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Research Article

Determining the Effectiveness of Three-Gene Pyramids Against *Aphis glycines* (Hemiptera: Aphididae) Biotypes

A. J. Varenhorst,[1,4] S. R. Pritchard,[2] M. E. O'Neal,[2] E. W. Hodgson,[2] and A. K. Singh[3]

[1]Plant Science Department, South Dakota State University, Brookings, SD 57007, [2]Department of Entomology, Iowa State University, Ames, IA 50011, [3]Agronomy Department, Iowa State University, Ames, IA 50011, and [4]Corresponding author, e-mail: adam.varenhorst@sdstate.edu

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Abstract

Since the discovery of *Aphis glycines* Matsumura (Hemiptera: Aphididae) in the United States, the primary management tactic has been foliar insecticides. Alternative management options such as host plant resistance to *A. glycines* have been developed and their effectiveness proved. However, the use of host plant resistance was complicated by the discovery of multiple, virulent biotypes of *A. glycines* in the United States that are capable of overcoming single *Rag* genes, *Rag1* and *Rag2*, as well as a two-gene pyramid of *Rag1+Rag2*. However, current models predict that the virulent allele frequency of *A. glycines* decreases in response to the use of pyramided *Rag* genes, suggesting that pyramids represent a more sustainable use of these traits. Previous research has demonstrated that virulent biotypes can be effectively managed using a three-gene pyramid of *Rag1+Rag2+Rag3*. Additional *Rag*-genes have been discovered (*Rag4* and *Rag5*), but whether the incorporation of these genes into novel three-gene pyramids will improve efficacy is not known. We tested single-gene (*Rag1* and *Rag2*) and pyramid cultivars (*Rag1+Rag2*, *Rag1+Rag2+Rag3*, *Rag1+Rag2+Rag4*) to multiple biotypes in laboratory assays. Our results confirm that the *Rag1+Rag2+Rag3* pyramid effectively manages all known *A. glycines* biotypes when compared with cultivars that are overcome by the associated biotype. Our results indicate that *Rag1+Rag2+Rag4* would be an effective management option for biotype-1, biotype-2, and biotype-3 *A. glycines*, but had a negligible impact on biotype-4.

Key words: soybean aphid, host plant resistance, *Rag* genes, virulent, avirulent

The first detection of *Aphis glycines* Matsumura (Hemiptera: Aphididae) in the United States occurred in 2000 and since then it has been detected in 30 states and three Canadian provinces (Ragsdale et al. 2011). *Aphis glycines* is now regarded as the most economically damaging insect of soybean, *Glycine max* (L.) Merr., in the Midwest due to its potential to reduce yields by as much as 40% (Ragsdale et al. 2011). Management of *A. glycines* has resulted in a dramatic increase of insecticide applications to soybean, which prior to its arrival were rarely treated using a foliar insecticide (Ragsdale et al. 2011). In response to population outbreaks of *A. glycines* during the early 2000s, researchers in the Midwest developed an economic threshold and economic injury level to assist farmers with making insecticide management decisions to reduce unnecessary insecticide applications (Ragsdale et al. 2007, 2011; Olson et al. 2008). Although insecticides provided an immediate solution to this serious pest of soybean, attempts to find alternative management strategies are also ongoing. An integrated pest management program for soybean aphid management should include multiple strategies for *A. glycines*. The need for an alternative to foliar insecticides has been realized in the Midwest with documented cases of pyrethroid-resistant *A. glycines* populations occurring in Minnesota and Iowa (Koch and Potter 2016). Genes from the soybean germplasm that provide host plant resistance toward *A. glycines* represent an alternative management strategy to foliar insecticides. These genes have been coined *Rag* (Resistance to *Aphis glycines*) genes (Hill et al. 2006). *Rag* genes have been found to be effective at managing *A. glycines* populations as single genes, a two-gene pyramid, a three-gene pyramid, and also in combination with natural enemies (McCarville and O'Neal 2012, McCarville et al. 2014, Ode and Crompton 2012, Varenhorst and O'Neal 2015, Ajayi-Oyetunde et al. 2016).

Within the first 10 years of *A. glycines*' presence in the United States, a virulent biotype to *Rag1* soybean (i.e., first research soybean aphid resistance gene) was discovered in Illinois (Kim et al. 2008), which has since been described as biotype-2 (Cooper et al. 2015). But this discovery did not coincide with area-wide adoption of *Rag* genes, and was actually observed at least 2 years prior to the first commercial release of a *Rag* soybean cultivar in 2010 (Ragsdale et a. 2011). This discovery does not follow the logic associated with

other insect pests overcoming resistance sources (Bourguet et al. 2005). Since the discovery of biotype-2, additional virulent biotypes have been confirmed in the United States; to date, one avirulent and three virulent biotypes of *A. glycines* have been identified. These biotypes are categorized based on their capability to survive and colonize soybean that contain *Rag* genes. Of the four *A. glycines* biotypes, biotype-1 is avirulent to all known *Rag* genes (Kim et al. 2008, Alt and Ryan-Mahmutagic 2013). Biotype-2 is virulent to *Rag1* soybean (Kim et al. 2008), biotype-3 is virulent to *Rag2* soybean (Hill et al. 2010), and biotype-4 is virulent to *Rag1*, *Rag2*, and *Rag1+Rag2* soybean (Alt and Ryan-Mahmutagic 2013). The discovery of virulent biotypes in the United States was unexpected because of limited commercial availability and adoption of *Rag* soybean cultivars (McCarville et al. 2012). In addition, Michel et al. (2011) determined that the populations of *A. glycines* in the United States experienced a genetic bottleneck, and the genetic diversity in the United States is much lower than in its native range in Asia. Often though, the development of resistance (i.e., virulence) to a management strategy can be described by a gene-for-gene interaction between the host and the pest (Smith and Boyko 2007, Harris et al. 2012, Smith and Clement 2012). However, this mode of inheritance was ruled out for *A. glycines* (Wenger and Michel 2013).

