Cowpea aphid resistance in cowpea line CB77 functions primarily through antibiosis and eliminates phytotoxic symptoms of aphid feeding

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ORIGINAL PAPER

Cowpea aphid resistance in cowpea line CB77 functions primarily through antibiosis and eliminates phytotoxic symptoms of aphid feeding

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Abstract

Cowpea (*Vigna unguiculata*) is one of the most important crops in semiarid areas of the world, where it thrives in hot, dry conditions. While cowpea is able to withstand abiotic stresses, it suffers serious losses from biotic antagonists, including infestation by the cowpea aphid (*Aphis craccivora*). Cowpea aphid infestations are highly destructive, especially on young plants. However, it is unclear whether cowpea aphid damage is the result of aphids having phytotoxic effects on their hosts, or simple density effects. To better understand cowpea aphid damage and the potential for resistance traits to mitigate aphid impacts, we evaluated phenotypic changes in cowpea in response to variable aphid densities and systemic versus local infestations. Low aphid densities induced leaf distortions and pseudogalling, suggesting that cowpea aphids are phytotoxic to cowpea. Resistance to the cowpea aphid has been previously identified in an African cowpea germplasm, and near isogenic lines (NILs) containing resistance quantitative trait loci (QTL) were generated in the California blackeye cultivar background. Using a series of performance assays, we determined that resistance conferred by the two QTL counteracts aphid phytotoxicity and severely limits aphid growth and fecundity. Using choice assays, a preference by cowpea aphids for the susceptible NIL was observed. Electrical penetration graph analysis revealed that the resistance phenotype includes weak surface level deterrence and strong phloem-based resistance that manifests during the sap ingestion phase. Our study provides evidence of phytotoxic traits in *A. craccivora* while identifying a viable means of counteracting aphid damage and reproductive potential through resistance.

Keywords Plant resistance · Electrical penetration graph · Phytotoxic · Insect performance · Antibiosis · Antixenosis

Key message

- Cowpea aphids are phytotoxic causing damage even at low aphid densities.
- Endogenous resistance to cowpea aphids is needed to better combat cowpea aphid infestation.
- Antibiosis was determined to be the main resistance mechanism in cowpea line CB77 and acts in the phloem.
- Antixenosis was determined to be the secondary resistance mechanism acting at the surface.

Introduction

Cowpea, *Vigna unguiculata* L. Walp (Fabales: Fabaceae), is a multi-functional crop that serves as a source of food and fodder, and as a cover crop, in semi-arid regions of the

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world (Singh et al. 2002; Langyintuo et al. 2003; Timko and Singh 2008). Cowpea is able to fill these many needs in arid locations because it can withstand harsh growing conditions such as heat and drought stress (Hall et al. 2002; Hall 2004) as well as low soil fertility and variable soil pH (Elawad and Hall 1987; Fery 1990). However, while cowpea is robust to many adverse abiotic conditions, it is vulnerable to several biotic stressors that curtail growth and yield potential.

The cowpea aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae), is one of the most important biotic antagonists of cowpea. Aphids feed by navigating long, flexible stylets between mesophyll cells to reach phloem sieve tube elements, where they subsequently engage in salivation and prolonged periods of passive sap ingestion (Tjallingii and Esch 1993; Tjallingii 2006). This process typically inflicts minimal cellular damage and does not involve tissue removal. However, aphid feeding can still facilitate negative outcomes for the host in several ways. Because aphids typically leave cells intact during feeding, and viruses require live cells to establish infections, aphid feeding is optimized for virus transmission. Cowpea aphids are broadly polyphagous and can transmit over 50 plant viruses, including the notorious cowpea-aphid-borne mosaic virus (genus *Potyvirus*, family *Potyviridae*) (Chan et al. 1991; Brady and White 2013; Singh 2014). Aphid feeding also results in leaves accumulating honeydew—the sugar water–fecal material excreted at high rates by aphids ingesting phloem sap. Honeydew acts as a carbon source for opportunistic molds, which subsequently block light from reaching leaf surfaces (Reynolds 1999). In cowpea, aphid densities can rapidly increase, resulting in an abundance of honeydew on the largest, most photosynthetically active leaves. Finally, aphids can inflict direct damage to plants by removing nutrients and continuously secreting salivary effectors that modify plant growth and development (Kaloshian and Walling 2016).

Effector-based mechanisms are hypothesized to underlie the severe damage that cowpea aphid inflicts on its host; feeding by cowpea aphids induces dramatic symptoms, including chlorosis, stunted growth and pseudogalling—a type of leaf rolling reminiscent of the gall structures induced by certain arthropods and fungi (Jackai and Daoust 1986; Goggin et al. 2017; Omoigui et al. 2017). Although not formally characterized as such, the damage phenotype induced by cowpea aphid resembles that induced by "phytotoxic" aphid species. An aphid is considered to be phytotoxic if it causes chlorosis or leaf deformity in host plants even at low densities. For example, two of the most economically important phytotoxic aphids are the Russian wheat aphid (*Diuraphis noxia* Kurdjumov) and greenbug (*Schizaphis graminum* Rodani) (Miles 1999; Nicholson et al. 2012; Nicholson and Puterka 2014). Both species induce chlorosis at low densities ($\leq$ 15 aphids), and Russian wheat aphid causes also pseudogalling. Both ultimately reduce grain yield (Burd and Burton 1992; Kieckhefer and Gellner 1992; Burd et al. 1993). In contrast, non-phytotoxic species, such as the pea aphid (*Acyrthosiphon pisum* Harris) and the green peach aphid (*Myzus persicae* Sulzer), cause little to no observable damage at low densities (Nicholson and Puterka 2014).

Resistance against cowpea aphid is a crucial management tactic given that cowpea is cultivated widely in areas of the world where insecticide use is cost prohibitive (Singh and Allen 1980; Obopile 2006; Souleymane et al. 2013). Without the use of pesticides, prolonged cowpea aphid infestations can cause over 50% losses in crop yield (Obopile 2006). This highlights the need for identifying endogenous cowpea resistance to cowpea aphids to better protect cowpea from aphid infestations. In 2013, a source of resistance was identified in an African breeding line (IT97K-556-6) (Souleymane et al. 2013). Using recombinant inbred lines (RILs), derived from a susceptible California blackeye cultivar (CB27) and the resistant African breeding line (IT97K-556-6), the sources of the resistance were mapped to two quantitative trait loci (QTLs). The resistance QTLs were found to be a major QTL, *QAC-vu7.1,* and a minor QTL, *QAC-vu1.1* (Huynh et al. 2015). The mechanism of the resistance mediated by these two QTLs remains unknown.

In the present study, we performed controlled density feeding experiments to determine if cowpea aphids are phytotoxic to their hosts at low densities. These experiments were carried out with both cowpea aphid-susceptible and resistant genetic material undergoing phenotypic evaluation for breeding potential. We performed behavioral and electrophysiological experiments to characterize the resistance phenotype associated with these two QTLs. Plant resistance to insects has been classified into three different subcategories: antibiosis, antixenosis and tolerance (Smith 1989). Antibiosis-based resistance affects the insect biology and growth, while antixenosis-based resistance affects the insect behavior. Tolerance on the other hand is the ability of the plant to withstand insect damage. Based on previous works with the resistant germplasm, we hypothesized that the type of resistance associated with QTL *QAC-vu7.1* and *QAC-vu1.1* may include both antixenotic and antibiotic effects (Souleymane et al. 2013; Huynh et al. 2015). To test this hypothesis while fully characterizing the cowpea damage phenotype, we obtained a pair of near isogenic lines (NILs) with and without the resistance trait. Using these lines, we quantified phytotoxic responses to controlled aphid feeding and characterized the nature of the resistance including its tissue location through electrical penetration graphing (EPG) (Tjallingii 2006).

