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PLANT RESISTANCE

Effects of Rag1 on the Preference and Performance of Soybean Defoliators

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ABSTRACT The Rag1 gene confers antibiotic resistance to soybean aphid, Aphis glycines Matsumura (Hemiptera: Aphididae), and in 2010, varieties expressing Rag1 were released for commercial use in the United States. We do not know how Rag1 varieties will influence the broader community of defoliating insects that inhabit soybean fields. In 2010 and 2011, the preference and performance of pest insects that defoliate soybeans [Glycines max (L.) Merr] were tested using Rag1 and aphid-susceptible varieties. Three coleopterans and four lepidopterans were used: northern corn rootworm, Diabrotica barberi Smith & Lawrence (Coleoptera: Chrysomelidae); southern corn rootworm, Diabrotica undecimpunctata howardi Barber (Coleoptera: Chrysomelidae); bean leaf beetle, Ceratoma trifurcata Förster (Coleoptera: Chrysomelidae); fall armyworm, Spodoptera frugiperda (J. E. Smith) (Lepidoptera: Noctuidae); corn earworm, Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae); soybean looper, Chrysodeix includens (Walker) (Lepidoptera: Noctuidae); and velvet-bean caterpillar, Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae). The preference of insects was evaluated in choice and no-choice tests using Rag1 and susceptible soybeans. Lepidopterans also were evaluated on Rag1 leaves using four nutritional indices: relative growth rate, approximate digestibility, and efficiency of conversion of ingested material. In the majority of preference tests, no effect of Rag1 was detected, and in cases where preferences were found, there was no consistent pattern of preference for Rag1 vs. susceptible leaf tissue. Helicoverpa zea demonstrated a preference for resistant leaf tissue, but this was dependent on the genetic background of the variety. Evaluations of nutritional indices indicated that three species of Lepidoptera, S. frugiperda, H. zea, and A. gemmatalis, displayed reduced conversion efficiency for Rag1 soybeans, suggesting effects of antibiosis.

KEY WORDS host-plant resistance, nutritional index, Rag1 gene, preference, soybean

Since its arrival in 2000, the invasive soybean aphid, Aphis glycines Matsumura (Hemiptera: Aphididae), has been recognized as an economically important pest of soybean in the United States (Venette and Ragsdale 2004). This insect has the ability to reduce plant height, pod set, seed size, and the amount of protein in seeds (Ragsdale et al. 2011) and has been known to reduce yields as much as 40% (Ragsdale et al. 2007). Before the arrival of the soybean aphid in the United States, <0.1% of soybean acreage in the North Central region reported any application of insecticide in the North Central region reported any application of insecticide to soybean crops (National Agricultural Statistics Service–U.S. Department of Agriculture [NASS/USDA] 2006). In 2006, >13% of soybean acreage was treated with insecticides (NASS/USDA 2006, Ragsdale et al. 2011). After the appearance of the soybean aphid, research was conducted to improve insecticide-based management (Myers et al. 2005, Johnson et al. 2009) and to establish an economic threshold for this pest (Ragsdale et al. 2007). Efforts were also made to discover sources of host-plant resistance in soybean. Host-plant resistance to insects operates through one of three mechanisms: antixenosis, antibiosis, and tolerance. Antixenosis, also referred to as nonpreference (Kogan and Ortman 1978, Panda and Khush 1995), is defined as a group of plant characters and insect responses that lead the insect away from the use of a particular plant variety for oviposition, food, shelter, or any combination of these. Antibiosis is defined as reduced fecundity, size, longevity, or survival as a result of consuming plant tissue. Tolerance is the ability of a host plant to preserve yield after suffering injury from herbivorous insects (Panda and Khush 1995, Schoonhoven et al. 2005).

Antixenosis and antibiosis have been discovered through germplasm screening in several soybean varieties (Hill et al. 2004, Díaz–Montano et al. 2006,
Hesler et al. 2007, Li et al. 2007), but aphid-resistant soybeans that are commercially available use only antibiotic in the form of the Rag1 gene (O’Neal and Johnson 2010). Hill et al. (2004) characterized the Dowling, Jackson, and PI 71,506 soybean varieties as being resistant to soybean aphid. Hill et al. (2004, 2006) also described the source of resistance in Dowling as a single, dominant gene that they designated Rag1. While expression of Rag1 produces antibiotic against the soybean aphid, the exact mechanism of this resistance is currently unknown.

