'sinks' for infectious conidia.

Additionally, the entire host population can be defined as:

$$H = X + Y + Z \tag{5}$$

where $H$ is the entire aphid population. However, a more useful characterization of the aphid population for pest management would be:

$$H^* = X + Z \tag{6}$$

where $H^*$ is the portion of the aphid population which can cause economic damage to the host. Because infectious aphids, $Y$, are no longer feeding or reproducing, they need not be considered in management decisions.

While equations (1), (2), (3), and (4) describe the soybean aphid-*P. neoaphidis* pathosystem, and were a necessary first step in understanding the relationships among the populations in the system, we are interested in determining the aphid density at which *P. neoaphidis* can establish and persist in the aphid population. Anderson and May (1981) defined the reproductive rate of a pathogen, $R$, as the number of expected new infections per time unit, with the time scale based on the natural rate at which hosts die or succumb to infection. Thus, $R$ is equivalent to the number of secondary infections caused by each primary infection caused by the pathogen. In the soybean aphid-*P. neoaphidis* system, the pathogen reproductive rate is a function of conidia production, $\lambda$, the lifespan of infectious cadavers, $1/\beta$, rate of production of infectious cadavers, $\alpha$, the lifespan of infecteds, $1/(\alpha+b)$, and the proportion of infective conidia leading to new infections, $vH^*/(vH^*+\mu)$. Therefore, the reproductive rate of *P. neoaphidis* is:

$$R = \lambda \frac{1}{\beta} \alpha \frac{1}{(\alpha + b)} \frac{vH^*}{(vH^* + \mu)} . \tag{7}$$

It follows then, that for *P. neoaphidis* to establish in an aphid population, a primary infection must lead to at least one secondary infection, or $R \geq 1$. Therefore, the $ED$ occurs when $R$=1. Setting $R$=1 and solving for $H$, yields the $ED$:

$$ED = \frac{\mu}{\nu} \frac{\beta(\alpha + b)}{\lambda\alpha - \beta(\alpha + b)}. \quad (8)$$

*Estimating Parameters.* Although there are eight parameters in the dynamic equations, only six of these parameters are included in (8) and therefore necessary for the calculation of the $ED$. Model analysis requires independent estimates of the central tendency, variability and distribution of each parameter. Each parameter was assumed to be normally distributed with a mean and standard deviation (Table 1). Independent estimates of the mean and standard deviation of $\nu$ (Xu and Feng 2003; Nakasuji et al. 1985), $\mu$ (Brobyn et al. 1985), $\beta$ (Hua and Feng 2003), and $\lambda$ (Sierotski et al. 2000) were calculated using data available in the published literature (Table 1). However, further analyses and data transformations were required to obtain these parameter estimates.

The transmission constant, $\nu$, was not available directly from the literature. Instead, the regression method utilized by Nakasuji et al. (1985) was applied to time-dose-mortality data of the infection of *Myzus persicae* by *P. neoaphidis* from Xu and Feng (2000) to obtain an estimate of its mean and standard deviation. Xu and Feng (2000) examined how *P. neoaphidis* conidia density, in conidia per $mm^2$, impacted the rate and latent period of infection of green peach aphid, *Myzus persicae*, over a seven day period. Throughout the experiment of Xu and Feng (2000) the conidia density, $W$, and host density, $H^*$, are held constant; however, aphids can move from susceptibles, $X$, to infecteds, $Z$, giving:

$$\frac{dX}{dt} = -\nu XW. \quad (9)$$

This equation is similar to (1), except that reproduction and natural death were eliminated because this took place under controlled, experimental conditions. Following Nakasuji et al. (1985), we can integrate (9) with respect to $X$ while holding $W$ constant, yielding:

$$X_t = X_0 e^{-\nu W t} \quad (10)$$

where $X_t$ is the number of healthy aphids at time $t$ and $X_0$ is the initial number of healthy aphids. We can then transform (10), yielding:

$$\frac{\ln(X_t / X_0)}{t} = -vW . \qquad (11)$$

Following this theory, we used data from Xu and Feng (2000) to perform a linear regression (R 2.9.2), using the slope of the regression line as an estimate of the mean and standard deviation for $v$ (Table 1). While the data does originate from a different species of aphid, we assume that resulting estimate of $v$ can be applied to soybean aphid infection by *P. neoaphidis* because green peach and soybean aphid are of similar size and because the study was performed under controlled conditions.

To estimate $\mu$, we used data published in Brobyn et al. (1985) which reported the infection rates of *Aphis fabae* after challenge with *P. neoaphidis* conidia which had been exposed to field conditions (i.e. solar radiation, changes in temperature and humidity) for up to 28 d. For the purposes of estimating the *ED*, we defined conidia as dead once they lost the ability to infect aphids. As a proxy estimate of conidial lifespan in the environment, we performed a probit analysis (PROC PROBIT, SAS 9.2) to calculate the $LT_{50}$ for *P. neoaphidis* conidia using length of conidial exposure and proportion of aphids becoming infected reported in Brobyn et al. (1985). We only used data from conidia located on the underside of the leaves because we assume aphids are located only on the underside of soybean leaves. The estimated $LT_{50}$ is actually an estimate of the lifespan of the conidia, whereas the parameter $\mu$ is the conidia death rate, the inverse of the lifespan. To correct this, we used the $LT_{50}$ as an estimate of the mean of and the 95% CI to estimate its standard deviation and when the *ED* was calculated, the parameter value for $\mu$ was inverted (Table 1).

There was no direct estimate of aphid cadaver decay rate provided in the literature; however, Hua and Feng (2003) reported that over 99% of spores produced by a *M. persicae* cadaver were released within the first 3 d of the aphid succumbing to *P. neoaphidis* infection. Of the 84,000 conidia produced per cadaver, 78.6% were released on the first day after aphid death, with 19.9% released on the second day, and 1.3% released on the third day post-death (Hua and Feng 2003). Sporulation was observed up to 4 d after aphid death, while the majority of conidia were released within 2 d of death (Hua and Feng 2003). Therefore, we estimated the mean of cadaver infectious period as 3 d and standard deviation as 1 d. Because the parameter $\beta$ is cadaver decay rate, the

estimate of the cadaver infectious period had to be inverted as part of the *ED* calculation (Table 1).

Conidia production by four isolates of *P. neoaphidis* was recorded as part of a study published by Sierotzki et al. (2000). Conidia produced per $mm^2$ was recorded for each isolate after 10 min. Because the time scale of the *ED* is per day, these conidial densities were converted to conidia produced per $mm^2$ per d. The mean and standard deviation of the daily conidia production of the four isolates was used as the estimate of the production of *P. neoaphidis*, $\lambda$, in conidia per $mm^2$ (Table 1).

The aphid death rate due to *P. neoaphidis* infection was estimated using data collected from a series of laboratory bioassays in which soybean aphids were exposed to *P. neoaphidis* conidia in order to estimate the latent period of infection. We exposed 500 soybean aphids, in ten replications of 50 aphids each, to actively sporulating *P. neoaphidis* cultures (KAK, unpub. data). Of the 500 exposed, 301 became infected by the fungus and the time, in d, elapsed between exposure and aphid death was used to calculate an estimate of the mean and standard deviation of the aphid death rate due to infection, $\alpha$, in $d^{-1}$.

The death rate of soybean aphid, not including death due to *P. neoaphidis* infection, was estimated using soybean aphid lifespan data from 192 individuals. Soybean aphids, which were protected from predation, were monitored in the field to determine their lifespan in degree days (B.P. McCornack, personal communication). Because the time scale of the *ED* estimate is in d, number of d at 20°C with a base of 8.6°C was calculated from degree days (McCornack et al. 2004). Mean and standard deviation of the lifespan in d of the 192 aphids was used as the estimate of the inverse of aphid death rate (B.P. McCornack, personal communication). The estimate was inverted during model calculation in order to convert it from aphid lifespan to natural aphid death rate.

*Model Analysis*. To obtain a robust estimate of the *ED*, we performed a nonparametric bootstrap analysis incorporating estimates of parameter means and standard deviations into equation (8) (R 2.9.2). The major benefit of this type of analysis is the ability to compensate for the uncertainty surrounding each parameter estimate. A random value for each parameter is selected from its distribution each time the *ED* was

calculated. We repeated this process 10,000 times, yielding 10,000 possible *ED* values. Because of the nature of equation (8), a small number of runs resulted in negative *ED* values, which were removed from the dataset because it is impossible to have a negative aphid density. For the purpose of discussion, we translated *ED* estimates from aphids per $mm^2$ to aphids per V7 plant by multiplying each *ED* value by a constant, 61,950, which is based on soybean leaf areas of 29.5 $cm^2$ reported in O'Neal et al. (2002). We approximated the *ED* of soybean aphid for the establishment of *P. neoaphidis* as the $ED_M$, which is the mean of all positive values resulting from the model analysis. We also calculated the 95% CI of $ED_M$ as a measure of variability.

*Sensitivity Analysis.* We performed a sensitivity analysis in order to determine how small changes in parameter estimates would change the $ED_M$. We increased and decreased each parameter estimate by 15%, while keeping the standard deviation and all other parameters constant, and recalculated the $ED_M$.

*Model Assumptions.* We have made assumptions about the soybean aphid-*P. neoaphidis* pathosystem in order to complete model development and analysis. First, the model assumes that all individual soybean aphids and all *P. neoaphidis* conidia are average individuals, meaning each individual exhibits identical behavior in the field. This is probably not exactly true, as there is evidence that alate aphids and apterous aphids are not equally susceptible to fungal infection (Yu et al. 1995); however, during the time that this model is applicable (i.e. when soybeans are in the V7 stage), the populations will be primarily composed of apterous aphids. There is no evidence that *P. neoaphidis* spores vary significantly, however, strains of this fungus do vary biologically (Sierotski et al. 2000). We also assume that laboratory data used to calculate $\alpha$, $\lambda$, $\beta$, and $v$ will be applicable in a field setting and that data collected from other aphid species to estimate $\lambda$, $\beta$, $v$, and $\mu$ will be applicable to soybean aphid. We assume that soybean aphids are located on the under-side of the leaves. Based on field observations, this is most often the case, with aphids only moving to the top of leaves in response to extreme crowding on the underside of the leaves (KAK, personal observation). However, early in the season aphid colonies tend to occur at the soybean growing tip. We also assume that leaf area is consistent in a population of V7 soybean plants. While this is likely not true, as variety, environmental conditions, and the growth stage of the plant affects the leaf

area of soybean plants, the true *ED* is on a $mm^{-2}$ spatial scale, so the calculated *ED* value can easily be adjusted to any soybean population. The most critical assumption is that environmental conditions are conducive for disease transmission. The experiments performed to determine the transmission parameter were done in conditions that were highly suitable for disease transmission. Because the *ED* should be relevant in the field, conditions will be much more variable and the ability of the fungus to cause infection will likely decrease, especially under hot and dry conditions (Sivčev and Draganić 1994; Sivčev and Manojlović 1995; Shah et al. 2002).

**Model Validation Field Experiment**

*Field Plots.* At the Sand Plain Research Farm at Becker, MN, soybean variety NK S19L7 was planted with a grain drill on 22 May 2009. Soybean was planted in 76.2 cm rows and at a seeding rate of 407,724 seeds per ha. Soybean seed was Roundup Ready and glyphosate was applied prior to soybean emergence to control early season weeds. We utilized mechanical weed control for the remainder of the season due to the presence of experimental cages which precluded further glyphosate applications. Soybean plants were irrigated due to the sandy soil at the research farm and in order to increase the humidity to encourage aphid disease progression. Irrigation occurred twice each week with solid set overhead irrigation. Pipes were laid parallel to soybean rows with nozzles occurring every 9.14 m. Irrigation frequency and duration was adjusted in the event of significant rainfall so that plants received approximately 4 cm of water each week.

Plots consisted of a single, caged soybean plant within a section of soybean 3.05 m long and 3 rows (2.29 m) wide. Cages included a wire tomato frame (1.37 m tall by 0.41 m wide; Glamos Wire, Hugo, MN) enclosed by a white no-see-um mesh sleeve (1.31 m tall by 0.66 m wide with 0.65 by 0.17 mm gaps; Eastex Products Inc., Holbrook, MA). Mesh sleeves prevented predator immigration and aphid emigration. Metal cage frames were installed around the soybean plant, the bottom of the mesh cage was buried, and the top was closed with a zip tie.

Three treatments were initiated once soybean plants reached the V7 stage with ten replications each. Plots were organized in a completely randomized design. Treatments included a low aphid density treatment in which *P. neoaphidis* inoculum was introduced

into an aphid population of approximately 50 aphids per plant, a moderate aphid density treatment in which inoculum was introduced into an aphid population of approximately 250 aphids per plant, and a high aphid density treatment in which inoculum was introduced into an aphid population of approximately 675 aphids per plant. The purpose of these treatments was to ensure that there would be a range of initial aphid densities across the 30 plots. It was essential that all plots receive inoculum at the same time to ensure that disease initiation and progression occurred under identical environmental conditions across all treatments. Therefore, 10 soybean aphids were added to the high aphid density treatment first, on 6 Jul 2009, plots receiving the moderate aphid density treatment were infested with 10 aphids on 8 Jul 2009, and the low aphid density was infested with 15 aphids on 13 Jul 2009. On 17 Jul 2009, *P. neoaphidis* inoculum was released into the cages as 10 soybean aphid cadavers killed by *P. neoaphidis*. Infected cadavers were produced by inoculating soybean aphids from a laboratory colony with an actively sporulating culture of *P. neoaphidis*. This culture was originally isolated from an infected pea aphid, *Acryrthosiphon pisum* Harris, collected from Rosemount, MN in 2008. The fungus was used to infect soybean aphid and was reisolated from soybean aphid twice prior to use in this experiment. The cultures used to produce cadavers were infected using the first subculture of the second reisolation from soybean aphid. Cultures of *P. neoaphidis* were maintained on Sabouraud dextrose agar (Difco™, Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with egg yolk and milk (SDAEYM) and were kept at 19 ± 1°C and 0:24 (L:D) h.

*Sampling.* Aphid density within each plot was determined by counting and recording the number of aphids on each node of the plant. This sampling method allowed for a precise measurement of aphid density as well as the spatial distribution of aphids on the plant. Aphid sampling was performed twice per week from 13 Jul 2009 through 6 Aug 2009.

Bioassays were also performed twice per week from 20 Jul 2009 through 6 Aug 2009 in order to determine if the *P. neoaphidis* inoculum released into the cages led to secondary infections and fungal establishment. Bioassays consisted of collecting 50 apparently healthy aphids from each cage, returning them to the laboratory, transferring them to excised soybean leaves (variety S19R5) grown in a greenhouse, and monitoring

them for 4 d. Any apparently infected aphid was transferred to 1% water agar to induce sporulation. Samples of conidia from all sporulating aphids were stained with aceto-orcein stain (Humber 1997) to confirm *P. neoaphidis* caused the infection. Conidia were identified via spore morphology at 200x magnification following Samson et al. (1988).

At the conclusion of the experiment, on 10 Aug 2009, plants from all cages were returned to the laboratory for examination. If aphid cadavers apparently infected by *P. neoaphidis* were located on the plant, they were placed on water agar to induce further sporulation. Conidia were identified following the same procedures utilized for the bioassays. This was done to confirm the status of *P. neoaphidis* establishment, as low levels of disease (<2%) could not be detected via the bioassay method. We concluded that *P. neoaphidis* establishment occurred if infection was confirmed via bioassays or plant examination.

*Statistical Analysis.* We performed a binomial regression of the $\log_2$ transformed aphid density in cages at the time of inoculation on establishment of *P. neoaphidis* (R 2.9.2). Following the delta method, we know:

$$\operatorname{logit}(\Pr(E)) = a + b * ED_{50} \tag{12}$$

where Pr(E) is the probability of *P. neoaphidis* establishment, which in this case Pr(E)=0.5, $a$ is the intercept calculated in the binomial regression model, $b$ is the model estimate for initial aphid density, and $ED_{50}$ is the aphid density at which there is a 50% chance of *P. neoaphidis* establishment and is the field estimate of *ED*. Solving for $ED_{50}$ yields:

$$ED_{50} = \frac{[\operatorname{logit}(0.5) - a]}{b} \tag{13}$$

and after taking the logit of 0.5 the equation is simplified to:

$$ED_{50} = \frac{-a}{b}. \tag{14}$$

We were able to calculate the $ED_{50}$ and its 95% CI using the delta method ('alr3' Package, R 2.9.2; Weisberg 2009), where our $a$ is equivalent to Weisberg's *b0* and our $b$ is equivalent to Weisberg's *b1*. Following identical procedures, we also calculated the $ED_{95}$ for the purposes of comparing the more conservative $ED_{95}$ to the already accepted soybean aphid ET and EIL.

## Results

### Model Estimate of *ED*

We calculated the $ED_M$ as 152 (95% CI: 144-160) soybean aphids per V7 plant (0.0025 soybean aphids per $mm^2$). The $ED_M$ is the mean of all positive model calculations ($n$=9,961), as 39 observations were removed from the dataset because they were less than zero. Model iteration results had a large range, with a minimum of 21 aphids per plant and a maximum of over 22,000 aphids per plant (Table 2). The median, 98 soybean aphids per plant, was lower than the $ED_M$, implying that results were right-skewed, which is confirmed in the frequency distribution of the central 98% of calculated values (Fig. 2). The frequency distribution also demonstrates that most of the calculated values fall between 0 and 1000 aphids per plant, which is a reasonable range for soybean aphids on V7 plants.

The sensitivity analysis indicated that small changes in the estimates of $\lambda$ (conidia production by *P. neoaphidis* per $mm^2$) and $\beta$ (decay rate of infectious aphids) can lead to relatively large changes in the predicted $ED_M$, its standard deviation, and its 95% CI (Table 3). Whereas identical perturbations of the estimates of $b$ (natural death rate) and $\alpha$ (death rate due to infection) lead to changes of less than 5% in the calculated $ED_M$, its standard deviation, and its 95% CI (Table 3). Changes in the final two parameters, $v$ (transmission constant) and $\mu$ (death rate of *P. neoaphidis* conidia), lead to moderate changes of 10-20% in $ED_M$, its standard deviation, and its 95% CI (Table 3).

### Field Estimate of *ED*

At the time of inoculation with *P. neoaphidis*, aphid density within plots ranged from 14 to 889 aphids per plant. The mean (±SE) aphid density was 57±10, 244±54, and 507±69 aphids per plant for the low, moderate, and high aphid densities, respectively. The mean soybean stage at the time of inoculation was V7.4±0.3. Thus, treatments were successful in that there was a wide range of aphid densities represented and inoculation occurred at soybean stage V7. However, three cages were eliminated from the analysis due to death of the soybean plant.

Successful *P. neoaphidis* establishment occurred in 20 of the 27 plots (Fig. 3), with 70% of successful establishments being detected via the biweekly bioassays and the remaining 30% of successful establishments being detected via plant examinations.

Infections in cages were first observed on 23 Jul 2009, with one plot exhibiting 2% infection. Most plots in which successful establishment occurred did not exhibit positive bioassays until after 3 Aug 2009, with disease levels not exceeding 20% in any plot. All infected soybean aphids recovered from experimental plots were infected with *P. neoaphidis*. Plots in which *P. neoaphidis* established had a mean aphid density of 324±51 aphids per plant, while plots without establishment had a mean aphid density of 50±13 aphids per plant. Results of the binomial regression predict that the $ED_{50}$ is 48 (95% CI: 30-78) aphids per V7 plant, while the $ED_{95}$ is 130 (95% CI: 53-318) (Fig. 3).

