Salivary Sheaths of the Asian Citrus Psyllid Show Signs of Degradation 3–4 Weeks Following Their Deposition into Citrus Leaves by the Feeding Psyllids

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Abstract

Background: Salivary sheaths, also known as stylet sheaths or stylet tracks, are essential features of the piercing-sucking feeding mechanism of plant-feeding hemipteran insects, many of which are vectors of economically important plant viral and bacterial pathogens. Although knowledge of their structure and function is incomplete, these salivary sheaths are frequently used by researchers to study hemipteran's feeding behavior, host preference, or host resistance, because these sheaths remain in the plant tissues after the insect withdraws its stylets following its feeding or probing on these tissues. However, in most cases, it is not known how long these salivary sheaths may last in plant tissues after their deposition by the feeding insects. An earlier report suggested that the salivary sheaths of the Asian citrus psyllid, *Diaphorina citri* (Hemiptera, Liviidae), vector of the devastating huanglongbing (citrus greening) disease bacterium, start to dissipate 1 week after their deposition in citrus leaves. **Methods and Results:** Here, using epifluorescence microscopy of cross sections in citron leaves, we found that *D. citri* salivary sheaths show signs of degradation in 3–4 weeks and become mostly degraded by 5–6 weeks, following their deposition by the psyllids into citrus tissues. Degradation of the salivary sheath starts at or near the "flange" area close to the leaf surface and continues gradually inward through the intercellular part of the sheath, within the mesophyll tissue, but apparently does not extend to the deeper or intracellular parts of the sheath in or near the phloem. Staining citron leaf sections with the fluorescent stain calcofluor white, which stains fungi, or propidium iodide (DNA/RNA stain) suggested that the degraded parts of the older salivary sheaths are not associated with fungi or bacterial accumulations. **Conclusion:** We speculate that degradation of the salivary sheaths may be due to enzymatic activities in the host plant, especially in the extracellular matrix of the mesophyll tissue.

Keywords: Citrus greening, hemiptera, huanglongbing disease, psyllids, salivary sheaths

Introduction

Plant-feeding hemipteran insects are important pests of agriculture, either directly through their feeding and excretion activities on their host plants[1,2] or indirectly as vectors of many economically important plant pathogens.[3,4] Hundreds of known plant viruses and several plant bacterial pathogens are transmitted by hemipteran vectors.[3-6] Hemipteran insects have piercing-sucking mouth parts that include a stylet bundle, composed of two mandibular and two interlocking maxillary stylets.[7,8] During probing and/or feeding, these insects produce salivary secretions, some of which solidify around the stylets and are termed "salivary sheaths," "stylet sheaths," or "stylet tracks." Although knowledge of their structure and function is incomplete, these salivary sheaths are used frequently to study which tissues in the host plant hemipteran insects have been probing and/or feeding on[1,8,9] and to assess or quantify hemipteran feeding damage.[10] Since feeding and probing on host plants are instrumental for pathogen transmission or disease induction by hemipteran insects, the salivary sheaths have been the subject of several studies to understand these mechanisms.[8,9,11-13]

Very few studies, however, were done to investigate how long these salivary sheaths may last following their

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deposition by hemipteran insects into various tissues of their host plants. Salivary sheaths of the beet leafhopper, *Circulifer (Neoalituris) tenellus* (Baker) (Cicadellidae. Hemiptera), were found in sugar beet and other host plant leaves 20–30 days after they had been fed on by these leafhoppers[14] (R. Creamer, personal communication). With the much larger glassy-winged sharpshooter leafhopper, *Homalodisca vitripennis* (Germar) (Cicadellidae, Hemiptera), their much thicker and tougher salivary sheaths persisted in grape vine leaves for 80 days before they were apparently dissolved or dissipated[11,12] (E. Backus, personal communication). On the other hand, Yang *et al*.[15] reported that the salivary sheaths/tracks of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Liviidae, Hemiptera), started dissipating from citrus (*Calamondin*) leaves 7 days after feeding by the psyllids and that the number of sheaths/tracks declined with increasing maturity of those leaves.

