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Resistance of True Citrus species to *Diaphorina citri*

Short running title: Resistance of True Citrus to *D. citri*

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ABSTRACT

BACKGROUND: Host genetic resistance is a promising strategy for the management of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), and consequently Huanglongbing (HLB). To date, no study has investigated the resistance to *D. citri* in the clonal and vegetatively propagated plants of the *Microcitrus*, *Eremocitrus,* and *Atalantia* genera. This study assesses Near and True Citrus genotype antixenosis and antibiosis against *D. citri*, with trichome density and volatile emission as possible mechanisms of resistance.

RESULTS: All genotypes were oviposited by *D. citri*, however, 8 of 14 genotypes were less oviposited than *Citrus* × *sinensis* 'Valencia' (susceptible control). *Diaphorina citri* nymphs had lower nymphal viability in *E. glauca* (31%) and *M. warburgiana* (58%) than that in *C.* × *sinensis* (77%). The behavioral assay showed that 30% of *D. citri* nymphs in the last instars evaded *E. glauca* shoots, whereas no nymphs evaded *C.* × *sinensis* shoots. A higher trichome density was observed in *E. glauca* shoots compared to the other genotypes. Chemical analysis revealed differences in the volatile profiles of *E. glauca* and *C.* × *sinensis*.

CONCLUSION: *Eremocitrus glauca* and *M. warburgiana* genotypes were more resistant to *D. citri* than *C.* × *sinensis*. Higher trichome density in the shoots may negatively influence the development of *D. citri* nymphs. *E. glauca* volatiles may also be involved in their resistance to *D. citri*.

KEYWORDS

Host plant resistance, Oceanian citrus species, Asian citrus psyllid, Trichomes, Huanglongbing, Volatiles.

1 INTRODUCTION

The Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), the most important citrus pest worldwide,[1] is the vector of the phloem-limited bacteria '*Candidatus* Liberibacter asiaticus' (*C*Las) and '*Ca.* Liberibacter americanus' (*C*Lam),[1,2] the putative causal agents of Huanglongbing (HLB) a devastating citrus disease.[1,3] *Diaphorina citri* and *C*Las are originated in Asia and have spread worldwide.[1,4] HLB incidence in sweet orange [*Citrus* × *sinensis* (L.) Osbeck] was approximately 100% in Florida, USA[5] and 22.37% in the São Paulo and Minas Gerais citrus belt in Brazil in 2021, which are the most important regions for sweet orange juice production worldwide.[6]

The spread of HLB is associated with the dispersal and feeding of *D. citri.*[7–9] Flushing shoots, young stems, and leaves of most True Citrus species (sensu)[10] and other citrus relatives are the feeding substrates of this psyllid. *D. citri* prefers to feed, oviposit, and develop on flushes at the initial stages of development.[11–13] Although different levels of resistance to *D. citri* exist in species of the family Rutaceae,[14] to date, there has been no resistance to the psyllid identified within the *Citrus* genus (Rutaceae: Aurantioideae).[15] The most economically important *Citrus* varieties are susceptible to *D. citri* and can succumb to *C*Las-bacterial infections.[16,17]

Disease management strategies include planting healthy nursery trees, scouting and eradicating HLB-symptomatic trees, and controlling insect vectors.[18,19] The most commonly used control method is chemical insecticides, primarily for the rapid and efficient reduction of *D. citri* populations.[20,21] However, frequent insecticide application increases the risk of secondary pest resurgence[22], selection of pest populations resistant to insecticides, including *D. citri,*[23,24] and environmental damage. Also, these management strategies have not been able to completely prevent HLB primary infection throughout citrus groves;[8,25] hence, the development of an effective and sustainable control tactic to complement chemical control in reducing the entry of *D. citri* into commercial orchards is crucial for HLB management.

Host plants resistant to *D. citri* can provide an effective, economical, and environmentally safe method of long-term management for HLB. Screening studies for resistance to *D. citri* in sexually compatible species with *Citrus* have revealed that some *Poncirus trifoliata* (L.) Raf. accessions are less colonized.[14,15,26–32] Oceanian citrus species, such as *Eremocitrus glauca* (Lindley) Swingle and *Microcitrus* hybrids, have been shown to be susceptible to oviposition by *D. citri* in a field screening experiment with 87 Rutaceae seed-source genotypes.[14] However, using seedlings of monoembryonic species implies that segregating individuals genetically different from the true-to-type mother genotypes were assessed, which could inflate data variation[29] and interfere in the host response to insects or the identification of these species as either resistant or susceptible to *D. citri*.

Several mechanisms may be involved in the resistance of Citrinae to *D. citri*. George and Lapointe[31] suggested that morphological and physiological barriers associated with access to the phloem sieve elements, poor nutritional quality, and deterrent chemical compounds may be involved in *P. trifoliata* resistance to *D. citri*. Trichome density is a morphological trait that may serve as a defense against insect herbivory[33–36] thereby interfering with insects landing, walking, and feeding on plant surfaces.[37,38] In *Citrus* and *Poncirus*, trichomes have little to no role in deterring oviposition by *D. citri.*[39] However, to the best of our knowledge, the influence of trichome density on the shoots of Citrinae species on *D. citri* development has not been studied.