Soybeans in the United States are attacked by several species of folivorous insects, and many of these have been the subject of research on host-plant resistance. Soybean pests such as corn earworm, Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae); soybean looper, Chrysodeix includens (Walker) (Lepidoptera: Noctuidae); and velvetbean caterpillar, Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) have been studied to find resistant varieties (Rowan et al. 1990, Warrington et al. 2008, Pinbelli et al. 2009). Research has also been conducted on varieties with resistance against the Japanese beetle, Popillia japonica Newman (Coleoptera: Scarabaeidae), and bean leaf beetle, Ceratoma trifurcata Förster (Coleoptera: Chrysomelidae), which are known for their ability to injure leaf tissue, reduce seed quality, and act as a disease vector (Delate et al. 2008, Kunihiko et al. 2010, Yesudas et al. 2010). Although it is clear that host-plant resistance against some of these pest insects exists in soybeans, we do not know how Rag1 will influence these species.

As Rag1 varieties become common in soybean production in the United States, growers may experience additional benefits if these varieties have antibiotic or antixenotic effects on additional pest species. However, Rag1 soybeans could complicate pest management in soybeans if they suffer greater feeding injury from other folivorous pest insects. The purpose of this study was to identify what preference, if any, leaf-feeding pests have for soybean varieties where Rag1 is present. This study also looked at how the presence of Rag1 influenced rates of leaf consumption and larval growth for larvae of four lepidopteran species found in soybeans.

**Materials and Methods**

**Plant Tissue.** Leaf tissue used in this study was collected from plants grown in the field and greenhouse. Field-grown leaf tissue was collected at the Neely–Kinyon and Johnson research farms of Iowa State University during the summer seasons of 2010 and 2011. Whole, undamaged leaflets were taken from soybean plants at the R5 stage (Fehr and Caviness 1977). Before use in an experiment, field-grown leaf tissue was stored in plastic, resealable bags and held at ~4°C for 24 h. Plants were grown in a greenhouse bay from July to September 2010 and April to September 2011 (26°C, range 18–37°C, 50% relative humidity [RH] range 32–80%; and a photoperiod of 16.8 [L:D] h), and leaf tissue was collected between the V4 and R3 stages; leaf tissue collected from greenhouse grown plants was used immediately in experiments. The six resistant varieties used in this study, which ranged between maturity groups 2.5 and 3, were: Res1 (LD05-1637), Res2 (Blue River Hybrids 29AR9), Res3 (Syngenta S25-F2), Res4 (Blue River Hybrids 25AR1), Res5 (LD05-16060, near isoline to Sus5), and Res6 (IA3027-RAG1, near isoline to Sus6). The six aphid-susceptible varieties used, also ranging between maturity groups 2.5 and 3, were Sus1 (Syngenta S25-T8), Sus2 (Blue River Hybrids 30A7), Sus3 (Syngenta S25-R3), Sus4 (Blue River Hybrids 26F0), Sus5 (SD76R, near-isoline to Res1), and Sus6 (IA3027, near-isoline to Res6). Varieties Res1, Res2, Sus1, and Sus2 were used in 2010, and the other eight varieties were added the following year to expand inferences made into the effect of Rag1 on preference and performance of the insects.

**Insects.** Coleoptera used in this study were collected during the summer of 2010; northern corn rootworm, Diabrotica barberi Smith & Lawrence (Coleoptera: Chrysomelidae), southern corn rootworm, Diabrotica undecimpunctata howardi Barber (Coleoptera: Chrysomelidae), and the bean leaf beetle, C. trifurcata were collected via sweep netting from soybean fields at Iowa State University research farms in Floyd, Cherokee, and Hancock counties. C. trifurcata, an occasional pest of soybean, is known to injure soybean leaves, stems, and pods as well as spread bean pod mottle virus (Giesler et al. 2002, Bradshaw et al. 2010). The two species of Diabrotica studied here are subeconomic pests in soybeans but are commonly found in corn–soybean cropping systems. In addition, the closely related western corn rootworm Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae) can display resistance to crop rotation and disperse into soybean fields where it feeds on leaf tissue before depositing eggs (Dunbar and Gassmann 2013). Insects were maintained on an artificial diet (western corn rootworm diet, Bio-Serv, Frenchtown, NJ) in a growth chamber (25°C; 65% RH; and a photoperiod of 16.8 [L:D] h).