D. citri is the natural vector of two of the bacterial pathogens, *Candidatus* Liberibacter asiaticus (CLas) and *Ca*. Liberibacter americanus, associated with huanglongbing or citrus greening, currently the most devastating disease of citrus worldwide.[6,16-19] In this work, we used epifluorescence microscopy to investigate the persistence and/or degradation of the salivary sheaths of *D. citri* in healthy and CLas-infected citrus leaves for periods extending up to 6 weeks following their feeding on these plants.

Materials and Methods

Healthy (non CLas infected) nymphs and adults of *D. citri* were taken from our laboratory colony that has been maintained for many generations on young healthy orange jasmine (*Murraya paniculata* [L.] Jack) or citrus (*Citrus macrophylla* Wester) plants in the greenhouse. Individuals from the colony were assayed with quantitative polymerase chain reaction (qPCR) tests every 3 months to ensure that the colony remained free of CLas. Healthy (non CLas infected) citron plants (*Citrus medica* L.) were grown from healthy seeds in the greenhouse. Infected citron plants used were infected with CLas by grafting several months earlier and tested positive for CLas in qPCR tests.[20]

To study the persistence and/or degradation of salivary sheaths in leaves of healthy or diseased citron plants, large groups of *D. citri* nymphs and/or adults (>100) were placed on each plant (which contained young flush leaves) for 1–2 weeks of feeding. After removal of these psyllids, 2–4 fully expanded leaves from two healthy or infected plants were collected biweekly at 1–2, 3–4, and 5–6 weeks postfeeding on each plant. These plants and psyllids were kept in growth chambers at 25°C, 70% humidity, and 14 h light/day. From each leaf, 4–5 pieces of the midrib and/or petioles (5 mm long each) were cut with a sharp razor blade, fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), and then washed 3 times in PBST (PBS + 0.1% Triton ×100). Each of these pieces was placed in a drop of PBS on a microscope slide and sectioned by hand using a sharp razor blade to the thinnest possible sections under a stereomicroscope (at ×20 or higher). These sections, determined by confocal microscopy to be ca. 50–70 µm thick,[8,21] were transferred gently (without staining) to a drop of Fluoro-Gel mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA) on another microscope slide and covered with a cover slip. Autofluorescence of the salivary sheaths and the surrounding leaf tissues was examined under ultraviolet light using an epifluorescence inverted microscope (Olympus IX70, with ×4 or ×10 objectives) fitted with a camera and an imaging program (CellSens software, Olympus, Tokyo, Japan). At least 100 sections were examined from each of the above time categories from healthy or infected plants.

To test if the degradation of older salivary sheaths was associated with fungi or bacterial accumulations, ca. 50 sections from leaves fed on 4–6 weeks earlier by *D. citri* were treated according to one of the following protocols before mounting and fluorescence microscopy:

a. Stain the sections with calcofluor white (CFW, ampules containing 0.5 ml, at 0.05% solution in distilled water) for 15–20 min, then wash 2 times for 10–15 min PBST. CFW is a fluorescent dye known to stain fungal hyphae as well as plant cell walls in blue[22]

b. Stain the sections with the nuclear, fluorescent, stain propidium iodide (PI) which is known to stain DNA and RNA (that may stain any aggregates of bacteria associated with the degraded parts of the salivary sheaths), before washing in PBST. These sections were examined both by epifluorescence and by laser scanning confocal microscopy as described earlier.[8]

To test if *D. citri* salivary sheaths lose their autofluorescence with time after their deposition by the psyllids, we conducted the following experiment: *D. citri* adults (males and females) were induced to produce their salivary sheaths *in āere* (in air) by caging them in "mock-feeding chambers" as described by Morgan *et al*.[23] Each chamber was composed of the bottom of a small plastic Petri dish (35 mm diameter and 10 mm deep) tightly covered with one layer of clear plastic "kitchen" wrap. Ten psyllid adults were caged per dish for a 24 h mock-feeding period without adding any feeding solution (three replicate dishes used). These dishes were placed in a large plastic container covered with a thin, clear green plastic sheet to stimulate feeding/probing. Following the 24 h mock-feeding period, the caged psyllids were discarded and the mock-feeding chambers, covered with the plastic wrap (that included the salivary sheaths deposited by the psyllids), were placed in the large container covered with a clear plastic sheet at 23°C–25°C under T5 grow light (14 h light/day) for several weeks. The salivary sheaths deposited through the plastic wrap were examined by epifluorescence microscopy (at ×20), 1 day, 2 weeks, 4 weeks, and 7 weeks following their deposition by the psyllids.

