

# An Assessment of Japanese Beetle Defoliation on Aphid-Resistant and Aphid-Susceptible Soybean Lines

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## ABSTRACT

As public institutions and seed companies incorporate soybean aphid (*Aphis glycines* Matsumura) resistance genes into soybean [*Glycine max* (L.) Merr.] cultivars, it is important to retain resistance to defoliators. This study compared Japanese beetle (*Popillia japonica* Newman) defoliation among *rag1b* and *rag3* aphid-resistant lines (E06901, E06905, and E06906) with a *Rag1* aphid-resistant line (LD05-16060) and aphid-susceptible lines (DKB27-53, SD01-76R, and Titan RR). Under natural insect pressure, the percentage of leaflets consumed by Japanese beetle was greater on *rag1b* and *rag3* lines (50–86%) than LD05-16060 (11%) and SD01-76R (5%). Defoliation on the three most damaged trifoliates was higher on *rag1b* and *rag3* lines (49–54%) and its aphid-susceptible parent Titan RR (35%) than on LD05-16060 (5%) and its aphid-susceptible parent, SD01-76R (1%). Similarly in laboratory choice and no-choice tests, greater leaf area was removed from *rag1b* and *rag3* lines. There was more feeding on LD05-16060 under no-choice conditions than under choice conditions, suggesting LD05-16060 was more attractive to Japanese beetle in the absence of a preferred line. At present, the differential susceptibility among these lines cannot be attributed to a specific compound or compounds until sufficient genetic and biochemical studies are conducted. This study shows the importance of monitoring Japanese beetle defoliation in breeding programs to determine the severity of threat posed by this insect on new lines.

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**Abbreviations:** MSU, Michigan State University; QTL, quantitative trait loci; *Rag*, resistance to *Aphis glycines*; TIFF, tagged image file format.

THE SOYBEAN APHID, *Aphis glycines* Matsumura, was discovered in North America in 2000 and is now a serious pest of soybean in the midwestern United States (Hill et al., 2004; Ragsdale et al., 2004). Starting in 2001, university-based soybean breeding programs rapidly began to screen soybean accessions for aphid resistance. Several sources of resistance were found, controlled by a single dominant *Rag* (resistance to *Aphis glycines*) gene, including *Rag1* (Hill et al., 2004, 2006; Li et al., 2007; Kim et al., 2010) and *Rag2* (Mian et al., 2008). At Michigan State University (MSU), Mensah et al. (2005) identified four aphid-resistant lines that originated in northern China. Resistance in one of these lines, PI 567598B, was controlled by two recessive genes (Mensah et al., 2008), currently named *rag1b*\_provisional and *rag3*\_provisional (Soybean Genetics Committee, 2009).

In 2007, a trial evaluating aphid resistance was conducted in Michigan as part of a wider multistate project. Three sister lines (E06901, E06905, and E06906), developed at MSU from PI 567598B, showed excellent aphid resistance in this trial (Chiozza, 2009). However, elevated feeding by Japanese beetle (*Popillia japonica* Newman) was observed on these lines, compared to other breeding lines and cultivars in the trial, which raised questions that resulted in the execution of the studies described in

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this paper. This was surprising because most commercial soybean cultivars have some resistance to defoliation by Japanese beetle (Hammond, 1994).

Japanese beetle is established in 28 U.S. states and Canada (NAPIS, 2009). It is a common destructive pest of turf and landscape plants, feeding on more than 300 species of wild and cultivated plants in 79 families (Potter and Held, 2002). On soybean, leaf feeding occurs from July through August in the midwestern United States, when plants are flowering and filling pods (Turnipseed and Kogan, 1976; Cook and Gray, 2004). Adults feed on tissue between leaf veins, usually of the upper and outermost leaves, leaving a characteristic lace-like appearance (Hammond, 1994; Cook and Gray, 2004). Although Japanese beetle adults are frequently present in Michigan soybean fields, feeding by this species alone is rarely enough to merit treatment (DiFonzo and Warner, 2010). Instead, producers consider overall defoliation from multiple insect species to make a treatment decision. Action thresholds for soybean defoliation in the Great Lakes region generally range from 30% to 40% prebloom, decreasing to 15% between bloom and pod fill and to 25% thereafter (Eisley and Hammond, 2007; DiFonzo and Warner, 2010; Krupke et al., 2011).

