Epifluorescence and Stereomicroscopy of Trichomes Associated with Resistant and Susceptible Host Plant Genotypes of the Asian Citrus Psyllid (Hemiptera: Liviidae), Vector of Citrus Greening Disease Bacterium

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Abstract

Background and Significance: Foliar trichomes (tiny hair-like structures) are part of the plant defense mechanisms that may confer resistance to some herbivore pests. Trifoliate orange, *Poncirus trifoliata*, is a genotype resistant to infestations by the Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae), vector of the economically important citrus greening (huanglongbing) disease bacterium. We discovered that dense trichomes are associated with young leaves of trifoliate orange plants and hypothesized that these might be responsible for reduced infestation by this psyllid. **Materials and Methods:** Epifluorescence and stereomicroscopy were used to study the density and structure of trichomes associated with young flush leaves and stems of trifoliate orange and of five other plant genotypes that are highly susceptible to colonization by the psyllid: lemon, grapefruit, sweet orange, curry leaf, and orange jasmine. **Results:** Simple unicellular trichomes were observed at moderate-to-large densities on young leaves and stems of each genotype except lemon and sweet orange, which had considerably fewer trichomes. Trichomes were generally abundant on young leaves of curry leaf and orange jasmine, two genotypes that are often heavily colonized by the psyllid. Although we did not quantify oviposition rates on these genotypes, we observed that psyllid females deposited eggs on young leaves, buds, and stems regardless of the density of trichomes present, sometimes directly within or close to a dense bed of trichomes. **Conclusions:** While trichomes were moderately abundant on young leaves of trifoliate orange, our results strongly suggest that these trichomes may play little or no role in reduced colonization by the psyllid on this genotype.

Keywords: Asian citrus psyllid, autofluorescence, citrus greening, huanglongbing, trichomes

INTRODUCTION

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is an important pest mainly because it transmits '*Candidatus* Liberibacter asiaticus' (*C*Las), the putative bacterial agent causing a serious worldwide disease of citrus known as citrus greening, also known as huanglongbing.[1] Citrus trees with this disease frequently become unthrifty to the point they are no longer economically viable.[2] Once a tree is infected, there is no cure for the disease. Growers attempt to prevent the introduction and spread of *C*Las primarily using intensive insecticide programs against the psyllid[3,4] but usually with limited success.[5] In addition to being a relatively ineffective management tactic against huanglongbing, the intensive use of insecticides against the vector is not sustainable based on both economic and environmental factors. Alternative short-term and long-term management strategies for the psyllid are needed. Host plant resistance to *D. citri* is one of the major alternative tactics considered.[2,3]

Host plant species utilized by the psyllid for food and reproduction fall almost exclusively within the plant family Rutaceae, subfamily Aurantioideae[4,6] While *Citrus* species are known to be among the most preferred genotypes for

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colonization by *D. citri*, there are closely related genotypes that are considerably less susceptible to infestations[7] and some of these can readily hybridize with *Citrus*. Trifoliate orange, *Poncirus trifoliata* (L.) Raf., is one such genotype, reported many years ago as having traits that discourage infestations of *D. citri*.[8] Trifoliate orange is an important germplasm source for citrus scion and rootstock improvement, especially with respect to cold hardiness and pest resistance.[9,10] Recent research reports support that trifoliate orange is an un-preferred (less colonized) host of *D. citri*.[11,12] although the level of resistance to the psyllid has been variable possibly due to environmental conditions, plant age, and other factors. Both antixenosis and antibiosis types of plant resistance to *D. citri* have been noted in trifoliate orange,[11,13] but the specific traits conferring resistance remain unclear. With respect to reduced oviposition rates on trifoliate orange, it has been thought that kairomones or secondary plant metabolites may be involved,[14] but factors related to plant morphology have not been investigated so far.

Field observations during the spring and summer of 2016 on low *D. citri* infestation densities associated with several trifoliate orange cultivars revealed that there were fairly dense trichomes (tiny hair-like structures) present on young leaves while comparable leaves from a sweet orange cultivar (*Citrus sinensis* [L.] Osbeck) were mainly glabrous (with none or very few trichomes present). Pubescence (having dense trichomes) in other plants has been reported to confer resistance to some insect herbivores.[15-21] A literature review indicated that a majority of botanical descriptions of trifoliate orange failed to mention leaf trichomes including a comprehensive review by Swingle and Reece.[22] We, therefore, decided to investigate trichomes associated with the psyllid-resistant trifoliate orange plants in comparison to five other genotypes regularly colonized by *D. citri*: lemon (*Citrus jambhiri* Lush.), grapefruit (*Citrus paradisi* Macf.), sweet orange (*C. sinensis*), curry leaf (*Bergera koenigii* L.), and orange jasmine (*Murraya paniculata* [L.] Jack).

