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RESEARCH

Phenotyping Techniques and Identification of Soybean Resistance to the Kudzu Bug

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ABSTRACT

The kudzu bug (KZB), *Megacopta punctatissima* Montandon 1896, is a newly invasive Asian pest of soybean [*Glycine max* (L.) Merrill] in the southeastern United States. Due to the unique biology of this soybean pest, six screening techniques were tested to identify host plant resistance (HPR) to KZB. Soybean lines previously characterized as resistant to either leaf-chewing or piercing–sucking insects were used to test screening techniques and to identify potential sources of KZB resistance. Four open field experiments and one field cage experiment were conducted in 2010 to 2013 to screen 'Benning' near-isogenic lines (NILs) for KZB resistance. These NILs possess different combinations of quantitative trait loci (QTLs) for resistance to leaf-chewing insects from PI 229358 (QTLs M, G, and H) and PI 227687 (QTL E); however, none of these lines were consistently effective at controlling KZB. Additionally, 30 plant introductions (PIs) with previously observed resistance to either the soybean aphid (*Aphis glycines* Matsumura) or the silverleaf whitefly (*Bemisia tabaci* Gennadius) were screened in the field for KZB resistance. Six lines were identified as potential sources of resistance to KZB from this field using a KZB index. A no-choice (antibiosis) assay was developed to confirm resistance observations from the field and to characterize the type of host plant resistance. PI 567336A and PI 567598B were confirmed as the most resistant of the screened PIs, and were characterized as having antibiosis resistance to KZB.

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Abbreviations: HPR, host plant resistance; IPM, integrated pest management; KZB, kudzu bug; NIL, near-isogenic line; PI, plant introduction; QTL, quantitative trait locus.

Soybean [*Glycine max* (L.) Merrill] is the second largest crop grown in the United States in terms of land area and cash value, with 33.9 million hectares planted, yielding US$40.3 billion in 2014 (USDA–NASS, 2015a, 2015b). Forty-eight percent of soybean planted in the United States was exported in 2014, making it the largest value export crop (ASA, 2014). Yet, soybean suffers from insect pests that cause significant reductions in both seed quality and yield. Insect damage in the US Southeast is greater than that in the rest of the United States due to the warmer climate and longer growing season. Even with integrated pest management (IPM) strategies, growers must apply large amounts of insecticides to control insect pests. In 2002, approximately 771 Mg of insecticide were applied to soybean fields nationwide (Gianessi and Reigner, 2006). Soybean insecticide use quadrupled between 2002 and 2012 due to the introduction of the soybean aphid (*Aphis glycines* Matsumura) in 2000 (Yang and Suh, 2015).

The kudzu bug (KZB), *Megacopta sp.* (Hemiptera: Plataspidae), is a recently introduced insect pest from Asia, where it is commonly known as the globular stink bug, lablab bug, or bean plataspid (Eger et al., 2010). The KZB was first found in Georgia in 2009 on kudzu [*Pueraria montana* (Lour.) Merr. variety *lobata* (Willd.)] and was thus named the kudzu bug.

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Since its introduction in 2009, KZB has quickly spread across the southeastern United States, becoming a major soybean pest in this region. The KZB is now established in 13 states, from the Atlantic Coast to the Mississippi River (Gardner, 2015). A prediction model based on the KZB native range shows its potential to spread north into major soybean production areas of the Midwest (Zhu et al., 2012). However these predictions may not hold, as a decrease in KZB populations across the Southeast was observed during 2014, which is attributed to the abnormally cold winter during 2013–2014 (Thompson, 2014).

While kudzu and soybean are the primary hosts for KZB, they are known to feed on other legumes as well (Medal et al., 2013; Zhang et al., 2012). Chloroplast DNA from loblolly pine (*Pinus taeda* L.), black walnut (*Juglans nigra* L.), red oak (*Quercus rubra* L.), and sweet gum (*Liquidambar stryraciflua* L.) has been detected in the guts of adult KZBs (Lovejoy and Johnson, 2014), which may indicate that these tree species serve as overwintering and migratory hosts when kudzu and soybean are not available. Adult KZB are known to overwinter near kudzu patches and soybean fields under leaf litter, behind tree bark, and in houses, where they become nuisance pests due to their stench and ability to stain white surfaces (Suiter et al., 2010).

In the spring, adults begin feeding and mating on kudzu and early-planted soybean (Pozo-Valdivia and Reisig, 2013). The KZB uses its piercing–sucking mouthparts to feed on the stems, leaves, and pod walls, which results in visible plant damage in the form of black feeding lesions, and ultimately in yield loss. The average yield loss due to KZB infestation in soybean fields in the Southeast is 19%, with losses up to 60% observed (Greene et al., 2012; Seiter et al., 2013). Therefore, growers face the added expense of controlling KZBs with application of a broad-spectrum insecticide.

Females can oviposit more than 100 eggs on the leaves or stems of a variety of hosts, but nymphs are only known to develop into adults on kudzu, soybean, cowpea [*Vigna unguiculata* (L.) Walp.], pigeon pea [*Cajanus cajan* (L) Millsp.], lima bean (*Phaseolus lunatus* L.), and common bean (*Phaseolus vulgaris* L.) (Medal et al., 2013; Zhang et al., 2012). First-instar nymphs ingest their gut endosymbiont bacteria, *Candidatus Ishikawaella capsulata* Mp., from small brown capsules deposited under the egg mass (Fukatsu and Hosokawa, 2002). KZB develop through five nymphal instars, each lasting approximately 6 to 10 d, before molting into sexually mature winged adults (Pozo-Valdivia and Reisig, 2013). Adult longevity is 23 to 77 d, so one to three generations can occur each year (Eger et al., 2010).