Although the presence of virulent biotypes in the United States could be problematic for the commercial use of *Rag* soybean cultivars, evidence from multiple studies indicate that there are fitness costs associated with virulence (Enders et al. 2014, Wenger et al. 2014, Varenhorst et al. 2015b). In addition, refuges of susceptible soybean may provide a suitable approach for sustaining low virulent allele frequencies in *A. glycines* populations in North America (Wenger et al. 2014, Varenhorst and O'Neal 2015). The deployment of *Rag* soybean cultivars should be carefully managed to reduce the likelihood of complete failure of the resistance gene. Research in other systems has determined that pest insects overcome deployments of single-gene sources of resistance more rapidly than deployments of pyramided-resistant gene combinations (Smith 1989). Prior to the discovery of biotype-4, it was believed that the *Rag1+Rag2* pyramid would be sufficient for managing avirulent and virulent biotypes (i.e., biotype-2 and biotype-3) of *A. glycines*. However, the presence of biotype-4 in North America suggests that additional combinations of *Rag*-genes other than the *Rag1+Rag2* pyramid may be needed for future management. The first objective of this study was to evaluate the efficacy of two separate three-gene *Rag* pyramids (i.e., *Rag1+Rag2+Rag3* and *Rag1+Rag2+Rag4)* against all known *A. glycines* biotypes. The second objective of this study was to evaluate the efficacy of susceptible cultivars and plant introductions that contained *Rag3*, *Rag4*, and *Rag5* to biotype-1 and biotype-5 *A. glycines* populations.

Materials and Methods

Aphid Colonies and Soybean Cultivars

A. glycines individuals that were used to form the colonies for this experiment were originally obtained from the Ohio State University and the University of Wisconsin. Four biotypes of *A. glycines* that are defined by their response to *Rag1*, *Rag2*, or *Rag1+Rag2* (i.e., biotype-1, biotype-2, biotype-3, and biotype-4) were used. The initial populations for biotype-1 and biotype-2 were found from individuals that were initially collected and identified in Illinois (Kim et al. 2008). Biotype-3 populations were also initially collected and identified in Illinois (Hill et al. 2010). The colonies of biotype-1, biotype-2, and biotype-3 were established at Iowa State University in 2011. Biotype-4 populations were initially collected and identified in Wisconsin (Alt and Ryan-Mahmutagic 2013). The biotype-4 colony was established at Iowa State University in 2013. The biotype identity (i.e., avirulent or virulent) of each colony was confirmed using detached leaf assays as described by Michel et al. (2010a). For rearing, biotype-1 *A. glycines* were raised on susceptible soybean (IA3027). Biotype-2 (IA3027RA1), biotype-3 (IA3027RA2), and biotype-4 (IA3027RA12) were raised on resistant soybean. The susceptible and resistant cultivars used for rearing and maintaining the biotypes are genetically closely related (≥75% of genes from the recurrent parent IA3027). The cultivars containing no *Rag* genes (IA3027), *Rag1* (IA3027RA1), or *Rag1+Rag2* (IA3027RA12) are near-isolines for the resistance genes *Rag1* and *Rag2* (≈93.75% genetically identical) (Mardorf et al. 2010, Brace and Fehr 2012). The soybean line containing only the *Rag2* gene is genetically similar to these lines with 75% of its genes derived from the recurrent parent line IA3027 (Wiarda et al. 2012).

Biotype Screening Protocol

We individually screened biotype-1, biotype-2, biotype-3, and biotype-4 *A. glycines* on soybean lines using an experimental protocol that was originally established by Varenhorst et al. (2015a). For each biotype assay, individual second vegetative growth stage (V2) soybean plants were infested with five mixed age *A. glycines* of a single biotype using a 000 fine tip paintbrush. The mixed age *A. glycines* used for infestations were removed from the leaves of colony soybean plants and immediately placed onto experimental plants. The mixed age *A. glycines* that were used for these assays consisted of third and fourth instar individuals. Early instar and adult *A. glycines* were not used for the infestations. The population density present on each soybean plant was measured 11 d after infestation (Varenhorst et al. 2015a).

For each biotype screen, we used 13 treatments in a growth chamber using individually potted soybean plants. Each treatment was replicated using a randomized complete block design with three blocks. The experiment was repeated three times (nine total experimental units per treatment). Individually potted soybean plants were grown in 16-cm diameter pots in a growth chamber (E41L2C9, Percival Scientific, Incorporated, Perry, IA) using a 14:10 light:dark cycle and a constant temperature of 27°C with a relative humidity of 60%. Each potted soybean was covered with a mesh net to prevent plant-to-plant movement of the *A. glycines* during the experiments. Each treatment was a single cultivar or line of soybean. Susceptible (IA3027), *Rag1* (IA3027RA1), *Rag2* (IA3027RA2), and *Rag1+Rag2* (IA3027RA12) cultivars were used. In addition, four experimental lines of *Rag1+Rag2+Rag3* and five experimental lines of *Rag1+Rag2+Rag4* were used. Each of the three-gene pyramid lines were BC4F2 with the recurrent parent IA3027. The presence of *Rag3* or *Rag4* in each of the lines was confirmed by Dechun Wang at Michigan State University using SNP markers. Data analysis for the multiple lines of each of the three-gene pyramids was pooled because of a lack of significance among the individual lines.