Development of soybean cultivars with insect resistance was a focus of U.S. soybean breeders for more than 30 yr (All et al., 1989). Although Japanese beetle feeding on soybean is not a severe threat in most soybean-growing regions in the United States, soybean lines were screened for defoliation as early as the 1940s. Coon (1946) assessed Japanese beetle defoliation on 26 soybean genotypes using a numerical damage scale and concluded that all were susceptible. However, based on his ratings, he confirmed that four cultivars (Chief, Viking, Illini, and Wilson) were less susceptible to Japanese beetle feeding than others. Furthermore, his studies confirmed that increased beetle feeding resulted in decreased yield. In the 1960s, the Japanese PI 229358 was one of the first found to be resistant to Mexican bean beetle, *Epilachna varivestis* Mulsant (Van Duyn et al., 1971, 1972). The PIs 229358, 171451, and 227687 showed greater resistance to this beetle in a choice test when planted with other genotypes (cultivars and lines). Furthermore, in a laboratory forced-feeding test, these same Plant Introductions were the least consumed among 29 genotypes, which Van Duyn et al. (1971) presumed to be due to either absence of feeding stimulants or presence of feeding deterrents. Mexican bean beetle feeding on these three Plant Introductions also had reduced longevity and fecundity. These three Plant Introductions were the main sources of defoliation resistance to several insects in soybean (Zhu et al., 2006, Lambert and Tyler, 1999) and served as donor parents to develop defoliation-resistant soybean in conventional breeding programs (Van Duyn et al., 1971, 1972). More recently, quantitative trait loci (QTL) identified from PI

229358 have been used in breeding programs to develop defoliation-resistant soybean lines (Zhu et al., 2006, 2007).

As public institutions and seed companies incorporate aphid-resistance genes into commercial cultivars, it is important to retain resistance to defoliators. The objective of this study was to determine whether aphid-resistant and aphid-susceptible lines had differential resistance to Japanese beetle feeding. Both field and detached-leaflet assays, under choice and no-choice conditions, were performed during the period from 2007 to 2010 with a number of aphid-resistant and aphid-susceptible lines. Japanese beetle defoliation levels were either visually assessed or calculated using digital images to determine any potential differential feeding behavior.

## MATERIALS AND METHODS

### Visual Estimates of Japanese Beetle Damage in the Field

#### Field Test 2007

In late July, defoliation was noticed in a study evaluating aphid resistance at the MSU Entomology Research Farm, East Lansing, MI. Based on scouting the only defoliator present was Japanese beetle; the pattern of damage (skeletonizing) was also consistent with Japanese beetle. This study, planted on 15 May 2007 at a rate of 253,669 seeds ha<sup>-1</sup>, consisted of 15.2 by 9.1 m 12-row plots arranged in a randomized complete block design with four replications of six soybean lines. Weeds were controlled using a combination of preemergence herbicides, Pursuit DG (102.2 mL ha<sup>-1</sup>; BASF Corporation) and Dual Magnum (105.12 mL ha<sup>-1</sup>; Syngenta), on 30 May 2007.

The aphid-resistant soybean lines evaluated were E06901, E06905, E06906, with aphid-resistance genes *rag1b*\_provisional and *rag3*\_provisional from PI 567598B, and LD05-16060 (developed by the University of Illinois), with resistance gene *Rag1* from cultivar Dowling. The aphid-susceptible lines were SD01-76R (developed by South Dakota State University), which is the aphid-susceptible parent line of LD05-16060, and DKB27-53, a commercial cultivar. On 24 Aug. 2007, the number of leaflets fed on by Japanese beetle, irrespective of the severity of damage, was recorded from four randomly selected plants per plot. The number of leaflets damaged was used to calculate the percentage of leaflets damaged per plant.

