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Research

Plant Resistance

Resistance of Soybean Plant Introductions to Three Colonies of Soybean Aphid (Hemiptera: Aphididae) Biotype 4

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Abstract

Soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), infestations of soybean, *Glycine max* (L.) Merr. (Fabales: Fabaceae), and the associated yield loss have led to a large dependence on insecticidal management in soybean throughout the Midwestern United States. However, several populations of pyrethroid-resistant soybean aphids have recently been found in Iowa, Minnesota, North Dakota and South Dakota, which highlights the importance of alternative management approaches. One such alternative method is host-plant resistance, which uses naturally occurring plant defenses in crop cultivars to reduce the potential for yield loss from a pest population. Current soybean aphid-resistant cultivars do not protect against all soybean aphids due to the presence of virulent biotypes. In particular, soybean aphid biotype 4 is virulent to *Rag1* and *Rag2* resistance genes both individually and in combination. However, we hypothesized that resistance to biotype 4 may exist in previously identified, but uncharacterized resistant soybean plant introductions (PIs). To test this, we evaluated 51 previously identified but uncharacterized soybean aphid-resistant PIs for their resistance to colonies of soybean aphid biotype 4 collected in separate site-years (Lomira, WI 2013; Volga, SD 2015, 2016). Free-choice tests identified 14 PIs with putative resistance to 'Lomira13', two to 'Volga15', and eight to 'Volga16' soybean aphid colonies. Follow-up, no-choice tests corroborated two to three resistant PIs per colony, and PI 437696, which was resistant to each of the three colonies and could aid in breeding efforts and an integrated approach to soybean aphid management.

Key words: host-plant resistance, virulence, crop protection, invasive species

Soybean aphid, *Aphis glycines* Matsumura, is a pest of soybean, *Glycine max* (L.) Merr., in the Midwestern United States and southern Canada (Ragsdale et al. 2011). In North America, an estimated $2.4 to $4.9 billion are lost annually due to soybean aphid feeding and management input costs (Song et al. 2006, Hill et al. 2012). Since the 2000 discovery of soybean aphids in North America, broad-spectrum foliar insecticides have been the primary method for managing this pest (Olson et al. 2008, Tilmon et al. 2011). The dependence on insecticidal management is undoubtedly factor that has led to the development of pyrethroid resistance in soybean aphid populations in Minnesota, Iowa, North Dakota, and South Dakota, which highlights the need for the adoption of integrated pest management for soybean aphids and also the need for alternative management tools (Koch et al. 2016, Hanson et al. 2017, Potter et al. 2017, Varenhorst et al. 2017b, Koch et al. 2018).

One alternative soybean aphid management tool is the implementation of host-plant resistance through the incorporation of *Rag* (i.e., *R*esistance to *Aphis glycines*) genes into soybean cultivars. In general, host plant resistance minimizes the impact of a pest population on a host plant by either disrupting pest survival and/or reproduction (i.e., antibiosis), deterring colonization (i.e., antixenosis), reducing the feeding impact (i.e., tolerance) or through a combination of these characteristics (Painter 1951, Beck 1965). To date, more than 10 *Rag* genes have been identified with antibiosis or antixenosis (Zhang et al. 2018). However, there are a limited number of cultivars commercially available and they either contain the single genes *Rag1*, *Rag2* or the pyramid of *Rag1*+*Rag2* (Chiozza et al. 2010, Michel et al. 2011, McCarville et al. 2012).

To further complicate the adoption of *Rag* soybean cultivars, in North America there are soybean aphid phenotypes that are

characterized by their ability to colonize (i.e., virulence) soybean that contain these *Rag1*, *Rag2* or *Rag1+Rag2* genes (Alt and Ryan-Mahmutagic 2013). To date, there have been four soybean aphid biotypes (i.e., phenotypes) described in North America that differ in their virulence (i.e., ability to colonize) to *Rag* genes. Of the biotypes, soybean aphid biotype 1 is avirulent (i.e., inability to colonize) to soybean containing *Rag1*, *Rag2*, *Rag1+Rag2* pyramid, *Rag3*, *Rag4*, *Rag1+Rag2+Rag3* pyramid or *Rag1+Rag2+Rag4* pyramid (Kim et al. 2008, Alt and Ryan-Mahmutagic 2013, Varenhorst et al. 2017a). Biotype 2 is virulent to *Rag1* soybean but avirulent to *Rag2* soybean (Kim et al. 2008). Biotype 3 is virulent to *Rag2* soybean but avirulent to *Rag1* soybean (Hill et al. 2010). In addition, negative cross-resistance was observed for biotype 2 on *Rag1* and biotype 3 on *Rag2* (Varenhorst et al. 2015b). The hypersensitivity in soybean aphid biotype 2 and biotype 3 to other resistance genes explain their inability to effectively colonize *Rag1+Rag2* pyramided soybean. Lastly, biotype 4 is virulent to soybean with *Rag1*, *Rag2*, *Rag1+Rag2* pyramid, *Rag4*, and the *Rag1+Rag2+Rag4* pyramid (Alt and Ryan-Mahmutagic 2013, Varenhorst et al. 2017a). However, biotype 4 is avirulent to soybean with the single gene *Rag3* and the *Rag1+Rag2+Rag3* pyramid (Varenhorst et al. 2017a). Although virulent biotypes exist in North America and their distribution is widespread (Michel et al. 2011), they make up a small percentage of the overall summer populations (Cooper et al. 2015, Alt et al. 2018). However, recent research has indicated that some virulent soybean aphids can manipulate host plants to make them suitable for avirulent biotypes (i.e., obviation of resistance) (Varenhorst et al. 2015a), which suggests a need for robust resistance sources to prevent successful soybean aphid establishment on *Rag* soybean. The recently developed three-gene pyramided sources of resistance offer broader management of soybean aphid biotypes, even to biotype 4 (e.g., *Rag1+Rag2+Rag3*) (Ajayi-Oyetunde et al. 2016, Varenhorst et al. 2017a, Zhang et al. 2018). However, it is possible that additional virulent biotypes of the soybean aphid have yet to be discovered.