Results

In cross sections of the petiole and midrib of citron plant

leaves, the fresh salivary sheaths (1–2 weeks old) of *D. citri* are known to autofluoresce in bluish green [Figure 1a], the xylem vessels fluoresce in light blue [Figure 1a-d], whereas the fibrous ring (sclerenchyma) fluoresces in deep blue, especially in older leaves [Figure 1a, c and d].[8,13] However, in older salivary sheaths of *D. citri* (3–4 weeks old), we observed that the outer part of the salivary sheath, at or near the "flange," begins to take a rusty (light brown) color, whereas the rest of the sheath (in the mesophyll and other tissues) still fluoresces in bluish green [Figure 1b]. In still older sheaths (5–6 weeks old), most of the intercellular parts of the sheath inside the mesophyll tissue take the rusty (light to dark brown) color, but the inner parts of the sheath near the fibrous ring or in the phloem still fluoresce in bluish green [Figures 1c, d, 2a and b]. These results equally applied to salivary sheaths deposited on leaves from healthy [Figure 1a-c] or CLas-infected [Figures 1d and 2a] citron plants.

In order to investigate whether the degradation of *D. citri* sheaths is associated with fungi or bacteria, sections of citron leaves with older salivary sheaths (5–6 weeks postdeposition) were stained with CFW or PI. CFW is known to stain fungal hyphae and cell walls with blue fluorescence[22] and PI is known to stain DNA and RNA in red. In the CFW-stained sections, cell walls of the mesophyll tissue were stained in blue as expected, but none of the rusty parts of the disintegrating sheaths were stained in blue [Figure 2b]. In the PI-stained sections, the degraded parts of the salivary sheath were not stained in red, or any other color (results not shown). These results suggest that fungi or bacterial accumulations are not associated with the degradation of *D. citri* salivary sheaths in host plant tissues.

To examine the possibility that the autofluorescence of the salivary sheaths fades with time following deposition, we induced *D. citri* adults to produce their salivary sheaths *in āere* (in air) by caging them in "mock-feeding chambers" as described earlier by Morgan *et al*.[23] Epifluorescence microscopy of these salivary sheaths, exposed to T5 Grow Light for up to 7 weeks, indicated that they were still strongly fluorescing in blue 2, 4, and 7 weeks following their deposition by the psyllids [Figure 3a-f].

Discussion and Conclusions

Yang *et al*.[15] reported that the number of salivary sheaths/tracks of *D. citri* in citrus (*Calamondin*) leaves started declining

