



Sugars and cuticular waxes impact sugarcane aphid (*Melanaphis sacchari*) colonization on different developmental stages of sorghum

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ABSTRACT

Sugarcane aphid (SCA; *Melanaphis sacchari*) is a devastating pest of sorghum (*Sorghum bicolor*) that colonizes sorghum plants at different growth stages. Leaf surface characteristics and sugars often influence aphid settling and feeding on host plants. However, how changes in cuticular waxes and sugar levels affect SCA establishment and feeding at different development stages of sorghum have not been explored. In this study, two- and six-week-old BTx623 plants, a reference line of sorghum, was used to evaluate plant-aphid interactions. Monitoring aphid feeding behavior using Electrical Penetration Graph (EPG) technique revealed that aphids spent more time in the sieve element phase of six-week-old plants compared to two-week-old plants. Significant differences were found in the time spent to reach the first sieve element and pathway phases between the two- and six-week-old plants. However, no-choice aphid bioassays displayed that SCA population numbers were higher in two-week-old plants compared to six-week-old plants. Differences in the abundance of wax and sugar contents were analyzed to determine how these plant components influenced aphid feeding and proliferation. Among the cuticular wax compounds analyzed, α -amyrin and isoarborinone increased after 10 days of aphid infestation only in six-week-old plants. Trehalose content was significantly increased by SCA feeding on two- and six-week-old plants. Furthermore, SCA feeding depressed sucrose content and increased levels of glucose and fructose in two-week-old but not in six-week-old plants. Overall, our study indicates that plant age is a determinant for SCA feeding, and subtle changes in triterpenoids and available sugars influence SCA establishment on sorghum plants.

1. Introduction

In recent years, sugarcane aphid (SCA; *Melanaphis sacchari*) has become one of the most important pests of sorghum (Bowling et al., 2016). Efforts to mitigate their impact on sorghum have increased substantially in the United States. The success of this pest is subject to

various aspects of its biology (Brewer et al., 2017) and ecology (Singh et al., 2004). The rapid growth of SCA populations, especially in warm environments and dry conditions (Neupane et al., 2020), its parthenogenetic reproduction and its effective dispersal strategy have ensured the success of this pest in almost all sorghum growing regions of the U.S. (Brewer et al., 2017). Each of these challenges has become a problem for

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the pest management of SCA in sorghum acreages. Insecticides are the most widely used management strategy to reduce SCA populations in sorghum (Szczepaniec 2018a). However, these strategies have become unfeasible when the number of aphids on plants increases drastically. Additionally, the production of sticky honeydew, the digestive waste products of aphids, highly affects the harvesting process and causes reduction in the crop yield (Szczepaniec 2018a).

Host plant resistance (HPR) is an alternative strategy to control SCA populations in the field (Bowling et al., 2016). HPR can exploit the ecology and biology of the pest leading to the development of control mechanisms that are friendlier to the environment and more effective against the pest. One of the biggest challenges with SCA management is its reproductive ability and high dispersion. It can colonize sorghum in vegetative stages in the southern U.S. and in the reproductive stages in the northern U.S. in the same year (Kiani and Szczepaniec, 2018). Thus, it is highly critical to understand the timing of SCA colonization for optimal pest management (Szczepaniec 2018a). Previously, it was shown that the developmental stages of sorghum plants impact SCA population sizes and are an important factor for the knowledge of the biology and ecology of the pest (Kiani and Szczepaniec, 2018). SCA populations were almost 2.5 times larger in plants near reproductive stages of about six-week-old plants compared to plants in the vegetative stage of about two-week-old, suggesting that timely management of SCA is needed in the field (Szczepaniec 2018a; Szczepaniec 2018b). However, HPR factors that affect SCA ability to colonize are poorly understood (Kiani & Szczepaniec, 2018).

Electrical Penetration Graph (EPG) technique is used as one of the most powerful tools to monitor insect feeding behavior (Tjallingii, 1988). This technique provides detailed information associated with different feeding activities on the plant, among them, saliva secretion, and sap ingestion (Tjallingii, 2006; Nalam et al., 2019; Zogli et al., 2020). Several studies have shown EPG as a robust biological validation technique for monitoring the feeding behavior associated with plant resistance to piercing-sucking insects. This information is crucial to determine the numerous resistance mechanisms that plants develop under aphid infestation/herbivory (Grover et al., 2019, 2020b, 2022a; Tjallingii, 2006).

Waxes play a fundamental role in several biological, physiological, and morphological processes of plants. The main function of waxes is to prevent water loss. In addition, being the first point of contact of the plant with the environment waxes also contribute to plant defense mechanisms, becoming a factor in determining the resistance or susceptibility of a plant to biotic stressors (Shepherd et al., 1999a). Waxes in plants are formed by two major layers, the epicuticular wax layer and the intracuticular wax layer (Shepherd et al., 1999a). The epicuticular wax layer is the main component of cuticular waxes. Epicuticular waxes are formed by a complex mixture of long chain aliphatic alcohols and acids, n-alkanes, wax esters, as well as low levels of triterpenoids, sterols, flavonoids, and phenolic substances (Wójcicka 2015, Kumar et al., 2017; Griffiths et al., 2000; Busta et al., 2021). Epicuticular waxes are involved in a variety of processes in plants, which includes the production of important secondary metabolites related to plant defenses (Eigenbrode and Espelie, 1995) and plant resistance upon herbivory (Sharma & Dhillon, 2005; Shepherd et al., 1999a; Shepherd et al., 1999b). Previous studies have linked epicuticular waxes with anti-xenotic effects in plants against aphids (Verdugo et al., 2007). Waxes can alter the aphid adherence (Pike et al., 2002; Smith, 1999; Gagic et al., 2016), and aphid movement (Bergman et al., 1991; Dixon et al., 1990). Moreover, Harris-Shultz et al. (2020) and Leszczynski et al. (2004) have found that the total number of aphids were lower on normal plants compared to bloomless plants.