#### Field Test 2008

Japanese beetle feeding was again observed in the same study site at the MSU Entomology Research Farm. Similar to 2007, this study was planted on 13 May 2008 at a rate of 253,669 seeds ha<sup>-1</sup> in 15.2 by 9.1 m 12-row plots arranged in a randomized complete block design with four replications of six soybean lines. Weeds were controlled by a combination of Dual Magnum (225 mL ha<sup>-1</sup>; Syngenta) and Authority First DF (292 mL ha<sup>-1</sup>; FMC Agricultural Products) applied on 13 May 2008.

The aphid-resistant lines were E06901, E06905, E06906, and LD05-16060. The aphid-susceptible lines were SD01-76R and Titan RR (the aphid-susceptible parent of the three MSU lines). On 15 Aug. 2008, the three most damaged trifoliates on

four randomly selected plants per plot were visually assessed for percent defoliation by Japanese beetle. Each leaflet of each trifoliolate was assessed separately by the same observer (nine leaflets total per plant). For each plant, a mean percent defoliation was calculated based on these nine leaflets. Since the majority of the defoliation was at the top of the plant, this method concentrated on the area of the plant with the most damage.

## Digital Estimates of Japanese Beetle Feeding in the Laboratory

In addition to visual estimates in the field, Japanese beetle feeding was assessed with both choice and no-choice tests in the laboratory using digital image analysis following methods described by O'Neal et al. (2002). This method used a desktop scanner and public domain software to compare "before" and "after" images of leaflets and provide a more accurate measure of defoliation.

### Choice Test 2008

On 30 July 2008, undamaged leaflets of aphid-resistant (E06901, E06905, E06906, and LD05-16060) and -susceptible (SD01-76R and Titan RR) lines were collected from the site of 2008 field test. From each line, 20 fully expanded trifoliates were collected from the top of 20 different plants; all plants were in the flowering (R1–R2) stage (Fehr and Caviness, 1977). The petiole of each leaflet was labeled with a unique identifier before scanning. The leaflets were scanned on a Hewlett-Packard Officejet Pro L7680 desktop scanner operating on a Dell Optiplex 755 computer. The original color images were saved as tagged image file format (TIFF) files for digital conversion to measure total leaf area. After scanning, petioles were wrapped with a moist piece of cotton. One leaflet from each of the six lines was randomly placed on a moist paper towel in a 20 by 30 cm aluminum foil cake pan. Twelve Japanese beetles were then placed on the moist paper towel (not on leaflets) in each pan. These beetles were collected by hand earlier the same day from an asparagus (*Asparagus officinalis* L.) field on the MSU campus and held in a cooler for 5 to 6 h before the experiment. Each pan was closed with a tight-fitting plastic lid to prevent escapes, and pans were left at room temperature under natural indirect light. After 48 h, individual leaflets were rescanned and saved as TIFF files for digital conversion to measure leaf area fed on by beetles. There were a total of 20 replicates (pans).

### Choice Test 2010

The choice test was repeated on 12 Aug. 2010 with only two aphid-resistant lines, each differing in the source of aphid resistance (E06906 and LD05-16060), and their aphid-susceptible parents (Titan RR and SD01-76R, respectively). To represent the *rag1* and *rag3* aphid-resistant lines in this study, only E06906 was included since E06901, E06905, and E06906 were sister lines arising from the same parent material (PI 567598B and Titan RR). A small block of each line was planted in early June to serve as a source of leaf material. From each line, 10 undamaged fully expanded trifoliates were collected from the top of 10 different plants; all plants were in the flowering (R1–R2) stage. Using the same methods for the 2008 choice test, leaflets from each of the four lines were placed in cake pans and

**Table 1. Soybean lines used for assessing Japanese beetle feeding in East Lansing, MI, 2007 through 2010.**

Soybean line or cultivar	Aphid resistance gene(s)	Field test		Choice test		No-choice test
		2007	2008	2008	2010	2008
E06901	<i>rag1b</i> and <i>rag3</i>	x	x	x	–	–
E06905	<i>rag1b</i> and <i>rag3</i>	x	x	x	–	–
E06906	<i>rag1b</i> and <i>rag3</i>	x	x	x	x	x
LD05-16060	<i>Rag1</i>	x	x	x	x	x
SD01-76R	none	x	x	x	x	x
DKB27-53	none	x	–	–	–	–
Titan RR	none	–	x	x	x	x

exposed to Japanese beetle feeding for 48 h. Before and after digital images were created as previously described.