Olfactory cues play a role in host plant selection by *D. citri*[40] since they discriminate between different host blends.[41] For example, the volatile emission profile of curry leaves (*Bergera koenigii* L.) is more attractive to *D. citri* than sweet orange.[42] Conversely, the volatile emission profile of non-host plants is not attractive[43,44] or deterrent[45,46] to psyllid; a non-attractive/deterrent volatile profile may explain resistance to the psyllid. For example, the lack of attractive compounds or the emission of repellent compounds may explain the resistance of

some *P. trifoliata* accessions to *D. citri* infestation.[47] Meanwhile, inducing the emission of *D. citri*-repellent volatile trans-caryophyllene by an *Arabidopsis thaliana* (L) Heynh. overexpressing sesquiterpenes or a sweet orange overexpressing the same gene can turn these genotypes repellent to *D. citri*.[44,48]

Insight into the resistance of *D. citri* within Oceanian citrus relatives resistant to *C*Las[49,50], such as *Microcitrus*, *Eremocitrus*, and their hybrids with *Citrus*, is important for breeding programs to develop commercial cultivars resistant to *D. citri*. This study assesses Near and True Citrus genotype antixenosis and antibiosis against *D. citri*, in addition to trichome density and volatile emission as possible mechanisms of resistance. To our knowledge, this is the first report of resistance to *D. citri* in clonal, vegetatively propagated plants of the *Microcitrus* and *Eremocitrus* species and hybrids. This study is also the first to report antixenosis and antibiosis responses to *D. citri* in *Eremocitrus* and *Microcitrus* genotypes through laboratory experiments, where we minimized the interference from biotic and abiotic factors, which could negatively influence the results. Our results suggest that a higher trichome density in *E. glauca* may be associated with resistance to *D. citri*. The volatile emission profile of *E. glauca* may also be related to deterrence to *D. citri*.

2 MATERIAL AND METHODS

2.1 Plant material

Bioassays with *D. citri* were performed on *Microcitrus, Eremocitrus, Atalantia, Citrus,* and intergeneric hybrids among them (Table 1). *C.* × *sinensis* (L.) Osbeck 'Valencia' was used as a susceptible control. Budwood of these genotypes was grafted as inverted T-budding onto nucellar seedlings of 'Rangpur' lime (*C.* × *limonia* Osbeck) sowed in 240 mL

plastic tubes filled with coir (Figure S1). Approximately 50 grafted plants were produced for each Citrinae genotype.

Plants were watered twice a week with a diluted solution of water-soluble fertilizers [nitrogen (92.5 mg/L), potassium (84.8 mg/L), phosphorus (30.15 mg/L), magnesium (56.7 mg/L), calcium (69.7 mg/L), sulfur (75.6 mg/L), iron (2.16 mg/L), copper (2.24 mg/L), zinc (0.54 mg/L), manganese (0.41 mg/L), boron (0.29 mg/L), and molybdenum (0.12 mg/L)]. Plants were maintained in a screenhouse at the Fund for Citrus Protection (Fundecitrus) in Araraquara, São Paulo State, Brazil, for approximately 1 year after grafting (until plants were pruned to carry out the experiments). The mean daily air temperature in the screenhouse ranged between 18.5 and 34.4 ºC under natural light sources.

2.2 Insects

Diaphorina citri adults were obtained from a colony free of *Ca.* Liberibacter sp., a batch initiated in 2009, and maintained in a climate-controlled room (26 ± 2 °C, relative humidity 60 ± 10%, and 14:10-h light:dark period) on *Murraya paniculata* L. plants.

2.3 Oviposition preference assay

A no-choice test to study *D. citri* oviposition preference in Citrinae genotypes was arranged in a completely randomized design with 20 plants (replicates) for each genotype (Table 1). All plants from the genotypes were pruned approximately 15 days before the assay and maintained under similar climatic conditions as the insect colony. Plants with a single flush shoot (1.5 to 2 cm in length, Figure S2) were used, and one 10- to 15-day-old mated female was confined onto the shoot using a tulle "sleeve" cage for 48 h. The number of eggs per shoot was counted under a stereoscopic microscope.

2.4 Antibiosis assay

An antibiosis assay to *D. citri* was performed on five Citrinae genotypes (*M. australis* hybrid, *M. warburgiana*, *E. glauca*, *E. glauca* × *C.* × *sinensis* hybrid, and *C.* × *sinensis* as a susceptible control). These genotypes were selected based on the results of the previous assay of *D. citri* oviposition preference (section 3.1) and due to their resistance to *C*Las.[50] Fifty plants of each genotype were pruned approximately 15 days before the assay and maintained under similar climatic conditions as the insect colony. Two 10- to 15-day-old mated females were confined on the single flush shoot (1.5 to 2 cm in length) of each plant using a tulle "sleeve" cage for 4 h. The number of eggs per shoot was counted without detaching the shoots from the plants 3 days after *D. citri* oviposition. Plants with 20 ± 10 eggs per flush shoot (≈ 20 plants per genotype) were selected to trace the development of the nymphs in a completely randomized design. The number of hatched nymphs and unviable eggs was counted 7 days post oviposition. Subsequently, a metallic cage with a tulle screen was used on the flush shoot of each plant until the emergence of adults. Adult emergence was observed daily, and the emerged psyllids were sexed to determine the sex ratio [♀/ (♀ + ♂)] and assessed for morphological deformities.