Foliar trichomes are highly variable appendages associated with the epidermis of plant leaves and stems. They may be unicellular, multicellular, glandular or nonglandular,[23,24] and have forms that are stellate, peltate, long-stalked, short-stalked, or capitate.[25] Different types of trichomes may occur together on some plant species such as tomatoes.[26] In addition to imparting resistance to insect herbivores, trichomes can contribute to the control of transpiration, leaf temperature, and protection against ultraviolet (UV)-B radiation.[20,27,28] The expression and density of leaf trichomes can vary with a leaf's age[29] and location on a plant.[30] Depending on the action of genes involved in trichome expression, the progeny of some pubescent plants may include both pubescent and nonpubescent individuals.[26]

The purpose of the research presented here was to determine if trichomes might be responsible for resistance in trifoliate orange to infestations by *D. citri*. We used epifluorescence, light, and stereomicroscopy to characterize the structure (unicellular/multicellular), shape, location, and density of trichomes associated with young leaves and stems of seedlings of the six aforementioned genotypes, and to investigate if Asian citrus psyllid avoids ovipositing in the vicinity of trichomes. In addition, a field survey of 5-year-old trees in a USDA grove in Florida was conducted to determine if foliar trichomes were associated with young leaves of one sweet orange cultivar, a tangor cultivar, six trifoliate orange cultivars, and four trifoliate hybrids.

MATERIALS AND METHODS

This study focused on trichomes associated with young developing leaves and stems associated with flush shoots, previously described by Hall and Albrigo.[31] Flush shoots were chosen because Asian citrus psyllid reproductive biology is highly dependent on young foliage being present.[4]

Trichomes associated with the six studied genotypes were investigated by surveying greenhouse seedlings (6–10 months old) growing in individual plastic pots (3.8 cm dia. by 21 cm) (SC-10 supercell "Cone-tainers," Stuewe and Sons, Tangent, OR) containing steamed potting mix (Pro-Mix BX, Premier Horticulture, Inc., Quakertown, PA). The seedlings were watered on an as-needed basis and fertilized weekly with a general purpose 20N-10P-20K water-soluble fertilizer mix (Peters Professional, The Scotts Company, Marysville, OH). The specific cultivars studied were "Duncan" grapefruit, "Rough" lemon, "Ridge Pineapple" sweet orange, and "Flying Dragon" trifoliate orange. The specific curry leaf cultivar was accession 3165 of the USDA-ARS National Clonal Germplasm Repository for Citrus and Dates (Riverside, CA). The specific orange jasmine cultivar was not known, but the seeds were obtained from plants growing locally at a USDA-ARS farm near Fort Pierce, FL.

The greenhouse seedlings were inspected for trichomes during late June and July 2016. Three flush shoots from each genotype were excised and examined to estimate trichome densities using a stereomicroscope (Leica MZ 9.5, with ×10 eyepieces and ×1.0 objective; Leica Microsystems, Inc., Wetzlar, Germany) fitted with a counting reticle (KR-410, 23 mm, 10 × 10 major grids; Klarmann Rullings, Inc., Litchfield, NH). When focused on a leaf or stem with the scope's zoom set at ~2.4X, each major grid covered a 0.11 mm² surface area. Trichome counts were made within three successive grids, and the total count per three grids was multiplied by three to estimate the total number of trichomes per mm². Trichomes were enumerated at locations similar to those studied by Bartimachi *et al*.[16] including trichome counts on leaf midribs and stems. The stem of each flush shoot was examined near the base, at the center, and near the distal end to count trichome densities per mm². Curry leaf and orange jasmine have compound leaves, so for these two species, the stem of a new leaf was examined. Depending on the plant genotype, trichomes were sometimes unequally distributed around the girth of a stem, so at each stem location a site was chosen where trichomes were generally most abundant. Next,

we examined the midrib and laminae of a young leaf (for curry leaf and jasmine, a leaflet) suitable for oviposition but expanded/unfolded enough to see the adaxial surface of the midrib. Trichome densities were estimated at three locations on the adaxial and abaxial leaf surfaces: near the base of the midrib; midway along the midrib between the base and leaf tip; and on the lamina to one side of the midrib midway between the leaf edge and midrib about halfway between the leaf's base and tip. Statistical comparisons of trichome densities among genotypes were made using analyses of variance (PROC GLM; SAS Institute, 2012)[32] in conjunction with the Ryan-Einot-Gabriel-Welsch multiple-range test. All statistical tests were conducted at the 0.05 level of significance.

