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Plant Resistance

The Effect of an Interspersed Refuge on *Aphis glycines* (Hemiptera: Aphididae), Their Natural Enemies, and Biological Control

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Abstract

Soybean production in the north central United States has relied heavily on the use of foliar and seed applied insecticides to manage *Aphis glycines* (Hemiptera: Aphididae). An additional management strategy is the use soybean cultivars containing *A. glycines* resistance genes (*Rag*). Previous research has demonstrated that *Rag* cultivars are capable of preventing yield loss equivalent to the use of foliar and seed-applied insecticides. However, the presence of virulent biotypes in North America has raised concern for the durability of *Rag* genes. A resistance management program that includes a refuge for avirulent biotypes could limit the frequency at which virulent biotypes increase within North America. To what extent such a refuge reduces the effectiveness of aphid-resistant soybean is not clear. We conducted an experiment to determine whether a susceptible refuge mixed into resistant soybean (i.e., interspersed refuge or refuge-in-a-bag) affects the seasonal exposure of aphids, their natural enemies, biological control, and yield protection provided by aphid resistance. We compared three ratios of interspersed refuges (resistant: susceptible; 95:5, 90:10, 75:25) to plots grown with 100% susceptible or resistant soybean. We determined that an interspersed refuge of at least 25% susceptible seed would be necessary to effectively produce avirulent individuals. Interspersed refuges had negligible effects on yield and the natural enemy community. However, there was evidence that they increased the amount of biological control that occurred within a plot. We discuss the compatibility of interspersed refuges for *A. glycines* management and whether resistance management can prolong the durability of *Rag* genes.

Key words: host–plant resistance, soybean aphid, refuge-in-a-bag

Since 2000, the soybean aphid, *Aphis glycines* Matsumura, has been an economically important pest of soybean in North America (Ragsdale et al. 2007, 2011). The current management strategy for *A. glycines* is the use of broad-spectrum insecticides (e.g., organophosphates, pyrethroids) (Olson et al. 2008, Ragsdale et al. 2011). One concern for the prolonged use of insecticides to manage *A. glycines* is the potential for insecticide-resistant populations to evolve, as observed in other aphid species (Furk and Hines 1993, Devonshire et al. 1998). Although additional classes of insecticides (e.g., ketoenoles) are effective against *A. glycines*, these products are not marketed for use on soybean (Ohnesorg et al. 2009, Varenhorst et al. 2012). An additional alternative to broad-spectrum insecticides is the implementation of resistant soybean cultivars. Resistance to aphids in soybean may be produced through the introduction of toxins (e.g., Bt proteins) into the plant genome (i.e., plant incorporated protectant [PIP]) (Chougule et al. 2013), or the incorporation of *A. glycines* resistance genes (*Rag*) from the soybean germplasm (McCarville et al. 2014). Regardless of the mechanism, an insect resistance management (IRM) program can extend the length of time that the resistance trait can be used (Tabashnik et al. 2013). Aspects of the biology and ecology of *A. glycines* in North America suggest that an IRM plan is needed, initially for *Rag*-genes and possibly for future PIPs.

Previous research has demonstrated that soybean cultivars containing either the single *Rag1* gene or a pyramid of *Rag1*+*Rag2* genes are effective at reducing *A. glycines* populations without an associated yield drag (Brace and Fehr 2012, McCarville et al. 2014). However, their availability and adoption is limited, and occasionally economically damaging populations of *A. glycines* are observed on cultivars containing *Rag1* (Michel et al. 2011, McCarville et al. 2012, Hesler et al. 2013). Although *A. glycines* populations are not reported to reach economically damaging levels on *Rag1*+*Rag2* cultivars, a potential limit to their adoption is the presence of virulent *A. glycines* biotypes. In North America, there are currently four recognized *A. glycines* biotypes. Biotype-1 is described as being avirulent toward both *Rag1*, *Rag2*, *Rag1*+*Rag2* genes (Kim et al. 2008, McCarville et al. 2014). Biotype-2 is virulent toward *Rag1* but not *Rag2* (Kim et al. 2008). Biotype-3 is virulent toward *Rag2* but not

Rag1 (Hill et al. 2010). Biotype-4 is virulent toward *Rag1*, *Rag2*, and the pyramid-containing both genes (Alt and Ryan-Mahmutagic 2013). Although their geographic distribution is largely unknown, it appears that these biotypes are widespread across North America (Michel et al. 2011).

A refuge of susceptible plants can reduce the frequency of virulent alleles in a population of insect herbivores. A refuge is defined as a habitat in which the target pest (i.e., *A. glycines*) is not under selection pressure due to the presence of a toxin/resistance gene (Bourguet et al. 2005). A refuge can either be wild host plants or also crop hosts that do not contain the source of the selection pressure (i.e., *Rag* genes). The underlying principle of a refuge is that resistant/virulent individuals emerging from the resistant host plants will mate with susceptible (i.e., avirulent) individuals emerging from the susceptible refuges (Bourguet et al. 2005). Although *A. glycines* do not mate on soybean (Ragsdale et al. 2011), it is possible that a refuge strategy may contribute to the aphids returning to *Rhamnus* spp., where sexual reproduction takes place. By increasing the amount of avirulent individuals on *Rhamnus spp.*, the frequency of virulence for *Rag* genes could be reduced through random mating occurring between avirulent and virulent individuals (Alstad and Andow 1995).