Materials and methods

Plant and aphid growth conditions

Two lines of cowpea (*V. unguiculata*), susceptible California blackeye (CB46) and its resistant NIL (CB77), were grown in UC Mix 3 soil (agops.ucr.edu/soil/) in 24 oz plastifoam cups and fertilized weekly with MiracleGro (18-18-21; Stern's MiracleGro Products). Plants and aphids were maintained and used in experiments in a pesticide free plant growth room at 28 ± 2 °C and 16:8 light:dark photoperiod unless otherwise noted.

The cowpea aphids (*A. craccivora*) were collected from a field in Riverside, California (CA), USA, in the summer of 2016. *Aphis gossypii* Glover (cotton melon aphid) was used as a conspecific non-specialist in damage experiments. The colony was established from aphids collected from squash about a decade ago near Reedley, CA, and was maintained on melon (*Cucumis melo* L. cv. Iroquois).

Aphid damage assay

One-week-old susceptible cowpea plants were evaluated under two different aphid densities, constant cowpea aphid density and variable cowpea aphid density. For both aphid densities, plants were infested with 15 mixed stages of 3rd and 4th instars and adults of cowpea aphids, or cotton melon aphids for 15 days. For each repetition of the experiment, plants received between 5 and 7 adult aphids (always the same number within an experiment, depending on availability) and a mixture of 3rd and 4th instars totaling together 15 aphids. Plants were encased in plastic pollination bags with minute holes (Seedburo, SKU: S27) to restrict aphids to the plant for 15 days. For the constant cowpea aphid density treatment, aphids were maintained at 15 aphids on the entire plant by removing neonates daily. Newborn nymphs were kept when the number of the original aphids dropped below 15. Fifteen aphids were used in the constant cowpea aphid density because previously it was identified to be the number of phytotoxic aphids needed to reach the threshold for significant yield loss (Kieckhefer and Gellner 1992). The variable cowpea aphid density and cotton melon aphid density were allowed to grow and reproduce without any numeric restriction. The presence or absence of symptoms induced by the aphids (chlorosis, pseudogalling and/or stunted growth) was monitored visually daily throughout the experiment. For each aphid density or species, 8–10 plants were used per aphid density and the experiment was repeated three times.

Local vs systemic damage assay

A single unifoliate leaf of one-week-old susceptible cowpea plants was infested with 15 mixed stages of 3rd and 4th instars and adults of cowpea aphids using a mesh sleeve bag. The aphids were left to reproduce without restriction. Plants were maintained for 15 days. On the uninfested areas of the plants, symptoms including chlorosis, pseudogalling and/or stunted growth were monitored daily throughout the 15-day period. After 15 days, the mesh sleeves were removed and the symptoms on the infested leaves as well as on the entire plants were documented. Three plants were infested on a single leaf per experiment, and the experiment was performed three times (total $n = 9$).

Aphid fecundity assay

Clip-cages were prepared using a hollowed 25-ml plastic beaker. The bottom of the beaker was replaced with a muslin fabric. Using a hot glue gun, a piece of foam attached to plastic was connected to the side of the beaker using a hairclip to enclose the beaker. Age-synchronized cohorts of one-day-old adult aphids were generated by clip caging adult apterous aphids to the adaxial side of a susceptible cowpea plant for 24 h to produce progeny. After this 24-h period, the adults were removed with a fine tip paint brush and the first instars were clip-caged and allowed to grow to maturity. Cowpea aphids feed on both sides of the leaf (adaxial and abaxial), so the clip-cage was positioned for each side to be accessible. Once they reached maturity, a single, one-day-old adult was transferred to a naïve two-week-old unifoliate leaf of either a susceptible or a resistant cowpea plant and clip-caged on the adaxial side with the abaxial side accessible to the aphid. The cages were monitored daily, for one week, and the survival of the adult and the number of nymphs produced were recorded. After counting, the offspring were removed. A single leaf per plant was infested, and a total of 16 plants of each cowpea line were used.

Aphid growth rate assay

Ten cowpea aphid adults were clip-caged onto the adaxial side of a susceptible or a resistant cowpea leaf. The clip-cage was positioned for access to both the adaxial and abaxial sides for the aphids. After 24 h, the adults were removed, and ten newborn nymphs were left on the leaf in the clip-cage. After six days, the surviving aphids were counted and pooled before weighing with a microbalance (M2P, Sartorius; ± 0.5 µg).

The mean relative growth rate (MRGR) was calculated as the difference of logarithms of the mean weight of day-old aphid nymphs and the mean weight of the surviving aphid nymphs divided by the number of days ($MRGR = (\log W_{surviving} - \log W_{day\text{-}old})/\text{Number of days}$). Twelve cages were used per cowpea line.

Electrical penetration graph

A DC-electrical penetration graph (DC-EPG system) was used for the EPG analysis (Tjallingii 1988). The EPG technique has been developed to monitor the probing activities of arthropods with piercing mouthparts when probing inside plants. The EPG waveforms are determined depending on the stylet tip positions in leaf tissue and the insect's behavior (Tjallingii 1988). An EPG is performed by securing an electrode onto an aphid and placing a second electrode in the soil next to plant roots. Cowpea aphids were tethered to a 12.5 µm gold wire on the dorsal side of the abdomen using a water-based, conductive silver glue (Cervantes and Backus 2018). After an hour-long starvation period, tethered aphids were placed on the abaxial side of a two-week-old unifoliate leaf and a second electrode was placed in the soil of the potted plant. Simultaneous recordings for eight aphids were performed on a Giga-8 DC-EPG amplifier for 8 h. The aphid–plant systems were housed in a Faraday cage in a climate-controlled room at 24 ± 1 °C. Each recording session had half of the aphids on susceptible plants and half on resistant plants. At the end, a total of 25 and 30 EPG recordings on susceptible and resistant plants, respectively, were obtained and analyzed. The analysis of the EPG variables and waveforms was performed with PROBE 3.5 software (EPG systems, www.epgsystems.eu) naming convention based (Ebert et al. 2015). These EPG parameters were based on six different waveforms corresponding to stylet pathways in plant tissues other than phloem and xylem (C), to potential drops (i.e., intracellular stylet punctures) (pd), to salivation in phloem elements (E1), to passive phloem sap ingestion (E2), to active xylem sap ingestion (G), and to derailed stylet mechanics (F). The calculations were performed with EPG-Calc 6.1 software (Giordanengo 2014).

Aphid no-choice assay

Two-week-old susceptible and resistant cowpea plants were infested with twenty apterous adults on a single unifoliate leaf and enclosed in a mesh sleeve bag. The plants were left undisturbed for six days after which the total number of aphids were counted on each leaf. A total of 15 plants were used for each cowpea line.

Aphid choice assay

A large, modified Petri dish (150 × 15 mm) arena was placed above two 2-week-old cowpea plants, one of each resistant and susceptible line, to evaluate aphid preference. The arena had two holes of 2 cm diameter cut out of the bottom of the plate, directly across from each other. When positioned over paired leaves from each treatment, the aphids were able to choose between equal tissue amounts of each leaf, spaced equidistant within the plate. Aphids were introduced to the arena through a third 2-cm hole directly in the center area between the two choice options. This hole was fitted on the underside (exterior) of the arena with a screw cap to which a modified (shortened) 50-mL conical tube containing the aphids could be affixed (Online Resource 1). A group of twenty apterous adults (starved for 1 h prior to use) was introduced to the arena through this release mechanism. The number of aphids feeding on each leaf was recorded at 2, 3, 6 and 24 h after release. Four to five new plant pairs were used per experiment, and the experiment was performed four times.