SCA infestation in sorghum in the U.S. can be highly influenced by plant age and development and as mentioned above, is a crucial factor for the survival of this species and its populations in sorghum. Additionally, Busta and co-workers (2021) have recently shown that the composition and coverage of epicuticular waxes in sorghum plants differ

greatly along plant development. Sorghum cuticular waxes also influence the host plant selection by SCA on young (three-leaf stage) sorghum plants (Cardona et al., 2023) (nullM). Elevated levels of 16-monoacylglycerols and 32-C-alcohols in wax components impact the aphid performance and feeding behavior on young sorghum plants, however, it may not be affecting the SCA reproduction, growth, and survival (Cardona et al., 2023) (nullM). However, little is known about how these cuticular waxes influence aphid performance at different sorghum developmental stages.

Plant phloem is a rich source of sugars and other nutrients and is the primary feeding site for aphids. Turgor pressure allows sap to flow passively via the aphid stylets inserted into the phloem, and excess fluids are excreted by the aphids as a sticky substance called honeydew (Louis and Shah, 2013; Nalam et al., 2019). Callose deposition, and changes in saccharide profiles in the phloem stream directly affects the feeding ability of the aphids by restricting flow in sieve elements and/or changing turgor of the phloem sap respectively (Singh et al., 2011; Varsani et al., 2019; Grover et al., 2022a). Plants can also modulate sucrose loading/concentration or increase disaccharides and oligosaccharides to change turgor under aphid pressure to make phloem sap less amenable/palatable to aphids (Louis & Shah, 2013; Nalam et al., 2019; Twayana et al., 2022).

Since pest management alternatives against SCA in sorghum are still limited, knowledge of HPR mechanisms is essential to better understand the performance of SCA in sorghum. In addition, the analysis of factors that can influence insect-plant interactions such as the role of cuticular waxes in these interactions is highly important for implementing sustainable pest management strategies. The goal of this study was to evaluate how callose accumulation, cuticular waxes, and sugars, will impact SCA feeding behavior and colonization on different developmental stages of sorghum. Thus, in this study, using EPG technique we monitored SCA feeding on two- and six-week-old sorghum BTx623 plants, which were further complemented by no-choice bioassays, monitoring the expression of the callose synthase-encoding genes, chemical composition of cuticular waxes, and levels of select sugars in SCA infested plants.

2. Materials and methods

2.1. Plant material

The sorghum (*Sorghum bicolor*) BTx623 genotype used in this study is considered as the reference line for sorghum with fully sequenced genome (Paterson et al., 2009). Two- and six-week-old sorghum plants were grown in the University of Nebraska-Lincoln (UNL) greenhouse with a 16-h-light/8-h-dark photoperiod, 25 °C, and 50–60% relative humidity. Seeds were sown in soil mixed with vermiculite and perlite (PRO-MIX BXBIOFUNGICIDE + MYCORRHIZAE, Premier Tech Horticulture Ltd., Canada) in Cone-Tainers (Ray Leach SC10; Stuewe & Sons, Inc., Tangent, OR). Experiments with two- and six-week-old plants were initiated at the same time when plants reached the appropriate age.

2.2. Insect colony

The SCA were reared as previously described (Grover et al., 2020a) and were maintained on BCK60 sorghum plants in a growth chamber with 16-h-light/8-h-dark photoperiod, 140 $\mu\text{E m}^{-2} \text{ s}^{-1}$ light quality, 23 °C, and 50–60% relative humidity. BCK60 sorghum plants needed for aphid rearing were grown to the 7-leaf stage in a greenhouse with new plants substituted for old, deteriorated plants in growth chamber, whenever necessary. Fine-bristled paintbrush was used to transfer the adult apterous aphids to experimental plants.

2.3. Aphid no-choice bioassay

No-choice assay was conducted with SCA using two- and six-week-

old potted BTx623 plants. A Completely Randomized Design was performed to determine the aphid growth and survival on both developmental stages. For this experiment, sixteen (16) two-week-old and six-week-old plants were randomly organized and infested with five adult apterous aphids. Adults were placed in the adaxial side of the leaves in two-week-old plants and at the top of the leaf whorl in six-week-old plants. Following infestation, two-week-old plants were caged with tubular clear plastic ventilated with organdy fabric on the top and sides of six-week-old plants were caged using cloth bags. Wooden stakes were used as plant supports. The total number of aphids including adults and nymphs were counted after 10 days of infestation on each developmental stage.

2.4. Aphid feeding behavior recording and analysis

Two- and six-week-old BTx623 plants were used for monitoring SCA feeding behavior. Adult apterous SCA was individually placed on each plant at the center of the adaxial leaf lamina. Aphid wiring and EPG experimental procedures were performed as described previously (Tetreault et al., 2019). Briefly, aphids were starved for 1 h in a plastic petri dish prior to EPG recording. Using a stereoscope, a brass nail with a gold wire attached (insect electrode) was glued to the dorsum of aphids using a silver conductive glue obtained by mixing 4 mL water with a single drop of Triton X-100, 4 mg water-soluble glue (Scotch clear paper glue, non-toxic; 3 M, St. Paul, MN, USA), and 4 g silver flake (99.95%, size, 8–10 μ m, Inframet Advanced Materials, Manchester, CT, USA). To complete the basic circuit, a plant electrode (stiff copper wire) was introduced into the soil surrounding the potted plant. For measurements, a Giga-8 EPG (EPG Systems, Wageningen, The Netherlands) with a $10^9 \Omega$ resistance amplifier was connected to each of the electrodes and an adjustable plant voltage were used for measuring feeding behavior of SCA on sorghum plants. Experiments for EPG were conducted at laboratory conditions at 22–24 °C and 40–45% relative humidity under continuous light. Four plants with same age were placed at a time randomly in a Faraday's cage for the recordings. The EPG recordings were started between 8 am and 10 am local time (U.S. Central Standard Time). Overall, 14 replications were used for each developmental stage of recordings for 8 h. EPG acquisition software (Stylet+, EPG Systems, Wageningen, The Netherlands) was used to record EPG waveforms. Different categorized EPG waveform phases/patterns considered in this study include, pathway phase, which corresponds to the penetration and removal of aphid stylets intercellularly; xylem phase represents water ingestion; sieve element phase (phloem phase) indicates ingestion of phloem sap (that includes the aphid salivation (E1) and passive ingestion (E2), respectively, were also monitored) (Grover et al., 2022a). The non-probing phase shows the relative interval of no/limited stylet movement. For more detailed results, additional parameters were measured: the number of potential drops that correspond to intracellular punctures. Another parameter measured was the time to first probe, which is the time difference between the starting of recording and the first style insertion into plant, and finally, the time to first sieve elements once the recording was initiated.