## Discussion

Both model, $ED_M$, and field, $ED_{50}$ and $ED_{95}$, estimates of *ED* fall below the soybean aphid ET and EIL, indicating that *P. neoaphidis* can establish in sub-economic aphid populations. The $ED_M$ of 152 aphids per V7 plant is also consistent with the $ED_{50}$ of 48 aphids per plant and the more conservative $ED_{95}$ of 130 aphids per plant. Thus, there is strong evidence that the *ED* model and parameters accurately describe the soybean aphid-*P. neoaphidis* pathosystem.

Although the $ED_M$ is well below the EIL, values calculated in the analysis span a wide range of 21 to over 20,000 aphids per plant. Because all calculated values are possible $ED_M$ estimates, having some knowledge of the probability that the actual *ED* is larger than the EIL is an important step in vetting model results in the context of soybean aphid IPM. Of the 9,961 positive model calculations, 10% of the values were larger than the ET of 250 aphids per plant and 1.5% of values were larger than the EIL of 674 aphids per plant. Thus, we have evidence that the actual *ED* will be less than the ET in 90% of soybean aphid populations and less than the EIL in 98.5% of aphid populations. Additionally, when the $ED_M$ is applied to V3, V5, V10, and V12 soybean plants, the estimated $ED_M$ becomes 65, 108, 217, and 260 soybean aphids per plant, respectively. Even on large, late-season V12 soybean plants, the $ED_M$ estimates that the *ED* is close to the ET and lower than the EIL.

However, the $ED_M$ was determined based on a simplified mathematical model with many assumptions about complex ecological events. Such simplistic representations have been criticized (Onstad et al. 1990; Onstad 1993); however, such models have

successfully described the dynamics of the gypsy moth, *Lymantria dispar*, and its viral and fungal pathogens (Dwyer and Elkinton 1993;Hajek et al. 1993). The purpose of our analysis was not to gain a complete understanding of all underlying ecological processes, rather, we attempted to quickly and economically determine if *P. neoaphidis* has the potential to contribute to aphid management when the aphid occurs on soybean and would therefore warrant further, more in depth study. Thus, while such simplification and its associated assumptions may be seen as a weakness, that we calculated reasonable estimates of *ED* which coincide with commonly observed field aphid densities without performing complicated experiments is a clear benefit of this application of mathematical modeling. Refining parameter estimates through further experimentation does have the potential to produce an $ED_M$ which is closer to the actual *ED*, but this would diminish key benefits of this methodology.

The model sensitivity analysis demonstrated that for most of the parameters, small perturbations in parameter mean had correspondingly small impacts on the calculated $ED_M$. However, changes in calculated $ED_M$ were relatively large when small changes were made to either $\lambda$, conidia production by *P. neoaphidis* per $mm^2$, or $\beta$, decay rate of cadaver (Table 3). Both of these parameters directly affect the density of *P. neoaphidis* conidia available to infect soybean aphid. Because the transmission constant, $v$, is so low (i.e. very few interactions between host aphids and fungal conidia lead to successful infection), high densities of conidia are required for pathogen persistence. Thus, a reduction in pathogen production, $\lambda$, or an increase in the rate at which cadavers cease producing spores, $\beta$, lead to a disproportionately large increase in the $ED_M$ and its standard deviation (Table 3). This may be due to an increase in large, outlier values for $ED_M$ as there were fewer negative values when $\lambda$ was increased by 15% and $\beta$ decreased by 15%, respectively. Thus the uncertainty of the model could be reduced with better estimates of $\lambda$ and $\beta$.

While including six parameters in a model does introduce uncertainty, comparing model results with field data can validate model results and mitigate such uncertainty. In this case, field data was able to validate model results as the $ED_{50}$ and $ED_{95}$ are both lower than the $ED_M$. However, there are weaknesses in the field validation experiment. First, we must consider that *P. neoaphidis* conidia are capable of escaping cages, entering

the environment, and providing additional inoculum to surrounding cages at a time when aphid densities had increased beyond the initial treatment levels; thus, lowering the estimate of *ED*. While this is possible, we believe it is unlikely to have impacted results of the experiment since only seven cages had established disease, and only at enzootic levels of 2-6%, prior to 10 Aug 2009, the final bioassay date. Thus, considering the spatial separation of each cage in addition to the low inoculum load, it is unlikely that such contamination occurred. Additionally, aphid densities in surrounding soybean remained below 60 aphids per plant throughout the experiment and no infected aphid cadavers were observed while sampling surrounding soybean for aphids. So it is unlikely that inoculum was being produced by aphid populations outside of experimental cages over the three weeks of the experiment.

Another potential weakness is that plots were closed systems and while alate aphids, both healthy and infected, would normally be able to leave an aphid population, they were unable to do so in this experiment. Because infected alate aphids can successfully migrate to new hosts and establish new colonies prior to succumbing to infection (Feng et al. 2007), it is likely that in an open aphid population, the actual inoculum load would be decreased by this emigration of infected alates, leading to artificially inflated inoculum levels within cages. We also think it is unlikely that this weakness significantly impacted results of the field validation experiment because again, disease was not detected in most cages until the final sampling date of the experiment. Thus, the inoculum load within cages was likely low throughout the experiment.

Most important to consider is that during the field experiment, disease did not become detectable in most plots until at least 17 d after inoculum was released. This is a significant lag time and aphid densities were able to increase substantially, with 20 of the 27 plots having aphid densities exceeding the EIL at the conclusion of the experiment. While part of this was due to the exclusion of predators and prevention of emigration, it suggests that while *P. neoaphidis* is capable of *establishing* in sub-economic aphid populations, it may not be capable of exerting enough pressure on aphid densities to prevent damage to soybean. However, we hypothesize that this lack of effective control may have been caused by abiotic conditions which were not ideal for the transmission of fungal disease. Typical *P. neoaphidis* epizootics begin in early August in Minnesota,

when aphid populations are nearing their peak densities, dew is frequently present on leaves for several hours a day, and the soybean canopy is closed (K.A.K. personal observation). Because this experiment was initiated in mid-July, dew was not yet commonly found on leaves and plants in experimental plots were caged, preventing canopy closure around the experimental plants. Climate data for this region of Minnesota indicate that while daily average temperatures were moderate, 18.4±2.1°C, for the duration of the experiment, daily average relative humidity was 65.3±5.4%, which is lower than the 96-100% required for *P. neoaphidis* transmission (NCDC 2011; Steinkraus 2006). Thus, the microclimate experienced by the aphid populations in experimental plots was probably less humid than would be ideal for *P. neoaphidis* transmission. Such abiotic factors likely contributed to the slow disease progression observed in spite of the high aphid densities.

One potential application of the modeling methods utilized in this paper is the screening of pathogens which are potential biological control agents of pests in agricultural, greenhouse, or natural systems. If enough is known about such pathogens, limited experimentation can yield an estimate of the minimum pest density required for establishment of the pathogen. Such knowledge can inform whether the pathogen is likely to establish in sub-economic pest populations. Because establishment of biocontrol agents is problematic, using this simple model can help prioritize those agents which may be most likely to establish and persist in pest populations. Once a pathogen has been selected for release, this technique can help biocontrol practitioners select the best pest populations for release of the agent. Because parasitoid wasps act similarly to pathogens in many aspects of the parasitoid life history, such methods may also be applicable to screening this group of natural enemies.

In summary, we found, via the application of a mathematical model confirmed by a field validation experiment, that *P. neoaphidis* is capable of establishing within in a sub-economic soybean aphid population of as low as 50-150 soybean aphids per V7 soybean plant. Thus, *P. neoaphidis* is a potential contributor to the natural regulation of soybean aphid populations. While further experimentation is required to determine if and when *P. neoaphidis* can successfully maintain aphid populations below the ET and EIL, results of these experiments confirm that such further experimentation is warranted. If

we can better understand the dynamics of the soybean aphid-*P. neoaphidis* pathosystem, we may be able to take advantage of *P. neoaphidis* as a management tool, similar to the utilization of *Neozygites fresenii* in cotton aphid, *Aphis gossypii,* management in southeastern US cotton fields (Hollingsworth et al. 1995). Results of these experiments indicate that further research exploring this possibility is warranted and may provide benefits to soybean growers and society as a whole via increased soybean yield and decreased insecticide use.

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**Table 1.** Values and sources of parameter estimates.

| Parameter | Mean ± SD (definition) | Source |
|---|---|---|
| $1/b$ | 20.5 ± 7.7 (aphid lifespan in d) | B.P. McCornack (pers. comm.) |
| $\lambda$ | 1,670 ± 619 (conidia produced per $mm^2$) | Sierotzki et al. 2000 |
| $\alpha$ | 0.35 ± 0.02 (aphid death rate from infection) | K.A. Koch (unpublished data) |
| $v$ | 0.014 ± 0.003 (transmission constant) | Xu and Feng 2000 |
| $1/\beta$ | 3 ± 1 (cadaver infectious period in d) | Hua and Feng 2003 |
| $1/\mu$ | 11.3 ± 2.7 (conidia lifespan in d) | Brobyn et al. 1985 |

**Table 2.** Summary of nonparametric bootstrap analysis of *ED* model estimating the per V7 plant soybean aphid density required for the establishment of *P. neoaphidis*.

| Descriptive Statistic | Value |
|---|---|
| *n* (positive values) | 9,961 |
| mean | 152 aphids per V7 soybean plant |
| standard deviation of mean | 401 aphids per V7 soybean plant |
| 95% confidence interval of mean | 144 -160 aphids per V7 soybean plant |
| minimum | 21 aphids per V7 soybean plant |
| $1^{st}$ quartile | 69 aphids per V7 soybean plant |
| median | 98 aphids per V7 soybean plant |
| $3^{rd}$ quartile | 153 aphids per V7 soybean plant |
| maximum | 22,179 aphids per V7 soybean plant |

**Table 3.** Results of the sensitivity analysis in which each parameter mean was increased (+15%) and decreased (-15%), while all other model parameters were left unchanged, while model iterations were repeated. All descriptive statistics presented in aphids per V7 soybean plant. Results of the base model analysis are included in the bottom row for comparison.

| Parameter | Change | Mean | St Dev | 95% CI | 1st Quartile | Median | 3rd Quartile |
|---|---|---|---|---|---|---|---|
| *b* | +15% | 155 | 409 | 147 – 163 | 71 | 101 | 157 |
| | -15% | 149 | 394 | 141 – 157 | 68 | 97 | 151 |
| $\lambda$ | +15% | 126 | 912 | 108 – 144 | 61 | 85 | 128 |
| | -15% | 229 | 2,422 | 142 – 238 | 79 | 117 | 190 |
| $\alpha$ | +15% | 149 | 394 | 141 – 157 | 68 | 97 | 151 |
| | -15% | 155 | 409 | 147 – 163 | 70 | 100 | 157 |
| $v$ | +15% | 127 | 337 | 120 – 133 | 59 | 83 | 128 |
| | -15% | 178 | 476 | 169 – 187 | 79 | 114 | 179 |
| $\beta$ | +15% | 234 | 2,354 | 188 – 280 | 80 | 117 | 188 |
| | -15% | 124 | 298 | 118 – 130 | 61 | 85 | 129 |
| $\mu$ | +15% | 179 | 473 | 169 – 189 | 81 | 116 | 181 |
| | -15% | 132 | 348 | 125 – 139 | 60 | 86 | 134 |
| Unchanged Model | | 152 | 401 | 144 – 160 | 69 | 98 | 153 |

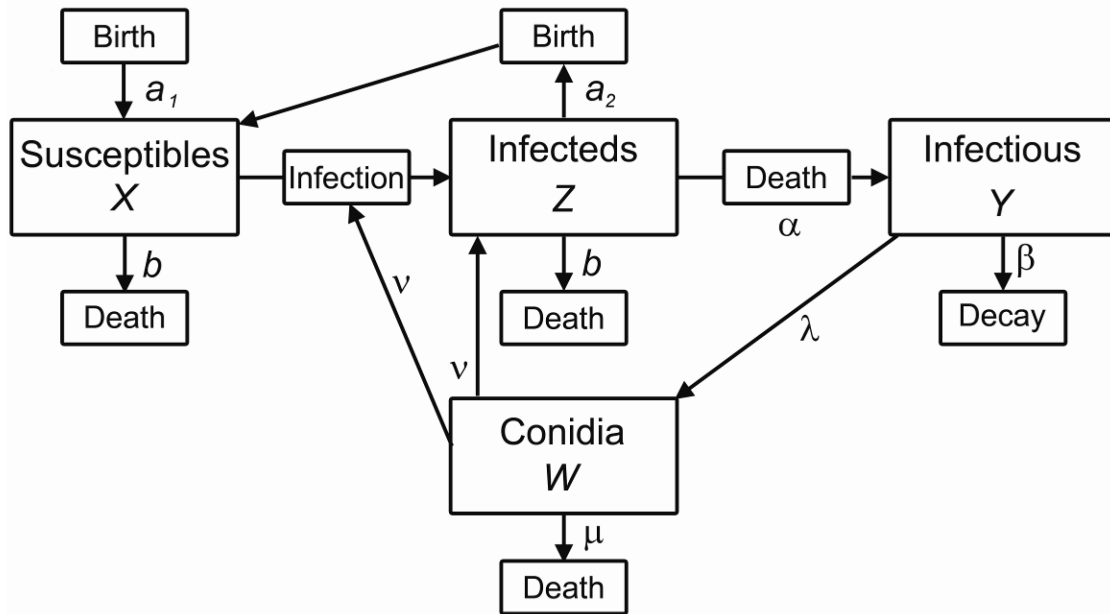


**Figure 1.** Conceptual model of the soybean aphid-*P. neoaphidis* pathosystem. Aphids fall into one of three categories, depending on their infection status, where $X$ is the population of susceptible aphids (susceptibles), $Z$ is the population of infected aphids (infecteds), and $Y$ is the population of infectious aphids (infectious). The pathogen, *P. neoaphidis*, is represented by $W$, which is the population of infective conidia (conidia). New susceptibles are formed at a rate of $a_1$, the per capita birth rate of susceptibles, and $a_2$, the per capita birth rate of infecteds. Susceptibles are lost at a rate of $b$, the natural death rate of aphids (due to causes other than *P. neoaphidis* infection) and due to infection, at a rate proportional to $v$, the transmission constant, at which time susceptibles become infecteds. Infecteds succumb to infection and become infectious at a rate of $\alpha$, the death rate due to infection. Infectious aphids produce infective conidia at a rate of $\lambda$, the rate of production of the pathogen. Infectious aphids cease producing spores at a rate of $\beta$, the decay rate of infectious aphids. Conidia lose infectiousness at a rate of $\mu$, the death rate of spores in the environment.

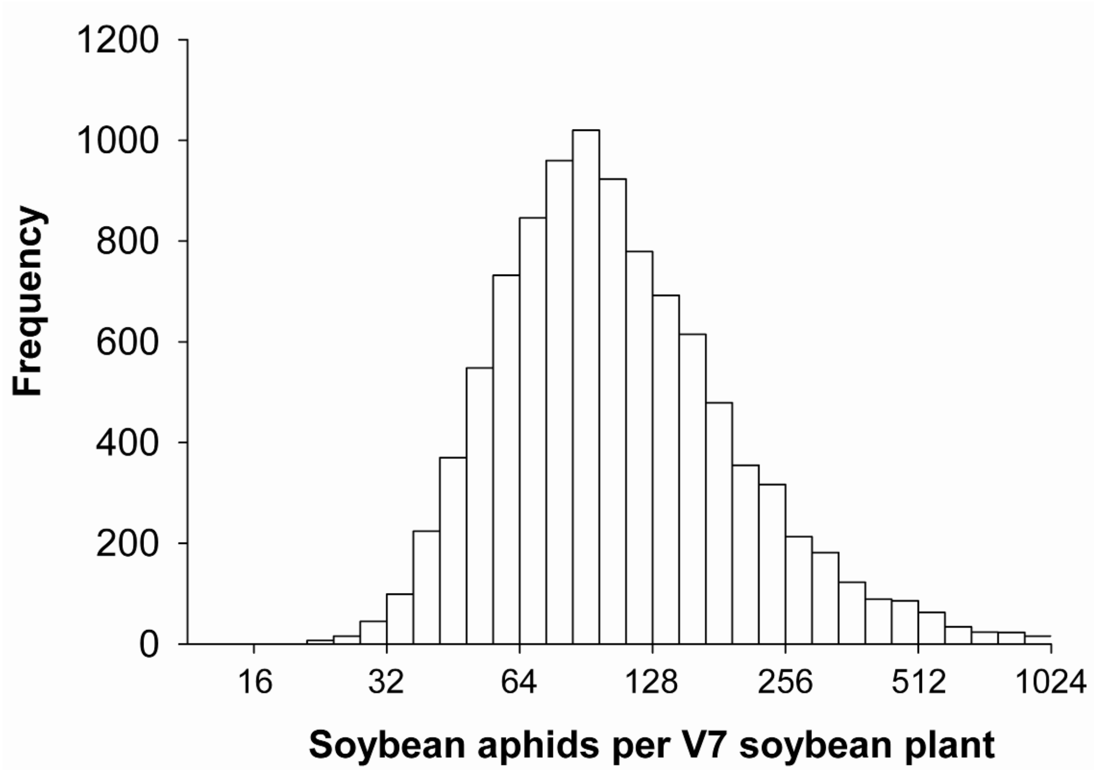


**Figure 2.** Results of nonparametric bootstrap ($n$=10,000) analysis of *ED* model which predicts the density of soybean aphid required for the establishment of *P. neoaphidis*. Only the central 98% of positive calculated values for *ED* are shown. Aphid values shown on the x-axis are presented on a $\log_2$ scale.

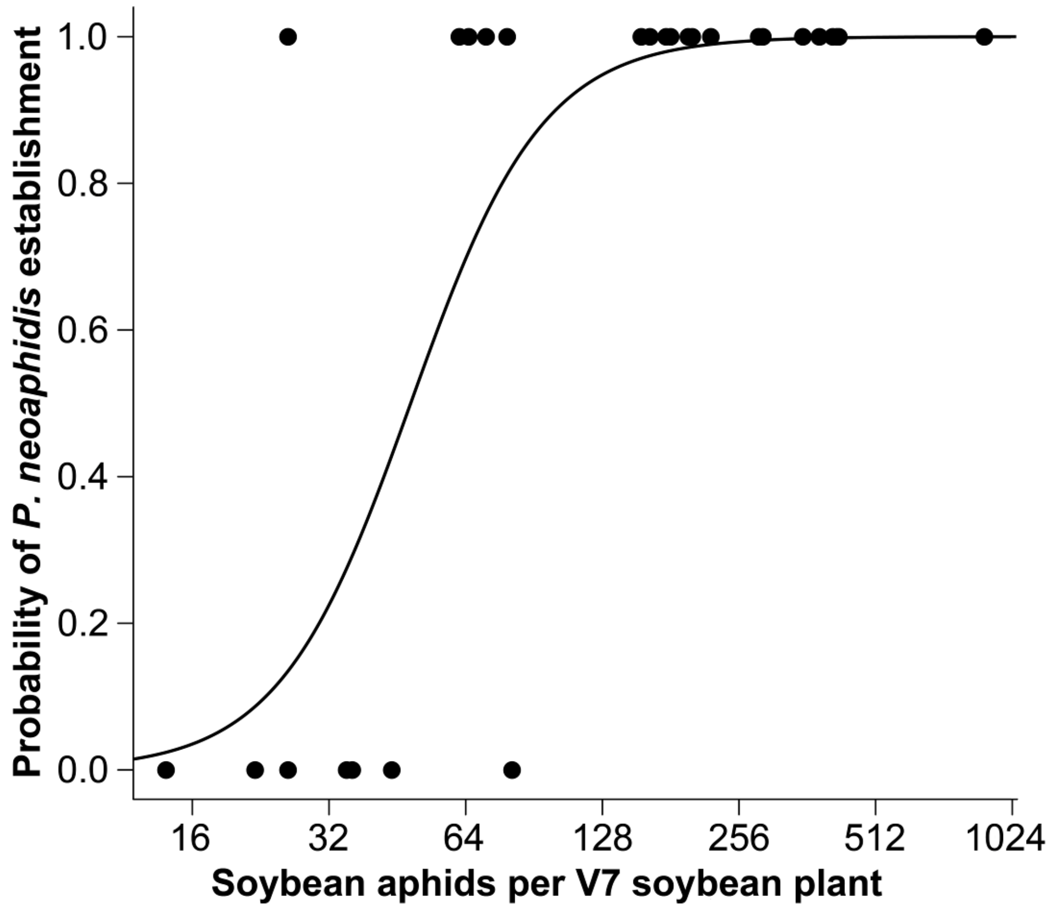


**Figure 3.** Results of the binomial regression predicting the probability of *P. neoaphidis* establishment from observed soybean aphid density at time of inoculation of field cages at Becker, MN 2009. Closed circles represent results of *P. neoaphidis* establishment in field cages, where circles at $Pr(E)$=1 represent cages in which the fungus successfully established and circles at $Pr(E)$=0 represent cages in which the fungus failed to establish. The solid line represents the predicted probability of *P. neoaphidis* establishment based on the binomial regression model. Aphid values shown on the x-axis are presented on a $\log_2$ scale.