Using primarily physical characteristics, the KZB present in the United States was originally identified as *Megacopta cribraria* (Fabricius) (Eger et al., 2010). *M. cribraria* originates from China, where it is not a major pest of soybean (Hosokawa et al., 2006). However, its Japanese sister species, *M. punctatissima* (Montandon), is considered a major soybean pest (Hosokawa et al., 2006). Based on comparison of an 8.7-kb mitochondrial DNA sequence from the native Japanese species with that of the US populations, the pest found in the United States is the Japanese species (Hosokawa et al., 2014). The same conclusion comes from genome sequencing of the gut microbial symbiont, *C. I. capsulata* (Brown et al., 2014), which determines host preference and pest status on soybean (Hosokawa et al., 2007).

Host plant resistance (HPR) is an integral part of IPM programs, and is considered by some to be the foundation of IPM strategies (Smith, 2005). HPR is recognized as a cost-effective, environmentally friendly way to control insect pests that has been deployed by breeders for controlling both leaf-chewing and piercing–sucking insect pests (Hill et al., 2006a, 2006b; Mensah et al., 2005; Mian et al., 2008; Zhu et al., 2006).

Painter (1951) proposed three categories for classifying HPR to insects: preference (antixenosis), antibiosis, and tolerance. Antixenosis refers to the host plant effect on insect behavior that deters oviposition and/or feeding. Natural field infestations, caged field plots, and greenhouse experiments that give insects a choice of plants to feed on are used to identify antixenosis (All et al., 1989; Hill et al., 2004; Rowan et al., 1991). Antibiosis refers to the adverse host plant effect on the physiology and life history of the insect. The adverse effect can be measured as increased mortality, slowed development, or decreased fecundity. No-choice experiments, which restrict the insect to feed on a single genotype, are used to measure antibiosis. These assays are usually conducted in controlled environments, such as growth chambers or greenhouses (Hill et al., 2004; Zhu et al., 2008).

Simplified visual plant damage ratings and indices have been established by breeders for resistance screening against important insect pests of soybean, such as a defoliation rating for leaf-chewing insects (All et al., 1989), a seed damage index for stink bug (Gilman et al., 1982), and the aphid index for soybean aphid (Hill et al., 2004; Mensah et al., 2005). These damage ratings facilitate quick and easy screening of lines that does not require tedious count measurements throughout the growing season. However, there is not an established rating for KZB. Xing et al. (2006, 2008) used a percent rating of black mildew on the stem and of purple spots on leaves to screen the Chinese soybean germplasm and map quantitative trait loci (QTLs) for resistance to *M. cribraria*. The rating was highly correlated ($r \geq 0.87$) with nymph counts.

This study tested screening techniques in the field, provides a simplified KZB index for screening lines, identified potential sources of resistance to the KZB in soybean, and characterized the resistance mechanism in growth chamber experiments.

MATERIALS AND METHODS

Soybean Entries

'Benning' (Boerma et al., 1997), 10 Benning near isogenic lines (NILs), and the elite breeding line G00-3213 were included in this study. The NILs were BC_6F_2–derived introgressions of defoliating-insect-resistant QTLs into Benning. QTLs M, G, and H are from plant introduction (PI) 229358 (Narvel et al., 2001) and QTL E is from PI 227687 (Hulburt, 2001). Four single QTL lines, Benning-M, -H, -G, and -E; four double-stack QTL lines, Benning-MH, -MG, -GH, and -EM; one triple-stack QTL line, Benning-MGH, and one quadruple-stack QTL line Benning-EMGH were tested (Warrington, 2006, Zhu et al., 2006, Zhu et al., 2008). Benning is a maturity group VII soybean that is susceptible to defoliating insects (Boerma et al., 1997). G00-3213 is a maturity group VII high yielding breeding line developed at the University of Georgia from a cross between 'N7001' (Carter et al., 2003) and 'Boggs' (Boerma et al., 2000).

An additional 30 soybean PIs were selected based on their resistance to known piercing–sucking insects (Table 1) and obtained from the USDA–ARS Soybean Germplasm Collection in Urbana, IL (USDA-ARS, 2015). Of those, 20 PIs were selected for having resistance to the soybean aphid; maturity groups below III were avoided to facilitate screening in Georgia. The remaining 10 PIs were observed to have fewer silverleaf whiteflies (*Bemisia tabaci* Gennadius) by David Walker (USDA–ARS, Urbana, IL) in Quincy, FL, during summer 2012 (personal communication, 2012). Two additional PIs (PI 229358 and PI 171451) were included as insect-resistant checks. These PIs have reported resistance to Coleopteran, Lepidopteran, and Hemipteran pests (Boethel, 1999).

HPR Tests with Benning NILs: 2010–2013

To test host plant resistance to KZB, 9 of the 10 Benning NILs and the susceptible check, Benning, were planted at the University of Georgia Plant Sciences Farm (Watkinsville, GA), each year from 2010 to 2013. The nine NILs included Benning-M, -H, -G, -E, -MH, -MG, -GH, -MGH, and -EMGH. The 2010 and 2011 tests included an additional entry, the null segregant, Benning-mgh, which has the Benning allele for each of the QTLs. The same randomized complete block experimental design with six replications was used each year, planted as 6.1-m two-row plots, with 76.2-cm row spacing and a 1.5-m alley. The test was surrounded by two border rows. These 9 lines were initially chosen to plant in 2010 as part of a field experiment to test

Table 1. Selected soybean accessions with resistance to piercing–sucking insects.

