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Soybean Aphid Biotype 4 Resistance in Soja and Soybean Plant Introductions

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SOYBEAN APHID BIOTYPE 4 RESISTANCE IN SOJA AND SOYBEAN PLANT INTRODUCTIONS

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Plant Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

ANOVA	Analysis of Variance
ARS	Agricultural Research Service
btm	bottom
C	Celsius
CA	California
CFIA	Canadian Food Inspection Agency
cm	centimeter
Co.	Company
e.g.	exempli gratia
ERS	Economic Research Service
et al.	et alia
etc.	etcetera
G.	*Glycine*
H_2SO_4	Dihydrogen sulfate
ht	height
i.e.	id est
IA	Iowa
IL	Illinois
Inc.	Incorporated
km	kilometer
KPEP	Kentucky Pesticide Education Program
L	liter
L.	Linnaeus
L:D	light: dark
Lomira13	Colony collected in Lomira, Wisconsin in 2013
LSMEANS	Least Square Means Procedure
MA	Massachusetts
Med	median
Merr.	Merrill

MG	maturity group
mL	milliliter
mm	millimeter
MN	Minnesota
MO	Missouri
N	number
n obs	number of observations
NA	not applicable
N/A	not available
NASS	National Agricultural Statistics Service
NCARL	North Central Agricultural Research Laboratory
No.	Number
NPGS	National Plant Germplasm System
Obs	observations
OH	Ohio
PI	plant introduction
PROC GLIMMIX	Generalized Linear Mixed Models Procedure
PROC MIXED	Mixed Model Procedure
R.	*Rhamnus*
SAS	Statistical Analysis System
SBA	soybean aphid
SD	South Dakota
SDSU	South Dakota State University
Sieb.	Siebold
U.S.	United States
USDA	United States Department of Agriculture
USSGC	United States Soybean Germplasm Collection
VC	vegetative cotyledon
VE	vegetative emergence
Volga15	Colony collected in Volga, SD in 2015
Volga16	Colony collected in Volga, SD in 2016

WI	Wisconsin
Zucc.	Zuccarini

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ABSTRACT

SOYBEAN APHID BIOTYPE 4 RESISTANCE IN SOJA AND SOYBEAN PLANT INTRODUCTIONS

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Soybean aphid, *Aphis glycines* Matsumura, is a major pest to soybean, *Glycine max* (L.) Merr. Host plant resistance is a management tactic that uses naturally occurring soybean plant defenses to limit soybean aphid pest damage. Virulent soybean aphid biotypes are able to successfully colonize on certain aphid resistant soybean. Soybean aphid biotype 4 is most virulent, overcoming all commercially available soybean aphid resistant soybeans (*Rag1*, *Rag2*, and *Rag1+Rag2*). Additional sources of resistance to avirulent biotypes have been identified in soja and soybean plant introductions (PIs). This study examined those resistant soja and soybean for resistance to the newly found soybean aphid biotype 4, using iso-female colonies of soybean aphid from three different site-years. Free-choice tests examined 20 soja and 50 soybean PIs for putative resistance to the three soybean aphid biotype 4 colonies. Promising PIs continued on in a follow-up, caged no-choice test with its respective colony. Soja PI 65549 and PI 101404A and soybean PI 437696 were found highly resistant to each of the three soybean aphid biotype 4 colonies and should be explored further as valuable sources of soybean aphid resistance.

LITERATURE REVIEW

Introduction

This thesis discusses the use of host plant resistance as a management approach for soybean aphid. The objective of this study was to evaluate soja and soybean plant introductions for resistance to three soybean aphid biotype 4 colonies.

Soybean and Soja

Characteristics: Soybean, *Glycine max* (L.) Merr., is an erect, annual legume from the plant family Fabaceae. Seed germination requires soil temperatures above 12°C (Karki 2017). As a legume, soybean adds atmospheric nitrogen to the soil through symbiotic relationships with bacteria. Time until maturation is dependent on photoperiod or day length requirements and ideal temperature; throughout the U.S. and Canada, latitudinal zones have distinct maturity groups based on these needs (Licht 2014). Soybean is a self-fertilizing plant with white or purple flowers. Seeds vary greatly in color, but most commercially-grown soybean has tan seed (Figure 1).

Wild soybean or soja, *Glycine soja* Sieb. and Zucc., is the closest relative to cultivated soybean (Carter et al. 2004), however, the evolutionary relationship is disputed (see Sedivy et al. 2017 for an in-depth review). Hypotheses on soybean domestication include models of a single origin, multiple origins, or an intermediate species complex (Sedivy et al. 2017), with the progenitor either being a common ancestor of soybean and soja (Kim et al. 2010) or an ancient form of soja (Sedivy et al. 2017).

Soja can often be found growing in natural conditions near roadsides and riverbanks of many Asian countries (Hymowitz 1970, CFIA 2012). Although soybean

and soja can hybridize easily (Carter et al. 2004), soja has many unique characteristics such as creeping, tendrilous growth, pods that shatter easily, and small black seeds (Figure 1) (Hymowitz 1970, Kim et al. 2010).

Fehr and Caviness (1977) developed a method for staging the growth of soybean, which is also applicable to soja. VE growth stage occurs when the seedling has emerged and the cotyledons are forming. VC growth stage begins when the cotyledons are fully formed while a pair of unifoliate leaves develop. After this time, vegetative plant growth for soybean and soja is measured by the number (n) of fully developed nodes or branches, denoted as V(n). Each vegetative node produces a trifoliate leaf. As flowers begin to form, the plant moves to the reproductive stages, designated R(n). There are eight R stages: R1 begins with full opening of first flower, R2 at full flowering, R3 developing pod, R4 pods begin seed development, R5 rapid seed filling, R6 pod with full green seed, R7 first fully mature pod, and R8 most pods have matured (Fehr and Caviness 1977, Licht 2014).

Soybean Origin: Due to limited archaeological information and molecular-based evidence, the precise time of soybean domestication is undetermined (Sedivy et al. 2017). However, the earliest known soybean cultivation can be traced back to China 4,000-5,000 years ago (Ma 1984), while its first cultivation in other Asian countries was closer to 2,500 years ago (Wu et al. 2004).

Soybean was not cultivated in North America until the 18th century. After collecting seed in China, Samuel Bowen introduced soybean, referred to by him as Chinese vetches, to the U.S. in 1765 (Hymowitz and Harlan 1983). In the 1804 Willich's

domestic encyclopedia, James Mease coined the word "soybean," after its use in soy sauce (Mease 1804, Hymowitz and Shurtleff 2005). Yet, it was not until the 1940s and 1950s that the U.S. overtook China in soybean production (Hymowitz 1970).

Soybean Production: By 1968, the U.S. was growing 76% of the total soybean produced worldwide, compared to China's 17% (Hymowitz 1970). Masuda and Goldsmith (2009) used global soybean production data to assess past trends and to estimate soybean projections. They found hectares harvested globally had quadrupled, from 24.0 million to 94.1 million, and yields doubled, from 1.14 tons/ha to 2.31 tons/ha, between 1961 and 2007. From 2005-2007, five countries were yielding 92.2% of the world's soybeans: the U.S. (37.0%), Brazil (24.8%), Argentina (19.0%), China (7.3%), and India (4.1%). With increases in soybean production expected to slow, they estimated 359.7 million tons will be harvested globally by 2030 (Masuda and Goldsmith 2009).

In 2016, 117.3 million tons of soybean were produced in the U.S. (USDA NASS 2017), second in production only to corn (Licht 2014). U.S. oilseed production is dominated by soy productivity, accounting for approximately 90% of all oilseed produced (USDA ERS 2017). In 2014, South Dakota was the seventh leading soybean-producing state and leading producer per capita in the U.S. with approximately 170,000 tons (Garcia 2015).

Soybean Uses: Soybean has many desirable qualities; it can be easily grown in an array of geographical areas and has many food, industrial, and medicinal applications

(Wu et al. 2004). Soybean seed is comprised of approximately 20% oil and 40% protein content (Raghuvanshi and Bisht 2010).

Soybean seed is considered an oilseed due to its high oil content (USDA ERS 2017) and is the second largest source of vegetable oil globally (Raghuvanshi and Bisht 2010). Soybean processing involves pressing seed to extract oil. Soybean oil can be used for printing ink and biodiesel (Raghuvanshi and Bisht 2010).

The by-product of soybean oil extraction is protein rich meal (USDA ERS 2017). Soybean meal is primarily used as the main source of compound livestock feed (Cromwell 2017). Its high protein content can be used as a supplement in other products; soy protein can be added into various foods for nutrition (e.g., wheat flour) or palatability (e.g., sausages). Soybean protein fiber can be blended with cotton or wool to create softer, higher quality fabrics (Raghuvanshi and Bisht 2010).

The raw material can be processed as bean curd and soybean milk, for human consumption. Technology has allowed isolation of other soybean compounds that have unique uses: lactoserum in cosmetics; oligosaccharides in laxatives; isoflavones in cancer therapy; phosphatide as a nutrition supplement used in food, medicine, and animal production; and polypeptides in many medicinal uses (Raghuvanshi and Bisht 2010).

Soybean Aphid

Biology and Life Cycle: The soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), is a greenish-yellow pear-shaped insect, that is approximately 1.5mm in length (Matsumura 1917, Wu et al. 2004). Soybean aphid feeds on sugary phloem sap using their piercing-sucking mouthparts (Ragsdale et al. 2011, Tilmon et al.

2011). Soybean aphid has a complex heteroecious holocyclic life cycle (Figure 2) (Takahashi et al. 1993).

Heteroecious refers to the alternation of host plant types required by soybean aphid each year (Ragsdale et al. 2004). Soybean aphid utilizes a primary host, buckthorn or *Rhamnus* species, for sexual reproduction and overwintering. During the summer months, soybean aphid reproduces asexually on a secondary host, *Glycine* species (Takahashi et al. 1993). The life cycle of soybean aphid in China and Japan is very similar to that in North America (Ragsdale et al. 2004). In Asia, the primary hosts are two buckthorn species, *Rhamnus davurica* Pallus and *R. japonica* Maxim (Takahashi et al. 1993). In North America, *R. cathartica* L., or common buckthorn (Figure 3a), is used as the main primary host of soybean aphid. *Rhamnus cathartica* is native to Europe, an invasive species in North America (Voegtlin et al. 2004), and prevalent throughout central U.S. and southern Canada. In Minnesota, for example, thousands of common buckthorn plants can be found per hectare (Ragsdale et al. 2004). In Asia, the secondary host can either be soybean or soja (Figure 3b), while in North America it is only soybean (Hill et al. 2004b, Wang et al. 1962, Wu et al. 2004).

Soybean aphid is holocyclic, meaning that the life cycle and reproduction pathway include both an asexual and sexual phase during the year. Soybean aphid is hemimetabolous insects with an egg, nymph, and a winged or non-winged adult life stages (Takahashi et al. 1993). A nymph is an immature, wingless, and pre-productive soybean aphid that closely resembles an adult; soybean aphid has four molts or instars before reaching maturity (Wu et al. 2004).

Soybean aphid overwinters as an egg on buckthorn, and can withstand laboratory temperatures as low as -34°C (McCornack et al. 2005). During spring, the egg hatches into a wingless female nymph. Upon reaching maturity, the soybean aphid then reproduces by giving live birth to all-female offspring without fertilization. After two or three generations on buckthorn, soybean aphid produces winged females. With increased temperature and photoperiod, winged migrants move on to find their secondary host, soybean (Ragsdale et al. 2004).

During summer, soybean aphid reproduces asexually on soybean with about 16 clonal generations under ideal conditions. As soybean aphid populations grow and overcrowding occurs, winged offspring arise and disperse to colonize other soybean plants (Ragsdale et al. 2004).

Soybean aphid reproduction is optimal at 27.8°C and slows as temperatures increase or decrease. Reproduction and growth ceases at temperatures greater than 34.9°C or less than 8.6°C (McCornack et al. 2004, Wu et al. 2004). With optimal conditions and absence of abiotic and biotic stressors, soybean aphid populations can double in less than two days (McCornack et al. 2004). However, average doubling times of field soybean aphid populations are closer to seven days (Ragsdale et al. 2007).

As temperature and photoperiod decrease in late summer, soybean aphids will begin producing winged female migrants. These migrants fly from deteriorating or senescing soybean to buckthorn and produce females capable of sexual reproduction. Concurrently, other soybean aphids on soybean produce winged males that migrate to buckthorn. The male mates with the sexual female, which lays fertilized eggs near bud shoots of buckthorn (Ragsdale et al. 2004, Wu et al. 2004).

Geographic Distribution and Pest Status: Soybean aphid is native to Asia and was first described in 1917 in Japan (Matsumura 1917). Soybean aphid has since been found to be native to China, Indonesia, Japan, Korea, Malaysia, The Philippines, Taiwan, and Thailand (Tilmon et al. 2011). Soybean aphid is also established in Canada, Russia, the U.S., and Vietnam (Wu et al. 2004).

While soybean aphid is a sporadic pest in Asia (Wu et al. 2004), it was not until it was discovered in North America in 2000 that soybean yields were substantially impacted over a wide production area (Liu et al. 2004, Ragsdale et al. 2004, Wu et al 2004). The first observation of soybean aphid in North America was in Wisconsin (Alleman et al. 2002). By 2004, soybean aphid had spread to 22 states and three Canadian provinces (Ragsdale et al. 2011). Soybean aphid is most successful in upper Midwestern states as well as the Canadian provinces of Manitoba, Ontario, and Québec (Tilmon et al. 2011). The prevalence of common buckthorn throughout North America has facilitated soybean aphid's wide abundance.

On soybean, soybean aphid feeding can reduce plant height, wrinkle foliage (Figure 4), decrease photosynthesis, stunt roots, reduce the number of pods, decrease seed size, lower seed oil and protein concentrations, and even kill plants (Beckendorf et al. 2008, Ragsdale et al. 2007, Wu et al. 2004). Soybean aphid can also vector plant viruses such as *Soybean mosaic virus* and *Alfalfa mosaic virus*, which may each cause additional yield loss (Hill et al. 2001). Excrement of soybean aphid is sticky and is often referred to as 'honeydew.' In areas with serious population outbreaks the honeydew excretions can cause sooty mold growth, which covers leaves and shoots and further affects

photosynthesis (Chen and Yu 1988). Early soybean aphid infestation can cause over 50% yield loss in soybean (Ragsdale et al. 2007). In North America, soybean aphid causes an estimated $2.4 to $4.9 billion annual loss due to both direct and indirect damage and input costs associated with management (Song et al. 2006, Hill et al. 2012).

Soybean Aphid Management Tactics

Biological Control: Natural enemies can be an efficient form of control of soybean aphid. The types of natural enemies are similar in both native and non-native habitats, with ground beetles, lady beetles, lacewings, parasitoids, pirate bugs, predatory flies, and entomopathogenic fungi (Ragsdale et al. 2011, Tilmon et al. 2011, Wu et al. 2004). In Asia, the effects of natural enemies were examined on caged and non-caged soybean aphid populations in northeast China (Liu et al. 2004, 2012). Although neither population surpassed the economic threshold (see Chemical Control section), soybean aphid populations exposed to natural predation experienced as much as a 60-fold decrease when compared to those in small-mesh cages (Liu et al. 2012).

In North America, more than 43 predatory taxa significantly suppress soybean aphid season-long (Rutledge et al. 2008). A 36- to 86-fold reduction was observed in U.S. soybean aphid field populations by natural enemies, with lady beetles (Coleoptera: Coccinellidae) being the dominant predators (Costamagna et al. 2008). Nonetheless, importation and release of natural enemies from Asia through classical biological control could play an important role in suppressing North American soybean aphid populations (Tilmon et al. 2011). In 2008, natural soybean aphid suppression was valued over $239

million per year to soybean producers in Iowa, Michigan, Minnesota, and Wisconsin (Landis et al. 2008).

Chemical Control: Currently, the most common approach to managing soybean aphid in the U.S. is by the application of insecticides to soybean (Tilmon et al. 2011). Insecticide use on soybean had increased 130-fold by 2006 in the northcentral U.S. after soybean aphid became a pest in 2000 (Ragsdale et al. 2011).

One form of insecticide application is seed treatment. By applying insecticides to seed before planting, the growing plant can take up and translocate the chemical systemically through the xylem (Magalhaes et al. 2009). Neonicotinoids are the only class of insecticide used on soybean seed for soybean aphid control (O'Neal and Johnson 2010). As a seed treatment, neonicotinoids can suppress soybean aphid populations for approximately three weeks, leaving the plants vulnerable for the majority of their growth and development (McCornack and Ragsdale 2006, Lundgren and Seagraves 2012, Krupke et al. 2017). This large, unprotected window often requires soybean growers to use foliar sprays later in the season to control soybean aphid outbreaks (Hodgson et al. 2012).

The second type of insecticide application for soybean aphid management is foliar spray. Foliar insecticides kill the pest through direct contact or prolonged contact with residues on the plant surface (KPEP 2016). Organophosphates, pyrethroids, and neonicotinoids are three classes of insecticide used as foliar sprays. Such sprays allow for soybean aphid populations to be controlled as they approach economically injurious levels (Hodgson et al. 2012).