### No-Choice Test 2008

A no-choice test was done to determine if Japanese beetles fed on nonpreferred lines (LD05-16060 and SD01-76R) in the absence of preferred lines (E06906 and Titan RR). The source of the leaf material was the same as the 2008 choice test. A list of lines and cultivar used for each test is given in Table 1.

From each line, 10 fully expanded trifoliates were collected from the top of 10 different plants on 30 July. Undamaged leaflets were labeled with a unique identifier and then scanned. Each detached leaflet was placed individually in a 15 by 150 mm diam. petri dish for a total of 40 dishes. Adult Japanese beetles were hand collected from an asparagus field that same day and held in a cooler for 5 to 6 h. Two beetles were placed in each petri dish for 48 h. Then leaflets were rescanned and images were saved as TIFF files for digital conversion to measure leaf area removed.

### Using Digital Images to Measure Leaf Area

To create digital images from scanned leaflets, the method described by O'Neal et al. (2002) was followed using the public domain software Scion Image 4.0.3 (Scion, 2001) to measure the surface area of leaflets in a digital format. The "before" images (TIFF) were converted from color to black and white. These converted images were used to measure total leaf area in square centimeters from leaflets before Japanese beetle feeding. The amount of leaf area removed was measured from inverted black and white "after" images (TIFF) in square centimeters. Total leaf area removed was calculated using measurements of the total surface area before feeding, and leaf area removed after feeding from each leaflet. Total leaf removed in square centimeters was used for analysis rather than the percent of leaf area consumed, because the leaflets of one line (SD01-76R) were significantly smaller than the leaflets of other lines ( $p < 0.01$ ).

### Data Analyses

Data from each field and laboratory test were analyzed separately with ANOVA in PROC MIXED and mean separations were computed using the LSMEANS statement ( $p = 0.05$ ) using SAS software version 9.1.3 (SAS Institute, 2004). A Satterthwaite solution was included in PROC MIXED to handle unequal variances where appropriate.

**Table 2. Visual estimates of Japanese beetle feeding on soybean aphid-resistant and aphid-susceptible soybean lines in a field trial in East Lansing, MI (2007 and 2008).**

Soybean line or cultivar	Aphid resistance gene(s)	Percent leaflets fed on in 2007	Percent defoliation on nine leaflets in 2008
E06906	<i>rag1b</i> and <i>rag3</i>	86.2 ± 3.7 a†	53.6 ± 5.3 a
E06905	<i>rag1b</i> and <i>rag3</i>	58.6 ± 6.9 b	49.7 ± 6.7 a
E06901	<i>rag1b</i> and <i>rag3</i>	49.9 ± 7.2 c	49.4 ± 4.3 a
Titan RR	none	NA†	34.6 ± 4.5 b
DKB27-53	none	15.0 ± 6.1 d	NA
LD05-16060	<i>Rag1</i>	11.1 ± 2.7 de	5.2 ± 1.8 c
SD01-76R	none	5.2 ± 1.2 e	1.2 ± 0.7 d

† Within each year, means followed by different letters are significantly different ( $p < 0.05$ ).

‡ NA, not applicable.