Accession	Insect	MG†	R-genes	Type of host resistance	Origin	Reference
PI 71506 ‡	Aphid	IV	–	Antixenosis	China	Hill et al., 2004
PI 200538	Aphid	VIII	*Rag2*	Antibiosis	Japan	Hill et al., 2004
PI 230977 ‡	Aphid	VII	–	Antibiosis	Japan	Hill et al., 2004; Hesler et al., 2007
PI 243540 ‡	Aphid	IV	*Rag2*	Antibiosis	Japan	Mian et al., 2008
PI 437696	Aphid	VI	–	Antibiosis	China	Hill et al., 2010
PI 548237 ‡	Aphid	VII	–	Antixenosis	USA	Hill et al., 2004
PI 548409 ‡	Aphid	IV	–	Antixenosis	Japan	Hill et al., 2004
PI 548445 ‡	Aphid	VII	–	Antixenosis	China	Hill et al., 2004; 'CNS'
PI 548480 ‡	Aphid	VII	–	Antibiosis	China	Hill et al., 2004
PI 548657 ‡	Aphid	VII	*Rag1*	Antibiosis	USA	Hill et al., 2004; 'Jackson'
PI 548663 ‡	Aphid	VIII	*Rag1*	Antibiosis	USA	Hill et al., 2004; 'Dowling'
PI 567318 ‡	Aphid	IV	–	Mod. resistant	China	Mian et al., 2008
PI 567324 ‡	Aphid	IV	–	Antixenosis	China	Mian et al., 2008
PI 567301B ‡	Aphid	IV	*Rag5*	Antixenosis	China	Mian et al., 2008; Jun et al., 2012
PI 567321A ‡	Aphid	IV	–	Mod. resistant	China	Mian et al., 2008
PI 567336A ‡	Aphid	IV	–	Mod. resistant	China	Mian et al., 2008
PI 567541B ‡	Aphid	III	*rag1c, rag4*	Antibiosis	China	Mensah et al., 2005; Zhang et al., 2009
PI 567543C ‡	Aphid	III	*Rag3*	Antixenosis	China	Mensah et al., 2005; Zhang et al., 2010
PI 567597C	Aphid	III	–	Antixenosis	China	Mensah et al., 2005
PI 567598B ‡	Aphid	III	*rag3, rag1b*	Antibiosis	China	Mensah et al., 2005; Bales et al., 2013
PI 605854B	Whitefly	V	–	Low infestation	Vietnam	D. Walker§
PI 605865B	Whitefly	V	–	Low infestation	Vietnam	D. Walker§
PI 605885B	Whitefly	V	–	Low infestation	Vietnam	D. Walker§
PI 605891A	Whitefly	V	–	Low infestation	Vietnam	D. Walker§
PI 606397B	Whitefly	V	–	Low infestation	Vietnam	D. Walker§
PI 606405	Whitefly	V	–	Low infestation	Vietnam	D. Walker§
PI 615445	Whitefly	V	–	Low infestation	Vietnam	D. Walker§
PI 417503	Whitefly	VI	–	Low infestation	Brazil	D. Walker§
PI 507009	Whitefly	VI	–	Low infestation	Japan	D. Walker§
PI 615437	Whitefly	VI	–	Low infestation	Vietnam	D. Walker§

† MG, maturity group.

‡ Lines included in the 2012 preliminary screening.

§ Personal communication in 2012 with D. Walker, USDA–ARS, Urbana, IL.

HPR to lepidopteran insects (Zhu et al., 2006, 2008), when the KZB appeared for the first time. Differences in KZB number were seen between the entries. Thus it was decided to examine these lines for KZB resistance as well as lepidopteran resistance in 2010, and to repeat the KZB HPR tests in subsequent years.

The 2010 test was planted on 6 July. Sweep-net samples were taken at 62 d after planting. The 2011 test was planted on 21 June, and sweep-net samples were taken at 64 d. The 2012 test was planted on 23 May and sampled at 111 d. Also, in 2012 a modified plant wash-bucket sampling technique (see below) was used at 118 d to collect the insects from each plant. Sweep-net sampling consisted of 20 sweeps taken while walking down the middle of each two-row plot. A smaller test was planted in 2013 to verify observations on QTL H. This test was planted on 14 May, and a wash-bucket sample was taken at 64 d, when the first population of KZB nymphs was developing.

The wash-bucket method is similar to a method used by Rummel and Arnold (1989) to sample western flower thrips (*Frankliniella occidentalis* Pergande) in cotton (*Gossypium hirsutum* L.). Ten plants were selected at random and clipped near the ground from the first row of each two-row plot. KZBs were dislodged from the plants by dipping and swirling the plants in an 18.9-L (5-gallon) bucket of soapy water for a few minutes. Plants were inspected to ensure that no KZBs remained on them before taking them out of the bucket. The insects were collected by pouring the bucket contents through a No. 35 mesh sieve (500 μm) into a funnel and into an empty bucket. The KZBs were then washed off the sieve and into a 133-mL sample cup using a spray bottle of 95% ethanol. Leaves were removed from the stems, which were brought back to the laboratory and frozen until it was possible to count feeding lesions. The nymphs and adults in the sample cups were counted by hand.