Based on the acceptance of farmers, it is possible that an interspersed refuge would be suitable for the implementation of an *A. glycines* IRM program. Gray (2011) determined that farmer willingness to incorporate a refuge was greatest when a 5% refuge was recommended. When purchased as an interspersed refuge (or refuge-in-a-bag [RIB]), it ensures that the farmer is compliant with this practice. Wenger et al. (2014) provide evidence that a refuge strategy may be appropriate for the management of virulent *A. glycines* biotypes. In a laboratory assay, an interspersed refuge with either 75% or 25% susceptible seed was evaluated for the production of virulent and avirulent biotypes. The inclusion of a refuge of susceptible seed increased the fitness of biotype-1 *A. glycines* while it decreased the fitness of biotype-3, resulting in the persistent population of aphids even when a resistant cultivar was the most dominant plant genotype used. To what extent an interspersed refuge that combines aphid-resistant and susceptible soybean in a field setting can allow for *A. glycines* to persist throughout the season is not known.

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

To our knowledge no research has been conducted to evaluate the effect of interspersed refuges of resistant and susceptible soybean in a field on *A. glycines* and their natural enemies. We conducted an experiment to determine an optimum interspersed refuge ratio in soybean that both prevents yield loss and conserves the natural enemy community in soybean. The objectives of this article were to examine the effect of interspersed refuges on 1) *A. glycines* abundance, 2) soybean yield, 3) natural enemy abundance, and 3) their biological control of *A. glycines*.

Materials and Methods

Field Site

The experiment was conducted at Iowa State University's Johnson Research Farm in 2012 and 2013, and at Iowa State University's Curtiss Research Farm and Northwest Research Farm in 2014. Both the Johnson and Curtiss Research Farms are in Story County, IA, and the Northwest Research Farm is in O'Brien County, IA. Conventional tillage practices were used at all locations during each year. Weed management was performed by hand, and at the Johnson and Curtiss Research Farms herbicides were not applied to the experiment. At the Northwest Research Farm, pre-emergent conventional herbicides (Enlite, DuPont, Wilmington, DE) (Sencor 4, Bayer CropScience, Research Triangle Park, NC) were applied to soil before soybean was planted. At the Northwest Research Farm in addition to hand weeding, a foliar conventional herbicide (Flexstar, Syngenta Crop Protection, LLC, Greensboro, NC) was applied when soybean reached the V5 growth stage (Fehr et al. 1971). Soybean were planted on 12 May 2012 and 18 June 2013 at the Johnson Research Farm. In 2014, soybean was planted at the Northwest Research Farm on 19 May and at the Curtiss Research Farm on 12 June.

Experimental Design

For this experiment we used a randomized complete block design with six blocks. In 2012, soybean were planted in six 23 by 15 m blocks. For 2013, soybean was planted in six 23 by 9 m blocks. Each block contained five adjacent plots that were 5 by 15 m in 2012, and 5 by 9 m in 2013. In 2014, soybean were planted at the Curtiss Research Farm in six 23 by 15 m blocks, while at the Northwest Research Farm they were planted in six 23 by 3 m blocks. Each block at the Curtiss Research Farm contained five adjacent plots that were 5 by 15 m and 3 by 15 m at the Northwest Research Farm. At all locations and years, plots were planted with 76 cm row spacing and a seed population of 370,000 seeds per hectare. At the Johnson and Curtiss Research Farms 9 m alleys separated each block, while at the Northwest Research Farm 6 m alleys separated blocks. All block and alley size variations were due to space constraints at the previously indicated research farms.

Interspersed Refuge Treatments

For this experiment we used two near-isogenic cultivars containing no *Rag* genes (IA3027, herein referred to as susceptible) or *Rag1*+*Rag2* (IA3027RA12, referred to as resistant). These two soybean cultivars are near isogenic, and are approximately 93.25% genetically identical (Wiarda et al. 2012). These cultivars were planted in the following treatments: 100% susceptible, 100% resistant, 25%:75% susceptible:resistant, 10%:90% susceptible:resistant, and 5%:95% susceptible:resistant, where the ratios were determined by seed weight. All seeds were weighed and mixed before being placed into cloth bags to ensure accurate ratios and even distribution of susceptible and resistant seed. Seed mixing was performed after the seed for each treatment had been weighed. The susceptible and resistant seed was poured into a cloth bag and mixed by hand for 3 min before being poured into the cloth bag used for storage.

Effect of Interspersed Refuges on *A. glycines* Populations and Soybean Yield

We hypothesized that increased amounts of the susceptible cultivar present in the interspersed refuges would positively affect *A. glycines* populations. We measured *A. glycines* populations on a weekly basis throughout each summer. Ten plants in the middle four rows of each plot were used to estimate *A. glycines* populations at the Johnson and Curtiss Research Farms. The middle two rows of each plot at the Northwest Research farm were used. All *A. glycines* (immatures and adults) present on those soybean plants were counted. When 100% of the 10 plants in the susceptible plot were infested 5 plants per plot were then scouted. Soybean exposure to *A. glycines* populations throughout a growing season was measured by calculating cumulative aphid days (CAD) (Hanafi et al. 1989).