For this reason, additional sources of resistance are needed to diversify *Rag* genes to ensure reliable and long-lasting protection against known and yet to be discovered virulent biotypes. Although previous studies have identified soybean plant introductions (PIs) with resistance to biotype 1, biotype 2 and biotype 3 (U.S. NPGS) it is unknown if these PIs may also confer resistance to biotype 4. If additional sources of resistance to biotype 4 are discovered, future breeding efforts could include them in pyramids to create more robust *Rag* soybean cultivars to provide a greater longevity for soybean aphid resistance. Furthermore, effective *Rag* pyramids that are bred into competitive soybean cultivars could reduce the dependency on foliar insecticides for soybean aphid management and reduce the likelihood of soybean aphids developing resistance to additional insecticide active ingredients or classes. The objective of this study was to assess a set of PIs that were previously identified as soybean-aphid resistant for their resistance to three independently-collected biotype 4 colonies through free-choice and no-choice tests.

Materials and Methods

This project was conducted at the USDA-ARS North Central Agricultural Research Laboratory (NCARL, Brookings SD). On-site aphid-free greenhouses were used to grow soybean plants that were used for colony rearing and also the experiments. A mixture of soil (2:1:1 mixture of Vienna soil [fine-loamy, mixed Calcic Hapludolls], coarse vermiculite [Perlite Vermiculite Packaging, North Bloomfield, OH], and sphagnum peat moss [Sun Gro Horticulture Distribution Inc., Agawam, MA]) was used for all plantings. Greenhouse plants were grown in conditions with a 16:8 (L:D) h photoregime and approximately 23:18°C (L:D) temperature. Colonies were reared in indoor growth chambers. The free-choice and no-choice tests were conducted in indoor growth chambers that were spatially separated from the colony growth chambers (CMP4030 Conviron, Winnipeg, Canada). All growth chambers were maintained with a 16:8 (L:D) h photoregime, 23:18°C (L:D) temperature, and approximately 50% relative humidity. For the free-choice and no-choice tests the methods that were used to evaluate resistance among the PIs were modified from Hesler et al. (2017a, 2017c).

Soybean Aphid Biotype 4 Colonies

The soybean aphid biotype 4 colonies were collected in three different site-years. The first colony was collected near Lomira, WI in 2013 ('Lomira13'). Isolates from the Lomira collection were maintained at the University of Illinois Urbana-Champaign before an infested soybean leaflet was sent to NCARL (Doris Lagos-Kutz, personal communication, 2016). The other two colonies were collected in at the South Dakota State University Volga Research Farm near Volga, SD in 2015 ('Volga15') and in 2016 ('Volga16'). The soybean aphid colonies were established and maintained in separate growth chambers at NCARL on IA2104RA12 (*Rag1+Rag2*). Each colony was maintained on approximately 10 plants per large pot (6 cm top diameter × 4 cm bottom diameter × 5.7 cm height; Myers Industries Inc., Earth City, MO) with 1 liter soil below and 300 ml soil above seeds. From each site-year collection, six apterous, adult females were arbitrarily chosen and transferred individually using a fine wetted paintbrush onto an IA2104RA12 colony plant (VC stage: developing first trifoliate; Licht 2014). Each female aphid was caged on a separate soybean plant (i.e., six soybean plants per site-year biotype 4 colony). Cages were made from a 0.6-cm thick clear extruded acrylic tube (12.7 cm outer diameter × 40.6 cm height, Ridout Plastics Co. Inc., San Diego, CA). Cages had two opposing holes drilled, 5.1-cm diameter, for ventilation. The holes and one end of the tube were covered and hot-glued with no thrips mesh screen (BioQuip, Rancho Dominquez, CA). The isolated female (iso-female) with the most clonal offspring after 2 wk was chosen to establish that colony, and all other aphids from that site-year were discarded. This process was completed for each of the three colonies (i.e., Lomira13, Volga15, Volga16). As previously mentioned, each of the iso-female colonies were reared in separate growth chambers and cared for separately to prevent colony cross-contamination.

Seed Acquisition

Seeds for the 51 soybean PIs (Table 1) were acquired from the U.S. Soybean Germplasm Collection (Urbana, IL). PIs were seed increased at NCARL as needed. Soybean cultivars with known soybean aphid resistance or susceptibility were used as checks: LD09-05484a (*Rag1*, Blue River Hybrids, Kelley, IA), 2880a (*Rag2*, Blue River Hybrids, Kelley, IA), LD14-8039 (*Rag3*, University of Illinois National Soybean Research Center, Urbana, IL), IA2104RA12 (*Rag1+Rag2*, Iowa State University Research Foundation Inc., Ames, IA), Brookings (susceptible [i.e., no *Rag* gene]; PI 667735; South Dakota State University, Brookings, SD), and IA2104 (susceptible, Iowa State University Research Foundation Inc., Ames, IA).

Free-Choice Tests

Five free-choice tests were conducted for each of the three biotype 4 iso-female colonies. Each free-choice test included 10 different test