Aphid dispersal assay

The rate of cowpea aphid dispersal in response to resistant cowpea was used to monitor aphid deterrence. Aphid dispersal was measured using a behavior dispersal assay similar to that described in Mauck et al. (2010). Bioassay arenas were constructed from 100 × 15 mm Petri dishes, with two conjoined, slightly overlapping holes (20 mm diameter) cut in the center. A unifoliate leaf from one of the two cowpea plant types being compared was exposed on one side of the conjoining holes, and the other choice option was immediately adjacent occupying the second conjoined hole (Online Resource 2). After a 15-min chill at 4 °C, twenty 4th stage instars and adult aphids were placed on a piece of filter paper. The filter paper with the chilled aphids was then placed directly on one of the exposed leaves. The placement of the filter paper on the leaf ensured that the aphids contacted the initial leaf they were placed on before dispersal. The filter paper was removed after one hour when all aphids had dispersed and the location of the aphids was documented at 1, 2, 6 and 24 h. The cowpea plants screened against each other included, susceptible with resistant, susceptible with susceptible and resistant with resistant. Five plant pairs were used per experiment and the experiment was performed twice.

Statistical analyses

We used one-way analysis of variance (ANOVA) followed by Tukey HSD tests to assess aphid density damage assay using GraphPad Prism version 8 (GraphPad Software, San Diego, California USA). The aphid no-choice experiment was analyzed using a two-tailed *t*-test using GraphPad Prism version 8 (GraphPad Software, San Diego, California USA). We used generalized linear models (GLM) with a likelihood ratio and Chi-square test (χ^2) to analyze adult aphid fecundity and survival rate using R software (version 3.6.0) (R Core Team 2019). Two-tailed *t*-test using GraphPad Prism version 8 (GraphPad Software, San Diego, California USA) was used to analyze the nymph survival and MRGR. Aphid

feeding behavior was analyzed using generalized linear models (GLM) with a likelihood ratio and Chi-square test. We had three types of data that are typical of EPG studies: (a) event duration, (b) frequency of penetration events and (c) time delay until the first occurrence of an event. Event durations were modeled using GLM with a gamma (link = "inverse") distribution, and frequency of penetration was modeled using GLM with poisson (link = "identity") distribution. Time delay data were modeled using the Cox proportional hazards (CPH) model, and we treated cases where the given event did not occur as censored. The assumption of validity of proportional hazards was checked using the functions "coxph" and "cox.zph," respectively (package R: "survival"). The fit of all generalized linear models was controlled by inspecting residuals and QQ plots. Aphid choice assays were analyzed using Friedman tests for the overall model and Kruskal–Wallis tests for each individual time point (Minitab v. 14). Aphid dispersal assays were analyzed using GLMs for the overall repeated measures model, followed by univariate tests to explore treatment effects within each time point (Minitab v. 14).

Results

Damage caused by cowpea aphids at point of feeding and distal sites

Two different cowpea aphid densities (constant and variable/increasing) were screened to observe the effect of damage and determine if symptoms consistent with phytotoxicity occur even at low densities. A third treatment, feeding by the generalist, cotton melon aphid was included because it is phylogenetically closely related to the cowpea aphid but is not adapted to cowpea and causes no damage to the plant (Song et al. 2016). In the variable cowpea aphid density, all three measured symptoms, chlorosis, pseudogalling, and stunted growth, were observed (Fig. 1). These symptoms first became apparent at the end of week 1 and increased in severity by the end of week 2. Damage symptoms were also seen in the constant cowpea aphid density, with most of the damage symptoms observed categorized as pseudogalling (Fig. 1B). Unlike the variable cowpea aphid density, where every plant exhibited at least one of the expected symptoms, only half of the constant cowpea aphid density plants exhibited a symptom(s) (Fig. 1D). Damage symptoms on the constant cowpea aphid density plants were localized to areas where aphids clustered (Online Resource 3). In a follow-up experiment quantifying local *vs.* systemic damage explicitly, no symptom was observed in the uninfested plant tissues. The only observed symptom(s) was on the infested unifoliate leaf (Online Resource 4).

As expected, no damage was observed on plants with the variable cotton melon aphid density (Fig. 1). After the 15-day infestation period, the total number of aphids was calculated for the variable aphid densities. The cotton melon aphid density was found to be on average less than 15 aphids (ranging from 0 to 52 cotton melon aphids), equivalent to the number of aphids used for the constant cowpea aphid density (Online Resource 5), while the variable cowpea aphid density had more than 15-fold higher aphids than either of the other two aphid densities.

Aphid performance metrics under no-choice conditions

Initial no-choice assays indicated a stark difference in aphid density growth on resistant (CB77) *vs.* susceptible (CB46) cowpea lines (Fig. 2). The cowpea aphids feeding on susceptible line had about fivefold higher numbers than those on the resistant plants. To determine if these effects may be due to an antibiosis resistance mechanism acting on adult aphids, single age-synchronized one-day-old adult aphids were clip-caged onto leaves and fecundity and survival were monitored for a week. Adults reared on the resistant cowpea had significantly lower fecundity than aphids reared on the susceptible line (Fig. 3A, GLM, $\chi^2 = 41.704$, $P < 0.001$). Adult aphid survival was also significantly lower on the resistant line compared to the susceptible (Fig. 3B, GLM, $\chi^2 = 8.049$, $P = 0.005$).

To investigate whether cowpea resistance affected aphid growth rates, ten neonates were caged onto susceptible or resistant lines. After six days, significantly more nymphs survived on the susceptible line compared to the resistant line (Fig. 4A). The nymphs reared on the susceptible line also had significantly higher MRGR (0.13) than the nymphs reared on the resistant line (0.04) (Fig. 4B). Together, these no-choice performance metrics suggest that the resistance in cowpea line CB77 involves antibiosis and has negative effects on the aphid's reproduction, survival and growth.

Electrical penetration graph

A representative EPG diagram of cowpea aphids feeding on both susceptible and resistant cowpea is shown in Online Resource 6. The differences in aphid feeding behavior on the two different cowpea lines start at the beginning of the probing process. The time to first probe (first stylet penetration into the plant) is significantly shorter for the aphids placed on the susceptible line compared to the resistant line (Table 1, $\chi^2 = 7.119$, $P = 0.008$). The aphids feeding on the susceptible line had a significantly longer probing period than the aphids feeding on the resistant line; aphid stylets were inserted in susceptible plant tissues for 30 more minutes than in resistant tissues (Fig. 5, Table 1, $\chi^2 = 4.649$,