## RESULTS AND DISCUSSION

### Visual Estimates of Japanese Beetle Damage in the Field

In 2007, the percentage of leaflets damaged by Japanese beetle in the field differed significantly among soybean lines ( $p < 0.01$ , Table 2). The three MSU aphid-resistant lines had a greater percentage of leaflets fed on (50–86%) compared to LD05-16060 (11%) and aphid-susceptible (SD01-76R, 5%, and DKB27-53, 15%) lines. Among the MSU lines, E06906 had significantly more leaflets damaged than E06905 and E06901. Similar results were obtained in 2008, with significantly greater defoliation of leaflets on the MSU lines (49–54%) and their parent, Titan RR (35%), compared to the *Rag1* line (5%) and its parent (1%) ( $p < 0.01$ ; Table 2). These visual assessments provided evidence of differential feeding on the aphid-resistant and aphid-susceptible material.

### Digital Estimates of Japanese Beetle Feeding in the Laboratory

#### Detached-Leaflet Choice Tests

In the 2008 choice test, a significant difference in Japanese beetle feeding was observed among soybean lines after 48 h in the laboratory ( $p < 0.01$ ; Fig. 1). Significantly more leaf area was removed from E06901, E06905, E06906, and Titan RR than from LD05-16060 or its aphid-susceptible parent (Fig. 2). E06901 leaflets were the most defoliated, and among the MSU aphid-susceptible and -resistant lines, feeding on E06901 was significantly more than on E06906 or Titan RR, the aphid-susceptible parent of the MSU lines. Defoliation on Titan RR was significantly greater than feeding on LD05-16060 or SD01-76R but not statistically different from feeding on two of the three MSU aphid-resistant lines. All the MSU lines had a greater percentage (90–100%) of leaflets fed on by Japanese beetle compared to the University of Illinois lines (50 and 70%). The choice test provided additional evidence that the MSU

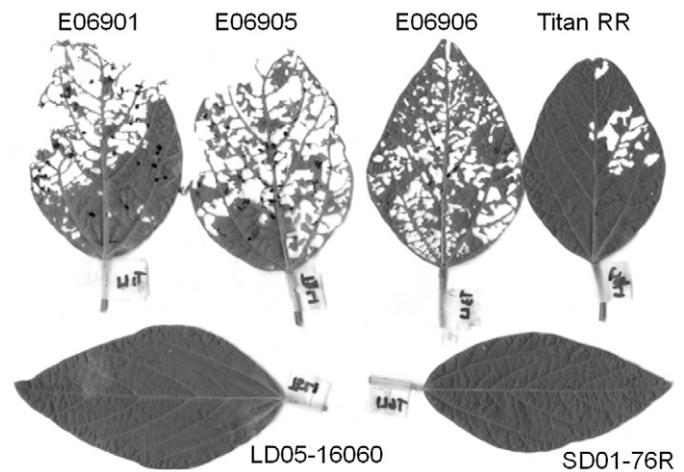


Figure 1. Digital images of leaflets from six soybean lines exposed to Japanese beetle for 48 h in a choice test in 2008. Top row from left to right: E06901, E06905, E06906 with *rag1b* and *rag3* aphid resistance, and Titan RR, the susceptible parent of these lines. Bottom left: LD05-16060, with *Rag1* aphid resistance. Bottom right: SD01-76R, the susceptible parent of LD05-16060.

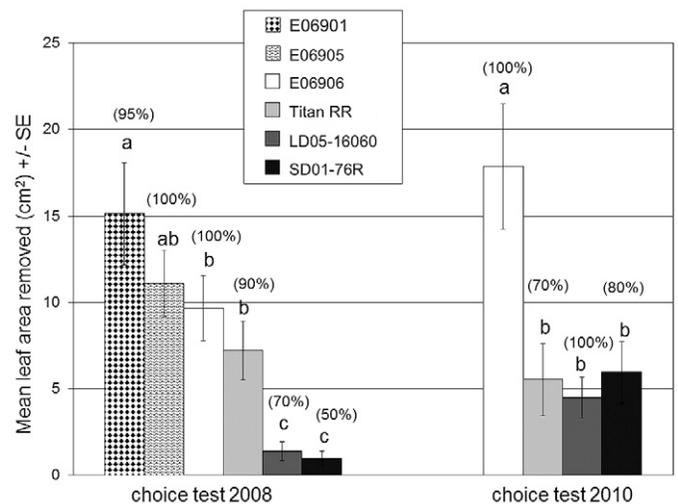


Figure 2. Mean leaf area (cm<sup>2</sup>) removed by Japanese beetle on leaflets of aphid-resistant and aphid-susceptible soybean lines in choice tests in 2008 and 2010. Within each year, means with different letters are significantly different ( $p < 0.05$ ; LSD). The value in parenthesis above each bar indicates the percentage of leaflets fed on out of the total leaflets (20 in 2008 and 10 in 2010) exposed to Japanese beetle.