Biotype Screens of the Three-Gene Pyramid Lines

We hypothesized that the three-gene pyramids (i.e., *Rag1+Rag2+Rag3* and *Rag1+Rag2+Rag4*) would decrease the population density of both avirulent (i.e., biotype-1) and virulent (i.e., biotype-2, biotype-3, and biotype-4) *A. glycines*, compared with populations on a susceptible soybean cultivar. We included cultivars of soybeans with single or two *Rag* genes to confirm the virulence status of the biotypes used in our assay. For example, we used a *Rag1*- or

Rag2-resistant soybean cultivars to confirm the virulence of biotype-2 or biotype-3, respectively. For biotype-2, the susceptible, *Rag2,* and *Rag1+Rag2* were utilized as negative controls to confirm each biotype's known inability to colonize the respective plants. For biotype-4, *Rag1*, *Rag2*, and *Rag1+Rag2* cultivars were used as positive controls to confirm its virulence. The susceptible cultivar was used as a negative control.

Susceptible Cultivars and Plant Introductions Screens

We observed the lowest population densities of biotype-4 on both the susceptible (IA3027) and the *Rag1+Rag2+Rag3* soybean cultivar during the biotype screens. However, we observed that the *Rag1+Rag2+Rag4* soybean cultivar had a negligible impact on the biotype-4 population density. To determine whether the reduced population densities of biotype-4 that were observed were the result of some here-to-fore unidentified resistance in our susceptible isoline, we utilized the protocol for biotype screening that was previously mentioned, but with multiple aphid-susceptible cultivars. We included a negative control (i.e., *Rag-3*-containing plant introduction) to confirm biotype-4's virulence status. We also included a plant introduction containing *Rag4* to evaluate its performance in the absence of *Rag1+Rag2*. In addition, we also included a plant introduction containing *Rag5* to evaluate its performance for biotype-1 and biotype-4. For this experiment, we utilized a total of 10 treatments in a growth chamber using individually potted soybean plants. Each treatment was replicated using a randomized complete block design with four blocks. The experiment was repeated three times (12 total experimental units per treatment). The treatments were IA3027, Williams 82 (PI 518671), Clark (PI 548533), Dwight (PI 597386), *Rag1* (IA3027RA1), *Rag2* (IA3027RA2), and *Rag1+Rag2* (IA3027RA12), *Rag3* (PI 567543 C), *Rag4rag1c* (PI 567541 B) (denoted throughout as *Rag4*), and *Rag5* (PI 567301 B). As previously described, each treatment was infested with either five mixed age biotype-1 or biotype-4 *A. glycines*. The population density of *A. glycines* was measured 11 d after infestation.

Statistical Analyses for Experiments

To address each of the hypotheses, we analyzed the number of *A. glycines* per plant at 11 d after infestation. All *A. glycines* population density data were log transformed to reduce heteroscadacity. Data for each individual biotype screen were analyzed using the PROC MIXED procedure with SAS statistical software version 9.3 (SAS Institute, Cary, NC). For all experiments, individual biotype screen data were analyzed using an analysis of variance. F-protected least-squares means tests using Tukey's honest significant difference method with a significance level of $P < 0.05$ were used to separate significant treatment effects. The statistical models used to analyze each experiment included the main effects of repetition, block, and soybean cultivar. The two-way interaction of repetition × block was included in the models.

Results

Overall, we observed that the main effect soybean cultivar was significant for each of the biotype screens (biotype-1: $F = 14.95$; df = 4, 34; $P < 0.0001$; biotype 2: $F = 15.74$; df = 5, 33; $P < 0.0001$; biotype-3: $F = 25.54$; df = 1, 34; $P < 0.0001$; biotype-4: $F = 16.38$; df = 1, 34; $P < 0.0001$).

Biotype Screens of the Three-Gene Pyramid Lines

Biotype-1

We observed lower population densities of biotype-1 *A. glycines* on both the *Rag1+Rag2+Rag3* and the *Rag1+Rag2+Rag4* three-gene pyramids (i.e., these pyramids are efficacious to biotype-1) for the biotype-1 screen. The population density of biotype-1 was significantly greater on the susceptible soybean cultivar when compared with the *Rag1* ($t = 7.73$; df = 1, 34; $P < 0.0001$), *Rag2* ($t = 4.91$; df = 1, 34; $P < 0.0003$), *Rag1+Rag2* ($t = 6.98$; df = 1,