2.5 Behavioral assay

Based on the antibiosis assay showing that *E. glauca* induced high *D. citri* nymphal mortality in their final development stages compared to *C.* × *sinensis* (susceptible control), a behavioral assay was conducted. Initially, mated *D. citri* females (10–15 days after emergence) were confined for 4 h in flush shoots (1.5 to 2 cm in length) of *E. glauca* and *C.* × *sinensis* for oviposition. These shoots were observed daily until the nymphs reached the third instar. Other *E. glauca* and *C.* × *sinensis* plants with a single uninfested flush shoot (≈ 7 cm in length) and black rectangular cardboard (25 cm wide × 40 cm length) were fitted using a metallic structure and adhesive tape. Entomological glue was placed on the black cardboard perimeter and around

the plant stem (Figure 1), following which groups of 10 third-instar *D. citri* nymphs from previously infested plants were transferred with a fine brush to each uninfested shoot of plants of the same genotype attached to the black cardboard (Figure 1). The number of live and dead nymphs on the flush shoot, black cardboard, and entomological glue arranged around the stem or on the black cardboard perimeter was assessed daily until adult emergence. Dead nymphs observed on the cardboard in the region near the stem or on entomological glue around the stem were considered nymphs that evaded the plant, while dead nymphs observed on the plant or on the cardboard central region were considered nymphs that died on the plant. Twenty plant replicates (10 nymphs per plant) arranged in a completely randomized design were used for each genotype, totaling 200 nymphs per genotype.

2.6 Trichome density analysis

The trichome density was determined for the five genotypes used in the antibiosis assay. In each genotype, 10 shoots of each age, 8- and 14-day-old, from different plants were evaluated (Figure S3), which had a mean length of 4.2 ± 0.23 cm and 7.5 ± 0.65 cm, respectively. To stimulate the emission of flushes, plants of each genotype were pruned and kept in a climate-controlled room under similar conditions as those used in the bioassays. Trichomes were quantified in three circular areas of 0.2 mm^2 of the same flush shoot on the median stem region of shoots and on both leaf sides (abaxial and adaxial) in the central region of the midrib (Figure 2). The assessed leaves were detached from the apical third of the shoots.

2.7 Volatile emission analysis

Based on the antibiosis and behavioral assays showing that *E. glauca* induced high *D. citri* nymphal mortality compared to *C.* × *sinensis* (section 3.2 and 3.3), a volatile emission analysis was performed. Headspace solid-phase adsorption and microextraction (HS-SPME) of

volatiles from *E. glauca* and *C.* × *sinensis* flushes (≈ 7 cm) were performed as previously described.[53] In brief, volatiles from approximately 400 and 100 mg of *E. glauca* and *C.* × *sinensis* flushes, respectively, accumulated in the headspace of Pyrex tubes for approximately 4 h at room temperature and subsequently adsorbed on 65 µm poly(dimethyl) siloxane/divinylbenzene fiber (Supelco Inc., Bellefonte, PA, USA) for 40 min at 22 °C. Volatile chromatography and analysis were performed at the Instituto de Biologia Molecular y Celular de Plantas (IBMCP) Metabolomics Platform. Volatile desorption and injection were performed using a 6890 N gas chromatograph (Agilent Technologies Inc., Las Rozas de Madrid, Spain) coupled to a 5975 B inert XL MSD mass spectrometer (Agilent Technologies Inc., Las Rozas de Madrid, Spain). We used a DB-5ms column (60 m × 0.25 mm i.d., 1-µm film thickness; J&W Scientific) and helium as the carrier gas at a flow rate of 1.4 mL min^{-1}. The temperature program was as follows: 40 °C hold for 2 min, then a 5 °C min^{-1} ramp to 250 °C, and a 5-min hold at 250 °C. Mass spectra were obtained at an ionization energy of 70 eV and a scan speed of 7 scans s^{-1}, with a mass-to-charge ratio scan range of 35–220. At least three independent pooled samples (a mix of flushes from at least three independent plants) from each genotype were analyzed. Compounds were identified comparing to a custom library generated using authentic standards as described by Gonzalez-Mas et al.[54] or to the NIST 2017 Mass Spectral library. Untargeted analysis and peak quantification were performed using Masshunter software (Agilent Technologies, Las Rozas de Madrid, Spain).

2.8 Statistical analyses

Data from the number of eggs per shoot (oviposition preference assay) and trichomes per 0.2 mm^2 in each shoot structure were analyzed using generalized linear models (GLM)[55] with quasi-Poisson distribution. Antibiosis assay data were analyzed using GLM with a quasi-Poisson distribution for the number of eggs, quasi-binominal distribution for nymphal viability,

sex ratio, and adult deformity, and Gaussian distribution for the egg to adult period. The goodness of fit for all variables described above was determined through a half-normal graph with a simulation envelope using the "hnp" package.[56] In cases of significant differences, multiple comparisons among treatments were performed using Scott-Knott test ($\alpha < 0.05$) for oviposition preference assay and Tukey test ($\alpha < 0.05$) for variables of the antibiosis assay and trichome density analysis. Behavioral assay data were analyzed using the Wilcoxon rank-sum test ($\alpha < 0.05$). All analyses were conducted using R statistical software version 3.6.1.[57] Additionally, a hierarchical clustering analysis was performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/).

3 RESULTS

3.1 Oviposition preference assay

Some *Microcitrus* species and hybrids, including both *M. australasica* genotypes, *E. glauca* × *Microcitrus* sp. hybrid, *Microcitrus* sp. hybrid, *M. inodora, M. warburgiana*, as well as *C. halimii* and *A. buxifolia*, were less oviposited by *D. citri* than *C.* × *sinensis*. In contrast, the *E. glauca* × *C.* × *sinensis* hybrid was the genotype most oviposited by *D. citri,* even more than both parents ($F = 3.94$; df = 13, 266; $P < 0.0001$) (Figure 3).

