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Plant–Insect Interactions

Seed Damage by the Neotropical Brown Stink Bug, *Euschistus heros* (F.) to Resistant Soybean Cultivars with the Block Technology Versus a Susceptible Cultivar

Weslley Pastorio Oliveira,[1] Tiago Lucini,[2] and Antônio Ricardo Panizzi[3,4,]

[1]Curso de Agronomia, Instituto Federal de Educação, Ciência e Tecnologia, Rodovia RS 135, Kilometer 32,5, Sertão, RS, 99170-000, Brasil, [2]Departamento de Zoologia, Universidade Federal do Paraná, Caixa Postal 19020, Curitiba, PR, 81531-980, Brasil, [3]Embrapa Trigo, BR 285 Kilometer 294, Caixa Postal 3081, Passo Fundo, RS, 99050-970, Brasil, and [4]Corresponding author, e-mail: antonio.panizzi@embrapa.br

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Abstract

Laboratory and greenhouse studies were conducted with the Neotropical brown stink bug, *Euschistus heros* (F.), to evaluate and compare adult damage at two infestation levels (2 and 4 adults/plant) and feeding behavior on seeds of three resistant soybean cultivars bearing the Block technology ('BRS 1003 IPRO', 'BRS 543 RR', and 'BRS 391') compared to a susceptible cultivar ('BRS 5601 RR'). No difference in number or weight of damaged seeds (as percentages) was found among the cultivars at either infestation level. Differences were only observed between infestation levels within cultivar; higher values were reported with 4 adults/plant, except for 'BRS 543 RR'. At 2 adults/plant, total seed area damaged (mm^2) and percentage of seed area damaged were significantly lower only on 'BRS 1003 IPRO'; significant differences among cultivars were found in damage to internal but not external seed surfaces. At 4 adults/plant, all Block cultivars differed from 'BRS 5601 RR' in overall seed damage, and greater percent damage occurred on both seed surfaces on 'BRS 5601 RR'. Electropenetrography (EPG) demonstrated that adults reached and fed in the seeds of all soybean cultivars. However, the feeding event duration in seeds of Block cultivars was much shorter than on 'BRS 5601 RR', which likely explains differences between internal and external seed damage. Furthermore, the total duration of feeding activities on seeds of Block cultivars was ca. 4–6 times shorter than on the susceptible cultivar; these two EPG feeding variables account for the lower seed damage observed for the Block cultivars. These cultivars represent an important new strategy for pest control on soybean.

Key words: stink bug, host plant resistance, soybean seed damage, EPG, electropenetrography

The Neotropical Region (Neotropics) is presently experiencing rapid growth of agricultural commodities, particularly soybean, *Glycine max* (L.) Merrill (Fabaceae) (source: http://www.fao.org/faostat/en/#data). Among the many insects that can damage the soybean crop in the Neotropics, stink bugs are the primary pests (Panizzi and Silva 2012). In Brazil alone, these bugs are responsible for at least $600 million (US) of soybean seed yield loss per year (CEPEA/ESALQ, and ANDEF 2017). Chemical control of stink bug pests is the standard method used by soybean growers in this region (e.g., Bortolotto et al. 2015; Bueno et al. 2020). Over the years, insecticides have caused several negative side effects (e.g., impact on natural biological control agents, increased costs in soybean production, appearance of resistant stink bug populations). To avoid or mitigate these problems, alternate control measures would be preferable. Use of soybean cultivars exhibiting resistance to stink bugs (e.g., cv. IAC 100) was previously attempted (Rossetto et al. 1995), but such cultivars did not thrive at that time due to low seed yield and other unsuitable agronomic characteristics. More recent research efforts, conducted by agronomists at the Embrapa Soybean Research Center, at Londrina, Paraná, Brazil, resulted in the release of soybean cultivars with the so-called 'Block technology'. These cultivars, developed through recurrent selection of soybean lines exposed to high levels of stink bug infestation, provide high yield and good seed quality and appear to display tolerance to stink bug attack (Arias et al. 2020).

The Neotropical brown stink bug, *Euschistus heros* (F.) (Hemiptera: Pentatomidae) is the most abundant species on soybean;

it feeds on several cultivated and non-cultivated plants but primarily on legumes (Fabaceae) (Smaniotto and Panizzi 2015). Lucini et al. (2021) examined the effect of soybean cultivars with the Block technology on *E. heros* biology and feeding behavior. The soybean Block cultivars did not affect nymphal or adult survivorship, nymphal development, or adult reproductive performance. However, using the electropenetrography (EPG) technique it was demonstrated that bugs spent less time feeding on seed endosperm of resistant plants compared with seed from a susceptible plant. Moreover, on seeds of Block cultivars, feeding behaviors included mostly laceration/maceration activities without ingestion of cell contents, whereas on the susceptible cultivar, ingestion was more frequent.

In this study, we aimed to evaluate in detail the damage caused by *E. heros* to seeds of soybean cultivars bearing the Block technology compared with a susceptible (conventional) cultivar. Percentage of damaged seeds, percentage of total weight (mg) of damaged seeds, seed area (mm^2) damaged/seed, and percentage of the seed area (mm^2) damaged/seed were evaluated, and damage to external and internal surfaces was compared. Additionally, an EPG study was conducted to determine counts and durations of the feeding activities performed by *E. heros* adults in seeds of the soybean cultivars tested. We predicted that seed damage overall would be higher in the conventional cultivar, with greater damage occurring on internal surfaces in this cultivar corresponding to increased feeding activity.

Materials and Methods

Stink Bug Colony in the Laboratory

During September/October 2020, *E. heros* adults were collected in the field from crop residues on the soil, and from various plants (cultivated and non-cultivated) at the Embrapa Wheat Field Experiment Station, located in Passo Fundo, Rio Grande do Sul state, Brazil (latitude 28° 15' 46' S, longitude 52° 24' 24' W). Adults were taken to the laboratory, placed in plastic rearing cages (25 x 20 x 20 cm) (Plasvale, Gaspar, SC, Brazil) in which the floor was lined with filter paper. Adults were fed a mixture of green bean pods, *Phaseolus vulgaris* L., raw shelled peanuts, *Arachis hypogaea* L. (Fabaceae), and mature seeds of soybean. Cages were kept in a locally built (no trademark available) walk-in chamber at 25 ± 1°C, 65 ± 10% relative humidity, and L14:D10 h photoperiod at the Embrapa Wheat Laboratory of Entomology.

Commercially available cotton balls and toilet paper were provided as oviposition substrates for adults. Egg masses were collected and placed inside plastic boxes (11 x 11 x 3.5 cm - floor lined with filter paper). Nymphs were fed the food mixture described above and reared to adults in the walk-in chamber. Nymphs that reached the adult stage were used for infesting caged soybean plants in the greenhouse. Studies in the greenhouse/laboratory were conducted from October 2020 to May 2021.