Field Cage with Benning NILs

Eight of the 10 insect-resistant Benning NILs, plus the two susceptible checks, Benning and G00-3213, were screened for resistance to the KZB in a field cage similar to that described for lepidopteran pests of soybean (Zhu et al., 2008). The eight NILs were Benning-M, -H, -G, -E, -MH, -GH, -MGH, and -EMGH. Fifteen replicates of each genotype were hand-planted in a randomized complete block design on 30 June 2013 at the University of Georgia Plant Sciences Farm (Watkinsville, GA). A plot consisted of one hill with six plants of the same genotype. Hills were planted on a 76-cm by 61-cm (between-row by within-row) spacing. A border hill of Benning was planted around the experiment. Drip irrigation was run along each row of hills. A week after planting, a Quonset-shaped frame was constructed with lumber and PVC pipe over the area. The frame was covered with a 0.9- by 0.9-mm nylon mesh to contain the test insects and prevent predators, parasitoids and other pests from entering the test area. During the experiment, weekly observations were made to identify and remove any other insect pests that appeared in the cage.

Once plants reached the V3 stage (Fehr and Caviness, 1977), infestations were initiated. Newly hatched KZB nymphs were collected from leaves of early-planted soybean plants in nearby fields that had not been sprayed with insecticide. Thirty first-instar nymphs were applied to each hill twice (22 July and 25 July) using small, natural-hair paint brushes. Visual counts were taken twice a week for 4 wk, starting 5 August and continuing through 27 August, to determine survival and movement between genotypes. Once the plants reached the R1 stage (Fehr and Caviness, 1977), approximately 46,000 adults were added to the caged plants, providing a yield-reducing artificial infestation (>25 adults plant^{-1}) (Seiter et al., 2013). The adult infestation was completed by evenly distributing adult KZBs in the cage four times over a 2-wk period (27 August through 6 September). Visual counts of adults were taken once a week for 4 wk starting on 9 September. On 5 November, after the plants matured, individual hills were harvested by hand. Plants were left indoors to dry for at least a week. Stem lesions were counted in the laboratory.

Field Evaluation of Plant Introductions with Aphid or Whitefly Resistance

Of the 20 selected PIs known to confer aphid resistance, three did not have sufficient seed to use immediately in field tests for KZB resistance. Thus, in 2012 a preliminary screen of 17 aphid-resistant lines was conducted at the University of Georgia Southeast Research and Education Center near Midville, GA (Table 1). The 17 lines were planted in unreplicated short rows (1.8 m) on 25 May 2012 due to low seed numbers. The remaining seed were sent to winter nursery for seed increase. Visual counts of KZB were taken on ten random plants per line on 24 July 2012.

The following year, all 30 aphid- and whitefly-resistant PIs (Table 1), two insect-resistant checks (PI 229358 and PI 171451), and the Benning susceptible check were evaluated in the field using a similar method to that described by Rowan et al. (1991) and Walker et al. (2004). The experimental plots were planted on 15 May 2013 at the University of Georgia Southeast Research and Education Center near Midville, GA. Each plot consisted of six hills of the same genotype, with six plants per hill. The six-hill plots were arranged two rows wide by three hills long on a 91-cm by 61-cm (row by within-row) spacing. Five replications of each plot were planted in a randomized complete block design. A border hill of susceptible Benning was planted between each replication and around the experiment. Drip irrigation was run along each row of hills to provide adequate soil moisture. Thirty-six 120 J s^{-1} (Watt) halogen floodlights mounted on 3-m poles provided 24-hour supplementary lighting to synchronize flowering across the maturity group III to VIII lines.

KZBs were observed in the field once plants reached the V3 stage, and the first visual counts were taken at this time. Visual counts of adults, nymphs, and egg masses were taken from the two middle hills once a month for three months (14 June, 19 July, and 21 August). Supplementary lighting was discontinued on 19 July to synchronize flowering in the field with that of other soybeans on the farm.

When the plants reached the R5 stage (9 September), all plants from the middle two hills were gently clipped and wash-bucket sampled as previously described. After washing the plants, stem and leaf lesions were rated for each hill on a scale of 1 to 9, where 1 = no feeding lesions, 3 = small black lesions near nodes, 5 = clusters of black lesions near nodes, 7 = large black lesions with dead tissue along the entire stem, and 9 = large clusters of black lesions along the entire stem surrounding dead (brown) tissue (Fig. 1). A black sooty mold growing from

Fig. 1. Kudzu bug lesion rating scale 1 to 9. Rating of 1 = no feeding lesions, 3 = small black lesions near nodes, 5 = clusters of black lesions near nodes, 7 = black lesions with dead tissue along the entire stem, and 9 = large clusters of black lesions along the entire stem surrounding dead (brown) tissue.

the honeydew excreted by KZBs was present on plants with a rating >7. The number of stem lesions per plant was counted for the susceptible check and the lines with the lowest and highest lesion rating for each replication. Sample cups from the wash-bucket procedure were brought back to the laboratory where adults and nymphs were counted.

