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***Rag1* soybean photosynthetic response to Biotype 2 aphid colonization and feeding**

Katy Hillard

April 1, 2017

Project format: Formal write-up of literature review, research results and conclusions
Objective: Fulfill requirement for MS project towards MS degree in Entomology

Abstract

Over the past ten years, the soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), has been one of the principal insect threats to commercial soybean, *Glycine max* (L.) Merr., production in the United States. Resistance genes identified from native soybean have been incorporated into commercial varieties to provide protection against aphid feeding damage. The biology of the pest lends itself to rapid buildup of biotypes capable of overcoming host plant resistance. Single gene resistance sources have displayed low levels of durability against aphids. The first aphid resistant commercial soybean line released in the United States contained the *Rag1* gene, which was almost immediately overcome by Biotype 2 aphids. Aphid resistant varieties containing *Rag1* have demonstrated increased yield compared to susceptible non-*Rag1* soybean varieties when infested with Biotype 2 aphids, indicating some level of a tolerance response. Photosynthetic capacity in non-*Rag1* soybeans has been observed to be significantly reduced by soybean aphid feeding. The photosynthetic impact of Biotype 2 aphids on *Rag1* soybeans has not been previously characterized. This experiment explored and compared photosynthetic capacity in *Rag1* and non-*Rag1* soybeans as impacted by Biotype 2 soybean aphids to determine whether higher photosynthetic capacities could be responsible for the tolerance observed in *Rag1* lines. While significant photosynthetic reductions were caused by Biotype 2 infestation, *Rag1* did not appear to provide any protection to photosynthesis or seed count in this instance, thus it cannot be determined whether photosynthesis plays a role in tolerance previously observed. Further studies are necessary to address this question.

Introduction

Soybean aphids (*Aphis glycines)*, native to Asia, emerged as a significant pest of United States (U.S.) soybean production in the early 2000's (Hartman et al., 2001) with observed yield losses as high as 50% (Wang et al., 1994), and commercial production loss estimates upwards of $2.4 billion (Song et al., 2006). Native, plant-based resistance is an integral part of an effective integrated pest management system and can offer environmental benefits through reduction of foliar-applied insecticides. Decreasing insecticide applications can reduce occurrence of insect-based insecticide resistance and preserve natural predatory insects within the field ecosystem (Thomas and Waage 1996). By the mid-2000's, multiple varieties were identified as having antibiosis resistance to soybean aphids, and *Rag1*, a single dominant gene from cultivar Dowling was the first to be characterized (Hill et al., 2006). Characterization enabled incorporation into commercial breeding programs, and the first aphid resistant varieties containing *Rag1* became commercially available in 2010 (Bruner et al., 2013).

In temperate regions, such as the North Central/Midwestern U.S., soybean aphids complete their lifecycle between two hosts (heteroecious), soybeans and buckthorn (*Rhamnus* spp.) (Tilmon et al., 2008). In the fall, soybean aphids complete the sexual phase of their lifecycle on primary host buckthorn, resulting in a genetically recombined progeny deposited as eggs, which overwinter (holocyclic), and in the spring/summer, nymphs emerge and remain on buckthorn where they feed to maturity (Tilmon et al., 2008). Winged adults (alatae) then migrate to soybean fields where parthenogenic reproduction characterized by telescoping generations ensues (Hartman et al., 2001). This reproductive strategy enables rapid buildup of clonal offspring, with aphid populations doubling every 1.5-2 days during optimal conditions (McCornack et al., 2004). The genetic diversity post sexual phase has enabled virulent populations to colonize large plantings of resistant soybean material quickly.

Predictably, virulence was observed shortly after initial field testing of *Rag1* material began (Kim et al., 2008), which was several years prior to commercial release of *Rag1* lines (Cooper et al., 2015). Soybean aphid populations with the ability to overcome *Rag1* are characterized as a unique biotype – Biotype 2 (Kim et al., 2014, Hill et al., 2009). As commercial *Rag1* varieties became popular with growers, Biotype 2 was observed more frequently colonizing commercial fields throughout the U.S. soybean growing region, and it became evident that, despite aphid populations exceeding the Economic Injury Level (EIL) of 675 ± 95 aphids per plant (Ragsdale et al., 2007), yield of *Rag1* varieties was not as severely impacted as susceptible (non-*Rag1*) varieties under similar aphid pressures. As a result, *Rag1* varieties have been described as displaying tolerance to soybean aphid Biotype 2 (Hesler et al., 2013).

Soybean aphid infestation and feeding causes reductions in soybean photosynthetic rates, which results in decreased yield (Macedo et al., 2003). Photosynthesis for *Rag1* and non-*Rag1* soybeans in the presence and absence of Biotype 2 soybean aphids has not been previously compared. Photosynthesis at the leaf level can be observed by collecting gas exchange data and plotting net CO_2 assimilation (A) against the concentration of intercellular CO_2 within the leaf (C_i) and modeling the response. Fitting a model to gas exchange data in this way allows estimation of V_{cmax} [maximum carboxylation rate allowed by ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco)], *J* [rate of photosynthetic electron transport (based on NADPH requirement)], *TPU* (triose phosphate use), *R*d (day respiration) and *g*m (mesophyll conductance). These are key metrics for exploring leaf-level photosynthesis (Sharkey et al., 2007).

Objectives

Rag1 soybeans have been described as displaying tolerance to soybean aphid Biotype 2 due to their ability to produce higher rates of grain as compared to non-*Rag1* lines under similar aphid pressures. The objective of this project was to observe whether photosynthetic differences can be detected between *Rag1* and non-*Rag1* soybeans in the presence and absence of Biotype 2 soybean aphids.

Methods

This experiment was designed during the fall 2013 semester in collaboration with researchers at Monsanto and was executed between December 2013 and February 2014. Data was analyzed between August 2016 and March 2017.

Soybean germplasm

Six F_4 soybean sister lines were selected from Monsanto's soybean breeding program, three *Rag1* lines and three non-*Rag1* lines. Presence/absence of the gene was evaluated through genotyping and phenotyping via SNP (TaqMan) molecular marker testing for the trait and growth chamber antibiosis assay using Biotype 1 aphid cultures, respectively. All lines thought to be *Rag1* positive tested positive for the molecular marker, while non-*Rag1* lines tested negative for the marker.

Phenotyping was performed in an individual plant antibiosis assay where seeds were pre-germinated in germination paper for three days and then planted into four inch pots of Fafard 4M potting media (Sun Gro Horticulture, Agawam, MA). Pots were placed in a Conviron (Winnipeg, Canada) growth room where water was provided twice daily via subirrigation. Each pot was over-planted with three seeds to ensure one healthy plant would be available for use in the assay. After approximately seven days of growth, plants had reached V1 stage with fully expanded unifoliate leaves, and pots were thinned down to one healthy plant each. At this stage, five apterous reproductive aphids were transferred to each plant using a fine point hobby paint brush, and cages were added such that infested plants were individually enclosed. Biotype 1 aphids used in the assay were taken from a colony maintained in a Conviron growth chamber at Monsanto (Saint Louis, MO) on a commercially available susceptible (non-*Rag1)* Asgrow soybean line.

Infested plants were allowed to incubate for approximately four weeks, after which ratings were performed based a 0-4 scale described by Mensah, et al (2005), where 0 = zero aphids, 1 = 1-100 aphids, 2 = 101-300 aphids, 3 = 301-800 aphids, and 4 = greater than 800 aphids (Table 1). Five replications were tested in the assay, and the mean rating was used for confirming presence or absence of *Rag1.* Lines were designated resistant if a mean rating less than 2 was observed, and susceptible if a rating greater than 3 was observed. All three *Rag1* lines (lines 1, 2, and 3) were confirmed to be resistant and thus *Rag1* positive, while two of the three non-*Rag1* lines (4 and 5) were found to be susceptible and thus *Rag1* negative, and one (line 6) displayed a moderately resistant reaction with a mean rating of 2.5. Data was collected on line 6 for the entirety of the experiment, yet it was omitted from the analysis (Table 2).

Table 1. Aphid rating scale based on number of aphids present per plant. Rating scale described by Mensah, et al (2005)

Rating	Aphid Count
0	0
1	1-100
2	101-300
3	301-800
4	> 800

Aphid cultures

Monsanto acquired Biotype 2 soybean aphids used in the study from Dr. Curtis Hill at the University of Illinois in 2011. Aphids were reared at Monsanto campus (Saint Louis, MO) in a Conviron (Winnipeg, Canada) growth chamber on *Rag1* positive soybean variety, Dowling until used in the study (25ºC; 60 ± 10% RH; 16-hour photoperiod). Biotype 2 aphids are virulent to *Rag1* containing soybeans, and were expected to colonize both *Rag1* and non-*Rag1* material in the study at similar rates. Biotype 1 aphids are avirulent to *Rag1* soybeans, meaning that they cannot colonize *Rag1* soybeans. Biotype 1 was not chosen for the study because the objective of the study was to determine whether *Rag1* provided protection to photosynthetic capacity when under infestation by Biotype 2, or *Rag1* virulent soybean aphids.