Greenhouse Study

Potted Plants

Seeds of soybean of cultivars with Block technology and a susceptible cultivar were seeded biweekly in pots (5L) in the greenhouse from October to November 2020. Seeds representing the Block cultivars were 'BRS 1003 IPRO', 'BRS 543 RR', and 'BRS 391'; seeds of 'BRS 5601 RR' represented the susceptible cultivar. For each cultivar, seeds were planted at a rate of five seeds per pot. Two weeks following emergence, seedlings were thinned to yield two seedlings per pot. Soybean plants were used at the R6 stage (full pod-filling) (Fehr et al. 1971). The environmental conditions in the greenhouse during the study period were temperature 19.6–22.1°C, relative humidity 61.3–77.7%, and 6.2–8.5 h. (solar radiation) (source: Embrapa Wheat—Meteorological information available at the website http://www.cnpt.embrapa.br/pesquisa/agromet/app/principal/agromet.php).

Infestation of Soybean Plants with *E. heros*

After reaching the R5 developmental stage (pod filling), only one plant (the healthiest) was kept in each pot. All plants were maintained with 40 pods/plant; excess pods were cut off. In the R6 stage, each plant was completely covered with a thin net supported by a metal frame. The plants were infested with 2 or 4 stink bug adults (2 to 3 weeks old) obtained from the established laboratory colony. The experiment was conducted in a completely randomized design with four treatments (soybean cultivars), two levels of infestation (2 and 4 stink bugs/plant) and 9 repetitions; in total, 72 potted plants were used. The stink bugs were caged on the plants for two weeks. Plants were inspected daily to remove any egg mass(es) laid by females to avoid nymphal emergence. At the end of the infestation period, stink bugs were removed, and plants were allowed to mature. At maturation, soybean plants were manually harvested.

Number and Weight of Damaged Seeds

After harvest, the pods were taken to the laboratory, and manually threshed. Initially, the seeds were separated into two groups: 1) seeds without any visual damage; and 2) seeds with damage. To aid in the determination of the presence or absence of damage (e.g., feeding punctures, seed malformation with chalky and dark spots), a stereomicroscope (LABMotic-SMZ500, São Paulo, SP, Brazil) was used. After separation, the total number of seeds with and without damage were counted and weighed using a precision electronic balance (Mettler Toledo MS 3002S/A01, Barueri, SP, Brazil). The data were used to calculate the percentage of damaged seeds (i.e., in relation to total number of seeds obtained), and percentage of the weight of damaged seeds (i.e., in relation to total weight of seeds).

$$\%\ damaged\ seeds = \left(\frac{number\ of\ damaged\ seeds}{total\ number\ of\ seeds}\right) . 100$$

$$\%\ of\ weight\ of\ damaged\ seeds = \left(\frac{weight\ of\ the\ damaged\ seeds}{total\ weight\ of\ the\ seeds}\right) . 100$$

Laboratory Study

Seed Area Damaged

To determine the seed area damaged by *E. heros* adults, 30 damaged seeds/infestation rate/cultivar were randomly chosen; the two main symptoms of damage were evidence of stylet insertion and discoloration of tissue (Fig. 1A and 1F). Seeds were subsequently kept inside a plastic box (11 x 11 x 3.5 cm) and, for 24 h, placed on a layer of wet cotton to induce swelling (Fig. 1B), which facilitated visualization of the damage and seed cut. After the 24 h period, a sharp razor blade was used to carefully cut each swollen seed along the length of the hypocotyl-radicle axis (Fig. 1C) to yield two similar pieces (cotyledons). On each cotyledon, we photographed the seed surface twice, with the external surface of the seed facing up and again with the internal surface facing up (Fig. 1D), using a digital camera (Nikon Canon Rebel T100, Tokyo, Japan). Images of each affected seed were recorded and analyzed using image processing software (ImageJ version 1.8.0). A ruler was included as a scale in each photo to calibrate the software (Fig. 1E). After an image of a seed with damage was uploaded and calibrated, we delineated the perimeter of the seed to obtain the total seed area (Fig. 1F—yellow dashed line); and then individually delineated the damaged areas on each seed surface (Fig. 1F—red continuous line). Using the annotated and calibrated