3.2 Antibiosis assay

Genotypes less-oviposited (*Microcitrus warburgiana*), equally-oviposited (*E. glauca*, *M. australis* hybrid), and more-oviposited (*E. glauca* × *C.* × *sinensis* hybrid) than *C.* × *sinensis* by *D. citri* in the previous assay (section 3.1) were selected for the antibiosis assay to *D. citri*, with *C.* × *sinensis* as the susceptible control. The initial number of eggs per flush shoot (16.8–20.7 eggs) ($F = 1.27$; df = 4, 104; $P = 0.2847$), egg viability (81.3%–91.4%) ($F = 2.37$; df = 4,

104; $P = 0.0570$), and initial number of nymphs per flush shoot (15.0–17.7 nymphs) (F = 1.27; df = 4, 104; $P = 0.2851$) were similar among the genotypes assessed. The lowest nymphal viability was observed in *D. citri* that developed on *E. glauca*. *Microcitrus warburgiana* also showed a lower nymphal viability than *C.* × *sinensis* but similar to *M. australis* hybrid (F = 20.02; df = 4, 104; $P < 0.0001$) (Figure 4). The sex ratio (0.43–0.56, F = 0.74; df = 4, 98; $P = 0.5644$) and percentage of deformed adults (0.8%–3.4%, F = 0.30; df = 4, 98; $P = 0.8745$) were similar among the tested genotypes.

3.3 Behavioral assay

Based on the antibiosis results, possible deterrence of *D. citri* nymphs was investigated in *E. glauca*. In the behavioral assay, 30.5 ± 7.5% of the nymphs (third to fifth instars) evaded *E. glauca* plants and died on the black cardboard in the region near the stem or on the entomological glue around the stem, whereas no nymphs evaded *C.* × *sinensis* (W = 300.00; $P = 0.0003$). Nymphal mortality in the flush shoots was also higher in *E. glauca* (22.0 ± 5.0% of mortality) than *C.* × *sinensis* (8.0 ± 1.56% of mortality) (W = 127.00; $P = 0.0374$); 2% and 0.5% of the nymphs on *E. glauca* and *C.* × *sinensis* shoots, respectively, were alive on the cardboard central region or dead on the entomological glue placed on the perimeter of the cardboard. These nymphs were not considered evaded or dead on plants.

3.4 Trichome density analysis

Trichome densities in both 8- and 14-day-old shoots were significantly higher in *E. glauca* than in the other genotypes regardless of the shoot structure assessed. Moreover, a higher trichome density was observed in the adaxial leaf surface and stem of *M. warburgiana* than in these same shoot structures of the genotypes *E. glauca* × *C.* × *sinensis* hybrid, *M. australis* hybrid, and *C.* × *sinensis* (Table 2).

3.5 Volatile emission analysis

The volatile emission profiles of the *E. glauca* and *C.* × *sinensis* flushes were different (Figure 5). Monoterpene and sesquiterpene compounds were predominant in *C.* × *sinensis*. In the *E. glauca* volatilome, monoterpene content was reduced, while few monoterpenes emitted by *C.* × *sinensis* [terpinen-4-ol, (*Z*)-sabinene hydrate, citronellal, α-thujene, isoterpinolene, cosmene, methyl geranate, geranyl acetate, geraniol, and 2-carene] were not detected in *E. glauca*. Among sesquiterpenes, only β-caryophyllene, α-humulene, and α-farnesene were detected in the *E. glauca* emission profile. The profile of fatty acid-derived volatiles also differed between the two genotypes. In addition, amino acid-derived volatile compounds were detected only in the *E. glauca* emission profile.

4 DISCUSSION

An oviposition preference assay of *D. citri* with clonal and vegetatively propagated plants of 14 genotypes (*Microcitrus*, *Eremocitrus*, *Atalantia*, and *Citrus* genera and their intergeneric hybrids) was performed under laboratory conditions. Interference from biotic and abiotic factors was minimized once the climatic conditions, insect density per flush shoot, insect age, oviposition period, and flush shoot size used in the assays were standardized, with no risk of egg predation in the laboratory, which could also negatively influence the results. All genotypes studied were oviposited by *D. citri,* corroborating previous findings, which showed that several citrus relatives within the Rutaceae family are oviposited by this psyllid species.[14,58,59] *Microcitrus* sp. hybrid, *M. australasica* 'Sanguinea' and 'True Sanguinea', *M. inodora*, *E. glauca* × *Microcitrus* sp. hybrid, *C. halimii*, *A. buxifolia*, and *M. warburgiana* were deterrents to *D. citri* oviposition in relation to *C.* × *sinensis*, decreasing the number of eggs in these Citrinae hosts by up to 40%. In a greenhouse experiment in which 15-day-old *D. citri* adults were confined to a single flush shoot (initial stages of development) for 72 h to lay eggs,

Microcitrus sp. hybrid and *A. buxifolia* were less oviposited than *C.* × *sinensis.*[58] In a previous study, *M. australis* hybrid, *M. australasica* 'Sanguinea', *M. inodora*, *E. glauca, C. halimii,* and *A. buxifolia* did not decrease *D. citri* oviposition compared to *C.* × *sinensis.*[14] This variation in the results may be attributed to experimental procedural differences. Westbrook et al.[14] conducted a field screening experiment with 87 seed-source genotypes in which the number of *D. citri* eggs, nymphs, and adults were quantified in the shoots of these genotypes monthly for 4 months. Under field conditions, several factors related to the environment, insect, and host plant, which could interfere with the results, cannot be controlled. Other studies have shown that *P. trifoliata* accessions within the subtribe Citrinae were also less oviposited by *D. citri* compared to *Citrus* accessions.[15,27–29,31] However, the defense mechanisms responsible for the deterrence of some Citrinae genotypes to *D. citri* oviposition remain to be elucidated.