Experimental design

Six soybean lines – 3 *Rag1* and 3 non-*Rag1* were tested in eight replications in two treatments (infested and uninfested), as shown in Table 2.

Seeds of the six soybean test lines were pre-germinated in germination paper envelopes for three days in the absence of light. Vigorous seeds with an emerged radicle were sown in eight inch pots containing Metro-Mix potting media (Sun Gro Horticulture, Agawam, MA). Eight replications of each line were planted for each of two treatments, one to be infested with Biotype 2 soybean aphids and an uninfested control. Plants were grown to V1 stage (first trifoliate fully expanded) in a growth room at Monsanto Saint Louis, MO (25ºC; 60 ± 10% RH; 16-hour photoperiod). Plants were then moved to an adjacent bench within the same growth room and into a bench-top Frame-World (Lake In The Hills, IL) insect cage enclosed on all sides with No-Thrips insect screen (Green-Tek, Edgerton, WI) and with plexiglass tops to allow light to penetrate cage from the top. Irrigation was applied via drip irrigation tubes to maintain desired soil moisture levels.

After placement into the cage, each plant was then inoculated with a small piece of infested leaf tissue containing approximately 10 apterous soybean aphids each (infested treatment only). Infested leaf material was taken from the Biotype 2 culture described above. One week after aphids were applied, it

was evident that the infestation was not effective, and a second round of infestation was performed. At the time the study was executed, the aphid colony used had been maintained on soybeans in the asexual phase of their lifecycle for approximately two years in a growth chamber. Lack of introduction of genetic diversity through sexual reproduction and the resulting high genetic homogeneity of the aphid colony coupled with the sudden movement of aphids from the colony chamber to the larger chamber where the study was executed likely contributed to lack of success in the first inoculation attempt. The growth chambers were programmed at identical set-points, yet biological organisms are often influenced by the subtlest of environmental changes. Prior to the second aphid infestation attempt, infested colony "stock" plants were enclosed in insect netting and transferred to the study chamber and allowed 48 hours to acclimate to the new environment before inoculation in order to increase chances of successful infestation.

The uninfested control plants were treated with Aria (active ingredient Flonicamid) via spray application on the same day that infestation occurred to ensure aphid feeding did not occur on control plants. Flonicamid offers long-term (2-3 week) systemic control against aphids and other piercing/sucking insects by disruption of feeding behavior (Morito et al., 2007). No evidence has been found in literature review indicating that Flonicamid impacts plant growth in any way.

Aphids were allowed to colonize the infested treatment for 30 days. After the 30-day interval, plant observations, aphid counts, and photosynthesis measurements using a LI-6400XT (Li-Cor, Lincoln, NE) were collected. Following data collection, plants were returned to cages and were grown to full maturity. Seeds were harvested and productivity (seed count) data collected.

Table 2. Two treatments of six soybean F_4 sister lines replicated eight times were planted for use in the study. Two lines were omitted based on quality control measures and plant growth issues. The final experimental design included two *Rag1* and two non-*Rag1* lines replicated four times in two treatments.

Soybean Line	Gene Class	Treatment	Targeted Replications	Actual Replications Measured	Comments
1	Rag1	Infested	8	4	
2	Rag1	Infested	8	4	
3	Rag1	Infested	8	1	Could not be used due to plant growth issues
4	Non-Rag1	Infested	8	4	
5	Non-Rag1	Infested	8	4	
6	Non-Rag1	Infested	8	4	Data omitted – gene class not confirmed by phenotyping
1	Rag1	Uninfested	8	4	
2	Rag1	Uninfested	8	4	
3	Rag1	Uninfested	8	1	Could not be used due to plant growth issues
4	Non-Rag1	Uninfested	8	4	
5	Non-Rag1	Uninfested	8	4	
6	Non-Rag1	Uninfested	8	4	Data omitted – gene class not confirmed by phenotyping

Photosynthesis measurements

Four Li-Cor 6400XT units were used to collect data for this experiment to allow for all measurements to be gathered on the same day. The top-most fully expanded trifoliate was consistently selected for measurement in each test plant. Plants were at R4-R5 growth stage when measured, which is the most critical period for determination of soybean yield, as dry matter is accumulating rapidly. Plants are said to be at R4, also known as full pod when they have developed one pod measuring ¾ inch long at one of top four nodes on the soybean main stem. Stage R5 soybeans, also known as beginning seed stage, have developed one seed that is 1/8 inch long on one of the top four nodes of the plant ("Growth stages: Growth and development : Soybean Production : University of Minnesota Extension", 2017).

Selected leaflets from the infested treatment were brushed gently with a fine hobby paint brush to remove any existing aphids and exoskeletons before measurement. Uninfested leaflets targeted for measurement were also gently brushed in the same way before clamping the Li-Cor chamber to reduce the variability between treatments.

CO_2 assimilation to intercellular CO_2, or A-Ci curves were collected using the A-Ci protocol as programmed on the Li-Cor 6400XT. CO_2 concentrations chosen to build the curve were: 400, 250, 100, 50, 0, 400, 650, 900, 1100, 400 ppm. A-Ci measurements were conducted at Photosynthetically Active Radiation (PAR) levels set to 750 µMol photons/ m^2/ s.

Data analysis

Infestation levels

Infestation rates between *Rag1* and non-*Rag1* lines were compared using a one-way ANOVA to test the hypothesis that no significant difference will be detected based on presence or absence of the gene. Analysis was conducted across sister lines in the same gene class. According to principles of inbred plant breeding, F_4 sister lines are 94% genetically homozygous (Singh & Singh, 2015).

Soybean productivity and gas exchange

An Excel curve fitting tool developed by Bernacchi was used where "plots of photosynthesis (A) vs. leaf intercellular [CO2] (Ci) were used to solve for Vc, max and Jmax using the equations of Farquhar et al., (1980). When necessary, measurements were corrected to 25° C using the temperature responses of Bernacchi et al., (2001) and Bernacchi (2003) for the Rubisco and RuBP-limited portions of the A vs. Ci curves, respectively, following the method outlined in Long and Bernacchi (2003)." Explanation quoted as recommended by Bernacchi in the tool information section.

Seed count and photosynthetic capacity (maximum rates of carboxylation, V_{cmax} and maximum rate of electron transport, J_{max}), as well as several photochemical measurements collected by the Li-Cor at 1100 CO_2 were compared based on presence or absence of the *Rag1* gene, treatment, and infestation level of infested treatment. Description of all metrics used for analysis can be found in Table 3. Measurements resulting from 1100 ppm CO_2 were used because it was the highest concentration used in A-Ci protocol, and at nearly triple the Earth's ambient CO_2 level, provides a significant test of the study plant photosynthetic capacity.

Comparisons considered:

The main hypothesis of this study was that *Rag1* soybean photosynthetic capacity and productivity would be increased as compared to non-*Rag1* soybeans when both gene classes were under similar colonization and aphid pressure. To test this hypothesis, infested Rag1 and non-Rag1 soybeans were compared using a one-way ANOVA based on photosynthesis metrics described above and seed count. The hypothesis would be supported if *Rag1* soybeans will produce significantly higher seed count and

have higher photosynthetic capacity (V_{cmax} and J_{max}) than non-*Rag1* lines despite similar aphid infestation rates.

Seed counts and photosynthetic capacity were also compared for uninfested *Rag1* and non-*Rag1* soybeans using a one-way ANOVA. This was a control experiment to test the hypothesis that no significant difference in yield or photosynthetic capacity will be observed based on gene class in healthy, uninfested soybeans.

A secondary experiment, another type of control experiment, was performed to compare uninfested and infested treatments across gene classes regardless of presence or absence of *Rag1.* The hypothesis that aphid infestation would significantly reduce soybean productivity (seed count) and photosynthesis across gene classes was tested based on knowledge that aphids significantly reduce soybean yield in the field (Wang et al., 1994), and have been observed to significantly reduce photosynthetic capacity (Macedo et al., 2003). One-way ANOVA was used to test this hypothesis. In addition to treatment effect across gene classes, treatment effects were also compared within each gene class. The hypothesis was that within each gene class, once again, aphid infestation would significantly reduce photosynthetic capacity and seed count.

All data analysis was performed using JMP version 12.0 software (JMP®, Version 12. SAS Institute Inc., Cary, NC).