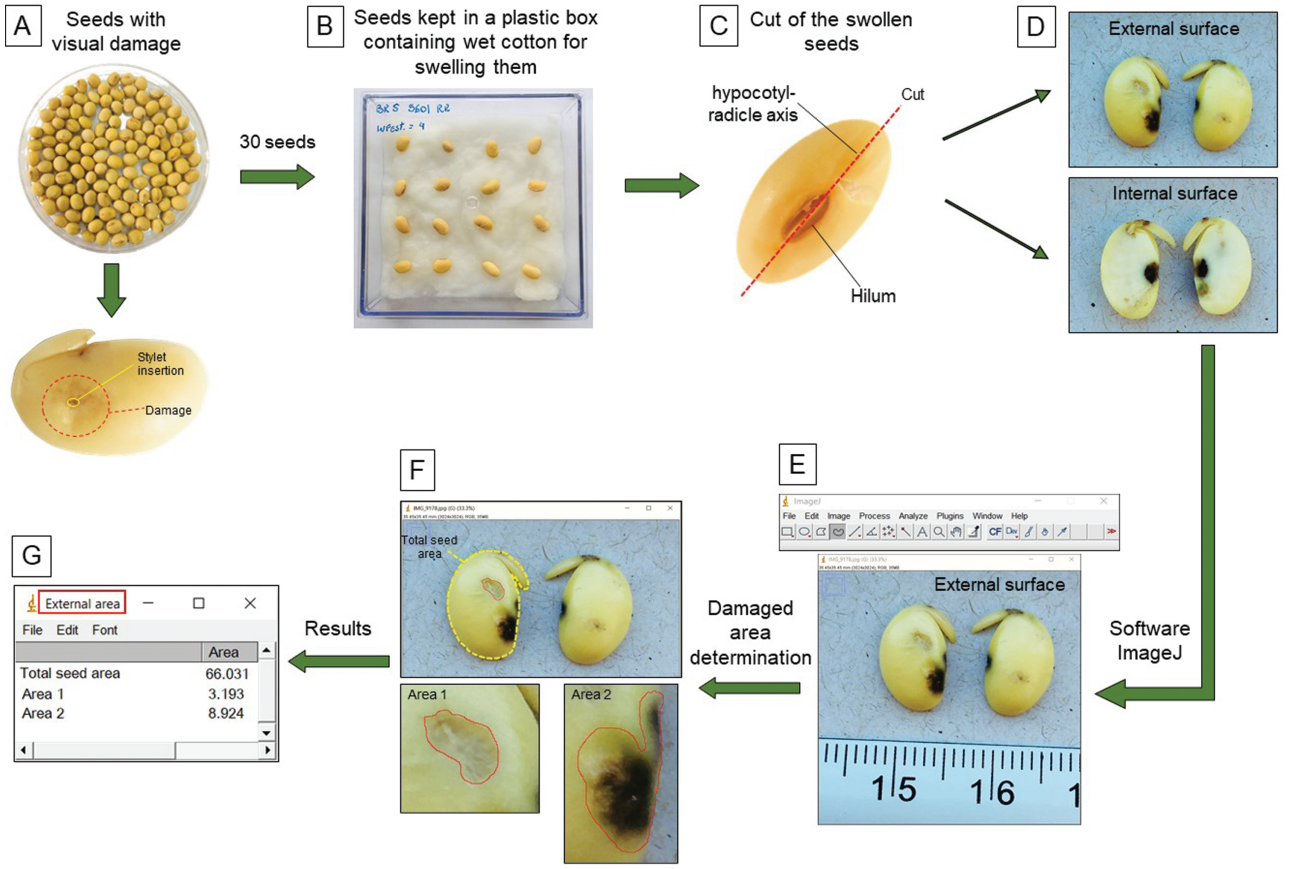

Fig. 1. Schematic illustration of the process used to calculate the seed area of different soybean cultivars damaged by *Euschistus heros*. Petri plate containing seeds for random selection of 30 seeds to assess visual damage (A); seeds in a plastic box containing a layer of wet cotton for swelling (B); cut of the swollen seeds in two pieces (cotyledons) (C); external and internal seed surfaces used to calculate the surface damaged area/seed (D); photos taken using a digital camera (Nikon Canon Rebel T100) with a ruler used as scale to calibrate the software ImageJ (E); total seed area (yellow dashed line), and each individual damaged area (red continuous line) (F); and different values of areas generated by the software (G) (See online version for color figure).

images, the image processing software generated values for the total seed area and the area(s) of affected tissue (Fig. 1G). These values were used to calculate 1) the damaged area (mm^2) on each seed surface (external versus internal), 2) the total damaged area (mm^2) per seed (summing external and internal surfaces for both cotyledon halves [Fig. 1D]), 3) the percentage of damage on each seed surface, and 4) the total percentage of the damage per seed (i.e., the proportion of the damage in relation to the total seed area, which was calculated as the sum of external plus internal surfaces). This process was repeated for each infestation rate and soybean cultivar.

$$Total\ seed\ area = total\ external\ area + total\ internal\ area$$

$$\%\ external\ damaged = \left(\frac{total\ external\ damage}{total\ external\ area}\right).100$$

$$\%\ internal\ damaged = \left(\frac{total\ internal\ damage}{total\ internal\ area}\right).100$$

$$Total\ \%\ seed\ damaged = \left(\frac{total\ external\ damage + total\ internal\ damage}{total\ seed\ area}\right).100$$

Electropenetrography (EPG) Procedures

To record the feeding behavior of adult *E. heros*, a four-channel EPG AC-DC monitor (Backus et al. 2019; EPG Technologies, Inc., Gainesville, FL, USA) was used. The equipment was adjusted to apply a voltage of 50 mV alternating current (AC) and an input impedance of 10^7 ohms in all channels (Lucini and Panizzi 2018). Changes in the system voltage during stylet activities were recorded and amplified at a rate of 100 Hz per channel using the WinDaq DI-710 equipment (DATAQ Instruments, Akron, OH) connected to a computer with the WinDaq Lite software installed.

Adult females (ca. 15-d old) were separated from the laboratory colony and starved for 4 h, in the presence of water. Subsequently, the insects were individually immobilized to attach the gold wire electrode (3 cm long; 0.1 mm in diameter) on their pronotum following the methodology of Lucini and Panizzi (2016). Wired stink bugs were again starved for 1 h; then, they were individually connected to the EPG head stage amplifier (channel) and positioned on the soybean pods. The plant electrode (a piece of copper wire) was inserted into the moistened soil containing the soybean plant to close the electrical circuit. To protect the system against external electrical noise, all EPG channels (amplifiers), insects, and plants were kept inside a Faraday cage.

The feeding behavior of stink bugs was monitored without interruption under laboratory conditions (a room maintained at ca. 25°C) and continuous light for a period of 15 hours. The experiment was conducted in a randomized complete block design with insects and soybean cultivars ('BRS 391', 'BRS 543 RR', 'BRS 1003 IPRO', and 'BRS 5601 RR') randomly assigned to one of the four EPG channels. Insects and plants were only used once in the experiment. Six adult females were successfully recorded on each soybean cultivar.

Statistical Analysis

Number and Weight of Damaged Seeds and Damaged Area

Preceding the analysis of variance (ANOVA), the data were first submitted to the Bartlett test for checking the homogeneity of variances ($P < 0.05$); data were transformed when required (see notation in the footnotes of tables and figures) to satisfy the pre-requisites of ANOVA, using a transformation that fitted for each case: $\sqrt{(x)}$ for seed damage area (mm^2), and arcsine $\sqrt{(x/100)}$ for percentage of seed damage. Means (± SE) of the different parameters evaluated (i.e., percentage of seeds damaged, percentage of weight (mg) of soybean seeds damaged, and damaged area of affected seeds) were separated, when applicable, using the Tukey test or Student's *t*-test ($P < 0.05$). The Tukey test was applied for comparisons among the four soybean cultivars on each individual infestation level and on each seed surface analyzed (external versus internal). Student's *t*-test ($P < 0.05$) was applied to compare the two infestation levels on each individual soybean cultivar. All statistical details are explained in the tables and figure footnotes. The statistical analyses were performed using the 'R' statistical program, v.4.1.0 (R Development Core Team 2018), by applying the functions available in this program, including 'Bartlett.test' to check homogeneity of variances and 'aov' for the one-way ANOVA model. The Tukey test was performed using the 'TukeyC' package (Faria et al. 2018) and Student's *t*-test by applying the 't.test' function.

Electropenetrography (EPG) Data

The feeding behavior of *E. heros* on soybean pods and their respective waveforms have already been characterized by Lucini and Panizzi (2018), who created an EPG waveform library containing 13 different waveforms, including non-feeding and feeding activities. In this study, our goal was to assess the effect of feeding activities of *E. heros* in the seeds of soybean; therefore, we considered only the waveform named 'Eh3' in our analysis. In summary, this waveform represents a combination of fast stylet movements (laceration) and watery salivation (maceration) to degrade the seed tissue, followed by the ingestion of the cell contents dissolved (further details in Lucini and Panizzi 2018).