2.5. RNA extraction and RT-qPCR analysis

Two- or six-week-old sorghum BTx623 leaf tissues (~100 mg) were used for RNA extraction. Total RNA extraction from sorghum leaves and subsequent RT-qPCR were performed as described previously (Grover et al., 2022b). The gene-specific primers used in this study are listed in [Supplemental Table S1](#).

2.6. Wax composition analysis

For wax composition analysis, plants were infested with aphids as described for the no-choice assays and leaf tissues were collected after 10 days of SCA feeding. SCA uninfested plants were used as the control

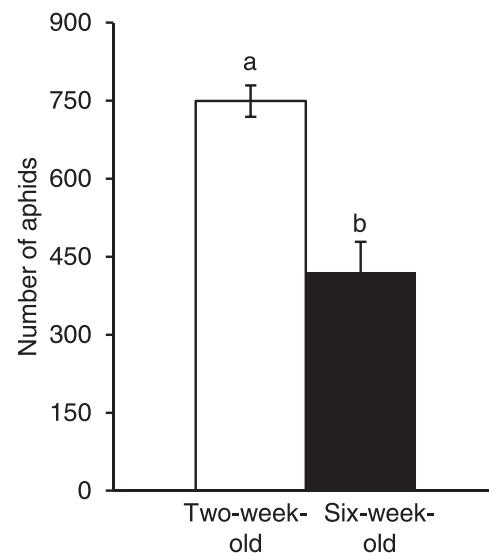


Fig. 1. Sugarcane aphids (SCA) colonize better on two-week-old BTx623 sorghum plants. Total number of SCA adults and nymphs per plant after 10 days of infestation in two-week-old and six-week-old BTx623 plants ($n = 16$). Error bars represent \pm SEM. Different letters above the bars represent significant differences from each other ($P < 0.05$). Aphid no-choice experiments were conducted twice with similar results.

plants for both developmental stages (two-week-old and six-week-old). For leaf sample collection, the second most developed leaf of the plant at the end of the experimental period (10 days) was carefully placed in a hole puncher of approximately 3 cm^2 in area, without manipulating or contaminating the collection area. Once the lamina was located in the hole puncher one leaf disc was punched out. A total of three leaf discs were considered a replication in each of the treatments. The leaf discs were placed directly into a vial of polypropylene cap and polyethylene liner (20 mL 28 \times 61 mm (with Cap) (Busta et al., 2021) and capped for storage. The protocol for the analysis of the epicuticular waxes present in each of the samples in the four different treatments via gas chromatography-mass spectrometry (GC-MS) was as described previously (Busta et al., 2021).

2.7. Sugar analysis

Quadruplicate sorghum leaf samples from each condition (100 mg fresh weight) were extracted with 1 mL of 80% ethanol:20% 18 M Ω water for 1 h at room temperature with shaking. After centrifugation, a 20 μ L aliquot of each extraction supernatant was dried in vacuo and samples were resuspended in 1 mL 18 M Ω water for analysis. Quantitative analysis of the extractable soluble oligosaccharide products (i.e. fructose, glucose, sucrose, raffinose, stachyose, and trehalose) from sorghum leaves was performed by HPAEC-PAD (high-performance anion-exchange chromatography-pulsed amperometric detection) due to its superior sensitivity, speed, and separation chemistry for soluble oligosaccharide components (Giannoccaro et al., 2008). Prior to injection, samples were maintained at 4 °C. Five μ L of each sample was injected on a Thermo Scientific ICS 5000 HPAEC-PAD (Thermo Scientific, Sunnyvale, CA) utilizing a PA-10 column (2 mm \times 250 mm, Thermo Scientific, Sunnyvale, CA) for separation using an isocratic 90 mM sodium hydroxide eluent at a flow rate of 250 μ L/min. Analytes (glucose, fructose, sucrose, raffinose, stachyose, and trehalose) were identified and quantified based on a 4-point standard curve generated from authentic standards run several times during course of analysis.