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

Effect of Refuges on Natural Enemy Density and Biological Control

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

Our fourth hypothesis addressed the impact of natural enemies on *A. glycines* in each treatment. Specifically we used sentinel plants that were artificially infested with *A. glycines* to measure the mortality from natural enemies within each treatment. To do this at the Johnson and Curtiss Research Farms, we planted four susceptible and resistant plants into the 25%:75% susceptible:resistant, 10%:90% susceptible:resistant, and 5%:95% susceptible:resistant treatments. In the 100% susceptible plots, four susceptible seeds were planted, and in the 100% resistant plots, four resistant seeds were planted. The soybean seeds were planted into the middle two rows at an arbitrary distance into each plot. When the sentinel soybean plants reached the V4 growth stage (30 July 2012, 31 July 2013; S1 July week 4) in the treatments containing interspersed refuges one susceptible and resistant soybean plant were caged according to methods described by McCarville et al. (2012), and the uncaged resistant and susceptible plants were marked with flags. Due to late maturing soybean in 2014, the first and second set of soybean were infested at V4 (2 August 2014; S1 August week 1). In 100% susceptible and resistant plots, one plant was caged and one uncaged plant was marked with a flag in each plot. All caged and uncaged flagged plants were infested with pieces of soybean leaves containing approximately 50 mixed-age *A. glycines*. Infested leaves were paper clipped to the top full trifoliate of each plant. The success of the infestation was checked after 24 h. The caged and uncaged plants were then counted 12 d after their initial infestation. This was repeated when the second set of sentinel soybean plants in each plot reached the V6 growth stage (9 August 2012, 13 August 2013; S1 August weeks 1 and 2).

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

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

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

Statistical Analyses for Experiments

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

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

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

We determined that the BSI data for our fourth hypothesis were not normally distributed using a Shapiro–Wilk test for normality in the PROC UNIVARIATE procedure with SAS statistical software version 9.3 (SAS Institute) ($P < 0.0001$). Data were then analyzed using the PROC NPAR1WAY procedure with SAS statistical software version 9.3 (SAS Institute). Data were analyzed using a Kruskal–Wallis test with the main effect interspersed refuge to determine if mean ranks were the same for each treatment. Due to differences observed among the mean ranks data were then analyzed using a Wilcoxon two-sample test. All comparisons were made for both susceptible and resistant treatments including the 100% susceptible, 100% resistant, 25%:75% susceptible:resistant, 10%:90% susceptible:resistant, and 5%:95% susceptible:resistant treatments.

Results

Effect of Interspersed Refuges on *A. glycines* Populations and Soybean Yield

Our results support the hypothesis that higher percentages of susceptible seed present in the interspersed refuges lead to greater *A. glycines* populations. To determine if the varying ratios of susceptible and resistant soybean seed present in the different interspersed refuge treatments had an impact on the seasonal exposure of soybean to *A. glycines*, we analyzed CAD data for the significance of the main effects location-year, block, and interspersed refuge ratio. The main effect of location-year was significant ($F = 474.40$; df = 3, 747; $P < 0.0001$) so data were analyzed by location-year. The main effect interspersed refuge ratio was significant for Johnson Research Farm in 2012 ($F = 8.29$; df = 4, 40; $P < 0.0001$), Johnson Research Farm in 2013 ($F = 19.49$; df = 4, 36; $P < 0.0001$), Northwest Research Farm in 2014 ($F = 6.19$; df = 4, 24; $P < 0.0014$), and Curtiss Research Farm 2014 ($F = 27.23$; df = 4, 28; $P < 0.0001$).

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

For the Johnson Research Farm in 2013, the CAD for the 100% susceptible treatment was significantly greater than the 25%:75% susceptible:resistant ($t = 3.78$; df = 4, 36; $P < 0.0049$), 10%:90% susceptible:resistant ($t = 4.73$; df = 4, 36; $P < 0.0003$), 5%: 95% susceptible:resistant ($t = 5.82$; df = 4, 36; $P < 0.0001$), and 100% resistant ($t = 8.56$; df = 4, 36; $P < 0.0001$) treatments (Fig. 1B). The CAD for the 100% resistant treatment were significantly lower than the 25%:75% susceptible:resistant ($t = 4.78$; df = 4, 36; $P < 0.0003$), and 10%: 90% susceptible: resistant ($t = 3.82$; df = 4, 36; $P < 0.0043$) treatments (Fig. 1B). There was no significant difference between the 5%:95% susceptible:resistant and 100% resistant treatments.

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

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

Effect of Refuges on Natural Enemy Density and Biological Control

The sweep net data supported the third hypothesis that varying interspersed refuges would affect the total abundance of natural enemies. In all four location-years, the 100% susceptible plots had more natural enemies than any of the other treatments; however, this was only significant for two of the four location-years. This difference was observed by analyzing data for the significance of the main effects location-year, date, block, interspersed refuge ratio, and the interaction of date by interspersed refuge ratio (location-year). The total abundance of natural enemies varied significantly by the main effects location-year ($F = 88.81$; df = 3, 906; $P < 0.0001$) and interspersed refuge ratio ($F = 34.31$; df = 4, 906; $P < 0.0001$). The significance of the main effects indicates that the total abundance of natural enemies varied by location-year and that the total abundance of natural enemies was not equal among the interspersed refuge treatments. This difference was further illustrated by the significant interaction of location-year by interspersed refuge