aphid-resistant lines and Titan RR were more susceptible to Japanese beetle than the *Rag1* line and its parent.

In the 2010 choice test, a significant difference in mean leaf area removed was again observed after 48 h ( $p < 0.01$ ; Fig. 2). As in 2008, leaflets of the MSU aphid-resistant line had significantly more leaf area removed (17.8 cm<sup>2</sup> ± 3.6 SE) compared to LD05-16060, SD01-76R, and even Titan RR (4.5–6.0 cm<sup>2</sup>). All leaflets of the MSU aphid-resistant line were fed on, in contrast to 70% of Titan RR and 80% of SD01-76R leaflets. All LD05-16060 leaflets were also fed on. Overall, the amount of feeding per leaflet in the

2010 choice test was greater compared to the 2008 choice test. For example, leaf area removed from E06906 averaged 9.5 cm<sup>2</sup> in 2008 and 17.8 cm<sup>2</sup> in 2010. Similarly, leaf area removed was just over 1.0 cm<sup>2</sup> for LD05-16060 and SD01-76R in 2008 and 4.5 to 6.0 cm<sup>2</sup> in 2010. This increase may have been an artifact of the test in 2010, which used the same size of cake pans, same number of beetles, and same 48 h exposure as in 2008 but two-thirds less leaf tissue from the MSU aphid resistant lines (one E06906 leaflet in 2010 compared to three E06901, E06905, and E06906 leaflets in 2008). After the E06906 leaflet was heavily defoliated, beetles may have started to feed on the less preferred LD05-16060 and SD01-76R leaflets. Nevertheless, the 2010 choice test continued to show preference for the MSU aphid-resistant line over other lines in the test.

### Detached-Leaflet No-Choice Test

In the no-choice test, a significant difference in feeding were observed among the four soybean lines ( $p = 0.03$ ; Fig. 3). E06906 had significantly greater defoliation than SD01-76R ( $p = 0.01$ ) and Titan RR ( $p = 0.02$ ; Fig. 3). The mean leaf area removed on LD05-16060 was numerically but not statistically different from E06906 in this test ( $p = 0.09$ ). However, only nine replications from each of LD05-16060 and SD01-76R were considered for this data analysis, as some leaflets from these two lines showed unhealthy appearance (turned yellow during trial period). As in the choice tests, all leaflets of E06906 and LD05-16060 had some feeding, but some Titan RR and SD01-76R leaflets were untouched. This test provided more evidence that the MSU aphid-resistant line(s) still suffered more damage by Japanese beetle compared with other lines.

Under choice and no choice conditions and using different methods measuring number, percentage, or area (cm<sup>2</sup>) of leaflets eaten, the MSU aphid-resistant lines

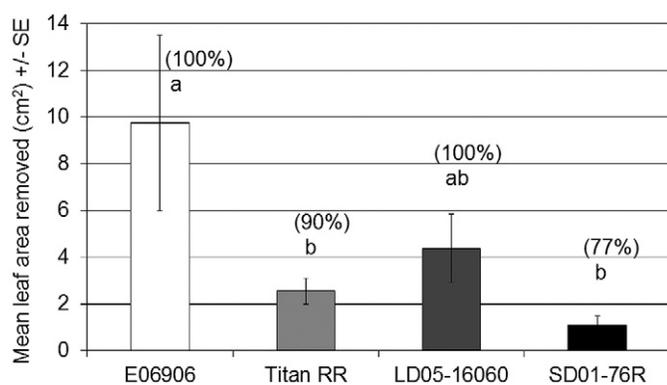


Figure 3. Mean leaf area (cm<sup>2</sup>) removed by Japanese beetle on leaflets of aphid-resistant and aphid-susceptible soybean lines in a no-choice test in 2008. Means with different letters are significantly different ( $p < 0.05$ , LSD). The value in parenthesis above each bar indicates the percentage of leaflets fed on out of the total leaflets exposed to Japanese beetle.