To facilitate comparison between soybean lines, a KZB index was computed by combining three resistance measurements: adult wash-bucket counts, peak nymph counts (19 July), and the lesion rating. The adult wash-bucket counts were transformed to normalize the data using $\text{Log}_{10}(x+1)$, and then they were scaled to fit a 0 to 3 scale. The nymph counts were recorded on a 0 to 3 scale, where 0 = 0 nymphs, 1 = 1 to 20 nymphs, 2 = 21 to 49 nymphs, and 3 = greater than 50 nymphs, to fit a normal distribution. The lesion rating was also scaled to fit a 0 to 3 scale. The three values were then added together to generate a KZB index, with values ranging from 0 to 9.

Kudzu Bug Colony

Adult KZBs were collected from soybean fields at the University of Georgia Plant Sciences Farm near Watkinsville, GA on 15 Oct. 2012 and the following year on 6 Sept. 2013. The colony was maintained on Benning plants growing in 3.4-L black plastic pots inside an insect rearing cage (Product No. 1466DV, Bio-Quip Product, Inc. Rancho Dominguez, CA). The colony was placed in an insecticide-free greenhouse during the late-spring and summer, and in a walk-in growth chamber set to 27°C, 14:10, light/dark, and 50 to 60% relative humidity in the winter. Plants were replaced every 4 wk to supply fresh plants and to curtail the growth of greenhouse pest populations.

Kudzu Bug Antibiosis Assay

A no-choice assay was performed using a procedure modified from Zhu et al. (2008). The growth chamber was maintained at 27°C and 60% relative humidity with a 14-h photoperiod provided by white fluorescent and soft white incandescent bulbs at an intensity of 40 μmol photons m^{-2} s^{-1}. Due to space constraints two lines, PI 567336A and PI 567598B, which had been verified as resistant, and two lines, PI 548409 and PI 71506, which had been verified as susceptible in the PI field evaluations, were screened in no-choice experiments, using Benning as a susceptible check. The assay was arranged in randomized complete block design with 12 replications. Newly expanded trifoliolate leaves of similar size were collected from V3-stage plants grown in 0.95-L (32-oz) polystyrene foam cups in the greenhouse, and placed into a clear 60-mL polypropylene cup with a 1-cm layer of moistened plaster of Paris in the bottom. Newly hatched first-instar nymphs were collected from the colony, waiting until 24-h after hatching to allow ingestion of the endosymbiont capsule (Hosokawa et al., 2008). Five KZBs were added to each trifoliolate leaf using a small paint brush made of natural hair. The surviving nymphs were counted twice weekly, and the instar recorded. Once per week, a new trifoliolate leaf of similar size was added to the cup and the old leaf was discarded. Cups were maintained until all nymphs either developed into adults or died.

Statistical Analyses

All statistical data were analyzed using the software program JMP, Version 11 (SAS Institute, 2007). Each experiment was analyzed independently. Insect count data did not fit a normal distribution, so a $\text{Log}_{10}(x+1)$ transformation was used to normalize count data before conducting an analysis of variance to determine if there were treatment effects. For graphical representation, back-transformed counts were used. If statistical differences ($P < 0.05$) were found in the analysis of variance, mean separation was calculated using Tukey's HSD at $\alpha = 0.05$ or a Student's t test as noted in the results.

RESULTS

'Benning' Near-Isogenic Lines

There were no consistent differences in KZB counts between Benning and the Benning NILs across years. The data showed a tendency for QTL H from PI 229358 to affect KZB population development (Table 2). The 2010 HPR test showed that Benning-G and Benning-H had significantly fewer ($\alpha = 0.05$) nymphs compared to Benning, and Benning-H and -MH had significantly fewer ($\alpha = 0.05$)

Table 2. Kudzu bug population and lesion damage on Benning near-isogenic lines in host plant resistance field tests.

	2010		2012	2013
Line	Nymph†	Adult†	Lesion‡	Nymph‡
Benning	5.5 a§	11.2 a	106.5 ab	49.4 a
Benning-mgh	3.2 abc	7.8 ab	–	–
Benning-M	1.5 abc	5.3 ab	93.0 b	–
Benning-H	0.8 bc	4.2 b	104.3 b	30.3 b
Benning-G	0.7 c	6.7 ab	157.3 a	–
Benning-E	2.7 abc	5.7 ab	121.3 ab	–
Benning-MG	3.0 abc	4.7 ab	105.0 b	–
Benning-MH	1.5 abc	2.8 b	130.3 ab	–
Benning-EM	4.8 ab	4.5 ab	116.0 ab	–
Benning-MGH	3.2 abc	6.2 ab	144.0 ab	57.6 a
Benning-EMGH	2.7 abc	6.3 ab	95.3 b	–

† Sampled with the sweep net method.

‡ Sampled with the wash-bucket method.

§ Values within columns followed by the same letter are not significantly different by Tukey's HSD ($\alpha = 0.05$).

adults compared to Benning. No differences in nymph and adult counts between lines could be detected in the 2011 and 2012 HPR tests; therefore, the data are not shown. Stem lesions were counted for the first time in the 2012 HPR test and showed a significant difference ($P = 0.0076$) between the Benning NILs, although none of the lines had significantly fewer ($\alpha = 0.05$) lesions than Benning. In the 2012 field, the last two replications had a strong border effect from a significantly higher KZB infestation ($P < 0.0001$; Mean KZBs per plot: 258 in Rep 1–4 and 766 in Rep 5–6) compared to the other part of the field. In the 2013 HPR test, Benning-H had significantly fewer nymphs ($\alpha = 0.05$) compared to Benning and Benning-MGH.