2.8. Statistical analyses

EPG data was analyzed using a non-parametric Kruskal-Wallis test in

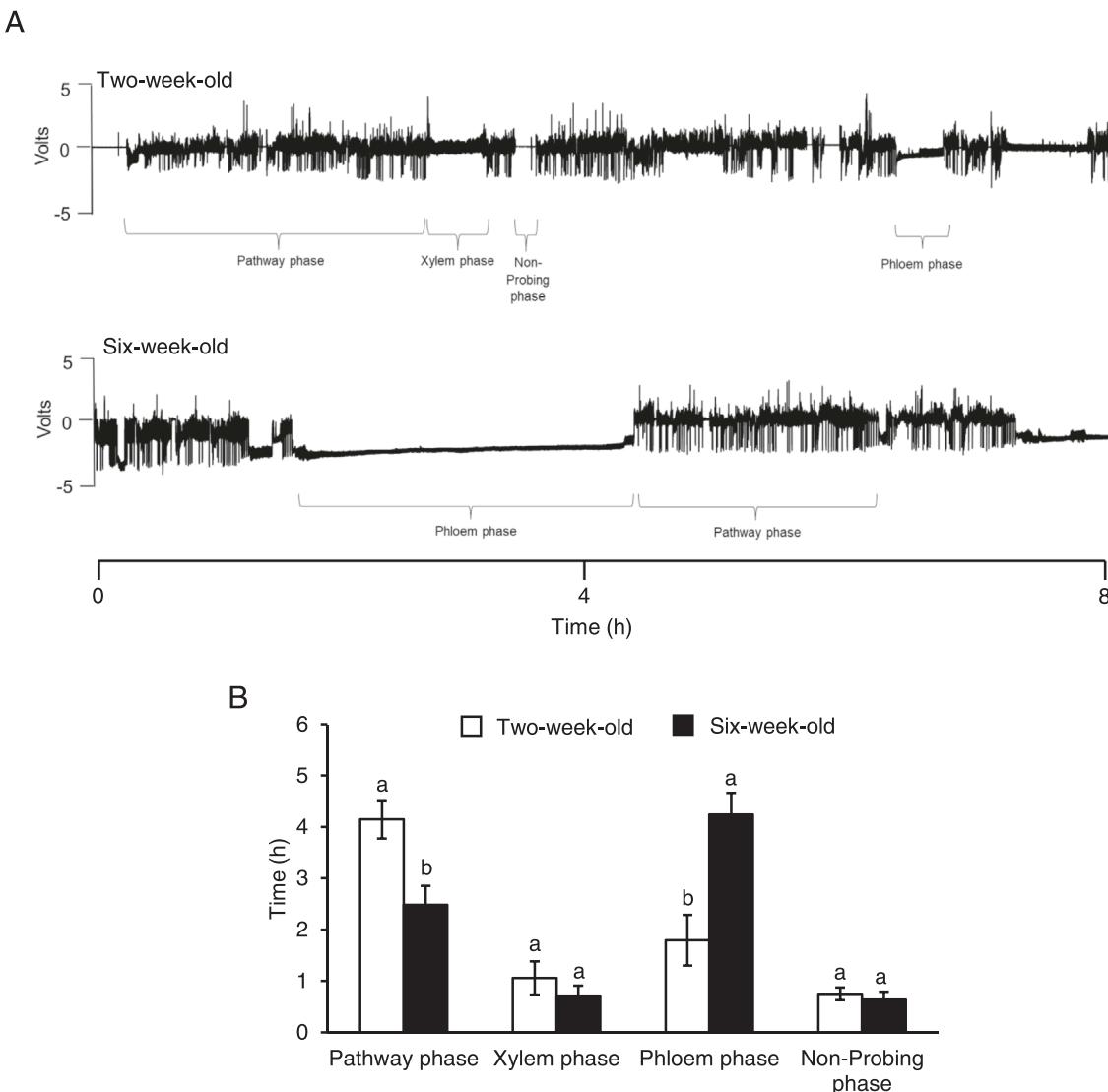


Fig. 2. Sugarcane aphids (SCA) fed more from the phloem sap of six-week-old BTx623 sorghum plants (A) Representative Electrical Penetration Graph (EPG) waveforms representing the SCA aphid feeding behavior in two-week-old (top panel) and six-week-old (bottom panel) sorghum BTx623 plants for 8 h. (B) SCA feeding behavior parameters in two- and six-week-old sorghum BTx623 plants for 8 h of feeding duration. The total time spent by SCA for different feeding behavior parameters in each of the developmental stages is shown ($n = 14$). Bars denote the mean values obtained for two-week-old and six-week-old sorghum BTx623 plants. Bars with different letters represent significant differences from each other ($P < 0.05$; Kruskal-Wallis test). Error bars represent \pm SEM.

nine different feeding phases/patterns for each developmental stage. Considering the non-normality distribution of the data, the PROC NPAR1WAY procedure was used. Multiple comparisons of different treatments between the means were performed using SAS. For the no-choice assay, RT-qPCR, sugar and wax composition data analysis, multiple comparisons were carried out using Tukey's honestly significant difference test. Values presented are least square means and standard error.

3. Results

3.1. SCA survival and reproduction was higher on two-week-old plants

SCA survival and reproduction was higher on two-week-old plants as compared to six-week-old plants 10 days post infestation (dpi) (Fig. 1). SCA population was almost twice the size (average mean population = 749) on two-week-old plants as compared to six-week-old plants (average mean population = 421.3) (Fig. 1).

3.2. SCA spent more time in phloem feeding on six-week-old plants

Representative EPG waveforms of SCA feeding on two-week-old and six-week-old plants are shown in Fig. 2A. These EPG data demonstrated that the aphids spent longer time in the sieve elements phase on six-week-old plants compared to two-week-old plants (Fig. 2B). On the other hand, aphids spent less time in the pathway phase on six-week-old plants compared to two-week-old plants. No significant differences were found in the xylem and non-probing phases between the two different plant developmental stages (Fig. 2B).