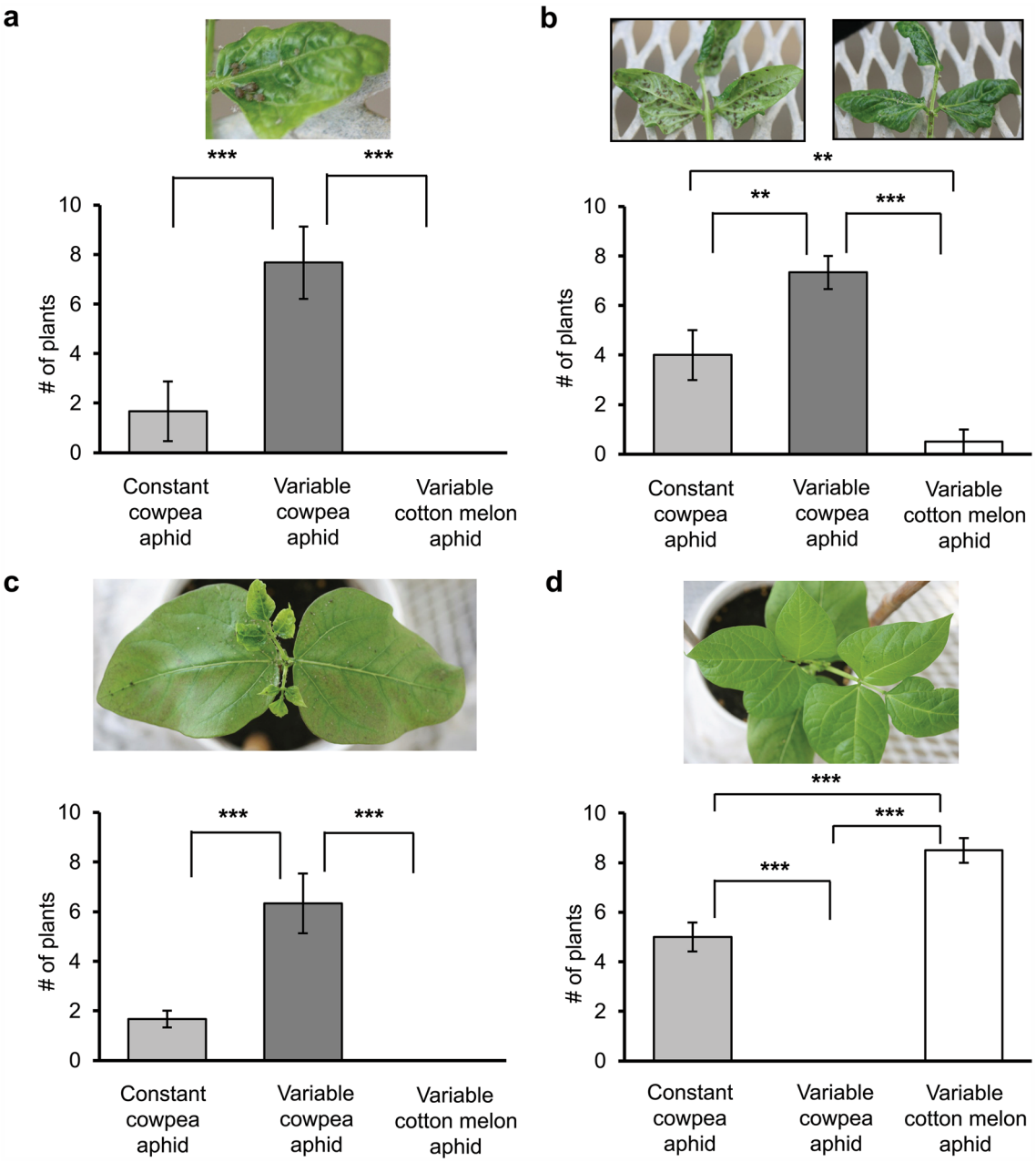

Fig. 1 Number of cowpea plants showing damage symptoms by cowpea aphids or cotton melon aphids. The symptoms observed were **a** chlorosis, **b** pseudogalling, **c** stunted growth and **d** no damage. A picture representing each symptom (or lack of it) is displayed above each graph. Pictures for **a**–**c** represent the variable cowpea aphid density, **d** represents constant cowpea aphid density. All photographs were taken on day 15 of the infestation with plants infested during the same replicate. The constant cowpea aphid density was maintained at 15 aphids for the entirety of an experiment. Aphids in the variable aphid density were allowed to grow without any restriction. The experiment was performed three times with 10 plants per aphid density except for one replicate where cotton melon aphid had only 8 plants. Constant cowpea aphid $n=30$, variable cowpea aphid $n=30$, variable cotton melon aphid $n=28$. Values are expressed as the mean ± SE. Analyses were performed with one-way ANOVA followed by Tukey's HSD test. Only significant differences were indicated with asterisks $^{**}P<0.01$, and $^{***}P<0.001$

$P=0.031$). The aphids feeding on the susceptible line spent one fewer hour in the pathway (C) phase than aphids feeding on the resistant line. This difference indicates that aphids on the resistant line are spending more time trying to reach the phloem than those on the susceptible line (Fig. 5, Table 1, $\chi^2=8.853$, $P=0.003$). The difference in the number of C phases is in line with the differences seen in the number of potential drops (pd), both parameters being greater for

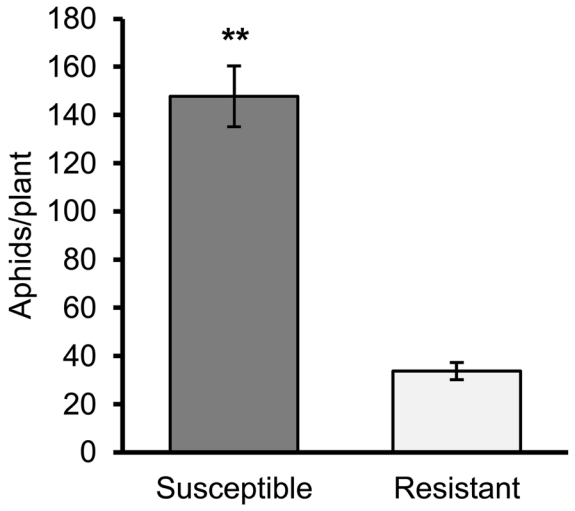

Fig. 2 Cowpea aphid density growth on susceptible and resistant cowpea NILs. Plants were infested with twenty adults for 6 days. The difference in aphid density between the two plant NILs ($n=15$) was analyzed using two-tailed t-test. Asterisks indicate significance $^{**}P<0.01$

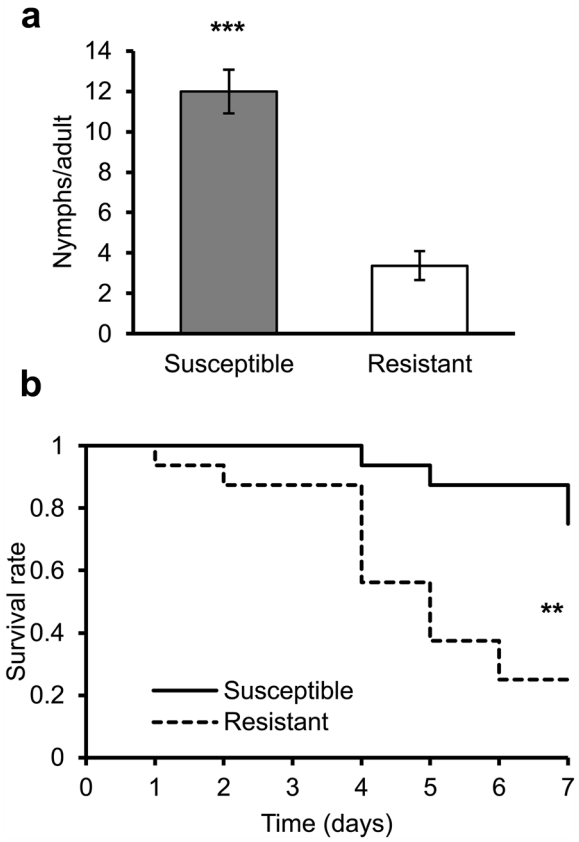

Fig. 3 Adult cowpea aphid performance on susceptible and resistant cowpea. **a** Fecundity of age synchronized one-day-old adult aphids on resistant or susceptible plants. Values are the means and SE of $n=16$. **b** Survival rate of adults on susceptible or resistant cowpea. Aphid performances were monitored for 7 days, $n=16$. The differences in aphid fecundity and survival rates were analyzed using generalized linear models (GLM) with a likelihood ratio and $\chi^2=41.704$. The asterisks indicate a significant difference between aphids on susceptible and resistant cowpea plants; $^{***}P<0.001$ for the daily fecundity and Cox model, $\chi^2=8.049$; $^{**}P=0.005$ for the aphid survival rate)

aphids feeding on the resistant cowpea than the aphids feeding on the susceptible cowpea.