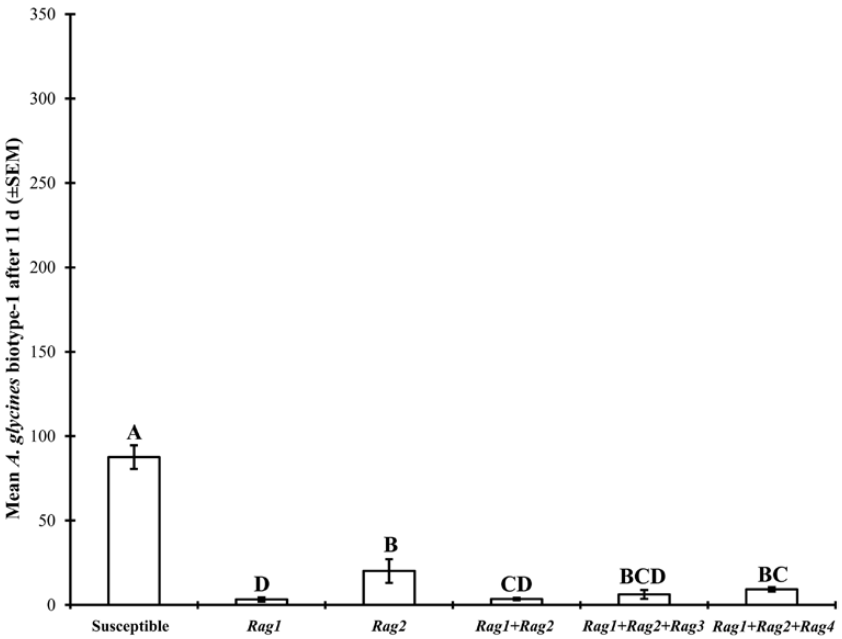

Fig. 1. Biotype-1 *A. glycines* reproduction reduced on *Rag1+Rag2+Rag3* and *Rag1+Rag2+Rag4* three-gene pyramids. Note that biotype-1 is avirulent to all tested aphid-resistant soybean cultivars. For this experiment, the susceptible soybean cultivar was IA3027, *Rag1* cultivar was IA3027RA1, *Rag2* cultivar was IA3027RA2, *Rag1+Rag2* cultivar was IA3027RA12, *Rag1+Rag2+Rag3* cultivar was RA123, and *Rag1+Rag2+Rag4* cultivar was RA124. Data were analyzed by soybean cultivar, and capital letters indicate significant differences among cultivars ($P < 0.05$).

34; $P < 0.0001$), *Rag1+ 2+3* ($t = 6.02$; df = 1, 34; $P < 0.0001$), and *Rag1+Rag2+Rag4* ($t = 5.36$; df = 1, 34; $P < 0.0001$) soybean cultivars (Fig. 1). There were significantly great populations of biotype-1 on the *Rag2* cultivar when compared with *Rag1* ($t = 4.59$; df = 1, 34; $P < 0.0002$) and the *Rag1+Rag2* ($t = 3.36$; df = 1, 34; $P < 0.0136$) soybean cultivars. In addition, there were significantly greater populations of biotype-1 on the *Rag1+Rag2+Rag4* pyramid when compared with the *Rag1* ($t = 3.85$; df = 1, 34; $P < 0.0029$) soybean cultivar (Fig. 1). There were no significant differences among any of the other *Rag*-containing soybean cultivars. In summary, these results suggest that for the biotype-1 *A. glycines* population that was tested, the single *Rag* genes and pyramids were effective at reducing its populations, i.e., *Rag1+Rag2+Rag4* appears to be comparable to *Rag1+Rag2+Rag3*.

Biotype-2

For our biotype-2 *A. glycines* screen, we observed reduced populations on both of the three-gene pyramids. Contrary to a previous study (Varenhorst et al. 2015b), the populations of biotype-2 on the susceptible and *Rag1* soybean cultivar were not significantly different ($t = 1.19$; df = 1, 33; $P = 0.8363$). However, the population density on the susceptible cultivar was significantly greater than that on *Rag2* ($t = 3.46$; df =1, 33; $P < 0.0173$), *Rag1+Rag2* ($t = 5.04$; df = 1, 33; $P < 0.0002$), *Rag1+Rag2+Rag3* ($t = 4.78$; df = 1, 33; $P < 0.0005$), or *Rag1+Rag2+Rag4* ($t = 5.27$; df = 1, 33; $P < 0.0001$) soybean cultivars. The population density of biotype-2 was also significantly greater on *Rag1* when compared with *Rag2* ($t = 4.61$; df = 1, 33; $P < 0.0008$), *Rag1+Rag2* ($t = 6.24$; df = 1, 33; $P < 0.0001$), *Rag1+Rag2+Rag3* ($t = 5.97$; df = 1, 33; $P < 0.0001$), and *Rag1+Rag2+Rag4* ($t = 6.46$; df = 1, 33; $P < 0.0001$) soybean cultivars (Fig. 2). There were no significant effects among the other *Rag* cultivars. These results suggest that both *Rag1+Rag2+Rag3* and *Rag1+Rag2+Rag4* would be potential management options for biotype-2 *A. glycines* population that was tested.

Biotype-3

We observed that population densities of biotype-3 *A. glycines* were reduced on both of the three-gene pyramids. For the biotype-3 screen, we observed significantly greater biotype-3 population densities on the *Rag2* cultivar when compared with the susceptible cultivar ($t = 3.60$; df = 1, 34; $P < 0.0119$), *Rag1* ($t = 9.17$; df = 1, 34; $P < 0.0001$), *Rag1+Rag2* ($t = 5.65$; df = 1, 34; $P < 0.0001$), *Rag1+Rag2+Rag3* ($t = 9.14$; df = 1, 34; $P < 0.0001$), and *Rag1+Rag2+Rag4* ($t = 7.48$; df = 1, 34; $P < 0.0001$) soybean cultivars (Fig. 3). In addition, the population density of biotype-3 was significantly greater on the susceptible cultivar when compared with *Rag1* ($t = 5.58$; df = 1, 34; $P < 0.0001$), *Rag1+Rag2* ($t = 4.09$; df = 1, 34; $P < 0.0360$), *Rag1+Rag2+Rag3* ($t = 5.54$; df = 1, 34; $P < 0.0001$), and *Rag1+Rag2+Rag4* ($t = 3.88$; df = 1, 34; $P < 0.0056$) soybean cultivars. We also observed significantly more biotype-3 *A. glycines* on the *Rag1+Rag2* pyramid when compared with *Rag1* ($t = 3.52$; df = 1, 34; $P < 0.0144$) and *Rag1+Rag2+Rag3* ($t = 3.49$; df = 1, 34; $P < 0.0158$) soybean cultivars. There were no significant differences observed among the other soybean cultivars. These results suggest that for the biotype-3 population that was tested both of the three-gene pyramids would be potentially suitable management options.