3.3. SCA took less time in reaching phloem phase on six-week-old plants

Aphids started feeding faster on six-week-old plants compared to two-week-old plants, suggesting that the SCA preferred to feed on sorghum plants at a more advanced stage of development compared to young sorghum plants (Fig. 3A). No significant differences were found in the time to first probe (Fig. 3A) and number of potential drops (Fig. 3B) between both developmental stages. Aphids started probing on six-

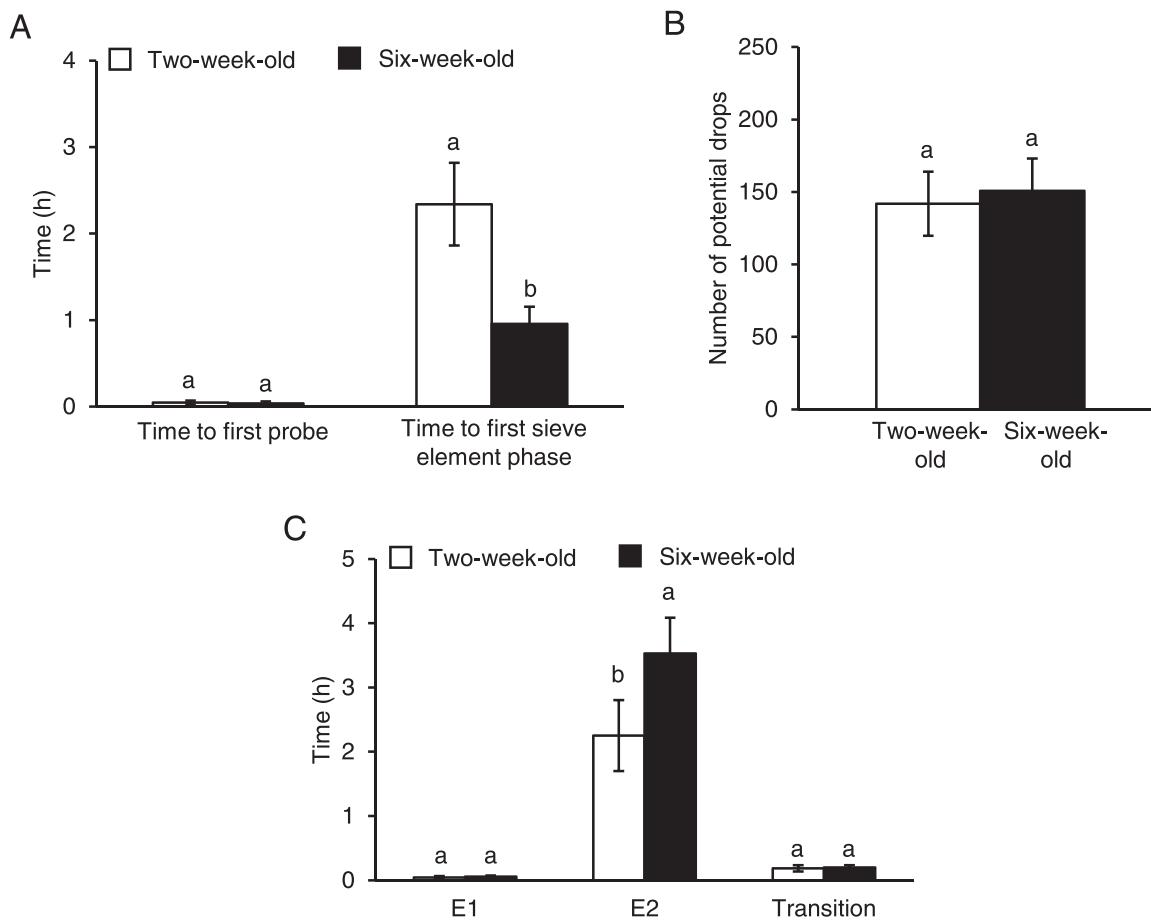


Fig. 3. Sugarcane aphid (SCA) took less time in reaching the phloem phase and more time in passive ingestion of phloem sap on six-week-old BTx623 sorghum plants (A) Time taken by SCA to first probe and time to first sieve element in two-week-old and six-week-old sorghum BTx623 plants; (B) Mean number of potential drops in two-week-old and six-week-old sorghum BTx623 plants in 8 h duration of SCA feeding. (C) Salivary (E1), passive ingestion (E2) and transition phases in six-week-old and two-week-old sorghum BTx623 plants in 8 h duration of SCA feeding. For experiments, A–C, n = 14. Bars denote the mean values obtained for two-week-old and six-week-old sorghum BTx623 plants. Bars with different letters represent significant differences from each other ($P < 0.05$; Kruskal-Wallis test). Error bars represent \pm SEM.

week-old and two-week-old sorghum BTx623 plants rapidly at the same time, and SCA made comparable number of probing attempts on both developmental stages of sorghum.

3.4. SCA spent more time in passive ingestion of phloem sap on six-week-old sorghum plants

Considering the differences in the phloem phase between both developmental stages, the phloem phase was analyzed in detail. This analysis was performed for the E1 phase or salivary phase, the E2 or passive ingestion phase, and a transition phase, which is an alternance between E1 and E2 phases (Will et al., 2007). Results confirmed that the aphids spent more time feeding in the passive ingestion phase of six-week-old plants (Fig. 3C). Aphids spent ~1 more hour during the passive ingestion in six-week-old plants compared to two-week-old plants. Although there were no significant differences in E1 or salivation phase between both developmental stages, salivation period was longer in six-week-old plants (Fig. 3C). No significant differences were found in the transition phase.

3.5. SCA feeding-induced callose deposition is independent of the sorghum growth stages

Aphid feeding on host plants induce the expression of the callose synthase-encoding genes and callose deposition (Mondal et al., 2018;

Varsani et al., 2019), which acts a defense mechanism to regulate aphid colonization. Here, we analyzed the expression of three putative sorghum callose synthase genes (Tuleski et al., 2020), *callose synthase*, *glucan synthase* and β -1,3 *glucanase*, in two- and six-week-old sorghum plants before and after SCA infestation for 10 days. Although SCA feeding significantly induced the expression of callose synthase gene in both two- and six-week-old plants compared to SCA-uninfested control plants, there was no significant difference between the transcript expression on both sorghum growth stages after SCA infestation for 10 days (Supp. Fig. S1A). Additionally, expression of *glucan synthase* and β -1,3 *glucanase* genes was comparable in two- and six-week-old plants before and after SCA infestation for 10 days (Supp. Fig. S1B and C). Collectively, our results suggest that callose deposition may not act as a major contributor in providing defense against SCA when comparing different developmental stages of sorghum.