Table 1. Soybean PIs known to be resistant to soybean aphids

PI	MG[a]	Origin	References	Known SBA[b] resistance gene
PI 153214	I	Belgium	Bhusal et al. 2014	
PI 189860	00	France	Hesler and Dashiell 2007	
PI 189946	I	France	Bhusal et al. 2014	
PI 194627	00	Sweden	Hesler and Dashiell 2007	
PI 194645	00	Sweden	Hesler and Dashiell 2007	
PI 200595	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 230977	VII	Japan	Hesler et al. 2007; Hill et al. 2004a,b	
PI 243540	IV	Japan	Hesler et al. 2011a, Mian et al. 2008a	*Rag2* (Rouf Mian et al. 2008)
PI 340034	IV	South Korea	Bansal et al. 2013	
PI 430491	00	China	Bhusal et al. 2013; Hesler and Dashiell 2007; Hesler et al. 2011a,b	
PI 436684	III	China	Hesler and Dashiell 2007	
PI 437075	I	Russian Federation	Bhusal et al. 2014	
PI 437282	I	Moldova	Hesler et al. 2017a,b,c	
PI 437353	I	Russian Federation	Hesler et al. 2017a,b,c	
PI 437658	I	China	Hesler et al. 2017a,b,c	
PI 437696	VI	China	Fox et al. 2014	*Rag1*+*Rag2* (Fox et al. 2014)
PI 437733	I	China	Hesler et al. 2017a,b,c	
PI 438118	I	China	Hesler et al. 2017a,b	
PI 464911	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 507713	—	Russian Federation	Hanson et al. 2016	
PI 518753	I	Former Serbia and Montenegro	Hesler et al. 2017a,b	
PI 524994	I	Russian Federation	Hesler et al. 2017a,b	
PI 548395	00	United States	Hesler and Dashiell 2007	
PI 548417	I	Italy	Hesler et al. 2017c	
PI 548530	I	United States	Hesler et al. 2017c	
PI 548544	00	Canada	Hesler and Dashiell 2007	
PI 587870	VII	China	Fox et al. 2014	*Rag1*+*Rag2* (Fox et al. 2014)
PI 588000	X	China	Fox et al. 2014	*Rag1*+*Rag2* (Fox et al. 2014)
PI 592389	I	United States	Hesler et al. 2017a,b	
PI 594573	VII	China	Fox et al. 2014	*Rag1*+*Rag2* (Fox et al. 2014)
PI 603326	I	China	Bhusal et al. 2014	
PI 603712	0	China	Bhusal et al. 2013; Hesler et al. 2011a,b	
PI 319535A	I	China	Hesler et al. 2017a,b	
PI 361088B	I	Romania	Hesler, unpublished data	
PI 438048B	I	China	Hesler et al. 2017a,b	
PI 512322B	I	Georgia	Hesler et al. 2017c	
PI 561285B	I	China	Hesler et al. 2017a,b	
PI 567250A	I	China	Bhusal et al. 2014	
PI 567301B	IV	China	Mian et al. 2008a,c	*Rag5* (Jun et al. 2012)
PI 567541B	III	China	Hesler and Dashiell 2007; Hesler et al. 2011a,b; Mensah et al. 2002; Mensah et al. 2005; Mian et al. 2008a,c	*rag1c* and *rag4* (Zhang et al. 2009)
PI 567598B	III	China	Hesler and Dashiell 2007; Mensah et al. 2002; Mensah et al. 2005; Mian et al. 2008a,b,c	*rag1b* and *rag3* (Bales et al. 2013)
PI 578388B	I	China	Hesler et al. 2017a,b	
PI 603339A	I	China	Bhusal et al. 2014	
PI 603426D	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 603432B	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 603546A	I	China	Bhusal et al. 2014	
PI 603587A	I	China	Bhusal et al. 2014	
PI 605765B	I	Vietnam	Hanson et al. 2016	
PI 606390A	IV	Vietnam	Bansal et al. 2013	
PI 612759B	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 612759C	I	China	Hesler et al. 2017a,b,c	

[a]MG, maturity group.
[b]SBA, soybean aphid.

PIs (50 PIs tested in total per colony) and the six checks (i.e., 16 experimental units per replicate). A randomized complete block design with eight blocks was used for each free-choice test. For each free-choice test, two soybean seeds for each PI and check were planted in small, square pots (8.25 cm top side × 6.5 cm bottom side × 7.62 cm height; International Greenhouse Co., Danville, IL) with 150-ml soil mixture below and 100 ml above them and grown in the greenhouse. Two weeks before testing, soybeans were at the VC growth stage

and they were thinned to one plant per pot and the soil surface was covered in sand (industrial quartz; Unimin Corporation, Le Seuer, MN) to facilitate aphid movement. A single plant for each of the PIs and checks were organized in plastic trays (26.5 cm width × 51 cm length × 6.5 cm height; T. O. Plastics Inc., Clearwater, MN), which were used as blocks.

For each free-choice test, two founder plants per block (i.e., plastic tray) were used as aphid inoculum. Two weeks prior to each free-choice test, founder plant (IA2104RA12, VC stage) were established by infesting soybean with 10 apterous, adult aphids from the appropriate iso-female colony plants using a fine-tipped, wetted paintbrush. The founder plants were maintained in a growth chamber (i.e., growth chamber separated from the growth chamber used for free-choice tests and those that were used for maintaining iso-female colonies) for 2 wk allowing the soybean aphid populations to increase to approximately 250 aphids per plant. At the beginning of each free-choice test, the founder plants were cut at the stem and the plants were placed upright back in their pots to wilt (i.e., to encourage aphid movement). Two pots containing a wilting founder plant were placed at foci equidistant from surrounding experimental plants within each experimental block (i.e., plastic tray).

After founder plants were placed in the plastic trays, each of the eight trays (i.e., blocks) were placed into a growth chamber. Fourteen days after the founder PI the test plants were at approximately V2 stage (two developed trifoliates; Licht 2014) and the soybean aphid populations for each plant were rated individually using a 0-to-6, 50 aphid-increment scale (i.e., 0: 0, 1: 1–50, 2: 51–100, 3: 101–150, 4: 151–200, 5: 201–250, 6: 250+ aphids; Hesler et al. 2017c). The 14-day experimentation period was selected for the free-choice tests as the soybean aphid populations can double approximately every 1.8 d under growth chamber conditions (McCornack et al. 2004) and the populations reached high densities on the IA2104RA12 (i.e., *Rag1*+*Rag2*) check. Free-choice PIs with mean and median ratings below 2.5 were considered putatively resistant to the respective colony and advanced for a set of no-choice tests specific for each of the colonies.

No-Choice Tests

The soybean tested in the no-choice tests consisted of the PIs selected from the free-choice tests as well as IA2104 (no *Rag* gene, positive control), IA2104RA12 (*Rag1*+*Rag2*, positive control), and LD14-8039 (*Rag3*; negative control; unpublished data, Varenhorst et al. 2017a). Plants were grown to the VC growth stage in small pots in a greenhouse. Twelve uniform plants of each line were then chosen for testing and transplanted in pairs into large pots (i.e., two plants of a single PI or check planted per pot) (6 cm top diameter × 4 cm bottom diameter × 5.7 cm height), and the soil surface of each pot was covered in sand to stabilize cages (as used for aphid colonies) that covered the two test plants. Each potted pair of the lines being tested were arranged in a complete randomized design with six replicates per line. Each of the test plants within a pot were infested with six apterous, adult soybean aphids that were transferred from the appropriate iso-female colony to the unifoliates of each test plant using a fine-tipped, wetted paintbrush and infested plants were then caged. Ten days after infestation, one of the two plants in each pot was randomly chosen, cut, and placed in an individual, labeled bag, which was then frozen. Twenty days after infestation, the remaining plant in each pot was cut, placed in a bag, and frozen. At this time, the sand and cage were examined for stray aphids, which were counted, and these data was included in the 20 d after-infestation counts. After ≥48 h, the 10 d after-infestation and 20 d after-infestation plants were thawed and all of the soybean aphids present on the plants and also in the bags were counted.