# Chapter 3: Impacts of thiamethoxam seed treatments and host plant resistance on the soybean aphid fungal pathogen *Pandora neoaphidis*

## Introduction

The discovery of soybean aphid, *Aphis glycines* Matsumura, in North America in 2000 (Ragsdale et al. 2004) has spurred the development of new soybean pest management tactics including insecticide seed treatments and aphid resistant soybean varieties. Because research has shown that natural enemies can be important regulators of soybean aphid populations (Fox et al. 2004; Nielsen and Hajek 2005; Costamagna et al. 2007; Xue et al. 2009), understanding how these new technologies impact the function of natural enemies in soybean fields is important if sound pest management programs for soybean aphid are to be developed and successfully implemented (Ragsdale et al. 2011).

Soybean aphid (Hemiptera: Aphididae) alternates between two host plants in order to complete sexual reproduction and overwinter (Ragsdale et al. 2004). In spring, eggs laid on the aphids' primary host, *Rhamnus cathartica* L. or common buckthorn (Voegtlin et al. 2004), hatch producing parthenogenetic fundatrices and as subsequent generations occur, an increasing proportion of individuals become alate and move to the aphid's secondary host, *Glycine max* or cultivated soybean (Ragsdale et al. 2004). Soybean aphids occurring on soybean also reproduce parthenogenetically, which enables populations to grow exponentially if adequate resources are available (Ragsdale et al. 2004). In mid- to late summer, when aphids are crowded or when host plants decline in quality, the proportion of aphids which are alate will increase and these migratory aphids will disperse to new host plants (Hodgson et al. 2005). In the fall, as day length shortens and temperatures cool, sexual forms of the aphid are produced. Female gynoparae and, approximately two weeks later, males leave soybean and return to buckthorn. Gynoparae produce oviparae which mate with winged males and overwintering eggs are laid near buckthorn buds (Dixon 1973; Ragsdale et al. 2004).

Entomopathogenic fungi belonging to the fungal order Entomophthorales have been documented in many cropping systems and are particularly important natural enemies of aphid pests in cotton (Steinkraus et al. 1995) and potato (Shands et al. 1962). These fungi are also present in soybean and are frequently associated with declines in soybean aphid densities (KAK, unpublished data). In addition, these pathogens move with aphids from the soybean field to common buckthorn, where rates of aphid fungal infection can be higher than those observed in soybean (Nielsen and Hajek 2005).

Therefore, preservation of these entomopathogenic fungi, particularly *Pandora neoaphidis*, the most common pathogen infecting soybean aphid in Minnesota (Koch et al. 2010), Michigan (Noma and Brewer 2007), and New York State (Nielsen and Hajek 2005), may be an important part of preventing severe aphid outbreaks.

Many studies have explored potential non-target impacts of neonicotinoid seed treatments on aphids and their natural enemies. These studies demonstrated significant negative impacts on survival and fecundity of both target aphids and non-target arthropod natural enemies (Daniels et al. 2009; Cutler et al. 2009; Moser and Obrycki 2009; Papachristos and Milonas 2008). On the other hand, *in vitro* and field studies have shown that thiamethoxam is compatible with non-entomophthoralean fungi including *Beauveria bassiana* and *Metarhizium anisopliae* (Neves et al. 2001; Filho et al. 2001). The disparity of these results highlights the need for specific research addressing how neonicotinoid seed treatments impact the entomophthoran fungi infecting soybean aphid.

The first aphid-resistant soybean varieties have the *Rag1* gene and have been shown to increase mortality and decrease fecundity and lifespan of soybean aphids (Li et al. 2004; Hill et al. 2006). Increased frequency of aphid departure from *Rag1* resistant soybean leaves indicate that resistance may also be due in part to antixenosis (Li et al. 2004). Host plant resistance is generally believed to be a pest management strategy that is 'safe' for non-target organisms. However, exposure to aphid resistant soybean reduced survival and lifespan of adult *H. axyridis*, but did not impact larval *H. axyridis* or *Orius insidiosus* (Lundgren et al. 2009). Survival of *Aphidius rhopalosiphi*, a parasitoid of *Sitobion avenae*, was reduced on resistant wheat cultivars, but *P. neoaphidis* was unaffected by wheat resistance (Fuentes-Contreras et al. 1998). Exploring the compatibility of soybean aphid pathogens with new resistant soybean varieties will be an important part of understanding aphid dynamics when resistant cultivars are grown on a large scale.

Steinkraus (2006) outlines several factors which can influence aphid fungal infections including agricultural chemicals (Koch et al. 2010) and host plant genotype (Fuentes-Contreras and Niemeyer 2000). Impacts of insecticides on fungal pathogens of aphids are usually indirect, by lowering aphid density below a level which can sustain infection (Steinkraus 2006). Host plant resistance will also reduce aphid density and may

cause changes in host plant structure and chemistry, potentially affecting pathogens (Duetting et al. 2003; Steinkraus 2006). While reductions in aphid density are expected when thiamethoxam seed treatments and/or aphid resistant soybean are utilized, the purpose of this study is to determine if such changes in aphid populations prevent establishment or significantly lower the prevalence of a common fungal pathogen of soybean aphid, *P. neoaphidis*.

## Materials and Methods

### Seed Treatment Experiments, 2008

*Field Plots*. At the University of Minnesota Outreach, Research, and Education Park at Rosemount, MN, soybean variety NK S19L7 (NK, Golden Valley, MN) and was planted in 76.2 cm rows at a seeding rate of 368,000 per hectare. Soybean was Roundup Ready® and glyphosate was applied as needed for weed control. Small soybean plots were planted on 5 Jun as four randomized complete blocks with two treatments, thiamethoxam-treated seed or untreated seed. Thiamethoxam (Cruiser®, Syngenta Crop Protection, Greensboro, NC) was applied to soybean seed at a rate of 50 g active ingredient per 100 kg seed. Plots were eight rows wide and 18.3 m long with 7.6 m fallow border separating each block and 2.4 m separating each plot within blocks.

At the Sand Plain Research Farm at Becker, MN, variety NK S19L7 was planted in 76.2 cm rows with a precision garden hand planter on 9 May 2008. Soybean was Roundup Ready and glyphosate was applied once prior to emergence. Afterward, mechanical weed control was utilized because the installation of cages precluded the use of application machinery in plots. Plots were completely randomized with thiamethoxam seed treatment, identical to the seed treatment at Rosemount, and an untreated control, replicated four times. Plots were four rows wide and 7.6 m long with a 1.5 m fallow border around each plot. Soybeans were irrigated twice per week via solid set overhead irrigation with nozzles every 9.14 m. Irrigation frequency and amount of water applied was adjusted if significant rainfall occurred so that soybeans received 4.3 ± 1.6 cm of water per week.

*Open Plot Experiment.* The open plot experiment was performed at Rosemount, MN. Per plant aphid densities were monitored beginning when plants emerged through 2

Sep with once weekly whole-plant aphid counts. Initially, 20 plants per plot were counted, but as the proportion of plants infested increased, the number of plants per plot counted decreased following the procedures of Ragsdale et al. (2007). Initially, plants were non-destructively sampled; however, once 50% of plants were infested, plants were destructively sampled.

To monitor aphid disease prevalence, 25 aphids were collected twice per week for three weeks from each plot beginning on 5 Aug. Aphids were returned to the laboratory for bioassay to determine disease prevalence. Aphids were transferred to excised soybean leaves in 100 mm polystyrene petri dishes and monitored for three days. Any apparently infected aphid was moved to 1% water agar in a 50 mm tissue culture plate to induce sporulation. If sporulation occurred, the aphid was considered infected and spores from all infected aphids were stained with an aceto-orcein stain (Humber 1997) to confirm pathogen identity using spore morphology illustrated in Samson et al. (1988).

*Cage Experiment.* The cage experiment was performed at both Rosemount and Becker. Cages consisted of a wire tomato frame (0.41 m x 1.37 m; Glamos Wire, Hugo, MN) surrounded by a no-see-um mesh sleeve (131 x 66 cm with 0.65 x 0.17 mm gaps; Eastex Products Inc., Holbrook, MA) placed around a single soybean plant. Cages were used to prevent emigration of aphids and immigration of aphid predators. Cages were held in place with three tent stakes and the base of the cage was buried. Two cages were installed in each plot at Becker and in each plot planted at Rosemount. Cages were installed when plants were in early reproductive stages (V9/R2) on 18 Jul and 30 Jul at Becker and Rosemount, respectively. Aphids collected from the surrounding field were used to initiate aphid populations within cages and cages were monitored periodically to remove any predators. When aphid populations reached approximately 700-1000 per plant, *P. neoaphidis*-infected soybean aphid cadavers were released into each cage to initiate an epizootic. Prior to release of inoculum, aphids were collected from each location to determine if disease was already present in the aphid population. At Becker, three infected cadavers were released into each cage on 31 Jul. At Rosemount, 25 laboratory inoculated soybean aphids were released into each cage on 5 Aug. Two different procedures were undertaken due to availability of inoculum. Inoculated aphids and infected cadavers were produced by inoculating soybean aphids from a laboratory

colony with an actively sporulating culture of *P. neoaphidis*. This culture was originally isolated from an infected pea aphid, *Acryrthosiphon pisum* Harris, collected from Rosemount, MN in 2008. The fungus was used to infect soybean aphid and was reisolated from soybean aphid prior to use in this experiment. Cultures of *P. neoaphidis* were maintained on Sabouraud dextrose agar (Difco™, Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with egg yolk and milk (SDAEYM) and were kept at 19 ± 1°C and 0:24 (L:D) h.

To monitor disease prevalence within cages, 50 aphids were collected from each cage every three to four days for two weeks beginning 11 Aug and 13 Aug at Becker and Rosemount, respectively. Aphids were returned to the laboratory and bioassayed utilizing similar procedures as those in the open plot experiment, except that aphids were monitored for four days and spores from one infected aphid per cage per day were examined to determine pathogen species. Aphid density within cages was estimated using a three node subsample (McCornack et al. 2008; Costamagna et al. 2010) once per week during the experiment.

***Rag1* Resistant Soybean Experiments, 2009**

*Field Plots.* At Rosemount, MN, a *Rag1* aphid resistant (LD05 16060) and susceptible (SD01 76R) variety were planted on 20 May in 76.2 cm rows at a seeding rate of 368,000 per hectare. Only the non-resistant isoline was Roundup Ready®. Soybean plots were 15.24 m long and 6 rows wide and were part of a randomized complete block design with the two varieties as treatments replicated six times. A 3.05 m fallow border surrounded each plot.

At Becker, MN, the same two soybean varieties planted at Rosemount, MN were planted on 22 May with a precision garden hand planter. Plots were 1 row wide and 15.24 m long, replicated five times, and completely randomized. Rows were planted 76.2 cm apart with one border row of SD01 76R surrounding the experimental plots. Irrigation methods were identical to those at Becker in 2008.

*Open Plot Experiment.* The open plot experiment was performed at Rosemount, MN. Methods used to estimate per plant aphid density and aphid disease prevalence followed those utilized in the open plot experiment in 2008, except aphids were collected for bioassay beginning on 4 Aug and were only sampled once during the weeks of 3 Aug

and 24 Aug and twice per week during the weeks of 10 Aug and 17 Aug. Sampling was suspended after 25 Aug due to low aphid densities.

*Cage Experiment.* The cage experiment was performed at Becker, MN. Two cages, identical to those used in the cage experiment in 2008, were placed in each plot. Methods utilized for aphid sampling for bioassays and estimation of within cage aphid densities followed those utilized in the 2008 cage study, except for the following details. Cages were set-up on 29 Jul, when plants were in the early reproductive stages (V7/R1). Five soybean aphid cadavers infected with *P. neoaphidis* were released into each cage on 13 Aug. Cadavers were produced using the same *P. neoaphidis* isolate and following the same procedures outlined for the 2008 cages experiments. Aphid sampling for bioassays began on 21 Aug was repeated twice per week for 2.5 weeks.

**Seed Treatment + *Rag1* Resistance Experiments, 2010**

*Field Plots.* At Rosemount, MN, a *Rag1* aphid resistant (LD05 1121) and susceptible (SD01 76R) variety were planted on 19 May in 76.2 cm rows at a seeding rate of 321,000 per acre. Both varieties were Roundup Ready®. Soybean plots were 41.14 m long and 32 rows wide and were part of a 2x2 factorial randomized complete block design with the first factor being soybean variety (resistant or susceptible) and the second being seed treatment (thiamethoxam-treated or untreated), with the susceptible and untreated plots as a control. Treatments were replicated four times. A 3.05 m fallow border surrounded each plot.

An identical 2x2 factorial design with the same two soybean varieties and seed treatment were planted at Becker, MN on 17 May with a precision garden hand planter. Plots were 1 row wide and 10.67 m long, replicated six times in a complete block design. Rows were planted 76.2 cm apart. Irrigation methods followed those at Becker in 2008 and 2009.

*Open Plot Experiment.* The open plot experiment was performed at Rosemount, MN. Methods used to estimate per plant aphid density and aphid disease prevalence followed those utilized in the open plot experiment in 2008 and 2009. Aphids were collected for bioassay beginning on 27 Jul and were sampled twice per week, except for the week of 9 Aug, when only one sample was taken. Sampling was suspended after 17 Aug due to low aphid densities.

*Cage Experiment.* Cage experiments were performed at Rosemount and Becker, MN. Two cages, identical to those used in the cage experiment in 2008 and 2009, were placed in each plot in Rosemount and one cage in each plot in Becker. Methods utilized for aphid sampling for bioassays and estimation of within cage aphid densities followed those utilized in the 2008 and 2009 cage studies, except for the following details. Cages were set-up on 26 Jul and 30 Jul and aphids were released into each cage on 4 Aug and 3 Aug, at Becker and Rosemount, respectively. Soybean plants were in the mid-reproductive stages (V9/R3) and aphid density within each cage was estimated once per week for the duration of the experiments. Five soybean aphid cadavers infected with *P. neoaphidis* were released into each cage on 9 Aug and 12 Aug. Cadavers were produced using the same *P. neoaphidis* isolate and following the same procedures outlined for the 2008 cages experiments. Aphid sampling for bioassays began on 16 Aug and 17 Aug, at Becker and Rosemount, respectively. Bioassays were repeated twice per week for 2.5 weeks.

**Statistical Analysis**

Treatment effects on aphid densities and aphid disease prevalence were determined using a repeated measures analysis of variance (PROC MIXED, SAS 9.2, SAS Institute Inc. 2008). The covariance structure was selected based on the methods of Littell et al. (2006), in which the Akaike Information Criterion (AIC) was minimized. In order to satisfy the assumptions of the models, several of the data sets required transformations. To normalize the mean and variance, aphid density and disease prevalence data collected from cage studies at Becker, MN in 2008 required square root transformations. Disease prevalence and aphid density data from cage studies at Rosemount, MN in 2008 required square root andnatural log transformations, respectively. For the 2009 data, aphid densities in cages at Becker, MN required a square root transformation and aphid disease prevalence in open plots at Rosemount, MN required an arcsine-root transformation. For the 2010 data, aphid densities in cages at Becker, MN and aphid disease prevalence in cages at Rosemount, MN required square root transformations.

Statistical models included block, seed treatment and/or aphid resistance, sampling date, and all pair-wise interactions as factors, as applicable for a given

experiment. Least squares means for significant factors were separated using the Tukey-Kramer correction, which adjusts p-values for multiple comparisons. The significance level was set at $\alpha=0.05$ and only significant pair-wise interactions are reported. Prevalence data are presented as percent of aphids infected ± SE and aphid densities are reported as the number of aphids per plant ± SE.

## Results

### Seed Treatment Experiment, 2008

*Open Plot Experiment.* Aphids were first found at Rosemount, MN on untreated and thiamethoxam-treated plants on 23 Jun and 9 Jul, respectively. Seed treatment did not significantly impact aphid densities as neither the main effect of seed treatment ($F_{1,60}=0.93$, $P=0.3381$) nor the interaction of sampling date and seed treatment were significant factors ( $F_{10,60}=1.68$, $P=0.1062$; Fig 1a). Sampling date was a significant factor ($F_{10,60}=23.03$, $P<0.0001$), with aphid densities increasing from 23 Jun through 5 Aug, when peak densities were observed (Fig. 1a). Aphid densities declined after 5 Aug until sampling was suspended on 2 Sep (Fig.1a).

Diseased soybean aphids were first identified at Rosemount on 5 Aug, at the time of peak aphid densities. Sampling date significantly impacted aphid disease prevalence ($F_{5,28}=3.60$, $P=0.0122$). After 5 Aug, disease continued to increase until the peak level was observed on 15 Aug, after which disease declined through 22 Aug, the final disease prevalence sampling date (Fig. 1a). Infected aphids were collected from all plots in the study and all aphid infections were caused by *P. neoaphidis*. Aphid disease prevalence was not significantly impacted by seed treatment ($F_{1,28}=0.90$, $P=0.3513$; Fig. 1a).

*Cage Experiments.* Aphid densities within cages at Becker, MN increased significantly throughout the 25 day experiment with a peak of 7,601 ± 1,642 aphids per cage on 25 Aug, the final sampling date ($F_{3,18}=81.14$, $P<0.0001$; Fig. 2a). Over the course of the experiment, untreated soybean had a mean of 2,411 ± 524 aphids per cage and seed-treated soybean had 3,526 ± 998 aphids per cage, a non-significant difference ($F_{1,6}=1.12$, $P=0.3310$).

Infected aphids were recovered from all cages at Becker, MN and all infections were caused by *P. neoaphidis*, the pathogen species originally released into the cages.

Aphid disease prevalence increased significantly until 21 Aug when a peak of 40.0 ± 5.6% of aphids infected was observed ($F_{4,24}$=31.05, $P$<0.0001; Fig. 2a). Effects of seed treatment on aphid disease levels were non-significant ($F_{1,6}$=0.01, $P$=0.9195), with 17.6 ± 3.6% of all aphids collected from cages with untreated soybeans and 17.2 ± 3.6% of all aphids collected from cages with seed-treated soybean becoming infected over the entire experiment.