Integrated pest management uses a combination of management techniques to provide long-term, economic, and sustainable pest control (Pedigo 2017). This approach may effectively manage soybean aphid while reducing unnecessary input costs to producers and limiting injury to beneficial insects. Thresholds take into account a pest population's doubling time, current yield averages, control costs, and market values to help growers prevent excessive and unwarranted pesticide use (Pedigo 2017). The economic threshold is met when a pest population reaches a high enough density that requires growers to take action in order to prevent economically significant injury. For soybean aphid, the economic threshold has been determined as approximately 250 soybean aphids per soybean plant on at least 80 percent of plants throughout a field (Ragsdale et al. 2007). Upon soybean aphid reaching the economic threshold, growers have a seven-day window before populations become high enough to cause economic losses (Ragsdale et al. 2007, Koch et al. 2016, Pedigo 2017). Although it takes populations one week to double in size on most soybean plants, soybean aphid-resistant soybean varieties (discussed in the Host Plant Resistance section) have a doubling rate of 10-14 days, postponing the time to when economic injury level is reached (Chiozza et al. 2010, Ragsdale et al. 2011). Many other biotic and abiotic factors can influence growth rates, and it is recommended that fields be reevaluated prior to spraying to ensure soybean aphid counts are a threat to yields. Soybean fields can experience areas of randomly concentrated aphid populations, known as "hot-spots," and can cause growers to prematurely treat fields before reaching the economic threshold.

Misuse of pesticides can allow pest species to develop tolerance to the chemicals (Hodgson et al. 2012). Currently, pyrethroid-resistant soybean aphids have been reported

in Minnesota, Iowa, North Dakota, and South Dakota (Hanson et al. 2017, Potter et al. 2017, Varenhorst et al. 2017b). Insecticide tolerance can be best avoided by scouting for soybean aphid populations meeting economic threshold to prevent un-needed sprays, not using more than one insecticide at a time, and rotating the modes of action of the chemicals applied (Hodgson et al. 2012).

Insecticide spray timing is also an important part of chemical control. Song et al. (2006) conducted a study throughout the North Central U.S. analyzing soybean aphid control treatments. They found that fields sprayed once during late July or early August, when soybeans bloom or develop pods (R1-R4), yielded better than soybeans sprayed in the latter part of August when seeds develop (R5-R6). Peak soybean aphid populations are usually found during growth stages R3 to R5 when the plant is developing its pods and seeds, concurrent to yields being most influenced (Ragsdale et al. 2007, Hodgson et al. 2012). If sprayed too early, soybean aphid resurgence is possible later in the season. Secondary pest problems are also a potential threat (Hodgson et al. 2012); for instance, two-spotted spider mite populations can increase dramatically when insecticidal sprays have eliminated the mite's natural enemies (Rice et al. 2007, O'Neal and Johnson 2010). If spraying late in the season, a certain amount of time, known as the pre-harvest interval, is required before the crop is safe for consumption. This usually ranges from 7 to 60 days before harvesting soybean, and the information is found on the insecticide product label (Hodgson et al. 2012).

Host Plant Resistance: Soybean and soja plants growing in the wild have unique genotypes altered by abiotic and biotic factors associated with their growing location.

Seed banks preserve the genetic diversity of these plants, maintain a stock collection of their seed, and assign each collection an individual plant introduction (PI) number. Each PI has its own set of traits: growth rate, leaf size, seed color, disease resistance, etc. Some PIs will have resistance against a particular pest.

Heritable plant characteristics that influence the level of damages caused by a pest are known as host plant resistance (Painter 1951, Beck 1965). Plant resistance reduces the need for insecticide and input costs while protecting beneficial insect communities and sparing non-target insects. Host plant resistance to insects occurs in the forms of tolerance, antixenosis, and antibiosis (Smith 1989). In soybean or soja plants, resistance can be observed through increased plant tolerance of soybean aphid-feeding (i.e., tolerance), reduction in ability for soybean aphids to survive and reproduce on a plant (i.e., antibiosis), or through unattractive or deterrent qualities to soybean aphid feeding (i.e., antixenosis) (Hill et al. 2004b, Tilmon et al. 2011). Tolerance is polygenic and allows the plant to withstand larger pest populations before yields are effected (Smith 2005); 'KS4202' is the only soybean with documented soybean aphid tolerance (Pierson et al. 2010). Antibiosis and antixenosis resistance come from individual genes within soybean and soja called *Rag* genes (Resistance to *Aphis glycines*) (Hill et al. 2006, Tilmon et al. 2011). As with many other genetic traits, there are dominant (R) and recessive (r) forms (Hill et al. 2012). Currently, 14 *Rag* genes have been identified (Table 1). After further testing, the provisional gene may be renamed. *Rag1* was the first host plant resistance gene to be used commercially in 2010 (Chiozza et al. 2010, Michel et al. 2011). By 2012, multiple genes (or a 'pyramid') *Rag1+Rag2* soybean cultivar became commercially available (McCarville et al. 2012). The addition of *Rag1+Rag2* resistance

has not shown to affect yields of soybean cultivars (i.e., no yield drag) (Brace and Fehr 2012, McCarville et al. 2014).

Host plant resistance, however, can select for biotypes that overcome specific resistance genes (Gallun 1972). Some soybean aphids are able to successfully feed on soybean plants with *Rag* genes while others cannot. Depending on a soybean aphid's ability to colonize and reproduce on resistant plants, it is considered to be of a particular biotype. Soybean aphids that are unable to colonize soybean containing *Rag* genes are referred to as avirulent, while soybean aphids that are able to colonize resistant soybean are referred to as virulent towards that particular *Rag* gene. Currently, there are four known soybean aphid biotypes in North America, each responding differently to plants with the *Rag1* and *Rag2* genes (Figure 5). Cooper et al. (2015) sampled the biotypic composition of soybean aphid in 10 states and one province over three years. Soybean aphid populations were comprised of 21% biotype 1, 54% biotype 2, 18% biotype 3, and 7% biotype 4. Furthermore, their data indicated greatest variability in soybean aphid virulence in Wisconsin, where soybean aphid was first detected in the U.S. (Cooper et al. 2015).

To date, *Rag3* resistance has not been well characterized with the U.S. soybean aphid biotypes. Alt and Ryan-Mahmutagic (2013) and Varenhorst et al. (2017a) reported lower soybean aphid biotype 4 populations on *Rag3* (PI 567543C) than for other soybean aphid biotypes. Meanwhile, Ajayi-Oyetunde et al. (2016) showed *Rag3* to be ineffective against each of the four biotypes (Ajayi-Oyetunde et al. 2016). Interestingly, these studies all used soybean aphid biotype 4 colonies collected in 2013 from Lomira, WI (Alt and Ryan-Mahmutagic 2013, Ajayi-Oyetunde et al. 2016; Varenhorst, et al. 2017a). Other

resistance genes have not been well documented to soybean aphid biotypes in the U.S. (Hesler et al. 2013; Hill et al. 2012).

Soybean aphid biotypes in China have been characterized using additional resistance genes (*Rag1*, *Rag2*, *Rag3*, *Rag5*, *Rag6*) (Zhong et al. 2014). Four China biotypes were classified by their virulence: China biotype 1 (*Rag1*, *Rag2*, and *Rag5* virulence), China biotype 2 (*Rag2* and *Rag6)*, China biotype 3 (*Rag2* and *Rag5*), and China biotype 4 (*Rag6*) (Zhong et al. 2014).

The characterization of biotypes is complicated by intrabiotypic variation (Pawlowski et al. 2015), induced soybean susceptibility (Varenhorst et al. 2015a), and fitness costs (Varenhorst et al. 2015b). Pawlowski et al. (2015) documented soybean aphid isolates of the same biotype to be successful on particular resistant soybean genotypes at significantly different rates; such intrabiotypic variants could, therefore, be significantly different in some studies. Varenhorst et al. (2015a) documented that initial feeding by a virulent biotype on a resistant plant can induce plant susceptibility and facilitate subsequent colonization by avirulent biotypes; thus, soybean aphid fitness may be associated with population density on resistance soybean (Varenhorst et al. 2015a). Additionally, Varenhorst et al. (2015b) reported reduced populations of virulent biotypes on a susceptible cultivar, suggesting a fitness cost to soybean aphid virulence that had not been observed by previous studies (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013).

Because genetic mapping is prohibitively time-consuming and expensive, very few PIs have been tested for the presence of *Rag* genes. Instead, researchers often allow soybean aphid biotype 1 to feed on different PIs to identify resistant sources among the

lines. To date, free-choice tests have found soybean aphid resistance in approximately one hundred soybean and soja PIs (Hill et al. 2004a, Hesler 2013, Hesler and Tilmon 2017, U.S. NPGS 2017). Further evaluation of the sources of resistance would support the continued protection of our soybean yields against a diversity of soybean aphid field populations.

Other Management Practices: Abiotic and biotic factors affect soybean aphid populations and resulting damage. Changing planting dates to avoid soybean aphid damage is not recommended often due to risks involved in planting too early or too late (e.g. bean leaf beetle pest, soil pathogens, higher soybean aphid populations). Rather, planting crops when they have the highest germination potential is recommended. Adjustments to row spacing also does not affect aphid populations because of soybean aphid's high mobility (Johnson 2010); soybean aphids can travel for up to 11 hours and 6.7 km in a single, tethered flight (Zhang et al. 2008). It is unrealistic to eradicate either its primary or secondary host. Spring soybean aphid populations would likely be unaffected by heavy reductions of common buckthorn overwintering plants (Ragsdale et al. 2004).

Soil nutrition levels may impact soybean aphid colonies. High potassium treatments showed fewer soybean aphids compared to potassium deficient plants (Walter and DiFonzo 2007). Nitrogen levels also play a part in soybean aphid levels, as nitrogen is often the limiting nutrient in many herbivorous insect diets (Mattson 1980). Therefore, high soybean nitrogen levels are correlated with more soybean aphid damage (Hu et al. 1992).

Odors may play a role in aphids' attraction to their hosts; large amounts of non-host plants in the area may hinder their ability to colonize soybean (e.g., grasslands) (Lundgren et al. 2013; Wu et al. 2004). Using a cover crop has proved to also be very beneficial when used correctly and can decrease the need for pesticides. Organic farming practices, could especially benefit from cover crop use as a profitable and chemical-free management tactic (Lundgren et al. 2013, Koch et al. 2015).

Research Objectives

Identification of three virulent soybean aphid biotypes (Kim et al. 2008, Hill et al. 2010, Alt and Ryan-Mahmutagic 2013) and reports of insecticide-resistant soybean aphid (Hanson et al. 2017; Potter et al. 2017; Varenhorst, et al. 2017b) create urgency for finding new, reliable soybean aphid management strategies. The objective of this study was to find soybean and soja PIs resistant to virulent soybean aphid biotype 4, so that strong sources of soybean aphid-resistance may be bred into high-yielding pyramid soybean cultivars, which could significantly reduce the need for insecticides for soybean aphid control while promoting beneficial insects.

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Table 1. List of known soybean aphid resistance genes.

Gene	**Reference**
Rag1	Hill et al. 2006
rag1b	Bales et al. 2013
rag1c	Zhang et al. 2009
Rag2	Mian et al. 2008
Rag2_PI 567301B	Jun et al. 2012
Rag3	Zhang et al. 2010
rag3	Bales et al. 2013
Rag3b	Zhang et al. 2013
Rag3c	Zhang et al. 2017b
Rag3d	Du 2016
Rag3e	Zhang et al. 2017a
rag4 or *Rag4*	Zhang et al. 2009, Varenhorst et al. 2017a
Rag5 (provisional)	Lee et al. 2017
Rag6	Xiao et al. 2013, Zhang et al. 2017a, 2017b

Figure 1. Single soybean seed (left) and multiple soja seeds (right).

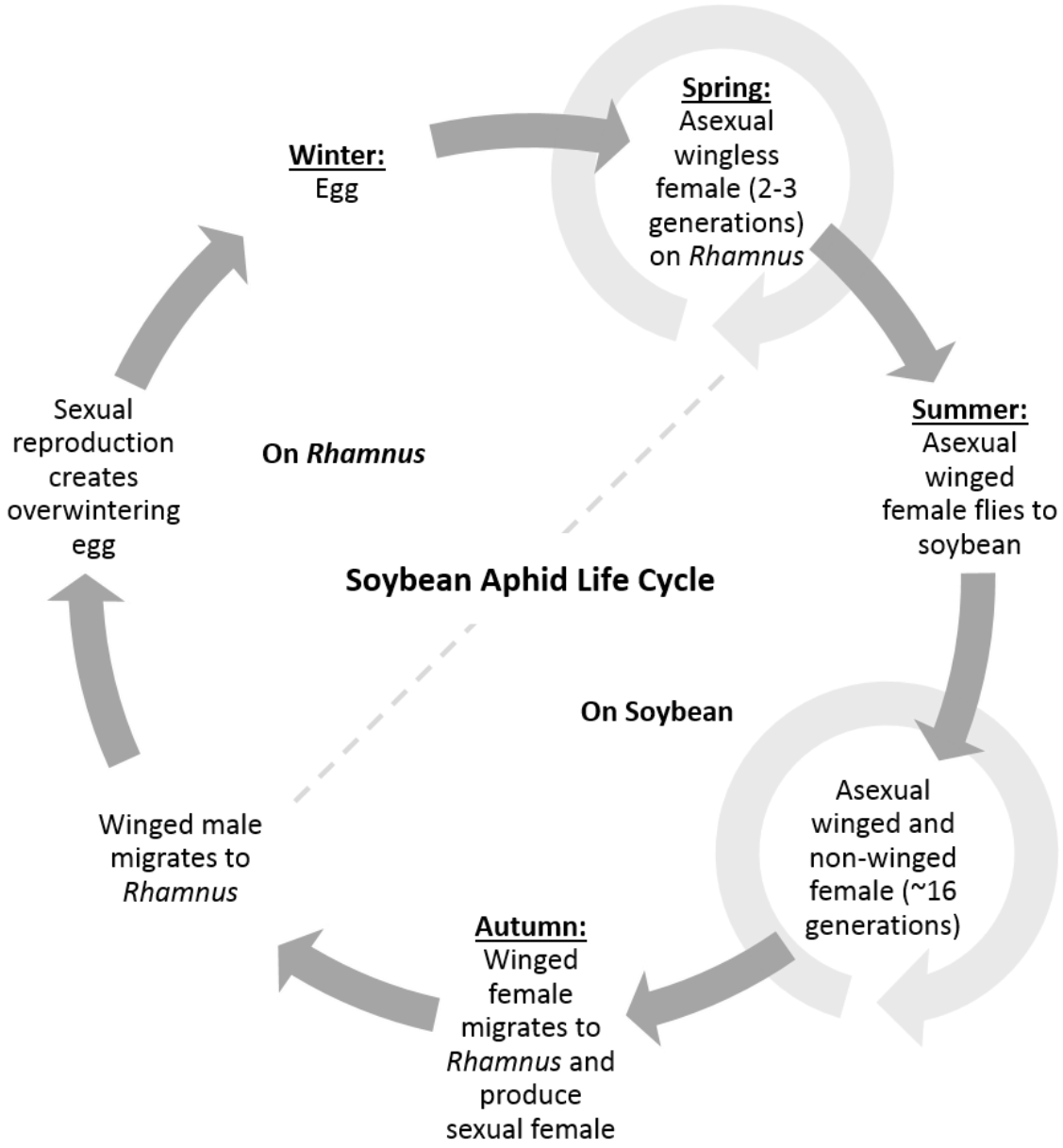

Figure 2. Soybean aphid annual life cycle.

Figure 3. (a) Soybean aphids on a primary host plant, *Rhamnus cathartica* and **(b)** soybean aphids on two secondary host plants of *Glycine soja* (Photo by Eric Beckendorf).

Figure 4. Soybean plant without aphids (left) and soybean with stunting and wrinkled leaves from soybean aphid infestation (right).