Biogtype-4

Biotype-4 population growth was inhibited by the *Rag1+Rag2+Rag3* pyramid, but not the by *Rag1+Rag2+Rag4* pyramid. For the biotype-4 screen, we observed significantly lower population densities on the susceptible cultivar when compared with the *Rag1* ($t = 4.24$; df = 1, 34; $P < 0.0021$), *Rag2* ($t = 3.47$; df = 1, 34; $P < 0.0167$), *Rag1+Rag2* ($t = 6.05$; df = 1, 34; $P < 0.0001$), and *Rag1+Rag2+Rag4* ($t = 4.43$; df = 1, 34; $P < 0.0012$) soybean cultivars. We also observed significantly lower population densities on the *Rag1+Rag2+Rag3* when compared with the *Rag1* ($t = 5.61$; df = 1, 34; $P < 0.0001$), *Rag2* ($t = 4.84$; df = 1, 34; $P < 0.0004$), *Rag1+Rag2* ($t = 7.42$; df = 1, 34; $P < 0.0001$), and *Rag1+Rag2+Rag4* ($t = 5.80$; df = 1, 34; $P < 0.0001$)

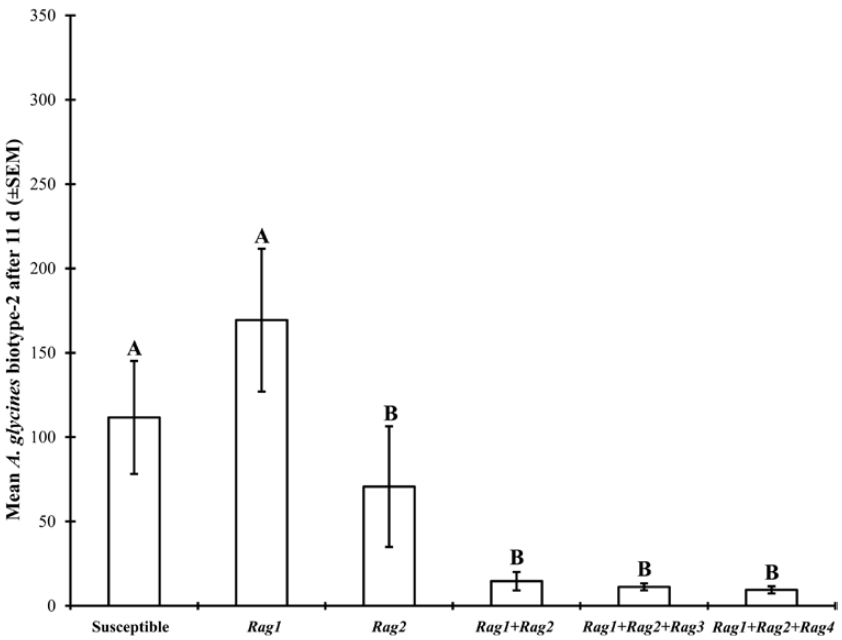

Fig. 2. Biotype-2 *A. glycines* reproduction reduced on *Rag1+Rag2+Rag3* and *Rag1+Rag2+Rag4* three-gene pyramids. Note that biotype-2 is virulent *Rag1* aphid-resistant soybean cultivars. For this experiment, the susceptible soybean cultivar was IA3027, *Rag1* cultivar was IA3027RA1, *Rag2* cultivar was IA3027RA2, *Rag1+Rag2* cultivar was IA3027RA12, *Rag1+Rag2+Rag3* cultivar was RA123, and *Rag1+Rag2+Rag4* cultivar was RA124. Data were analyzed by soybean cultivar, and capital letters indicate significant differences among cultivars ($P < 0.05$).

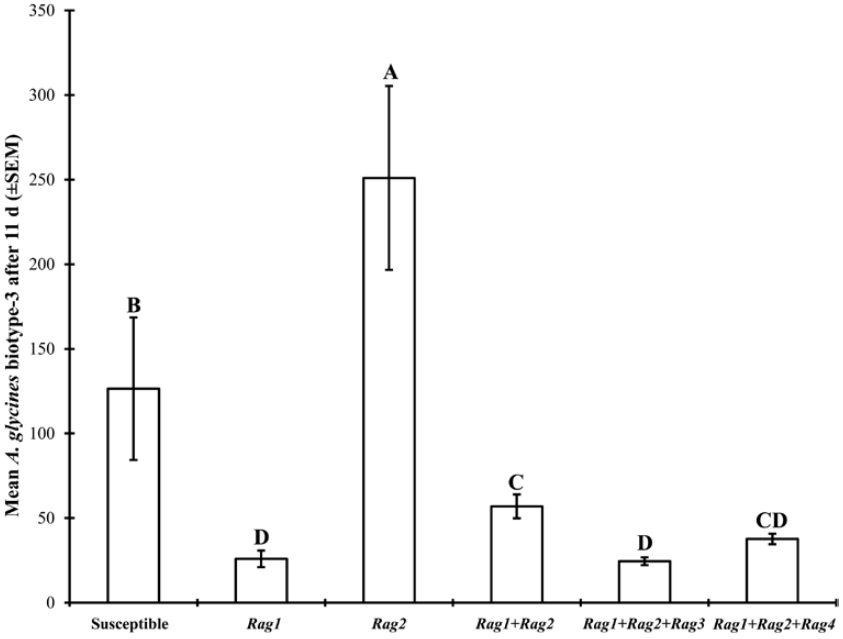

Fig. 3. Biotype-3 *A. glycines* reproduction reduced on *Rag1+Rag2+Rag3* and *Rag1+Rag2+Rag4* three-gene pyramids. Note that biotype-3 is virulent to *Rag2* aphid-resistant soybean cultivars. For this experiment, the susceptible soybean cultivar was IA3027, *Rag1* cultivar was IA3027RA1, *Rag2* cultivar was IA3027RA2, *Rag1+Rag2* cultivar was IA3027RA12, *Rag1+Rag2+Rag3* cultivar was RA123, and *Rag1+Rag2+Rag4* cultivar was RA124. Data were analyzed by soybean cultivar, and capital letters indicate significant differences among cultivars ($P < 0.05$).