In addition to surveying the greenhouse seedlings for trichomes, observations on the presence of trichomes associated with flush shoots were made in a field planting of 5-year-old trees in a USDA grove in Fort Pierce, Florida. These trees included six trifoliate orange cultivars ("Argentina," "Flying Dragon," "Large Flower," "Pomeroy," "Rich 16-6," and "Rubidoux"); four trifoliate hybrids ("C-35," "Carrizo," "Norton" and "Uvalde"); "Hamlin" sweet orange; and "Temple" tangor (*Citrus reticulata × C. sinensis)*. The purpose of this field survey was merely to determine whether or not trichomes were present. During late May and again in mid-June, two flush shoots with leaves suitable for oviposition were collected from several trees of each cultivar and examined with a stereomicroscope to confirm that trichomes were present on young leaves and stems of older trees in the field.

To study the structure, length, and distribution of trichomes at higher magnifications, young (unfolded) and very young (folded) leaves and young stems were studied using epifluorescence microscopy, exploiting the blue autofluorescence phenomenon that we observed on these trichomes during our preliminary work for this study. These plant parts were excised from young shoots and fixed in 4% paraformaldehyde overnight, then washed in phosphate buffered saline (3 times, 15–20 m each). Some of the young stems and midribs were then hand-sectioned as described earlier.[33] Cross sections in the stem and midrib, as well as whole parts of the leaf blade, were mounted on glass slides under coverslips using Gel Mount (Sigma-Aldrich, Saint Louis, MO). They were then examined, without staining, using an epifluorescence microscope (Olympus IX70; Olympus Corp., Center Valley, PA) at total magnifications of ×40–100. Under UV light, the trichomes autofluoresced in light blue color. In some cases, the incident white light was used in addition to UV light to show other structures of the leaves or stems more clearly. To make sure that this autofluorescence method can reveal not only the shape but also the structure (unicellular/multicellular) of trichomes, tomato leaves which are known to have multicellular trichomes[26] were similarly processed and examined as controls in these experiments. Digital images were taken using CellSens Suite (Olympus Corp.). The length of trichomes on various genotypes was measured by tracing their images with the mouse curser using ImageJ

program (NIH, Bethesda, MD). Statistical comparisons of trichome lengths among genotypes were made using analyses of variance (PROC GLM) in conjunction with the Ryan-Einot-Gabriel-Welsch multiple range test. All statistical tests were conducted at the 0.05 level of significance.

Finally, an experiment was conducted to determine if Asian citrus psyllid females would oviposit near or within beds of trichomes associated with young leaves of each of the six genotypes from the greenhouse. On June 17, 2016, the terminal part of young flush shoots was excised from seedlings and the cut end was placed into a small microfuge tube (1 mL) filled with water, after which Parafilm® 'M' laboratory film (American National Can, Chicago, IL, USA) was stretched around the openings of the tube anchoring the shoot and closing the opening. Each shoot in its tube was then placed into a 50 mL tube with ventilated cap,[34] and then two female and two male psyllids were introduced into each tube for 24 h, except for trifoliate orange where the egg laying was allowed to continue for 5 days because it was observed earlier that much fewer psyllid eggs are laid on this genotype compared to other citrus-related genotypes.[7,11,12] These tubes were placed into an environmental chamber at 25°C, 14 h daily illumination, ambient humidity. The number of eggs and their location relative to trichomes on each flush shoot was determined under stereomicroscopy.