For each no-choice test, soybean aphid counts were log-transformed, and an analysis of variance with a generalized linear mixed model (PROC GLIMMIX, SAS Institute 2014) assessed treatment factors for test line, sample day, and test line-by-sample day interaction. If a result was significant ($P < 0.05$), a least squares means (LSMEANS) procedure with Bonferroni adjustment was used for multiple comparisons of the test lines. In cases with a significant test line-by-sample day interaction, test lines were compared among one another for each sample day. A test line was considered resistant to a respective colony when the mean number of soybean aphids per plant was significantly different (i.e., lower) than that of the soybean aphid populations present on LD14-8039 (i.e., *Rag3* or negative control).

Results

Free-Choice Tests

For the Lomira13 colony, 14 of the 55 PIs were identified as being putatively resistant to soybean aphid biotype 4 based on mean and median infestation ratings <2.5 (Table 2, bolded values). Four were observed in the first (PI 437696, PI 588000, PI 594573, and PI 606390A), third (PI 430491, PI 438118, PI 567250A, and PI 603426D) and fourth tests (PI 438048B, PI 512322B, PI 603339A, and PI 603712), and two (PI 567541B and PI 605765B) in the fifth test. For the Volga15 colony, two PIs from the first test (PI 437696 and PI 567598B) were identified as being putatively resistant (Table 2). For the Volga16 colony, eight PIs were rated as putatively resistant (Table 2): four (PI 437696, PI 567598B, PI 588000, and PI 606390A) from the first test, one (PI 430491) in the third test, one PI from the fourth test (PI 603712), and two PIs (PI 567541B and PI 605765B) from the fifth test. The PIs that were identified as being putatively resistant were then used in the no-choice tests for the appropriate iso-female colony.

No-Choice Tests

For the no-choice tests, resistance to biotype 4 soybean aphids was determined if the iso-female population density on the tested PI line had no significant difference or had a population density that was significantly different (i.e., lower) than LD14-8039 (*Rag3*) (i.e., negative check).

Lomira13 Colony.

For the Lomira13 iso-female colony a total of three no-choice tests were conducted due to growth chamber space limitations. In the first Lomira13 no-choice test, the soybean line, sampling day, and the line-by-sample day interaction significantly affected the number of soybean aphids per plant (Table 3). At 10 d post-infestation, PI 437696 had significantly fewer soybean aphids per plant than LD14-8039 (Fig. 1a). Although PI 567541B had fewer soybean aphids than LD14-8039 it was not significantly different, but it did have significantly fewer soybean aphids than IA2104RA12 (Fig. 1a). Based on our definition of resistance, at 10 d post-infestation, only PI 437696 and PI 567541B were considered resistant to Lomira13 biotype 4 soybean aphids.

At 20 d post-infestation, PI 437696 had significantly fewer aphids than all other soybean lines (Fig. 1a). Although PI 567541B, PI 430491, and PI 603712 were not significantly different from LD14-8039 they had significantly fewer aphids when compared to IA2104RA12. For first no-choice test, the results indicate that PI

Table 2. Mean and median ratings of soybean free-choice tests using three colonies of soybean aphid biotype 4

	Colony					
	Lomira13		*Volga15*		*Volga16*	
Line	Mean	Median	Mean	Median	Mean	Median
Test 1						
PI 230977	3.0	2.5	3.6	4.0	3.5	3.5
PI 340034	5.6	6.0	3.5	3.5	5.9	6.0
PI 436684	4.8	5.5	4.3	4.5	5.7	6.0
PI 437696	**1.0**	**1.0**	**0.9**	**1.0**	**1.1**	**1.0**
PI 567301B	3.8	3.0	3.0	2.0	5.6	6.0
PI 567598B	3.8	3.0	**0.9**	**1.0**	**1.9**	**2.0**
PI 587870	4.9	5.5	4.3	5.0	5.9	6.0
PI 588000	**2.0**	**2.0**	**1.0**	**1.0**	6.0	6.0
PI 594573	**1.8**	**2.0**	4.3	4.0	5.3	6.0
PI 512322B	N/A[a]	N/A	4.3	4.0	5.3	6.0
PI 606390A	**1.3**	**1.0**	**1.5**	**1.0**	2.7	3.0
Brookings (susceptible)	3.8	4.0	4.3	5.0	6.0	6.0
IA2104 (susceptible)	5.0	6.0	5.0	6.0	6.0	6.0
LD09-05484a (*Rag1*)	5.8	6.0	5.1	6.0	5.3	6.0
2880a (*Rag2*)	4.9	6.0	4.5	5.0	5.7	6.0
IA2104RA12 (*Rag1+Rag2*)	3.0	2.0	3.6	3.5	3.1	3.0
LD14-8039	3.8	4.0	3.1	2.5	5.3	6.0
	Lomira13		*Volga15*		*Volga16*	
	Mean	Median	Mean	Median	Mean	Median
Test 2						
PI 189946	4.5	5.0	5.4	6.0	6.0	6.0
PI 319535A	4.0	4.0	5.7	6.0	6.0	6.0
PI 361088B	2.8	2.5	3.4	4.0	5.4	5.0
PI 437282	3.5	4.0	4.7	5.0	6.0	6.0
PI 548417	4.5	4.5	3.6	4.0	6.0	6.0
PI 548530	4.0	4.5	5.0	5.0	6.0	6.0
PI 561285B	4.3	5.0	4.1	5.0	6.0	6.0
PI 578388B	3.0	3.0	4.7	5.0	5.6	6.0
PI 592389	3.5	3.5	5.6	6.0	6.0	6.0
PI 603326	4.5	4.5	4.7	5.0	5.6	6.0
Brookings (susceptible)	4.2	4.0	5.3	6.0	6.0	6.0
IA2104 (susceptible)	5.8	6.0	5.4	6.0	5.6	6.0
LD09-05484a (*Rag1*)	5.2	5.5	5.1	6.0	5.7	6.0
2880a (*Rag2*)	5.2	5.5	3.9	4.0	6.0	6.0
IA2104RA12 (*Rag1+Rag2*)	2.3	2.0	3.4	4.0	5.4	6.0
LD14-8039 (*Rag3*)	3.2	3.0	2.9	2.0	5.6	6.0
	Lomira13		*Volga15*		*Volga16*	
	Mean	Median	Mean	Median	Mean	Median
Test 3						
PI 200595	2.8	2.5	3.9	3.5	5.3	6.0
PI 430491	**1.4**	**1.0**	**2.0**	**2.0**	N/A	N/A
PI 588000	N/A	N/A	N/A	N/A	2.4	2.5
PI 437658	4.1	5.5	5.5	6.0	5.3	6.0
PI 437733	2.6	2.0	4.3	6.0	5.5	6.0
PI 438118	**1.4**	**1.0**	4.6	6.0	5.1	5.5
PI 518753	3.8	4.0	4.9	6.0	5.9	6.0
PI 524994	4.4	5.5	5.8	6.0	6.0	6.0
PI 567250A	**1.5**	**1.0**	2.9	2.0	2.8	2.5
PI 603426D	**1.9**	**1.0**	5.3	6.0	5.6	6.0
PI 612759B	3.8	4.5	4.1	4.0	5.9	6.0
Brookings (susceptible)	1.6	1.5	5.9	6.0	6.0	6.0
IA2104 (susceptible)	2.9	2.0	3.3	3.0	5.9	6.0
LD09-05484a (*Rag1*)	3.0	3.0	4.8	6.0	5.5	6.0