Physical, morphological, and chemical mechanisms may be involved in host selection for insect oviposition.[60] Trichome density was a morphological trait assessed in the genotypes used in the antibiosis assay. Although *E. glauca* had a significantly higher trichome density than *C.* × *sinensis,* which in turn was considered glabrous (without trichomes), they were both oviposited similarly, suggesting that trichome density did not influence *D. citri* oviposition, which corroborates the results in the literature comparing this trait in *Citrus* types versus *P. trifoliata, M. paniculata,* and *B. koenigii.*[39] Other morphological mechanisms, such as shoot architecture and tissue hardness, may be involved in *D. citri* oviposition preference.[61,62] Chemical compounds produced by the secondary metabolism of citrus plants and the nutritional quality of shoots are other possible mechanisms involved in the deterrence to *D. citri* oviposition in some of the True Citrus species.[31]

In the antibiosis assay, egg viability and the number of hatched nymphs were the same among all genotypes assessed. Thus, all genotypes had similar insect densities at the beginning of the assay. Using different insect densities in host selection assays may interfere with the

insect response to the host, which influence the results.[38] *Diaphorina citri* nymphal viability on *E. glauca* and *M. warburgiana* was lower than that on *C.* × *sinensis*, indicating that suitable hosts for *D. citri* oviposition (Figure 3) may not be appropriate for nymphs feeding or development. The lowest nymphal viability in *E. glauca* likely occurred due to the higher trichome densities in its flush shoots compared to the other genotypes. In *E. glauca* flush shoots, trichomes were more evenly distributed across their perimeter, which likely interfered negatively with nymph feeding and/or development. Such trichomes are defined as appressed grayish hairs by Swingle and Reece.[51] No studies have associated the resistance of Citrinae genotypes to *D. citri* with trichome density. In a previous study, trichome density and sizes of six cultivars of the Rutaceae species had little to no role in reducing *D. citri* oviposition,[39] although the authors did not assess the behavior and development of nymphs. Hence, to the best of our knowledge, this is the first study to suggest that high trichome densities in flush shoots may affect *D. citri* nymphal viability in Citrinae genotypes.

Microcitrus warburgiana showed a higher trichome density than *C.* × *sinensis* but significantly lower than *E. glauca*, while no trichomes were observed on the abaxial side of *M. warburgiana* leaves; they were distributed only in the midrib of the leaf adaxial side and shoot stem. The lower nymphal viability in *M. warburgiana* shoots may have occurred due to other defense mechanisms, such as chemical compounds, low nutrient contents present in the leaf, or others morphological traits. Previous studies have shown that *P. trifoliata* has antibiosis effects on *D. citri*[27,29,31], which may be related to several factors including morphological and physiological barriers[31]. Histological work on *P. trifoliata* illustrated the presence of a sclerenchymatous fibrous ring around the vascular bundle, which may act as a physical barrier to prevent psyllid stylets from reaching the phloem[63,64]. *P. trifoliata* leaves contain flavonoid compounds[65], an important group of plant defense metabolites that may negatively interfere with insect feeding, oviposition, and development.[66–68] Histological and metabolomics studies

may help further elucidate the defense mechanisms involved in the resistance of *E. glauca* and *M. warburgiana* to *D. citri*.

In this study, although not quantified, *D. citri* nymphs were observed on the leaves of the shoot terminal portion and between the axillary bud and stem in *C.* × *sinensis*, *E. glauca* × *C.* × *sinensis* hybrid, *M. warburgiana,* and *M. australis* hybrid. In different psyllid hosts (*M. paniculata*, *C. jambhiri* Lush, *C. aurantium* L., and *C. paradisi* Macfad.), nymphs are also often found in the shoot terminal portion.[62] Interestingly, nymphs on *E. glauca* were often observed along the stem instead of the shoot terminal portion of leaves, which suggests that this genotype accumulates deterrent compounds to the psyllid specifically or more abundantly in young leaves than in stems.

In *E. glauca,* unlike the other genotypes, *D. citri* nymph mortality was higher in the last stages of development (third to fifth instars), while dead nymphs were usually found in the confinement cage. To gain insight into this, a behavioral assay was performed. Approximately 52% of *D. citri* nymphs of the third to fifth instars did not develop, while 22% had died on shoots and more than 30% transferred to *E. glauca* evaded the shoots and were stuck on the black cardboard entomological glue around the stem, suggesting deterrence of the shoots to *D. citri* feeding, since nymphs evaded the plant, probably trying to find a more suitable feeding sites. *Diaphorina citri* nymphs in the early development stages are less mobile than those in later stages,[62] which explains the higher nymph evasion in third- to fifth-instar nymphs. The demand for better nymph feeding sites may be attributed to the higher trichome densities, which could interfere with the feeding and/or development of *D. citri* nymphs. Alternatively, some non-terpene volatiles detected in the *E. glauca* profile but absent in sweet orange could be deterrent to the psyllid. This may be the case of 3-hexenyl butyrate, also identified in the non-host *Anacardium occidentale* L., which has been previously postulated as a putative *D. citri* repellent.[41]

All Citrinae genotypes caused low adult deformity (< 3.4%), usually observed in the wings. Other studies on *D. citri* biology in Rutaceae species have also shown low morphological deformities in emerging adults.[28,69] The sex ratio of the adults that emerged in the assessed hosts was approximately 0.5, similar to that reported in other studies.[28,69–71]

In this study, we identified sources of resistance to *D. citri* in *Eremocitrus* and *Microcitrus* species sexually compatible with *Citrus* species, and, for the first time, showed that higher trichome densities may influence the behavior of *D. citri* nymphs, hampering their development. The volatile profile of *E. glauca* may be related to its deterrence of *D. citri*. *Eremocitrus glauca* and *M. warburgiana* genotypes showed the potential to generate genetic resistance against *D. citri* if used in breeding programs aimed at developing commercial *Citrus* or *Citrus*-like cultivars resistant to psyllid.