In addition to the QTL combinations from the HPR field trials, the 2013 field cage experiment evaluated an additional QTL combination, namely GH. Based on the ANOVA, there were no significant differences ($P = 0.4576$) between nymph counts on the Benning NILs and checks, but there were significant differences ($P = 0.0459$) between adult counts, with Benning-MGH and G00-3213 having significantly ($\alpha = 0.05$; Student's *t* test) fewer adults compared to Benning (Fig. 2). Stem lesion counts were significantly different ($P = 0.0009$) between lines in the cage experiment (Fig. 3). Benning-GH, -MGH, -EMGH, -H, -M, and G00-3213 had significantly fewer ($\alpha = 0.05$; Student's *t* test) stem lesions compared to Benning.

Wash-Bucket Validation

To determine the accuracy of the wash-bucket sampling method for quantifying the number of KZBs per plant, two Benning plants from each of the five replicated plots in the 2013 HPR experiment were wash-bucket sampled, and each plant was visually inspected to count the remaining KZBs. Thirty-two adults were washed off the 10 Benning plants, and only one adult remained on visual inspection of the plants. One thousand seventy-four nymphs were washed off the 10 Benning plants, and only 20 nymphs remained on visual inspection of the plants. With 97.5% of adults (32 of 33) and 98.2% of nymphs (1074 of 1094) collected from these plants, the wash-bucket sampling method was considered to be more accurate than counting insects while they move about on the plant.

Field Evaluation of Plant Introductions with Aphid or Whitefly Resistance

The 2012 preliminary screening of 17 aphid-resistant PIs showed that PI 548409 had the highest KZB population, and PI 567324, PI 567301B, PI 567321A, PI 567543C, and PI 567318 had the lowest KZB infestation, with less than 25 KZBs per row counted (Fig. 4). The following year, when more seed was available, there were significant differences ($P < 0.0001$) in the KZB index between the lines (Fig. 5). Additionally, an ANOVA was performed for each component of the KZB index. Both the $\text{Log}_{10}(x+1)$ adult counts and the scaled nymph scores

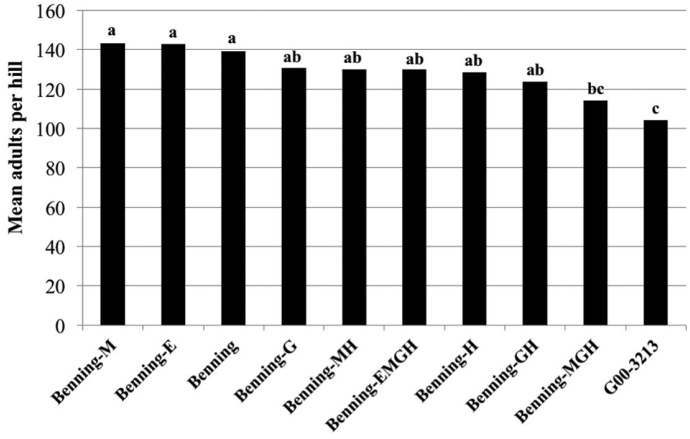


Fig. 2. Adult kudzu bugs on the Benning NILs in the 2013 field cage. Lines with the same letter are not significantly different according to a Students *t* test ($\alpha = 0.05$).

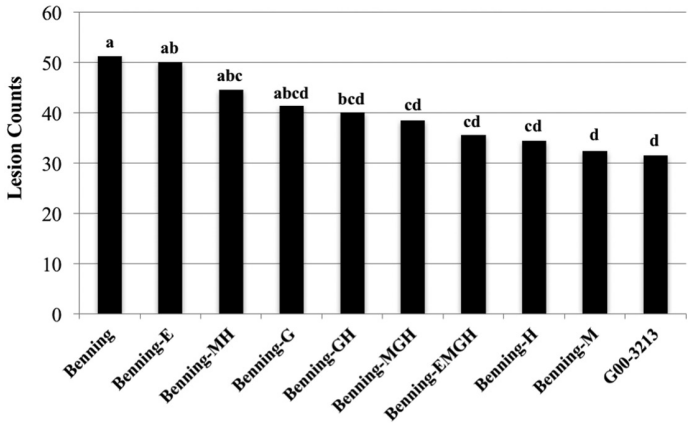


Fig. 3. Stem lesion counts on the Benning NILs in the 2013 field cage. Lines with the same letter are not significantly different according to a Students *t* test ($\alpha = 0.05$).

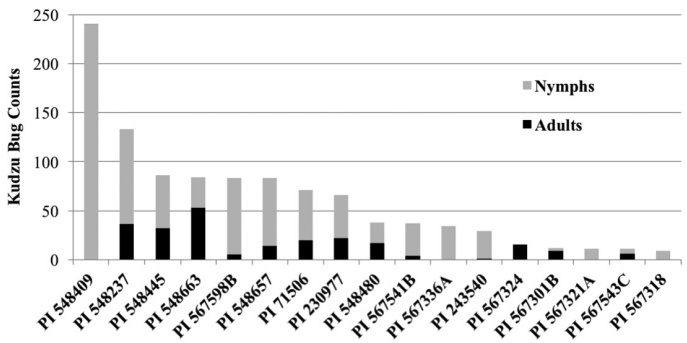


Fig. 4. Kudzu bug counts from the 2012 Preliminary PI evaluation.

showed significant differences among lines at $P < 0.0001$, and the lesion rating was different at $P = 0.1357$ among lines. A Student's *t* test ($\alpha = 0.05$) on the KZB index indicated that six lines were significantly lower than that of Benning. These were PI 437696, PI 567543C, PI 567597C, PI 567301B, PI 567598B, and PI 567336A, thus supporting the low KZB population observed for PI 567301B and PI 567543C in the 2012 preliminary test.