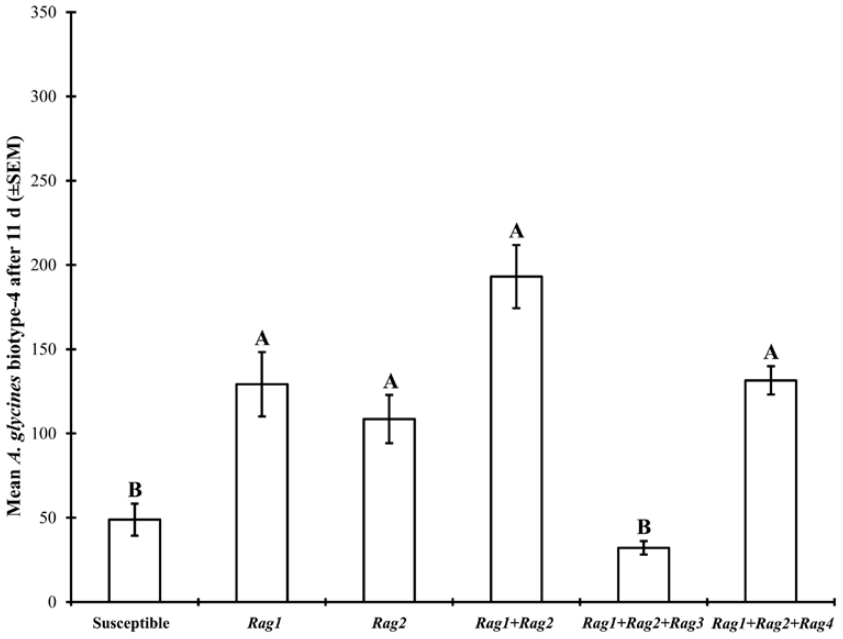

Fig. 4. Biotype-4 *A. glycines* reproduction reduced on *Rag1+Rag2+Rag3* three-gene pyramid. Note that biotype-4 is virulent to *Rag1*, *Rag2*, and *Rag1+Rag2* aphid-resistant soybean cultivars. For this experiment, the susceptible soybean cultivar was IA3027, *Rag1* cultivar was IA3027RA1, *Rag2* cultivar was IA3027RA2, *Rag1+Rag2* cultivar was IA3027RA12, *Rag1+Rag2+Rag3* cultivar was RA123, and *Rag1+Rag2+Rag4* cultivar was RA124. Data were analyzed by soybean cultivar, and capital letters indicate significant differences among cultivars ($P < 0.05$).

soybean cultivars (Fig. 4). We observed no significant difference between the susceptible and *Rag1+Rag2+Rag3* cultivars. These results indicate that the *Rag1+Rag2+Rag3* pyramid would potentially be an effective management tool for the tested biotype-4 *A. glycines* population. These results also suggest that the *Rag1+Rag2+Rag4* pyramid is not effective for managing biotype-4 populations.

Susceptible Cultivars and Plant Introductions Biotype-1 and Biotype-4 Screens

We did not observe evidence for any differences of susceptibility of the screened susceptible cultivars for either biotype-1 or biotype-4 population densities. However, the resistant plant introductions that were screened significantly affected the population densities of both

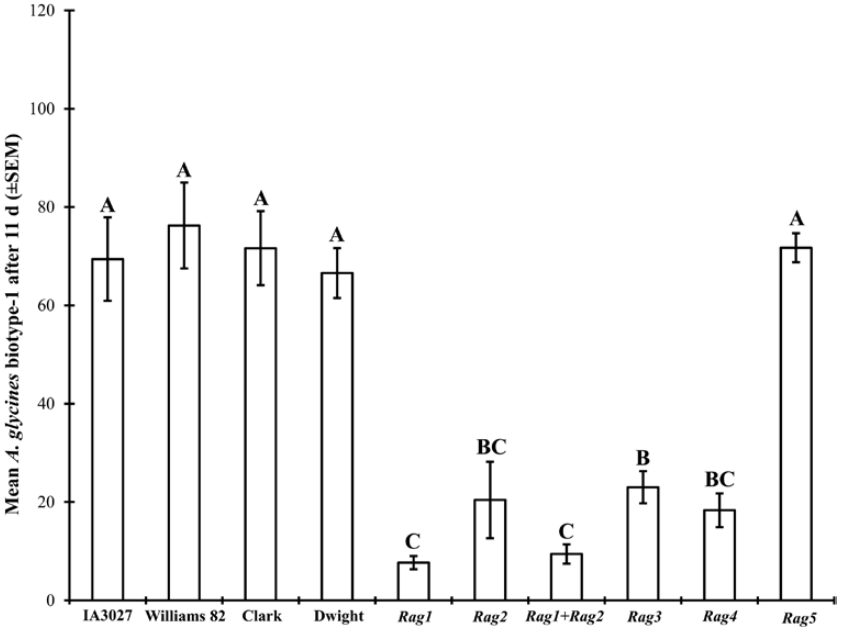

Fig. 5. Biotype-1 *A. glycines* reproduction is similar on multiple susceptible soybean cultivars. Note that biotype-1 is avirulent to all tested aphid-resistant soybean cultivars. For this experiment, the susceptible soybean cultivars were IA3027, Williams 82, Clark, and Dwight. The *Rag1* cultivar was IA3027RA1, *Rag2* cultivar was IA3027RA2, *Rag1+Rag2* cultivar was IA3027RA12, *Rag3* cultivar was PI 567543 C, *Rag4* cultivar was PI 567541 B, and *Rag5* cultivar was PI 567301 B. Data were analyzed by soybean cultivar, and capital letters indicate significant differences among cultivars ($P < 0.05$).

biotypes (biotype-1: $F = 26.60$; df = 9, 75; $P < 0.0001$ and biotype-4: $F = 15.41$; df = 9, 74; $P < 0.0001$).