The counts and durations of Eh3 waveform on each soybean cultivar were manually performed using the WinDaq/Waveform Browser software (DATAQ Instruments, Akron, OH). Three non-sequential EPG variables were analyzed and calculated: 1) number of Eh3 events performed per insect (NWEI); 2) duration (min) of each Eh3 event performed per insect (WDEI); and 3) total duration (min) of Eh3 waveform performed per insect (WDI) (Backus et al. 2007). These variables were recorded for *E. heros* in a previous publication on the cultivars we tested (Lucini et al. 2021). Herein, we also added calculations of the percentage of probes containing Eh3 event(s) on each cultivar. The term 'probe' includes all activities performed by the insect, from the stylet insertion into the pod tissue until its complete removal; in turn, the term 'event' of the waveform Eh3 represents a continuous and uninterrupted occurrence of this waveform within a probe.

Values for these EPG variables were generated and analyzed using the Backus 2.0 program for running on the SAS statistical program (SAS Institute 2009) (Backus 2.0 program is available at http://www.crec.ifas.ufl.edu/extension/epg). Mixed model analysis of variance using restricted maximum likelihood estimation (REML-ANOVA) was performed via the SAS procedure GLIMMIX to verify differences in the EPG variables between the four soybean cultivars evaluated. When required, counts and duration data were transformed via $\sqrt{(x)}$ and $\log(x)$, respectively, to satisfy assumptions of ANOVA. The means were separated using the least significant difference test (LSD; $\alpha = 0.05$).

Results

Greenhouse Study

Number and Weight of Damaged Seeds

Considering the percentage of damaged seeds (in relation to total number of seeds obtained), results indicated that, for both infestation levels of *E. heros* tested (2 and 4 stink bugs/plant), no significant differences were observed among the resistant Block cultivars and the susceptible cultivar (2 insects/plant: $F_{3,32} = 0.86$, $P = 0.47$; 4 insects/plant: $F_{3,32} = 0.75$, $P = 0.53$). With 2 stink bugs/plant, percentage of damaged seed varied from ca. 14 to 20%; with 4 stink bugs/plant these values almost doubled (range ca. 24 to 31%). Comparisons between infestation levels on each cultivar showed that for the majority of cultivars, the percentage of damaged seed was significantly higher with 4 stink bugs/plant ('BRS 1003 IPRO': $t_{calc} = 4.14$, $P < 0.001$; 'BRS 391': $t_{calc} = 2.50$, $P = 0.03$; 'BRS 5601 RR': $t_{calc} = 2.42$, $P = 0.03$), except for 'BRS 543 RR' ($t_{calc} = 1.39$, $P = 0.18$) (Table 1).

Regarding the percentage weight of damaged seeds (in relation to total weight of seeds obtained), similar results were obtained; i.e., no significant differences were observed for either infestation level tested, among the Block cultivars and the susceptible cultivar (2 insects/plant: $F_{3,32} = 0.55$, $P = 0.65$; 4 insects/plant: $F_{3,32} = 0.84$, $P = 0.48$). With 2 stink bugs/plant, percentage weight of damaged seed varied from ca. 14 to 18%; with 4 stink bugs/plant these values substantially increased (range ca. 21 to 28%). Also, the percentage weight of damaged seeds on each cultivar was significantly higher with 4 stink bugs/plant on most cultivars ('BRS 1003 IPRO': $t_{calc} = 3.20$, $P = 0.006$; 'BRS 391': $t_{calc} = 2.53$, $P = 0.03$; 'BRS 5601 RR': $t_{calc} = 2.59$, $P = 0.02$), except for 'BRS 543 RR' ($t_{calc} = 1.37$, $P = 0.19$) (Table 2).

Laboratory Study

Area Damaged/Seed

The mean (± SE) of the total seed surface area (mm^2) damaged by *E. heros* with 2 stink bugs/plant was significantly greater on the susceptible cultivar 'BRS 5601 RR' than on the Block cultivar 'BRS

Table 1. Mean percentage (± SE) of soybean seeds damaged in resistant Block technology cultivars and a susceptible cultivar after exposure to different infestation levels of *Euschistus heros* per potted greenhouse plant during 14 d at R6 stage (full pod-filling)

		Infestation level/plant[a,b]	
Trait	Cultivar	2 stink bugs	4 stink bugs
Resistant Block	'BRS 1003 IPRO'	14.5 ± 2.4 aB (1045)	27.9 ± 2.2 aA (1063)
	'BRS 543 RR'	16.9 ± 3.1 aA (845)	23.8 ± 3.8 aA (813)
	'BRS 391'	20.0 ± 1.7 aB (946)	30.4 ± 3.8 aA (874)
Susceptible	'BRS 5601 RR'	17.7 ± 2.5 aB (943)	31.1 ± 4.9 aA (911)

[a]Percentage values followed by the same lowercase letter within a column (among cultivars within each infestation level), and values followed by the same uppercase letter within a row (within each cultivar between infestation levels) do not differ significantly using the Tukey test and Student's *t*-test ($P < 0.05$), respectively.

[b]Total number of seeds used for each percentage calculated in parentheses.

1003 IPRO' ($F_{3,116}$ = 4.12, P = 0.008); however, relative to the other two Block cultivars, 'BRS 543 RR' and 'BRS 391', the total seed area damaged only tended to be numerically greater on 'BRS 5601 RR' (Fig. 2A). However, with the higher infestation level (4 stink bugs/plant) the area damaged on the susceptible cultivar was significantly higher compared with those on all Block cultivars ($F_{3,116}$ = 11.07, P < 0.001) (Fig. 2A). Comparisons performed between infestation levels within each cultivar showed that only on 'BRS 5601 RR' was the damaged area significantly higher with 4 stink bugs/plant (t_{calc} = 3.70, P < 0.001); whereas, on Block cultivars, no significant differences were observed within cultivars ('BRS 1003 IPRO': t_{calc} = 1.83, P = 0.07; 'BRS 391': t_{calc} = 1.46, P = 0.15; 'BRS 543 RR': t_{calc} = 1.69, P = 0.10) (Fig. 2A).

Analyzing each seed surface separately, we observed that with 2 stink bugs/plant the seed damaged (mm^2) on the internal surface, was significantly greater on the susceptible cultivar 'BRS 5601 RR' than on the Block cultivar 'BRS 1003 IPRO' ($F_{3,116}$ = 5.01, P = 0.003); the other two Block cultivars 'BRS 543 RR' and 'BRS 391' were intermediate (Fig. 3A) and numerically lower than 'BRS 5601 RR'. Regarding damage on the external surface, no significant differences were obtained ($F_{3,116}$ = 0.99, P = 0.40) among soybean cultivars. In contrast, for the higher infestation level (4 stink bugs/plant), differences among cultivars were highlighted. On external surface, the damage area was significantly higher on the susceptible cultivar 'BRS 5601 RR' than on all Block cultivars tested; for internal surface, damage was numerically lower on the Block cultivars, but only BRS 1003 IPRO and BRS 543 RR differed significantly from the susceptible cultivar (external: $F_{3,116}$ = 10.11, P < 0.001; internal: $F_{3,116}$ = 5.47, P = 0.002) (Figs. 3B and 4).