At Rosemount, aphid density in cages significantly changed over time and peaked on 13 Aug with 2,832 ± 632 aphids per cage ($F_{2.12}$=14.31, $P$=0.0007; Fig. 2b). However, densities declined slightly by the final sampling date, 18 Aug, with 2,680 ± 578 aphids per cage. The effect of seed treatment on aphid densities depended on the sampling date ($F_{2,12}$=5.19, $P$=0.0237); however, after correcting for multiple comparisons, there were no significant differences between treatments within any sampling date. Thus, seed treatment had a negligible impact on aphid density.

Infected aphids were recovered from 12 of 16 cages at Rosemount, MN. All infected aphids recovered were infected by *P. neoaphidis* and generally, lower levels of disease were observed in cages at Rosemount, the non-irrigated site, compared to Becker, the irrigated site, although locations could not be compared statistically. Disease levels fluctuated over the sampling dates and peaked in the final sample on 22 Aug at 11.8 ± 3.0% of aphids infected and differences among sample dates was significant ($F_{3,18}$=3.79, $P$=0.0287; Fig. 2b). Seed treatment did not significantly impact disease levels within cages ($F_{1,18}$=1.13, $P$=0.3019), with 7.8 ± 1.9% and 4.8 ± 1.4% of all aphids infected in cages with untreated and seed-treated soybean, respectively.

***Rag1* Resistant Soybean Experiments, 2009**

*Open Plot Experiment.* Aphid colonization of *Rag1*-resistant plants occurred three weeks later, on 23 Jun, than that of susceptible plants, on 2 Jun. Over the course of the season, *Rag1* resistance significantly lowered aphid densities compared to a susceptible variety, but this effect depended on sampling date ($F_{13,130}$=2.61, $P$=0.0029). Peak aphid density, occurring on 11 Aug, was significantly lower on resistant plants than on susceptible plants ($t_{130}$=5.81; $P$<0.0001; Fig. 3a).

Diseased aphids were collected from all plots and *P. neoaphidis* was the only pathogen species identified. Impacts of resistance on aphid disease depended on

sampling date, with disease in susceptible plots being significantly higher than resistant plots on 14 Aug, three days after peak aphid densities were observed ($F_{5,50}$=9.27, $P$<0.0001; Fig. 3a). Aphid disease for both treatments peaked five days later on 19 Aug with 44.7 ± 2.8% and 29.3 ± 3.7% of aphids infected in susceptible and resistant soybean, respectively.

*Cage Experiment.* Aphid densities within cages were similar between treatments, with a mean of 2,060 ± 368 and 2,180 ± 328 aphids per plant in the susceptible and *Rag1* resistant soybean cages, respectively ($F_{1,8}$=0.16, $P$=0.7034). Aphid densities did change significantly with sampling date, with densities increasing from 25 per cage at the initiation of the experiment to a peak of 3,512 ± 677 aphids per cage 33 days later ($F_{5,40}$=61.41, $P$<0.0001). At the conclusion of the experiment, aphid densities declined slightly to 3,077 ± 938 aphids per cage (Fig. 4a).

Diseased aphids were recovered from all plots, but not all cages. In two susceptible plots, infected aphids were recovered from only one of the two cages. All infected aphids collected from cages were infected with *P. neoaphidis*, the pathogen originally released. Both susceptible and *Rag1* resistant soybean had similar levels of aphid disease within the cages, with 29.6 ± 3.5% and 26.5 ± 3.8% of aphids infected in susceptible and resistant plots, respectively ($F_{1,8}$=0.23, $P$=0.6423). Aphid disease changed significantly over the sampling dates with disease levels increasing from 7.7 ± 3.5% of aphids infected on the first sampling date to a peak of 45.2 ± 5.4% ten days later ($F_{4,32}$=16.81, $P$<0.0001; Fig. 4a).

**Seed Treatment + *Rag1* Resistance Experiments, 2010**

*Open Plot Experiment.* Aphid densities were significantly impacted by *Rag1* resistance; however, this effect was dependent upon sampling date ($F_{9,117}$=6.33, $P$<0.0001; Fig. 3b). Resistance did not impact the relatively low aphid densities at the beginning and end of the experiment, but significantly lowered aphid densities, compared to the susceptible variety, on the date of peak density, 5 Aug (Fig. 3b). The effect of resistance was independent of the effect of seed treatment ($F_{1,117}$=0.09, $P$=0.7648) and seed treatment did not have a significant impact on aphid density ($F_{1,117}$=1.07, $P$=0.303; Fig. 1b).

Observed aphid disease prevalence in 2010 was generally much lower than that observed in 2009, though this difference could not be explored statistically. Infected aphids were recovered from all plots and all infections observed were caused by *P. neoaphidis*. Effect of resistance depended on sampling date ($F_{5,65}$=2.85, $P$=0.0219) and aphids on susceptible soybean had significantly higher levels of disease compared to those on resistant plots on 5 Aug and 8 Aug (Fig. 3b). The effect of resistant soybean was not related to seed treatment ($F_{1,65}$=0.08, $P$=0.7774) and seed treatment had no significant impact on disease prevalence ($F_{5,65}$=0.08, $P$=0.7774; Fig. 1b).

*Cage Experiments.* At Becker, MN, aphid-resistant soybean harbored significantly fewer aphids than susceptible soybean, but this effect depended on sampling date ($F_{4,84}$=4.26, $P$=0.0035). For the first two sampling dates, after initiation of the experiment on 4 Aug, susceptible and resistant plants had similar aphid densities, 931 ± 89 and 1294 ± 201 aphids per plant on 9 Aug and 16 Aug, respectively. Treatment differences are significant on the final two sampling dates when resistant plants had 892 ± 280 and 803 ± 412 aphids per plant while susceptible plants had 2,673 ± 467 and 2,486 ± 771 aphids per plant on 23 Aug and 30 Aug, respectively (Fig. 4b). Seed treatment had no significant effect on aphid density in the cages ($F_{1,84}$=0.04, $P$=0.8418; Fig. 2c).

At Becker, MN, neither seed treatment nor aphid resistance significantly impacted aphid disease in cages. On seed-treated plants, 11.6 ± 2.4% of aphids became infected, while on untreated plants 9.3 ± 1.8% of aphids became infected, which was a non-significant difference ($F_{1.73}$=0.22, $P$=0.6408; Fig. 2c). Aphids on susceptible plants exhibited a higher infection rate, 14.6 ± 2.4% , compared to resistant plants, 5.7 ± 1.5%; however, this difference was not significant ($F_{1.73}$=3.81, $P$=0.0548; Fig. 4b). Aphid disease levels were consistent over time, with sampling date also being a non-significant factor ($F_{4.73}$=2.45, $P$=0.0533).

At Rosemount, MN, aphid densities were significantly higher in susceptible cages on 12 Aug, 17 Aug, and 24 Aug, the first three sampling dates after initiation of the experiment on 3 Aug; but not the final sampling date, 31 Aug; thus, the effect of resistance depended on sampling date ($F_{4,48}$=8.78, $P$<0.0001; Fig. 4c). Aphid densities on 12 Aug were 2,563 ± 161 and 1,395 ± 212 aphids per plant in susceptible and resistant cages, respectively. These densities increased to 3,871 ± 439 and 1,662 ± 190 aphids per

plant on 17 Aug, for susceptible and resistant cages, respectively. On 24 Aug, aphid densities in both treatments declined slightly to 2,956 ± 294 and 1,331 ± 275 aphids per susceptible and resistant cage, respectively. The seed treatment by resistance interaction was significant ($F_{1,48}$=10.49, $P$=0.0022), results of the mean separation indicate that both non-resistant treatments had significantly higher aphid densities than both resistant treatments, regardless of seed treatment. In addition, both non-resistant treatments had similar aphid densities while both resistant treatments also had similar aphid densities. Thus, seed treatment had a negligible impact on the aphid densities in cages (Fig. 2d).

Aphid disease prevalence was significantly lower, 20.5 ± 3.1%, in cages with resistant plants compared to cages with susceptible plants, 32.6 ± 3.3% ($F_{1,48}$=5.25, $P$=0.0264; Fig. 4c). Seed-treatment did not significantly impact aphid disease prevalence in cages at Rosemount, MN, with 26.8 ± 2.7% and 27.1 ± 4.1% of aphids infected on untreated and seed-treated plants, respectively ($F_{1,48}$=0.35, $P$=0.5545; Fig. 2d). Disease prevalence also significantly changed over time, with peak prevalence occurring on 24 Aug with 38.5 ± 5.7% of aphids infected ($F_{4,48}$=11.77, $P$<0.001).

## Discussion

Results confirm that *Rag1*-resistant soybean significantly lowers aphid densities compared to a susceptible variety in open plots. This reduction was consistent across seasons, even though one year had aphid densities which exceeded the economic injury level (EIL) of 674 aphids per plant (2009), while the next year saw peak aphid densities falling under the economic threshold (ET) of 250 aphids per plant (2010; Fig. 2). Results of open plot studies consistently showed reductions in aphid density in resistant plots accompanied by significant reductions in aphid disease prevalence (Fig. 3). In spite of low aphid densities in 2010, *P. neoaphidis* was able to establish, persist, and cause measurable levels of aphid mortality (Fig. 3b).

In both locations of the 2010 cage studies, significantly lower aphid densities were observed in resistant plots, compared to susceptible plots. Host plant resistance led to significantly lowered aphid disease prevalence in the Rosemount cage study, but not the Becker cage study. This may be due to differences in agronomic practices between the two locations, as the Becker location was irrigated while the Rosemount location was

not. Additionally, the difference between the resistant and susceptible plots was nearly significant ($P = 0.0548$), with higher levels of disease observed in susceptible plots, 14.7 ± 2.4%, compared to resistant plots, 5.7 ± 1.5%. Thus, the trend of lowered levels of aphid density and aphid disease were consistent between location-years.

For the 2009 cage study, aphid resistant and susceptible soybean had equivalent aphid densities (Fig. 4a). While this is inconsistent with the open plot and 2010 cage studies, it does allow us to compare aphid disease prevalence between treatments without aphid density as a confounding factor. Because aphid disease prevalence was also equivalent between resistant and susceptible soybean (Fig. 4a), we have evidence that reductions in *P. neoaphidis* prevalence did not result from changes in aphid susceptibility caused by feeding on resistant plants. Furthermore, it supports the conclusion that observed differences in disease prevalence in the open plot and 2010 cage studies were driven by aphid density, rather than soybean genotype.

Often called the disease triangle, three conditions must be met for disease to occur: infective propagules of the pathogen (i.e. *P. neoaphidis* conidia) must be present, hosts (i.e. soybean aphids) must be present, and abiotic conditions must be appropriate (i.e. moderate temperatures and high relative humidity). Results from the 2009 cage study indicate that *Rag1*-resistant soybean does not impact the pathogen portion of this triangle. Instead, aphid resistance is more likely to affect the host portion of the triangle, by reducing aphid densities. Anderson and May (1981) theorized that there is a minimum host density required to support infection by pathogens. Previous work has indicated that this minimum host density is relatively low (<200 aphids per plant) (KAK, unpub. data). However, if host plant resistance lowers aphid densities below this threshold host density, *P. neoaphidis* will not be able to persist in the aphid population. In the case of *Rag1*-resistant soybean, *P. neoaphidis* prevalence was significantly lower in resistant plots; however, the fungus was not completely excluded. Thus, host plant resistance is likely compatible with the action *P. neoaphidis*.

Thiamethoxam seed treatment had a negligible effect on aphid density and disease prevalence (Figs. 1, 2) and this result was consistent across all studies. This is likely due to the limited residual effect of the seed treatment beyond 40 days after planting (McCornack and Ragsdale 2006). Seed treatment can delay soybean aphid infestation,

and this was the case for the 2008 open plot experiment for which seed treatment delayed aphid infestation for 16 days (Fig. 1a). However, once insecticide levels within the plant decrease and infestation occurs, aphid densities can increase rapidly, somewhat compensating for the delay caused by seed treatment. Thus, by the time *P. neoaphidis* arrived in early August, aphid densities were large enough in treated soybean to allow for equivalent establishment and proliferation of the fungus in treated and untreated plots.

Past studies of fungal pathogens of soybean aphid on soybean have not definitively described the relative importance of pathogens in soybean aphid biological control (Nielsen and Hajek 2005; Noma and Brewer 2007; Koch et al. 2010). It is difficult to conduct a controlled experiment to tease out the role of fungal infection in naturally regulating aphid populations because of the difficulties associated with excluding the pathogen in controls, excluding other natural enemies which also utilize soybean aphid as a resource, and ensuring that abiotic conditions are appropriate for disease transmission. However, there is evidence that fungal pathogens are important regulators of soybean aphids occurring on buckthorn. We found that 39.9 ± 5.5% of gynoparae collected from buckthorn in Lamberton, MN in Sep 2009 were infected (KAK, unpublished data) and Nielsen and Hajek (2005) found that 27.3 to 86.8% of gynoparae on buckthorn were infected. While infection levels of oviparae were much lower (0-5% infected) (KAK, unpublished data), the shortened lifespan of the infected gynoparae limits production of oviparae, likely leading to fewer eggs being deposited.

Feng et al. (2007) demonstrated that fungal pathogens can be disseminated via the movement of infected alate aphids and up to one third of these infections were caused by *P. neoaphidis*. If *P. neoaphidis* is present in soybean aphid populations on soybean late in the season, which is supported by our results, it is likely that infections occurring in buckthorn originated in soybean as infections of immature or newly emerged gynoparae. Therefore, preserving *P. neoaphidis* and other aphid fungal pathogens when they occur in soybean may prevent damaging aphid populations the following year. Because thiamethoxam seed treatment and *Rag1* aphid resistance do not prevent *P. neoaphidis* from colonizing soybean aphid populations, these new technologies are likely compatible with soybean aphid fungal pathogens.

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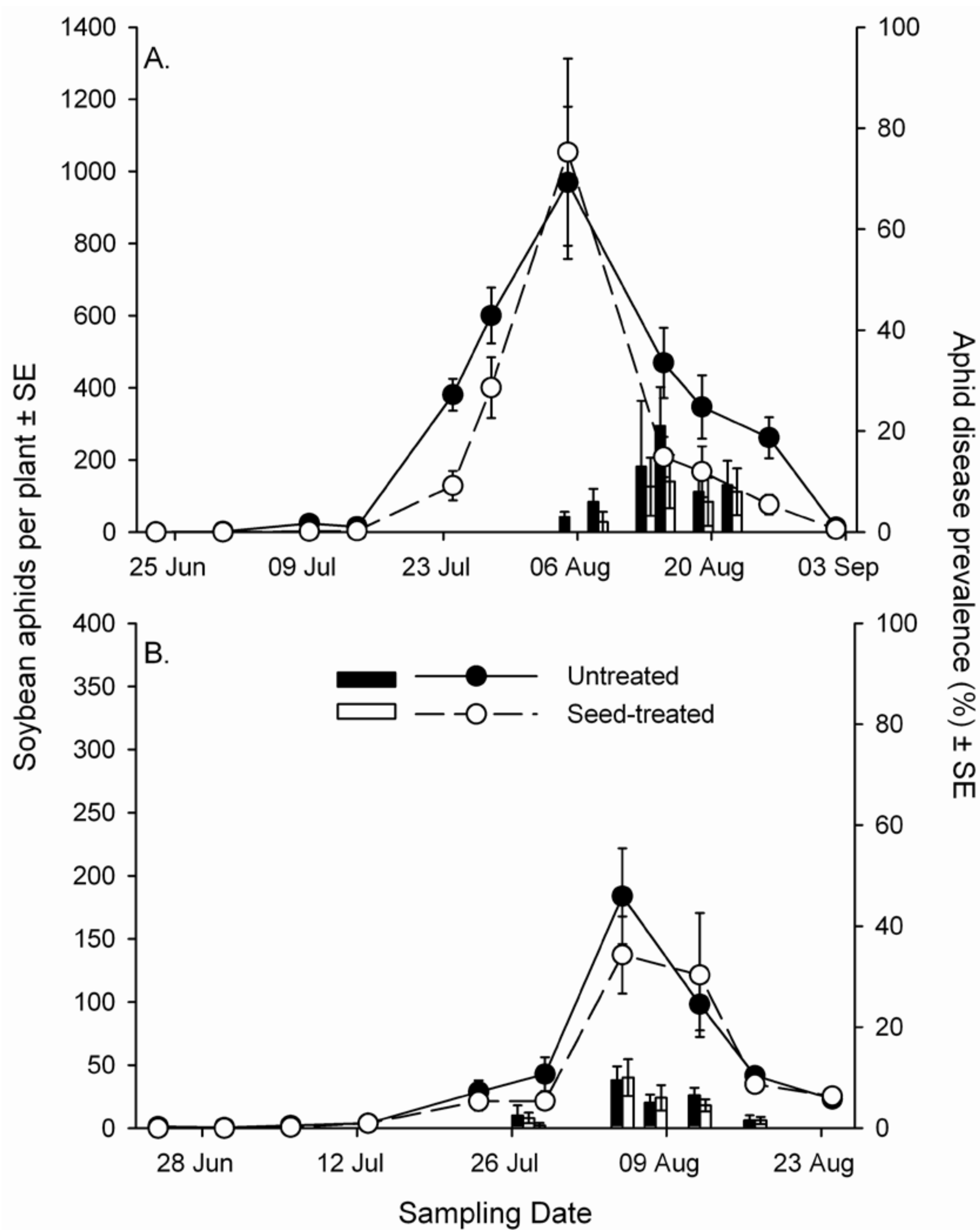


**Figure 1.** Per plant aphid densities (± SE; line plots) and aphid disease prevalence (± SE; bar plots) for open plot experiments at Rosemount, MN in 2008 (Panel A) and 2010 (Panel B). Seed treatment did not significantly impact aphid density or aphid disease prevalence in either year of the study.

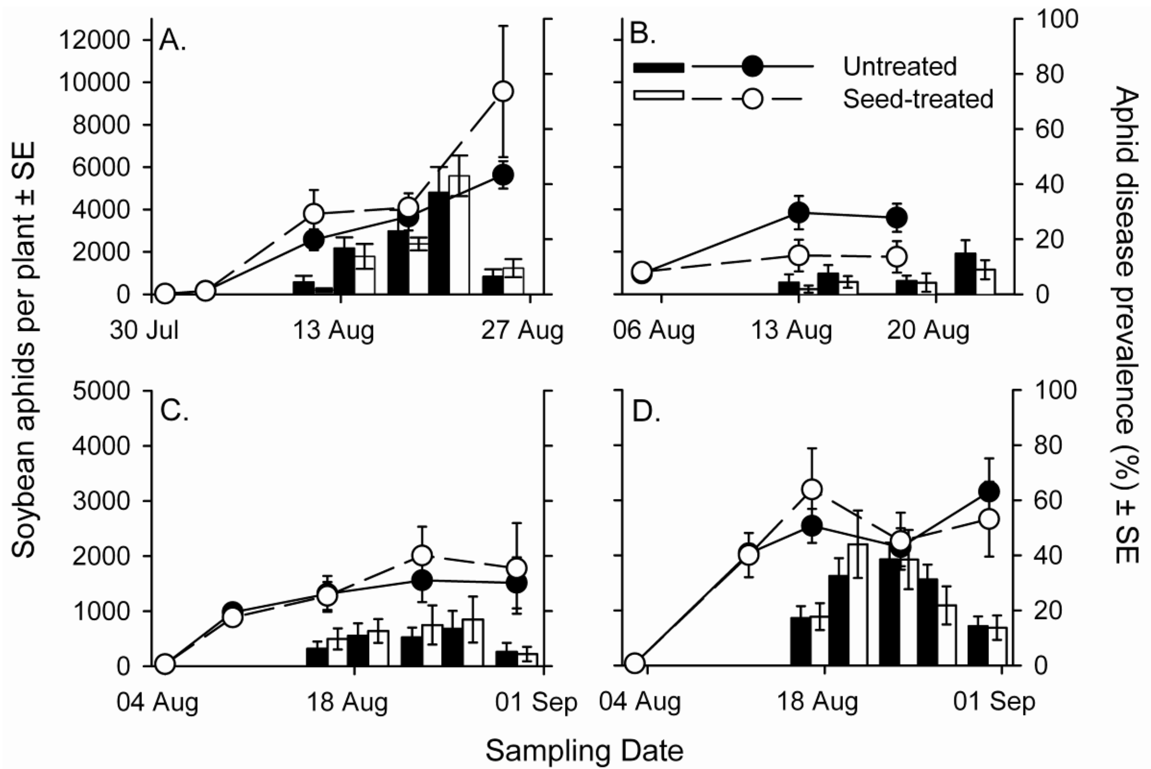


**Figure 2.** Per plant aphid densities (± SE; line plots) and aphid disease prevalence (± SE; bar plots) for cage experiments at Becker, MN in 2008 (Panel A) and 2010 (Panel C) and at Rosemount, MN in 2008 (Panel B) and 2010 (Panel D). Seed treatment had no significant impact on aphid densities or aphid disease prevalence in any location-year.