Dataset Attrition and Quality Control

Data attrition occurred based on unexpected factors inherent in working with biological systems in addition to targeted removal of data to control quality of the dataset. This experiment was designed for data collection on 8 replications of each genotype in each treatment. Attrition occurred with all genotypes due to plant health and quality, suboptimal aphid infestation, and physical damage due to movement of plants out of cage to measurement staging area. As a result, data was collected on 4 reps of each genotype or line in each treatment, with plant growth complications occurring in one line resulting in survival of only one plant per treatment (line 3 as indicted in Table2).

Further attrition occurred during quality control before data analysis, as some replications were found to show severely negative intercellular CO_2 concentrations. This was likely a result of excessive scrubbing during A-Ci curve data collection (equipment operator error) causing relative humidity (RH) to be extremely low causing an effect on stomatal conductance. Reps found to have negative Ci values were all found to have been subjected to RH less than 30%, and these reps were removed from the dataset. Three additional plant measurements were removed due to an error in A-Ci protocol execution where Li-Cor light setpoints were inaccurately set to 500 µMol photons/ m²/ s where the rest of the measurements were taken at PAR of 750 µMol photons/ m²/ s.

While these errors during gas exchange data collection resulted in a reduced dataset for data analysis relating to photosynthetic capacity, these data concerns did not impact aphid infestation levels or

soybean productivity (seed count) measurements. Infestation and soybean productivity data was analyzed by full dataset, and analysis was repeated a second time to include only reps with higher quality gas exchange data. This allowed a look at the more robust dataset as well as, for consistency purposes, the reduced dataset on which gas exchange analysis was performed. Results were included for both datasets (see Results section and Tables 4 and 5). Gas exchange data analysis was performed on only the high quality, reduced dataset. Throughout the paper, non-*Rag1* material is synonymous with "no gene" germplasm class.

Plant quality in the growth chamber was sufficient for growth, aphid infestation, and reproduction, yet plant vigor was reduced as compared to plants grown in the field. A parallel field growth experiment was not performed using the same varieties, yet a general observation of plants used in this study is that they were less hearty and vigorous than typical field-grown soybeans. Plexiglass topped insect cages were used to enable as much light to penetrate plants as possible, and this result was much improved as compared to plants grown in full insect cages which tend to greatly reduce light and produce weak and spindly plants. While plants were noted to be less vigorous than typical field-grown soybeans, experimental design successfully maximized the plant quality within the confines of the facilities used to fulfill the experiment objectives.

Table 3. Photochemical metrics used in this study to assess photosynthetic performance of soybean plants based on genetics and/or infestation by soybean aphids.

Metric	Description	Units	Source
Photo	Carbon exchange rate; Photosynthetic Rate	µmoles/m²/s	Measured by Li-Cor 6400xt
Ci	Intercellular CO_2 Concentration	µMol/mol	Measured by Li-Cor 6400xt
Cond	Conductance to H_2O	Mol/m²/s	Measured by Li-Cor 6400xt
ETR	Electron Transport Rate	µmoles/m²/s	Measured by Li-Cor 6400xt
PhiCO_2	Apparent Quantum Yield of CO_2 assimilation	Ratio	Measured by Li-Cor 6400xt
PhiPS2/PhiCO_2	Quantum efficiency of CO_2 fixation; Ratio of electrons passed through PSII per CO_2 fixed; Operating Quantum Efficiency (Phi PSII) / Apparent Quantum Yield (PhiCO_2)	Ratio	Calculated using Li-Cor data
Fv'/Fm'	Maximum light adapted PSII efficiency	Ratio	Measured by Li-Cor 6400xt
PhiPSII	Apparent Quantum Yield of Photosystem II	Ratio	Measured by Li-Cor 6400xt
qN	Non-Photochemical Quenching	Number	Measured by Li-Cor 6400xt
qP	Photochemical Quenching	Number	Measured by Li-Cor 6400xt
TE	Transpiration Efficiency; Photo/Trans (umol CO_2/mol H_2O transpired)	Ratio	Calculated using Li-Cor data
Trans	Transpiration rate	µmol/m²/s	Measured by Li-Cor 6400xt
WUE	Instantaneous Water Use Efficiency; Photo/Cond (umol CO_2/mol H_2O)	Ratio	Calculated using Li-Cor data
Vcmax	Maximum rate of carboxylation allowed by ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco)	µmol/m²/s	Product of A-Ci curve fitting process
Jmax	The maximum photosynthetic rate of a C_3 A-Ci curve	µmol/m²/s	Product of A-Ci curve fitting process

Results

Aphid Infestation

Aphid infestation rates did not reach the Economic Injury Level (EIL) of 675 ± 95 aphids per plant (Ragsdale et al., 2007) during the study. Although the EIL was developed based on field conditions, the levels observed in this study were two to three times lower. As shown in Table 4, mean counts per plant on *Rag1* and non-*Rag1*, or "no gene" germplasm (± SE) were 127 (± 62.70) and 196 (± 122.26) aphids respectively. Mean aphid numbers for the reduced data set were 280 (± 6.32) and 360 (± 136.83) for *Rag1* and non-*Rag1* material respectively. Analysis using a one-way ANOVA indicated that no significant difference existed between aphid infestation levels on *Rag1* and non-*Rag1* soybeans with $P = 0.5976$ for the full dataset and $P = 0.7214$ for the reduced dataset used for the gas exchange data analysis.

Seed count

As shown in Table 5, within the infested treatment, no significant difference was found in seed count based on presence or absence of *Rag1* gene ($P = 0.7009$), with the mean number of seeds per plant produced by *Rag1* and non-*Rag1* soybeans in the full dataset (± SE) being 60 (± 5.87) and 45 (± 13.60) seeds respectively. In the reduced dataset, no significant difference was detected in seed count based the presence or absence of the *Rag1* gene in the infested treatment ($P = 0.5218$), with seed count per plant observed to be 68 (± 9.50) and 54 (± 15.50) seeds for *Rag1* and non-*Rag1* respectively.

The uninfested treatment produced a mean (± SE) of 43 (± 4.97) and 48 (± 3.93) seeds per plant for *Rag1* and no gene germplasm respectively, and seed count was not found to be significantly impacted by presence or absence of *Rag1* gene ($P = 0.4305$). Gas exchange data QC measures did not impact replication numbers for the uninfested treatment, and as a result, only one analysis was performed.

As shown in Table 5, differences in seed count between infested versus uninfested lines across both gene classes were also not significant at the $\alpha = 0.05$ level set for the study with a P value just greater than 0.05 at $P = 0.0588$. The infested treatment actually produced a greater mean number of seeds per plant compared to the uninfested treatment.

Table 4. Soybean aphid infestation level (aphid count) for *Rag1* and non-*Rag1* (no gene) material in Infested treatment shown by full dataset (all) and reduced dataset. Reduced dataset only was used for gas exchange data analysis in this study.

	Infested		
	No gene	**Rag1**	*P*[a]
	Mean ± SE		
Aphid Count - full data	196 ± 122.26	127 ± 62.70	0.5976
Aphid Count - reduced	360 ± 136.83	280 ± 6.32	0.7214

[a] *P* value for ANOVA observing impact of presence or absence of *Rag1* on aphid infestation (aphid count) for infested treatment

Table 5. Soybean productivity (seed count) for *Rag1* and non-*Rag1* (no gene) material for Infested and Uninfested treatments shown by full dataset (all) and reduced dataset. Reduced dataset only was used for gas exchange data analysis in this study.

	Infested (61 ± 6.23, n=4)			**Uninfested (46 ± 3.46, n=13)**			***P*[c] = 0.0588**
	No gene	**Rag1**		**No gene**	**Rag1**		
	Mean ± SE		*P*[a]	Mean ± SE		*P*[b]	
Seed Count - all	45 ± 13.60	60 ± 5.87	0.7009	48 ± 3.93	43 ± 4.97	0.4305	
Seed Count - reduced	54 ± 15.50	68 ± 9.50	0.5218	N/A	N/A	N/A	

[a] *P* value for ANOVA observing impact of presence or absence of *Rag1* on productivity (seed count) for infested treatment

[b] *P* value for ANOVA observing impact of presence or absence of *Rag1* on productivity (seed count) for uninfested treatment

[c] *P* value for ANOVA comparing impact of treatment (infested or uninfested) on productivity (seed count)

Gas Exchange

***Rag1* vs. non-*Rag1* comparison by Treatment**

One-way ANOVA assessing gas exchange metrics as impacted by presence or absence of *Rag1* resulted in no significant differences for both infested and uninfested treatments as analyzed separately (Appendix 1). A-Ci curves are shown for infested treatment and uninfested treatment in Figure 1 and Figure 2 respectively. Standard error for Photosynthesis (A) are much higher in Infested treatment, particularly for *Rag1* material (Figure 1). Figure 2 shows very little difference between A-Ci curves for *Rag1* and no gene material in infested treatment.