Percentage Area Damaged/Seed

The mean total percentage (± SE) of the seed area damaged by *E. heros* on soybean Block cultivars ('BRS 1003 IPRO', 'BRS 543 RR', and 'BRS 391') and on a susceptible cultivar ('BRS 5601 RR') during 14 d with 2 and 4 stink bugs/plant followed, in general, the results for the total area (mm^2) damaged/seed (2 insects/plant: $F_{3,116}$ = 5.74, P = 0.001; 4 insects/plant: $F_{3,116}$ = 16.16, P < 0.001). The differences between the Block cultivars and the susceptible cultivar were not very pronounced with 2 stink bugs/plant. However, at the higher infestation level (4 stink bugs/plant), differences between the susceptible and Block cultivars were very conspicuous, with over two-fold more area damaged on the former compared with the latter (resistant) cultivars (Fig. 2B). Again, only 'BRS 5601 RR' presented significantly more total damaged area with 4 stink bugs/plant (t_{calc} = 4.06, P < 0.001) compared to 2 stink bugs/plant. Block cultivars did not show significant differences between the infestation levels ('BRS 1003 IPRO': t_{calc} = 1.47, P = 0.15; 'BRS 391': t_{calc} = 0.32, P = 0.75; 'BRS 543 RR': t_{calc} = 1.64, P = 0.11) (Fig. 2B).

Results obtained on each seed surface separately were similar to those obtained for seed area damaged (mm^2) at both infestation levels. With 2 stink bugs/plant, the percentage of seed damaged on the internal surface was significantly higher on the susceptible cultivar 'BRS 5601 RR' than on the Block cultivars 'BRS 1003 IPRO' and 'BRS 543 RR' ($F_{3,116}$ = 6.08, P < 0.001); the Block cultivar 'BRS 391' was intermediate (Fig. 3C). Again, damage on the external surface did not shows significant differences among cultivars ($F_{3,116}$ = 2.12, P = 0.10). On the other hand, with 4 stink bugs/plant, differences among cultivars were strongly expressed on both external and internal surfaces. The percentage of damage was significantly higher (3-4 times more area damaged) on the susceptible cultivar 'BRS 5601 RR' than on all Block cultivars tested (external: $F_{3,116}$ = 14.12, P < 0.001; internal: $F_{3,116}$ = 7.91, P < 0.001) (Figs. 3D and 4).

Table 2. Mean percentage (± SE) weight (mg) of soybean seeds damaged in resistant Block technology cultivars and a susceptible cultivar after exposure to different infestation levels of *Euschistus heros* per potted greenhouse plant during 14 d at R6 stage (full pod-filling)

		Infestation level/plant[a,b]	
Trait	Cultivar	2 stink bugs	4 stink bugs
Resistant Block	'BRS 1003 IPRO'	13.8 ± 3.29 aB (151)	25.9 ± 1.82 aA (295)
	'BRS 543 RR'	14.7 ± 2.95 aA (141)	21.2 ± 3.65 aA (189)
	'BRS 391'	18.2 ± 1.59 aB (190)	28.0 ± 3.51 aA (264)
Susceptible	'BRS 5601 RR'	14.6 ± 2.49 aB (167)	28.5 ± 4.90 aA (289)

[a]Percentage values followed by the same lowercase letter within a column (among cultivars within each infestation level), and values followed by the same uppercase letter within a row (within each cultivar between infestation levels) do not differ significantly using the Tukey test and Student's t-test (P < 0.05), respectively.

[b]Number of damaged seeds used for each percentage calculated in parentheses.

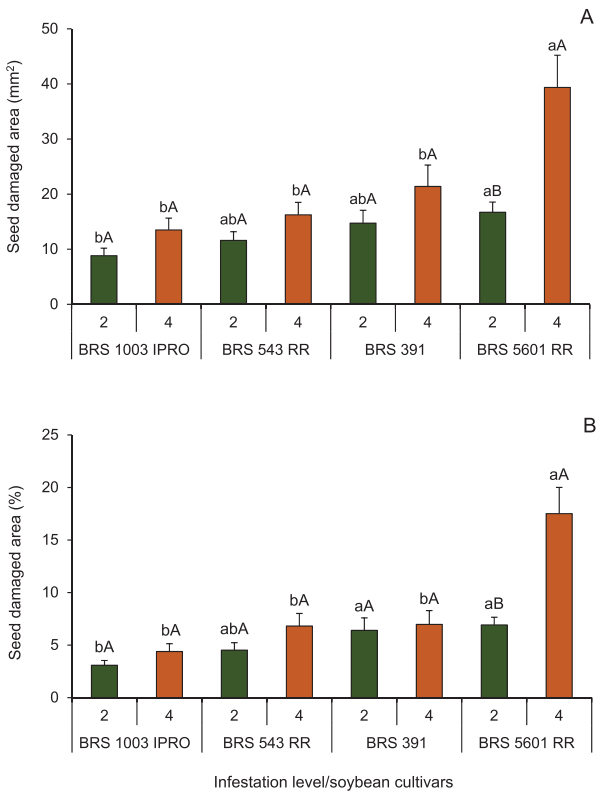

Fig. 2. Total seed area damaged by *Euschistus heros* on soybean Block technology cultivars ('BRS 1003 IPRO', 'BRS 543 RR', and 'BRS 391') and on a susceptible cultivar ('BRS 5601 RR') during a 14 d feeding period with 2 and 4 stink bugs/plant. Mean (± SE) damaged area of the seed (mm^2; n = 30 seeds per infestation level/cultivar) (A); mean (± SE) percentage of seed damage area (B). Columns with the same lowercase letter (among cultivars within each infestation level) and with the same uppercase letter (within each cultivar between infestation levels) do not differ significantly using the Tukey test and Student's t-test (P < 0.05), respectively. Original data presented [for analysis and means separation, seed damage area (mm^2) and percentage of damage area were transformed to √(x) and arcsine √(x/100), respectively] (See online version for color figure).