Aphids also differed in feeding parameters describing interactions with phloem sieve elements (the E phases). The potential E index is calculated as the percentage of time spent in the E phases after the subtraction of the time needed to first reach phloem sap and is a measure of how consistently insects feed following the first phloem contact, with higher numbers indicating more consistent feeding (Van Helden and Tjallingii 1993). The potential E index was significantly higher for aphids feeding on the susceptible line relative to aphids feeding on the resistant line (Table 1, $U=163$, $P=0.0014$). Aphids feeding on the susceptible line also salivated (E1) more frequently than on the resistant line but not for longer periods of time (Fig. 5, Table 1, $\chi^2=14.388$, $P<0.001$). One of the most notable differences observed in the phloem sieve element (E) phases was the inability of over half the aphids feeding on the resistant line to reach the phloem sap ingestion phase (E2) while 96% of aphids feeding on the susceptible line were able to do so (Table 1, $P<0.001$). When comparing only the aphids that successfully reached phloem sap ingestion, individuals on the susceptible line were able to do so over an hour sooner compared to those on the resistant line and ingested sap for over an hour longer (Fig. 5, Table 1, $\chi^2=22.619$, $P<0.001$).

Host selection behavior

At the 2, 3 and 6 h time points for the two-way choice test, aphid participation was low (e.g., only a few aphids had emerged into the arena and/or made a choice), and aphids were equally present on resistant or susceptible leaves. However, at 24 h, aphid participation was higher (most aphids had selected a feeding location) revealing a significant preference for the susceptible line over the resistant line (Fig. 6; Online Resource 1, $H=12.58$, $df=1$, $P<0.001$).

In a different type of assay, we monitored aphid dispersal after the aphids were directly released on either susceptible or resistant cowpea and another plant (susceptible or resistant) directly adjacent to it (Online Resource 2). Aphids released on both susceptible and resistant cowpea had low initial dispersal levels (Fig. 7A, $F=0.17$, $df=3$, $P=0.914$). However, after 2 h significant aphid dispersal was observed when the aphids were initially released on the resistant line screened with the susceptible line (Res–Susc) (Fig. 7B; $F=4.15$, $df=3$, $P<0.05$). This dispersion level was not observed in the other plant combinations.

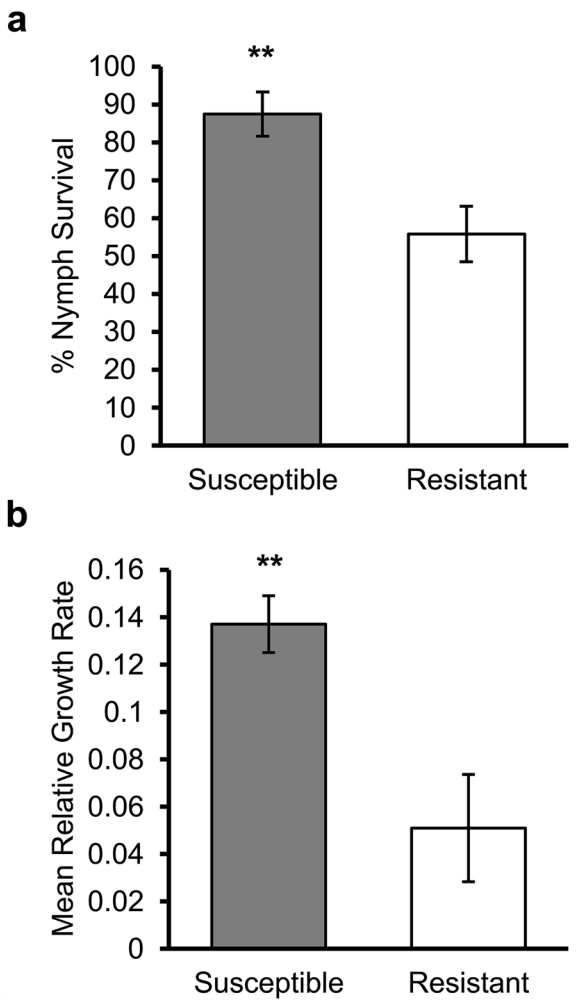

Fig. 4 Cowpea aphid nymphal survival and growth rate on susceptible and resistant cowpea. Ten neonates were clip-caged onto a single leaf of susceptible or resistant plants for 6 days. Values are the means ± SE of $n = 12$. **a** Nymphal survival. **b** Mean relative growth rate (MRGR) (MRGR = (Log($Avg_{surviving\ nymphs}$) − Log($Avg_{day\text{-}old\ nymphs}$))/Days on Plant)/Days on Plant). Average weight of 20-day-old nymphs were used as base value. Analysis was performed by two-tailed *t*-test. Asterisks indicate significance $^{**}P < 0.01$

The difference in dispersion levels became even more significant after 24 h (Fig. 7D; $F = 11.61$, $df = 3$, $P < 0.01$). Only about a third of the aphids that were initially released on resistant cowpea when screened with susceptible (Res–Susc) remained on the initial plant indicating a high level of aphid dispersal. In comparison, almost two thirds of the aphids were remaining on the susceptible when initially released on susceptible cowpea leaf adjacent to a resistant cowpea leaf (Susc–Res). A similar level of dispersion was observed when the two cowpea lines were compared with each other (Susc–Susc, Res–Res) after 24 h. For both assays, we observed a lower level of dispersion for aphids released on the resistant line when it was adjacent to the susceptible line (Res–Susc). The aphids initially released on the resistant line when screened with resistant (Res–Res) had a higher level of dispersal than aphids initially released on the susceptible line screened with resistant (Susc–Res).

Discussion

Cowpea aphid infestation produces direct damage on susceptible cowpea plants that severely compromises yields. By controlling for infestation density, we were able to explore induction of phytotoxic symptoms and their relationship with aphid density. Our results revealed that even low density of cowpea aphids can induce symptoms consistent with phytotoxicity on susceptible plants; half of the plants in controlled density treatments exhibited at least one symptom (chlorosis, pseudogalling, or stunting). With larger cowpea aphid numbers (variable density treatment) phytotoxic effects were more consistent, with all plants exhibiting at least one of the three symptoms, but no new, unique phytotoxic effects were apparent. The appearance of phytotoxic symptoms even at low cowpea aphid numbers (15 aphids) indicates that this species should be considered as a phytotoxic aphid like the Russian wheat aphid and greenbug (Kieckhefer and Gellner 1992; Miles 1999; Nicholson et al. 2012; Nicholson and Puterka 2014). However, unlike these well-known examples, our experiments suggest that the morphological and coloration-based symptoms induced by cowpea aphid feeding are localized to the infestation sites and are not systemic (Deol et al. 2001).