S. frugiperda, H. zea, Ch. includens, and A. gemmatalis, were purchased from Benzon Research (Carlisle, PA), and these insects were allowed to develop to the third instar on the artificial diet (multiple species lepidopteran diet, Southland Products Incorporated, Lakeville, AR). They were separated to minimize any density-dependent stress or cannibalism. Each of these four species is a pest of soybeans throughout the southern and southeastern United States (Buschman et al. 1977, Young 1979, Walker et al. 2000). Initial data indicated that lepidopterans preferred feeding on Rag1 plants, thus, additional experiments focused primarily on Lepidoptera.

**Choice Tests.** Leaf disks (10 mm²) were excised from whole leaflets using a size 6 cork borer. Insects were offered one leaf disk from a resistant variety and one from a susceptible variety. The experimental unit consisted of a petri dish (diameter = 10 cm) lined with filter paper (#2 Whatman) moistened with enough deionized water to soak the filter paper but leaving no
standing water. Leaf disks were placed in the petri dishes with a distance of 2 cm between disks. Insects were placed equidistant from each disk and given 48 h to feed. *D. barberi, D. undecimpunctata howardi, C. trifurcata,* and *S. frugiperda* were evaluated on two varieties of *Rag1* soybeans (Res1 and Res2) and two varieties of aphid-susceptible soybeans (Sus1 and Sus2) using leaf tissue collected from the field. These preference tests were repeated 40 times. *S. frugiperda* and *H. zea* were evaluated against Res1 to Res6 and Sus1 to Sus6. *A. gemmatalis* and *C. includens* were evaluated against two pairs of *Rag1* and susceptible soybeans with the same genetic background (Res5 and Sus5; and Res6 and Sus6). Each test was repeated 32 times.

No-Choice Tests. The methodology of no-choice tests closely followed the choice tests. Leaf disks were excised and placed in a petri dish with moistened filter paper as in the choice tests, however, because only one leaf tissue disk was offered to an insect in a no-choice test, each disk was placed directly in the center of the dish. When an insect was introduced to the petri dish, it was placed on the filter paper surrounding the disk. As with choice tests, the insect was allowed 48 h to consume the leaf tissue and was then removed. An insect was used only once in a no-choice test.

In both choice and no-choice tests, once insects were removed from the petri dish, leaf tissue area consumed was measured using a digital scanner (HP ScanJet G4050 flatbed scanner, Hewlett-Packard Company, Palo Alto, CA) and image analysis software (Adobe Photoshop CS5.1, Adobe Systems Incorporated, San Jose, CA).

Tests of Performance. Relative growth rate, approximate digestibility, and efficiency of conversion of ingested materials (Waldbauer 1968, Raubenheimer and Simpson 1992, Beaupre and Dunham 1995, Raubenheimer 1995) were tested for *S. frugiperda, H. zea,* *C. includens,* and *A. gemmatalis* on the Res5, Res6, Sus5, and Sus6 varieties. The initial wet mass of insects and leaf tissue were measured to the nearest 0.001 mg on a microbalance (XS205 Dual Range analytical balance, Mettler Toledo, Columbus, OH). The unit of replication was a petri dish (10 cm²) lined with moistened filter paper. A single larva was placed in a petri dish and received one whole leaflet (average 0.34 g) on the first day and at least 0.2 g of additional leaf tissue each following day. Larvae were allowed to feed for 7 d, after which larvae were weighed. Then, larvae and the remaining leaf tissue were dried for 4 d at 60°C and weighed. The evaluation for each species consisted of 32 replications.