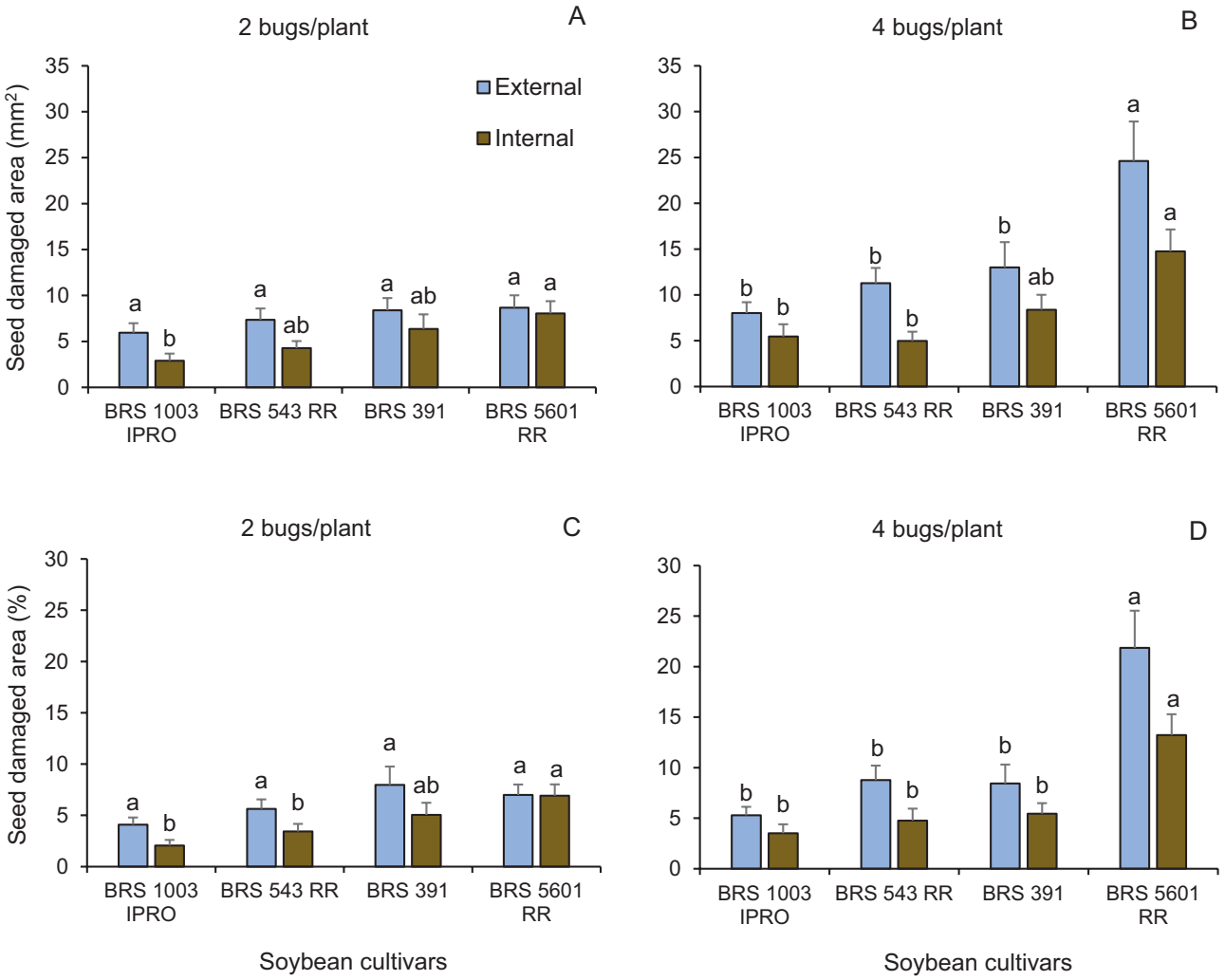

Fig 3. Seed area damaged by *Euschistus heros* on external and internal seed surfaces of soybean Block technology cultivars ('BRS 1003 IPRO', 'BRS 543 RR', and 'BRS 391') and on a susceptible cultivar ('BRS 5601 RR') during a 14 d feeding period with 2 and 4 stink bugs/plant. Mean (± SE) damage area of the seed (mm^2; $n = 30$ seeds) with 2 stink bugs/plant (A) and 4 stink bugs/plant (B). Mean (± SE) percentage of seed damage area with 2 stink bugs/plant (C) and 4 stink bugs/plant (D). Columns with the same letter (among cultivars within each seed surface) do not differ significantly using the Tukey test ($P < 0.05$). Original data presented [for analysis and means separation, seed damage area (mm^2) and percentage of damage area were transformed to $\sqrt{(x)}$ and arcsine $\sqrt{(x/100)}$, respectively] (See online version for color figure).

Electropenetrography (EPG)

The percentage of probes exhibiting waveform Eh3 was similar among the soybean cultivars tested, varying from ca. 58 to 61% (Fig. 5A). The number of events of the waveform Eh3 per insect (NWEI) did not show significant differences among cultivars ($F_{3,20} = 2.07$, $P = 0.1361$), although, the number of Eh3 events was numerically higher on 'BRS 1003 IPRO' than all other cultivars (Fig. 5B). Alternatively, the duration of each Eh3 event per insect (WDEI) was significantly shorter for all Block cultivars compared with the susceptible cultivar ($F_{3,20} = 7.90$, $P = 0.0011$). The cultivar 'BRS 1003 IPRO' showed the shortest event duration (ca. 7 min./event), followed by 'BRS 391' and 'BRS 543 RR' with ca. 16 and 30 min./event, respectively. The duration on the susceptible 'BRS 5601 RR' was over twice as long than that observed for any of the Block cultivars (Fig. 5C). This wide difference in the event duration directly impacted the total duration of the seed activities per insect (WDI). All Block cultivars presented significantly ($F_{3,20} = 5.09$, $P = 0.0089$) shorter total Eh3 duration (< 60 min./insect) compared with the susceptible cultivar (ca. 190 min.) (Fig. 5D).

Discussion

Our greenhouse and laboratory results demonstrate that the Neotropical brown stink bug, *E. heros*, feeds on and explores both soybean resistant Block cultivars and a susceptible cultivar as food sources. The number and weight percentages of damaged seeds on these cultivars did not differ at either level of stink bug infestation. Electropenetrography (EPG) studies conducted in the laboratory confirmed the greenhouse test results. The EPG results revealed the lack of differences in the total percentage of probes containing feeding activities on seeds (i.e., Eh3 waveform), and the number of feeding events on seeds performed per insect. Thus, in general, feeding activity occurs on both types of cultivars and feeding occurs with similar frequency.