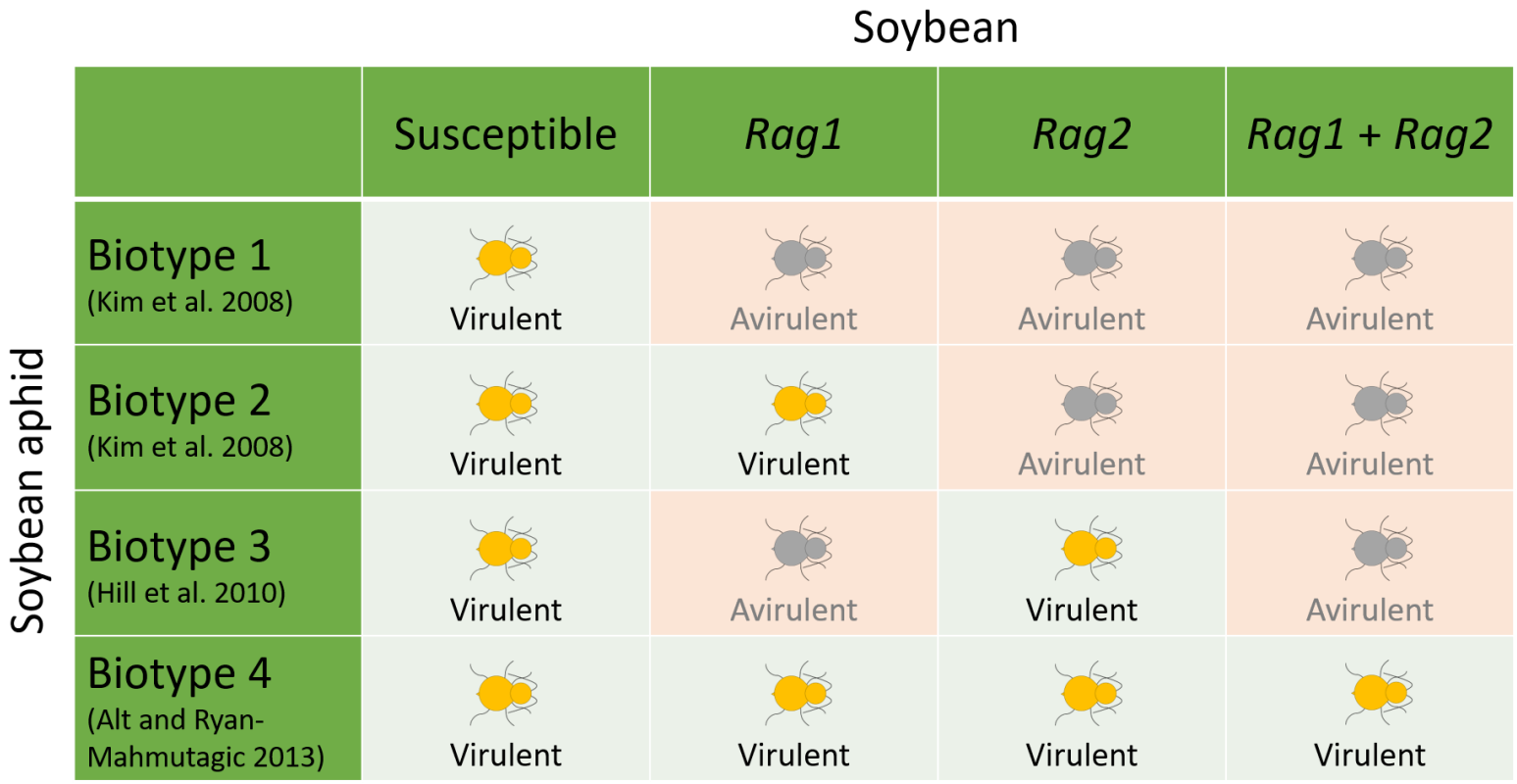

Figure 5. Ability (virulence) or inability (avirulence) of soybean aphid biotypes to heavily infest soybean plants in relation to two main soybean aphid resistance genes.

CHAPTER 1. RESISTANCE TO SOYBEAN APHID BIOTYPE 4 AMONG SELECTED SOJA PLANT INTRODUCTIONS

Abstract

Host plant resistance in soybean, *Glycine max* (L.) Merr., can be used to suppress soybean aphid, *Aphis glycines* Matsumura, populations without the use of insecticides. Of the known biotypes of soybean aphid, biotype 4 is the most virulent and is capable of overcoming all commercially available resistant soybean cultivars. Identifying sources with resistance to soybean aphid biotype 4 is necessary to improve soybean aphid management in an integrated pest management approach. Soja, *Glycine soja* Sieb. and Zucc., plant introductions (PIs) with known resistance to an avirulent soybean aphid biotype were investigated against three iso-female colonies of soybean aphid biotype 4. The biotype 4 colonies were established from three different site-years in Lomira, WI (2013) and Volga, SD (2015 and 2016). Six, three, and eight soja PIs showed putative resistance in free-choice tests to colony 'Lomira13,' 'Volga15,' and 'Volga16,' respectively. Free-choice tests identified two soja PIs with putative resistance to all three colonies: PI 101404A and PI 65549. Six, two, and six soja PIs were resistant to Lomira13, Volga15, and Volga16 no-choice populations, respectively. PI 65549 and PI 101404A suppressed each of the three biotype 4 colonies significantly, which may serve as a valuable source of soybean aphid resistance for future breeding efforts.

Introduction

Plant resistance uses naturally occurring plant defenses to manage pests (Painter 1951, Beck 1965). Genes conferring resistance to soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), have been found in plant introductions (PIs) of soybean, *Glycine max* (L.) Merr. Currently, *Rag* (Resistance to *Aphis glycines*) genes (Hill et al. 2006, Tilmon et al. 2011) are available in some commercial soybean cultivars as *Rag1* alone, *Rag2* alone, or a *Rag1+Rag2* pyramid (Diers 2017).

However, some soybean aphids are able to overcome these *Rag* genes. Currently, there are four soybean aphid biotypes that are characterized by their ability to colonize soybean with various *Rag* genes. Biotype 1 is avirulent, i.e., unable to successfully colonize, soybean containing any known *Rag* genes (Kim et al. 2008). Biotype 2 is virulent on, or able to successfully colonize, soybean containing *Rag1*, but is avirulent on *Rag2* (Kim et al. 2008). Biotype 3 is avirulent on *Rag1*, but is virulent on *Rag2* (Hill et al. 2010). Lastly, soybean aphid biotype 4 is virulent on soybean with *Rag1*, *Rag2*, and *Rag1+Rag2* (Alt and Ryan-Mahmutagic 2013). Recently, broader resistance has been documented (Ajayi-Oyetunde et al. 2016, Varenhorst et al. 2017) in three gene pyramids with *Rag1+Rag2+Rag3* and *Rag1+Rag2+Rag4* with resistance to four (biotype 1, biotype 2, biotype 3, and biotype 4) and three (biotype 1, biotype 2, and biotype 3) biotypes, respectively.

The response of soybean aphid biotype 4 to *Rag3* soybean has varied among recent studies. Reduced biotype 4 populations were reported on PI 567543C with *Rag3* resistance (Alt and Ryan-Mahmutagic 2013, Varenhorst et al. 2017). However, *Rag3* resistance was ineffective at controlling biotype 4 in soybean line ‘LD14-8006’ (*Rag3*

donor: PI 567543C) (Ajayi-Oyetunde et al. 2016). *Rag3* resistance was found to be moderately resistant to soybean aphid biotype 4 in soybean line 'LD14-8039' (*Rag3* donor: PI 567543C) during preliminary studies (S.R.C., unpublished data).

Intrabiotypic variability is defined as quantitative variation among isolates within a biotype of a pest species (Claridge and Den Hollander 1983, Futuyma and Peterson 1985, Pawlowski et al. 2015). Quantitative virulence variation of soybean aphid biotype 3 on resistant soybean genotypes has been documented (Pawlowski et al. 2015). The varied performance of soybean aphid biotype 4 on *Rag3* soybean lines mentioned above suggests that variability exists within this biotype.

Soja, *Glycine soja* Sieb. and Zucc., is the closest living relative to cultivated soybean, and has been theorized to be a promising source of soybean aphid resistance (Sun et al. 1990, Hill et al. 2004, Hesler 2013). Hesler (2013) and Hesler and Tilmon (2017) tested 501 soja plant introductions (PIs) against soybean aphid biotype 1, and identified 17 highly resistant and 9 moderately resistant soja PIs (Hesler 2013, Hesler and Tilmon 2017).

Soja resistant to soybean aphid biotype 1 may also confer resistance for other, more virulent soybean aphid biotypes. The first objective of this study was to evaluate 20 soja PIs for resistance to soybean aphid biotype 4. The second objective of this study was to evaluate three colonies of soybean aphid biotype 4 on the soja PIs. To investigate this, the soybean aphid biotype 4 colonies from three different site-years underwent comprehensive free-choice and no-choice tests.

Materials and Methods

Soybean Aphid Biotype 4 Colonies

The first of the three soybean aphid biotype 4 colonies acquired was 'Lomira13.' This colony was initially collected by University of Wisconsin (Madison, WI) researcher Michael Crossley in August 2013 near Lomira, WI, the original site from which soybean aphid biotype 4 was identified (Alt and Ryan-Mahmutagic 2013). Isolates were brought to Urbana, IL and maintained by Doris Lagos-Kutz at University of Illinois Urbana-Champaign on a pyramid *Rag1+Rag2* line 'LD12-12734a.' In 2016, biotype 4 isolates from the Lomira13 collection were obtained and reared by the USDA-ARS North Central Agricultural Research Laboratory (NCARL) in Brookings, SD. The second colony, 'Volga15,' was collected near Volga, SD on a South Dakota State University (SDSU, Brookings, SD) research farm. The soybean aphids were found on a pyramid soybean breeding line 'LD12-15805Ra' containing the *Rag1+Rag2* resistance genes during September 2015 by Swapna Purandare and MacKenzie Mattern from SDSU. Lastly, 'Volga16' was collected on LD12-15805Ra in August 2016 at a similar location to that of Volga15 by Eric Beckendorf and S.R.C. at NCARL and SDSU, respectively.

All subsequent work was conducted at NCARL. Aphid-free greenhouses had a 16:8 (Light:Dark) photoregime and temperature regime of approximately 23:18°C (L:D). Growth chambers (CMP4030 Conviron, Winnipeg, Canada) held soybean aphid-infested plants under similar conditions of photoperiod 16:8 (L:D), temperature 23:18°C (L:D), and relative humidity of 50%. At NCARL, soybean aphids were continually reared in growth chambers on soybean line 'IA2104RA12' containing the *Rag1+Rag2* pyramided resistance genes (Table 2). Colony plants were first grown in greenhouses with ten seeds

per large pot with potting soil (Table 3 and 4). After approximately four weeks, colony plants were transferred to growth chambers and infested with soybean aphid biotype 4.

Once a soybean aphid biotype 4 colony was established, six apterous (wingless) adults were chosen arbitrarily, caged individually, and kept in a separate growth chamber. Cages were made from clear 0.6cm thick extruded acrylic tube (12.7cm outer diameter x 40.6cm height; Ridout Plastics Co. Inc., San Diego, CA, item number: ACREXT5.000X4.750). Two opposing holes, 5.1cm diameter, were drilled into the tube, and no-thrips resistant screens (screen hole size: 0.150mm^2, thread size: 15mm, BioQuip, Rancho Dominquez, CA) were hot-glued to cover the holes and one end of the tube. After two weeks, the isolated female that reproduced the most clonal offspring was chosen for that associated iso-female biotype 4 colony; all other aphids from that collection were discarded. Each of the three iso-female colonies was maintained in a separate growth chamber and assigned its own caretaker to avoid cross-colony contamination.

Seed Acquisition

Soja PIs were obtained as seeds from the USDA-ARS U.S. Soybean Germplasm Collection (USSGC) in Urbana, IL. The PIs were chosen based on their ability to suppress soybean aphid biotype 1 in past research (Table 5) (Hesler 2013, Hesler and Tilmon 2017). PIs perpetuated by USSGC in 1999 may have experienced seed mislabeling (Hesler and Tilmon 2017). Since the discovery of this mishap, soja PIs from 1999 have been discarded at USSGC, and the seed we had in-house was no longer recognized as its labelled PI number. For that reason, we renamed these PIs with a “99-

PI" in front of the PI number it was originally thought to be (Hesler and Tilmon 2017). Both soybean and soja lines were used as checks (Table 2). The soybean aphid-resistant soybean checks were chosen because of known *Rag* genes associated with them, whereas the genetic bases of resistance has not been determined for the resistant soja checks.

PIs were seed-increased at NCARL as needed. All soja seeds were treated for 20 minutes in sulfuric acid (H_2SO_4, Fisher Chemical Catalog No. A300-212), scarifying the hard seed coat to promote germination (Lenis et al. 2011).

Free-Choice Tests

We hypothesized that some soja PIs with resistance to soybean aphid biotype 1 would also show putative resistance to soybean aphid biotype 4. A free-choice test procedure was used, based on methods from Hesler (2013).

Soja test PIs and soybean checks were planted in a greenhouse. The soja test PIs were first planted in soaked peat pellets (Table 4, Figure 6) with two seeds per pellet. Soybean checks were planted first in small pots (Table 3) with two to three seeds per pot. Peat pellets and small pots were reduced to a single plant prior to experimentation.

Test lines were grown for approximately two weeks to intermediate VC stage (vegetative cotyledon stage: developed unifoliate leaves, developing first trifoliate) (Fehr and Caviness 1977, Licht 2014). Twenty-four to 48 hours before free-choice tests commenced, uniform seedlings of test lines were chosen. Soja plants were transplanted into small pots by removing the mesh lining of the pellet and then covering the peat with potting soil. The soil surface of experimental pots was covered with about a 0.5cm layer of sand (Table 4) to help regulate soil moisture and facilitate aphid dispersal among test

PIs and checks. A set of 16 plants (usually 10 test PIs and 6 checks) per replicate was placed into a plastic tray (Table 3). Each free-choice test used a randomized complete block design with seven to eight replicate trays. A 35cm x 4mm (height, diameter) bamboo stake was placed adjacent to soja plants as needed to support their tendrils.

Founder plants were used to infest free-choice test plants and checks with soybean aphids. IA2104RA12 was used for founder plants, with one plant per small pot (Table 3). Founder plants were grown in greenhouses for approximately two weeks to the intermediate VC stage. Founder plants were infested with soybean aphid biotype 4 from colony plants that had been cut and placed in acrylic tubes with the open top side covered in Parafilm M® (Pechiney Plastic Packaging, Mensha, WI). As the cut colony plants dried, soybean aphids crawled off of them, up the tube, and on to the Parafilm. A wetted fine-tipped paintbrush was used to transfer 5 apterous adult aphids from the Parafilm to each unifoliate leaf of a founder plant (10 aphids per plant). Based on preliminary testing, this initial infestation rate produced approximately 250 aphids per founder plant two weeks later; and two founder plants with this level of aphids was adequate for infesting each set of test PIs and checks in the free-choice test. The stems of founder plants were cut and the detached stems were placed back in the center of their pot upright (Figure 7). A founder plant was placed at one of two focal points per tray, each equidistant from surrounding lines (Figure 8). As founder plants dried, soybean aphids dispersed from them and colonized test PIs and checks.

Individual free-choice tests were run for two weeks in a growth chamber, whereupon individual plants were rated on a 0-to-6 scale based on 50 aphid-increments (Table 6). Means and medians of the ratings for the respective PIs and checks were

determined using the PROC MEANS procedure, which was part of the SAS statistical software package (SAS Institute, 2014). Two free-choice tests were run for each of the three colonies. PIs with both mean and median ratings <2.5 were advanced for follow-up in a no-choice test.

No-Choice Tests

We next hypothesized that some putatively resistant soja from the free-choice tests would continue to significantly suppress soybean aphid biotype 4 in no-choice tests. Methods from Hesler et al. (2017) were used for follow-up testing, initial aphid infestation numbers were modified. Soja PIs were planted in soaked peat pellets, and soybean checks were planted in small pots (Table 3). Test lines were grown until intermediate VC stage. Twenty-four to 48 hours before infestation, twelve uniform plants were chosen for each line and transplanted into large pots (Table 3), with each pot containing two plants of a particular soja PI or soybean check. For the soja PIs, the mesh lining of the peat pellets was removed before transplanting. Potting soil was used to fill the pots, and the soil surface was sanded 0.5cm deep to stabilize acrylic tubes that were used to confine aphids on test plants.

Aphid colony plants were cut and dried in tubes to facilitate the availability of soybean aphids for infesting test plants. Three apterous adult soybean aphids were transferred onto each unifoliate, or six soybean aphids per test plant. After infestation, each pair of test lines within a pot was covered with an acrylic tube to confine aphids (Figure 9).

Ten days after infestation, one of the two plants in each tube was chosen at random, cut, placed in its own labelled bag, and stored in a freezer. Twenty days after infestation, the remaining plant was cut, bagged, and frozen. The tube and sandy surface were examined, and any live soybean aphids were recorded. Later, plants were thawed, and soybean aphids counted.

One no-choice test was completed for each of the three colonies; each test included three soybean checks and the particular PIs identified as resistant in the free-choice tests for each respective colony (Table 2). Test lines had six replications each.

The number of aphids per plant was treated as discrete, Poisson response variables in a generalized linear mixed model (PROC GLIMMIX; SAS Institute, 2014) in an analysis of variance with test line, sample day, and test line-by-sample day interaction as treatment factors. Following a significant (P <0.05) result, a least squares mean (LSMEANS) procedure with Bonferroni adjustment was used for multiple comparisons of the mean number of aphids per plant among test lines. If the line-by-sample day interaction was significant for a particular no-choice test, test lines were compared separately within each sample day. An individual PI was considered highly resistant to a respective colony when its mean number of aphids per plant was significantly lower than that of the moderately resistant *Rag3* check (LD14-8039).

Results

Free-Choice Tests

Results of the free-choice tests confirmed our first hypothesis, that some soja PIs with resistance to soybean aphid biotype 1 showed putative resistance to soybean aphid

biotype 4. Mean and median aphid infestation ratings differed by test lines for free-choice tests for each colony (Table 7 and 8). For Lomira13, soja lines PI 101404A, PI 135624, PI 342618A, PI 549046, and PI 65549 were resistant in the first free-choice test; and 99-PI 81762 was resistant in the second free-choice test. For Volga15, PI 101404A and PI 65549 were resistant in the first free-choice test, and PI 407299 was resistant in the second. For Volga16, PI 101404A, PI 135624, PI 342618A, PI 407205, PI 549046, and PI 65549 were resistant in the first free-choice test, and PI 407299 and 99-PI 81762 in the second free-choice test.

No-Choice Tests

Our second hypothesis was confirmed in no-choice tests, as some resistant free-choice soja PIs continued to significantly suppress soybean aphid biotype 4 in no-choice tests.