For tests of performance, the mass of egested material, the mass of leaf tissue consumed, and the mass of leaf tissue digested were calculated. The mass of egested materials was calculated as the difference between the dry mass of filter paper at the start of the experiment and the dry mass of filter paper and frass after the 7 d period. The mass of leaf tissue consumed was calculated as the difference between an average of whole dried leaf tissue disk and final dry mass of leaf tissue remaining.

Statistical Analysis. Choice tests were analyzed using a Student’s t-test (PROC TTEST, SAS 9.2, SAS institute Inc., Cary, NC). No-choice tests were analyzed with an analysis of variance in PROC GLM (SAS Institute 2008). Tests of performance were analyzed with an analysis of covariance (ANCOVA) (PROC GLM). Relative growth rate was tested with final larval dry mass as the response variable, soybean variety as categorical variable, and initial wet mass as a covariate. Efficiency of conversion of ingested leaf tissue was analyzed with the response variable of final dry mass of the larvae, the categorical variable of soybean variety, and the covariate of the mass of leaf tissue consumed. Data were log-transformed as needed to meet the assumption for normality.

Results

Choice Tests. Of the coleopteran species evaluated, only *D. barberi* demonstrated a significant preference, consuming more *Rag1* soybean than susceptible soybean leaf tissue when given the choice of the Res2 and Sus2 varieties (Table 1), whereas *D. undecimpunctata howardi* and *C. trifurcata* demonstrated no preference. Among the lepidopteran species, *H. zea* demonstrated a preference for Sus1 (Table 1); however, this insect also demonstrated a preference for Res5 and Res6 (Table 1). *C. includens* showed preference for the Sus6 variety over its resistant counterpart (Table 1), and *S. frugiperda* did not show preference for any variety offered.

No-Choice Tests. In no-choice testing, *S. frugiperda* consumed more of Res2 when compared with the Res1 and Sus1 varieties (Fig. 1; Table 2). *C. trifurcata* also consumed more Res2 compared with Res1 and Sus1 varieties (Fig. 1; Table 2). No other insects included in this experiment demonstrated any preference for or against *Rag1* soybeans.

Tests of Performance. Analysis of relative growth rate shows that *S. frugiperda, H. zea,* and *A. gemmatalis* had significantly lower dry mass on *Rag1* leaf tissue when corrected for initial wet mass of the larvae (Fig. 2; Table 3). Analysis of the conversion efficiency of ingested materials showed that all three of these insects had significantly lower larval dry mass, when corrected for consumption, on at least one resistant variety (Fig. 3; Table 3), suggesting the presence of antibiosis. *C. includens* did not display any significant effects in this set of experiments.

Discussion

The results of this study imply that *Rag1* soybean has little if any effect on feeding preference of adult Coleoptera and larval Lepidoptera but does have antibiotic based resistance to some lepidopteran larvae. Introducing resistance genes (*e.g., Rag1*) into elite lines can have unintended effects if linkage transfers additional genes from the original resistant variety into the elite line. These effects may be difficult to predict without a complete knowledge of the genetic background of the parents (Campbell et al. 2002, Kim and
Fig. 1. No-choice tests for (A) *S. frugiperda* consuming soybeans from unrelated backgrounds, (B) *C. trifurcata* consuming soybeans from unrelated backgrounds, (C) *H. zea* consuming resistant and susceptible varieties from unrelated backgrounds, and (D) *H. zea* consuming resistant and susceptible varieties from the same genetic background.
Diers 2008). Panthee et al. (2006) found several quantitative trait loci associated with methionine, glutamine, leucine, and serine on the same molecular linkage group that the Rag1 gene occupies. The composition of amino acids is also influenced by the presence of the Rag1 gene (Chiozza et al. 2010), and this interaction may affect the performance of herbivores. In the field, effects of resistant or susceptible tissue may depend upon the species of the insect and the genetic background of the soybean variety.