The seed area damaged and its percentage related to the total seed surface observed reveal contrasting differences between the soybean resistant Block cultivars and the susceptible cultivar, mostly under the high infestation level. Results on the total percent area damaged/seed, compared to those on the area damaged/seed, better demonstrate that the resistant cultivars show a greater total reduced percentage damaged area (< 7%) compared to the susceptible cultivar (ca. 18% of

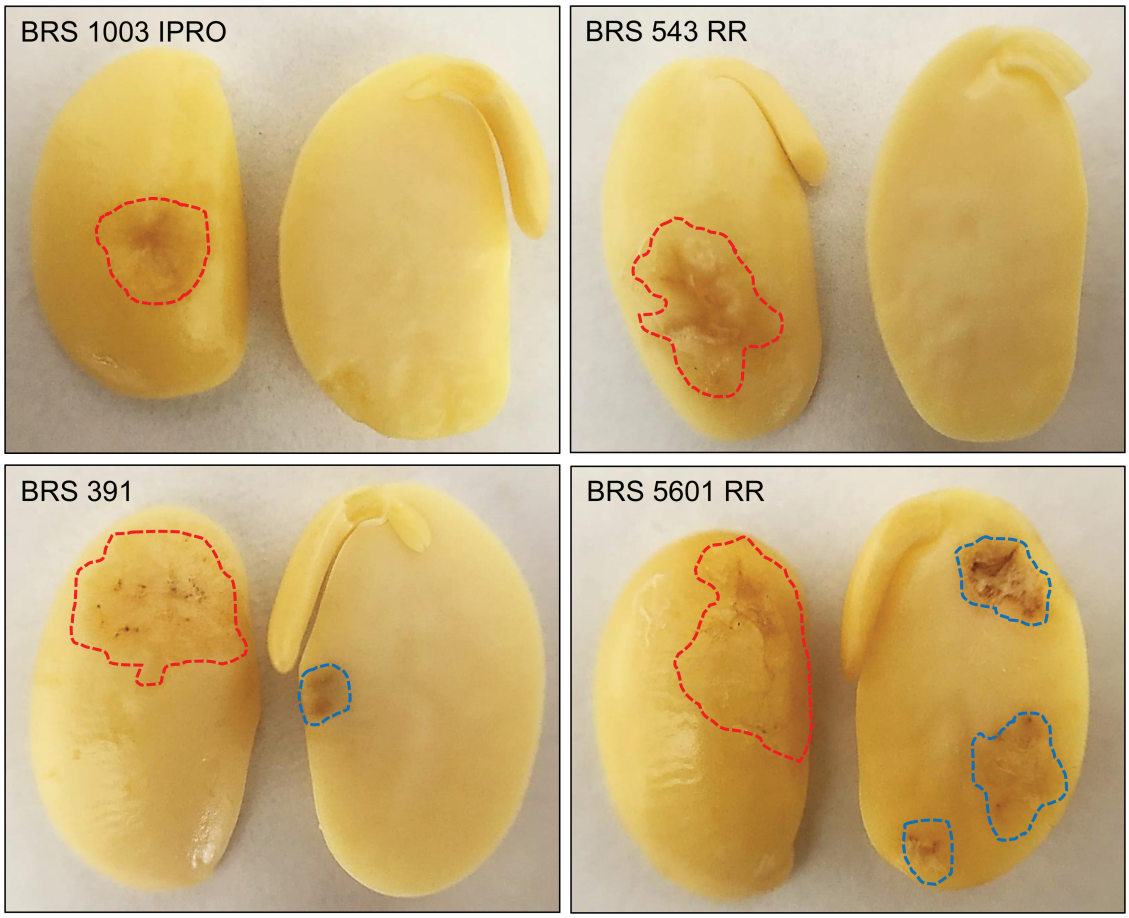

Fig. 4. Damage caused by *Euschistus heros* on soybean seeds of resistant Block technology cultivars ('BRS 1003 IPRO', 'BRS 543 RR', and 'BRS 391') compared with a susceptible cultivar ('BRS 5601 RR') under high infestation level (4 stink bugs/plant). Note the contrasting difference between resistant and susceptible cultivars with the damaged areas circled with red dashed lines (external surface) and blue dashed lines (internal surface of the cotyledons) (See online version for color figure).

the seed area surface). These differences between the two methods of assessing damage (area and %) occurred because the seeds of some cultivars are smaller, so the damage proportion is variable.

It is interesting to note that a relatively great amount of the seeds (selected to quantify the damage—ca. 37%) of the cultivar 'BRS 1003 IPRO' did not show visual damage in the internal surface of any cotyledon analyzed, i.e., the damage was restricted to the external surface of the seed. This also occurred to a lesser extent for the remaining two Block cultivars. Under low infestation level, there were no significant differences in external damage but only internal damage, likely due to the shorter probes and thus failure of stylets to reach that tissue. Moreover, the possible action of tissue destructive enzymes of the saliva (chemical damage) was inhibited deep inside the seeds in particular in the Block cultivars, which may explain the greater damage external than internally on the seeds.

This discrepancy between resistant and susceptible cultivars can also be explained by the results obtained with the EPG studies that indicated much shorter duration of each feeding event on seed reserve tissue, as well as the briefer total duration of feeding performed per insect on seeds of the Block cultivars compared with the susceptible cultivar.

Lucini et al. (2021) demonstrated that the most relevant differences in the feeding behavior of *E. heros* adults on Block cultivars was observed when analyzing the feeding activities on seeds. In our study, the number of times that bugs reached the seed was numerically similar among soybean cultivars; however, insects dedicated much less time to feeding on seeds of Block cultivars compared to the susceptible one. On resistant cultivars, the feeding event duration and the overall duration on seeds was over 3 times shorter compared to the susceptible cultivar. The EPG results coupled with biological studies suggest that the resistant cultivars have no effect on *E. heros* nymphal and adult biology (i.e., no antibiosis); however, these cultivars showed an antixenotic effect with lower preference for feeding (i.e., feeding deterrence), primarily on seed reserve tissue.

To elucidate which factors could explain the results obtained on the contrasting differences in seed damaged areas between the Block resistant cultivars versus the susceptible cultivar, we may consider various physical and chemical factors expected to occur in the pod walls and seeds of soybean. Physical factors such as hardness of the pod wall and seed coat could create difficulty for the bugs to introduce their stylets, decreasing their feeding in Block cultivars compared to the susceptible cultivar. However, EPG data from a previous study (Lucini et al. 2021) suggest that bugs did not encounter such a physical barrier. In fact, a waveform (Eh1c) has been directly related to the presence of a physical barrier in the wall of soybean pods (sclerenchyma cell layer) that a stink bug must overcome to reach the seed (see Lucini and Panizzi 2018 for further details). However, no significant differences in frequency or duration of this waveform were observed among resistant and susceptible cultivars; actually, frequency and durations were numerically