(Continued)

Table 2. Continued

	Colony					
	Lomira13		*Volga15*		*Volga16*	
Line	Mean	Median	Mean	Median	Mean	Median
2880a (*Rag2*)	2.6	2.0	3.8	3.5	6.0	6.0
IA2104RA12 (*Rag1+Rag2*)	1.4	1.0	3.3	3.0	4.6	4.5
LD14-8039 (*Rag3*)	1.5	1.0	2.8	2.5	3.4	3.0
	Lomira13		*Volga15*		*Volga16*	
	Mean	Median	Mean	Median	Mean	Median
Test 4						
PI 153214	4.9	6.0	4.9	6.0	6.0	6.0
PI 512322B	**2.3**	**2.0**	N/A	N/A	N/A	N/A
PI 243540	N/A	N/A	4.4	5.0	N/A	N/A
PI 430491	N/A	N/A	N/A	N/A	2.6	2.0
PI 438048B	**1.8**	**1.0**	3.1	3.0	3.9	3.5
PI 464911	3.4	2.5	4.3	5.0	5.5	6.0
PI 507713	2.8	2.5	4.1	4.0	3.8	4.0
PI 603339A	**1.9**	**1.5**	3.0	2.0	3.5	3.5
PI 603546A	5.6	6.0	5.4	6.0	5.8	6.0
PI 603587A	2.5	1.5	4.0	4.0	5.5	6.0
PI 603712	**1.1**	**1.0**	**1.1**	**1.0**	3.1	3.0
PI 612759C	2.8	3.0	3.7	3.0	5.1	6.0
Brookings (susceptible)	2.4	1.0	3.7	5.0	5.8	6.0
IA2104 (susceptible)	2.6	1.5	4.6	6.0	6.0	6.0
LD09-05484a (*Rag1*)	4.1	4.0	3.9	4.0	5.5	6.0
2880a (*Rag2*)	2.1	1.5	4.7	5.0	4.4	5.0
IA2104RA12 (*Rag1+Rag2*)	2.0	1.5	2.9	3.0	5.0	5.0
LD14-8039 (*Rag3*)	1.1	1.0	2.6	3.0	3.0	3.0
	Lomira13		*Volga15*		*Volga16*	
	Mean	Median	Mean	Median	Mean	Median
Test 5						
PI 189860	5.5	6.0	5.0	6.0	4.9	5.5
PI 194627	4.9	5.0	3.9	5.0	5.0	6.0
PI 194645	5.1	6.0	4.7	6.0	5.3	6.0
PI 437075	6.0	6.0	5.7	6.0	5.9	6.0
PI 437353	3.0	2.5	4.1	5.0	4.8	5.5
PI 548395	5.5	5.5	5.0	6.0	5.5	6.0
PI 548544	5.0	5.0	4.1	4.0	5.9	6.0
PI 567541B	**2.4**	**2.0**	**2.3**	**1.0**	3.1	3.0
PI 603432B	5.3	6.0	N/A	N/A	5.5	6.0
PI 603426B	N/A	N/A	5.6	6.0	N/A	N/A
PI 605765B	**2.1**	**1.5**	**2.1**	**2.0**	4.4	4.0
Brookings (susceptible)	4.4	5.0	5.3	6.0	5.6	6.0
IA2104 (susceptible)	5.9	6.0	5.9	6.0	6.0	6.0
LD09-05484a (*Rag1*)	5.1	6.0	5.1	6.0	5.9	6.0
2880a (*Rag2*)	5.0	5.5	5.0	5.0	4.5	4.0
IA2104RA12 (*Rag1+Rag2*)	3.6	4.0	4.4	5.0	4.3	5.0
LD14-8039 (*Rag3*)	3.6	4.5	3.4	4.0	3.8	3.0

Ratings were based on 50 aphid-increments from 0 (no aphids) to 6 (>250 aphids). Eight observations were recorded per soybean line unless otherwise noted. Soybean plant introductions indicated by bold ratings were considered putatively resistant and were selected for no-choice tests; check lines are indicated by italics.

[a]Indicates that the colony was not tested against the particular PI. In some cases, PIs were substituted based on seed availability.

437696, PI 567541B, PI 430491, and PI 603712 are resistant to the Lomira13 biotype 4 colony.