Figure 1. Mean Photosynthesis by Intercellular CO2 concentration for *Rag1* and no gene material within Infested treatment

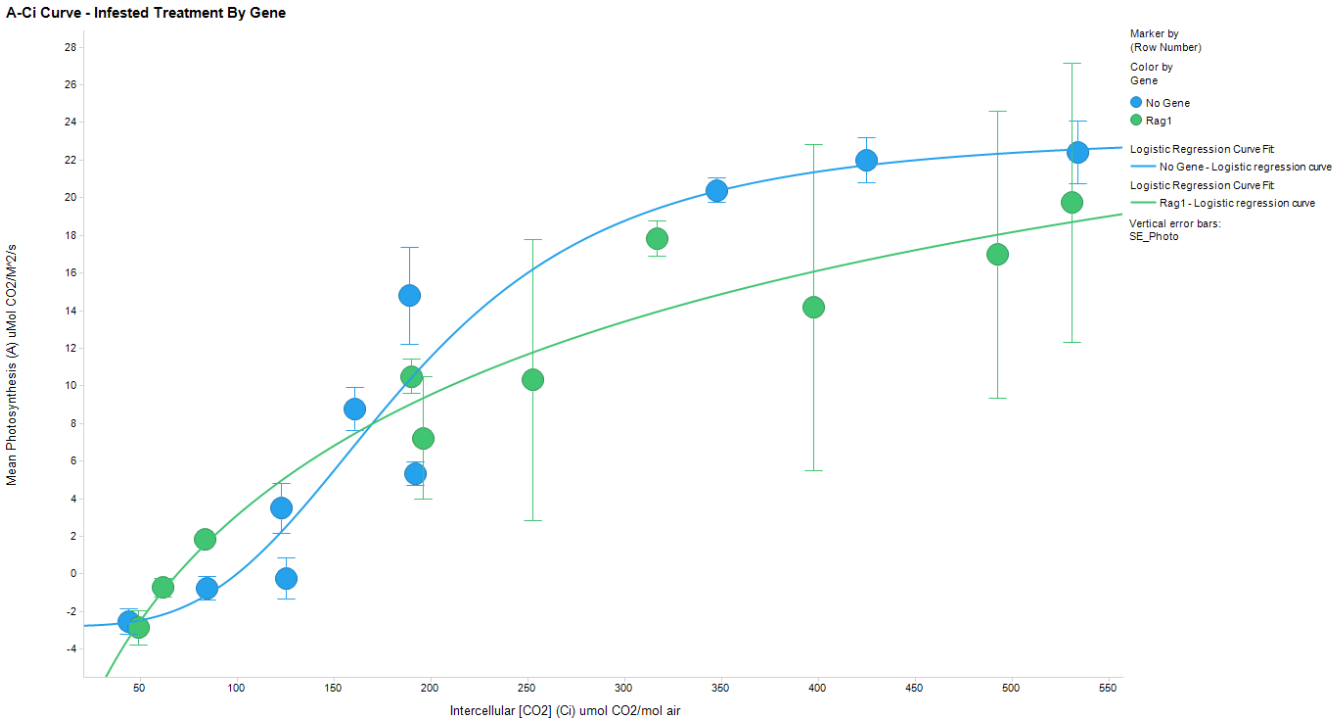
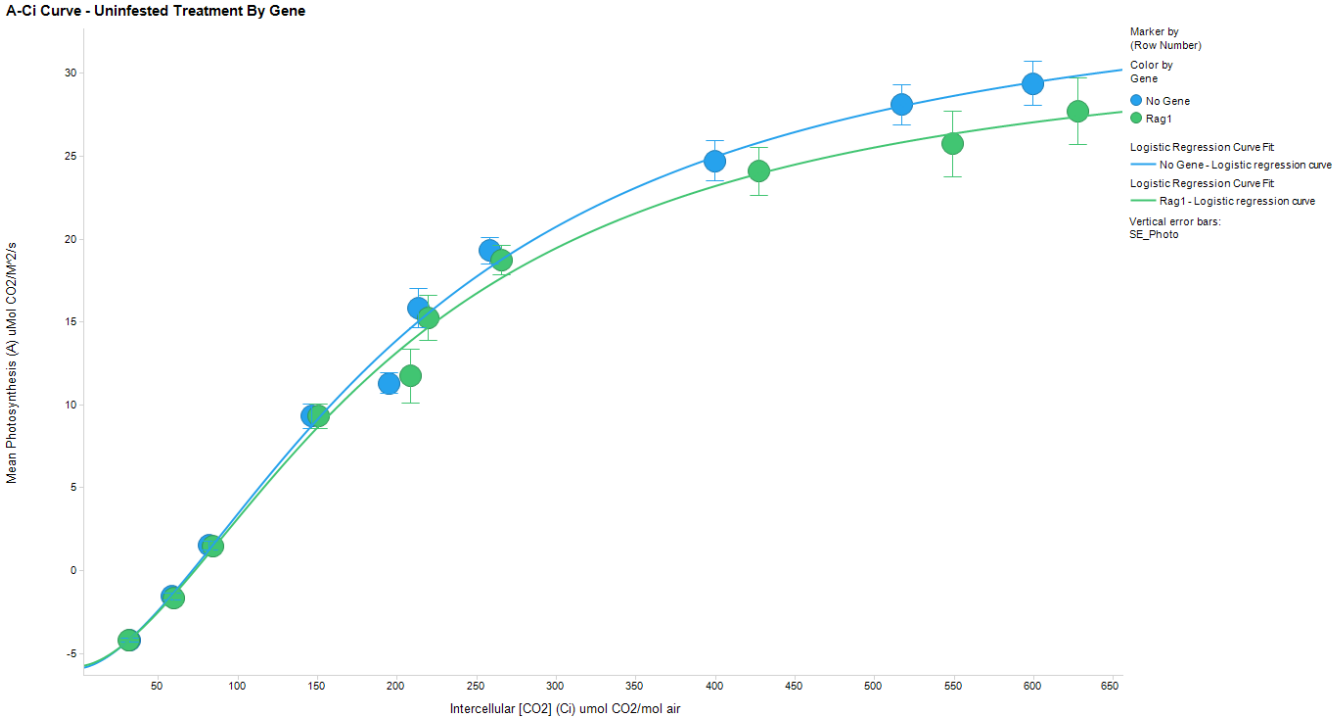


Figure 2. Mean Photosynthesis by Intercellular CO2 concentration for *Rag1* and no gene material within Uninfested treatment



Infested vs. Uninfested Treatment comparison

Several gas exchange metrics collected at 1100 ppm CO_2 were found to differ significantly between Infested and Uninfested material including: photosynthetic rate, apparent quantum yield of CO_2 assimilation, transpiration rate (all lower in infested treatment), and quantum efficiency of CO_2 fixation (higher in infested treatment). Additionally, the maximum photosynthetic rate and maximum rate of carboxylation were also found to be significantly different between treatments (reduced in infested treatment). ANOVA *P* values for these analyses can be found in Table 6. *P* values for all metrics analyzed as influenced by treatment can be found in Appendix 2. A-Ci curve comparison is shown in Figure 3, and illustrates the photosynthetic impact of infestation.