This pattern of phytotoxic effects is consistent with other reports of pseudogalling symptom induction by members of the *Aphis* genus when feeding on their main crop hosts. For example, the cotton melon aphid induces pseudogalling on cotton (*Gossypium hirsutum* L.) and melon (Miles 1990; Goggin et al. 2017). And the spirea aphid (*Aphis spiraecola*) can cause pseudogalling on apple (Maloideae) (Blackman and Eastop 1994; Goggin et al. 2017). It is unknown exactly how pseudogalling is induced by aphids, but it likely does confer benefits. For instance, the presence of pseudogalls can provide shelter and protection from natural enemies and insecticide exposure (Flint 2013). In all cases, pseudogalling symptoms are induced on hosts that are highly suitable and easily exploited by each respective species. Our results, combined with those for other *Aphis* species, suggest that pseudogalling could be a general phytotoxic effect of feeding on compatible hosts by members of the *Aphis* genus.

Understanding the suite of symptoms induced by cowpea aphids on susceptible germplasm, and their relation to aphid proliferation, facilitated characterization of the resistance mechanism in a NIL derived from germplasm that does not exhibit damage symptoms in response to cowpea aphid feeding (Souleymane et al. 2013). To

Table 1 Cowpea aphid probing and feeding parameters on the susceptible (S) and resistant (R) cowpea NILs

	S ($n=25$)	R ($n=30$)	Stats
Probing phase			
Time to first[a] (h)	0.02 ± 0.01	0.06 ± 0.02	**Chi-sq = 7.119; *P* = 0.008****
# Brief probes (< 3 min)[b]	9.72 ± 2.51	8.267 ± 1.05	Chi-sq = 3.214; *P* = 0.073 NS
#	22.04 ± 3.42	27.73 ± 2.24	**Chi-sq = 17.738; *P* < 0.001*****
Duration[c] (h)	7.07 ± 0 .19	6.48 ± 0.19	**Chi-sq = 4.649; *P* = 0.031***
Pathway phase (C)			
#	25.04 ± 3.46	30.63 ± 2 .21	**Chi-sq = 15.305; *P* < 0.001*****
Minimum time (C) to first E in probe containing E (h)	0.40 ± 0.05	0.37 ± 0.05	Chi-sq = 0.367; *P* = 0.544 NS
Duration (h)	3.32 ± 0.31	4.54 ± 0 .25	**Chi-sq = 8.853; *P* = 0.003****
Potential drops (Pd)			
#	163.12 ± 19.28	203.10 ± 14.89	**Chi-sq = 118.88; *P* < 0.001*****
Sieve element salivation (E1)			
#	5.64 ± 0.79	3.85 ± 0.42	**Chi-sq = 14.388; *P* < 0.001*****
Time to first E from first probe (h)	1.99 ± 0.40	2.86 ± 0.64	Chi-sq = 2.797; *P* = 0.094 NS
Duration (h)	0.62 ± 0.12	0.92 ± 0.17	Chi-sq = 2.226; *P* = 0.136 NS
n[d]	100% (25/25)	90% (27/30)	*P* = 0.242 NS
Phloem sap ingestion (E2)			
#	3.67 ± 0.56	2.5 ± 0.44	**Chi-sq = 34.286; *P* < 0.001*****
Time to first (h)	2.31 ± 0.38	3.94 ± 0.67	**Chi-sq = 22.619; *P* < 0.001*****
Time to first sustained (h)	2.92 ± 0.43	4.24 ± 0.69	**Chi-sq = 21.632; *P* < 0.001*****
Potential E index (%E after 1st E)[e]	57.33 ± 6.41	29.04 ± 5.72	***U* = 163; *P* = 0.0014****
Duration (h)	2.90 ± 0.45	1.43 ± 0.39	**Chi-sq = 4.089; *P* = 0.043***
n	96% (24/25)	46.7% (14/30)	***P* < 0.001*****
Xylem (G)			
#	1 ± 0	1 ± 0	Not enough repetition
Duration (h)	0.92 ± 0.20	0.96 ± 0.22	Not enough repetition
n[c]	12% (3/25)	10% (3/30)	
Stylet derailment (F)			
#	2 ± 0.52	1.75 ± 0.41	Chi-sq = 0.005; *P* = 0.943
Duration (h)	0.79 ± 0.19	1.08 ± 0.21	Chi-sq = 0.293; *P* = 0.589
n	24% (6/25)	26.67% (8/30)	*P* = 1 NS

Feeding and probing parameters measured from the aphids feeding on the two cowpea NILs, n = number of individual aphids, and # = number of phases measured

The statistics used are the following: [a]Cox model, [b]GLM (family = poisson), [c]GLM (family = Gamma), [d]Fisher's exact test, and [e]Mann–Whitney U tests

All values in bold are statistically significant

produce the NIL used in our study, resistance in an African breeding line that lacked phytotoxic symptoms following cowpea aphid feeding was mapped to two QTLs and introgressed into an elite susceptible California blackeye cultivar (our susceptible line) (Huynh et al. 2015; Huynh et al. submitted). Our experiments confirm that the resistance is associated with the previously identified QTLs, as the resistant NIL did not express phytotoxic symptoms in response to cowpea aphid infestation in any of our experiments, whereas the susceptible line nearly always exhibited at least one of the three symptoms (chlorosis, pseudogalling, or stunting) (Fig. 1). Aphids also failed to thrive and reproduce on the resistant line; density growth was minimal, and survival was about half of that seen for cowpea aphids on the susceptible line (Figs. 2 and 3).

This difference in density growth between the two cowpea lines is similar to what has been reported for soybean aphid (*Aphis glycines* Matsumura) growth on susceptible and resistant soybean (*Glycine max* L.) over a similar time period (Studham and MacIntosh 2013). This difference in aphid density growth can be attributed to soybean aphid mortality due to the antibiosis resistance gene *Rag1* (Hill et al. 2004; 2006a; b). In addition to aphid survival, aphid growth rate has been used as a parameter to identify antibiosis.

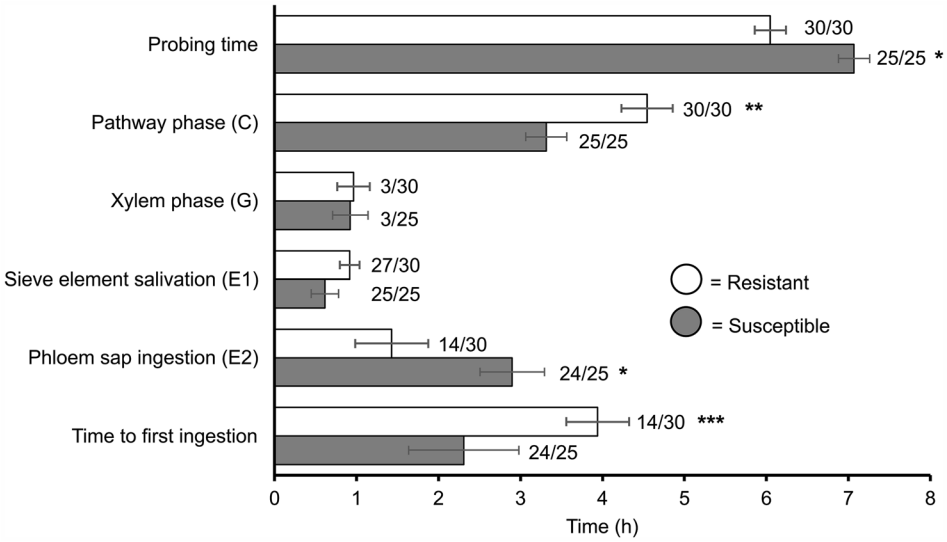

Fig. 5 Histogram of aphid activities during electrical penetration graphs (EPGs) analysis of cowpea aphids feeding on susceptible and resistant cowpea. Probing time indicates the time the aphid stylets were in the plant. The pathway phase indicates the time the aphid stylets are in the mesophyll or parenchyma cells (C phase + potential drops). The xylem phase indicates the time the aphid stylets are in the xylem (G phase). The sieve element salivation is the time the aphid is salivating into the sieve element (E1 phase). The phloem sap ingestion is the time the aphid is ingesting the plant phloem sap (E2 phase). The time to first ingestion is the average time it took for the aphid to first ingest phloem sap. Data are based on 25 and 30 aphids tested on susceptible and resistant cowpea NILs, respectively. The asterisks indicate a significant difference between the plants (GLM models, $*P<0.05$, $**P<0.01$, $***P<0.001$)