For the second Lomira13 no-choice test, soybean aphid population density varied significantly by plant line and sample day. However, the soybean line-by-sampling day interaction was not significant, and the 10 d post-infestation and 20 d post-infestation data were combined for the analysis (Table 3). Of the tested lines, only PI 588000 had significantly fewer aphids per plant when compared to LD14-8039 (Fig. 1b). Although the population density of soybean aphids on PI 567250A did not differ significantly from those on LD14-8039 they were significantly lower than on those observed on IA2104RA12 (Fig. 1b). The results from second no-choice

Table 3. Analyses of variance output for the mean number of soybean aphids per plant in soybean no-choice tests of three soybean aphid biotype 4 colonies

Colony	Test	Effect	df	*F* value	*P* value
Lomira13	Test 1	Line	9, 98	92.36	<0.0001
		Day	1, 98	146.04	<0.0001
		Line × day	9, 98	8.3	<0.0001
	Test 2	Line	8, 88	16.01	<0.0001
		Day	1, 88	154.52	<0.0001
		Line × day	8, 88	1.95	0.0618
	Test 3	Line	4, 50	32.34	<0.0001
		Day	1, 50	547.24	<0.0001
		Line × day	4, 50	2.27	0.0751
Volga15	Test 1	Line	4, 48	141.37	<0.0001
		Day	1, 48	66.58	<0.0001
		Line × day	4, 48	6.07	0.0005
Volga16	Test 1	Line	6, 70	240.41	<0.0001
		Day	1, 70	301.48	<0.0001
		Line × day	6, 70	3.95	0.0018
	Test 2	Line	6, 70	16.75	<0.0001
		Day	1, 70	106.7	<0.0001
		Line × day	6, 70	0.83	0.5532

test indicate that PI 588000 and PI 567250A are resistant to the Lomira13 biotype 4 colony.

For the third Lomira13 no-choice test, soybean aphid population density varied significantly by soybean line and sampling day. However, the line-by-sample day interaction was not significant, and the 10 d post-infestation and 20 d post-infestation data were combined for the analysis (Table 3). None of the PIs had soybean aphid densities that were significantly lower than LD14-8039 (Fig. 1c). However, the soybean aphid populations observed on PI 603339A were significantly lower than those on IA2104RA12. The results for the third no-choice test indicate that PI 603339A was resistant to the Lomira13 biotype 4 colony.

Of the 14 PIs that were tested for resistance, only seven were determined to be resistant to the Lomira13 biotype colony (i.e., PI 437696, PI 567541B, PI 430491, PI 603712, PI 588000, PI 567250A, and PI 603339A). Of the seven identified PIs, only two significantly outperformed the negative check (i.e., PI 437696 and PI 588000).

Volga15 Colony.

For the Volga15 iso-female colony, only one no-choice test was conducted. For this no-choice test, soybean aphid population densities varied significantly by soybean line, sampling day and the soybean line-by-sampling day interaction (Table 3). For both the 10 d post-infestation and 20 d post-infestation counts, PI 437696 and PI 567598B had significantly fewer aphids when compared to LD14-8039 (Fig. 2). The results for the no-choice test indicate that PI 437696 and PI 567598B are resistant to the Volga15 biotype 4 colony.

Volga16 Colony.

For Volga16 iso-female colony, two no-choice tests were conducted. In the first no-choice test, soybean aphid population densities varied significantly by soybean line, sampling day and the soybean line-by-sample day interaction (Table 3). At 10 d post-infestation, PI 437696 and PI 567598B had significantly fewer aphids per plant than LD14-8039 (Fig. 3a). Although population densities on PI 430491 were not significantly different from those on LD14-8039, they were significantly lower than those observed on IA2104RA12 (Fig. 3a). At 10 d post-infestation, PI 437696, PI 567598B PI and PI 430491 were considered resistant to the Volga16 colony. However, at 20 d post-infestation only PI 437696 and PI 567598B had significantly fewer aphids per plant than LD14-8039. For the first no-choice test, the results indicate that PI 437696 and PI 567598B are resistant to the Volga16 colony.

In the second no-choice test, soybean aphid densities varied significantly by soybean line and sampling day. However, the line-by-sample day interaction was not significant, and the 10 d post-infestation and 20 d post-infestation data were combined for the analysis (Table 3). Of the tested PIs, only PI 567541B had significantly fewer aphids per plant than LD14-8039 (Fig. 3b). However, PI 588000 had significantly lower soybean aphid densities than IA2104RA12 (Fig. 3b). For the second no-choice test, the results indicate that PI 567541B and PI 588000 are resistant to the Volga16 colony.

Out of the eight PIs that were tested, only four were considered resistant to the Volga16 biotype 4 colony (i.e., PI 437696 and PI 567598B, PI 567541B and PI 588000). Of these four, three significantly outperformed the LD14-8039 negative check (i.e., PI 437696, PI 567598B, and PI 567541B).

Discussion

Fifty-five soybean PIs, which were previously identified with resistance to other soybean aphid biotypes, were examined for resistance to three distinct soybean aphid biotype 4 iso-female colonies (i.e., Lomira13, Volga15, and Volga16). For the Lomira13 colony, seven (i.e., PI 437696, PI 567541B, PI 430491, PI 603712, PI 588000, PI 567250A, and PI 603339A) of the 14 soybean lines that were identified as being putatively resistant in free-choice tests were also identified as being resistant in no-choice tests (i.e., significantly fewer aphids than LD14-8039, the negative control). Of these, two (i.e., PI 437696 and PI 588000) had significantly lower soybean aphid populations than the negative check (i.e., LD14-8039). For Volga15 colony, both of the PIs that were identified as being putatively resistant in the free-choice test also were identified as being resistant in the no-choice test (i.e., PI 437696 and PI 567598B). For Volga16 colony, of the eight PIs that were identified as putatively resistant in free-choice tests four were identified as being resistant in the