tested in this study were consistently more preferred by Japanese beetle. This confirmed the first visual observation of elevated Japanese beetle feeding in the field in 2007. There was more feeding on LD06-16060 under no-choice conditions than under choice conditions, suggesting LD05-16060 was more attractive to Japanese beetle in the absence of a preferred line. Nevertheless, LD05-16060 continued to show significantly less beetle defoliation than MSU aphid-resistant lines under choice conditions in both the field assessments and in detached-leaflet assays.

It does not appear that *Rag1* aphid resistance is genetically linked to defoliation resistance, because both LD05-16060 and its susceptible parent SD01-76R were less preferred by beetles. However, it cannot be concluded that defoliation susceptibility is genetically linked to *rag1b* and *rag3* aphid resistance. MSU aphid-resistant lines and their susceptible parent Titan RR all suffered moderate to high levels of defoliation (although Titan RR had statistically less feeding than one or more MSU lines in each study), suggesting that Japanese beetle susceptibility could be inherited from the aphid-susceptible Titan RR. However, a firm conclusion on the origin of Japanese beetle susceptibility in MSU aphid-resistant lines cannot be made until both parents of MSU aphid-resistant germplasm are monitored for feeding. Therefore measuring Japanese beetle defoliation also on the aphid-resistant parents, Dowling (source of *Rag1*) and PI 567598B (source *rag1b* and *rag3*), would help in determining the potential linkage between aphid-resistant sources and defoliation.

The basis for differential Japanese beetle susceptibility among the tested lines is likely biochemical, not physical. Volatiles are released when herbivores feed on plants, and these chemicals may in turn trigger aggregation of the defoliating species. Common volatiles induced by Japanese beetle feeding are euginols, geraniols, jasmynes, and phenyl-acetonitriles (Loughrin et al., 1996a, 1996b). Japanese beetles locate hosts primarily by olfaction, so soybean lines with more feeding in this study could have naturally elevated levels of these volatile compounds compared to less preferred lines. Once a Japanese beetle finds a host, host selection occurs by olfaction and/or by chemoreception (taste) (Potter and Held, 2002). In general, several plant-derived sugars, including fructose, glucose, maltose, and sucrose, are strong phagostimulants for Japanese beetle (Ladd 1987, 1988; Potter and Held, 2002). In soybean specifically, secondary plant compounds such as flavonoids, isoflavonoids, and phytoalexins serve as feeding deterrents to herbivorous insects (Caballero et al., 1986; Chen, 2008; Dakora, 1995, Hart et al., 1983; Treutter, 2006), including Japanese beetle. At present, the differential susceptibility of the soybean lines tested in this study cannot be attributed to a specific compound or compounds until sufficient biochemical analyses are conducted.

In addition to understanding the biochemical mechanisms for differential susceptibility by Japanese beetle, it is important to understand the genetic mechanisms leading to differential susceptibility to defoliators. Quantitative trait loci conferring resistance to several soybean defoliators were recently reported (Rector et al., 2000; Zhu et al., 2006, 2008). Moreover, Yesudas et al. (2010) identified QTL from seven chromosomes conferring resistance specifically to Japanese beetle in a recombinant inbred population. Ongoing research at MSU on a breeding population derived from E06906 (*rag1b* and *rag3*) × LD05-16060 (*Rag1*) identified potential QTL for Japanese beetle resistance (Chandrasena et al., 2012). Candidate genes involved in the flavonoid biosynthesis pathway were found within these QTL regions (unpublished data, 2012). Therefore, flavonoids may play a key role in explaining differential defoliation by Japanese beetle on these aphid-resistant lines.

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