Results and Discussion

In this study, trichomes were found on flush shoots of the greenhouse seedlings of the six studied genotypes, but there were significant differences among genotypes in trichome density/abundance [Table 1 and Figures 1, 2]. Trichomes were most abundant on trifoliate orange, curry leaf and orange jasmine and moderately abundant on grapefruit. However, much fewer trichomes were observed on sweet orange and lemon seedlings, rendering them nearly glabrous. Trichomes on young leaves and stems autofluoresced in blue, and were morphologically similar among the six genotypes, all apparently simple, unicellular, hair-like structures often bent or curved [Figure 1]. The average length of trichomes ranged from 86.7 μm for trifoliate orange to 199.6 μm for curry leaf seedlings [Table 2 and Figure 1]. Trichomes on the abaxial midrib surface were generally longer than trichomes on either the adaxial leaf surface or on the stem. The autofluorescence method we used here readily distinguished between unicellular trichomes [Figure 1] and multicellular trichomes of tomato leaves used as control (results not shown).

Trichomes were observed on both the adaxial and abaxial surfaces of midribs of each genotype as well as on the stems of flush shoots and sometimes on the blades of young leaves or leaflets [Table 1 and Figures 1-3]. Even tiny new or folded leaves and leaflets often had at least a few trichomes along the midrib [Figures 1i, 3c, and e]. Younger leaves and stems normally had more trichomes than older ones in most cases and genotypes. On midribs of young leaves and leaflets,

Table 1: Trichome densities on the stems and leaves of young flush shoots from greenhouse seedlings of six genotypes of *Diaphorina citri* hosts. The densities on stems reflect counts made at the base, middle and distal. Laminae samples reflect counts on one side of the midrib at the center of a leaf or leaflet midway between the leaf edge and the midrib. Genotypes are arranged descendingly according to mean number of trichomes on the stem[z]

Genotype	Mean number of trichomes/mm²						
	Stem	Adaxial midrib base	Adaxial midrib middle	Adaxial lamina	Abaxial midrib base	Abaxial midrib middle	Abaxial lamina
Trifoliate orange	101.4[a]	165.2[a]	88.1[a]	1.0[a]	25.0[b,c]	13.0[a,b]	0.0[a]
Curry leaf	92.1[a]	64.1[b,c]	26.0[b]	1.0[a]	49.0[a]	23.0[a]	0.0[a]
Orange jasmine	66.7[a, b]	101.1[a,b]	5.0[b]	0.0[a]	32.0[a,b]	16.0[a,b]	1.0[a]
Grapefruit	24.7[b,c]	36.0[b,c]	17.0[b]	1.0[a]	11.0[b,c]	3.0[a,b]	2.0[a]
Lemon	13.0[c]	5.1[c]	2.0[b]	0.0[a]	5.0[c]	0.0[b]	0.0[a]
Sweet orange	0.0[c]	3.0[c]	1.0[b]	0.0[a]	4.0[c]	1.0[b]	0.0[a]