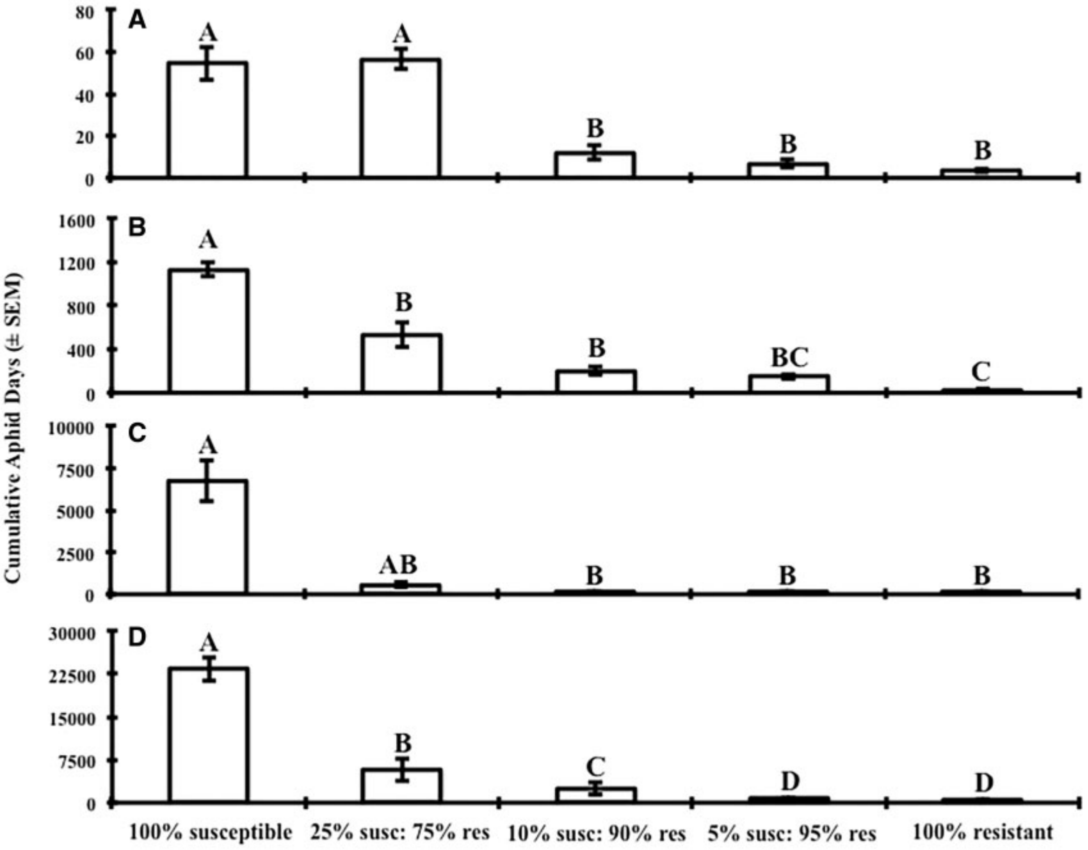

Fig. 1. A comparison of plant exposure to *A. glycines* within plots grown with varying ratios of susceptible and resistant soybean at the Johnson Research Farm in 2012 (A), Johnson Research Farm in 2013 (B), Northwest Research Farm in 2014 (C), and Curtiss Research Farm in 2014 (D). Exposure was measured in cumulative aphid days (CAD). The susceptible soybean cultivar used for this experiment was IA3027, and the resistant cultivar was IA3027RA12. Capital letters indicate significance among treatments ($P < 0.05$).

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

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

To further evaluate the effect that the varying interspersed refuge ratios had on specific aphidophagous natural enemies, we evaluated *H. axyridis* and *O. insidiosus* due to the previous research that demonstrated their importance as predators of *A. glycines*. We also evaluated the natural enemies that were most abundant across each of the four location-years; Araneae, Chrysopidae, and Nabidae (Table 1). Only at the Curtiss 2014 location year did the abundance of *H. axyridis* vary among the treatments, with more in the 100% susceptible treatment than the 25%:75% susceptible:resistant ($t = 4.40$; df = 4, 194; $P < 0.0002$), 10%:90% susceptible:resistant ($t = 4.80$; df = 4, 194; $P < 0.0001$), 5%:95% susceptible:resistant ($t = 5.02$; df = 4, 194; $P < 0.0001$), and 100% resistant ($t = 5.08$; df = 4, 194; $P < 0.0001$) treatments (Table 2B). There were no significant differences in *H. axyridis* abundance among the other treatments. For *O. insidiosus* (Table 2C) and Araneae (Table 2D), there were no differences in abundance during any of the four location-years among any of the treatments. We observed significant differences in Chrysopidae abundance among the treatments at Johnson 2013 and Curtiss 2014. For Johnson 2013, the abundance of Chrysopidae was significantly greater in the 10%:90% susceptible:resistant treatment than the 5%:95% susceptible:resistant ($t = 2.92$; df = 4, 232; $P < 0.0310$) resistant and 100% resistant ($t = 3.19$; df = 4, 232; $P < 0.138$) treatments (Table 2E). There were no other differences in Chrysopidae abundance for Johnson 2013. For Curtiss 2014, there were significantly more Chrysopidae in the 100% susceptible treatment than the 25%:75% susceptible:resistant ($t = 4.47$; df = 4, 203; $P < 0.0001$), 10%:90% susceptible:resistant ($t = 4.99$; df = 4, 203; $P < 0.0001$), 5%:95% susceptible:resistant ($t = 5.46$; df = 4, 203; $P < 0.0001$), and 100% resistant ($t = 5.34$; df = 4, 203; $P < 0.0001$) treatments (Table 2E). During the Johnson 2013 location-year there were significantly more Nabidae in the 100% susceptible treatment than the 5%:95% susceptible:resistant ($t = 2.98$; df = 4, 232; $P < 0.0266$)