For Lomira13, the mean number of soybean aphids per plant varied significantly (Table 9) by line, sample day, and line-by-sample day interaction (Figure 10 and 13). After 10 days, Lomira13 soybean aphid populations were significantly lower on five PIs (PI 101404A, PI 135624, PI 549046, PI 65549, 99-PI 81762) than on the *Rag3* check LD14-8039. Populations decreased between sample day 10 and day 20 on four lines (PI 101404A, PI 135624, PI 549046, and 99-PI81762), but increased on PI 342618A and soybean checks. All six free-choice resistant PIs (PI 549046, PI 101404A, PI 135624, 99-PI 81762, PI 65549, PI 342618A) had significantly lower mean number of aphids per plant than on LD14-8039 on sample day 20, and thus were resistant in the no-choice test.

Volga15 populations varied significantly (Table 9) by line and sample day (Figure 11 and 13). Aphid counts increased between sample day 10 and day 20 across all test entries, and thus the line-by-sample day interaction was not significant. PI 65549 and PI 101404A had significantly lower populations than LD14-8039.

Populations of Volga16 soybean aphids varied significantly (Table 9) by line and sample day (Figure 12 and 13). The mean number of soybean aphids per plant increased from day 10 to day 20 on all test entries, and thus the line-by-sample day interaction was not significant. Six soja (PI 549046, PI 135624, 99-PI 81762, PI 65549, PI 407299, PI 101404A) had significantly lower numbers of Volga16 soybean aphids compared to LD14-8039.

Soybean checks susceptible to soybean aphid biotype 4 typically had greater numbers of aphids per plant than the soja test lines. The mean number of soybean aphids per plant was always greater on IA2104 (no *Rag* genes) than on any of the soja PIs. In addition, the mean number of soybean aphids per plant was generally greater on IA2104RA12 (*Rag1+Rag2*) than on the soja PIs, except that the numbers of Volga16 aphids per plant did not significantly differ between PI 407205 and IA2104RA12.

Discussion

Soybean Aphid Biotype 4 Colonies

Our findings indicate that two soja PIs were resistant to all three colonies during free-choice tests: PI 101404A and PI 65549. Both PI 101404A and PI 65549 had significantly lower no-choice populations than on the *Rag3* soybean check and therefore were considered resistant to the three colonies of soybean aphid biotype 4.

For this research, each biotype 4 iso-female colony presented a unique set of responses to biotype 1-resistant soja. Free-choice tests identified six, three, and eight PIs that showed resistance to Lomira13, Volga15, and Volga16 soybean aphids, respectively. The Lomira13 and Volga16 colonies had higher mean ratings (i.e., larger populations) in free-choice tests on *Rag1* check 'LD09-05484a' than the *Rag2* check, while the Volga15 colony had higher mean ratings on *Rag2* check '2880a' than on the *Rag1* check. No-choice tests identified six, two, and six PIs that were resistant to Lomira13, Volga15, and Volga16 colonies, respectively. For Volga16, one PI was not significantly different than the *Rag1+Rag2* check IA2104RA12. Colonies differed in reproductive rates on soybean no-choice checks, and Volga16 populations nearly double that of Lomira13, with intermediate reproductive rates for Volga15 soybean aphids.

Quantitative differences between isolates of a soybean aphid biotype have also been documented in previous research (Michel et al. 2010, 2011; Pawlowski et al. 2015). The cause of variability among isolates is still unknown. Possible factors contributing to these differences include endosymbiotic diversity, i.e., bacteria influencing soybean aphid host specificity, nutritional uptake, and defensive qualities (Wenger and Michel 2013, Cassone et al. 2015, Wulff and White 2015), or complex polygenic mechanisms, i.e., genes working in combination, thus causing virulency to occur on a gradient (Diehl and Bush 1984, Wenger and Michel 2013).

Soja Plant Introductions

Although free-choice tests are conducted over two weeks, timing of no-choice tests among research groups varies from 7 days (Alt and Ryan-Mahmutagic 2013) to 21

days (Hesler 2013). We chose to collect no-choice counts 10 and 20 days after infestation.

Variability within soybean aphid biotypes creates greater challenges for soybean protection. However, the large percentage of soja that moved on to follow-up testing for each colony was especially promising: 31.6% in Lomira13 testing, 15% in Volga15, and 40% in Volga16. Because this research evaluated phenotypic traits, our reasoning for causes of resistance (i.e., molecular) in soja is limited to speculation.

Soja is a wild plant in Asia (CFIA 2012). In the U.S., soybean aphid's contact with soja has largely been limited to controlled environment experimentation (Hill et al. 2004, Hesler 2013, Hesler and Tilmon 2017). Therefore, soybean aphid may not be as evolutionarily adept at utilizing soja as a secondary host.

As the likely ancestor to cultivated soybean (Carter et al. 2004), soja has great genetic diversity in pest and disease resistance (Guo 2012, Hajjar and Hodgkins 2007). Currently, only one soja line has been genetically analyzed for soybean aphid resistance: soja germplasm '85-32' was mapped with *Rag6* and *Rag3c* genes (Zhang et al. 2017). Pyramided resistance genes can provide broader soybean aphid protection (McCarville et al. 2014, Ajayi-Oyetunde et al. 2016). If there are multiple resistance genes in many of these soja PIs, it would explain the large proportion of resistance found in our soja PIs.

Larger soybean aphid populations were anecdotally observed on taller soja compared to shorter soja of the same PI. Count differences could be due to the higher carrying capacity of taller soja or may be caused by differences in resource allocation of the plant. Allocating more resources to growth rather than defense or reproduction could

leave the plant more susceptible to pest outbreaks (Lerdau and Gershenzon 1997, Mithöfer and Boland 2012).

Conclusions

The lack of research on soja makes it difficult to discern the significance of our results. Resistance in a growth chamber setting does not guarantee success in a field setting. However, the large proportion of soja that continued to suppress soybean aphid biotype 4 populations in no-choice testing was very promising. It was especially encouraging that two soja PIs suppressed the three colonies, collected from different site-years. PI 65549 and PI 101404A are of maturity group II, collected in Heilongjiang, China, and showed strong resistance to our biotype 4 colonies.

Future research should include investigation of soybean aphid polygenic mechanisms or endosymbiotic communities that may be involved in soybean aphid variability. PI 65549 and PI 101404A should continue on for genetic testing to identify resistance genes. Breeding of soja resistance may add the necessary diversity to high-yielding soybean cultivars to increase durability of host plant resistance.

One purpose of this research was to revise the current pool of resistant soja to the most recently discovered soybean aphid biotype. We eliminated soja susceptible to three iso-female soybean aphid biotype 4 colonies collected in three site-years. In the process, we observed remarkable soybean aphid variability within biotype 4. We hope breeders explore PI 65549 and PI 101404A further to identify the cause of their strong resistance. Host plant resistance in soybean is a tool that limits input costs, controls pest damage, protects beneficial insect populations, and reduces environmental impacts. With

continued research, we can better understand the complexity of this plant-pest interaction, not only improving soybean protection but to hopefully extend this knowledge to future pest introductions.

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Table 2. Characteristics of soybean and soja plants used as experimental checks.

Check Type	**Species**	**Line**	**Provider**	**Pedigree**
Susceptible, Free-choice check	*Glycine soja*	PI 522212B	U.S. Soybean Germplasm Collection, Urbana, IL	None
Susceptible, Free-choice check	*Glycine max*	'Brookings' (PI 667735)	South Dakota State University, Brookings, SD	A00-711063 x SD98-595
Susceptible, No-choice check	*Glycine max*	'IA2104'	Iowa State University Research Foundation Inc., Ames, IA	IA3027 x Soygenetics F40412C
Rag1, Free-choice check	*Glycine max*	'LD09-05484a'	Blue River Hybrids, Kelley, IA	undisclosed
Rag2, Free-choice check	*Glycine max*	'2880a'	Blue River Hybrids, Kelley, IA	undisclosed
Rag3, Free-choice and No-choice check	*Glycine max*	'LD14-8039'	University of Illinois National Soybean Research Center, Urbana, IL	[Titan(5) x E10005] x [Titan(5) x F1 (LD08-12446a x LD05-30588a)]
Rag1+Rag2, Free-choice and No-choice check; Colony and founder plants	*Glycine max*	'IA2104RA12'	Iowa State University Research Foundation Inc., Ames, IA	undisclosed

Table 3. List of containers used in planting.

Product	**Size**	**Soil above/ below seed**	**Use**	**Manufacturer**
Small pot	8.25cm x 6.5cm x 7.62cm (top side, bottom side, ht.)	100mL/ 150mL	Founder plants Free-choice tests	International Greenhouse Co., Danville, IL
Large pot	6cm x 4cm x 5.7cm (top diam., bottom diam., ht.)	300mL/ 1L	Colony plants No-Choice tests	Myers Industries Inc., Earth City, MO
Tray	26.5cm x 51cm x 6.5cm (width, length, ht.)	Not applicable	Holds 18 small or 6 large pots	T.O Plastics Inc., Clearwater, MN

Table 4. Soil media used for growing soybean and soja.

Media	**Elements**	**Source**
Potting Soil (2:1:1 mixture)	Vienna soil (Fine-loamy, mixed Calcic Hapludolls)	Brookings, SD
	Horticultural coarse vermiculite	Perlite Vermiculite Packaging, North Bloomfield, OH
	Canadian sphagnum peat moss	Sun Gro Horticulture Distribution Inc., Agawam, MA
Peat Pellets	Jiffy-7® Horticultural Peat Pellet	Jiffy Products of America Inc., Tea, SD
Sand	Industrial quartz	Unimin Corporation, Le Sueur, MN

Table 5. Soja plant introductions (PI) with reported resistance to soybean aphid.

PI	**MG[1]**	**Country of origin**	**References**
PI 101404A	II	China	Hesler and Tilmon 2017
PI 135624	II	China	Hesler and Tilmon 2017
PI 342618A	II	Russian Federation	Hesler and Tilmon 2017
PI 407032B	IV	Japan	Hesler and Tilmon 2017
PI 407205	IV	South Korea	Hesler and Tilmon 2017
PI 407299	II	China	Hesler and Tilmon 2017
PI 468399C	IV	China	Hesler 2013
PI 479747	III	China	Hesler 2013
PI 479749	III	China	Hesler 2013
PI 483464A	III	China	Hesler 2013
PI 507756	00	Russian Federation	Hesler 2013
PI 507786	III	Russian Federation	Hesler 2013
PI 522228	I	Russian Federation	Hesler 2013
PI 522232	I	Russian Federation	Hesler 2013
99-PI 522233	NA[2]	NA[2]	Hesler and Tilmon 2017
99-PI 522235C	NA[2]	NA[2]	Hesler and Tilmon 2017
PI 549032	III	China	Hesler and Tilmon 2017
PI 549035B	III	China	Hesler and Tilmon 2017
PI 549046	IV	China	Hesler 2013, Hesler and Tilmon 2017
PI 65549	II	China	Hesler and Tilmon 2017
99-PI 81762	NA[2]	NA[2]	Hesler and Tilmon 2017

[1]MG, maturity group; [2]NA, not applicable

Table 6. Rating scale for free-choice tests. Plants were individually rated after two weeks based on a 50 soybean aphid-increment scale.

Rating	Soybean aphids per plant
0	0
1	1-50
2	51-100
3	101-150
4	151-200
5	201-250
6	250+

Table 7. Mean and median ratings of first soja free-choice test results for each soybean aphid biotype 4 colony. Checks (italicized) and soja plant introduction (PI) plants were rated individually on a 0-to-6 soybean aphid scale. Susceptible PIs indicated in blue and resistant PIs indicated in purple.

Soybean aphid colony

Lomira13 Soja 1

Line	Mean	Med
PI 101404A	**1.8**	**1**
PI 135624	**1.4**	**1**
PI 342618A	**2.3**	**2**
PI 407205	5.5	6
PI 468399C	5.4	6
PI 479749	4	4.5
PI 549032	4.3	4.5
PI 549035B	5.1	5.5
PI 549046	**1**	**1**
PI 65549	**1.6**	**1.5**
LD09-05484a	*5.6*	*6*
2880a	*5.3*	*6*
LD14-8039	*4.4*	*4.5*
IA2104RA12	*3.5*	*3.5*
PI 522212B	*4.3*	*4.5*
Brookings	*5.4*	*6*

Volga15 Soja 1

Line	Mean	Med
PI 101404A	**1.4**	**1**
PI 135624	2.5	2
PI 342618A	2.6	2
PI 407205	5.8	6
PI 468399C	5	6
PI 479749	4.3	4.5
99-PI 522233	6	6
PI 549035B	5.1	6
PI 549046	2.8	3
PI 65549	**2**	**1**
LD09-05484a	*5.5*	*6*
2880a	*5.9*	*6*
LD14-8039	*5.5*	*6*
IA2104RA12	*3.9*	*3.5*
PI 522212B	*5.1*	*6*
Brookings	*5.3*	*6*

Volga16 Soja 1

Line	Mean	Med
PI 101404A	**1**	**1**
PI 135624	**1**	**1**
PI 342618A	**2**	**1**
PI 407205	**2.1**	**2**
PI 468399C	3.9	4
PI 479749	5.4	6
PI 549032	3.4	3
PI 549035B	4.1	4
PI 549046	**1.5**	**1**
PI 65549	**1**	**1**
LD09-05484a	*5.8*	*6*
2880a	*4.6*	*5*
LD14-8039	*3.9*	*4*
IA2104RA12	*4.3*	*4*
PI 522212B	*5.1*	*6*
Brookings	*6*	*6*

Eight replications were observed for all test lines in each free-choice test.

Test lines were repeated in the Soja 1 tests for all colonies unless insufficient germination: Volga15 PI 549032: not tested, Volga16: 99-PI 522233 not tested, Volga 15: 99-PI 522233 tested in Soja 2 (Table 6).

Table 8. Mean and median ratings of second soja free-choice test results for each soybean aphid biotype 4 colony. Plants were rated individually on a 0-to-6, 50 soybean aphid-increment scale for all soybean checks and plant introductions (PIs). Susceptible PIs indicated in blue and resistant PIs indicated in purple.

Soybean aphid colony

Lomira13 Soja 2

Line	Mean	Med
PI 407299	3.8	4
PI 479747	5.4	5.5
PI 483464A	5	5.5
PI 507756	5.4	6
PI 507786	4.9	5.5
PI 522232	5	6
99-PI 522233	5.8	6
99-PI 522235C	6	6
99-PI 81762	**1.6**	**1**
IA2104	*5.5*	*6*
LD09-05484a	*4.8*	*6*
2880a	*4.6*	*5*
LD14-8039	*2.6*	*2*
IA2104RA12	*2.9*	*3*
PI 522212B	*4.8*	*5*
Brookings	*4*	*4.5*

Volga15 Soja 2

Line	Mean	Med
PI 407032B	4	5
PI 407299	**1.7**	**1**
PI 479747	4.4	6
PI 483464A	4.9	6
PI 507756	5	6
PI 507786	3.7	4
PI 522228	5.4	5
PI 522232	4.9	6
99-PI 522235C	5.1	6
99-PI 81762	3.4	3
LD09-05484a	*4.7*	*6*
2880a	*6*	*6*
LD14-8039	*5.4*	*6*
IA2104RA12	*3.9*	*5*
PI 522212B	*4.9*	*6*
Brookings	*4.7*	*5*

Volga16 Soja 2

Line	Mean	Med
PI 407032B	3	2
PI 407299	**1.1**	**1**
PI 479747	4.6	4.5
PI 483464A	2.6	2
PI 507756	6	6
PI 507786	4.3	5
PI 522228	5.9	6
PI 522232	6	6
99-PI 522235C	5.3	5.5
99-PI 81762	**1**	**1**
LD09-05484a	*6*	*6*
2880a	*4.5*	*4.5*
LD14-8039	*4.3*	*4*
IA2104RA12	*4.6*	*5*
PI 522212B	*5.3*	*6*
Brookings	*5.9*	*6*

Eight replications were observed for each line in Lomira13 and Volga16 tests; seven replications were observed for Volga15 lines. Test lines were repeated in the Soja 2 tests for all colonies unless insufficient germination: Lomira13 PI 522228: not tested, Lomira13 PI 407032B: not tested.

Table 9. ANOVA output for mean no-choice counts of soja plant introductions in each of the three soybean aphid colonies.

Colony	Effect	DF[1] (line, error)	*F* value	*P* value
Lomira13	Line	8, 86	84.95	<.0001
	Sample day	1, 86	7.47	0.0076
	Line-by-Sample day	8, 86	9.75	<.0001
Volga15	Line	5, 60	27.02	<.0001
	Sample day	1, 60	143.54	<.0001
	Line-by-Sample day	5, 60	1.16	0.3401
Volga16	Line	10, 110	34.59	<.0001
	Sample day	1, 110	110.54	<.0001
	Line-by-Sample day	10, 110	0.62	0.7955

[1]DF, degrees of freedom

Figure 6. Plastic tray with peat pellets and emerging soja plants, sown at two seeds per pellet.

Figure 7. Founder plants used as sources of soybean aphid inoculum in free-choice tests. The founder plants were grown in small pots. After infestation, aphid populations on founder plants grew to approximately 250 per plant after two weeks. Plants, such as the one held here, were cut at the stem and positioned upright in the center of their pot, which was positioned at two foci within each tray used in free-choice tests.

1	2	3
4	Founder plant	5
6	7	8
9	10	11
12	Founder plant	13
14	15	16

Figure 8. Actual (right) and schematic (left) spatial arrangement of plants in one of eight soja free-choice test replications. Each replicate consisted of test lines (10 per experiment), checks (six), and soybean aphid-infested founder plants (two). Infested founder plants were positioned equidistantly from test lines with randomly assigned location numbers for each replication.