[z]Means in the same column followed by the same letter are not significantly different (*P*>0.05), Ryan-Einot-Gabriel-Welsch multiple range test
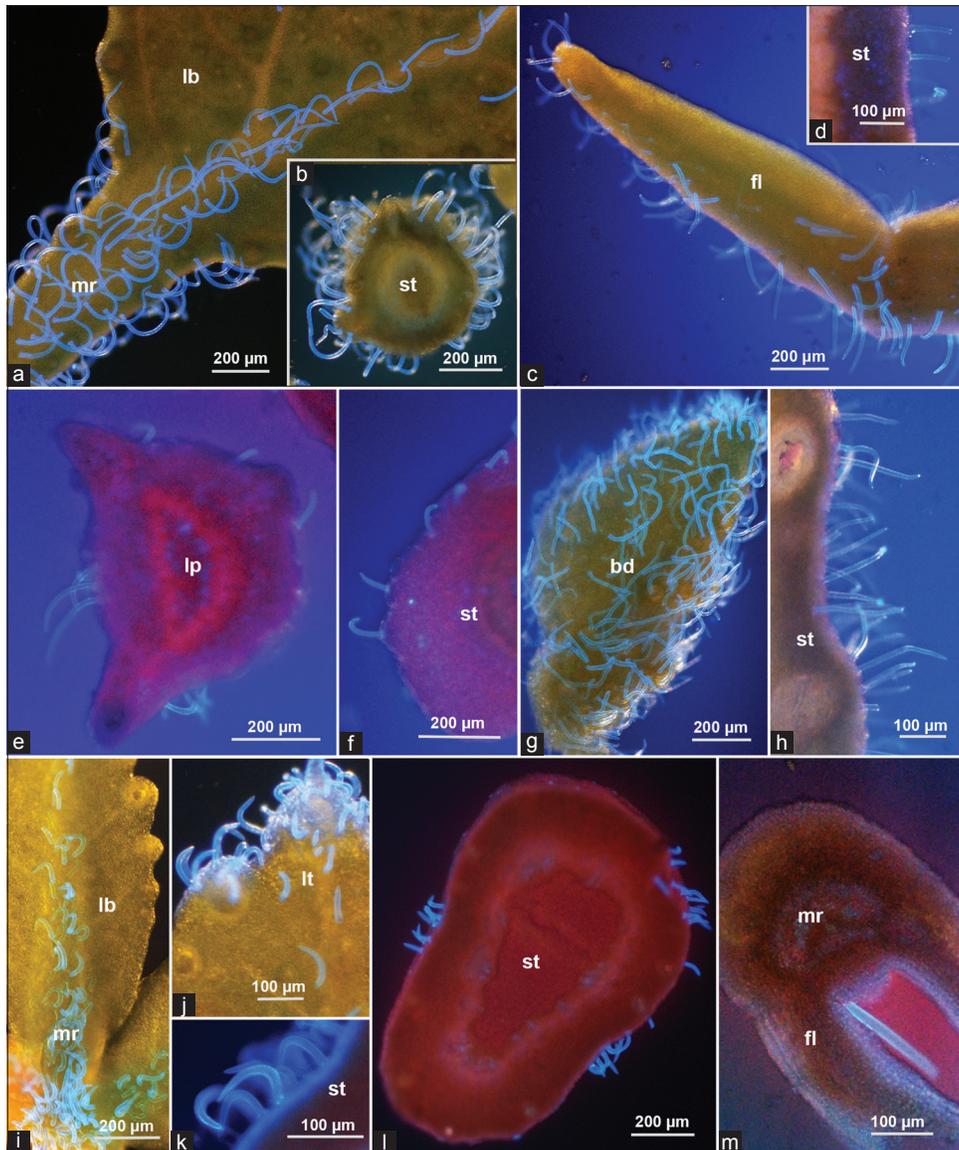

Figure 1: Epifluorescence micrographs showing trichomes on young leaves, buds or stems of curry leaf (a and b), grapefruit (c and d), lemon (e and f), orange jasmine (g and h), trifoliate orange (i-l) and sweet orange (m). Trichomes are auto-fluorescent (in light blue); incident light (in addition to UV) was used to show the leaf blades or stems in a-d and g-j. Abbreviations: bd: Bud; fl: Folded leaf; lb: Leaf blade; lt: Leaf tip; mr: Midrib; st: Stem