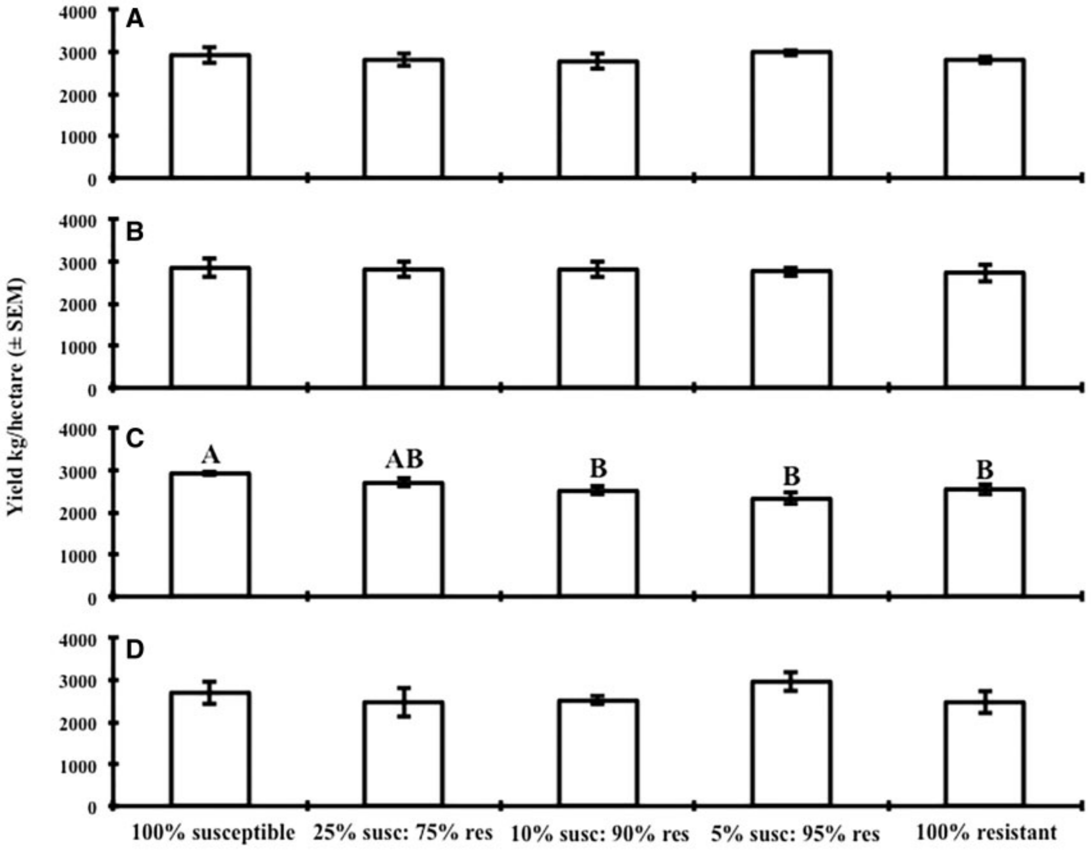

Fig. 2. A comparison of yield (average kg/ha) from plots grown with varying ratios of susceptible and resistant soybean at the Johnson Research Farm in 2012 (A), Johnson Research Farm in 2013 (B), Northwest Research Farm in 2014 (C), and the Curtiss Research Farm in 2014 (D). The susceptible soybean cultivar used for this experiment was IA3027, and the resistant cultivar was IA3027RA12. Capital letters indicate significance among treatments ($P < 0.05$).

and 100% resistant ($t = 3.25$; df = 4, 232; $P < 0.0114$) treatments (Table 2F). There were no significant differences among the other treatments. For the individual taxa that were evaluated, in all instances of significant differences, there were more of the individuals in the 100% susceptible treatment than the 5%:95% susceptible:resistant and 100% resistant treatments.

The BSI data supported the fourth hypothesis that varying interspersed refuge ratios would affect predation of *A. glycines* by natural enemies. This was observed by analyzing the BSI calculated for each year using a Kruskal–Wallis test. For 2012 ($H = 19.58$; df = 1; $P < 0.0065$) and 2013 ($H = 27.62$; df = 1; $P < 0.0003$) there was evidence that the BSI values among the treatments were not equal. In 2014 ($H = 8.32$; df = 1; $P < 0.3049$), there was no significant differences in the BSI values among the treatments. To determine differences among treatments for 2012 and 2013, we analyzed the data using a Wilcoxon two-sample test. For 2012, we observed the highest BSI values for the resistant soybean in the 5%:95% susceptible:resistant and susceptible soybean in the 25%:75% susceptible:resistant treatments. The lowest BSI was observed for susceptible soybean in the 100% susceptible treatment (Fig. 3A). The BSI value for the resistant soybean in the 5%:95% susceptible:resistant treatment was significantly greater than the 100% susceptible ($W = 200$; $P < 0.0001$), 100% resistant ($W = 176$; $P < 0.0432$), and the resistant soybean in the 25%:75% susceptible:resistant ($W = 124$; $P < 0.0432$) treatments. The BSI value for the susceptible soybean in the 25%:75% susceptible:resistant treatment was significantly greater than the 100% susceptible ($W = 124$; $P < 0.0432$), 100% resistant ($W = 100$; $P < 0.0001$), resistant soybean in the 25%:75% susceptible:resistant ($W = 124$; $P < 0.0432$) treatments.