Figure 9. Soja no-choice test plants in large pots caged with acrylic tubes and infested with six soybean aphids per plant. Ten days after initial infestation, one plant per pot was chosen at random, cut, and aphids on it were counted. After 20 days, the remaining plant was cut and aphids were counted.

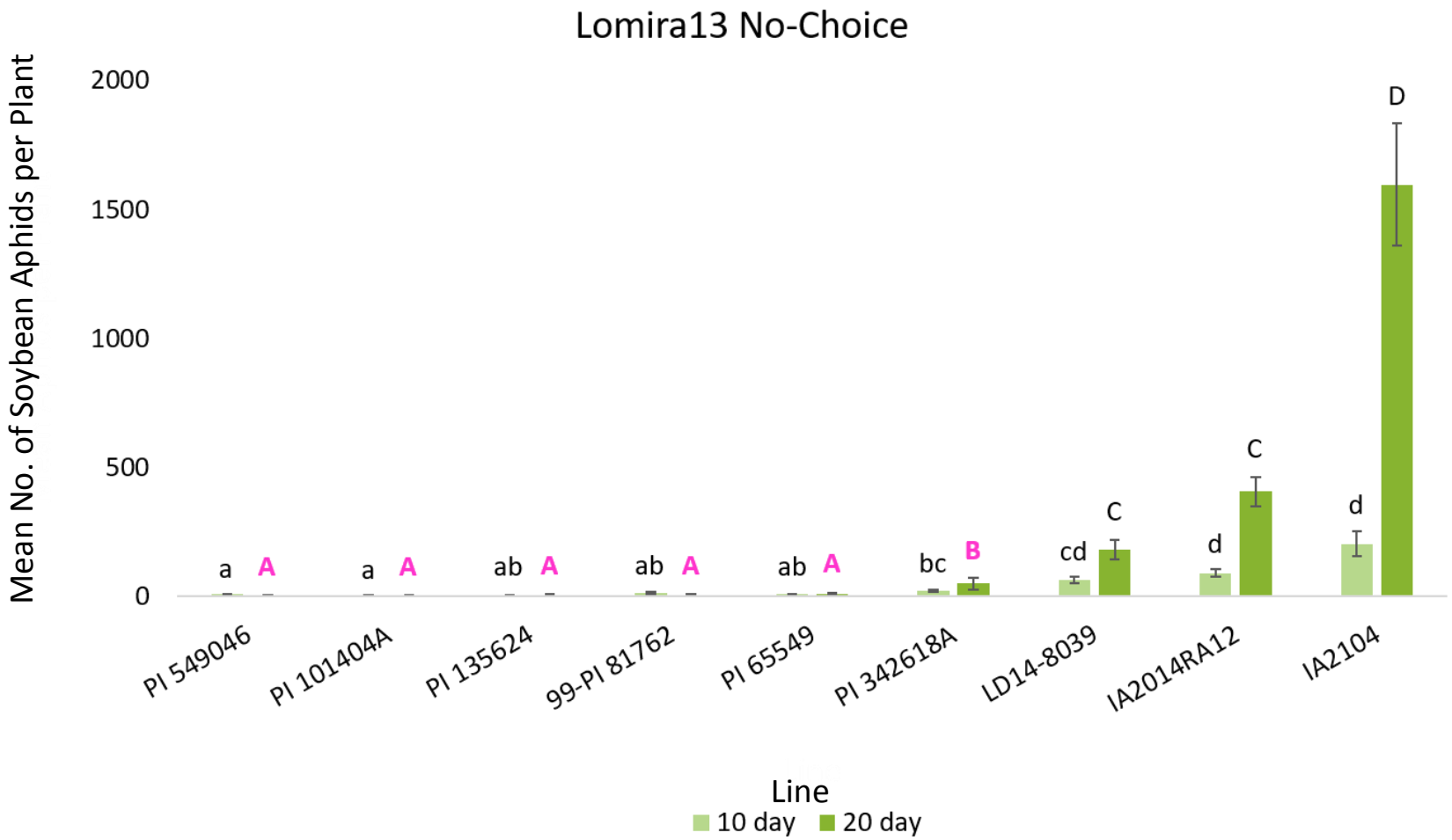

Figure 10. Lomira13 soja no-choice test, mean ± SEM number of soybean aphids per plant 10 and 20 days post-infestation. Bars with different letters above them indicate statistically significant differences. Pink letters signify plant introductions with significantly lower mean aphids per plant than the moderately resistant check (LD14-8039) after 20 days.

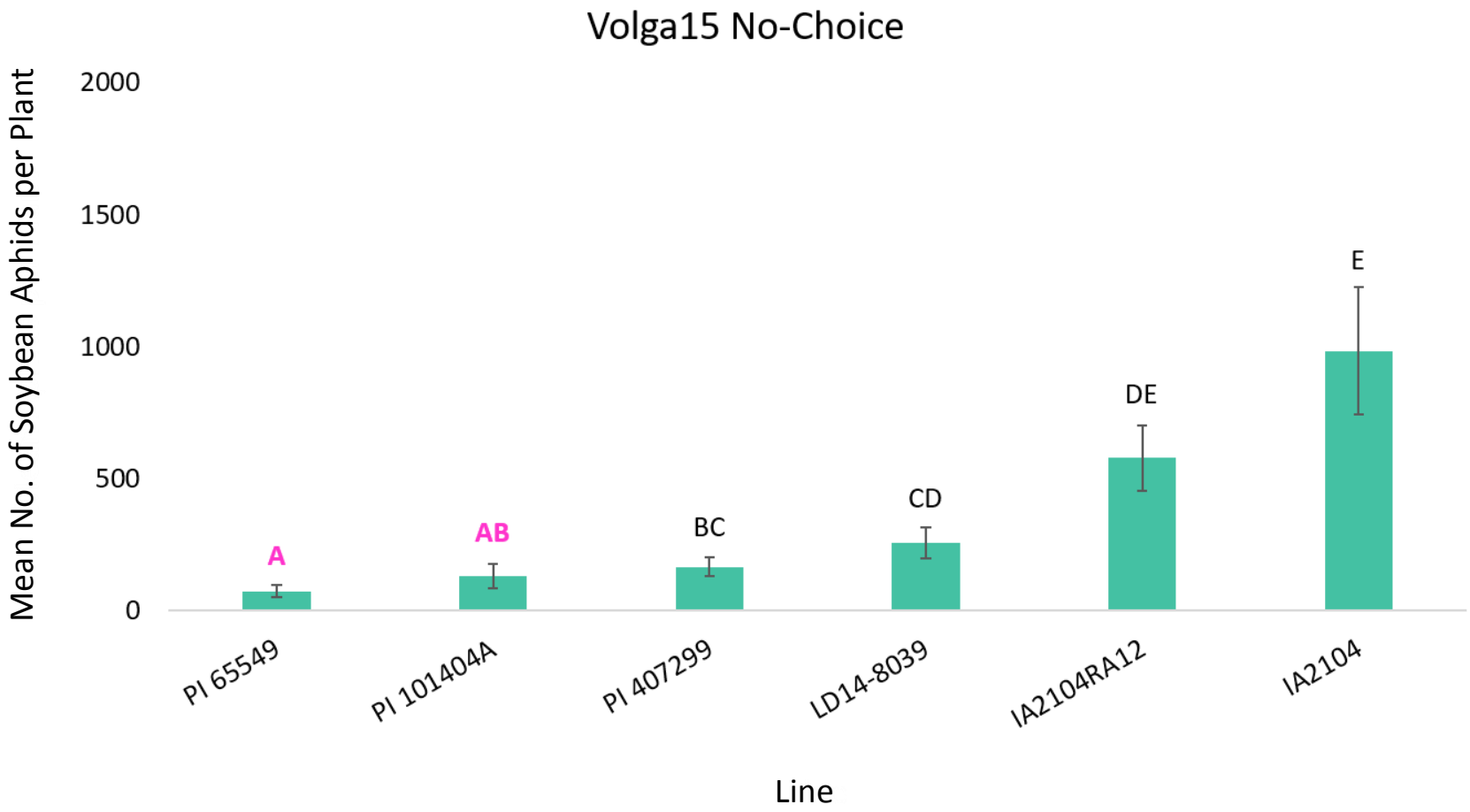

Figure 11. Volga15 soja no-choice test, combined mean ± SEM number of soybean aphids per plant 10 and 20 days after infestation. Pink lettering signify plant introductions with aphid populations statistically lower than those on the *Rag3* check LD14-8039.

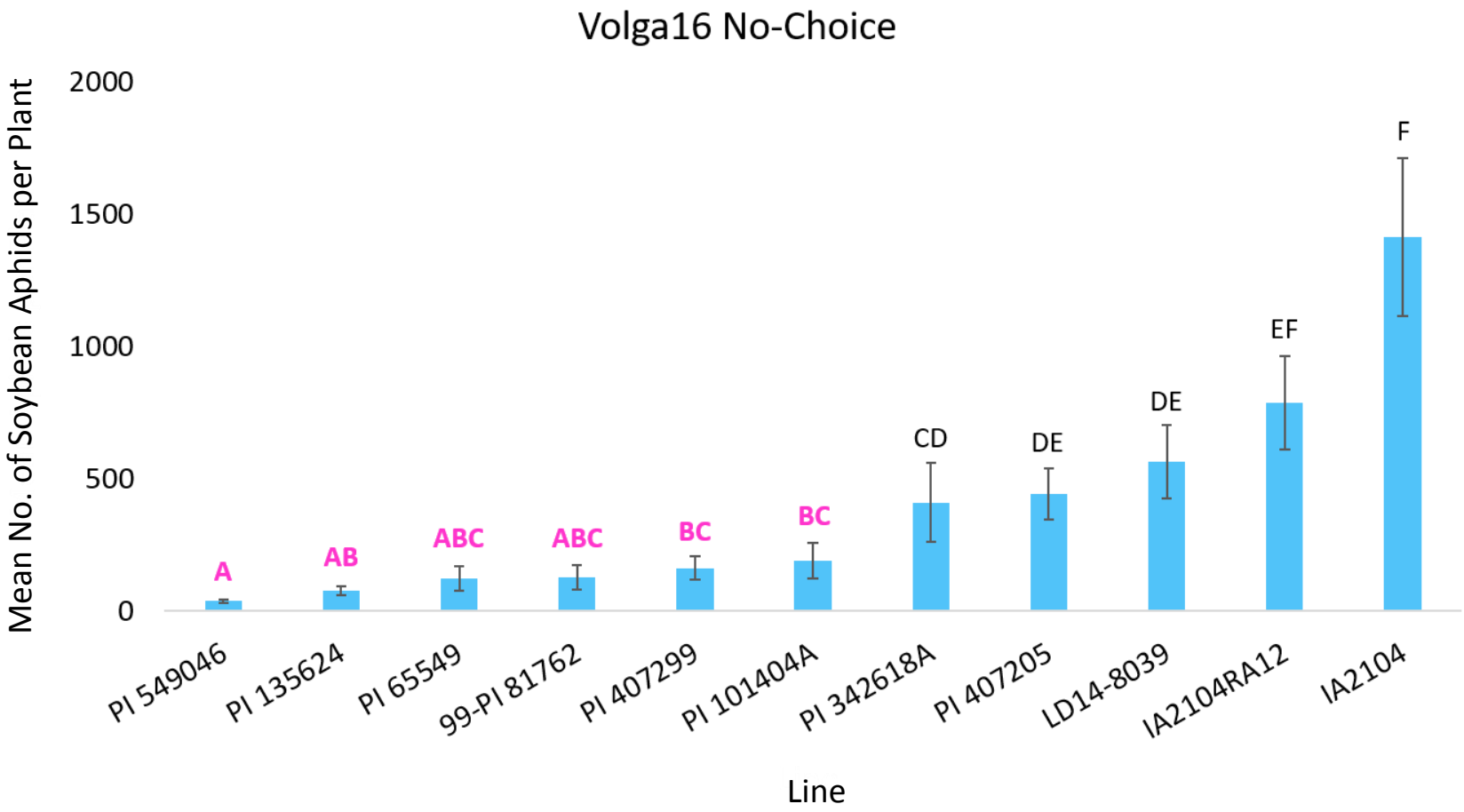

Figure 12. Volga16 soja no-choice test, mean ± SEM of soybean aphids per plant of combined 10 and 20 sample days. Pink lettering represents plant introductions with significantly lower mean aphid populations than on the LD14-8039 check.

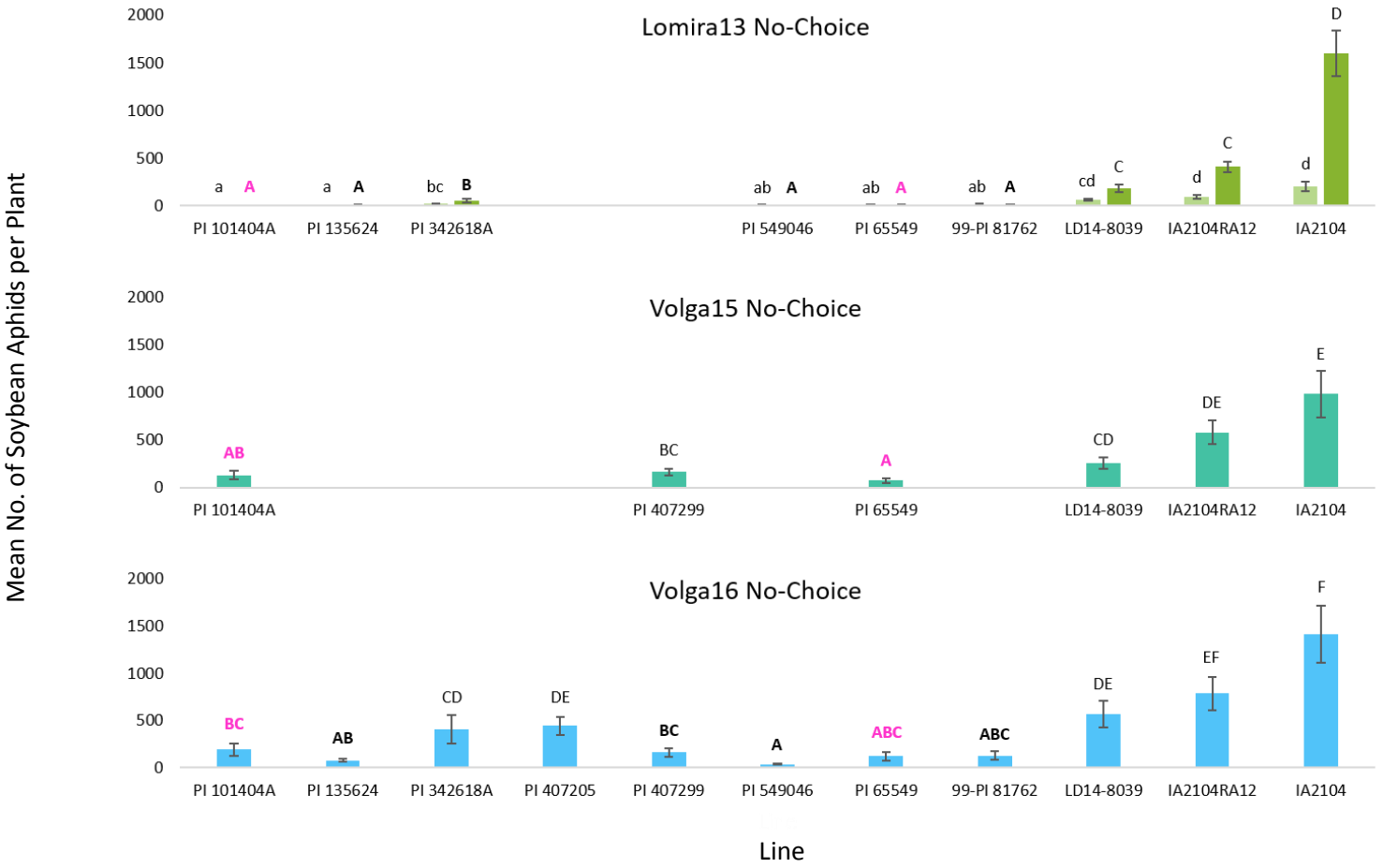

Figure 13. Soja no-choice tests, mean ± SEM soybean aphids per plant of each of the biotype 4 colonies. The Lomira13 colony (top) showing 10 and 20 sample day aphid counts accounting for line-by-sample day interaction; the Volga15 (middle) and Volga16 (bottom) colonies showing combined means. Bars with different letters indicate statistically significant differences, bold lettering represents significantly lower populations than on LD14-8039, and pink lettering signifies the PIs resistant to each of the colonies.

CHAPTER 2. SOYBEAN APHID BIOTYPE 4 RESISTANCE AMONG SELECTED SOYBEAN PLANT INTRODUCTIONS

Abstract

Host plant resistance in soybean, *Glycine max* (L.) Merr., can be an effective management tool for soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae). However, virulent soybean aphid biotypes challenge this management tactic. Of the four identified biotypes, soybean aphid biotype 4 is the most virulent and is capable of overcoming all commercially available soybean cultivars with soybean aphid resistance. By discovering sources of resistance to soybean aphid biotype 4, host plant resistance may continue to be a dependable method of management, ultimately reducing insecticide reliance. To identify biotype 4 resistance, approximately 50 soybean plant introductions (PIs) with known resistance to avirulent soybean biotypes were tested against three iso-female soybean aphid biotype 4 colonies. Colonies were collected from three separate site-years from Lomira, WI in 2013 and Volga, SD in 2015 and 2016. Fourteen soybean PIs indicated putative resistance in no-cage free-choice tests to 'Lomira13,' whereas only two PIs indicated putative resistance to 'Volga15,' and eight to 'Volga16.' Two, two, and three of the identified resistant PIs in free-choice tests also demonstrated resistance to Lomira13, Volga15, and Volga16 colonies, respectively in caged no-choice tests. Of the tested plant introductions, PI 437696 significantly suppressed each of the three soybean aphid biotype 4 colonies, and should be explored further for soybean aphid resistance efforts.