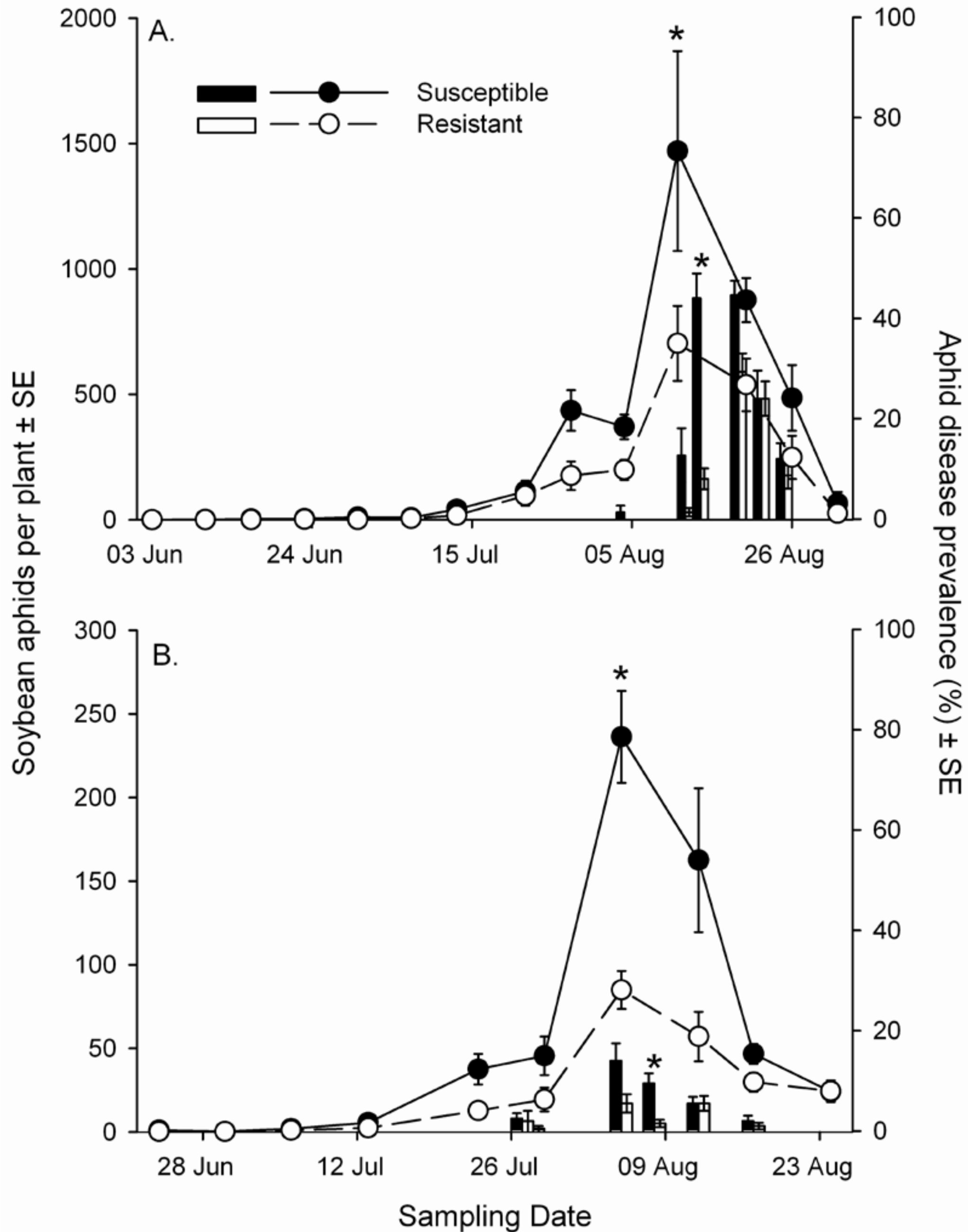


**Figure 3.** Per plant aphid densities (± SE, line plot) and aphid disease prevalence in percent of aphids infected (± SE, bar plot) in open plots at Rosemount, MN in 2009 (Panel A) and 2010 (Panel B). Asterisks above error bars indicate significant differences between susceptible (black circles and bars) and resistant (open circles and bars) soybean plots in aphid densities on 11 Aug 2009 ($F_{13,130}$=2.61, $P$=0.0029) and 5 Aug 2010 ($F_{9,108}$=6.07, $P$<0.0001) and in aphid disease prevalence on 14 Aug 2009 ($F_{5,50}$=9.27, $P$<0.0001) and 8 Aug 2010 ($F_{5,60}$=8.02, $P$<0.0001).

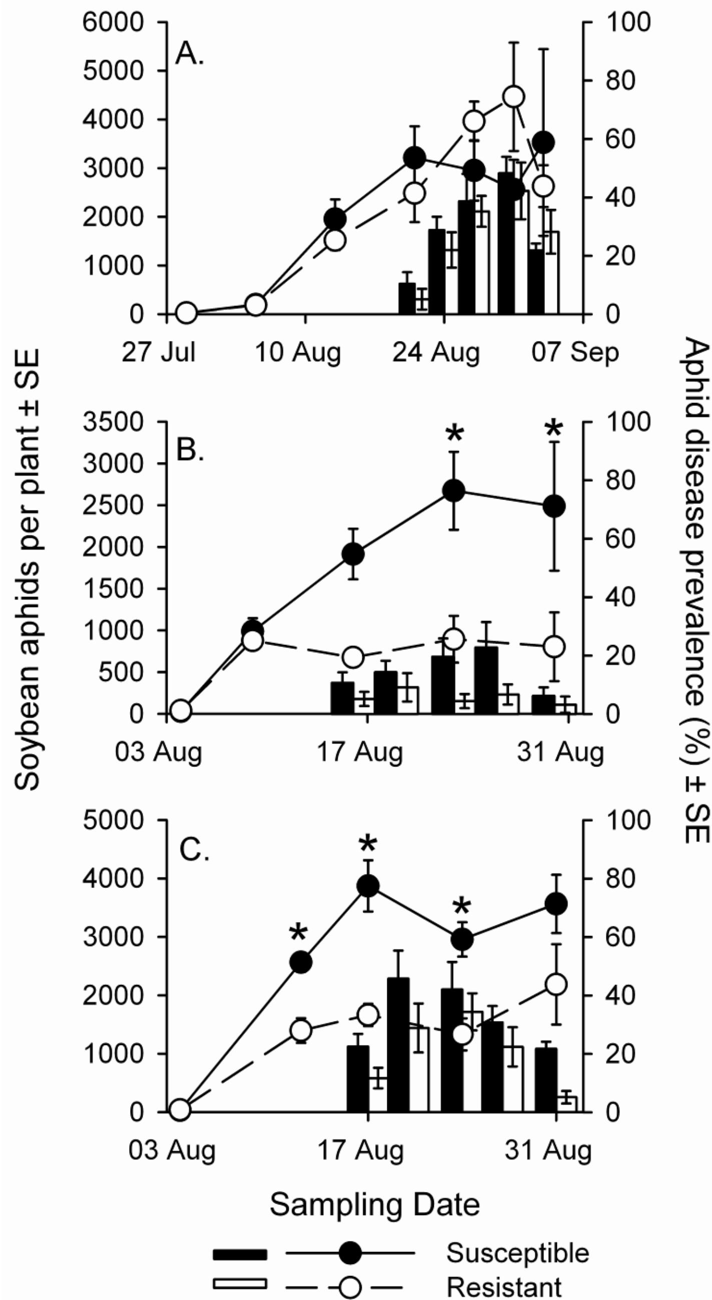


**Figure 4.** Per plant aphid densities (± SE; line plots) and aphid disease prevalence (± SE; bar plots) for cage experiments at Becker, MN in 2009 (Panel A) and 2010 (Panel B) and at Rosemount, MN in 2010 (Panel C). Asterisks above error bars indicate significant differences between susceptible (black circles and bars) and resistant (open circles and bars) cages. In both locations in 2010, aphid resistance significantly lowered aphid densities in cages on multiple sampling dates. However, disease prevalence was not significantly affected in any location-year.

# Chapter 4: Compatibility of a predator, *Harmonia axyridis*, and pathogen, *Pandora neoaphidis*, of soybean aphid

**Introduction**

Outbreaks of soybean aphid, *Aphis glycines* Matsumura, an invasive arthropod pest of cultivated soybean, have caused economic damage via increased insecticide use and decreased yield since the aphids' discovery in North America in 2000 (Ragsdale et al. 2004, 2007). However, resident natural enemies, including predators, parasitoids, and pathogens, have responded to the aphid's invasion and have been shown to contribute to aphid population regulation (Rutledge et al. 2004, Nielsen and Hajek 2005, Noma and Brewer 2008), sometimes providing significant aphid control (Fox et al. 2004, Costamagna et al. 2007). One of the most commonly cited generalist predators of soybean aphid is *Harmonia axyridis* (Pallas), the multicolored Asian lady beetle, another non-native, and sometimes invasive, insect species (Koch 2003, Costamagna and Landis 2007, Schmidt et al. 2008). While *H. axyridis* has been shown to be an effective soybean aphid predator (Costamagna and Landis 2007, Costamagna et al. 2008, Xue et al. 2009), the beetle can cause problems via its propensity for intraguild predation (IGP), which occurs when beetles consume other beneficial organisms which also utilize soybean aphid as a resource. If IGP is frequent, *H. axyridis* may limit populations of other aphid natural enemies, potentially leading to lowered levels of aphid control. Understanding how *H. axyridis* affects other important soybean aphid natural enemies can provide information which may improve soybean aphid management.

Fungal pathogens, the most common of which is *Pandora neoaphidis*, are frequently found infecting soybean aphids across North America (Nielsen and Hajek 2005, Noma and Brewer 2007, Koch et al. 2010) and likely provide significant levels of control under certain conditions. Adding generalist coccinellid predators to the soybean aphid-*P. neoaphidis* patho-system may enhance aphid infection by dispersing fungal conidia or reduce aphid infection via the elimination of fungal inoculums resulting from IGP of infected aphids. The aphid predator *Coccinella septempunctata* has been shown to consume pea aphid, *Acyrthosiphon pisum*, cadavers infected with *P. neoaphidis* (=*Erynia neoaphidis*), particularly when they were starved for 48 hr; however, uninfected aphids were preferred over infected cadavers (Pell et al. 1997). Attenuating the negative impact of cadaver consumption was that *P. neoaphidis*-inoculated *C. septempunctata* adults were able to initiate infection in pea aphid colonies after foraging on the same

plant (Pell et al. 1997). In a more complicated, multiple plant environment, these results were confirmed in that *C. septempunctata* adults and larvae consumed infected pea aphids and again, starved beetles fed on infected aphids more frequently than non-starved individuals (Roy et al. 2003). Whether *C. septempuntata* larvae and adults were artificially inoculated with *P. neoaphidis* conidia or naturally inoculated by foraging near sporulating pea aphid cadavers, the beetles were able to initiate infection in previously healthy pea aphid colonies when foraging on the same plant (Roy et al. 2001).

Similar studies examining how *H. axyridis* interacts with *P. neoaphidis*-infected aphids further confirms previous results; however, *H. axyridis* did not tend to prefer healthy pea aphids over infected pea aphids and more frequently consumed the entire cadaver, where, in the same study, *C. septempunctata* rarely consumed infected aphids (Roy et al. 2008). However, in a later study, transmission of *P. neoaphidis* was higher when coccinellids, *H. axyridis* or *C. septempunctata*, were present, but there were no differences in the frequency of transmission between the two coccinellid species (Wells et al. 2011). Thus it remains unclear if the benefit of increased transmission in the presence of *H. axyridis* outweighs the costs of IGP of *P. neoaphidis*-infected aphid cadavers.

In this study we attempted to determine if *H. axyridis*, a key soybean aphid predator, affects *P. neoaphidis*, the most common soybean aphid pathogen. In the field, we utilized single soybean plant cages to confine the two natural enemies with their shared resource, soybean aphid. By monitoring aphid densities and aphid infection, we attempted to determine if adding *H. axyridis* adults to aphid colonies exposed to *P. neoaphidis* would have a positive, negative, or no effect on *P. neoaphidis* prevalence in soybean aphid colonies and the density of those colonies.

## Materials and Methods

At the Sand Plain Research Farm at Becker, MN, soybean variety NK S19L7 (NK, Golden Valley, MN) was planted on 22 May 2009 and 17 May 2010. Soybean was Round Up Ready® and planted in 76.2 cm rows at a seeding rate of 408,000 seeds per ha. Glyphosate was applied once prior to plant emergence and mechanical weed control was used for the remainder of the season because installation of cages precluded the use of

spray equipment in plots. Soybean fields were irrigated twice each week using solid set overhead irrigation. Nozzles were placed every 9.14 m along field borders. In the event of significant rainfall, irrigation frequency and duration were reduced so that fields received approximately 4.5 cm of water per week.

In 2009, we utilized a 3 x 2 factorial design with the first factor being *H. axyridis* density, no *H. axyridis* adults, 1 *H. axyridis* adult (low *H. axyridis* treatment), or 2 *H. axyridis* adults (high *H. axyridis* treatment), and the second factor being the release of *P. neoaphidis* inoculum, either inoculum was released or not released into plots. Plots were organized into three blocks, with each block representing one of the *H. axyridis* densities. Within each block, plots were completely randomized by *P. neoaphidis* treatment, for a total of seven replications of each treatment combination. While this is technically pseudoreplication, the field in which the experiment took place was small (12 rows by 30.5 m) and homogenous, thereby limiting any random effects the blocking may have had on results. Plots were 3 rows by 3.1 m and a single soybean plant cage was installed around a randomly selected plant in the center row of the plot. Cages consisted of a wire frame (137 x 41 cm; Glamos Wire, Hugo, MN) surrounded by a no-see-um mesh sleeve (131 x 66 cm with 0.65 x 0.17 mm gaps; Eastex Products Inc., Holbrook, MA) and the bottom of the mesh sleeve was buried to prevent aphid emigration and exclude predators.

In 2010, we utilized identical treatments; however, plots were arranged ten blocks of three plots, each randomly assigned to one of the *H. axyridis* treatments. Five of the ten blocks were assigned to receive *P. neoaphidis* inoculum. Blocks were 6 rows wide by 10.7 m long and plots within the block were 1 row wide by 10.7 m long. Cages, identical to those used in 2009, were placed around randomly selected plants in the second, third, and fourth rows from the edge of the field plot. We changed our design in this way to maximize the space between cages receiving *P. neoaphidis* inoculum and those not receiving the inoculum to prevent contamination of the latter.

On 29 July 2009, cages were infested with 25 soybean aphids and on 21 July 2010 cages were infested with 30 soybean aphids. On 6 August 2009, additional aphids had to be added to cages due to poor establishment of the initial infestation. *Pandora neoaphidis* inoculum was released into cages on 6 August 2009 and 28 July 2010 as five soybean aphid cadavers which had been killed by *P. neoaphidis*. The *P. neoaphidis*

culture used had been originally isolated from *Acyrthosiphon pisum* Harris, pea aphid, collected in Rosemount, MN in 2008. We then infected soybean aphids with the original isolate and reisolated the fungus from soybean aphid prior to use in these experiments. Aphid cadavers were produced by exposing soybean aphids from a laboratory colony with actively sporulating *P. neoaphidis* cultures which were the first subculture of a reisolate. Cultures of *P. neoaphidis* were maintained on Sabouraud dextrose agar (Difco™, Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with egg yolk and milk (SDAEYM) and were kept at 19 ± 1°C and 0:24 (L:D) h. After allowing for proliferation of the fungus in cages, the *H. axyridis* treatments were initiated on 21 Aug 2009 and 4 Aug 2010, in which adult female beetles were released into the cages, according to the assigned treatment. In 2009 beetles were reared in the laboratory before release into the cages, but in 2010 ample individuals were available and field caught individuals were used. If any other predators were observed in cages, including eggs deposited by the released females, they were removed immediately.

Sampling consisted of estimating the per plant soybean aphid density within cages using a node-based sampling plan in which all aphids on three specific nodes of each plant were counted. These counts were used to estimate the whole plant density following procedures developed in McCornack et al. (2008) (for non-caged plants) and Costamagna et al. (2010) (for caged plants). Aphid densities were estimated at least once per week for the duration of the experiments. Aphid disease prevalence was estimated twice per week beginning on the date beetles were released into the cages. To measure aphid disease prevalence, 25 aphids were collected out of each cage and returned to the laboratory for bioassay. Aphids were transferred to clean, excised soybean leaves in 100 mm polystyrene petri dishes. Leaves were kept fresh by placing the petiole in moistened floral foam (Oasis® General Purpose Floral Foam, Smithers-Oasis Company, Kent, OH). Aphids were monitored for 4 d and over the course of this time any apparently infected aphids were removed from the dish and transferred to 50 mm tissue culture plate lined with 1% water agar to induce sporulation. If sporulation occurred, the aphid was considered infected. To ensure that aphids were infected with *P. neoaphidis*, the pathogen released into cages, spores from at least one infected aphid per plot per day was stained with an aceto-orcein stain (Humber 1997) and examined under 200x

magnification. Pathogen identity was confirmed via spore morphology as shown in Samson et al. (1988).

Aphid densities and disease prevalence was compared among treatments with a repeated measures analysis of variance (PROC MIXED, SAS Institute 2008). Data from the two years were analyzed separately. The covariance structure was chosen based on the methods of Littell et al. (2006) in which the Akaike Information Criterion was minimized. Disease prevalence data from 2010 required an arcsine transformation to fulfill the assumptions of the model. Main factors included *H. axyridis* treatment, *P. neoaphidis* treatment, and sampling date. All two-way interactions and the *H. axyridis* treatment x *P. neoaphidis* treatment x sampling date three-way interaction were also included in the model. Least squares means of significant factors and interaction terms were compared using a Tukey-Kramer adjustment to compensate for multiple comparisons. The significance level was set at $P < 0.05$ and adjusted $P$-values are reported. Aphid density is reported as the number of aphids per soybean plant ± SE and aphid disease prevalence is reported as the percent of aphids infected ± SE.

## **Results**

In spite of the lack of intentionally introduced inoculum in half of the cages in each year of the study, all cages in both 2009 and 2010 produced soybean aphids infected by *P. neoaphidis*. All spores taken from infected aphid cadavers and examined were confirmed as *P. neoaphidis* conidia.