trichome densities were generally greater near the base of the leaf [Figures 1a, i and 2j, p]. Regardless of genotype, fewer trichomes were observed on leaf laminae compared to stems or midribs [Table 1 and Figures 1-3]. Curry, orange jasmine, and trifoliate orange often had trichomes along the edges or tip of the leaflets [Figures 1j and 3e]. On stems, young flush shoots of grapefruit or trifoliate orange usually had a narrow strip of trichomes running along two opposing sides of the stem with the rest of the stem's girth being glabrous [Figures 1l and 2f, r]. This was in contrast to young flush shoots of curry leaf and orange jasmine, on which trichomes were more uniformly distributed around the girth of the stem [Figures 1b, h, and 2l, o]. Among the flush shoots observed, grapefruit, trifoliate orange, and orange jasmine had higher trichome densities on the adaxial side of the midrib while curry leaf had higher densities on the abaxial side [Table 1].
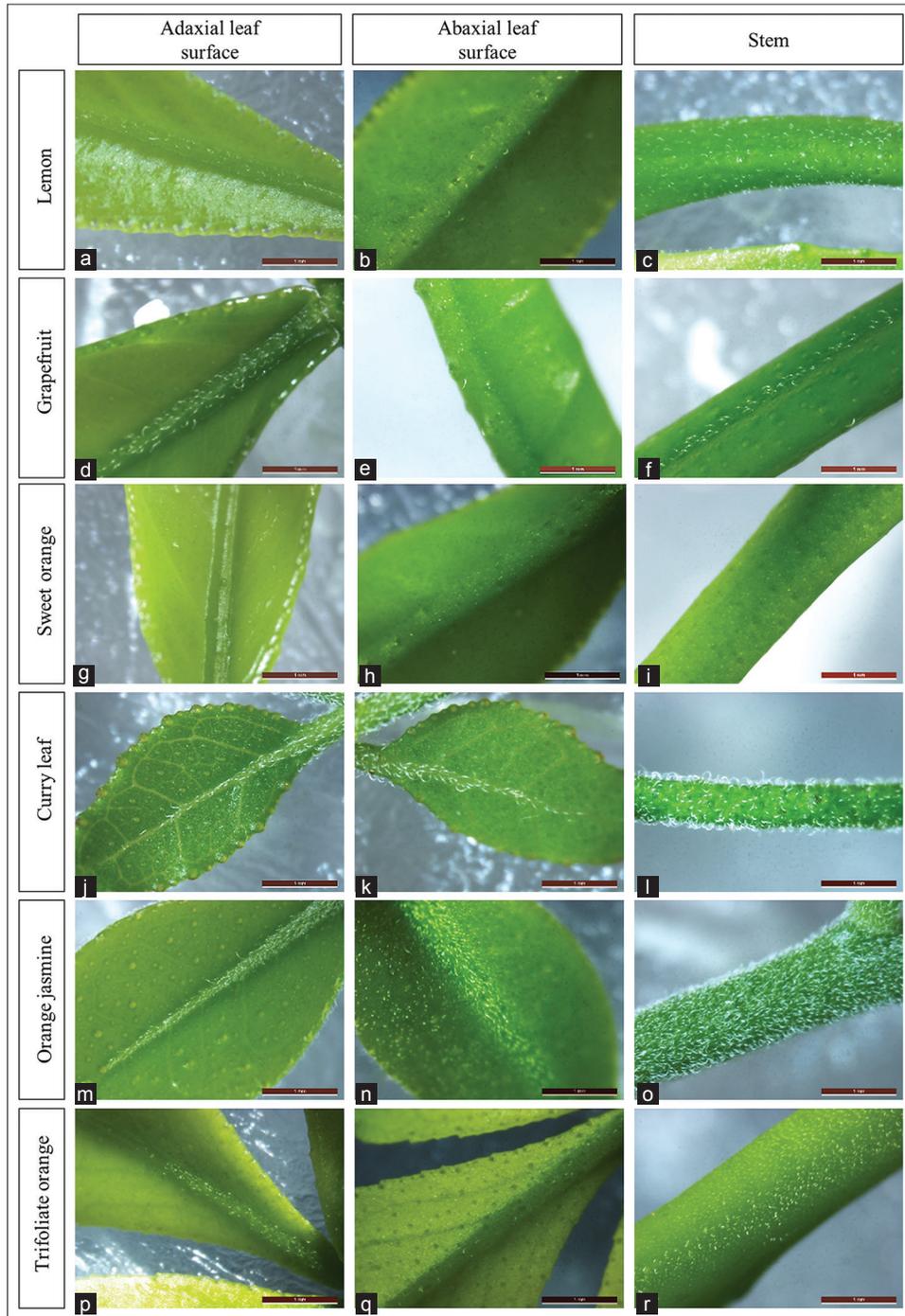