Figure 3. Mean Photosynthesis by Intercellular CO2 concentration for Infested and Uninfested Treatments

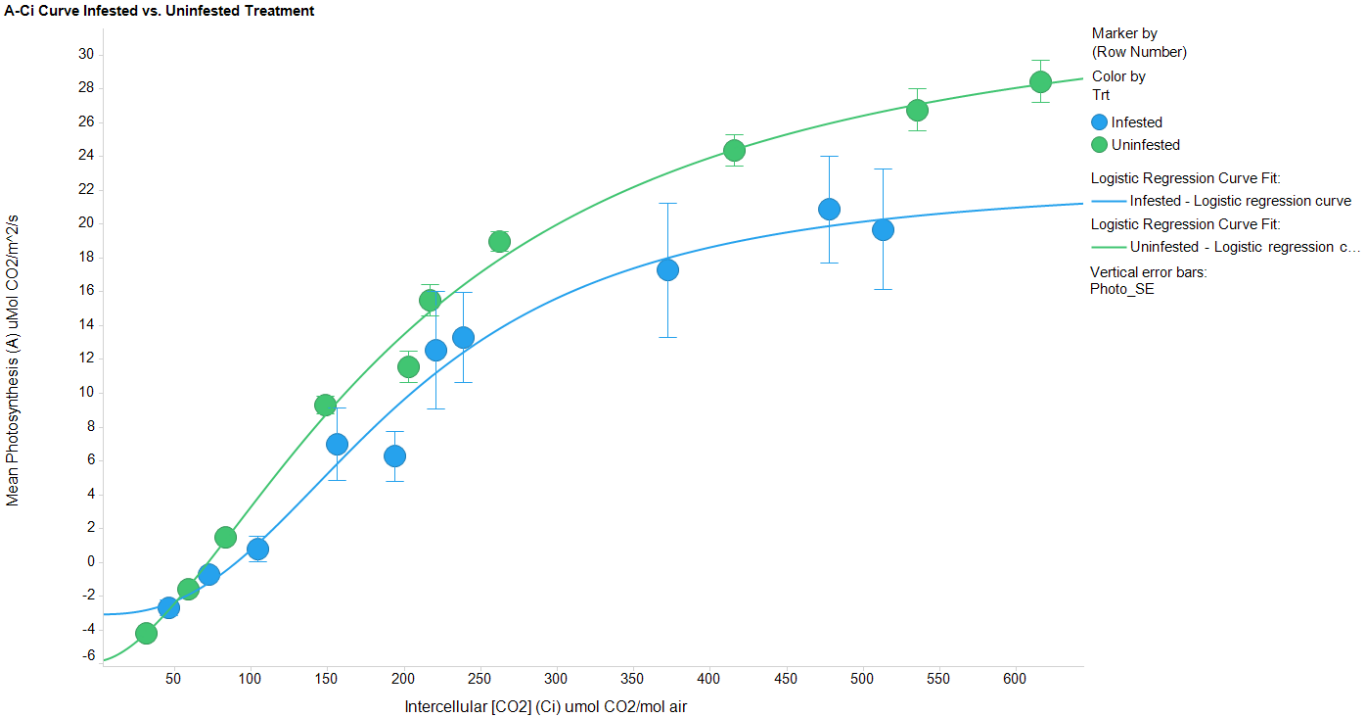


Table 6. Gas Exchange Metrics by Treatment and Gene Class

	Infested		Uninfested		P^a
	No gene	***Rag1***	**No gene**	***Rag1***	
	Mean ± SE		Mean ± SE		
Photo [b k]	21.99 ± 1.17	19.73 ± 7.41	29.4 ± 1.33	27.07 ± 2.18	
Photo [b k] **I vs U**	20.86 ± 2.54		28.15 ± 1.41		**0.0242**
Phi CO_2 [c k]	0.03 ± 0.002	0.03 ± 0.01	0.04 ± 0.002	0.04 ± 0.003	
Phi CO_2 [c k] **I vs U**	0.030 ± 0.0039		0.041 ± 0.002		**0.0244**
Trans [d k]	0.001 ± 0.0002	0.002 ± 0.0009	0.003 ± 0.0001	0.003 ± 0.0004	
Trans [d k] **I vs U**	0.0016 ± 0.0004		0.0026 ± 0.0002		**0.0465**
PhiPS2/Phi CO_2 [e k]	16.74 ± 0.11	20.42 ± 6.74	12.89 ± 0.62	14.08 ± 1.06	
PhiPS2/Phi CO_2 [e k] **I vs U**	18.58 ± 1.67		13.53 ± 0.93		**0.0185**
Vcmax [f]	79.1 ± 5.43	65.03 ± 17.54	102.18 ±7.66	90.43 ± 7.68	
Vcmax [f] **I vs U**	72.06 ± 9.62		95.85 ± 5.34		**0.0473**
Jmax [g]	161.28 ± 6.65	123.73 ± 52.75	238.81 ± 20.82	205.50 ± 23.38	
Jmax [g] **I vs U**	142.51 ± 27.87		220.87 ± 15.46		**0.0266**
Cond [h k]	0.056 ± 0.030	0.072 ± 0.030	0.106 ± 0.018	0.114 ± 0.016	
Cond [h k] **I vs U**	0.064 ± 0.021		0.110 ± 0.011		0.0623
Ci [i k]	424.85 ± 99.30	530.47 ± 99.30	599.70 ± 49.97	605.02 ± 46.27	
Ci [i k] **I vs U**	477.66 ± 59.93		602.57 ± 33.24		0.0884
WUE [j k]	393.48 ± 64.08	325.91 ± 64.08	279.32 ± 31.75	277.11 ± 29.40	
WUE [j k] **I vs U**	359.70 ± 38.20		278.13 ± 21.19		0.0815

[a] *P* values resulting from a one-way ANOVA comparing Uninfested and Infested treatments
[b] Photosynthetic Rate
[c] Apparent Quantum Yield of CO_2 assimilation
[d] Transpiration rate
[e] Quantum efficiency of CO_2 fixation; Ratio of electrons passed through PSII per CO_2 fixed
[f] Maximum rate of carboxylation allowed by Rubisco
[g] Maximum photosynthetic rate of a C_3 A-Ci curve
[h] Conductance to H_2O
[i] Intercellular CO_2 concentration
[j] Instantaneous water use efficiency
[k] Measurement resulted from application of 1100 ppm CO_2

Infested vs. Uninfested Treatment comparison by Gene Class (presence/absence of *Rag1)*

Rag1 material did not show any significant differences in seed count or gas exchange between infested and uninfested treatments. A-Ci curves for this comparison are shown in Figure 4. *Rag1* infested lines displayed high standard error. Non-*Rag1* material did not demonstrate significant differences in seed count by treatment, yet several gas exchange metrics had *P* values less than 0.05 including photosynthetic rate, apparent quantum yield of CO_2 assimilation, transpiration rate, quantum efficiency of CO_2 fixation, intercellular CO_2 concentration, transpiration efficiency, water use efficiency, and conductance to H_2O, all as measured at an input of 1100 ppm CO_2. Non-*Rag1* material also demonstrated significant differences in maximum photosynthetic rate and maximum rate of carboxylation, and the A-Ci curves in Figure 5 demonstrate the impact of aphid infestation on non-*Rag1* material. *P* values for these analyses resulting in significant differences in non-*Rag1* gene class are shown in Table 7. *P* values for all metrics analyzed can be found in Appendix 3.

Figure 4. Mean Photosynthesis by Intercellular CO2 concentration for *Rag1* material by Infested (I) and Uninfested (U) Treatment

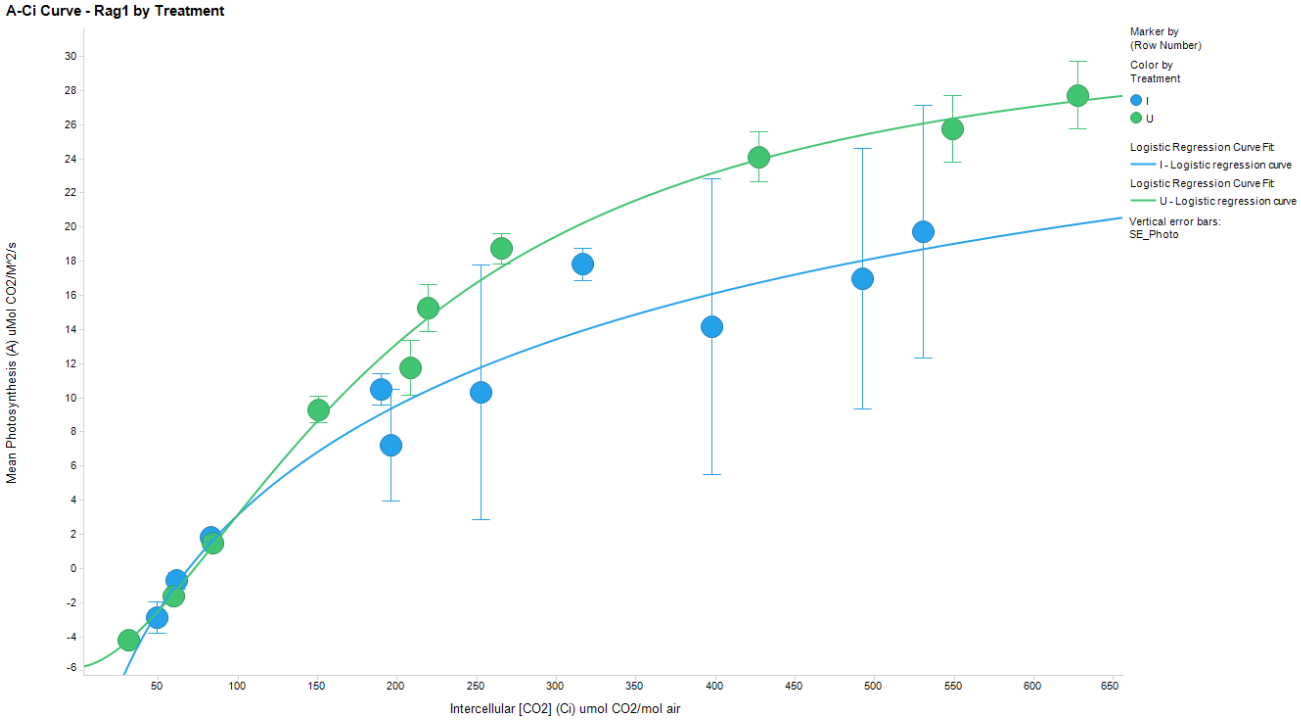


Figure 5. Mean Photosynthesis by Intercellular CO2 concentration for non-*Rag1* or no gene material by Infested (I) and Uninfested (U) Treatment