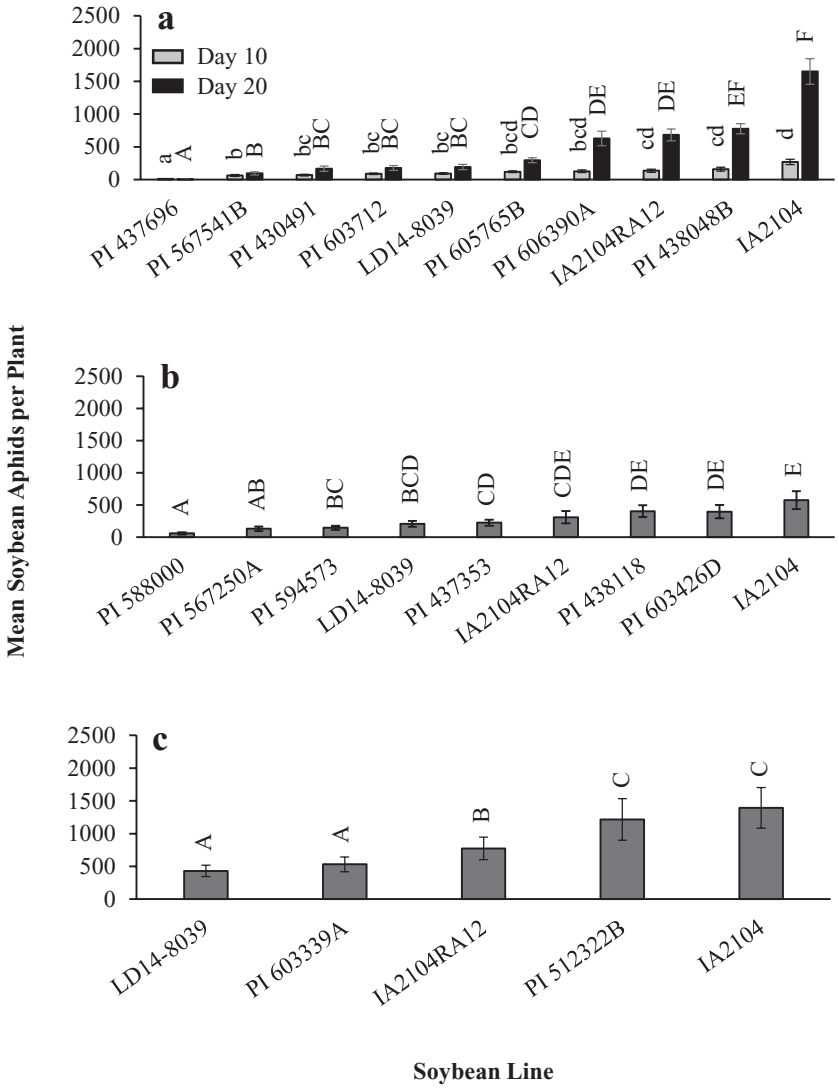

Fig. 1. Mean number (±SEM) of soybean aphids per soybean plant in three no-choice tests using the Lomira13 iso-female colony of soybean aphid biotype 4. For each test, IA2104 (susceptible) and IA2104RA12 (*Rag1*+*Rag2*) were positive checks, and LD14-8039 (*Rag3*) was a negative check. In (a), different lowercase letters indicate significant differences in means among soybean lines at 10 d post-infestation, and different uppercase letters indicate significant differences at 20 d. In (b) and (c), bars with different uppercase letters indicate means that differ significantly when combined across both days in test 2 and test 3, respectively.

no-choice tests (i.e., PI 437696 and PI 567598B, PI 567541B and PI 588000). Of these, three had significantly lower soybean aphid populations when compared to the negative check (i.e., PI 437696, PI 567598B, and PI 567541B). Of the identified resistant PIs, only PI 437696 was found to have resistance to each of the three unique soybean aphid biotype 4 colonies.

Although each soybean aphid colony was phenotypically defined as biotype 4 (i.e., able to colonize soybean plants with *Rag1*, *Rag2* or *Rag1*+*Rag2* genes), these colonies differed in their abilities to colonize a battery of PIs that were previously identified as resistant against other soybean aphid biotypes (U.S. NPGS 2017). Variation in resistance of the 55 PIs among our three soybean aphid colonies indicates that they differ in their virulence toward the *Rag* genes or other aphid-resistance genes found in these PIs. The genetics of the observed aphid-resistance characteristics have not been characterized for the majority of PIs we tested (i.e., genes or genes responsible for observed resistance). Only eight of these lines (PI 243540, PI 437696, PI 567301B, PI 567541B, PI 567598B, PI 587870, PI 588000, PI 594573) are characterized by the *Rag* genes present.

PI 243540 and PI 567301B each have a single aphid-resistance gene, *Rag2* (Rouf Mian et al. 2008) and *Rag5* (Jun et al. 2012), respectively. Previous research found the individual resistance of *Rag2* in PI 243540 and *Rag5* in PI 567301B to be ineffective against other colonies of biotype 4 that were collected near Lomira, WI (Alt and Ryan-Mahmutagic 2013, Varenhorst et al. 2017a). Similarly, these PIs did not suppress populations of any of the three biotype 4 colonies in our free-choice tests.

PI 567541B has two aphid-resistance genes, namely *rag1c* and *rag4* (Zhang et al. 2009). Although previous studies found it to be ineffective (Alt and Ryan-Mahmutagic 2013, Varenhorst et al. 2017a, Hill et al. 2017), our free-choice test results indicate that PI 567541B was resistant to the Lomira13 and Volga16 colonies, but not to the Volga15 colony. However, the results from the no-choice tests determined that PI 567541B was only resistant to the Volga16 colony. These results indicate varying responses, in terms of virulence, for the Lomira13 and Volga15 colonies toward *rag1c* and *rag4* and a lack of virulence toward one or both genes in the Volga16 colony.

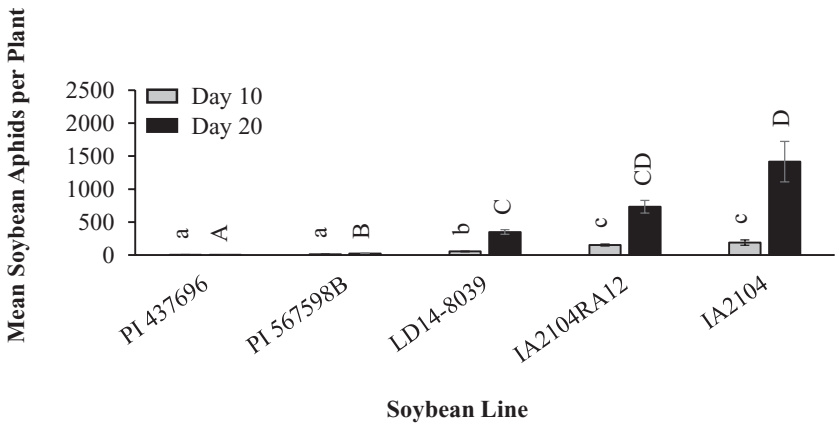

Fig. 2. Mean number (±SEM) of soybean aphids per soybean plant in a no-choice test using the Volga15 iso-female colony of soybean aphid biotype 4. Different letters indicate significant differences in means among the soybean lines at 10 (lowercase) and 20 (uppercase) days of infestation. IA2104 (susceptible) and IA2104RA12 (*Rag1*+*Rag2*) were positive checks, and LD14-8039 (*Rag3*) was a negative check.