Figure 2: Stereomicroscopy images showing abundance and location of trichomes on young leaves and stems of flush shoots associated with greenhouse seedlings. Each row is for a separate genotype, as indicated on the panels at the left. The adaxial and abaxial leaf surfaces are shown in the first two columns from the left and the surface of stems is shown in the third column. For the abaxial photo of a grapefruit leaf (e), only one leaf lamina and the midrib are visible because this was a very young folded leaf (scale bar = 1 mm)
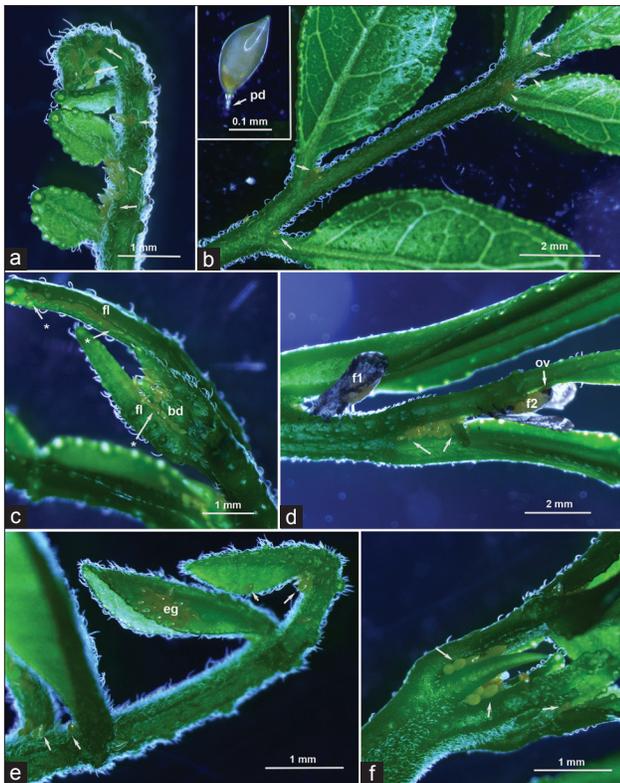
Figure 3: Stereomicroscopy images showing psyllid eggs (arrows and eg) laid on trichome-rich areas on young buds (bd), leaves and stems of curry leaf (a and b), grapefruit (c), lemon (d), orange jasmine (e), and trifoliate orange (f). The inset in panel B shows an egg with the pedicel (pd) that is usually inserted inside citrus epidermal layers during oviposition; in panel D, one female (f1) is apparently feeding and a second (f2) is laying eggs; note that the ventral side of f2 female is shown and her ovipositor (ov) is bent toward a leaf, apparently to anchor the eggs into citrus tissue. In panel C, asterisks indicate eggs laid inside the folded blades of tiny young leaves, and in E, eggs are laid on the outside of a folded leaf. Additional abbreviations: fl: Folded leaf

Foliar trichomes had previously been reported to be associated with the leaves and stems of curry leaf[35-37] and orange jasmine.[38] Trichomes associated with curry leaf were previously reported to be unicellular, bent or curved.[35] A single cell, nonglandular trichomes were reported to be associated with both curry leaf and orange jasmine.[23] Mbagwu *et al*.[39] reported that leaf trichomes were absent in four species within the genus *Citrus* (Mexican lime, *C. aurantiifolia* [Christm.] Swingle; lemon; grapefruit; and sweet orange); however, the age of leaves examined was not reported. Ogundare and Saheed[40] reported that trichomes were associated with grapefruit but not with lemon, sweet orange, or the mandarin *Citrus reticulata* Blanco. With respect to reports of trichomes associated with trifoliate orange, a review by Dianziang *et al*.[41] indicated that short trichomes are associated with the mid-vein of young leaves, but no information was presented on what types of trichomes were present nor on trichome density/abundance.

Among 5-year-old trees examined in the field planting, trichomes were relatively abundant on young flush shoots from multiple trees of the six trifoliate cultivars and the four trifoliate hybrid cultivars examined, whereas flush shoots from "Hamlin" sweet orange and "Temple" tangor trees were glabrous. The trifoliate hybrids, "C-35" and "Carrizo" are hybrids with sweet orange as the seed parent,[42,43] which suggests that the trichome density trait was inherited from the trifoliate pollen parent. The hybrid 'Norton' is likewise a cross between a sweet orange and *P. trifoliata*.[44,45] These observations indicated that genetic traits conferring trichome density could be readily transferred from trifoliate orange to hybrids with sweet orange or other genotypes.

When two egg-laying female psyllids (with two males) were held for 24 h on an individual flush shoot from various genotypes, the total number of eggs laid per shoot ranged from 72 to 134 eggs on curry leaf, grapefruit, lemon, orange jasmine, and sweet orange. In contrast, on trifoliate orange, a total of 28 eggs were laid in 5 days (=5.6 eggs/day). Although we intended to observe where eggs were laid relative to trichomes, not to quantify oviposition rates, these results are consistent with previous reports that the Asian citrus psyllid lays much fewer eggs on trifoliate orange than on other genotypes.[7,11,12] Psyllid females were found to lay their eggs on very young leaves, buds, and stems regardless of their trichome density, sometimes directly within or close to a bed of dense trichomes [Figure 3]. Females appeared to favor more protected areas for egg laying, such as junctions between leaves and stems [Figure 3a, b, and d-f] as well as on the inside of tiny folded leaves [Figure 3c]. This is probably to protect their eggs from predators and/or from the sticky honeydew excreted by psyllid males.[46]