In 2013, the resistant soybean in the 25%:75%, 10%:90%, and 5%:95% susceptible:resistant treatments had significantly greater BSI values than the susceptible counterparts (Fig. 3B). The BSI value for the resistant soybean in the 25%:75% susceptible:resistant treatment was significantly greater than the 100% susceptible treatment ($W = 85.0$; $P < 0.0013$), and the susceptible soybean in the 25%:75% ($W = 82.0$; $P < 0.0001$), 10%:90% ($W = 203$; $P < 0.0001$), and 5%:95% ($W = 100$; $P < 0.0222$) susceptible:resistant treatments. For the resistant soybean in the 10%:90% susceptible:resistant treatment, the BSI was significantly greater than the 100% susceptible treatment ($W = 88.0$; $P < 0.0025$), and the susceptible soybean in the 25%:75% ($W = 88.0$; $P < 0.0025$), 10%:90% ($W = 197$; $P < 0.0025$), and 5%:95% ($W = 106$; $P < 0.0533$) susceptible:resistant treatments. The resistant soybean in the 5%:95% treatment had a significantly greater BSI than the 100% susceptible treatment ($W = 87.5$; $P < 0.0021$), and the susceptible soybean in the 25%:75% ($W = 86.5$; $P < 0.0017$), 10%:90% ($W = 200$; $P < 0.0013$), and 5%:95% ($W = 102$; $P < 0.0259$) susceptible: resistant treatments.

Discussion

Results from these experiments indicate that an interspersed refuge composed of aphid-susceptible and aphid-resistant soybean is capable of reducing the exposure of *A. glycines* populations similar to

Table 1. Natural enemy community collected with a sweep net at each Iowa research site

Aphidophagous natural enemies

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

Nonaphidophagous natural enemies

Order	Family	Species	% of total abundance			
			Johnson 2012	Johnson 2013	Northwest 2014	Curtiss 2014
Diptera	Asilidae		0.15	0.00	0.00	0.00
	Tachinidae		0.00	0.12	0.00	0.00
Hemiptera	Reduviidae		0.31	0.70	0.00	0.00
Hymenoptera	Chalcididae		0.00	0.00	0.00	0.00
	Ichneumonidae		0.68	6.23	0.00	0.20
	Pteromalidae		0.37	9.25	0.00	0.00

what is observed in plots grown with only a resistant cultivar (Fig. 1). Although we observed fewer *A. glycines* on the interspersed refuges and 100% resistant treatments, we did not observe yield differences when compared to the 100% susceptible treatment which experienced the greatest exposure. For the Johnson 2012 and Johnson 2013 location-years, we attribute this to limited exposure to *A. glycines* populations, as the CAD accumulated were too low to affect yield, (Fig. 1A and B). For the Curtiss 2014 location-year, we attribute the absence of yield loss to an epizootic fungal outbreak that greatly reduced *A. glycines* populations within a 2-wk period (Supp Fig. 1 [online only]). So although the average number of aphids per plant resulted in significantly greater exposure than what was observed in 2013, the length of this exposure was likely too short to negatively effect yield. At the Northwest 2014 location-year, we observed a significantly greater yield in the 100% susceptible treatment. This is remarkable because the CAD experienced on the 100% susceptible treatment was great enough over several weeks that we anticipated yield loss compared with the other treatments. It is unclear why the yield was significantly lower in three of the interspersed refuge treatments at the Northwest Research Farm (Fig. 2C). This difference was significant, and accounts for a 14% difference between the 100% susceptible and 100% resistant treatments. Although this result could be explained by a yield drag due to the occurrence of *Rag* genes within the resistant cultivar, Brace and Fehr (2012) report no negative agronomic impacts associated with the inclusion of *Rag1* and *Rag2* resistance genes.

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

Despite having the most natural enemies, the years and treatments with the most aphids did not have the most biological control as estimated by BSI. The year with the largest *A. glycines* populations (i.e., 2014), we observed the lowest BSI values and no significant differences among the treatments. Within those years (i.e., 2012 and 2013) in which BSI varied significantly among the treatments, biological control provided was generally greater in plots containing a mixture of susceptible and resistant soybean than those containing only susceptible soybean (Fig. 3). Furthermore, there was not a consistent trend in the biological control observed on either susceptible versus resistant soybean plants within a treatment (e.g., Fig. 3A, susceptible versus resistant on plants within the 25%:75% treatment). During 2012 and 2013, half of the comparisons of BSI measured on either resistant or susceptible soybeans within a given treatment were not significant (4 out of 8). For three of the remaining four comparisons, BSI was higher on the resistant plants than the susceptible plant. Overall, this suggest that biological control of soybean aphids is not inherently greater on soybean plants with the most aphids. Therefore, we suggest that when compared with aphid

Table 2. Comparison of natural enemy populations collected in a sweep net during 2012–2014