Introduction

Primary reliance on insecticides for management of soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), has led to pyrtheroid-resistant soybean aphids in four states in North Central U.S. (Hanson et al. 2017; Potter et al. 2017; Varenhorst, et al. 2017b). Research groups have identified soybean, *Glycine max* (L.) Merr, plant introductions (PIs) from across the world with naturally developed resistance to soybean aphid, providing an alternative from insecticides for soybean aphid management. Plant resistance can make colonization of the plant more difficult for the pest, either from deterrent qualities (i.e., antixenosis) or through prevention of pest growth and reproduction (i.e., antibiosis) (Smith 1989, Hill et al. 2004b, Tilmon et al. 2011). Thirty resistant and 46 moderately resistant soybean PIs have been identified as soybean aphid resistant sources (U.S. NPGS 2018).

Genes that confer resistance to soybean aphid are named *Rag* (Resistance to *Aphis glycines*) genes (Hill et al. 2006, Tilmon et al. 2011). However, soybean aphid biotypes are capable of overcoming particular *Rag* genes. Soybean aphid biotype 1 is avirulent (i.e., unable to successfully colonize) on soybean containing any of the known *Rag* genes (Kim et al. 2008); since biotype 1 is avirulent, it is often used for identifying sources of soybean aphid resistance. Soybean aphid biotype 2 is virulent (i.e., able to successfully colonize) on *Rag1* resistant soybean, but is avirulent on *Rag2* resistant soybean (Kim et al. 2008). Soybean aphid biotype 3 is avirulent on *Rag1* soybean, but virulent on *Rag2* soybean (Hill et al. 2010). Lastly, soybean aphid biotype 4 is virulent on *Rag1*, *Rag2*, and *Rag1+Rag2* pyramided resistant soybeans (Alt and Ryan-Mahmutagic 2013). Recently, three-gene pyramid lines have been found to provide even broader soybean aphid

management; pyramid *Rag1+Rag2+Rag4* was found to confer resistance to biotype 1, biotype 2, and biotype 3 (Varenhorst et al. 2017a) while *Rag1+Rag2+Rag3* confers resistance to biotype 1, biotype 2, biotype 3, and biotype 4 (Ajayi-Oyetunde et al. 2016, Varenhorst et al. 2017a).

Soybean cultivars with *Rag1* and *Rag2* soybean aphid resistance genes, both individually and combined, are available commercially (Diers 2017), and have been identified as a reliable alternative to foliar insecticides (McCarville et al. 2014). Yet, *Rag1+Rag2* cultivars are ineffective for controlling soybean aphid biotype 4 (Alt and Ryan-Mahmutagic 2013). *Rag3* soybeans have been evaluated for resistance to biotype 4. Reduced soybean aphid biotype 4 populations have been reported on *Rag3* soybean, PI 567543C, by Alt and Ryan-Mahmutagic (2013) as well as Varenhorst et al. (2017a); while *Rag3* line 'LD14-8006' (*Rag3* donor: PI 567543C) was shown to be ineffective for biotype 4 control by Ajayi-Oyetunde et al. (2016). *Rag3* line 'LD14-8039' (*Rag3* donor: PI 567543C) (Table 10) was found to be moderately resistant to soybean aphid biotype 4 in preliminary studies (S.R.C., unpublished data).

Intrabiotypic variability occurs when isolates of a biotype experience quantitative variability (Claridge and Den Hollander 1983, Futuyma and Peterson 1985, Pawlowski et al. 2015). Quantitative variation in soybean aphid virulence has been documented in soybean aphid biotype 3 on resistant soybean genotypes (Pawlowski et al. 2015). The varied performance of biotype 4 on *Rag3* soybean lines, mentioned above, suggests that variability exists within this soybean aphid biotype. Continued investigation is required to find reliable sources of resistance to a larger spectrum of soybean aphid diversity.

Sources of resistance identified against avirulent soybean aphids may confer resistance for other, more virulent soybean aphid biotypes. The first objective of this research was to evaluate 50 soybean PIs, with known resistance to avirulent soybean aphids, for their resistance to soybean aphid biotype 4. The second objective of this study was to evaluate variants of soybean biotype 4 on the resistant sources. To explore this, soybean aphid biotype 4 colonies of three separate site-years underwent comprehensive free-choice tests; follow-up testing was completed for promising PIs.

Materials and Methods

Soybean Aphid Biotype 4 Colonies

Three colonies of soybean aphid biotype 4 were used in this study. First, the 'Lomira13' colony was collected near Lomira, WI in 2013 by Michael Crossley from the University of Wisconsin (Madison, WI). Lomira13 soybean aphids were collected near the original site biotype 4 was identified (Alt and Ryan-Mahmutagic 2013). Doris Lagos-Kutz at the University of Illinois (Urbana-Champaign, IL) maintained the colony from the Lomira collection on 'LD12-12734a,' a pyramid *Rag1+Rag2* breeding line. In January 2016, Lomira13 soybean aphids were mailed to the USDA-ARS North Central Agricultural Research Laboratory (NCARL, Brookings, SD). Lomira13 was resent to NCARL in August 2017 to complete the third no-choice test with that colony. The second soybean aphid biotype 4 colony was obtained from a collection by Swapna Purandare and MacKenzie Mattern of South Dakota State University (SDSU, Brookings, SD). This colony, referred to as 'Volga15,' originated at a SDSU research farm near Volga, SD in September 2015 on soybean *Rag1+Rag2* pyramid line 'LD12-15805Ra.'

Like Volga15, the third soybean aphid biotype 4 colony was also collected on LD12-5805Ra in Volga, SD in August 2016, thus named 'Volga16,' by Eric Beckendorf of NCARL and S.R.C. of SDSU.

Experiments were conducted at NCARL. Colony and test plants were first grown in aphid-free greenhouses, 16:8 photoregime (Light:Dark) and 23:18 °C (L:D) temperature. Each colony was maintained in a growth chamber (CMP4030 Conviron, Winnipeg, Canada) and provided with 16:8 (L:D) photoperiod, 23:18°C (L:D) temperature, and 50% relative humidity. Colonies were provided a continual diet of soybean pyramid *Rag1+Rag2* cultivar 'IA2104RA12' (Table 10). These soybeans, used for colony maintenance, were planted with ten seeds per large pot with potting soil (Table 11 and 12) and grown for approximately four weeks in a greenhouse before being transferred to a growth chamber and infested with a colony.

Once a soybean aphid collection was brought to NCARL, the colony was established on IA2104RA12. Six apterous (wingless) adults were chosen, caged individually with a potted soybean plant, and kept in a separate growth chamber. Cages were made from 0.6cm thick clear extruded acrylic tube, 12.7cm x 40.6cm (outer diameter x height) in size (Ridout Plastics Co. Inc., San Diego, CA, item number: ACREXT5.000X4.750). Two 5.1cm diameter drilled holes and one end of the tube were covered and hot-glued with no-thrips resistant screens (screen hole size: 0.150mm^2, thread size: 15mm, BioQuip, Rancho Dominquez, CA). Two weeks after infestation, the isolated soybean aphid female (iso-female) with the most clonal offspring was chosen as the respective progenitor for that biotype 4 colony, and all other aphids were discarded.

Each iso-female colony was assigned its own caretaker and maintained in a separate growth chamber to prevent cross-colony contamination.

Seed Acquisition

Seeds of the soybean PIs were obtained from the USDA-ARS Soybean Germplasm Collection (USSGC) in Urbana, IL. All PIs were chosen because of their resistance to avirulent soybean aphid biotypes in past research (Table 13). PIs were seed-increased as needed at NCARL. Soybean lines with known aphid resistance or susceptibility were used as checks for all research (Table 10).

Free-Choice Tests

We hypothesized that some soybean PIs with resistance to avirulent soybean aphid biotypes would also show putative resistance to soybean aphid biotype 4 in free-choice tests. Methods for the free-choice tests were modified from Hesler et al. (2017a).

Founder plants were used to infest free-choice tests with soybean aphids. Cultivar IA2104RA12 was used for founder plants, planted using small pots with potting soil (Table 11 and 12) in a greenhouse. After approximately two weeks, the plants had developed unifoliate leaves with a developing first trifoliate (VC stage) (Fehr and Caviness 1977, Licht 2014). At that stage, the pots were thinned to one plant each and the soil surface was sanded (Table 12). Founder plants were then infested with aphids. Colony plants were cut and placed into acrylic tubes that were covered with Parafilm M® (Pechiney Plastic Packaging, Mensha, WI) on the top open end. As the cut colony plants dried, aphids crawled off of them, up the tube, and on to the Parafilm. Five apterous,

adult aphids were transferred from the Parafilm, using a fine-tipped, wetted paintbrush, to each founder plant unifoliate. After two weeks in a growth chamber, the ten aphids per plant had reproduced to approximately 250 aphids.

Soybean PIs (Table 13) and checks (Table 10) were grown in small pots in the greenhouse per free-choice experiment. After approximately two weeks, plants were at intermediate VC stage. Twenty-four to 48 hours before free-choice tests began, plants were thinned to one seedling per pot, and eight replicates of each line were chosen based on uniformity. No-cage free-choice lines were arranged according to a randomized complete block design. The soil surface of experimental pots was covered with a layer of sand, approximately 0.5cm deep. Founder plants, with approximately 250 aphids, were cut at the stems (Figure 14), and detached stems were placed back in the center of their pot upright. Two cut founder plants were positioned in consistent, equidistant locations around free-choice test lines for each test replicate (Figure 15). Free-choice tests ran for two weeks in a growth chamber, allowing time for the founder plants to dry and the aphids to roam and reproduce on preferred test lines. Test lines were rated from 0-to-6 based on a 50 aphid-increment scale (Table 14).

The five soybean free-choice tests were repeated for each of the three iso-female soybean aphid biotype 4 colonies; each test included 10 PIs and 6 checks, with 6 to 8 replications. Conservative mean and median aphid ratings (PROC MEANS; SAS Institute, 2014) of less than 2.5 determined putatively resistant soybean PIs that would continue on for follow-up no-choice testing.

No-Choice Tests

Next, we hypothesized that some resistant soybean PIs from free-choice tests would continue to suppress soybean aphid biotype 4 in follow-up no-choice tests. No-choice testing methods were modified from Hesler et al. (2017c). Three soybean checks and the resistant soybean PIs were grown in small pots until intermediate VC stage. Twenty-four to 48 hours before infestation, 12 uniform soybeans plants of each PI and cultivar (Table 10) were chosen and transplanted into large pots with two plants per soybean line per pot. Pots were labelled, completely randomized, and the soil surface was covered with a 0.6cm layer of sand for tube stability.

Aphid colony plants were cut and dried before apterous adult aphids were transferred from Parafilm to test plant unifoliates. No-choice test plants were infested with six apterous, adult aphids per plant and each pair of test lines within a pot was covered with an acrylic tube to confine aphids (Figure 16). Ten days later, one of the two plants in each tube was chosen randomly, cut, and placed in its own labelled bag, and stored in a freezer. Similarly, 20 days after infestation, the remaining plant in each tube was cut, bagged, and frozen. The tube and sandy surface were examined and any live aphids were counted. Later, plants were thawed, and aphids counted.

Based on the numbers of PIs advanced from the free-choice tests, three, one, and two soybean no-choice tests were respectively completed for the Lomira13, Volga15, and Volga16 colonies. Tests included the susceptible (IA2104), *Rag1+Rag2* (IA2104RA12), and moderately-resistant *Rag3* (LD14-8039) checks, with six replications for both sampling days.

The number of aphids per plant was treated as discrete, Poisson response variables in a generalized linear mixed model (PROC GLIMMIX; SAS Institute, 2014) in an analysis of variance with test line, sample day, and test line-by-sample day interaction as treatment factors using an $\alpha = 0.05$ level of significance. A least squares mean (LSMEANS) procedure with Bonferroni adjustment was used for multiple comparisons of the mean number of aphids per plant among test lines. If the line-by-sample day interaction was significant for a particular no-choice test, test lines were compared separately within each sample day. PIs with mean counts significantly lower than those on the *Rag3* check (LD14-8039) were considered resistant to the respective soybean aphid biotype 4 colony.

Results

Free-Choice Tests

Mean and median ratings of soybean aphid populations varied among soybean lines in each free-choice test for each colony (Tables 15-19). However, not all free-choice tests had putatively resistant lines, determined as PIs with mean and median aphid ratings below 2.5 (Table 14).

For Lomira13, free-choice tests showed 14 of the 50 soybeans to be resistant to the soybean aphids in free-choice test 1 (PI 437696, PI 588000, PI 594573, PI 606390A), test 3 (PI 430491, PI 438118, PI 567250A, PI 603426D), test 4 (PI 438048B, PI 512322B, PI 603339A, PI 603712), and test 5 (PI 567541B, PI 605765B). For Volga15, 2 of the 50 soybeans were resistant, both in free-choice test 1 (PI 437696, PI 567598B). For Volga16, 8 of the 50 PIs were resistant in free-choice test 1 (PI 437696, PI 567598B, PI

588000, PI 606390A), test 3 (PI 430491), test 4 (PI 603712), and test 5 (PI 567541B, PI 605765B).

No-Choice Tests

For Lomira13 soybean aphids, the mean number of aphids per plant varied significantly by test line, sample day, and the test line-by-sample day interaction for no-choice tests 1 and 2 (Table 20, Figure 17). In no-choice test 3, the mean number of aphids per plant varied significantly by test line and sample day but the line-by-sample day interaction was not significant (Table 20, Figure 17). In test 1, PI 437696 was resistant to Lomira13, with lower counts on sample day 20 than on day 10. In test 2, PI 588000 was resistant to Lomira13 soybean aphids with significantly lower populations than on the *Rag3* check for sample day 20; but PI 588000 was not significantly different from the *Rag3* check on day 10. The soybean PIs in test 3 did not experience lower mean aphid counts than on the *Rag3* check, and were not resistant to the Lomira13 colony.

For Volga15 soybean aphids, mean aphid counts differed significantly by test line, sample day, and the line-by-sample day interaction (Table 20, Figure 18). PI 567598B and PI 437696 were resistant to Volga15 aphids at both 10 and 20 days. The mean number of aphids on PI 437696 was lower on sample day 20 than day 10.

For Volga16 soybean aphids, the mean number of aphids per plant differed significantly by test line, sample day, and the test line-by-sample day interaction in no-choice test 1, but only by test line and sample day in no-choice test 2 (Table 20, Figure 19). In test 1, populations on PI 437696 and PI 567598B were resistant to Volga16 on

both 10 and 20 sample days. In test 2, mean aphids per plant were significantly lower on PI 567541B than on the *Rag3* check for the combined sample day counts.

The mean number of soybean aphids per plant was always greater on IA2104 (no *Rag* genes) than on any of the soybean PIs. Four PIs in Lomira13 tests (PI 438048B, PI 438118, PI 603426D, and PI 512322B) and one PI in Volga16 tests (PI 606390A) experienced higher mean aphid counts than on IA2104RA12 (*Rag1+Rag2*), and were not significantly different from the check.

Discussion

Soybean Aphid Biotype 4 Colonies

One soybean line, PI 437696, was resistant in free-choice tests to each of the three soybean aphid biotype 4 colonies. PI 437696 experienced significantly lower populations than on the *Rag3* moderately-resistant check, LD14-8039, in no-choice tests and was considered resistant to the three colonies.

Each biotype 4 colony exhibited unique responses to soybean test lines, which were previously identified as resistant to avirulent soybean aphid biotypes in past research. Free-choice tests identified fourteen, two, and eight soybean PIs with resistance to Lomira13, Volga15, and Volga16 soybean aphids, respectively. More than four times as many PIs expressed resistance in free-choice tests to Lomira13 than to Volga15, Volga16 being intermediate. No-choice tests identified two, two, and three PIs that were resistant to Lomira13, Volga15, and Volga16 colonies, respectively. For Lomira13, five no-choice PIs were not significantly different from the susceptible check, IA2014.

Although soybean aphids are classified by biotypes, quantitative variability has been documented among isolates of a biotype in past research (Michel et al. 2010, 2011; Pawlowski et al. 2015). Reasons for differences among isolates of a biotype are still unknown. Two factors that may influence aphid variability are differences in endosymbiotic diversity, i.e., bacteria affecting soybean aphid host specificity, nutritional uptake, and defensive qualities (Wenger and Michel 2013, Cassone et al. 2015, Wulff and White 2015) and complex gene mechanisms, i.e., many genes working in combination, causing soybean aphid virulency to occur on a gradient (Diehl and Bush 1984, Wenger and Michel 2013).