Abundance of the triterpenoids α -amyrin and isoarborinone increased after aphid infestation in six-week-old sorghum plants

The relative abundance ($\mu\text{g}/\text{cm}^2$) of each of the wax components present in the leaf sample (3 cm^2) between two- and six-week-old plants before (control) and 10 days after SCA infestation were quantified. In total, 15 compounds were detected from the samples collected for each developmental stage (Fig. 4 and Supp. Fig. S2). On both two-week-old and six-week-old plants, the compounds with the highest abundance

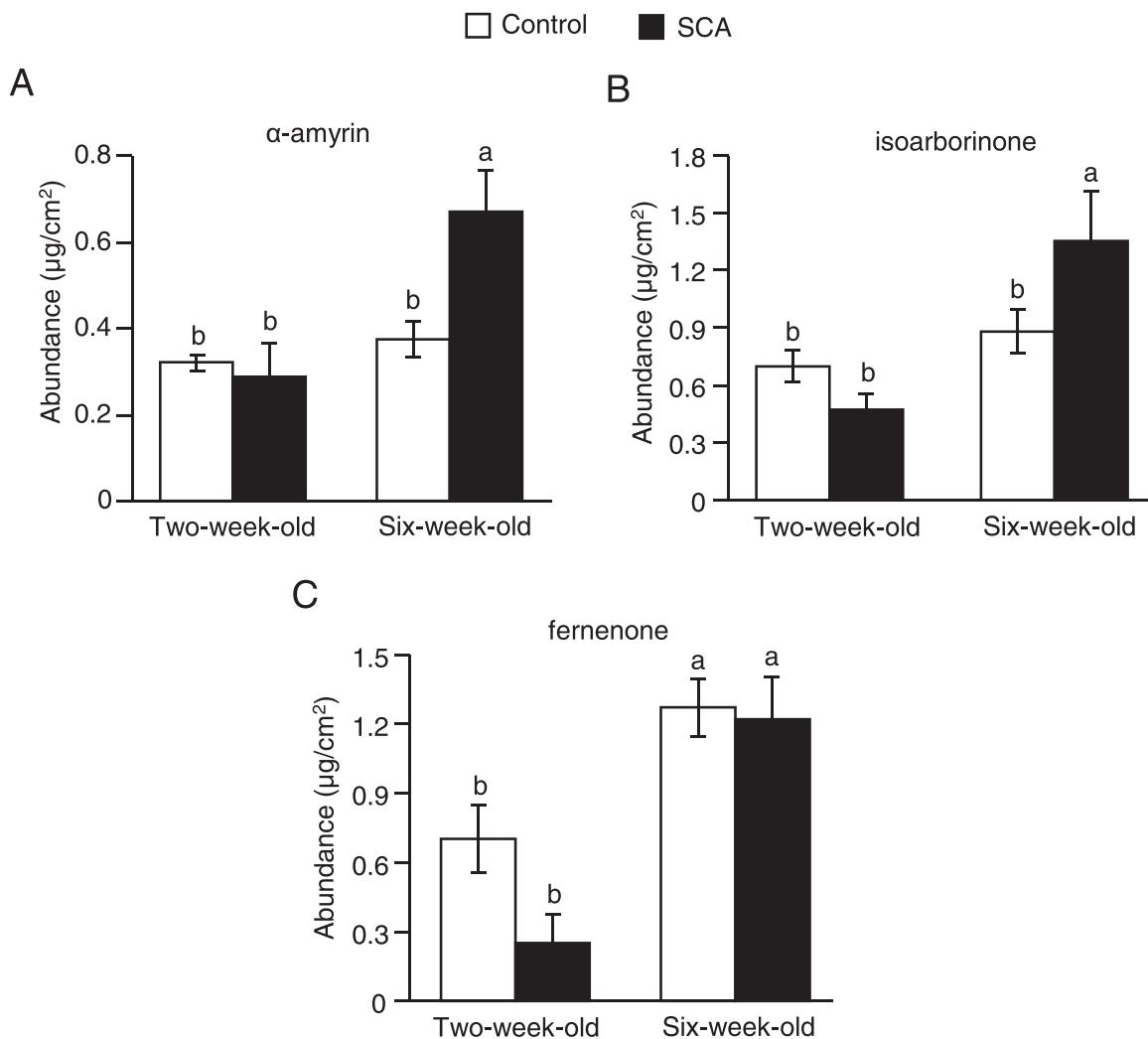


Fig. 4. Sugarcane aphid (SCA) feeding enhanced cuticular wax triterpenoids in six-week-old BTx263 sorghum plants. Abundance ($\mu\text{g}/\text{cm}^2$) of (A) α -amyrin, (B) isoarborinone, and (C) fernenone present in the leaf sample (3 cm^2) between two-week-old and six-week-old plants before (control) and 10 days after SCA infestation ($n = 6$). Bars with different letters represent significant differences from each other ($P < 0.05$). Error bars represent $\pm \text{SEM}$.

were fatty alcohols and triterpenoids. Significant increases in response to SCA infestation were evidenced only in six-week-old plants for two compounds, the triterpenoids α -amyrin and isoarborinone (Fig. 4A and B). We also observed a decrease in fernenone abundance after aphid feeding on the two-week old plants (Fig. 4C). Although significant differences in the direct comparison of controls and SCA infested plants at the same developmental stage were not observed for the other components, nine detected compounds were significantly higher in SCA infested six-week-old plants as compared to the SCA infested two-week-old plants (Supp. Fig. S2). In many of these cases, the cuticular wax components were depressed in the SCA infested two-week-old plants and elevated in the SCA infested six-week-old plants (Supp. Fig. S2).