	Johnson 2012	Johnson 2013	Northwest 2014	Curtiss 2014
A. Mean total all natural enemies				
100% susceptible	8.17 ± 1.76	12.28 ± 1.87a*	1.07 ± 0.57	21.3 ± 6.04a
25% susceptible: 75% resistant	6.63 ± 1.44	10.02 ± 1.32ab	0.40 ± 0.30	7.04 ± 1.70b
10% susceptible: 90% resistant	6.70 ± 1.53	10.11 ± 1.40ab	0.43 ± 0.33	4.96 ± 1.38b
5% susceptible: 95% resistant	7.90 ± 1.62	9.09 ± 1.22b	0.57 ± 0.62	4.71 ± 1.05b
100% resistant	6.61 ± 1.35	7.48 ± 1.05b	0.37 ± 0.43	4.04 ± 1.11b
B. *H. axyridis*				
100% susceptible	0.01 ± 0.01	0.87 ± 0.20	0	6.96 ± 2.16a
25% susceptible: 75% resistant	0	0.70 ± 0.13	0	1.24 ± 0.44b
10% susceptible: 90% resistant	0.02 ± 0.01	0.69 ± 0.13	0	0.72 ± 0.32b
5% susceptible: 95% resistant	0.03 ± 0.02	0.85 ± 0.17	0	0.39 ± 0.16b
100% resistant	0.02 ± 0.01	0.63 ± 0.15	0	00.30 ± 0.16b
C. *O. insidiosus*				
100% susceptible	0.74 ± 0.18	0.63 ± 0.16	0.17 ± 0.12	0.92 ± 0.33
25% susceptible: 75% resistant	0.80 ± 0.17	0.83 ± 0.16	0.03 ± 0.03	0.87 ± 0.30
10% susceptible: 90% resistant	0.86 ± 0.16	0.63 ± 0.17	0.03 ± 0.03	0.68 ± 0.25
5% susceptible: 95% resistant	0.66 ± 0.15	0.63 ± 0.14	0.13 ± 0.13	0.62 ± 0.23
100% resistant	0.67 ± 0.14	0.65 ± 0.14	0.10 ± 0.10	0.77 ± 0.25
D. Araneae				
100% susceptible	3.08 ± 0.35	0.63 ± 0.07	0.43 ± 0.18	0.26 ± 0.08
25% susceptible: 75% resistant	2.52 ± 0.26	0.70 ± 0.09	0.23 ± 0.11	0.26 ± 0.08
10% susceptible: 90% resistant	2.58 ± 0.32	0.74 ± 0.09	0.10 ± 0.06	0.22 ± 0.09
5% susceptible: 95% resistant	3.08 ± 0.36	0.80 ± 0.10	0.20 ± 0.09	0.57 ± 0.22
100% resistant	2.53 ± 0.29	0.57 ± 0.10	0.13 ± 0.10	0.30 ± 0.10
E. Chrysopidae				
100% susceptible	1.32 ± 0.24	1.66 ± 0.26ab	0.07 ± 0.05	4.77 ± 1.24a
25% susceptible: 75% resistant	1.02 ± 0.21	1.70 ± 0.25ab	0.07 ± 0.05	1.65 ± 0.42b
10% susceptible: 90% resistant	1.02 ± 0.20	2.35 ± 0.35a	0.17 ± 0.08	1.48 ± 0.40b
5% susceptible: 95% resistant	2.2 ± 0.25	1.35 ± 0.22b	0.07 ± 0.05	1.69 ± 0.26b
100% resistant	1.74 ± 0.22	1.26 ± 0.28b	0	2.56 ± 0.39b
F. Nabidae				
100% susceptible	0.58 ± 0.10	4.35 ± 0.59a	0.03 ± 0.03	0.64 ± 0.19
25% susceptible: 75% resistant	0.59 ± 0.12	3.39 ± 0.42ab	0.03 ± 0.03	0.43 ± 0.16
10% susceptible: 90% resistant	0.57 ± 0.10	3.20 ± 0.45ab	0	0.35 ± 0.11
5% susceptible: 95% resistant	0.77 ± 0.13	2.76 ± 0.40b	0	0.39 ± 0.13
100% resistant	0.74 ± 0.13	2.61 ± 0.36b	0.07 ± 0.05	0.33 ± 0.11

*Letters indicate significance among treatments for a given location-year ($P < 0.05$).

susceptible soybean the aphid-resistant soybean may experience similar biological control, although *A. glycines* populations are lower.

We note that the natural enemies we observed were mostly predators of *A. glycines* and the biological control measured within our experiments is likely due to their activity. We did not observe parasitized aphids in our study, nor did we observe parasitoids that could use *A. glycines*. To date, differences have been observed in parasitism of *A. glycines* on aphid-resistant versus aphid-susceptible soybean (Ballman et al. 2012, Ghising et al. 2012, Ode and Crompton 2012, Hopper and Diers 2014). If importation biological control programs targeting *A. glycines* become successful in North America, this experiment may need to be repeated.

The presence of virulent biotypes in North America may suggest that sustainable use of *Rag* genes is unlikely. However, there is increasing evidence (Wenger et al. 2014; Varenhorst et al. 2015a,b) that widespread use of *Rag* genes may not lead to an increase in the frequency of virulent biotypes such that *Rag* genes are no longer useful. Wenger et al. (2014) observed an increase in an avirulent biotype population on *Rag2* soybean when simultaneously coinfested with a virulent biotype. Varenhorst et al. (2015a) observed a similar effect with *Rag1* soybean and determined that a virulent biotype can obviate (i.e., remove) *Rag*-resistance allowing for survival of an avirulent biotype. A simple, single-gene deterministic model revealed that the frequency of alleles conferring virulence to *Rag*-genes was decreased by this obviation of resistance, prolonging the durability of *Rag* genes (Varenhorst et al. 2015a). Furthermore, Varenhorst et al. (2015b) observed fitness costs associated with virulent biotypes on susceptible soybean (i.e., soybean without *Rag* genes). When the model was expanded to include the obviation of resistance and fitness costs associated with virulence, *Rag1* was predicted to be effective for 18 years and *Rag1*+*Rag2* for more than 25 years when 25% of the landscape was composed of susceptible soybean. However, this model did not specify if susceptible soybean was grown in a block or interspersed refuge.