Research into host-plant resistance against soybean defoliators has documented antibiotic effects similar to those seen in our study. Hammond et al. (2001) observed that adult beetles had reduced feeding on foliar tissue when tested against lines known to slow the growth and increase the mortality of chrysomelid larvae. Soybean varieties known to produce antibiosis against several noctuid moths, including tobacco budworm, Heliothis virescens, and cabbage looper, Trichoplusia ni, inhibit digestion through plant secondary compounds and in the case of A. gemmatalis, increase susceptibility to multiple nucleopolyhedrosis virus (Pinelli et al. 2009). Soybean varieties with resistance to H. zea and Ch. includens reduced larval mass and leaf defoliation, but these varieties often produced significantly lower yields than conventional varieties (Warrington et al. 2008).

In our study, we found that S. frugiperda, H. zea, and A. gemmatalis experienced reduced larval mass on Rag1 varieties when corrected for initial wet mass and amount of leaf tissue consumed. The reduction in relative growth rate and conversion efficiency of ingested materials indicates that these insects did not convert Rag1 tissue into larval biomass as efficiently as susceptible leaf tissue, signifying an effect of antibiosis (Fig. 3; Table 3; Panda and Khush 1995, Ahn et al. 2011). Previous research has suggested that the use of detached leaves could result in a reduction in the effect of Rag1 in soybean leaf tissue (Michel et al. 2010). Michel et al. (2010) found that the activity of resistance in detached leaves was dependent upon the variety used in their experiment, and the results of the tests of performance conducted in this study indicate that, at least in the case of these resistant and suscep-

<table>
<thead>
<tr>
<th>Species</th>
<th>Varieties offered</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. trifurcata</td>
<td>Res1, Sus1, Res2, Sus2</td>
<td>3, 74</td>
<td>2.60</td>
<td>0.0567</td>
</tr>
<tr>
<td>D. barberi</td>
<td>Res1, Sus1, Res2, Sus2</td>
<td>3, 74</td>
<td>1.38</td>
<td>0.2359</td>
</tr>
<tr>
<td>D. undecimpunctata howardi</td>
<td>Res1, Sus1, Res2, Sus2</td>
<td>3, 69</td>
<td>1.49</td>
<td>0.2227</td>
</tr>
<tr>
<td>S. frugiperda</td>
<td>Res1, Sus1, Res2, Sus2</td>
<td>3, 60</td>
<td>14.59</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Res3, Sus3, Res4, Sus4</td>
<td>3, 60</td>
<td>2.14</td>
<td>0.1043</td>
</tr>
<tr>
<td></td>
<td>Res5(^b), Sus5(^b), Res6(^b), Sus6(^b)</td>
<td>3, 124</td>
<td>0.18</td>
<td>0.9081</td>
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<tr>
<td>H. zea</td>
<td>Res1, Sus1, Res2, Sus2</td>
<td>3, 60</td>
<td>2.56</td>
<td>0.0634</td>
</tr>
<tr>
<td></td>
<td>Res3, Sus3, Res4, Sus4</td>
<td>3, 60</td>
<td>0.77</td>
<td>0.5472</td>
</tr>
<tr>
<td></td>
<td>Res5(^b), Sus5(^b), Res6(^b), Sus6(^b)</td>
<td>3, 60</td>
<td>2.76</td>
<td>0.0499</td>
</tr>
</tbody>
</table>

\(^a\) Leaf tissue offered are from the resistant varieties (1) LD05-1637, (2) Blue River Hybrids 29AR9, (3) Syngenta S25-F2, (4) Blue River Hybrids 25AR1, (5) LD05-16060, and (6) IA3027-RAG1. Susceptible leaf tissue was taken from the varieties (1) Syngenta S25-T8, (2) Blue River Hybrids 30A7, (3) Syngenta S25-R3, (4) Blue River Hybrids 26F0, (5) SD76R, and (6) IA3027.

\(^b\) Leaf tissue produced within a greenhouse set to 80°C, 50% RH, and a photoperiod of 16:8 (L:D) h; all other plants were grown in the field.

![Fig. 2. Analysis of relative growth rate for (A) S. frugiperda, (B) H. zea, (C) A. gemmatalis, and (D) Ch. includens when consuming resistant and susceptible varieties that shared the same genetic background. Pairs with the same number (e.g., Res5 and Sus5) share the same genetic background. No significant difference was detected for the growth rate of Ch. includens.](image-url)
tible near-isolines, the presence of this gene had a significant effect on nutritional indices using detached leaf tissue. Further research should examine if the effects seen in this study are present with whole plants.