In 2009, aphid densities changed significantly over time, as sampling date was a significant factor ($F$=60.12; df=7, 252; $P$<0.0001) (Fig. 1A). Aphid densities increased from the date of infestation, 29 July 2009, until they peaked on 24 August or 27 August 2009, depending on treatment (Fig. 1A). After the peak, densities generally declined through the final sampling date of 3 September 2009 (Fig. 1A). The impacts of *H. axyridis* treatment on aphid density depended on sampling date, as the *H. axyridis* treatment by sampling date interaction term was significant ($F$=6.06; df=14, 252; $P$<0.0001) (Fig. 1A). Aphid densities varied significantly among the *H. axyridis* treatments on 24 August and 3 September 2009. On 24 August 2009, the no *H. axyridis* treatment had a significantly higher soybean aphid density than either the low ($t$=5.53;

df=252; *P*<0.0001) or high *H. axyridis* treatments (*t*=4.76; df=252; *P*=0.0008) (Fig. 1A), while on 3 September 2009, the no *H. axyridis* treatment had a significantly lower aphid density compared to the low *H. axyridis* treatment (*t*=4.29; df=252; *P*=0.0058) and the high *H. axyridis* treatment had an aphid density similar to both the no and low *H. axyridis* treatments. The effect of *P. neoaphidis* treatment also depended on sampling date as their interaction was also significant (*F*=2.36; df=7, 252; *P*=0.0237); however, after correcting for multiple comparisons, there were no significant differences among treatments within any sampling date. The *P. neoaphidis – H. axyridis* interaction also depended on the sampling date as their three-way interaction was significant (*F*=2.12; df=14, 252; *P*=0.0116). On 24 August 2009, the no *P. neoaphidis* inoculum/no *H. axyridis* treatment had a significantly higher aphid density, 6,109 ± 1,019 aphids per plant, than the no *P. neoaphidis* inoculum/low *H. axyridis* treatment, 2,758 ± 364aphids per plant (*t*=4.09; df=252; *P*=0.041), the no *P. neoaphidis* inoculum/high *H. axyridis* treatment, 2,689 ± 629 aphids per plant (*t*=4.17; df=252; *P*=0.0298), and the with *P. neoaphidis*/low *H. axyridis* treatment, 2,712 ± 146 aphids per plant (*t*=4.15; df=252; *P*=0.0329). On 3 September 2009, the with *P. neoaphidis*/high *H. axyridis* treatment, 6,258 ± 1,238 aphids per plant had a significantly higher aphid density than the no *P. neoaphidis* inoculum/no *H. axyridis* treatment, 2,524 ± 422 aphids per plant (*t*=4.56; df=252; *P*=0.0069), the no *P. neoaphidis* inoculum/high *H. axyridis* treatment, 1,709 ± 432 aphids per plant (*t*=5.55; df=252; *P*<0.0001), and the with *P. neoaphidis*/no *H. axyridis* treatment, 2,244 ± 484 aphids per plant (*t*=4.90; df=252; *P*=0016). The no *P. neoaphidis* inoculum/low *H. axyridis* treatment, 5,116 ± 1,146 aphids per plant, also had a significantly higher aphid density than the no *P. neoaphidis* inoculum/high *H. axyridis* treatment (*t*=4.16; df=252; *P*=0.0314).

Aphid disease prevalence in 2009 also significantly changed over time as sampling date was a significant factor (*F*=28.54; df=4, 143; *P*<0.0001). Aphid disease increased from the first sampling date of 21 August 2009 until the peak prevalence was observed on 31 August 2009 (Fig. 1A). The *P. neoaphidis* treatment also had a significant impact on aphid disease; however, this effect depended on sampling date as their interaction was significant (*F*=28.54; df=4, 143; *P*=0.0494). On 27 August 2009, cages without *P. neoaphidis* inoculum actually had higher disease prevalence, 45.7 ±

4.8% of aphids infected, compared to cages with *P. neoaphidis* inoculum, 25.9 ± 5.1% of aphids infected (*t*=3.33; df=143; *P*=0.0357). While the *H. axyridis* treatment by sampling date interaction was significant, after correcting for multiple comparisons, there were no significant within-sampling date differences among the three treatments (*F*=3.20; df=8, 143; *P*=0.0023). The two-way interaction between *P. neoaphidis* treatment and *H. axyridis* treatment and the three-way interaction among *P. neoaphidis* treatment, *H. axyridis* treatment and sampling date were not significant.

In 2010, sampling date significantly impacted aphid densities, with aphid densities peaking on 4 or 11 August 2010, then declining as the experiment concluded (*F*=15.29; df=3, 72; *P*<0.0001) (Fig. 1B). The other main factors of *P. neoaphidis* treatment and *H. axyridis* treatment did not have a significant impact on aphid densities. Similarly, no two-way interactions were significant. The three-way interaction among *P. neoaphidis* treatment, *H. axyridis* treatment, and sampling date was significant (*F*=3.13; df=6, 72; *P*=0.0088); however, after correcting for multiple comparisons there were no significant differences among treatments within any of the sampling dates.

Aphid disease prevalence in 2010 significantly changed over time, with peak prevalence occurring 9-11 August 2010 (*F*=14.31; df=4, 93; *P*<0.0001) (Fig. 1B). *Pandora neoaphidis* treatment had a significant impact on aphid disease as the cages receiving inoculum had significantly higher overall aphid disease, 45.0 ± 3.2% of aphids infected, compared to cages not receiving inoculum, 31.4 ± 3.3% of aphids infected (*F*=4.77; df=1, 93; *P*=0.0316). The impact of the *H. axyridis* treatment on aphid disease depended on sampling date, as their interaction was significant (*F*=2.24; df=8, 93; *P*=0.0315). However, after the multiple comparisons correction, no within-sampling date significant differences among treatments could be detected (Fig. 1B).

## Discussion

With these studies, we attempted to determine if the co-occurrence of *P. neoaphidis* and *H. axyridis*, two soybean aphid natural enemies, would impact *P. neoaphidis* infection rate or soybean aphid density due to the IGP of *P. neoaphidis* by *H. axyridis*. We found significant impacts of the *H. axyridis* treatment on soybean aphid densities in 2009 but the same effects were not repeated in the 2010 data (Fig. 1). In

2009, those cages which did not contain *H. axyridis* adults had significantly more aphids than both low and high *H. axyridis* treatments on 24 August, just 3 d after the release of the beetles into the cages; however, 10 d later, on the final sampling date, cages lacking *H. axyridis* had significantly fewer aphids than cages in the low *H. axyridis* treatment and had fewer, though not significantly so, than the high *H. axyridis* treatment (Fig. 1A). These results indicate that adding *H. axyridis* to a soybean aphid colony already infected by *P. neoaphidis* actually reduces aphid control, though a high density of *H. axyridis* may somewhat mitigate that effect. When considering results from 2010, we find that on the final sampling date, cages without *H. axyridis* have the highest aphid density and those in the high *H. axyridis* treatment had the lowest aphid density, though these differences were not significant (Fig. 1B). Thus, implying that the addition of *H. axyridis* to soybean aphid colonies already infected with *P. neoaphidis* increased aphid control-directly conflicting with results from 2009.

While we intended to have cages both with and without *P. neoaphidis* infection, this did not occur and all cages in both years produced infected soybean aphids. Due to the widespread infection, we can neither estimate the impact of *P. neoaphidis* or *H. axyridis* alone on soybean aphid densities, nor can we make comparisons between impacts of each natural enemy. However, results we do have are valuable in beginning to understand how these natural enemies impact soybean aphid populations in tandem. Previous work has shown that coccinellids, including *H. axyridis*, have potentially disparate impacts on aphid fungal pathogens. On one hand, these beetles engage in asymmetrical IGP of *P. neoaphidis* (Roy et al. 2008, Wells et al. 2011). Negative impacts of this type of interaction solely fall on the fungus, as such fungi, at least in this case, are not capable of infecting the beetle predator. On the other hand, beetles may pick-up fungal conidia and transport them to healthy aphid colonies where the fungus will benefit from new hosts (Roy et al. 2008, Wells et al. 2011). The relative frequency and effect of these two potential interactions will determine if the overall impact of *H. axyridis* on *P. neoaphidis* will be positive, negative, or neutral. Our results indicate that while the presence of *H. axyridis* may impact the level of aphid control exerted by the two natural enemies, it does not seem to significantly impact the ability of *P. neoaphidis* to infect hosts. This lack of an impact could be due to the small spatial scale of the

experiment- a single soybean plant.  Considering the rapidity with which the fungus contaminated all the cages in both years of the study, we can assume that spreading throughout a single soybean plant would also occur rapidly, discounting any positive impact of coccinellid transport of conidia.

*Pandora neoaphidis* is density dependent and changes in aphid disease prevalence often closely follow changes in aphid density (unpublished data).  In 2009, prior to treatment initiation, the no *H. axyridis* treatment had a larger aphid population than either of the *H. axyridis* treatments.  This subtle difference seems to have led to increased aphid disease prevalence in the no *H. axyridis* treatment on 24 August 2009.  While this difference was non-significant statistically, it may have been biologically significant, leading to lower aphid densities in that treatment by the end of the experiment (Fig. 1A). This is plausible considering that in 2010, the no *H. axyridis* treatment had the lowest aphid density when the treatments were initiated and subsequently, at the conclusion of the experiment, had the highest density (Fig. 1B).

Another potential explanation for the differences in results between 2009 and 2010 are the differences in aphid disease prevalence.  In 2009, disease prevalence peaked at around 40% of aphids infected, while in 2010, peak disease was above 50% of aphids infected.  This was likely due to differences in abiotic conditions, as the same amount of inoculum was released into cages with similar aphid densities in both years.  In 2009, mean daily temperatures during 18 d in which aphids were exposed to *P. neoaphidis* inoculum was 17.0 ± 2.5°C (±SD), with high temperatures never exceeding 28°C; however, in 2010 mean daily temperatures during the 20 d of the experiment was 22.7 ± 2.4°C, with high temperatures exceeding 32°C on 5 d (NCDC 2011); thus, temperatures were more conducive for *P. neoaphidis* infection in 2009 (Shah et al. 2002).  However, rain was recorded on 8 of the 20 d of the experiment in 2010, for a total of 11.8 cm of rain, while in 2009 rain was observed on 7 of 18 d for a total of only 4.4 cm (NCDC 2011).  Even if irrigation was adjusted to compensate for the additional rainfall in 2010, it is possible that the wetter conditions contributed to the increased level of *P. neoaphidis* infection observed (Feng et al. 1991).  If *H. axyridis* was participating in IGP, we would expect negative impacts of the reduction in inoculum would be more severe in an environment with fewer infected cadavers, as was the case in 2009; thus contributing to

the differences in impacts of *H. axyridis* on aphid densities between the two years of the study.

Finally, in spite of the inconsistencies in the data between 2009 and 2010, it is interesting that in both years, aphid densities seemed to somewhat level off as the experiments were concluding, implying that at least some level of control was exerted by the two natural enemies. Aphid densities in cages were still above the economic injury level; however, if the experiments could have continued, aphid densities may have been reduced to below this level. That these natural enemies can respond and impose significant levels of mortality is promising, especially considering that in the field, they would likely be present prior to aphids reaching such a high density, allowing them to exert control earlier in the outbreak. This is especially apparent in the high *H. axyridis* treatment in 2010, where in less than 2 wk aphid densities were reduced from more than 4,000 per plant to approximately 1,000 per plant (Fig. 1B).

In summary, in spite of differences between 2009 and 2010 results, we have no evidence that the presence of *H. axyridis* in a soybean aphid colony reduces the prevalence of *P. neoaphidis* infection. Thus, it is unlikely that the co-occurrence of these two aphid natural enemies will negatively impact aphid control. In fact, when *P. neoaphidis* prevalence is high enough, as in 2010, aphid control may be enhanced by the presence of *H. axyridis* (Fig. 1B). However, further research is needed to determine how high levels of *P. neoaphidis* infection, as observed in 2010, might impact the fitness of *H. axyridis*, as infected aphids have been noted to be a less suitable food source for aphid predators than healthy aphids (Simelane et al. 2008). Additionally, further work comparing the efficacy of soybean aphid control by *H. axyridis* both in association with and independent of *P. neoaphidis* would further elucidate the interactions between the beetle and the fungus.

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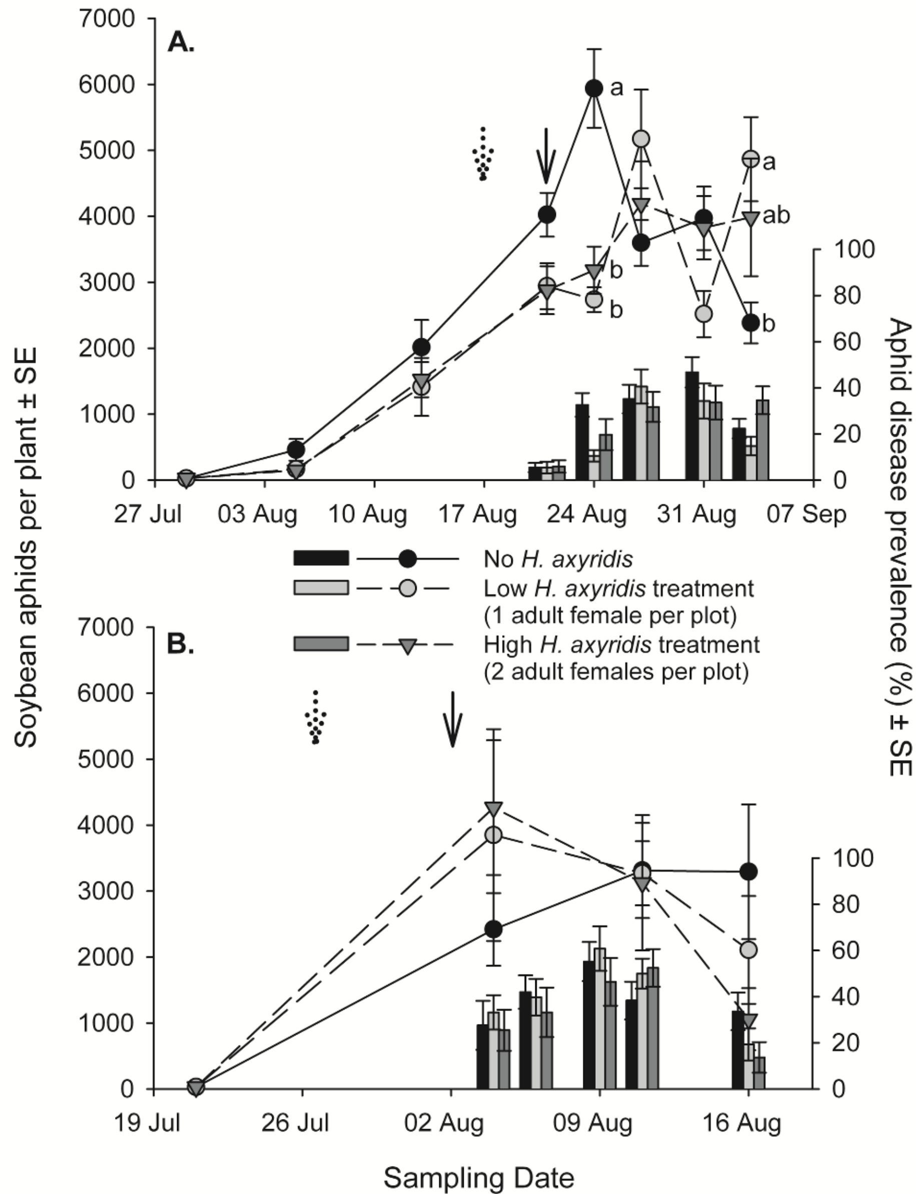


**Figure 1.** Impact of *H. axyridis* on soybean aphid density and disease prevalence illustrating per plant soybean aphid densities (line plots) and percent of soybean aphids infected (bar plots) in plots with no *H. axyridis* adults, 1 *H. axyridis* adult per plant, or 2 *H. axyridis* adults per plant at Becker, MN in 2009 (A) and 2010 (B). Dashed arrows indicate the date of release of *P. neoaphidis* inoculum and the solid arrows indicate the date of release of the *H. axyridis* individuals. *Harmonia axyridis* density had a significant impact on aphid density on 24 August and 3 September 2009. Within sampling dates, data points indicated by the same letter are not significantly different.

# Chapter 5: Evaluating the potential for a fungal pathogen to impact soybean aphid populations on the primary host, *Rhamnus cathartica*

## Introduction

Soybean aphid is a serious arthropod pest of soybean in the North Central US, causing yield loss when populations exceed the economic injury level (EIL) of 674 aphids per plant (Ragsdale et al. 2007). Economic damage is also caused when aphid densities exceed the economic threshold (ET) of 250 aphids per plant; which triggers broad spectrum insecticide applications to prevent yield loss (Ragsdale et al. 2007). There has been an extensive amount of research into the role of natural enemies in the management of soybean aphid populations on soybean (reviewed in Ragsdale et al. 2011), the aphid's secondary host. However, much less is known about the role of natural enemies in the population regulation of soybean aphid on its primary host, common buckthorn, *Rhamnus cathartica*. Because soybean aphid populations on buckthorn are the initial source of soybean infestations each summer, understanding how populations are impacted by natural enemies on the primary host can inform aphid management on the more economically relevant secondary host.

Soybean aphid has a complicated heteroecious, holocyclic lifecycle in which the aphid alternates between a primary, *Rhamnus cathartica*, and secondary, *Glycine max*, host with sexual reproduction occurring on the primary host in late summer and early autumn (Ragsdale et al. 2004; Voegtlin et al. 2005). Coinciding with buckthorn bud break in early spring, soybean aphid eggs hatch producing fundatrices, which are viviparous, parthenogenetic females which produce viviparous nymphs. As generations progress on buckthorn, an increasing proportion of individuals are winged and capable of migrating to the secondary host, cultivated soybean (Dixon 1973). Such spring migrants initiate colonies of parthenogenetic, viviparous females on soybean where populations can increase exponentially due to the aphid's high reproductive rate. Subsequently, as crowding increases and host plant quality decreases, summer migrants, winged parthenogenetic viviparae, are produced and disperse to colonize additional hosts (Dixon 1973). As day length shortens and temperatures cool, gynoparae are produced on soybean. These female sexual migrants move to buckthorn where they produce oviparae, the egg-laying morph. Approximately two weeks later, males are produced on soybean and also migrate to buckthorn where they mate with oviparae. Oviparae deposit eggs along the buckthorn buds, where the eggs remain throughout the winter (Dixon 1973).

Thus, buckthorn can be seen as a bottle-neck for soybean aphid populations, especially in regions where buckthorn is relatively rare. Management efforts targeted to aphid populations on buckthorn in the autumn has the potential to reduce management efforts on soybean the following summer.

Because soybean is the economically important host of soybean aphid, the majority of research on the management of the pest is focused on aphid populations occurring on soybean during the summer months. Because buckthorn serves as a temporal and spatial bottle-neck for these populations, understanding how soybean aphid is regulated on its primary host can offer new management strategies which may limit or delay soybean infestations the following summer. Before such strategies can be developed, however, we must better understand the dynamics of soybean aphid and its natural enemies on buckthorn. Such studies are complicated by the inconsistencies in aphid populations on buckthorn in time and space (i.e. Welsman et al. 2007) and the difficulties in confirming causes of mortality. However, we do know that pathogenic fungi utilize soybean aphids on buckthorn, with higher infection rates observed in populations on buckthorn compared to soybean (Nielsen and Hajek 2005). In the case of fungal infections, the cause of mortality can be confirmed in the field via observations of mycosed aphid cadavers, or in the lab via microscopic examination of fungal conidia; thus offering a partial solution. Additionally, fungal pathogens, most commonly *Pandora neoaphidis* (Remaudière & Hennebert) Humber, have been observed in soybean aphid populations on soybean and on buckthorn (Nielsen and Hajek 2005; Noma and Brewer 2007; Koch et al. 2010). Therefore, further examination of the role of such fungal pathogens in aphid population regulation on the primary host is warranted.