For the biotype-1 screen, we did not observe significant differences among the susceptible soybean cultivars and *Rag5* (Fig. 5). We did, however, observe significant differences among these treatments, the other *Rag*-containing soybean cultivars, and plant introductions that were tested. We observed significantly more biotype-1 *A. glycines* on IA3027 susceptible soybean when compared with *Rag1* ($t = 7.97$; df = 1, 75; $P < 0.0001$), *Rag2* ($t = 6.96$; df = 1, 75; $P < 0.0001$), *Rag1+Rag2* ($t = 7.93$; df = 1, 75; $P < 0.0001$), *Rag3* ($t = 4.37$; df = 1, 75; $P < 0.0015$), and *Rag4* ($t = 5.18$; df = 1, 75; $P < 0.0001$) soybean cultivars. Williams 82 susceptible soybean also had significantly greater biotype-1 population densities than *Rag1* ($t = 8.35$; df = 1, 75; $P < 0.0001$), *Rag2* ($t = 7.34$; df = 1, 75; $P < 0.0001$), *Rag1+Rag2* ($t = 8.30$; df = 1, 75; $P < 0.0001$), *Rag3* ($t = 4.74$; df = 1, 75; $P < 0.0004$), and *Rag4* ($t = 5.56$; df = 1, 75; $P < 0.0001$). Similarly, significantly more biotype-1 were observed on Clark susceptible soybean when compared with *Rag1* ($t = 7.78$; df = 1, 75; $P < 0.0001$), *Rag2* ($t = 6.81$; df = 1, 75; $P < 0.0001$), *Rag1+Rag2* ($t = 7.74$; df = 1, 75; $P < 0.0001$), *Rag3* ($t = 4.30$; df = 1, 75; $P < 0.0020$), and *Rag4* ($t = 5.08$; df = 1, 75; $P < 0.0001$) soybean cultivars (Fig. 5). We also observed significantly greater biotype-1 population densities on Dwight susceptible soybean when compared with *Rag1* ($t = 8.02$; df = 1, 75; $P < 0.0001$), *Rag2* ($t = 7.01$; df = 1, 75; $P < 0.0001$), *Rag1+Rag2* ($t = 7.98$; df = 1, 75; $P < 0.0001$), *Rag3* ($t = 4.42$; df = 1, 75; $P < 0.0013$), and *Rag4* ($t = 5.23$; df = 1, 75; $P < 0.0001$) soybean cultivars. In addition, we also observed significantly greater biotype-1 population densities on *Rag5* soybean when compared with *Rag1* ($t = 8.19$; df = 1, 75; $P < 0.0001$), *Rag2* ($t = 7.21$; df = 1, 75; $P < 0.0001$), *Rag1+Rag2* ($t = 8.15$; df = 1, 75; $P < 0.0001$), *Rag3* ($t = 4.69$; df = 1, 75; $P < 0.0005$), and *Rag4* ($t = 5.49$; df = 1, 75; $P < 0.0001$) soybean cultivars (Fig. 5). We also observed that there were significantly more aphids present on *Rag3* when compared with the *Rag1* ($t = 3.36$; df = 1, 75; $P < 0.0384$) and *Rag1+Rag2* ($t = 3.31$; df = 1, 75; $P < 0.0433$) soybean cultivars (Fig. 5). We did not observe significant differences among *Rag2*-, *Rag3*-, or *Rag4*-resistant soybean cultivars. These results suggest that for biotype-1 *A. glycines*, regardless of the cultivar, susceptible soybean genotypes are viable host plants. These results also suggest that the source of resistance present in *Rag5* may not be a suitable source of resistance for the tested biotype-1 population. Although *Rag1* had significantly lower populations of biotype-1 when compared with *Rag3*, it is likely that *Rag3* is still a suitable source of resistance for this biotype.

We did not observe evidence that any of the susceptible cultivars negatively affected biotype-4 populations due to the lack of significant differences among the susceptible soybean cultivars IA3027, Williams 82, Clark, Dwight, and the resistant cultivars *Rag1*, *Rag2*, *Rag1+Rag2*, *Rag4*, and *Rag5*. However, we did observe significantly lower biotype-4 population densities on the *Rag3* cultivar when compared with IA3027 ($t = 8.49$; df = 1, 74; $P < 0.0001$), Williams 82 ($t = 9.14$; df = 1, 74; $P < 0.0001$), Clark ($t = 8.46$; df = 1, 74; $P < 0.0001$), Dwight ($t = 6.78$; df = 1, 74; $P < 0.0001$), *Rag1* ($t = 8.63$; df = 1, 74; $P < 0.0001$), *Rag2* ($t = 10.25$; df = 1, 74; $P < 0.0001$), *Rag1+Rag2* ($t = 7.87$; df = 1, 74; $P < 0.0001$), *Rag4* ($t = 6.60$; df = 1, 74; $P < 0.0001$), and *Rag5* ($t = 8.95$; df = 1, 74; $P < 0.0001$) soybean cultivars (Fig. 6). These results are in agreement with Ajayi-Oyetunde et al. (2016) that *Rag3* is effective at reducing biotype-4 *A. glycines* populations. In addition, these results suggest that the *Rag4* and *Rag5* resistance genes are not viable for biotype-4 management.