In the field, antibiotic effects on lepidopteran larvae might be manifest as a reduced number of noctuid larvae on soybean plants. Field populations consuming \( \text{Rag1} \) varieties may produce fewer or smaller adults and population growth rates may be lowered. In addition, larvae on \( \text{Rag1} \) plants may be more susceptible to natural enemies (Price et al. 1980). Although the maturity of the plants used in this study would not be grown across the full habitat range for the noctuid pests studied here, the presence of effects from \( \text{Rag1} \) in multiple genetic backgrounds indicates that the results of these experiments would likely extent to other varieties. Future research directions could evaluate the effects of \( \text{Rag1} \) on lepidopteran larvae in the field.

The possible effects of \( \text{Rag1} \) offer several potential benefits to growers managing aphid populations; however, it is important to take into account the composition of defoliators in a given field and the effects this gene may have on preference as well as performance. It is also important to consider this work in light of different soybean aphid biotypes (Kim et al. 2008, Hill et al. 2010). As the durability of \( \text{Rag1} \) is reduced by virulent aphid biotypes, growers will continue to look for sources of host-plant resistance that can reduce damage by soybean aphid populations, and research is already being undertaken to identify new aphid-resistant soybean varieties (Bansal et al. 2013). Future research should assess effects on non-target insects for these novel forms of host-plant resistance.

Table 3. Analysis of variance for nutritional indices measured with larval Lepidoptera offered \( \text{Rag1} \) and susceptible soybean leaf tissue

<table>
<thead>
<tr>
<th>Species</th>
<th>Nutritional index</th>
<th>df</th>
<th>( F^b )</th>
<th>( P )</th>
<th>( F^d )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S. \ frugiperda )</td>
<td>RGR</td>
<td>1, 98</td>
<td>13.21, ***</td>
<td>0.11, NS</td>
<td>3.79, *</td>
<td>0.001, NS</td>
</tr>
<tr>
<td></td>
<td>ECI</td>
<td>1, 98</td>
<td>12.35, ***</td>
<td>1.73, NS</td>
<td>3.98, *</td>
<td>1.59, NS</td>
</tr>
<tr>
<td>( H. \ zea )</td>
<td>RGR</td>
<td>1, 98</td>
<td>13.33, ***</td>
<td>3.95, *</td>
<td>0.69, NS</td>
<td>0.88, NS</td>
</tr>
<tr>
<td></td>
<td>ECI</td>
<td>1, 58</td>
<td>12.13, ***</td>
<td>1.54, NS</td>
<td>6.12, *</td>
<td>1.16, NS</td>
</tr>
<tr>
<td>( A. \ gemmatalis )</td>
<td>RGR</td>
<td>1, 34</td>
<td>8.70, **</td>
<td>11.51, ***</td>
<td>0.001, NS</td>
<td>3.19, *</td>
</tr>
<tr>
<td></td>
<td>ECI</td>
<td>1, 34</td>
<td>8.03, **</td>
<td>10.24, **</td>
<td>0.34, NS</td>
<td>0.001, NS</td>
</tr>
</tbody>
</table>

* Asterisks indicate the significance of each \( F \)-statistic. Each factor was found to be significant at the \( P < 0.01(*) \), \( P < 0.05(**) \), \( P < 0.001(***) \), or not significant (NS).

\( F \)-statistic for the effect of \( \text{Rag1} \).

\( F \)-statistic for the genetic background of the soybean variety.

\( F \)-statistic of the interaction between genetic background and \( \text{Rag1} \).

\( F \)-statistic of the associated covariate of each analysis.

Fig. 3. Conversion efficiency of ingested materials by (A) \( S. \ frugiperda \), (B) \( H. \ zea \), and (C) \( A. \ gemmatalis \) when fed resistant and susceptible varieties that share a genetic background. Pairs with the same number (e.g., Res5 and Sus5) share the same genetic background.
Acknowledgments

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