Soybean Plant Introductions

Through genetic analyses, resistance genes have been mapped in eight of our soybean test lines. Single genes in PI 567301B (*Rag5*) (Jun et al. 2012) and in PI 243540 (*Rag2*) (Rouf Mian et al. 2008) were not successful in their respective free-choice tests and were not advanced for follow-up testing. Pyramided sources of resistance have been associated with six of our test lines. Two PIs that were resistant to Volga16 were PI 567541B with resistance genes *rag1c* and *rag4* (Zhang et al. 2009) and PI 567598B with *rag1b* and *rag3* (Bales et al. 2013). However, Alt and Ryan-Mahmutagic (2013) and Varenhorst et al. (2017a) found one or both of these PIs to be ineffective against their own biotype 4 colonies collected near Lomira, WI in 2013. PI 437696, PI 587870, PI 588000, and PI 594573 were found to have significant genetic marker associations with *Rag1* and *Rag2*, with significant interactions found between the two gene regions in both PI 437696 and PI 588000 (Fox et al. 2014). Yet, PI 588000 and PI 437696 experienced

significantly lower populations than on our *Rag1+Rag2* check in at least two of the three colonies.

Pyramided resistance is known to protect soybeans against more soybean aphid biotypes (McCarville et al. 2014, Ajayi-Oyetunde et al. 2016). Stacking host plant resistance genes may also provide synergistic effects (La Mantia et al. 2018), where combined genes create greater pest resistance than the sum of their individual resistance. Currently, pyramid *Rag1+Rag2+Rag3* resistance provides the greatest protection to soybean against all known soybean aphid biotypes (Ajayi-Oyetunde et al. 2016, Varenhorst et al. 2017a). Identifying and breeding strong sources of resistance into high-yielding cultivars will likely sustain host plant resistance as an effective management tactic for soybean aphid.

Findings from Fox et al. (2014) indicated that PI 437696 may have pyramided *Rag1+Rag2* resistance. PI 437696 was resistant to each of the three iso-female soybean aphid biotype 4 colonies in free-choice and no-choice tests. To our knowledge, this is the first report of soybean aphid biotype 4 resistance in PI 437696. We recommend that this PI be further evaluated for resistance genes and to continue on for soybean aphid host plant resistance research and breeding efforts.

Conclusions

Soybean aphid iso-female biotype 4 colonies, collected from three site-years, were used to evaluate soybean PIs resistant to avirulent soybean aphid biotypes in past research. Significant variability was observed among the soybean aphid biotype 4 colonies especially during non-caged free-choice tests. Caged no-choice tests assessed

resistant PIs further for each of the colonies. PI 437696 showed strong resistance to each of the three biotype 4 colonies. Although genetic markers linked resistance genes to this PI previously (Fox et al. 2014), we believe that reevaluation of PI 437696 would reveal additional sources of resistance.

Current soybean aphid resistance cultivars are being challenged by soybean aphid diversity. Although researching the cause of soybean aphid variability is important (i.e., endosymbiotic or polygenic mechanisms), our greatest goal is to provide reliable and sustainable sources of resistance against a greater diversity of soybean aphid. PI 437696 was extremely successful in suppressing each of our soybean aphid biotype 4 colonies. We recommend that breeders explore this PI further in the hopes of providing improved protection to our high-yielding soybean crops. Soybean aphid resistance in soybean limits input costs while preventing unnecessary damage to soybean, beneficial insect communities, and our environment. With continued research, we can further our knowledge of this plant-pest interaction and advance soybean crop protection.

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Table 10. Soybean plants with known soybean aphid susceptibility or resistance genes.

Check Type	**Cultivar or PI[1]**	**Provider**	**Pedigree**
Susceptible, Free-choice check	'Brookings' (PI 667735)	South Dakota State University, Brookings, SD	A00-711063 x SD98-595
Susceptible, No-choice check	'IA2104'	Iowa State University Research Foundation Inc., Ames, IA	IA3027 x Soygenetics F40412C
Rag1, Free-choice check	'LD09-05484a'	Blue River Hybrids, Kelley, IA	undisclosed
Rag2, Free-choice check	'2880a'	Blue River Hybrids, Kelley, IA	undisclosed
Rag3, Free-choice and No-choice check	'LD14-8039'	National Soybean Research Center, Urbana, IL	[Titan(5) x E10005] x [Titan(5) x F1 (LD08-12446a x LD05-30588a)]
Rag1+Rag2, Free-choice and No-choice check; Colony and founder plants	'IA2104RA12'	Iowa State University Research Foundation Inc., Ames, IA	undisclosed

[1]PI: Plant Introduction

Table 11. List of containers used to grow soybeans.

Container	**Size**	**Soil above / below seed**	**Use**	**Manufacturer**
Small pot	8.25cm x 6.5cm x 7.62cm (top side, bottom side, ht.)	100mL / 150mL	Founder plants Free-choice tests	International Greenhouse Co., Danville, IL
Large pot	6cm x 4cm x 5.7cm (top diam., bottom diam., ht.)	300mL / 1L	Colony plants No-Choice tests	Myers Industries Inc., Earth City, MO
Tray	26.5cm x 51cm x 6.5cm (width, length, ht.)	Not applicable	Holds 18 small or 6 large pots	T.O Plastics Inc., Clearwater, MN

Table 12. Soil media used for growing soybeans.

Media	**Elements**	**Source**
Potting Soil (2:1:1 mixture)	Vienna soil (Fine-loamy, mixed Calcic Hapludolls)	Brookings, SD
	Horticultural coarse vermiculite	Perlite Vermiculite Packaging, North Bloomfield, OH
	Canadian sphagnum peat moss	Sun Gro Horticulture Distribution Inc., Agawam, MA
Sand	Industrial quartz	Unimin Corporation, Le Sueur, MN

Table 13. Soybean plant introductions (PIs) with resistance to soybean aphid.

PI	MG[1]	Origin	References	Known soybean aphid resistance gene
PI 153214	I	Belgium	Bhusal et al. 2014	
PI 189860	00	France	Hesler and Dashiell 2007	
PI 189946	I	France	Bhusal et al. 2014	
PI 194627	00	Sweden	Hesler and Dashiell 2007	
PI 194645	00	Sweden	Hesler and Dashiell 2007	
PI 200595	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 230977	VII	Japan	Hesler et al. 2007; Hill et al. 2004a, 2004b	
PI 243540	IV	Japan	Hesler et al. 2011a, Mian et al. 2008a	*Rag2* (Rouf Mian et al. 2008)
PI 340034	IV	South Korea	Bansal et al. 2013	
PI 430491	00	China	Bhusal et al. 2013; Hesler and Dashiell 2007; Hesler et al. 2011a, 2011b	
PI 436684	III	China	Hesler and Dashiell 2007	
PI 437075	I	Russian Federation	Bhusal et al. 2014	
PI 437282	I	Moldova	Hesler et al. 2017a, 2017b, 2017c	
PI 437353	I	Russian Federation	Hesler et al. 2017a, 2017b, 2017c	

PI	**MG**[1]	**Origin**	**References**	**Known soybean aphid resistance gene**
PI 437658	I	China	Hesler et al. 2017a, 2017b, 2017c	
PI 437696	VI	China	Fox et al. 2014	Associated with *Rag1* and *Rag2* (Fox et al. 2014)
PI 437733	I	China	Hesler et al. 2017a, 2017b, 2017c	
PI 438118	I	China	Hesler et al. 2017a, 2017b	
PI 464911	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 507713	N/A[2]	Russian Federation	Hanson et al. 2016	
PI 518753	I	Former Serbia and Montenegro	Hesler et al. 2017a, 2017b	
PI 524994	I	Russian Federation	Hesler et al. 2017a, 2017b	
PI 548395	00	United States	Hesler and Dashiell 2007	
PI 548417	I	Italy	Hesler et al. 2017c	
PI 548530	I	United States	Hesler et al. 2017c	
PI 548544	00	Canada	Hesler and Dashiell 2007	
PI 587870	VII	China	Fox et al. 2014	Associated with *Rag1* and *Rag2* (Fox et al. 2014)
PI 588000	X	China	Fox et al. 2014	Associated with *Rag1* and *Rag2* (Fox et al. 2014)

PI	MG[1]	Origin	References	Known soybean aphid resistance gene
PI 592389	I	United States	Hesler et al. 2017a, 2017b	
PI 594573	VII	China	Fox et al. 2014	Associated with *Rag1* and *Rag2* (Fox et al. 2014)
PI 603326	I	China	Bhusal et al. 2014	
PI 603712	0	China	Bhusal et al. 2013; Hesler et al. 2011a, 2011b	
PI 319535A	I	China	Hesler et al. 2017a, 2017b	
PI 361088B	I	Romania	Hesler unpublished	
PI 438048B	I	China	Hesler et al. 2017a, 2017b	
PI 512322B	I	Georgia	Hesler et al. 2017c	
PI 561285B	I	China	Hesler et al. 2017a, 2017b	
PI 567250A	I	China	Bhusal et al. 2014	
PI 567301B	IV	China	Mian et al. 2008a, 2008c	*Rag5* (Jun et al. 2012)
PI 567541B	III	China	Hesler and Dashiell 2007; Hesler et al. 2011a, 2011b; Mensah et al. 2002; Mensah et al. 2005; Mian et al. 2008a, 2008c	*rag1c* and *rag4* (Zhang et al. 2009)
PI 567598B	III	China	Hesler and Dashiell 2007; Mensah et al. 2002; Mensah et al. 2005; Mian et al. 2008a, 2008b, 2008c	*rag1b* and *rag3* (Bales et al. 2013)
PI 578388B	I	China	Hesler et al. 2017a, 2017b	

PI	MG[1]	Origin	References	Known soybean aphid resistance gene
PI 603339A	I	China	Bhusal et al. 2014	
PI 603426D	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 603432B	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 603546A	I	China	Bhusal et al. 2014	
PI 603587A	I	China	Bhusal et al. 2014	
PI 605765B	I	Vietnam	Hanson et al. 2016	
PI 606390A	IV	Vietnam	Bansal et al. 2013	
PI 612759B	0	China	Bhusal et al. 2013, Hesler et al. 2011a	
PI 612759C	I	China	Hesler et al. 2017a, 2017b, 2017c	

[1]MG: Maturity Group; [2]N/A: Not Available

Table 14. Rating scale for free-choice tests. Soybean plants were rated individually after two weeks. Ratings were based on a 50 soybean aphid-increment scale.

Rating	Soybean aphids per plant
0	0
1	1-50
2	51-100
3	101-150
4	151-200
5	201-250
6	250+

Table 15. Mean and median ratings of soybean free-choice Test 1 for each soybean aphid biotype 4 colony. Soybean checks (italicized) and plant introduction (PI) plants were rated individually on a 0-to-6 soybean aphid scale. Susceptible PIs indicated in blue and putatively resistant PIs indicated in pink.

Soybean aphid colony

Lomira13 Soy 1

Line	Mean	Median
PI 230977	3	2.5
PI 340034	5.6	6
PI 436684	4.8	5.5
PI 437696	**1**	**1**
PI 567301B	3.8	3
PI 567598B	3.8	3
PI 587870	4.9	5.5
PI 588000	**2**	**2**
PI 594573	**1.8**	**2**
PI 606390A	**1.3**	**1**
LD09-05484a	*5.8*	*6*
2880a	*4.9*	*6*
LD14-8039	*3.8*	*4*
IA2104RA12	*3*	*2*
Brookings	*3.8*	*4*
IA2104	*5*	*6*

Volga15 Soy 1

Line	Mean	Median
PI 230977	3.5	3.5
PI 340034	5.9	6
PI 436684	5.7	6
PI 437696	**1.1**	**1**
PI 512322B	6	6
PI 567301B	5.6	6
PI 567598B	**1.9**	**2**
PI 587870	5.9	6
PI 594573	5.3	6
PI 606390A	2.7	3
LD09-05484a	*5.3*	*6*
2880a	*5.7*	*6*
LD14-8039	*5.3*	*6*
IA2104RA12	*3.1*	*3*
Brookings	*6*	*6*
IA2104	*6*	*6*

Volga16 Soy 1

Line	Mean	Median
PI 230977	3.6	4
PI 340034	3.5	3.5
PI 436684	4.3	4.5
PI 437696	**0.9**	**1**
PI 512322B	4.3	4
PI 567301B	3	2
PI 567598B	**0.9**	**1**
PI 587870	4.3	5
PI 588000	**1**	**1**
PI 606390A	**1.5**	**1**
LD09-05484a	*5.1*	*6*
2880a	*4.5*	*5*
LD14-8039	*3.1*	*2.5*
IA2104RA12	*3.6*	*3.5*
Brookings	*4.3*	*5*
IA2104	*5*	*6*

Eight observations per line in Soy 1 free-choice test for Lomira13 and Volga16; seven observations per line in Soy 1 Volga15. Lomira13 PI 512322B: Soy 4 (Table 8), Volga16 PI 594573: not included due to germination rate, Volga15 PI 588000: Soy 3 (Table 7).

Table 16. Mean and median ratings of soybean free-choice Test 2 for three soybean aphid biotype 4 colonies. Plants were individually rated on a 50 soybean aphid-increment, 0-to-6 scale for the soybean checks (italicized) and test plant introductions (PIs). Susceptible PIs indicated in blue.

Soybean aphid colony

Lomira13 Soy 2

Line	Mean	Median
PI 189946	4.5	5
PI 319535A	4	4
PI 361088B	2.8	2.5
PI 437282	3.5	4
PI 548417	4.5	4.5
PI 548530	4	4.5
PI 561285B	4.3	5
PI 578388B	3	3
PI 592389	3.5	3.5
PI 603326	4.5	4.5
LD09-05484a	*5.2*	*5.5*
2880a	*5.2*	*5.5*
LD14-8039	*3.2*	*3*
IA2104RA12	*2.3*	*2*
Brookings	*4.2*	*4*
IA2104	*5.8*	*6*

Volga15 Soy 2

Line	Mean	Median
PI 189946	6	6
PI 319535A	6	6
PI 361088B	5.4	5
PI 437282	6	6
PI 548417	6	6
PI 548530	6	6
PI 561285B	6	6
PI 578388B	5.6	6
PI 592389	6	6
PI 603326	5.6	6
LD09-05484a	*5.7*	*6*
2880a	*6*	*6*
LD14-8039	*5.6*	*6*
IA2104RA12	*5.4*	*6*
Brookings	*6*	*6*
IA2104	*5.6*	*6*

Volga16 Soy 2

Line	Mean	Median
PI 189946	5.4	6
PI 319535A	5.7	6
PI 361088B	3.4	4
PI 437282	4.7	5
PI 548417	3.6	4
PI 548530	5	5
PI 561285B	4.1	5
PI 578388B	4.7	5
PI 592389	5.6	6
PI 603326	4.7	5
LD09-05484a	*5.1*	*6*
2880a	*3.9*	*4*
LD14-8039	*2.9*	*2*
IA2104RA12	*3.4*	*4*
Brookings	*5.3*	*6*
IA2104	*5.4*	*6*

Six, seven, and eight observations per test line in Lomira13, Volga15, and Volga16 colony free-choice tests, respectively.

Table 17. Mean and median ratings of soybean free-choice Test 3 for soybean aphid biotype 4 colonies. Soybean checks (italicized) and plant introduction (PI) plants were rated individually on a 0-to-6, 50 aphid-increment scale. Susceptible PIs indicated in blue font and resistant PIs indicated in pink.

Soybean aphid colony

Lomira13 Soy 3

Line	Mean	Median
PI 200595	2.8	2.5
PI 430491	**1.4**	**1**
PI 437658	4.1	5.5
PI 437733	2.6	2
PI 438118	**1.4**	**1**
PI 518753	3.8	4
PI 524994	4.4	5.5
PI 567250A	**1.5**	**1**
PI 603426D	**1.9**	**1**
PI 612759B	3.8	4.5
LD09-05484a	*3*	*3*
2880a	*2.6*	*2*
LD14-8039	*1.5*	*1*
IA2104RA12	*1.4*	*1*
Brookings	*1.6*	*1.5*
IA2104	*2.9*	*2*

Volga15 Soy 3

Line	Mean	Median
PI 200595	5.3	6
PI 437658	5.3	6
PI 437733	5.5	6
PI 438118	5.1	5.5
PI 518753	5.9	6
PI 524994	6	6
PI 567250A	2.8	2.5
PI 588000	2.4	2.5
PI 603426D	5.6	6
PI 612759B	5.9	6
LD09-05484a	*5.5*	*6*
2880a	*6*	*6*
LD14-8039	*3.4*	*3*
IA2104RA12	*4.6*	*4.5*
Brookings	*6*	*6*
IA2104	*5.9*	*6*

Volga16 Soy 3

Line	Mean	Median
PI 200595	3.9	3.5
PI 430491	**2**	**2**
PI 437658	5.5	6
PI 437733	4.3	6
PI 438118	4.6	6
PI 518753	4.9	6
PI 524994	5.8	6
PI 567250A	2.9	2
PI 603426D	5.3	6
PI 612759B	4.1	4
LD09-05484a	*4.8*	*6*
2880a	*3.8*	*3.5*
LD14-8039	*2.8*	*2.5*
IA2104RA12	*3.3*	*3*
Brookings	*5.9*	*6*
IA2104	*3.3*	*3*

Eight observations per Soy 3 line for each of the three colonies.
Volga15 PI 430491: Soy 5 (Table 9).

Table 18. Mean and median ratings of soybean free-choice Test 4 soybean aphid biotype 4 colonies. Plants rated individually on a 0-to-6 scale based on soybean aphid populations of soybean checks (italicized) and plant introductions (PIs). Susceptible PIs indicated in blue and putatively resistant PIs indicated in pink.