Results of this study show that trichomes were generally most abundant on flush shoots from curry leaf and orange jasmine, and least abundant on those from lemon and sweet orange. All four genotypes are good hosts of *D. citri*,[7,11,12] which indicates that there is no correlation between trichome density on these genotypes and their resistance or susceptibility to colonization by the psyllid. Based on our egg-laying observations in conjunction with results of previous investigations,[7,11,12] trichomes associated with these genotypes may have little influence on infestations levels by immature stages of Asian citrus psyllid, as curry leaf and orange jasmine (both highly pubescent) are each highly susceptible to colonization by the psyllid and are regarded as good plant hosts for rearing the psyllid.[47] On these and other pubescent genotypes, it is possible that oviposition may be reduced in areas where trichomes are dense, and nymphs may be forced to feed at locations with fewer trichomes. However, our experiment on oviposition revealed that the psyllid can and will oviposit directly within a bed of foliar trichomes [Figure 3]. Additional studies are needed to fully investigate oviposition rates and sites, egg hatchability rates and nymphal survival of *D. citri* on *Citrus* spp. and related genotypes with differing trichome densities. In addition, further studies are needed to investigate other possible structural or physiological reasons for the resistance of trifoliate orange to colonization by *D. citri*.

Table 2: Mean length of trichomes on the stems and leaves of young flush shoots from greenhouse seedlings of six genotypes of *Diaphorina citri* hosts, arranged in the same order as in Table 1

	Trichome mean length (μm)[z]			
Genotype	Young stems	Adaxial midrib of young leaves	Abaxial midrib of young leaves	Over all samples
Trifoliate orange	90.6[c]	86.7[c]	129.3[c]	98.1[d]
Curry leaf	120.7[a,b]	153.2[a]	199.6[a]	180.1[a]
Orange jasmine	139.4[a]	151.2[a]	164.1[b]	134.2[b]
Grapefruit	94.9[c]	122.5[b]	137.0[c]	123.1[b,c]
Lemon	100.8[b,c]	100.4[c]	171.9[b]	116.4[c]
Sweet orange	97.4[c]	-y	102.1[d]	120.4[b,c]

[z]Means in the same column followed by the same letter are not significantly different (*P*>0.05), Ryan-Einot-Gabriel-Welsch multiple range test. y: Not measured, very few trichomes observed

Our survey for foliar trichomes did not include mature foliage, but we have observed trichomes on mature leaves of some genotypes, though at lower densities than on younger leaves or stems. The higher density of trichomes on younger leaves and stems of the same species or genotype may be a defense mechanism of the plant to protect these younger and more vulnerable tissues against some other herbivores and/or adverse environmental conditions. In addition to possessing trichomes on young leaves, unlike sweet orange and other citrus types, trifoliate orange cultivars produce fruit covered with trichomes.[22] Further assessments of the influence of trichomes on infestations of insect and mite pests of citrus could give insight into the possibilities of promoting pubescence as a general insect pest management tactic in citrus. Finally, the epifluorescence method we used here to study the structure and density of trichomes without any staining or lengthy processing, exploiting their blue autofluorescence, is a simple, fast, and promising technique to study trichomes on young leaves and shoots of various other plants.

Conclusions

Trifoliate orange is a genotype resistant to infestations by the Asian citrus psyllid, *D. citri*, vector of the economically important citrus greening (huanglongbing) disease bacterium. Host plant resistance to *D. citri* is one of the major tactics considered to combat this devastating worldwide citrus disease. To investigate whether trichomes found on this genotype are related to its resistance to colonization by *D. citri*, we compared the density of trichomes on flush shoots of trifoliate orange and on those from five other genotypes that are readily colonized by this psyllid. The study results strongly suggest that these trichomes may play little or no role in reduced colonization by *D. citri* on trifoliate orange. This is supported by our observations of an abundance of trichomes on curry leaf and orange jasmine, two genotypes highly susceptible to psyllid

colonization. Thus, further studies are needed to investigate other possible structural or physiological reasons for the resistance of trifoliate orange to colonization by *D. citri*.

In addition, the trichome autofluorescence method we used here to study the structure and density of trichomes, without staining, is a simple and promising technique to study trichomes on young leaves and shoots of other plants.

Conflicts of interest

There are no conflicts of interest.

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