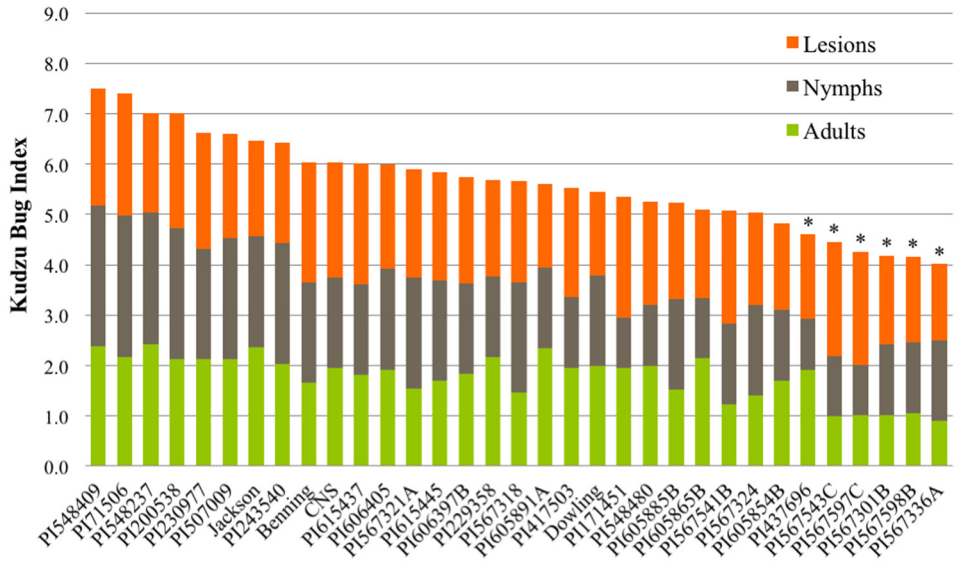


Fig. 5. Kudzu bug resistance index for the 2013 PI evaluation. Lines with an asterisks (*) are significantly lower than Benning according to a Student's *t* test ($\alpha = 0.05$).

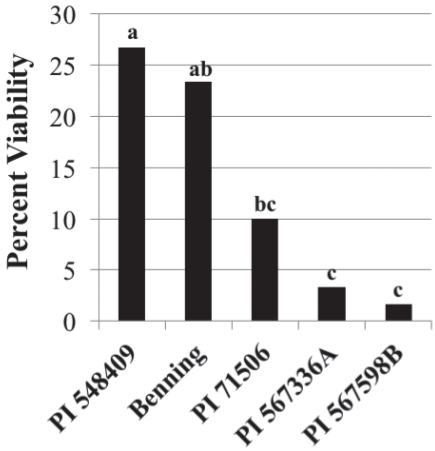
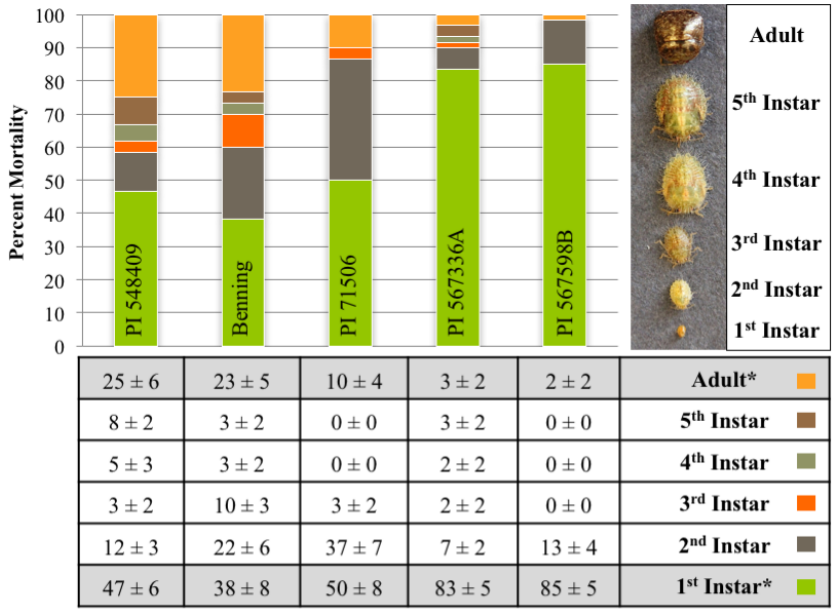


Fig. 6. Viability of kudzu bug nymphs in the antibiosis assay. Lines with the same letter are not significantly different according to Tukey's HSD ($\alpha = 0.05$).



PI 548409	Benning	PI 71506	PI 567336A	PI 567598B	
25 ± 6	23 ± 5	10 ± 4	3 ± 2	2 ± 2	Adult*
8 ± 2	3 ± 2	0 ± 0	3 ± 2	0 ± 0	5th Instar
5 ± 3	3 ± 2	0 ± 0	2 ± 2	0 ± 0	4th Instar
3 ± 2	10 ± 3	3 ± 2	2 ± 2	0 ± 0	3rd Instar
12 ± 3	22 ± 6	37 ± 7	7 ± 2	13 ± 4	2nd Instar
47 ± 6	38 ± 8	50 ± 8	83 ± 5	85 ± 5	1st Instar*

Fig. 7. Nymphal mortality and adult emergence in the antibiosis assay. Values shown are percentage ± one standard deviation. Significant differences denoted with an asterisk (*) were found at $\alpha = 0.05$ using Tukey's HSD for first instar mortality and adult emergence.