Because soybean aphids found on buckthorn in the autumn originate on soybean, it is also likely that fungal pathogens infecting soybean aphids on buckthorn originated on soybean. Multiple studies by Feng and colleagues indicate that winged aphids infected with entomophthoralean fungi are capable of locating a new host plant and producing offspring prior to succumbing to infection (Feng and Chen 2002; Feng et al. 2004; Feng et al. 2007). Even more intriguing is that up to one third to winged aphids captured over a four year period in China were infected with a pathogen and 80% of the infections were caused by *P. neoaphidis* (Feng et al. 2007). Thus, fungal infections

causing mortality to sexual soybean aphid morphs on buckthorn may originate in gynoparae on soybean, implying late-season conditions in soybean may potentially impact the disease-induced mortality of soybean aphids on buckthorn.

In the following studies, we attempt to determine if soybean aphids transport fungal pathogens, particularly *P. neoaphidis*, when moving from soybean to buckthorn in the autumn and, if so, how fungicide use in soybean may impact the level of aphid disease on buckthorn. Fungicide use in soybean is increasing due to the threat of Asian soybean rust, a soybean disease caused by the fungus *Phakopsora pachyrhizi* and recently introduced to the continental US (Schneider et al. 2005). Additionally, fungicide use on soybean has also increased due to applications for 'plant health effects', in which growers will apply fungicide- not to target a specific plant disease- but to increase the vigor of uninfected plants. In such cases, growers often tank-mix the fungicide with other required plant protection products as a form of low cost 'insurance' for their crop (Swoboda and Pedersen 2009). Fungicides are known to negatively impact soybean aphid fungal infection on soybean (Koch et al. 2010). Therefore, it follows that negative impacts on fungal pathogens may extend to aphid populations on buckthorn if healthier aphids are originating from fungicide-treated soybean.

In order to better understand dynamics of the soybean aphid-*P. neoaphidis* pathosystem on buckthorn, we compared susceptibility to *P. neoaphidis* among viviparous and sexual soybean aphid morphs. Understanding which morphs are most susceptible to *P. neoaphidis* infection will shed light on when this pathogen can most effectively control aphid populations. Previous studies indicate that alate *Myzus nicotianae* and *Sitobian avenae* are more susceptible to *P. neoaphidis* infection than their apterous counterparts (Yu et al. 1995; Dromph et al. 2002). Differences in susceptibility between viviparous morphs and sexual morphs of a host-alternating aphid to entomophthoran fungi is, as yet, unknown. However, there is evidence of differential susceptibility among morphs of host-alternating aphids as gynoparae and males of *Rhopalosiphum padi* had higher natural rates of infection than fundatrices, all of which occur on the primary host, *Prunus padus* (Nielsen and Steenburg 2004).

We utilized large plot field studies to assess if soybean aphids are capable of transporting pathogenic fungi from soybean to buckthorn and if so, to determine if

negative impacts of foliar fungicide treatments on fungal pathogens in aphids on soybean extend to pathogens occurring in aphids on buckthorn. To further understand the dynamics of soybean aphid and its fungal pathogens on buckthorn, we utilized a series of laboratory bioassays to determine the relative susceptibility of viviparous and sexual soybean aphid morphs to the common soybean aphid fungal pathogen, *P. neoaphidis*.

## Materials and Methods

### Field Experiments, 2009-2010

At the University of Minnesota Outreach, Research, and Education Park at Rosemount, MN, we planted soybean varieties NK 23N7 and Pioneer 91Y90 in 0.76 m rows on 19 May 2009 and 24 May 2010, respectively. Varieties were similar in plant size, structure, and maturity group. Soybean used in the experiment were selected from a bulk-planted field and treated with glyphosate as needed for weed control to simulate conditions in a conventionally managed grower field. Eight plots of 20 rows (15.2 m) by 30.5 m were measured, marked, and randomly assigned as fungicide-treated or untreated (the control), so that each treatment was replicated four times in each year.

Fungicide treatment consisted of three scheduled foliar applications over the course of the season, beginning when plants reached the R2 reproductive stage (full flower) and continuing on 14 d intervals (R2, R2 + 14 d, and R2 + 28 d). This treatment schedule corresponded to fungicide applications being made on 23 Jul, 5 Aug, and 21 Aug in 2009 and on 16 Jul, 3 Aug, and 17 Aug in 2010. The first two applications were made with trifloxystrobin + propiconazole, a strobilurin-triazole mixture (Stratego®, Bayer CropScience, Research Triangle Park, NC), applied at 0.52 and 0.74 liters of product per ha in 2009 and 2010, respectively. Rates were changed between years to comply with the maximum labeled field rate. The final fungicide application was made with chlorothalonil, a chloronitrile (Bravo WeatherStik®, Syngenta Crop Protection, Greensboro, NC), applied at 2.3 liters of product per ha in both years of the study. Fungicides were applied with a tractor-mounted 3-point hitch $CO_2$ sprayer with a 3.0 m boom and at a volume of 187 liters per ha.

Soybean aphids on soybean were sampled weekly beginning 22 Jul 2009 and 25 Jun 2010. Per plant aphid density was estimated using destructive whole-plant aphid

counts on five to 20 plants per plot. When less than 50% of plants were infested, 20 plants per plot were sampled, once plots reached 50-85% infested, sampling effort was reduced to ten whole-plant counts per plot and once infestation levels were >85%, sampling was further reduced to five whole-plant counts per plot. Aphid disease was also monitored throughout the season, sampling beginning on 4 Aug 2009 and 13 Jul 2010. Aphid disease prevalence was determined via bioassays in which 25 aphids were collected from each soybean plot, returned to the laboratory, transferred to excised soybean leaves in 100 mm polystyrene petri dishes, and monitored for three days for any sign of infection. Any moribund aphids observed were transferred to 50 mm polystyrene petri dishes with a tight-fit lid (BD Falcon™, Becton, Dickinson and Company, Franklin Lakes, NJ) containing 1% water agar (1 g agarose in 100 ml water) to induce sporulation. If sporulation occurred, spores were stained with aceto-orcein stain and examined under 200x magnification (Humber 1997). Pathogen identity was confirmed via spore morphology according to Samson et al. (1988). Spore samples from all apparently infected aphids were examined. Bioassays were performed once per week until disease was confirmed. Once disease was confirmed in the aphid population, bioassay frequency increased to twice per week for the remainder of the sampling period.

Small buckthorn, *Rhamnus cathartica*, shrubs were collected and transferred to large nursery pots for use in field experiments. In 2009, buckthorn individuals were collected from wind breaks at the University of Minnesota Outreach, Research, and Education Park at Rosemount, MN and in 2010, buckthorn individuals were collected from wind breaks at the Sand Plain Research Farm at Becker, MN. After transplanting, potted buckthorn was kept in the tree nursery on the St. Paul campus of the University of Minnesota and was fertilized and watered as needed. On 28 Aug 2009 and 25 Aug 2010, four potted buckthorn plants were randomly assigned to and set-out in each plot. Four 1 $m^2$ areas of soybean were cleared in each plot and buckthorn plants were set in the middle of the cleared area so that buckthorn foliage was not in contact with soybean foliage. Cleared areas were located in the fifth and sixth rows and the fourteenth and fifteenth rows of each plot and were 6.1 m from either edge of the plot. Buckthorn plants were checked twice per week for aphid infestations and any aphids observed were counted, collected, and returned to the lab to determine disease prevalence. The procedure for

these bioassays was similar to that for the aphids collected from soybean, except that all aphids observed were collected, so each plot did not necessarily have the same number of individuals in each bioassay. Monitoring for aphids on buckthorn ceased once all leaves dropped from soybean plants, on 18 Sep 2009 and 13 Sep 2010.

**Relative Susceptibility Experiment**

Eight different age-morph treatments were challenged in a laboratory bioassay to estimate the relative susceptibility of each morph to infection by *P. neoaphidis* (Entomophthorales: Entomophthoraceae). Treatments included small viviparous nymphs ($1^{st}$-$2^{nd}$ instar viviparae), large apterous nymphs ($3^{rd}$-$4^{th}$ instar apterous viviparae), large alatoid nymphs ($3^{rd}$-$4^{th}$ instar alate viviparae), apterous adult viviparae, alate adult viviparae, adult gynoparae, oviparae ($2^{nd}$-$3^{rd}$ instar), and adult males. All aphids used in the experiment originated from a laboratory colony of soybean aphids which was initiated in June 2010 with field collected aphids from the Sand Plain Research Farm at Becker, MN. The colony was maintained on soybean seedlings (variety S19R5) in a growth chamber at 25 ± 1°C and 16:8 (L:D) h. In order to generate sexual morphs (gynoparae, ovipare, and males), viviparae from the original laboratory colony were placed in two growth chambers under short day, 10:14 (L:D) h, and cool, 16 ± 1°C, conditions (Yoo et al. 2005). Under these conditions, gynoparae were produced in approximately 28 d and males in approximately 42 d. Plants with viviparae were added to one of the chambers each week so that both gynoparae and males would be available at the same time for use in the experiment. Aphids were selected for each treatment group based on external morphology; however, gynoparae and alate viviparae could not be separated morphologically. Therefore, gynoparous status was confirmed when suspected gynoparae moved to buckthorn leaves placed in chambers and deposited oviparae.

The *P. neoaphidis* culture used had been originally isolated from *Acyrthosiphon pisum* Harris, pea aphid, collected in Rosemount, MN in 2008. We then infected soybean aphids with the original isolate and reisolated the fungus from soybean aphid four times prior to use in this experiment. Actively sporulating *in vitro* cultures of *P. neoaphidis* maintained on Sabouraud dextrose agar (Difco™, Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with egg yolk and milk (SDAEYM) were used to inoculate aphids at the onset of the bioassays (Papierok 1997). All cultures used in the

bioassays were kept at 19 ± 1°C and 0:24 (L:D) h and were the first subculture taken from a *P. neoaphidis* reisolate originating from a single soybean aphid infected by *P. neoaphidis*. This was done to ensure that cultures were still infective and as uniform as possible. Subculturing of the original isolate to obtain the required number of cultures for the bioassays ocurred 35-40 d prior to initiation of the experiment as *P. neoaphidis* cultures are slow-growing and sporulation is generally not observed for several weeks after subculture. Subculturing involved transferring a 3 mm diameter portion of actively growing *P. neoaphidis* mycelium to a sterile SDAEYM plate. Once sporulation was evident, when spores were observed on the lid of petri dishes, cultures were ready to be used in the experiment. Cultures exhibiting similar levels of sporulation were selected for use in a single bioassay replication to ensure similar levels of aphid exposure to conidia for each treatment.

Bioassays consisted of selecting 25 aphids from each age-morph treatment, transferring these aphids to sterile, 100 mm polystyrene petri dishes, inverting actively sporulating *P. neoaphidis* cultures over the aphids for three hours, and monitoring aphids every 24 hr for 5 d after exposure. After inoculation, dishes were sealed with laboratory film (Parafilm®, Bemis Flexible Packaging, Neenah, WI) and were kept sealed for the duration of the 5 d observation period. This prevented aphid escape and maintained proper environmental conditions within dishes. Any apparently infected aphid was moved to a 50 mm tissue culture plate with 1% water agar to induce sporulation. Conidia from a subsample of infected aphids, at least one aphid per treatment per day, were stained with aceto-orcein stain and examined under 200x magnification to confirm the cause of the infection. The entire bioassay was replicated 12 times between Jan 2011 and Apr 2011.

Small viviparous nymphs, large apterous nymphs, large alatoid nymphs, apterous adult viviparae, and alate adult viviparae were maintained on excised, greenhouse-grown soybean leaves (variety S19R5) throughout the bioassays. Adult gynoparae, oviparae, and adult males were maintained on excised, greenhouse-grown and surface sterilized buckthorn leaves throughout the bioassays. Both leaf types were kept fresh by placing the petiole in a small block of moistened floral foam (Oasis® General Purpose Floral Foam, Smithers-Oasis Company, Kent, OH). Petri dishes were lined with moistened

filter paper (Fisherbrand$^{TM}$ P5 Qualitative-Grade Filter Paper, Fisher Scientific, Pittsburgh, PA) to maintain the high levels of relative humidity required for fungal disease transmission. Floral foam and filter paper were rewetted as necessary throughout the 5 d observation period. All aphids for all replications were kept in the same growth chamber at 16:8 (L:D) hr and 24.0 ± 0.4°C for the duration of the experiments.

**Statistical Analysis**

Aphid densities and aphid disease prevalence on soybean were compared between treatments, fungicide-treated and untreated, with a repeated measures analysis of variance (PROC MIXED, SAS 9.2, SAS Institute Inc. 2008). Data from each year of the field experiment were analyzed separately and aphid densities for both years required a natural log transformation in order to fulfill the assumptions of the statistical model. Covariance structure was chosen using the methods in Littel et al. (2006) so that the Akaike Information Criterion (AIC) was minimized. Models included treatment, sampling date, and the treatment-sampling date interaction as factors. Least squares means for significant treatment-sampling date interactions were separated using a Tukey-Kramer correction to compensate for multiple comparisons. For aphids counted and collected from the potted buckthorn outplants, aphid density, number of gynoparae, number of oviparae, and aphid disease prevalence was compared between treatments with an analysis of variance (ANOVA) (PROC GLM, SAS 9.2, SAS Institute Inc. 2008). Mean aphid density and aphid disease prevalence from the four buckthorn plants in each plot was used in the analysis. Because aphids were found on buckthorn on only a single sampling date, repeated measures analysis was not required.

For the relative susceptibility experiment, the proportion of aphids infected was compared among the eight treatments with ANOVA (PROC GLM, SAS 9.2, SAS Institute Inc. 2008), with each dish being the experimental unit, for a total of 12 observations per treatment. Replication and treatment were factors in the model and least squares means were separated with a Tukey correction for multiple comparisons. For all analyses, the significance level was set at 0.05 and, when multiple comparisons were performed, adjusted *P*-values are reported. Aphid densities are reported as mean (± SE) aphids per plant and aphid disease prevalence is reported as mean (± SE) percent of aphids infected.

## Results

### Field Experiments, 2009-2010

Soybean aphids were found on all sampling dates in both years of the study. In 2009, aphid densities increased from 22 Jul 2009 until the peak density was observed on 18 Aug 2009, after which densities decreased until sampling was concluded on 2 Sep 2009 (Fig. 1a). Aphid densities significantly changed over time, with sampling date being a significant factor in the model ($F$=129.24, df=6, 36, $P$<0.0001) (Fig. 1a). However, treatment ($F$=0.32, df=1, 36, $P$=0.5748) and the treatment by sampling date interaction ($F$=2.03, df=6, 36, $P$=0.0868) were not significant. Peak aphid densities for both treatments was similar, 762 ± 290 and 765 ± 135 aphids per plant for untreated and fungicide-treated soybean, respectively (Fig. 1a).

In 2010, aphid densities increased from the first sampling date, 25 Jun 2010, to the peak density on 5 Aug 2010 (Fig. 1b). After reaching the peak, aphid densities declined through the final sampling date on 24 Aug 2010 (Fig. 1b). The effect of fungicide-treatment on aphid densities depended on the sampling date, as the treatment-sampling date interaction was a significant factor in the model ($F$=2.97, df=9, 54, $P$=0.0061). Peak aphid densities for untreated and fungicide-treated soybean were 363 ± 71 and 222 ± 37 aphids per plant, respectively. However, after correcting for multiple comparisons, aphid densities did not significantly vary between treatments within any sampling date ($P$>0.05) (Fig. 1b). Aphid densities did significantly change over time, as sampling date was also a significant factor ($F$=196.55, df=9, 54, $P$<0.0001) (Fig. 1b). The main effect of treatment was non-significant ($F$=1.34, df=1, 54, $P$=0.2522).

Aphid infection was first observed on 11 Aug 2009 in both treatments and diseased aphids were identified in both untreated and fungicide-treated plots for all remaining sampling dates until sampling was suspended on 8 Sep 2009 (Fig. 1a). All soybean aphid infections were caused by *P. neoaphidis*. The effect of fungicide treatment depended on sampling date, as the treatment by sampling date interaction was significant ($F$=2.30, df=8, 48, $P$=0.0360). On 18 Aug 2009, aphids collected from untreated plots had a significantly higher disease prevalence, 23.0 ± 10.5% of aphids infected, compared to aphids collected from fungicide-treated plots, 5.0 ± 2.5% of aphids infected ($t$=3.73, df=48, $P$=0.0441) (Fig. 1a). Main effects of treatment ($F$=10.93, df=1,

48, $P$=0.0018) and sampling date ($F$=7.03, df=8, 48, $P$<0.0001) were also significant, with disease in untreated plots being significantly higher, 10.3 ± 1.9% of aphids infected across all sampling dates, compared to only 4.6 ± 1.0% of aphids infected across all sampling dates in fungicide-treated plots.

Aphid infections were first observed on 23 Jul 2010; however disease was not observed after 20 Aug 2010 and sampling was suspended on 27 Aug 2010 (Fig. 1b). All infections of soybean aphids were found to be caused by *P. neoaphidis*. The treatment by sampling date interaction term was significant; thus, the impact of fungicide treatment depended on sampling date ($F$=2.63, df=10, 60, $P$=0.0101). Specifically, on 5 Aug, untreated plots had aphids with significantly higher disease prevalence, 28.0 ± 5.4% of aphids infected, than fungicide-treated plots, 13.0 ±6.8% of aphids infected ($t$=4.89, df=60, $P$=0.0015) (Fig. 1b). The main effect of sampling date was also significant ($F$=15.71, df=10, 60, $P$<0.0001); though treatment was not ($F$=1.63, df=1, 60, $P$=0.2066).

In 2009, soybean aphids were recovered from potted buckthorn set out in each plot; however, no aphids were found on potted buckthorn in 2010. All aphids were found and collected on 18 Sep 2009. A total of 287 aphids were collected from buckthorn in untreated soybean plots and 306 aphids were collected from buckthorn in fungicide-treated plots. Samples from one of the untreated plots were lost and were not included in the analysis. Similar densities of gynoparae ($F$=2.28, df=1, 6, $P$=0.1916), oviparae ($F$=0.17, df=1, 6, $P$=0.6983), and total aphids ($F$=0.34, df=1, 6, $P$=0.5851) were found on buckthorn in both untreated and fungicide-treated plots. No males were found on buckthorn in any plot. Of aphids collected, 10.8 ± 1.1% and 2.9 ± 2.3% of aphids from buckthorn were infected, in untreated and fungicide-treated plots, respectively. This difference in disease prevalence was significant, with buckthorn in untreated plots harboring aphids with a significantly higher disease prevalence compared to those aphids on buckthorn in fungicide-treated plots ($F$=7.84, df=1, 6, $P$=0.0380). All infections of soybean aphids on buckthorn were found to be infected by *P. neoaphidis*. The majority of the infections were found in gynoparae, with 38.3 ± 9.2% and 20.8 ± 12.1% of gynoparae collected from untreated and fungicide-treated plots, respectively, being infected. Of ovipare collected, only 3.9 ± 2.2% from untreated plots were infected, while 0.2 ± 0.2% from fungicide-treated plots were infected.