The challenge for the sustainable use of *Rag*-genes is the production of a refuge that is compatible with soybean production. Farmer adoption of a refuge will determine to a large extent how successful this approach to IRM will be. We suggest that an interspersed refuge would be ideal for an *A. glycines* IRM program, as this method ensures greater compliance by farmers to plant a refuge. The combined results of our study indicate that interspersed refuges composed of susceptible and resistant soybean may provide a viable method for the management of *A. glycines*. Our results indicate that an interspersed refuge allows for the production of aphids throughout the

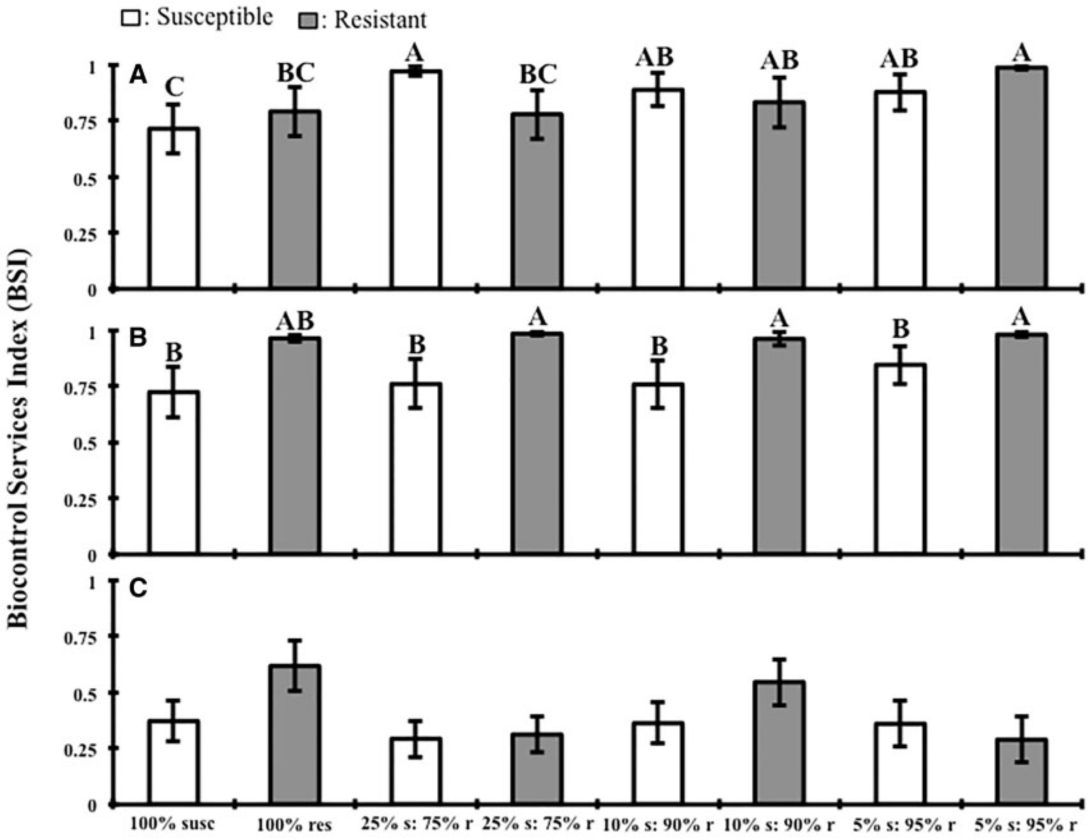

Fig. 3. A comparison of the Biocontrol Services Index (BSI) ratings within plots of varying ratios of susceptible and resistant soybean at the Johnson Research Farm in 2012 (A), Johnson Research Farm in 2013 (B), and the Curtiss Research Farm in 2014 (C). A value of one indicates a level of 100% biological control, and a value of zero indicates the absence of biological control. The susceptible soybean cultivar used for this experiment was IA3027, and the resistant cultivar was IA3027RA12. Capital letters indicate significance among treatments ($P < 0.05$).

growing season without interfering with yield or biological control of *A. glycines*.

For a refuge to be successful, avirulent *A. glycines* populations must exist in soybean fields, albeit at populations that would not cause economic loss. While our study evaluated the effectiveness of interspersed refuges for managing *A. glycines* in a single season, we did not evaluate the proportion of avirulent:virulent individuals produced within these refuges. Future research should investigation the biotype composition of *A. glycines* in interspersed refuge plots to determine if interspersed refuges are appropriate. We do not know if aphids within plots comprised 100% resistant soybean were all virulent, and that aphids in the treatments receiving a percentage of susceptible soybean produced only avirulent aphids. However, assuming that the refuge produces only avirulent aphids, then a goal would be to select the ratio that would produce more avirulent than virulent aphids. The CAD for the treatments containing 10% or 5% susceptible soybean were frequently not significantly different from that of the 100% resistant treatment (Fig. 1), suggesting that only virulent aphids may have survived within them. Therefore, a 25% refuge (or greater) of susceptible soybean may be required to produce significantly more avirulent aphids than what comes from a plot sown with 100% resistant soybeans.

Based on how *A. glycines* responds to conditions at the landscape scale (Gardiner et al. 2009, Schmidt et al. 2011), it is possible that in the future area-wide management may be possible. Area-wide suppression of an insect pest through the adoption of pest-resistant cultivars has been observed for other field crops (Carrière et al. 2003, Wu et al. 2008, Hutchinson et al. 2011). For example the use of Bt-containing maize cultivars on only 63% of corn acres in the United States has resulted in area-wide suppression of the *Ostrinia nubilalis*, providing yield and quality benefits to non-Bt maize (Hutchinson et al. 2011). Similar results have been observed for cotton pests in the North America (Carrière et al. 2003) and China (Wu et al. 2008). Large-scale adoption of *Rag* soybean could greatly reduce *A. glycines* populations over a large geographic area. However, this potential success of *Rag*-soybean will require an effective IRM plan to combat an increase in the occurrence of virulent biotypes.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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