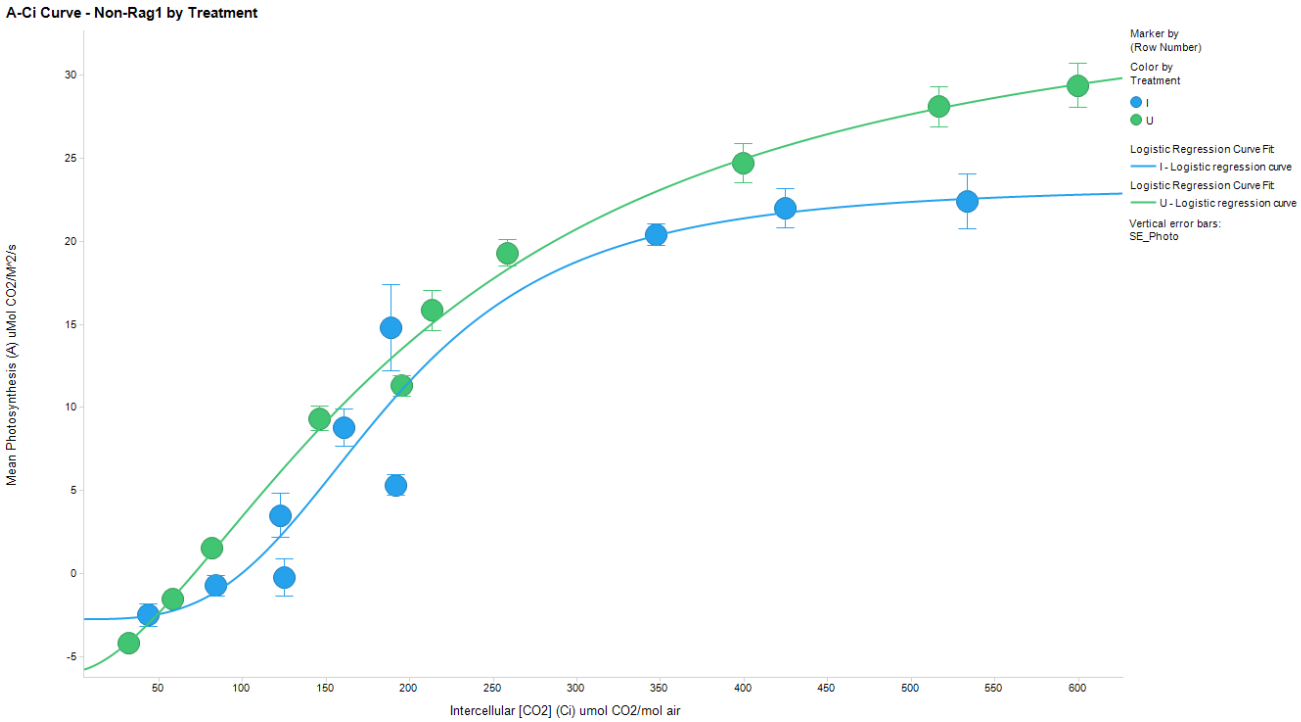


Table 7. Soybean productivity and Gas Exchange Metrics by Gene Class and Treatment. *P* values significant at α = 0.05 are shown in bold text.

	No gene			*Rag1*		
	Infested	**Uninfested**	*P*[a]	**Infested**	**Uninfested**	*P* [m]
	Mean ± SE			Mean ± SE		
Seed Count	54 ± 15.5	43 ± 4.97	0.4092	68 ± 9.5	48 ± 3.93	0.0636
Photo[b h]	21.99 ± 1.17	29.4 ± 1.33	**0.025**	19.73 ± 7.41	27.07 ± 2.18	0.2106
PhiCO2[c h]	0.03 ± 0.002	0.04 ± 0.002	**0.0245**	0.03 ± 0.01	0.04 ± 0.003	0.2131
Trans[d h]	0.001 ± 0.0002	0.003 ± 0.0001	**0.0048**	0.002 ± 0.0009	0.003 ± 0.0004	0.3823
PhiPS2/PhiCO2[e h]	16.74 ± 0.11	12.89 ± 0.62	**0.0141**	20.42 ± 6.74	14.08 ± 1.06	0.118
Vcmax[f]	79.1 ± 5.43	102.18 ±7.66	0.1558	65.03 ± 17.54	90.43 ± 7.68	0.1756
Jmax[g]	161.28 ± 6.65	238.81 ± 20.82	0.0883	123.73 ± 52.75	205.50 ± 23.38	0.1541
Cond [i h]	0.056 ± 0.006	0.11 ± 0.007	**0.0064**	0.072 ± 0.042	0.11 ± 0.02	0.3866
Ci [j h]	424.85 ± 33.54	599.70 ± 22.70	**0.0076**	530.47 ± 136.37	605.02 ± 59.63	0.5867
TE [kh]	14993.69 ± 796.96	11390.62 ± 501.68	**0.0108**	11797.04 ± 1884.42	11233.89 ± 1210.20	0.8289
WUE [l h]	393.48 ± 22.15	279.32 ± 14.30	**0.0066**	325.91 ± 87.87	277.11 ± 37.92	0.5771

[a] *P* values resulting from a one-way ANOVA comparing Uninfested and Infested treatments within no gene or non-*Rag1* gene class by itself.
[b] Photosynthetic Rate
[c] Apparent Quantum Yield of CO_2 assimilation
[d] Transpiration rate
[e] Quantum efficiency of CO_2 fixation; Ratio of electrons passed through PSII per CO_2 fixed
[f] Maximum rate of carboxylation allowed by Rubisco
[g] Maximum photosynthetic rate of a C_3 A-Ci curve
[h] Measurement resulted from application of 1100 ppm CO_2
[i] Conductance to H_2O
[j] Intercellular CO_2 concentration
[k] Transpiration efficiency
[l] Instantaneous water use efficiency
[m] *P* values resulting from a one-way ANOVA comparing Uninfested and Infested treatments within *Rag1* gene class by itself

Discussion

Studies comparing insect feeding impact on resistant *Rag1* and susceptible non-*Rag1* plant genotypes present challenges in maintaining similar infestation rates on resistant and susceptible material throughout the study when infesting with an avirulent biotype, such as Biotype 1 (*Rag1* resistance gene is effective). Additionally, *Rag1* confers antibiosis resistance to soybean aphids, and has been observed to cause disruptions in aphid feeding patterns such that it takes significantly longer for aphid stylets to reach the sieve element feeding phase, and feeding events are short and frequent with many disruptions as compared to feeding behavior observed on susceptible lines (Diaz-Montano et al., 2007). Variability and complexity exist in determining the mechanism for resistance as related to photosynthesis in studies of this nature.

A benefit of the current experimental design was the ability of Biotpye-2 aphids to colonize both genetic classes (*Rag1* and non-*Rag1* lines). During the study, aphid infestation rates were relatively stable across gene classes, and the need did not arise for equalization of aphid populations between *Rag1* and non-*Rag1* lines. Biotype 2 aphids have been observed to display reduced vigor on both *Rag1* and non-*Rag1* lines in the form of decreased reproductive and colonization rates. This indicates that there is a fitness cost associated with ability to overcome antibiotic resistance which persists even when a susceptible food source is available (Varenhorst et al., 2015). This study did observe reduced vigor of Biotype 2 aphids, as compared to expectations (parallel test not performed), given they did not colonize any of the plants in this study high enough to reach the EIL. Biotype 1 soybean aphids have been frequently observed to reach infestation rates in excess of 1,000 aphids per plant in similar growth chamber environments over shorter incubation periods (personal observation). Mean aphid infestation on non-*Rag1* material was observed to be higher than that of *Rag1* lines (comparison not significant. P = 0.5976).