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6 CONFLICT OF INTEREST

The authors declare no conflict of interest.

7 AUTHOR CONTRIBUTIONS

Conceptualization: MPM, WIE, LP, and HLXV. Investigation: WIE, RBG, BA, and AER. Formal Analysis: WIE, BA, and AER. Funding Acquisition: MPM and LP. Writing – Original Draft Preparation: WIE. Writing – Review & Editing: WIE, MPM, HLXV, RBG, EAG, BA, AER, and LP. All authors critically revised the intellectual content and approved the final version to be published.

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9 FIGURE LEGENDS

Figure 1. Schematic illustration of the behavioral assay to assess possible deterrence of *Diaphorina citri* nymphs to *Eremocitrus glauca*. Genotype plants with flush shoots of 1.5 to 2 cm in length were oviposited by *D. citri* females. Groups of 10 third-instar nymphs that hatched from these eggs were transferred to an uninfested shoot of a plant of the same genotype. Before *D. citri* nymphs were transferred to the uninfested shoot, a black rectangular cardboard was fit together in each plant below the uninfested shoot. In the cardboard, an entomological glue was applied on the perimeter and around the stem.

Figure 2. Stereomicroscopy images showing the assessment region of trichomes on 14-day-old flush shoots. Each row is used for a separate genotype, as indicated at the left. The median stem region of the shoots is shown in the first column. The abaxial and adaxial leaf surfaces are shown in the second and third columns, respectively (scale bar = 0.5 mm).

Figure 3. Number of eggs per flush shoot (1.5 to 2 cm in length) laid by one *Diaphorina citri* on Citrinae genotypes in 48 h. Bars (mean ± SEM, n = 20) followed by the same letter did not differ significantly by ANOVA using GLM with quasi-Poisson distribution, followed by post hoc Scott-Knott test ($\alpha = 0.05$). The white column is the susceptible control treatment.

Figure 4. Nymphal viability of *Diaphorina citri* on Citrinae genotypes. Bars (mean ± SEM, n = 20) followed by the same letter did not differ significantly by ANOVA using GLM with quasi-binomial distribution, followed by post hoc Tukey test ($\alpha = 0.05$). The white column is the susceptible control treatment.

Figure 5. Heatmap clustering representation of volatile diversity between *Eremocitrus glauca* (EG) and *Citrus* × *sinensis* (CS) flush shoots. Identified volatiles are in rows, while samples in columns, in which numbers 1 to 4 indicate biological replicates. Monoterpene (and derivatives) and sesquiterpenes are represented by red and green letters, respectively, while fatty acid and amino acid derivatives are indicated in blue and yellow letters, respectively. Compounds identified by comparison with chemical standards are indicated with an asterisk. Remaining compounds were identified based on NIST library comparison.

Resistance of True Citrus species to *Diaphorina citri*

Wellington Ivo Eduardo*; Marcelo Pedreira Miranda; Haroldo Xavier Linhares Volpe; Rafael Brandão Garcia; Eduardo Augusto Girardi; Berta Alquezar; Ana Espinosa Ruiz; Leandro Peña

Eremocitrus glauca reduced *Diaphorina citri* nymphal viability by 60% compared to *Citrus* × *sinensis*. Trichome density and volatile compounds of *E. glauca* may be the resistance mechanisms to *D. citri*.