Cowpea aphid resistance in *Medicago truncatula* Gaert. was determined to have an antibiosis component by monitoring MRGR of cowpea aphid nymphs (Kamphuis et al. 2012). Similar to our study, the aphids feeding on the resistant *M. truncatula* also had a significantly lower MRGR than those on susceptible *M. truncatula.*

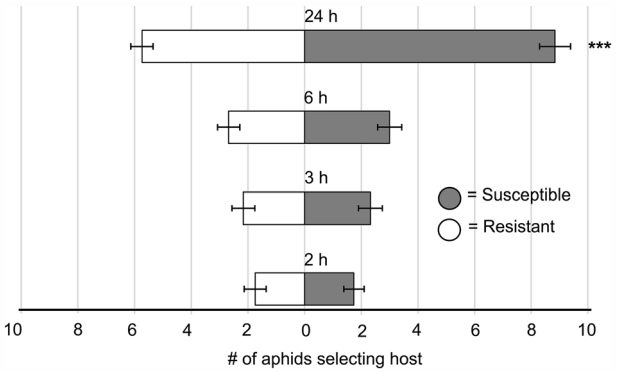

Fig. 6 Aphid choice assay. Twenty cowpea aphids were introduced into a large Petri dish arena to choose between a unifoliate leaf from susceptible or resistant cowpea NILs on either side. Values are the mean ± SE of $n=19$. The number of adults on either leaf line was documented at the indicated time points. Analysis with both Friedman (full model) and Kruskal–Wallis (individual time points) tests was performed. Asterisks indicate significance $*P<0.05$, $**P<0.01$, $***P<0.001$

Our aphid performance experiments demonstrate that resistance conferred by the resistant line has an antibiosis component that affects both survival and fecundity. To determine where in the feeding process this resistance operates, we employed the EPG technique to quantify differences in stylet activities and acquisition of plant fluids (phloem sap, xylem contents) between the resistant and susceptible lines. This technique can also provide some insight into whether antixenosis is a component of the resistance mechanism. To parse these mechanisms, we analyzed specific parameters for each phase of the aphid–plant interaction. Surface-level resistance traits that operate through antixenosis will be evident if insects are delayed in initiating their first probe into plant tissue (Van Helden and Tjallingii 1993; Alvarez et al. 2006). Resistance traits in peripheral layers of plant tissue (epidermis/mesophyll) may be operating if insects carry out significantly more short test probes on resistant lines, perform a larger number of intracellular punctures, or if they take significantly longer to traverse the mesophyll to initiate phloem contact (Gabrys et al. 1997; Schwarzkopf et al. 2013). Resistance at the phloem level is evident if the insects take longer to reach their first sustained bout of sap ingestion, spend a reduced percentage of the total recording time ingesting sap, and if fewer insects successfully engage in sap ingestion (Van Helden and Tjallingii 1993; Alvarez et al. 2006). Resistance may also alter salivation patterns at the site of the phloem. Bouts of salivation (E1) are typically followed by sap ingestion (E2) but on resistant or incompatible

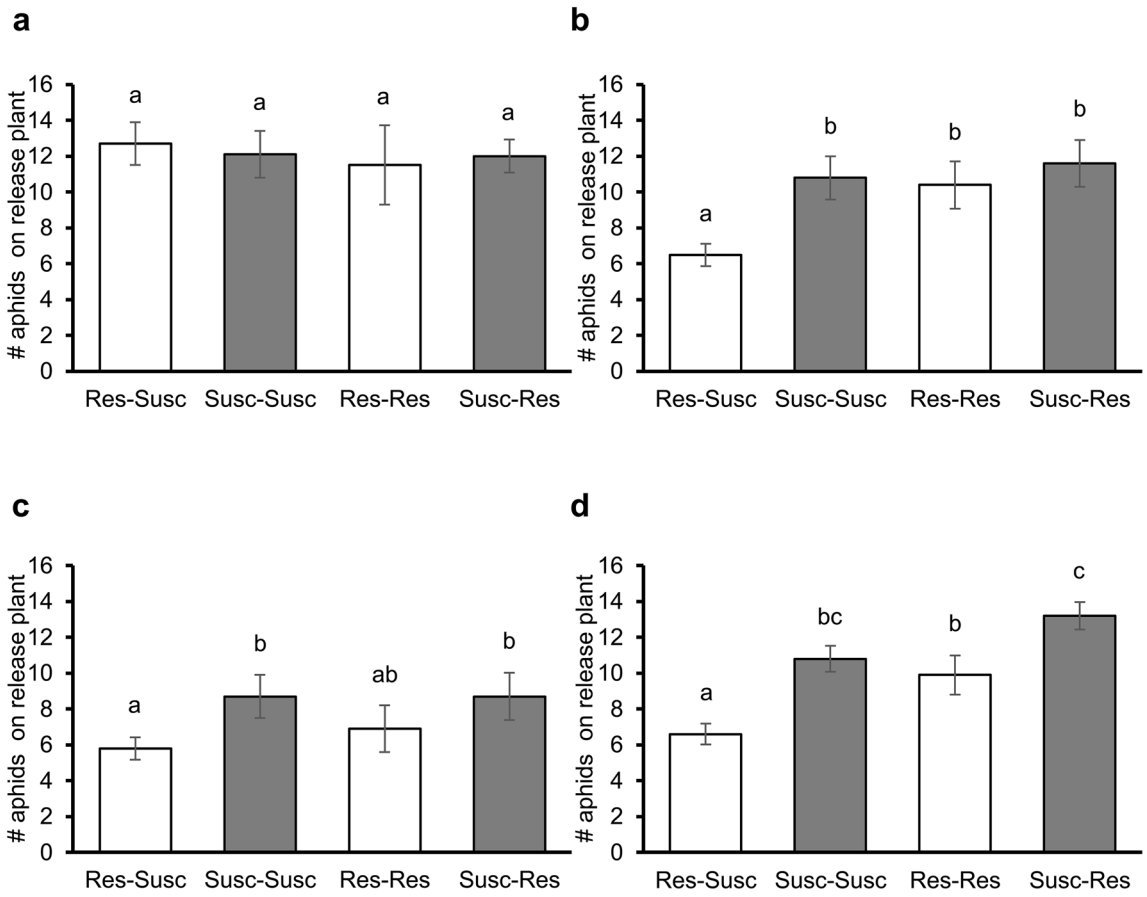

Fig. 7 Cowpea aphids remaining on susceptible (Susc–Susc, Susc–Res) or resistant (Res–Susc, Res–Res) cowpea after initial release. Twenty cowpea aphids were placed on a piece of filter paper and introduced to a resistant cowpea unifoliate leaf in a Petri dish arena. Either a susceptible or resistant cowpea leaf was placed opposite in the arena. The location of the aphids was monitored at **a** 1 h, **b** 2 h, **c** 6 h, and **d** 24 h. Values are the mean ± SE of $n = 10$. Dispersal level was compared to the appropriate baseline dispersal through generalized linear models (GLMs) followed by Tukey's HSD test. Different lowercase letters indicate significant differences at $P < 0.05$

plants this transition is not successful and instead of ingestion it results in phloem occlusion (Medina-Ortega and Walker 2015). The legume specialist pea aphid was able to ingest sap on fava bean (*Vicia faba* L.) without causing forisome occlusion (Walker and Medina-Ortega 2012). However, when two generalist aphids, the green peach aphid and potato aphid (*Macrosiphum euphorbiae* Thomas), fed on fava bean they had an incompatible interaction and stylet penetration led to forisome occlusion and little sap ingestion (Medina-Ortega and Walker 2015).