3.6. Saccharide levels were altered by SCA feeding on sorghum plants

SCA herbivory impacted levels of different sugars (Fig. 5). Trehalose levels were significantly enhanced by SCA infestation on both two- and six-week-old plants (Fig. 5A). For the other sugars measured, sucrose levels were significantly lower, and glucose and fructose levels were significantly greater only in SCA infested two-week-old plants as compared to the uninfested controls (Fig. 5 B-D). Two oligosaccharides of the raffinose family (RFOs) were also quantitated. Raffinose levels were significantly lower in SCA-infested two-week-old sorghum plants, and stachyose levels were significantly lower in SCA infested six-week-

old plants (Fig. 5E and F). These differential changes in sugar levels suggests potential age-based modulation of plant responses to the SCA.

4. Discussion

This study provides detailed information of the SCA feeding behavior on sorghum BTx263 plants at two different developmental stages: at a vegetative stage (two-week-old plants) and a stage approaching the reproductive period (six-week-old plants). Plants may possess epidermal and phloem-based defenses to protect the integrity of sieve tubes and hinder the nutrient accessibility to aphids. EPG data revealed that SCA established a prolonged feeding duration on the phloem sap of the six-week-old plants, and additionally spent reduced time in the pathway phase in search for a possible spot to get the nutrients from the plants. SCA also spent longer time in the passive ingestion phase, a phase in which nutrients are obtained by the SCA from the plants (Fartek et al., 2012). Our results also suggest that SCA were able to locate the necessary nutrients relatively faster in six-week-old plants compared to two-week-old plants, which was not unexpected, given the larger size of the plants where it may be potentially easier to locate phloem. In contrast, results from the no-choice study demonstrated that SCA proliferation were higher on plants during the vegetative stage. This result contrasts with a previous study, where higher number of aphids were associated with the reproductive stages of sorghum plants (Kiani and

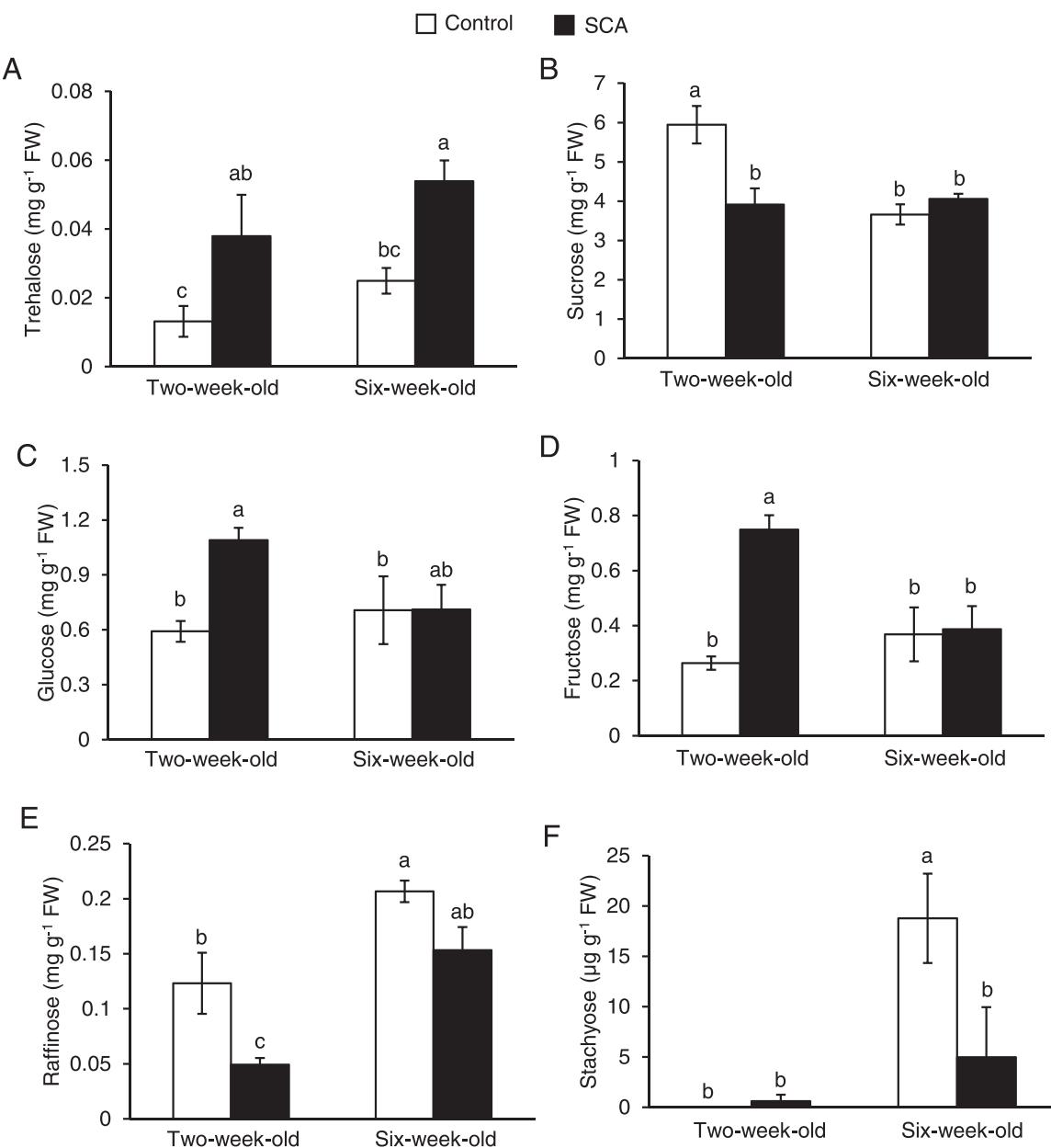


Fig. 5. Sugarcane aphid (SCA) feeding altered sugar levels on sorghum plants. Free sugar concentrations of (A) trehalose, (B) sucrose, (C) glucose, (D) fructose, (E) raffinose, and (F) stachyose, in two- and six-week-old BTx623 plants before and after SCA infestation for 10 days. Aphid uninfested plants were used as controls ($n = 3-4$). Bars with different letters represent significant differences from each other ($P < 0.05$). Error bars represent \pm SEM.