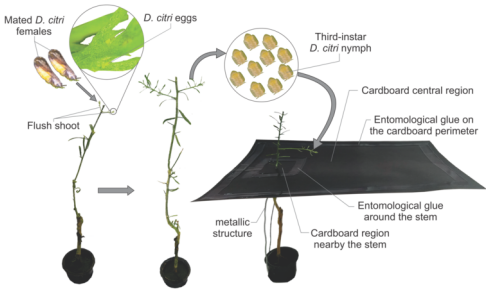


Fig 1.tif

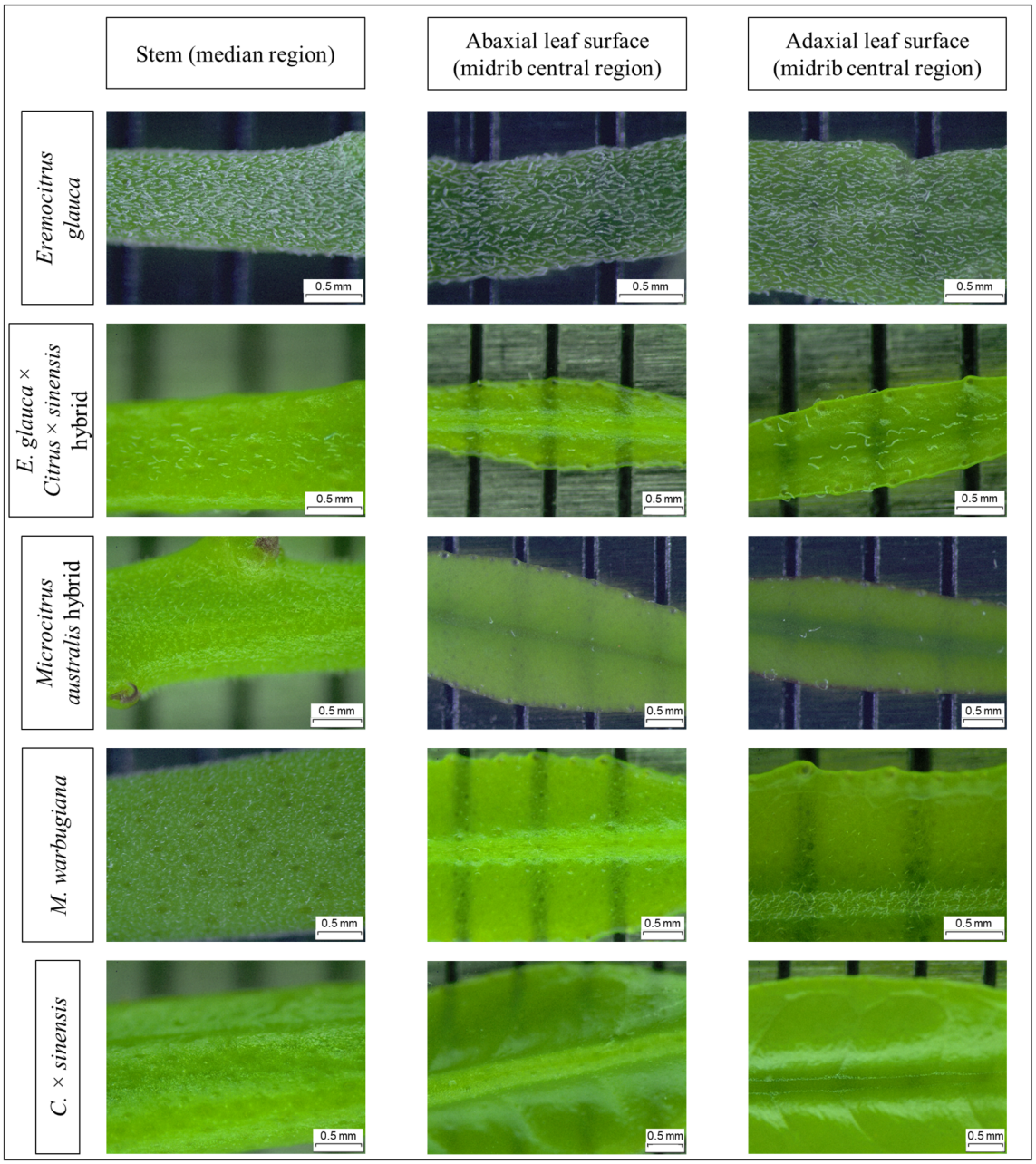


Fig 2.tif

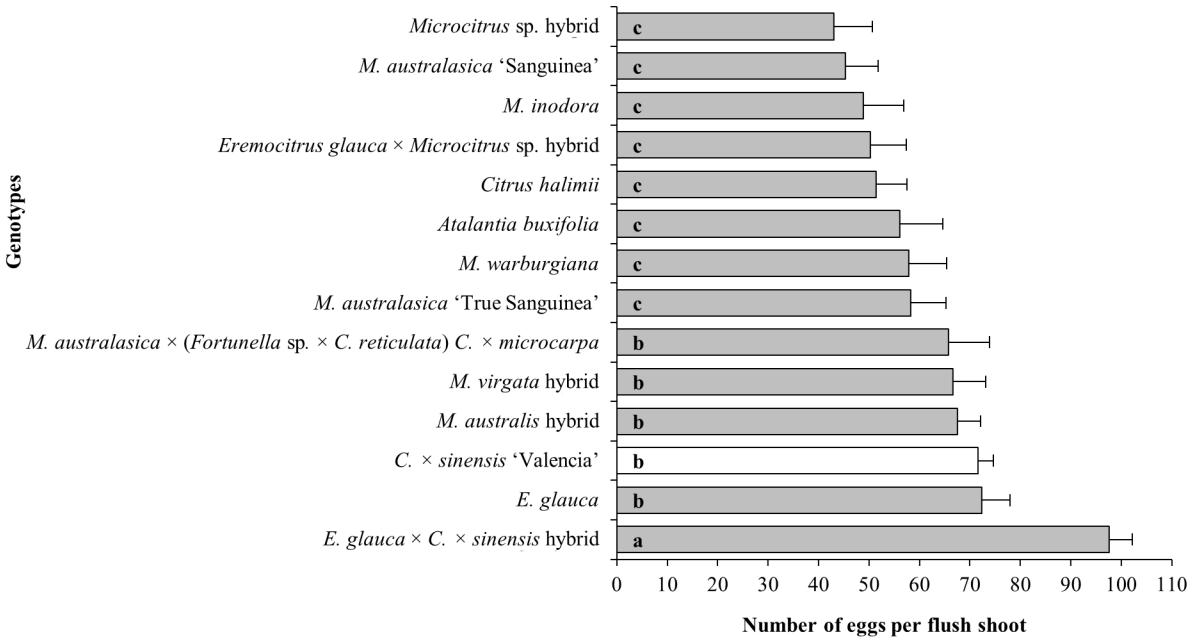


Fig 3.tif

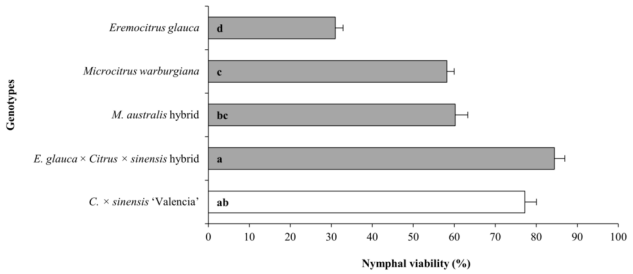


Fig 4.tif

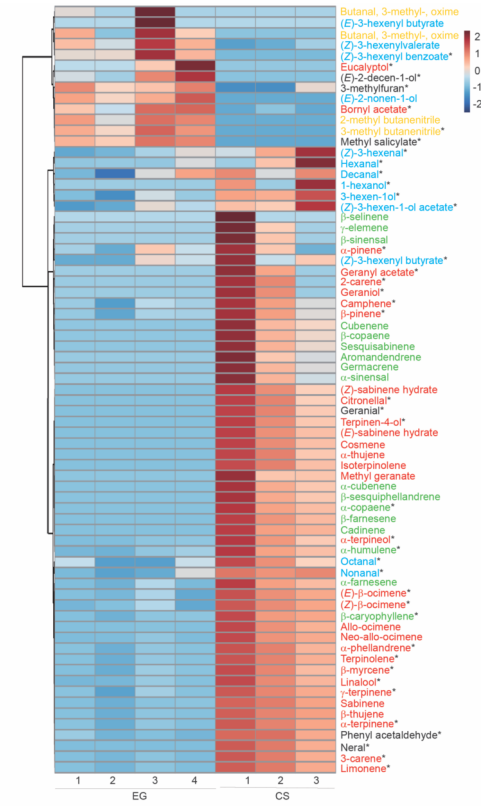
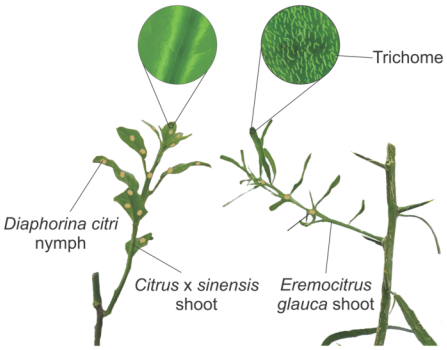


Fig 5.tif



Graphical abstract.tif

Table 1. Citrinae genotypes (embryony classification) used in the bioassays to assess resistance to *Diaphorina citri*.

Citrinae genotypes[a]	Common name
Microcitrus australasica (F. Muell.) Swingle 'Sanguinea' **(M[c])**	'Sanguinea' Australian finger lime
M. australasica 'True Sanguinea'**(M)**	'True Sanguinea' Australian finger lime
M. inodora (F.M. Bail) Swingle **(M)**	Australian large-leaf wild lime
M. australis hybrid **(M)**	Australian round lime hybrid
M. virgata hybrid **(PM[d])**	'Sydney' hybrid
M. warburgiana (F.M. Bailey) Tanaka **(M)**	New Guinean wild lime
Eremocitrus glauca (Lindl.) Swingle **(M)**	Australian desert lime
E. glauca × *Microcitrus* sp. hybrid **(PM)**	Australian desert lime hybrid BGC 682[b]
Microcitrus sp. hybrid **(PM)**	Australian finger lime-like hybrid BGC 695[b]
E. glauca × *Citrus* × *sinensis* (L.) Osbeck hybrid **(P[e])**	Eremorange
C. halimii B.C. Stone **(M)**	'Mountain' citron
Atalantia buxifolia (Poir.) Tenore	Chinese box orange (brachytic form)
M. australasica × (*Fortunella* sp. × *C. reticulata* Blanco) *C.* × *microcarpa* (Bunge) Wijnands **(PM)**	'Faustrimedin' hybrid 'Calamondin'
C. × *sinensis* 'Valencia' **(P)**	'Valencia' sweet orange