Experiments are sometimes less rigorously assessed at $\alpha = 0.1$ based on potential for variability inherent in the system being studied. Ample room for noise, variability, and complexity exists in these data based on the nature of biological plant and insect systems in addition to the complex nature of plant chemistry data. Both levels of rigor will be discussed in the following sections – both $\alpha = 0.05$ and $\alpha = 0.1$.

Rag1 **vs. non-*Rag1* by treatment**

Infested Treatment

Infested *Rag1* lines demonstrated higher mean seed counts than infested non-*Rag1* lines, but the difference was not significant at $\alpha = 0.05$. High standard error in photosynthesis metrics was observed in the infested treatment, due in part to incredibly small sample size (n=2) for each gene class, and this could be coupled with the variability inherent in insect feeding behavior. The hypothesis that *Rag1* would demonstrate an increased photosynthetic capacity as compared to non-*Rag1* material when

under similar aphid infestation levels was not supported by the data, but the question could be further explored with a more robust dataset to more thoroughly vet the question.

Uninfested Treatment

No significant differences were observed between uninfested *Rag1* and non-*Rag1* material, and standard errors were not as high as was observed in the infested treatment. There were more replications available with high quality gas exchange data for uninfested treatment analysis (*Rag1* uninfested n=7, non-*Rag1* uninfested n=6). A-Ci curves were very similar between gene classes indicating that photosynthesis in healthy plants that are not under feeding pressure by aphids have similar photosynthetic capacity regardless of presence or absence of *Rag1* gene.

Infested vs. Uninfested Treatment

Analysis of gas exchange metrics between infested and uninfested treatments across both gene classes demonstrated reduced photosynthetic capacity due to aphid feeding despite infestation rates never reaching the EIL. Even under lower aphid infestations, significant impacts on yield enabling processes of the plant were sustained. Mean productivity per plant was found to be higher in infested plants than uninfested plants. While this finding was not significant at the highest level of rigor, $\alpha = 0.05$, it worth noting that this response is significant at the $\alpha = 0.1$ with $P = 0.0588$. This is an interesting observation, and was not the expected outcome. Insect induced plant defense responses can sometimes be stimulatory to plant yield (Poveda et al., 2010). Additional studies could explore potential mechanisms explaining this outcome, for example, observation of source: sink relationships in the plant to understand whether plants are capable of more efficient allocation of photosynthetic products despite reduced photosynthetic rates caused by aphid feeding.

The photosynthetic capacity of soybeans under aphid pressure is clearly reduced. Metrics that were found to be significantly reduced at $\alpha = 0.05$ include photosynthetic rate, apparent quantum yield of CO_2 assimilation, transpiration rate, maximum rate of carboxylation allowed by Rubisco, and maximum photosynthetic rate. These responses indicate that stomatal conductance is impacted by aphid feeding. Significant reduction in quantum efficiency of CO_2 fixation (ratio of electrons passed through PSII per CO_2 fixed) was observed at $\alpha = 0.1$. This indicates that the light reactions may have been impacted in addition to stomatal conductance impacting the Calvin cycle and thus photosynthetic capacity.

Light reactions may be impacted if complications occur in light energy capture in PS I and II. Soybean aphids feeding does not generally result directly in leaf chlorosis. Build up of aphid secretions, or honeydew could potentially interfere with light reaching the leaf surface, particularly if sooty mold growth occurs based on presence of honeydew. Honeydew build up and sooty mold growth was not observed in infested plants in this experiment. This indication that light reactions are impacted by infestation was not expected for this reason.

Additional metrics supporting evidence of aphid infestation's significant stomatal limitation to photosynthesis include decreased conductance to H_2O, intercellular CO_2 concentration (Ci), and increased instantaneous water use efficiency (WUE) in the infested treatment as compared to uninfested plants. These metrics were found to be significant at $\alpha = 0.1$. WUE is equal to CO_2 assimilation divided by transpiration rate, thus WUE is expected to increase with reduced stomatal conductance, which was observed in the data.

Infested vs. Uninfested Treatment compared separately by Gene Class

No gene or non-*Rag1* soybeans appeared to be more severely impacted by aphid infestation as observed through significant differences in various gas exchange metrics. Ci, conductance, transpiration, and apparent quantum yield of CO_2 are all significantly decreased in the infested non-*Rag1* material based on $\alpha = 0.05$. This could be an indication that infestation resulted in stomatal limitations to photosynthesis. The apparent quantum yield of CO_2 assimilation efficiency is actually higher in infested non-*Rag1* lines than in the uninfested treatment. This means that the ratio of electrons passed through Photosystem II (PSII) was higher per CO_2 fixed, which could be a result of reduced CO_2 fixation with unimpacted light reactions. This supports the expectation that soybean aphid feeding does not impact light reactions. Mean seed count in the no gene infested treatment was found to be higher than the infested treatment. While not significant, this finding indicates that soybeans could potentially have a defense mechanism that is activated in response to aphid feeding that results in overcompensation and enhanced yield potential.

Rag1 material did not display any significant differences in metrics compared between infested and uninfested treatments at $\alpha = 0.05$ – this is not to say that *Rag1* material performed higher than sister lines without the gene. In actuality, mean gas exchange metrics indicated that non-*Rag1* material may have a higher photosynthetic capacity than *Rag1* material both in the presence and absence of aphids. As in the no gene analysis, the *Rag1* gene class infested treatment resulted in an increased mean seed count as compared to uninfested *Rag1* lines. This comparison was significant at $\alpha = 0.1$. This observation of increased productivity in infested plants was a consistent trend in the study, and as noted before, it was surprising, and was contrary to the expectation that infestation would significantly reduce not only photosynthetic rate, but also plant productivity.

Limitations to photosynthesis

Limitations to photosynthesis may be of stomatal, mesophyll, or biochemical nature. For optimum photosynthesis, adequate CO_2 must enter the leaf through stomata into leaf intercellular spaces, and must successfully diffuse to the leaf mesophyll and the stroma of the chloroplasts where carbon is fixed in the Calvin cycle reactions (light-independent reactions). CO_2 fixation in the Calvin Cycle requires ATP and NADPH that resulting from the light reactions that occur in the thylakoid membranes of the

chloroplasts. Photosynthesis can be limited if CO_2 cannot enter the leaf due to stomatal limitations, cannot diffuse to the stroma, or if energy is limited from the light reactions. Energy provided by light reactions can be limited if light cannot penetrate the chloroplasts due to physical interference in the case where light is blocked, or chloroplasts are damaged and cannot perform processes in Photosystems I and II. If energy from the light reactions (ATP and NADPH) is limited, the Calvin Cycle will be impacted and will not be able to fix CO_2 as efficiently.

This experiment points to aphid feeding induced stomatal limitations to photosynthesis. This is not only shown in results of analysis, but can also be observed in comparison of A-Ci curves between infested and uninfested treatments (Figures 3 through 5). The biggest differences between curves occur at higher [CO_2], which further supports reduced CO_2 conductance as the underlying factor causing reduced photosynthetic capacity.

During the Calvin Cycle, CO_2 and ribulose-1, 5-bisphosphate (RuBP) react to form phosphoglyceraldehyde (PGAL) in an energy requiring reaction that is catalyzed by the enzyme ribulose-1, 5-bisphosphare carboxylase/oxygenase (Rubisco). Some PGAL may be used to form sugars, while others stay in the cycle and are used to regenerate RuBP so that it can continue to react with incoming CO_2 and keep the cycle moving. RuBP regeneration may be impacted under situations where photorespiration occurs. This generally happens during heat stress, but could also happen in cases where the ratio of O_2: CO_2 is high due to reduced CO_2 entering the leaf and reduced O_2 leaving the plant through transpiration causing RuBP to react with O_2 based on increased availability in the stroma. Photorespiration results in decreased RuBP regeneration, decreased carbon fixation and CO_2 loss. Evidence of aphid feeding impacting RuBP regeneration was not observed in this study.

Conclusion

This study found that Biotype 2 aphid feeding significantly reduces photosynthetic capacity due to stomatal conductance limiting CO_2 availability as an input to the Calvin Cycle. In this instance, presence of the *Rag1* gene in Biotype 2 aphid infested soybeans did not appear to provide any level of aphid tolerance through increased soybean photosynthesis or productivity. Evidence remains that *Rag1* soybeans are capable of demonstrating a tolerance response to Biotype 2 soybean aphids, and further research into the mechanism of tolerance would be worthwhile, including further characterization of photosynthetic response. Soybean germplasm lacking the *Rag1* gene showed significantly reduced photosynthetic response to aphid infestation pointing to stomatal limitations. *Rag1* material appeared to be less affected by aphid infestation, as significant differences were not detected between *Rag1* infested and uninfested photosynthesis. Seed count was observed to be higher in infested *Rag1* plants which could imply that *Rag1* soybean defense response stimulates plant productivity and yield.