Szczepaniec, 2018; Szczepaniec, 2018a). Plausibly, differences in sorghum genotypes used could have influenced these results, but more experiments are needed with other genotypes of sorghum to evaluate interactions of plant age and aphid fecundity.

Cuticular waxes can influence the aphid preference/performance on host plants (Gorb and Gorb, 2017; Gorb et al., 2017). Previously, it was shown that there are differences in sorghum epicuticular waxes at different growth stages (Busta et al., 2021). Sorghum plants in vegetative stages (two-week-old) had higher wax coverage but lower abundance of wax components compared to plants close to reproductive stages (six-week-old). In contrast, six-week-old sorghum plants had lower wax coverage, but higher abundance of wax components compared to two-week-old plants (Busta et al., 2021). In addition, we have recently shown that 16-monoacylglycerols and long-chain 32-C-alcohols present in wax components may influence the host plant selection by SCA (Cardona et al., 2023) (nullM). The crystal structures, in addition

to the components, present in the waxes (White and Eigenbrode, 2000) may be related to the facilitation of adherence of insects in plants (Friedemann et al., 2015).

In our study, the compounds α -amyrin and isoarborinone, both belonging to the family of triterpenoids, were increased after SCA feeding on six-week-old plants compared to two-week-old plants. Triterpenoids have been reported in several studies as a major factor that modulates insect-plant interactions (González-Coloma et al., 2011). Previously, it has been reported that triterpenoid derivatives from *Manilkara subsericea* act as potent growth inhibitors of hemipteran nymphs (Fernandes et al., 2013). Additionally, α -amyrin was found to be toxic/repellent against *Anopheles stephensi*, *Plutella xylostella*, *Spodoptera frugiperda*, *Sitophilus zeamais*, and *Aedes aegypti* (Chenniappan et al., 2009, Chenniappan & Kadarkarai, 2012; Eigenbrode and Pillai, 1998, Bezerra et al., 2019; Sugauara et al., 2022). In contrast, few studies have suggested that α -amyrins act as feeding stimulants for insects (Tamura

et al., 2004; Robertson et al., 1991; Shepherd et al., 1999a; Eigenbrode & Espelie, 1995). Based on our results, it is highly likely that reduced SCA fecundity on six-week-old plants may be due to increased triterpenoid derivatives. In addition, composition of cuticular waxes indicated that under SCA pressure, two-week-old BTx623 sorghum plants responded with decreased wax biosynthesis (at least by composition) as compared to SCA infested six-week-old plants. These results indicate age-related changes in the biosynthetic capacity of BTx623 plants, although how they affected SCA reproduction remains to be solved.

The results presented here indicate that the time taken by SCA to reach the first sieve element or phloem phase (Fig. 3A) and the total duration of sieve element phase (Fig. 2B), were lower and higher, respectively, on six-week-old plants compared to two-week-old plants. On the contrary, SCA performed better on two-week-old plants compared to six-week-old plants (Fig. 1). Taken together, our results suggest that although the aphids preferred to feed on six-week-old plants during the 8 h duration of EPG experiments, the difference in aphid numbers were only observed later (i.e., 10 dpi) during aphid colonization. It is plausible that the metabolites (e.g., triterpenoids) have begun accumulating once the aphids started feeding on the plants. Furthermore, the extended time spent by the aphids in the sieve elements of six-week-old plants may have resulted in increased consumption of these metabolites and further impacted aphid's proliferation on six-week-old plants at a later time (i.e., 10 dpi) compared to two-week-old sorghum BTx623 plants.

Sugar analyses provided evidence for common and unique responses of the two- and six-week-old sorghum plants to SCA infestation. Plants of both ages had a significant increase in trehalose in response to SCA, which is a metabolite responsive to aphid feeding (Singh et al., 2011; Grover et al., 2022a). In fact, trehalose was shown to have a toxic effect on SCA (Grover et al., 2022a). Notably, sucrose levels of two-week-old control plants were significantly greater than those of six-week-old control plants, and following SCA infestation, sucrose levels decreased substantially only in the two-week-old plants. However, the levels of sucrose following SCA infestation were similar to data obtained from both control and SCA-infested six-week-old plants, indicating that sucrose levels could be among factors that influenced SCA populations in the no-choice studies. Furthermore, the decrease in sucrose in SCA-infested two-week-old sorghum were consistent with the increased levels of glucose and fructose. All of the measured sugars, including the RFOs, can variably impact aphid growth and modulate plant responses as well (Cao et al., 2013; Singh et al., 2011). Despite the lower number of aphids on six-week-old plants, as mentioned above, SCA feeding enhanced the triterpenoids levels in six-week-old plants compared to two-week-old plants and may have contributed to stronger antibiotic effects. Also, it is likely that reduced SCA numbers on six-week-old plants might not be enough to alter sugar metabolism to a greater extent compared to two-week-old sorghum plants.

Our study describes the possible defensive responses of sorghum to SCA infestation at two developmental stages and highlights the similarities and differences of the plant responses that appear to be dependent on plant age to this economically devastating pest. Continued knowledge and description of mechanisms that link physiological and morphological changes in plants upon herbivory can hasten development of new strategies for pest management through mechanisms based on HPR.

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CRediT authorship contribution statement

J.B.C. and J.L. conceived the research. J.B.C., S.G., L.B., and J.L. designed experiments. J.C., S.G., L.B., M.J.B., and P.K. conducted the experiments, collected and analyzed the data. G.S., K.G.K., and S.E.S. contributed reagents and provided guidance on experiments. J.B.C. and J.L. wrote the first draft of the manuscript. All authors reviewed and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111646.

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