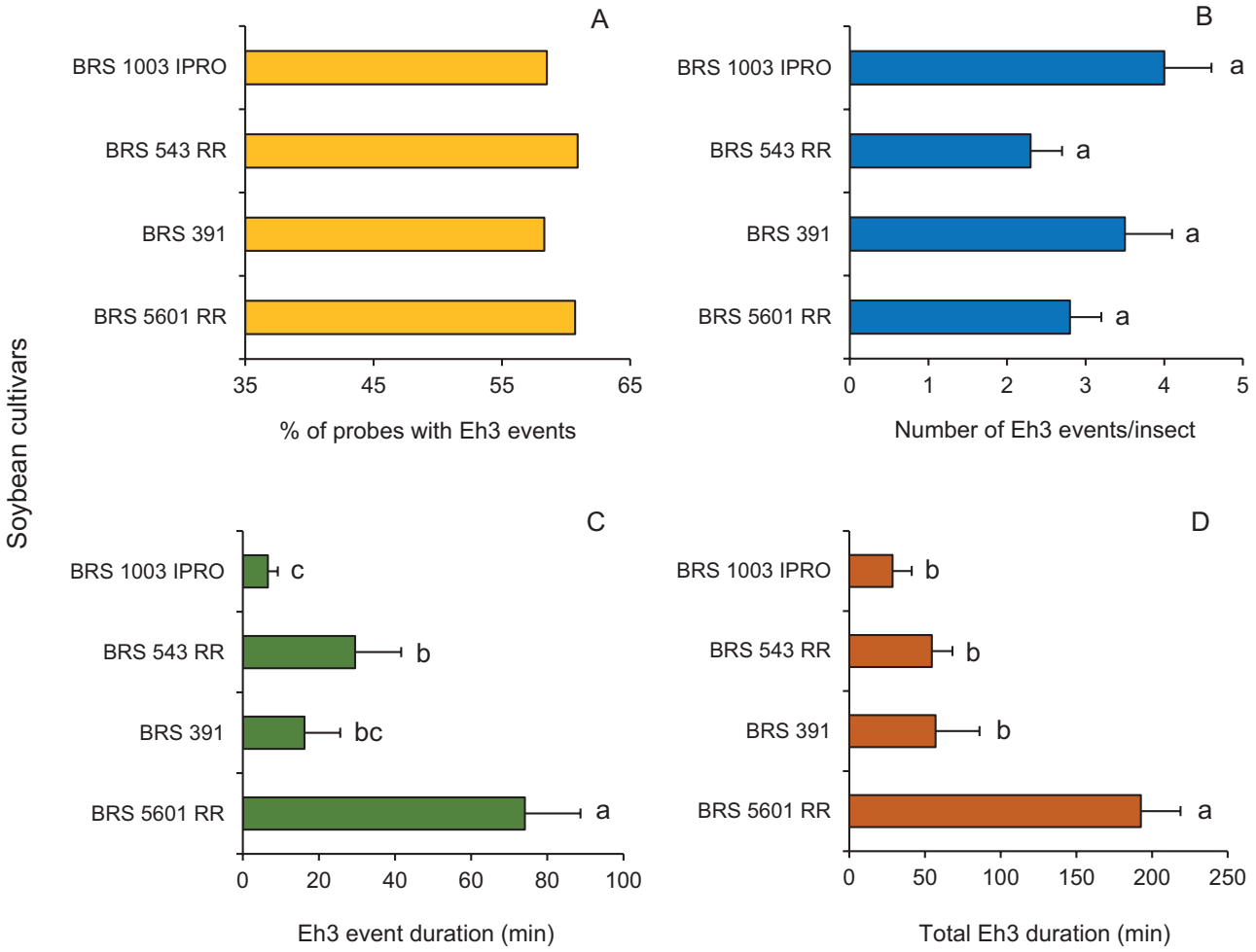

Fig. 5. Nonsequential EPG variables of the seed activities (waveform Eh3) performed by *Euschistus heros* on soybean Block technology cultivars ('BRS 1003 IPRO', 'BRS 543 RR', and 'BRS 391') and a susceptible cultivar ('BRS 5601 RR') (n = 6 insects per cultivar). Percentage of probes containing waveform Eh3 (A); number of Eh3 events per insect (B); duration (min.) of each Eh3 event performed per insect (WDEI) (C); total duration (min.) of Eh3 waveform performed per insect (WDI) (D). Means followed by the same letter, for each EPG variable, are not significantly different at $\alpha = 0.05$ (LSD means test). Original raw data presented [for analysis, counts and durations were transformed in √(x) and log(x), respectively] (See online version for color figure).

lower on Block cultivars (Lucini et al. 2021). Therefore, a physical barrier would not seem to be the reason for the differences in observed seed damage. Other physical traits, such as hairiness on the pods, may present difficulties for stylet penetration but can likely be discounted since the Block cultivars and on the susceptible cultivar possess similar hair densities.

Regarding chemical factors, multiple substances can play an important role in plant defenses against attack by insect pests (War et al. 2012). These chemicals include a complex of secondary substances, such as terpenoids, alkaloids, and some proteins; the latter include chitinase, lectins, proteinase inhibitors, and so on. These substances can occur constitutively in the plants or might be promptly synthetized and released in response to pest attack, i.e., induced defensive responses (see Habib and Fazili 2007 for further details). For example, the feeding activities of *Lygus lineolaris* (Palisot de Beauvois) on pin-head size cotton squares (determined using the EPG) triggered a release of tannins, where tannin production and release were positively correlated with the number of feeding events performed by *L. lineolaris*. However, the feeding activities of the insects were not deterred in response to tannin production (Cervantes et al. 2017).

Soybean seeds are known to contain several chemicals (allelochemicals), and some can be potentially toxic to insects or can modify their behavior. For example, isoflavonoid content in soybean seeds seems to influence the preference of *E. heros* to oviposit on pods. Cultivars with higher concentrations of isoflavonoids were less preferred for oviposition compared with those having lower concentrations (Silva et al. 2013). Other allelochemicals such as saponins (Hussain et al. 2019), phytic acid, and protease inhibitors (Galão et al. 2014) are known to be toxic. For example, seeds of soybean present the protease trypsin inhibitor which expresses antinutritional and deleterious effects on the biology of several insects (see Habib and Fazili 2007, and references therein).

Although the chemical profile of allelochemicals present in the seeds of soybean Block cultivars has not been determined, these and other compounds might act on the bugs' feeding behavior (affecting cell laceration) and/or on the digestive enzymes injected (enzymatic inhibitors affecting cell maceration). This likely explains the significant decrease of the seed feeding activities by *E. heros* adults, which culminated in a lower seed damaged area. Clearly, further investigation to screen the chemical compounds present in the seeds/pods of those resistant cultivars is needed to fully explain this 'blocked feeding'.

The current findings allow researchers to conclude that the Block cultivars, by virtue of much-reduced percentage of seed damaged area and reduced feeding event duration and seed activities by stink bugs compared with the susceptible 'BRS 5601 RR', constitute a new important strategy to manage pest stink bugs on the soybean crop.

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