A potential next step would be to assess photosynthetic response of *Rag1* and non-*Rag1* to Biotype 2 soybean aphids in a more robust dataset to improve power of the study, and in parallel, explore into source: sink relationships as impacted by aphid feeding.

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Appendix 1. ANOVA *P* values based on analysis Independent variable = presence or Absence of *Rag1* vs. Dependent Variables including Productivity (seed count), infestation level (aphid count), and several different gas exchange metrics. Full dataset (all data) and reduced dataset (RH >30) are shown side-by-side.

			Infested	Infested	Uninfested	Uninfested
Independent Variable (X)	Dependent Variable (Y)	Description of Dependent Variable	P value (All Data)	P value (RH>30)	P value (All Data)	P value (RH>30)
Aphid R Gene	Seed count	Productivity; count of seeds per plant	0.7009	0.5218	0.4305	0.4305
Aphid R Gene	Aphid count	Count of aphids per plant	0.3090	0.7214	N/A	N/A
Aphid R Gene	Photo@ 1100 ppm CO_2	Carbon exchange rate; Photosynthetic Rate	0.6816	0.7919	0.4005	0.4005
Aphid R Gene	Cond@ 1100 ppm CO_2	Conductance to H_2O	0.6230	0.7481	0.7588	0.7588
Aphid R Gene	Ci@ 1100 ppm CO_2	Intercellular CO_2 Concentration	0.6033	0.5305	0.9391	0.9391
Aphid R Gene	Fv'/Fm'@ 1100 ppm CO_2	Maximum light adapted PSII efficiency	0.3895	0.6096	0.3746	0.3746
Aphid R Gene	PhiPS2@ 1100 ppm CO_2	Apparent Quantum Yield of Photosystem II	0.4057	0.6326	0.3566	0.3566
Aphid R Gene	PhiCO_2@ 1100 ppm CO_2	Apparent Quantum Yield of CO_2 assimilation	0.6731	0.7946	0.3941	0.3941
Aphid R Gene	qP@ 1100 ppm CO_2	Photochemical Quenching	0.5210	0.6972	0.4059	0.4059
Aphid R Gene	qN@ 1100 ppm CO_2	Non-Photochemical Quenching	0.3904	0.6109	0.3690	0.3690
Aphid R Gene	ETR@ 1100 ppm CO_2	Electron Transport Rate	0.4295	0.6273	0.3915	0.3915
Aphid R Gene	Trans@ 1100 ppm CO_2	Transpiration rate	0.6107	0.7471	0.9175	0.9175
Aphid R Gene	PhiPS2/Phi CO_2@ 1100 ppm CO_2	Quantum efficiency of CO_2 fixation; Ratio of electrons passed through PSII per CO_2 fixed; Operating Quantum Efficiency (Phi PSII) / Apparent Quantum Yield (Phi CO_2)	0.6194	0.6397	0.3767	0.3767
Aphid R Gene	TE@ 1100 ppm CO_2	Transpiration Efficiency; Photo/Trans (umol CO_2/mol H_2O transpired)	0.1720	0.2586	0.9125	0.9125
Aphid R Gene	WUE@ 1100 ppm CO_2	Instantaneous Water Use Efficiency; Photo/Cond (umol CO_2/mol H_2O)	0.5924	0.5336	0.9602	0.9602
Aphid R Gene	Vcmax	Maximum rate of carboxylation allowed by Rubisco	0.4948	0.5237	0.3050	0.3050
Aphid R Gene	Jmax	The maximum photosynthetic rate of a C3 A-Ci curve	0.4707	0.5532	0.3174	0.3174

Appendix 2. ANOVA *P* values based on analysis Independent variable = Treatment vs. Dependent Variables including Productivity (seed count) and several different gas exchange metrics. Full dataset (all data) and reduced dataset (RH >30) are shown side-by-side.

Independent Variable (X)	Dependent Variable (Y)	Description of Dependent Variable	P value (all data), significance	P value (RH >30), significance
Treatment	Seed count	Productivity; count of seeds per plant	0.0385	0.0588
Treatment	Photo@ 1100 ppm CO_2	Carbon exchange rate; Photosynthetic Rate	0.3202	0.0242
Treatment	Cond@ 1100 ppm CO_2	Conductance to H_2O	0.0032	0.0623
Treatment	Ci@ 1100 ppm CO_2	Intercellular CO_2 Concentration	0.0003	0.0884
Treatment	Fv'/Fm'@ 1100 ppm CO_2	Maximum light adapted PSII efficiency	0.0607	0.2084
Treatment	PhiPS2@ 1100 ppm CO_2	Apparent Quantum Yield of Photosystem II	0.0226	0.1873
Treatment	PhiCO_2@ 1100 ppm CO_2	Apparent Quantum Yield of CO_2 assimilation	0.3142	0.0244
Treatment	qP@ 1100 ppm CO_2	Photochemical Quenching	0.0179	0.2323
Treatment	qN@ 1100 ppm CO_2	Non-Photochemical Quenching	0.0717	0.2825
Treatment	ETR@ 1100 ppm CO_2	Electron Transport Rate	0.0249	0.1951
Treatment	Trans@ 1100 ppm CO_2	Transpiration rate	0.0194	0.0465
Treatment	PhiPS2/PhiCO_2@ 1100 ppm CO_2	Quantum efficiency of CO_2 fixation; Ratio of electrons passed through PSII per CO_2 fixed; Operating Quantum Efficiency (Phi PSII) / Apparent Quantum Yield (PhiCO_2)	0.4755	0.0185
Treatment	TE@ 1100 ppm CO_2	Transpiration Efficiency; Photo/Trans (µmol CO_2/mol H_2O transpired)	0.0096	0.1516
Treatment	WUE@ 1100 ppm CO_2	Instantaneous Water Use Efficiency; Photo/Cond (µmol CO_2/mol H_2O)	0.0003	0.0815
Treatment	Vcmax	Maximum rate of carboxylation allowed by Rubisco	0.3089	0.0473
Treatment	Jmax	The maximum photosynthetic rate of a C3 A-Ci curve	0.3282	0.0266

Appendix 3. ANOVA *P* values based on analysis Independent variable = Treatment vs. Dependent Variables including Productivity (seed count) and several different gas exchange metrics analyzed by Gene Class (Presence/Absence of *Rag1*)

Independent Variable (X)	Dependent Variable (Y)	Description of Dependent Variable	*P* value - *Rag1* (RH >30)	*P* value - non-*Rag1* (no gene)
Treatment	Seed count	Productivity; count of seeds per plant	0.0636	0.4092
Treatment	Photo@ 1100 ppm CO2	Carbon exchange rate; Photosynthetic Rate	0.2106	0.025
Treatment	Cond@ 1100 ppm CO2	Conductance to H2O	0.3866	0.0064
Treatment	Ci@ 1100 ppm CO2	Intercellular CO2 Concentration	0.5867	0.0076
Treatment	Fv'/Fm'@ 1100 ppm CO2	Maximum light adapted PSII efficiency	0.189	0.7004
Treatment	PhiPS2@ 1100 ppm CO2	Apparent Quantum Yield of Photosystem II	0.239	0.5188
Treatment	PhiCO2@ 1100 ppm CO2	Apparent Quantum Yield of CO2 assimilation	0.2131	0.0245
Treatment	qP@ 1100 ppm CO2	Photochemical Quenching	0.3999	0.4305
Treatment	qN@ 1100 ppm CO2	Non-Photochemical Quenching	0.2189	0.7931
Treatment	ETR@ 1100 ppm CO2	Electron Transport Rate	0.2364	0.5469
Treatment	Trans@ 1100 ppm CO2	Transpiration rate	0.3823	0.0048
Treatment	PhiPS2/PhiCO2@ 1100 ppm CO2	Quantum efficiency of CO2 fixation; Ratio of electrons passed through PSII per CO2 fixed; Operating Quantum Efficiency (Phi PSII) / Apparent Quantum Yield (PhiCO2)	0.118	0.0141
Treatment	TE@ 1100 ppm CO2	Transpiration Efficiency; Photo/Trans (umol CO2/mol H2O transpired)	0.8289	0.0108
Treatment	WUE@ 1100 ppm CO2	Instantaneous Water Use Efficiency; Photo/Cond (umol CO2/mol H2O)	0.5771	0.0066
Treatment	Vcmax	Maximum rate of carboxylation allowed by Rubisco	0.1756	0.1558
Treatment	Jmax	The maximum photosynthetic rate of a C3 ACi curve	0.1541	0.0883