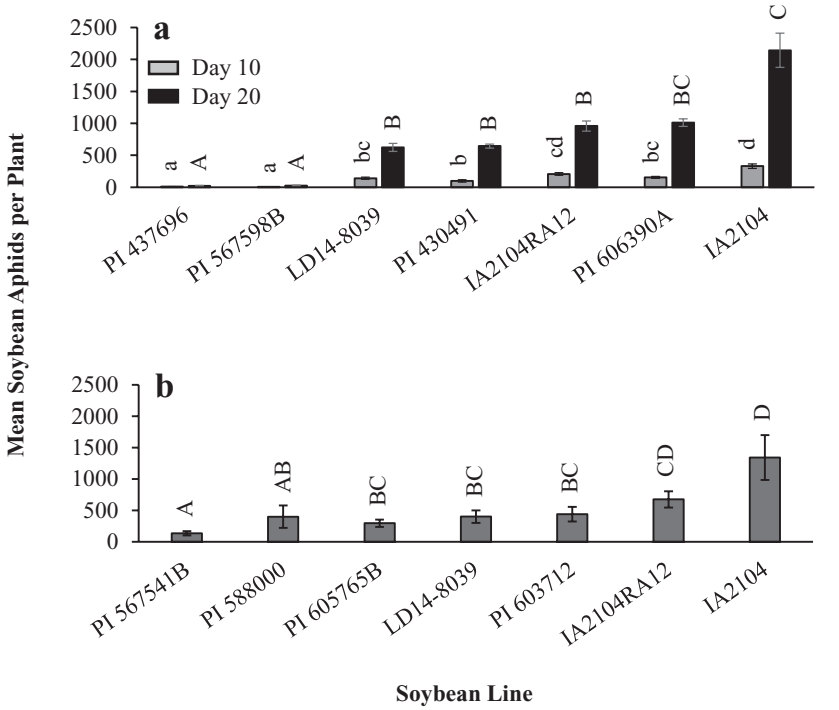

Fig. 3. Mean number (± SEM) of soybean aphids per soybean plant in two no-choice tests using the Volga16 iso-female colony of soybean aphid biotype 4. In (a), bars with different lowercase letters indicate significantly different means 10 d after infestation, and different uppercase letters indicate significant differences 20 d after infestation. In (b), bars with different uppercase letters indicate means that differ significantly when combined across sample days. IA2104 (susceptible) and IA2104RA12 (*Rag1*+*Rag2*) were positive checks, and LD14-8039 (*Rag3*) was a negative check.

PI 567598B has two aphid-resistant genes, *rag1b*, and *rag3* (Bales et al. 2013). Previous research found PI 567598B to be ineffective against other biotype 4 colonies that were collected near Lomira, WI (Alt and Ryan-Mahmutagic 2013, Hill et al. 2017). Our results showed that PI 567598B had strong resistance toward soybean aphids in the Volga15 and Volga16 colonies but lacked resistance against Lomira13 soybean aphids. Thus, soybean aphids from the Lomira13 colony appear to be virulent to *rag1b* and *rag3*, whereas Volga15 and Volga16 soybean aphids lack virulence toward one or both genes.

Resistance to soybean aphid biotype 1 was significantly associated with genetic markers for *Rag1* and *Rag2* regions in PI 437696, PI 587870, PI 588000, and PI 594573 (Fox et al. 2014). There was also significant genetic interaction between the two regions in PI 437696, PI 587870, and PI 588000 (Fox et al. 2014). However, relatively low R^2 values suggested that little of the total phenotypic variation in resistance of PI 437696 and PI 588000 against soybean aphid biotype 1 was explained by the markers associated with *Rag1* and *Rag2* (Fox et al. 2014). Additionally, our studies found PI 588000 to have significantly lower soybean aphid populations than on LD14-8039 for two of the three colonies. PI 437696 had lower soybean aphid biotype 4 populations for all three of our colonies, which matched results from a previous study (Hill et al. 2017). These findings suggest that one or more additional resistance genes may

be present in PI 437696 to account for the high levels of observed resistance.

Our results suggest that the characterization of our three colonies as biotype 4 may understate the scope of their virulence toward *Rag* / *rag* genes. Moreover, the differential responses of our three colonies toward various PIs with resistance to soybean aphids strongly suggest that a wider battery of *Rag* / *rag* genes needs to be employed against isolates of soybean aphid in the future to improve characterizations regarding the spectrum of their virulence (Zhong et al. 2014). The results of this study also suggest that in the future, soybean lines that are being bred for soybean aphid resistance should be screened against multiple different colonies of the known soybean aphid biotypes to ensure that a combination of genes is utilized that provide robust resistance with the greatest longevity.

In addition, the obviation of resistance effect that allows avirulent biotypes to feed on otherwise resistant soybean when virulent biotypes are also present on the same plant (Varenhorst et al. 2015a) suggests that a wide spectrum of resistance genes may be needed to develop effective soybean aphid-resistant soybean cultivars. Accordingly, the development and evaluation of pyramided soybean cultivars having three or more *Rag* / *rag* genes (Ajayi-Oyetunde et al. 2016, Hill et al. 2017, Varenhorst et al. 2017a, Zhang et al. 2018) may be useful for managing soybean aphids with host-plant resistance (Hill et al. 2017, Zhang et al. 2018). Operationally, a more thorough knowledge of soybean aphid responses to *Rag* / *rag* genes will improve efforts to develop soybeans cultivars with durable resistance to soybean aphid and enhance concepts about the nature of biotypes. Additional research should be conducted on PI 437696 to determine what the source is for the high level of resistance that was observed in the study against all three biotype 4 colonies.

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