Kudzu Bug Antibiosis Assay

The no-choice test indicated that KZB development (viability) on PI 567336A and PI 567598B was significantly ($\alpha = 0.05$) lower than that on PI 548409 and Benning (Fig. 6). First instar mortality was higher than mortality in other instars for all lines, and significantly ($\alpha = 0.05$) higher on PI 567598B and PI567336A compared to the susceptible lines (Fig. 7).

DISCUSSION

The arrival of the Japanese KZB poses several challenges for the identification of genetic sources of resistance. Herein we test new screening techniques and identify potential sources of resistance to the KZB. Wash-bucket sampling proved to be more accurate than sweep net sampling at quantifying the number of KZBs on soybean plants in the field. A field cage allowed for a heavy infestation of KZB to screen for resistance in the field. Even with the improved screening techniques, QTLs M, G, H, and E, identified for their resistance to leaf-chewing insects,

are not an effective form of resistance to the KZB. When additional insect resistant germplasm were screened and evaluated in the field with the KZB index, six lines were identified as potential sources of KZB resistance. Two lines from the field with the lowest KZB index were verified to have antibiosis resistance to KZB using a no-choice nymph survival assay.

Sweep-net sampling is typically used as an IPM tool to quickly sample fields to determine which insect pests are present and when to apply insecticide. Sweep-net sampling has since been shown to be more effective at only low KZB populations (Stubbins et al., 2014). Therefore, a wash-bucket sampling method was implemented to more accurately count the high number of KZBs on plants. Additionally, feeding lesions were counted on the stems of these plants to determine the amount of KZB feeding throughout the season. However, these new resistance measurements were not significantly lower for any of the NILs when compared to those of Benning (Table 2). Inconsistent results, along with the edge effects observed in 2012, indicated that a more controlled environment is necessary to maintain a heavy infestation and to reduce variability in the KZB population across the field. Therefore, a field cage was also planted in 2013.

The field cage allowed for a heavy, uniform infestation (>25 adults plant^{-1}) on the plants. Switching to hill plantings allowed an increase in the number of replications to 15, which increased the precision of the measurements. Furthermore, the adult population on all lines in the cage increased by an average of 23 adults per hill over the 4-wk period, with none of the lines having fewer adults at the end of the experiment than at the beginning. For both the open field experiments and the field cage experiment, no statistical association between adult counts and lesion counts could be identified.

Since the defoliator-resistance QTLs were not effective sources of resistance to the Japanese KZB, a broader range of germplasm was tested. Thirty lines with known resistance to soybean aphid and silverleaf whitefly were screened for KZB resistance. Six lines previously identified for aphid resistance, PI 437696, PI 567543C, PI 567597C, PI 567301B, PI 567598B, and PI 567336A, were found to be potential sources of resistance to KZB using the KZB index (Fig. 5).

By combining adult and nymph population counts with a stem lesion (damage) rating, the KZB index offers a broader picture of the plant-insect interactions, and can help breeders differentiate resistant from susceptible lines in the field. Based on the KZB index for the PI field experiment, the scaled adult counts were similar to the nymph scale described previously, where 0 = 0 adults, 1 = 1 to 20 adults, 2 = 21 to 49 adults, and 3 = greater than 50 adults. Additionally, based on the lesion counts taken from the highest- and lowest-rated plants from each replication and the Benning checks, the scaled lesion rating fits a 0 to 3 scale, where 0 = 0 lesions, 1 = 1 to 50 lesions, 2 = 51 to 100 lesions, and 3 = greater than 100 lesions. The KZB index can be used as a visual rating like the soybean aphid index established by Mensah et al. (2005), thus greatly reducing phenotyping time for KZB resistance.

Using the KZB index, PI 567598B and PI 567336A were identified to have the lowest rating in the field (Fig. 5). To test these lines for KZB resistance an antibiosis (no-choice) assay was developed to measure nymphal development and survival to adult. Nymphal mortality in the first instar was twice as high on PI 567336A and PI 567598B compared to that on Benning (Fig. 7), and adult emergence was 10-fold lower on these lines (Fig. 6 and 7). The impaired nymphal development confirms that these PIs show antibiosis to KZB.

This latter study was enabled through the establishment of a KZB colony. These proved remarkably easy to rear as long as fresh plant tissue was provided regularly to curtail the growth of other pests. The KZBs in the colony continued to reproduce and multiply during the winter and spring, indicating that there is no pre-set number of generations in a growing season before the insects need to overwinter. When couples were isolated, it became evident that a single female could lay up to 150 eggs in five batches over her lifetime. Such a reproduction rate is consistent with the insect's rapid expansion prior to the killing winter of 2014.

CONCLUSIONS

There are genotypes of soybean that have effective resistance to the KZB. Such resistance can be screened using both field-based and lab-based methods. It is not yet known if the resistance to KZB has the same or a different genetic basis as the soybean aphid resistance QTL previously described for PI 567598B (Mensah et al., 2005; Bales et al., 2013). Elucidation of the genetic control of this resistance should make it easier to deploy it in breeding programs, or even to enhance it by pyramiding these genes with transgenes.

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