Discussion

The results we presented on the efficacy of *Rag1+Rag2+Rag3* to biotype-1, biotype-2, biotype-3, and biotype-4 *A. glycines* are in agreement with those observed by Ajayi-Oyetunde et al. (2016). Our results also suggest that the *Rag1+Rag2+Rag4* three-gene pyramid is potentially capable of reducing biotype-1, biotype-2, and biotype-3 *A. glycines* populations. However, from the findings presented, it seems

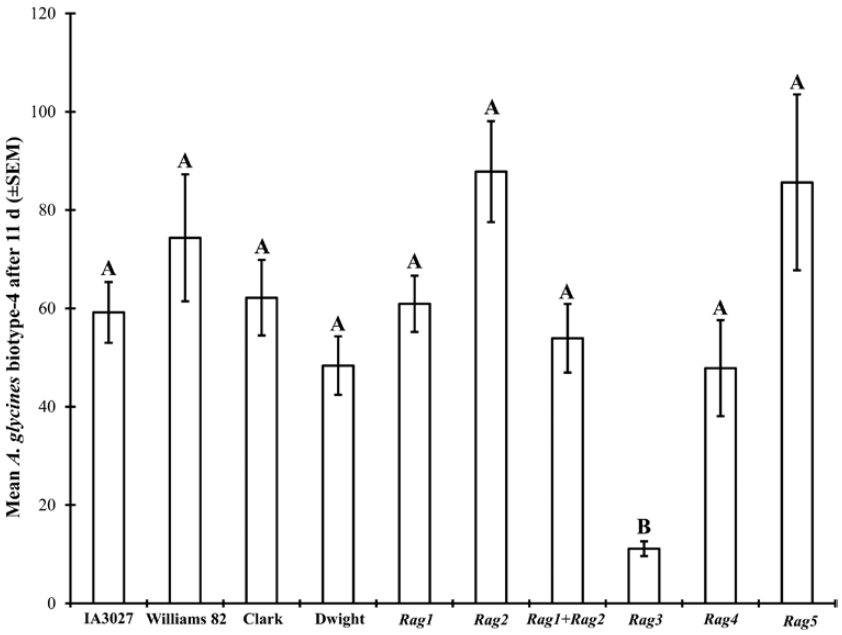

Fig. 6. Biotype-4 *A. glycines* reproduction is similar on multiple susceptible soybean cultivars. Note that biotype-4 is virulent to *Rag1*, *Rag2*, and *Rag1+Rag2* aphid-resistant soybean cultivars. For this experiment, the susceptible soybean cultivars were IA3027, Williams 82, Clark, and Dwight. The *Rag1* cultivar was IA3027RA1, *Rag2* cultivar was IA3027RA2, *Rag1+Rag2* cultivar was IA3027RA12, *Rag3* cultivar was PI 567543 C, *Rag4* cultivar was PI 567541 B, and *Rag5* cultivar was PI 567301 B Data were analyzed by soybean cultivar, and capital letters indicate significant differences among cultivars ($P < 0.05$).

that *Rag4* alone or in a three-gene pyramid (*i.e.*, *Rag1+Rag2+Rag4*) is not capable of effectively reducing biotype-4 *A. glycines* populations. To our knowledge, this is the first report of the failure of *Rag4* resistance toward biotype-4 populations. According to the previous findings regarding virulence of *A. glycines* to resistance sources by Kim et al. (2008), Hill et al. (2010), and Alt and Ryan-Mahmutagic (2013), the criteria used to describe a virulent biotype is their ability to successfully colonize a soybean cultivar containing a *Rag* gene or *Rag* gene combination. By this definition, biotype-4 should be described as being virulent to *Rag4* and *Rag1+Rag2+Rag4* resistance. Our results also suggest that both biotype-1 and biotype-4 should be described as being virulent to *Rag5* resistance.

In addition, our results indicate that the presence of fitness costs associated with the virulence of biotype-3 and biotype-4 on susceptible soybean observed during the three-gene pyramid are in agreement with Varenhorst et al. (2015b) (i.e., reduced population densities on susceptible soybean when compared with associated *Rag* soybean) are removed. However, our findings that there were no fitness costs associated with biotype-2 or biotype-4 virulence (populations that were tested during the susceptible cultivar screen) and were not in agreement with previous research (Varenhorst et al. 2015b). There are several possible explanations for why fitness costs were not observed in these experiments, but were observed in previous studies. The most likely explanation is that colonies of *A. glycines* are under occasional stress due to transferring, and other colony-related activities. Michel et al. (2010b) observed that laboratory colonies of *A. glycines* lack genetic diversity and demonstrate extreme genetic differentiation from colony to colony compared with field-collected populations. It is possible that in the elapsed time between the experiments, the colony may have been stressed, or that the presence of fitness costs may vary within a colony.

With initial discoveries of virulent *A. glycines* biotypes, there was much concern regarding the use of *Rag* gene as a management strategy. However, as previously mentioned, there are factors that will likely diminish the impact that these virulent biotypes will have on *Rag* success. These factors include fitness costs and induced susceptibility. The success of *Rag* soybean will also depend on the deployment of the available genes and the distribution of virulent biotypes. The utilization of a single-gene deployment method and the subsequent deterioration of the effectiveness of the source of resistance has been demonstrated in other cropping systems (Pan et al. 2011, Harris et al. 2012). In soybean, it has previously been suggested that virulence of *A. glycines* is not inherited in this way (Wenger and Michel 2013). We should be cautious in the deployment of single *Rag* gene soybean cultivars and put a concerted effort forward to encourage the deployment of pyramids. In addition, the geographical distribution of virulent biotypes is likely widespread (Michel et al. 2011, Crossley and Hogg 2015). However, the allele frequency of virulent biotypes in the environment is currently unknown but assumed to be low (Varenhorst et al. 2015a). The incorporation of multiple pyramided *Rag* gene soybean cultivars such as those tested here are needed to address whether resistant genes for virulent biotypes management need to be incorporated into an integrated pest management program to reduce the selection pressure of foliar insecticides and single-gene *Rag* cultivars.

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