Considering the parameters described above, our EPG recordings provide evidence of both antixenosis and antibiosis-based resistance in non-vascular tissues. Aphids took almost three times longer to initiate the first probe into plant tissue on the resistant line despite no apparent anatomical differences between the lines, which is consistent with surface-level antixenosis traits, as has been observed for surface-level antixenosis resistance to the green peach aphid in multiple *Solanum* species (Alvarez et al. 2006). Once probing was initiated, aphids on the resistant line performed more probes in a shorter total probing period but did not perform significantly more brief probes less than three minutes. This indicates that aphids do not have difficulty traversing the epidermis and immediately adjacent mesophyll, as most probes proceeded deeper than the first few cell layers. Aphids also performed slightly more pathway events (traversing the mesophyll layer to reach the phloem) and took longer to complete the pathway phase but did not differ in the minimum time needed to reach the phloem within pathway events that ultimately proceeded to phloem contact. During the pathway phase, aphids also performed significantly more intracellular punctures (potential drops) on the resistant line, during which they presumably sampled cell contents (Martin et al. 1997). Overall, these results suggest that some resistance traits are operating in the surface and non-vascular cell layers because aphids perform more non-nutritive activities on the resistant line (starting new probes, traversing cell layers, intracellular punctures). However, it

is unlikely that these factors are the main drivers of the dramatic reductions in aphid performance observed in our other experiments because 90% of the aphids on the resistant line reached the phloem, and we did not observe any differences in stylet derailment during the pathway phase.

Resistance factors that directly explain the reduced survival and growth metrics are strongly evident at the level of the phloem, specifically for phloem sap ingestion phase. Only half of the aphids recorded feeding on the resistant line successfully engaged in phloem sap ingestion. Of those that were successful, they took more than an hour longer to initiate sap ingestion, engaged in fewer ingestion events, and spent about half as much time ingesting after the first successful phloem contact (Table 1). These results suggest that the most effective resistance traits are operating at the level of the phloem sieve elements. It is interesting to note that this resistance operates almost exclusively in the ingestion phase, with few effects on salivation. Often, phloem-based resistance mechanisms are associated with differences in salivation occurrence and duration (Kamphuis et al. 2012; Sun et al. 2018; Peng and Walker 2020). For example, green peach aphids feeding on a resistant line of pepper (*Capsicum* spp.) salivated more frequently, and for longer bouts, relative to those on susceptible pepper (Sun et al. 2018). Similarly, cotton melon aphids feeding on resistant melon salivated for 2.8 times longer than cotton melon aphids on susceptible melon (Peng and Walker 2020). In contrast with this, a significantly shorter salivation time was reported for cowpea aphids feeding on resistant *M. truncatula* compared to susceptible (Kamphuis et al. 2012). In our study, the number of salivation events was slightly higher among aphids feeding on susceptible line, but the time to the first salivation (E1) event and duration of salivation (E1) in phloem elements did not differ across the two lines.

Phloem-based resistance has been reported in multiple studies that only observed differences in ingestion (E2) phase duration, similar to our results. Phloem-based resistance in *M. truncatula* to both the bluegreen aphid (*Acyrthosiphon kondoi* Shinji) (Klinger et al. 2005) and pea aphid (Gao et al. 2008) resulted in significantly shorter periods of ingestion (E2) without an observed difference in salivation (E1) phases compared to susceptible *M. truncatula*. This difference in only ingestion time could be the result of deposition of phloem proteins or callose acting as physical blockages to sap ingestion, or indicative of the biosynthesis of resistance factors locally in response to aphid feeding (Klinger et al. 2005; Kehr 2006; Furch et al. 2007; Gao et al. 2008). Identifying the mechanism of phloem-based resistance will require further EPG experiments combined with histological studies by microscopy to visualize occlusions that occur following specific durations of phloem contact by aphids (Medina-Ortega and Walker 2015; Peng and Walker 2020).

Aphid choice assays support EPG evidence suggesting a surface-level antixenosis resistance phenotype in addition to the phloem level antibiosis. Aphids preferred to settle and feed on susceptible leaves over resistant leaves in two-way choice tests (Fig. 6). And aphids initially released on a resistant leaf preferred to disperse to susceptible leaves over short time frames (two hours post-release) (Fig. 7B). This dispersion level was not observed when a resistant line release leaf was paired with resistant line choice leaf (Res–Res) at the same time point, indicating the presence of additional attractive (susceptible) or repellent (resistant) factors that affect aphid behavior. These differences in aphid preference and dispersal are consistent with the surface-level resistance identified by the delayed first probe on the resistant line observed in the EPG analysis (Table 1) demonstrating the cowpea aphids' reluctance to initiate feeding on the resistant plants. This kind of antixenosis could be mediated by differences in constitutive volatile emissions from resistant line vs. susceptible line hosts, or by surface-level morphologic characteristics such as epicuticular waxes, pilosity or glandular trichomes (Berlinger 1986; Jan et al. 1995; Canassa et al. 2020).

Conclusion

Cowpea aphids are a serious threat to food security in areas that rely on cowpea as a staple food crop because of the unique damage condition they induce on their host plants. The best way to prevent this damage in the field is through the development of resistant cowpea lines, as pesticides are expensive and harmful to the environment (Souleymane et al. 2013). Characterization of the resistant cowpea line CB77, developed from an African cowpea and introgressed into elite California blackeye cultivar, indicates that the resistance mechanism acts mainly via antibiosis, with a secondary antixenosis effect evident from behavioral assays. The antibiosis component appears to function at the level of the phloem to block sap ingestion, ultimately, affecting both aphid growth and fecundity. The antixenosis component may be mediated by volatiles, leaf surface characteristics or a combination of these two aspects and acts to deter aphids from initiating settling and feeding on resistant hosts. Further investigations of the resistant and susceptible cowpea NILs (CB77 and CB46, respectively), using EPG combined with microscopy, global gene expression analysis and defense hormone profiles, may shed light on phloem-mediated resistance mechanisms acting over short time frames as well as defense signaling pathways operating over prolonged host-aphid interactions. Ultimately, this information will inform future breeding efforts to develop and deploy elite

cultivars that provide robust cowpea resistance under a variety of crop conditions.

Author contributions

IK conceived the project. IK, KM and PN designed the experiments. JM and QC conducted the experiments. KM, JM, IK, QC and PN analyzed the data. IK, KM, and JM wrote the manuscript with input from all other authors.

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Availability of data and material The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval No approval of research ethics committees was required for this study because experimental work was conducted using an unregulated invertebrate species.

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