[a]The nomenclature used follows that of sensu Swingle and Reece[51] and Bayer et al.[10] [b]Accession number at the Citrus Germplasm Bank (BGC) of EMBRAPA Cassava & Fruits in Cruz das Almas, Bahia, Brazil. [c]M, monoembryonic; [d]PM, possibly monoembryonic; [e]P, polyembryonic. Polyembryony was classified according to Swingle and Reece[51] and Bitters.[52]

Table 2. Number of trichomes per 0.2 mm^2 (mean ± SEM) on structures (abaxial and adaxial sides of leaves from apical part and in the median stem region) of 8- and 14-day-old shoot from Citrinae genotypes.

Genotypes	Structure shoot/number of trichomes per 0.2 mm^2					
	8-day old shoot			14-day old shoot		
	Abaxial leaf surface	Adaxial leaf surface	Stem	Abaxial leaf surface	Adaxial leaf surface	Stem
Eremocitrus glauca	132.4 ± 8.39 a	185.9 ± 6.51 a	140.2 ± 8.43 a	134.5 ± 6.89 a	157.8 ± 4.08 a	72.1 ± 4.31 a
Microcitrus warburgiana	0.6 ± 0.31 b	87.8 ± 6.20 b	91.7 ± 7.12 b	0.6 ± 0.22 b	54.3 ± 5.74 b	36.9 ± 6.22 b
E. glauca × *Citrus* × *sinensis* hybrid	2.0 ± 0.44 b	2.0 ± 0.74 c	49.4 ± 4.42 c	2.1 ± 0.64 b	3.6 ± 1.64 c	17.3 ± 1.46 c
M. australis hybrid	1.5 ± 0.22 b	1.0 ± 0.21 c	23.2 ± 3.81 d	0.4 ± 0.16 b	0.9 ± 0.18 c	11.7 ± 3.18 c
Citrus × *sinensis*	0.2 ± 0.20 b	0.5 ± 0.17 c	0.0 ± 0.00 e	0.2 ± 0.20 b	0.4 ± 0.16 c	0.0 ± 0.00 d
F	480.08	604.46	146.20	566.03	322.47	67.50
df	4, 45	4, 45	4, 45	4, 45	4, 45	4, 45
P	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Means followed by the same letter for each shoot structure did not differ significantly based on the Tukey test (α = 0.05).