**Relative Susceptibility Experiment**

All conidia samples from infected aphids were found to be *P. neoaphidis*. The effect of morph on the susceptibility of soybean aphid to *P. neoaphidis* was significant, with treatment being a significant factor ($F$=13.16, df=7, 95, $P$<0.0001). Disease rates varied from a minimum of 0.3 ± 0.3% of large apterous nymphs infected to a maximum of 39.0 ± 4.8% of gynoparae infected (Fig. 2). Gynoparae were significantly more susceptible than all other morphs except males (Fig. 2). Males were significantly more susceptible than all the apterous morphs, including apterous adults, large apterous nymphs, oviparae, and small nymphs (Fig. 2). Alate adults and alatoid viviparous nymphs were significantly more susceptible than large apterous nymphs and small nymphs (Fig. 2). The large apterous nymphs and small nymphs were the least susceptible and were significantly less susceptible than all the alate morphs, including gynoparae, males, alate adults, and large alatoid nymphs (Fig. 2).

Because treatment was significantly correlated with the host plant (soybean or buckthorn), wing status (apterous or alate), and age (nymph or adult) of the aphid morph, we were not able to include these factors in the statistical model. However, it is interesting to note that generally, aphid morphs found on buckthorn, alate aphids, and adult aphids had higher rates of infection than aphid morphs found on soybean, apterous aphids, and immature aphids. Aphid morphs found on buckthorn, also the sexual morphs, had an infection rate of 24.8 ± 3.3%, while the viviparous morphs on soybean had an infection rate of only 10.0 ± 1.8%. Morphs with wings were more susceptible, with 26.6 ± 2.7% of aphids infected, than morphs lacking wings, with 4.5 ± 1.1% of aphids infected. Immature aphids seem to be less susceptible with an infection rate of 6.6 ± 1.6%, compared to 24.5 ± 2.7% for adult aphids.

## Discussion

The purpose of this research was to determine if soybean aphid was capable of transporting fungal pathogens from soybean to buckthorn in the autumn. While we only observed aphids on our potted buckthorn plants in 2009, we were able to confirm that gynoparae, which had presumably migrated from the surrounding soybean to the buckthorn, were infected at a rate, 38.3 ± 9.2% and 20.8 ± 12.1% for untreated and

fungicide-treated plots, respectively, that was higher than was observed on the final sampling date in soybean, ten days earlier (Fig. 1a). Thus, providing evidence that gynoparae are capable of carrying entomopathogenic fungi from soybean to buckthorn. Because we also observed infection in oviparae collected from buckthorn, these infected gynoparae were also able to pass the infection on to the egg-laying generation. Reductions in oviparae density are likely to subsequently reduce the aphid's overwintering population. Nielsen and Hajek (2005) found that 27.3 – 86.8% of gynoparae found on buckthorn in New York State were infected with an entomophthoraceous fungus and our observation of 38.3± 9.2% of gynoparae from untreated plots being infected fits within this range.

There is also evidence that fungicide applications to soybean may negatively impact entomopathogenic fungi moving to buckthorn with gynoparae in the autumn. Aphids recovered from potted buckthorn plants set out in untreated soybean plots had significantly higher levels of fungal infection than gynoparae recovered from buckthorn in fungicide-treated soybean plots. Such delayed impacts of fungicide applications on aphid fungal disease were also observed on spinach in which *Myzus persicae* disease prevalence was significantly reduced in fungicide-treated plots 21 d and 41 d after application (McLeod and Steinkraus 1999). However, we were only able to find aphids on the potted buckthorn in 2009. End of season aphid densities on soybean in 2010 were very low and likely prevented buckthorn colonization (Fig. 1b); therefore, we do not have an additional year of data with which to compare the 2009 results. Additionally, sample sizes were small and all data was collected on a single sampling date, 18 Sep 2009. While the data supports the hypothesis that fungicide treatments on soybean can have long-lasting impacts on the fungal pathogens of soybean aphid, even after aphids move to their primary host, common buckthorn, additional studies are needed to confirm our observations.

Soybean aphid fungal disease prevalence in soybean was significantly reduced by the application of foliar fungicides in both 2009 and 2010. These results confirm earlier observations of negative impacts of fungicide use in soybean on fungal pathogens of soybean aphid (Koch et al. 2010). In 2009, aphid disease in untreated plots exhibited a typical epizootic wave in which disease was first detected at low levels, increased to a

peak level of prevalence, 24.0 ± 6.5%, and then decreased, corresponding to changes in aphid density (Fig. 1a). However, the fungicide-treated plots did not exhibit a similar pattern, with aphid disease prevalence having lower peak prevalence, 16.0 ± 1.6%, which occurred eight days sooner than in untreated plots. Thus, fungicide applications altered the dynamics of the epizootic wave. Fungicide-treated plots had significantly lower aphid disease, 5.0 ± 2.5% of aphids infected, than untreated plots, 23.0 ± 10.5%, on 18 Aug 2009 (Fig. 1a). In 2010, peak aphid disease in both treatments coincided with peak aphid density, on 5 Aug 2010; however, fungicide-treated plots had significantly lower aphid disease, 13.0 ± 6.8% of aphids infected, compared to the untreated plots, 28.0 ± 5.4% (Fig. 1b). In this case, fungicide applications reduced peak aphid disease, but did not seem to alter the dynamics of the epizootic wave, which may be due to the reduced aphid densities observed in 2010. In both years of the study, significant reductions in aphid disease were observed between the second and third fungicide application (Fig. 1). While this may indicate that the strobilurin-triazole mixture was more hazardous to aphid fungal pathogens, it is more likely that this observation was an artifact of our application schedule. Considering fungal pathogens are density-dependent, pathogens will become most prevalent when aphid densities are highest and, in both 2009 and 2010, aphid densities peaked at a similar soybean growth stage corresponding to the full pod stage (R4) which occurred 14-28 d after soybean plants reached full flower (R2).

Traditionally, decisions to apply foliar fungicides were made considering current disease pressure and environmental conditions. However, recently, fungicide use in the absence of disease pressure has increased due to the plant physiological benefits purportedly provided by fungicides. When considering the potential negative impacts to beneficial fungi, such treatments for plant health effects may inadvertently exacerbate pest problems by reducing the efficiency of this group of natural enemies. Based on results presented here and research suggesting that plant physiological benefits of fungicide applications are minimal (Swodoba and Pedersen 2009), foliar fungicide applications should be utilized only when managing a specific fungal disease. Carefully considering risks of non-target impacts or environmental harm and economic costs of each fungicide application can prevent many of the negative consequences of fungicide use.

Soybean aphid densities were not significantly impacted by fungicide treatment; although, in 2010, a low aphid year, untreated plots showed a trend of higher aphid densities compared to fungicide-treated plots (Fig. 1b). However, in spite of a significant interaction between sampling date and treatment, after correcting for multiple comparisons, significant differences between treatments within sampling dates could not be detected. While we are unsure as to why such differences, significant or not, may have occurred, it is important to note that throughout the 2010 growing season, aphid densities remained below the EIL of 674 aphids per plant, even at peak densities (Fig. 1b). In 2009, when peak aphid densities exceeded the EIL, aphid densities in fungicide-treated plots actually declined more slowly, remaining above the ET until the final sampling date of 2 Sep 2009 (Fig. 1a). Thus it is unlikely that fungicide use in soybean contributes to reductions in aphid density, which is consistent with previous results in soybean (Koch et al. 2010).

Results of the relative susceptibility experiment reveal that soybean aphid susceptibility to *P. neoaphidis* varies significantly among age-morph treatments (Fig. 2). Gynoparae were the most susceptible with 39.0 ± 4.8% of individuals becoming infected. Male soybean aphids became infected frequently as well, with an infection rate of 29.3 ± 4.7%. Thus, those soybean aphid morphs moving from soybean to buckthorn in the autumn are the most susceptible to infection by *P. neoaphidis*. Furthermore, the other migratory morph, alate adult viviparae, also became infected quite frequently, 19.3 ± 5.6% of the time. Thus, supporting observations by Feng and colleagues that migratory aphids carry fungal entomopathogens when moving from host to host (Feng and Chen 2002; Feng et al. 2004; Feng et al. 2007). Interestingly, oviparae are much less susceptible than the other sexual morphs and are about as susceptible as apterous adult viviparae. That winged aphids are more susceptible than apterous aphids has been observed before (Yu et al. 1995; Dromph et al. 2002); however, to our knowledge this is the first study comparing the susceptibility of viviparous, summer morphs with sexual morphs. A study by Scorsetti et al. (2010) suggests that nymphs, apterous adults, and alate adults of *Nasonovia ribisnigri*, an aphid pest of lettuce in Argentina, are equally susceptible to *P. neoaphidis*. While differences may not have been statistically significant, their data indicate that approximately 50% of all observed apterous and alate

adults were infected, while only about a third of all observed nymphs were infected, implying that nymphs may be less susceptible than either apterous or alate adults (Scorsetti et al. 2010). This supports our observation that immature soybean aphids were less susceptible than their adult counterparts (Fig. 2). One potential cause of the reduced susceptibility of immature aphids is evasion of infection as a result of ecdysis. If the timing is right, immature aphids may be able to shed germinating *P. neoaphidis* conidia when their cuticle is shed at the conclusion of a stadium. Because adults no longer undergo ecdysis, they are unable to evade infection once conidia have successfully penetrated the cuticle.

One of our assumptions in the buckthorn out-plant study is that the aphids found on the buckthorn plants migrated from soybean plants within the same field plot. We can not be certain that this is the case as little is known about the migration of soybean aphid between its primary and secondary hosts. Additionally, the significant reduction in fungal infection we observed in aphids on buckthorn from fungicide-treated plots may have been caused by reduced inoculum availability within these plots, rather than healthier migrating gynoparae. When epizootics caused by entomophthoralean fungi occur, conidia enter the air space above fields (Steinkraus et al. 1996; Hemmati et al. 2001) and, in 2009, the epizootic of soybean aphids on soybean was ongoing even after buckthorn had been placed in the field on 28 Aug 2009 (Fig. 1a). Thus, it is possible that fungicide use in soybean reduced the inoculum load in the air around treated plots, leading to reduced conidial deposition on potted buckthorn and subsequently to lowered infection rates in soybean aphids which migrated to those buckthorn plants. However, conidial loads in the air decline rapidly as the epizootic declines (Steinkraus et al. 1996) and, by the time gynoparae were located on buckthorn plants, on 18 Sep 2009, the epizootic had been over for almost ten days. The final sampling date documenting the epizootic occurred on 8 Sep 2009 and indicated that only $1.0 \pm 0.7\%$ of aphids on soybean were infected (Fig. 1a). Thus few conidia likely remained in the air above plots by the time aphids migrated to buckthorn, which must have occurred between 14 Aug and 18 Aug 2009 since we did not observe aphids on buckthorn when in the field on 14 Aug 2009. Additionally, any conidia that had landed on buckthorn from the epizootic on soybean, which ended around 8 Sep 2009, would have lost the ability to infect aphids

prior to gynoparae arriving on the buckthorn, between 14 Aug and 18 Aug 2009. Using an in-field data logger, we found that relative humidity and temperature were 85.1 ± 17.9% and 19.0 ± 5.9°C between 8 Aug and 14 Aug 2009. Because these environmental conditions lead to the relatively swift inactivation of entomophthoralean conidia (Brobyn et al. 1987; Steinkraus and Slaymaker 1994), it is unlikely any infective conidia remained on buckthorn once gynoparae arrived. Therefore, it is likely that gynoparae became infected while still on soybean, where infected viviparae likely still occurred at undetectable levels (i.e. >1% infected), days prior to arriving on the buckthorn plants.

In summary, our results demonstrate that soybean aphid gynoparae are able to transport their fungal pathogens, particularly *P. neoaphidis*, from soybean to their primary host, buckthorn, at the end of the growing season. Once on buckthorn, gynoparae likely contaminate their offspring, oviparae, with conidia causing fungal infection in this morph as well. Foliar fungicide applications to soybean not only negatively impact aphid fungal pathogens on soybean, but also those same pathogens when they infect aphids on buckthorn in the autumn. Field results of increased infection rates in gynoparae were confirmed by a series of bioassays in which gynoparae were found to be the most susceptible to *P. neoaphidis* under controlled, laboratory conditions. Other alate morphs, including males and adult alate viviparae, were also highly susceptible. The apterous morphs were less susceptible, with apterous nymphs being the least susceptible. Thus, soybean aphid morph, and possibly aphid age, significantly impacts susceptibility to *P. neoaphidis*, the most commonly observed soybean aphid pathogen in the field studies.

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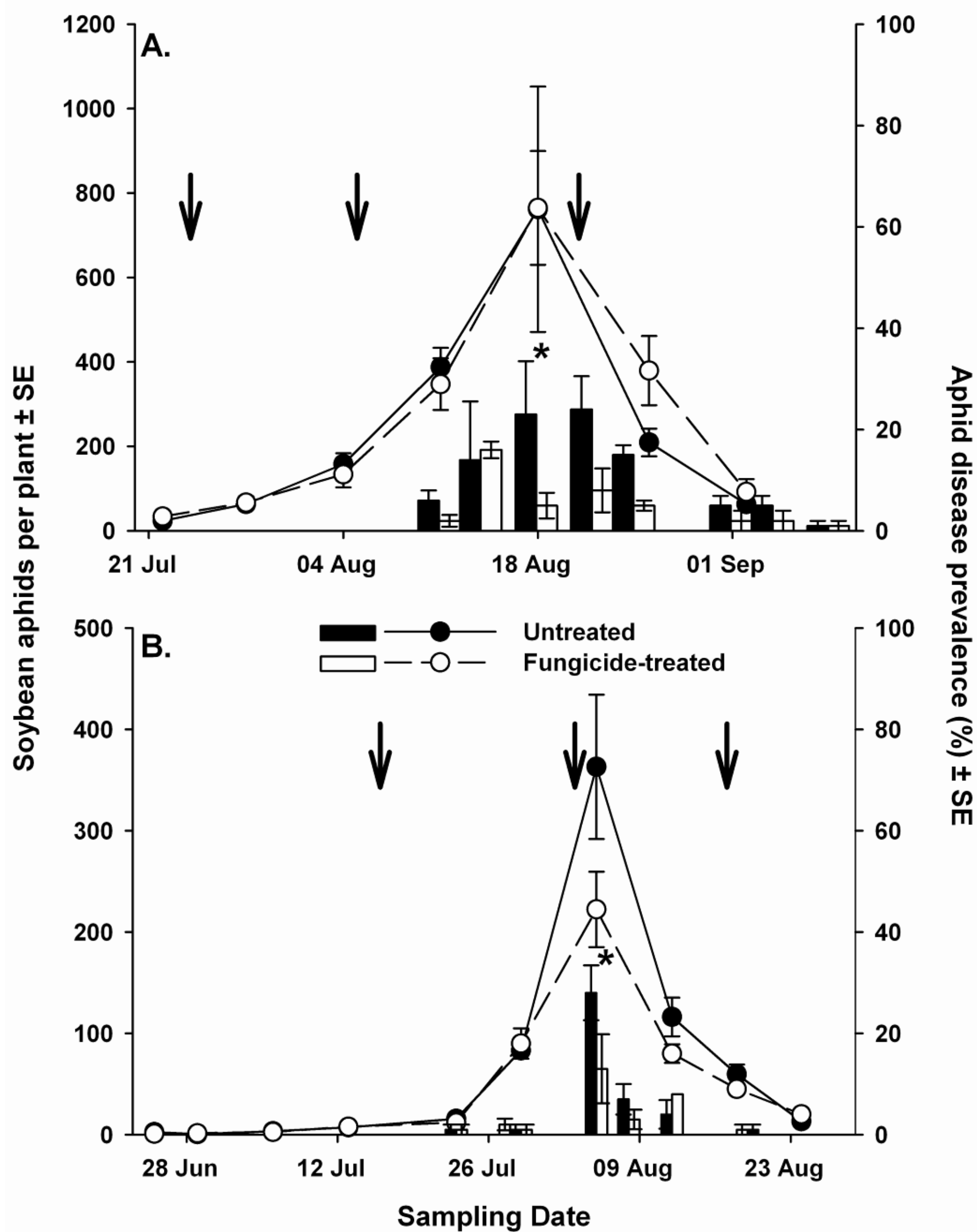


**Figure 1.** Results of field studies at Rosemount, MN in 2009 (A) and 2010 (B) including the per plant soybean aphid density (± SE) (line graphs) and the percent prevalence of aphid disease (± SE) (bar graphs). Arrows indicate foliar fungicide applications, with the first two in each year being trifloxystrobin + propiconazole and the third being chlorothalonil. Asterisks above bars indicate a significant treatment effect on that sampling date.

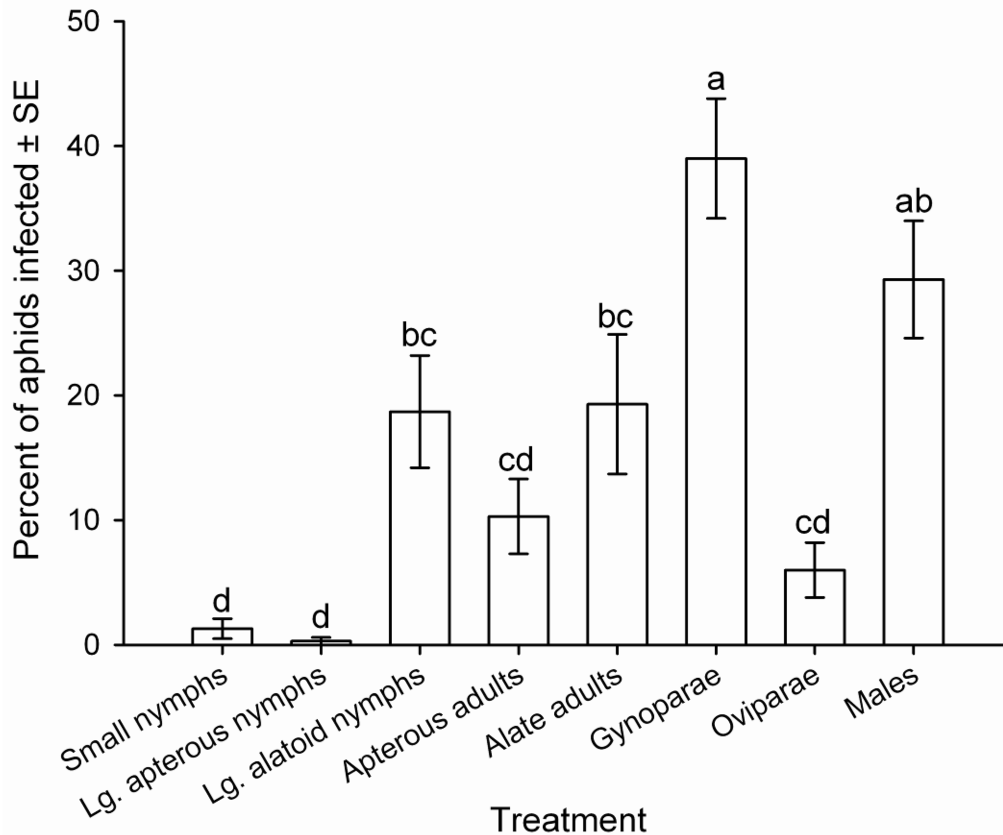


**Figure 2.** Percent infection (mean ± SE) of soybean aphid treatments included in relative susceptibility experiment with N=12 replications of n=25 soybean aphids per morph per replication. Where small nymphs are $1^{st}$-$2^{nd}$ instar viviparous nymphs, lg. apterous nymphs are $3^{rd}$-$4^{th}$ instar apterous, viviparous nymphs, lg. alatoid nymphs are $3^{rd}$-$4^{th}$ instar alatoid, viviparous nymphs, apterous adults are apterous, viviparous adults, alate adults are alate, viviparous nymphs, gynoparae are adult gynoparae (alate sexual migrants), oviparae are $2^{nd}$-$3^{rd}$ instar oviparae (apterous egg-laying morph), and males are alate, adult males. Bars indicated with the same letter are not significantly different.

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