Soybean aphid colony

Lomira13 Soy 4

Line	Mean	Median
PI 153214	4.9	6
PI 438048B	**1.8**	**1**
PI 464911	3.4	2.5
PI 507713	2.8	2.5
PI 512322B	**2.3**	**2**
PI 603339A	**1.9**	**1.5**
PI 603546A	5.6	6
PI 603587A	2.5	1.5
PI 603712	**1.1**	**1**
PI 612759C	2.8	3
LD09-05484a	*4.1*	*4*
2880a	*2.1*	*1.5*
LD14-8039	*1.1*	*1*
IA2104RA12	*2*	*1.5*
Brookings	*2.4*	*1*
IA2104	*2.6*	*1.5*

Volga15 Soy 4

Line	Mean	Median
PI 153214	6	6
PI 430491	2.6	2
PI 438048B	3.9	3.5
PI 464911	5.5	6
PI 507713	3.8	4
PI 603339A	3.5	3.5
PI 603546A	5.8	6
PI 603587A	5.5	6
PI 603712	3.1	3
PI 612759C	5.1	6
LD09-05484a	*5.5*	*6*
2880a	*4.4*	*5*
LD14-8039	*3*	*3*
IA2104RA12	*5*	*5*
Brookings	*5.8*	*6*
IA2104	*6*	*6*

Volga16 Soy 4

Line	Mean	Median
PI 153214	4.9	6
PI 243540	4.4	5
PI 438048B	3.1	3
PI 464911	4.3	5
PI 507713	4.1	4
PI 603339A	3	2
PI 603546A	5.4	6
PI 603587A	4	4
PI 603712	**1.1**	**1**
PI 612759C	3.7	3
LD09-05484a	*3.9*	*4*
2880a	*4.7*	*5*
LD14-8039	*2.6*	*3*
IA2104RA12	*2.9*	*3*
Brookings	*3.7*	*5*
IA2104	*4.6*	*6*

Eight observations per Soy 4 test line in Lomira13 and Volga15, seven observations per line in Volga16.
Volga16 PI 243540: not tested in other two colonies.

Table 19. Mean and median ratings of soybean free-choice Test 5 for three colonies of soybean aphid biotype 4. Plants of soybean checks (italicized) and plant introductions (PIs) were rated individually from 0-to-6 based on a 50 soybean aphid-increment scale. Susceptible PIs indicated in blue and putatively resistant PIs indicated in pink.

Soybean aphid colony

Lomira13 Soy 5

Line	Mean	Median
PI 189860	5.5	6
PI 194627	4.9	5
PI 194645	5.1	6
PI 437075	6	6
PI 437353	3	2.5
PI 548395	5.5	5.5
PI 548544	5	5
PI 567541B	**2.4**	**2**
PI 603432B	5.3	6
PI 605765B	**2.1**	**1.5**
LD09-05484a	*5.1*	*6*
2880a	*5*	*5.5*
LD14-8039	*3.6*	*4.5*
IA2104RA12	*3.6*	*4*
Brookings	*4.4*	*5*
IA2104	*5.9*	*6*

Volga15 Soy 5

Line	Mean	Median
PI 189860	4.9	5.5
PI 194627	5	6
PI 194645	5.3	6
PI 437075	5.9	6
PI 437353	4.8	5.5
PI 548395	5.5	6
PI 548544	5.9	6
PI 567541B	3.1	3
PI 603432B	5.5	6
PI 605765B	4.4	4
LD09-05484a	*5.9*	*6*
2880a	*4.5*	*4*
LD14-8039	*3.8*	*3*
IA2104RA12	*4.3*	*5*
Brookings	*5.6*	*6*
IA2104	*6*	*6*

Volga16 Soy 5

Line	Mean	Median
PI 189860	5	6
PI 194627	3.9	5
PI 194645	4.7	6
PI 437075	5.7	6
PI 437353	4.1	5
PI 548395	5	6
PI 548544	4.1	4
PI 567541B	**2.3**	**1**
PI 603426B	5.6	6
PI 605765B	**2.1**	**2**
LD09-05484a	*5.1*	*6*
2880a	*5*	*5*
LD14-8039	*3.4*	*4*
IA2104RA12	*4.4*	*5*
Brookings	*5.3*	*6*
IA2104	*5.9*	*6*

Eight observations for Lomira13 and Volga15 Soy 5 lines; seven observations for Volga16 lines.

Table 20. ANOVA output for mean number of soybean aphids per plant for various soybean no-choice tests.

Colony	Test	Effect	df[1]	*F* value	*P* value
Lomira13	1	Line	9, 98	79.21	<0.0001
		Sample day	1, 98	147.51	<0.0001
		Line-by-Sample day	9, 98	7.05	<0.0001
	2	Line	8, 88	12.63	<0.0001
		Sample day	1, 88	229.12	<0.0001
		Line-by-Sample day	8, 88	2.70	0.0105
	3	Line	4, 50	40.52	<0.0001
		Sample day	1, 50	452.94	<0.0001
		Line-by-Sample day	4, 50	1.86	0.1328
Volga15	1	Line	4, 50	114.66	<0.0001
		Sample day	1, 50	98.38	<0.0001
		Line-by-Sample day	4, 50	12.59	<0.0001
Volga16	1	Line	6, 70	238.31	<0.0001
		Sample day	1, 70	430.03	<0.0001
		Line-by-Sample day	6, 70	3.42	0.0051
	2	Line	6, 70	11.50	<0.0001
		Sample day	1, 70	211.00	<0.0001
		Line-by-Sample day	6, 70	1.57	0.1696

[1]df, degrees of freedom

Figure 14. Soybean seedling used as a founder plant for free-choice tests. Each founder plant had approximately 250 soybean aphids and served as sources of aphid inoculum in free-choice tests. Founder plants were cut at the base of the stems and positioned upright in the center of their pot to dry in order to facilitate aphid dispersal onto test plants. Pots with desiccating founder plants were placed at foci with each tray of test plants.

1	2	3
4	Founder plant	5
6	7	8
9	10	11
12	Founder plant	13
14	15	16

Figure 15. Actual (right) and schematic (left) arrangement of soybean free-choice test plants in one of eight replicates. Soybean aphid-infested founder plants were surrounded equidistantly by test lines (10 PIs and 6 checks) that were assigned a randomized location for each replication.

Figure 16. Soybean no-choice test lines in large pots, caged with an acrylic tube after being infested with six aphids per plant. Ten days after infestation, one soybean from each pot was chosen at random, the stem cut, plant removed, and aphids on the plant were counted. After 20 days, the remaining plant was cut and aphids were counted.

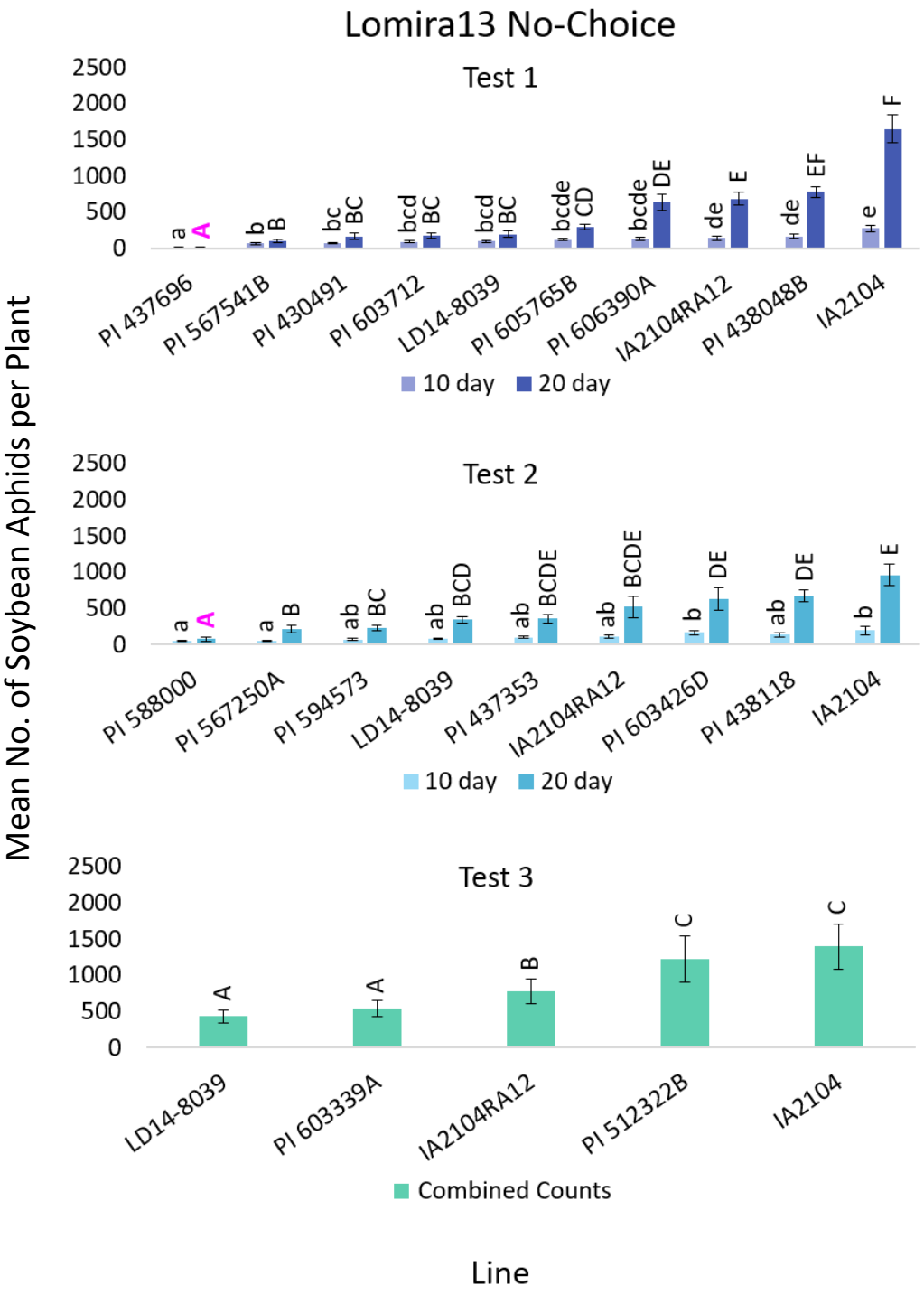

Figure 17. Lomira13 soybean no-choice tests with mean ± SEM number of soybean aphids per plant. Ten and 20 day counts shown for Test 1 (top) and Test 2 (middle); combined counts shown for Test 3 (bottom). Pink lettering represent PIs with significantly lower 20 day populations than on the respective *Rag3* check, LD14-8039.

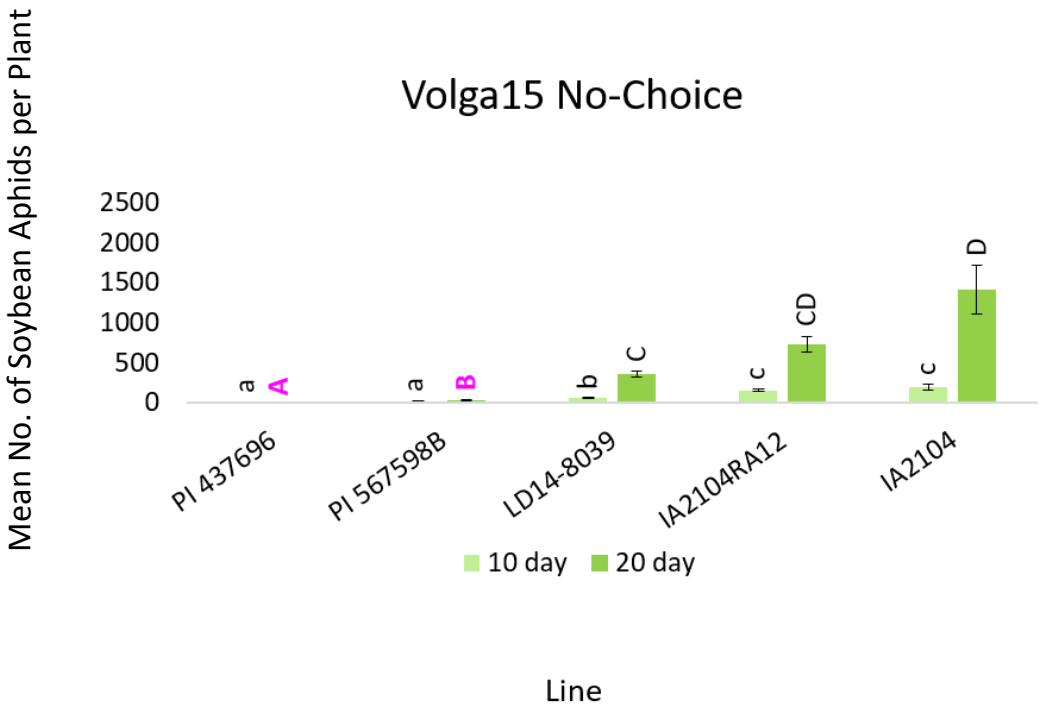

Figure 18. Volga15 soybean no-choice test with mean ± SEM number of soybean aphids per plant at 10 and 20 days post-infestation. Bars with different letters above them indicate significant differences. Letters colored pink represent 20 day populations with significantly lower counts than those on the *Rag3* check, LD14-8039, after 20 days.

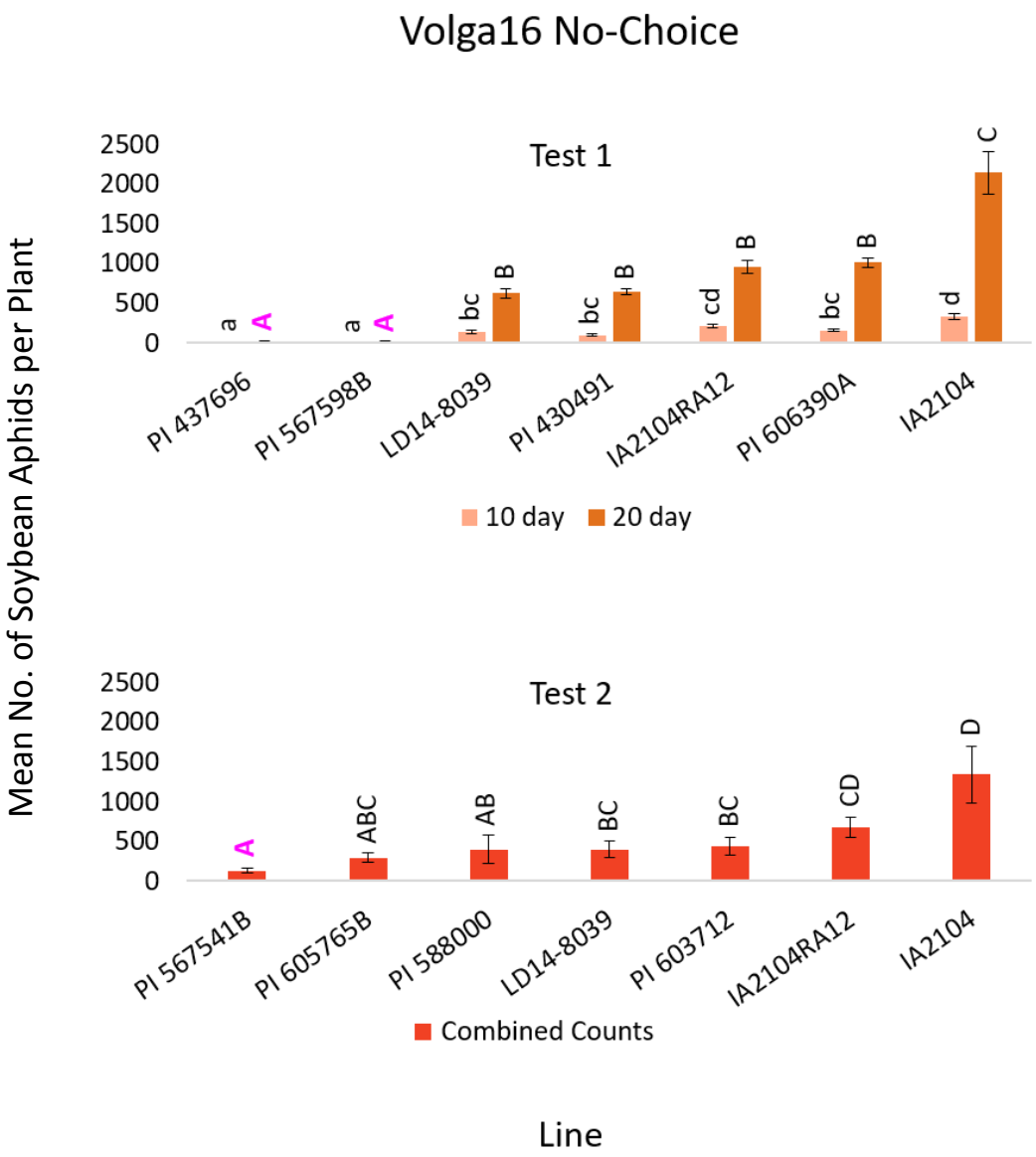

Figure 19. Volga16 soybean no-choice tests with mean ± SEM number of soybean aphids per plant. Test 1 (top) shows 10 and 20 day counts; Test 2 (bottom) shows combined 10 and 20 day counts. Pink lettering signifies counts that were significantly lower than LD14-8039 after 20 days (Test 1) or in combined counts (Test 2).