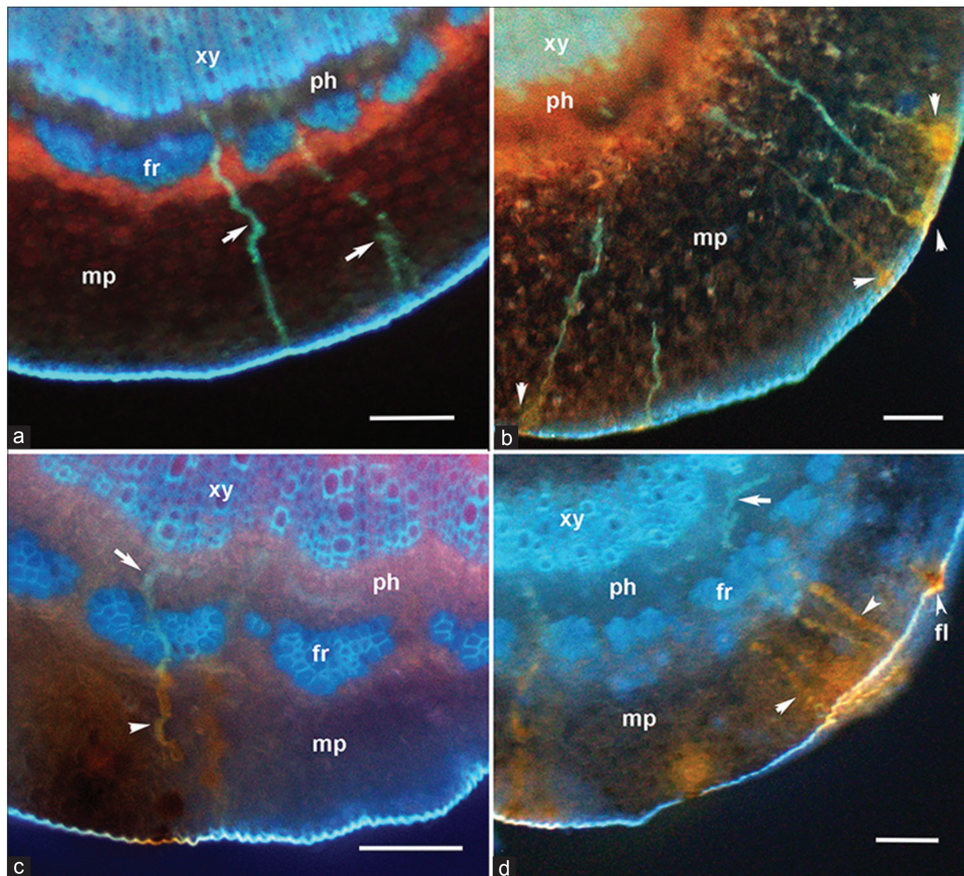

Figure 1: Fluorescence micrographs of cross sections in petioles and midribs of healthy (a-c) or *Candidatus liberibacter* asiaticus-infected (d) citron leaves 1–2 weeks (a), 3–4 weeks (b), or 5–6 weeks (c and d) postfeeding of *Diaphorina citri* psyllids. Arrows indicate normal color (bluish-green) salivary sheaths and arrowheads indicate rusty/brownish (degrading) parts of the salivary sheaths. Note that degradation starts at or near the flange (fl) close to the leaf surface and that inner parts of the sheaths in the phloem (ph) are still normal in color. Fl: Flange, fr: Fibrous ring, mp: Mesophyll parenchyma, ph: Phloem, xy: Xylem; Scale bars = 100 μm

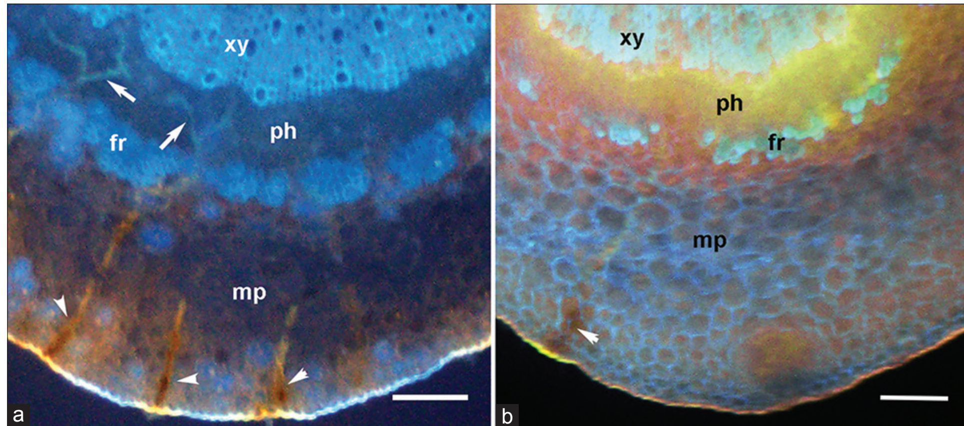

Figure 2: Fluorescence micrographs of older salivary sheaths, deposited 5–6 weeks earlier by *Diaphorina citri* in the petioles and midribs of *Candidatus Liberibacter* asiaticus-infected (a) or healthy (b) citron leaves. Note that most of the brownish (degrading) parts of the salivary sheaths are in the mesophyll, but those in the phloem are still bluish green (arrows). The section in B was stained with Calcofluor White, which stains fungi and cell walls in blue; only cell walls were stained in blue, but the rusty part of the salivary sheath (arrowhead) was not. Fr: Fibrous ring, mp: Mesophyll parenchyma, ph: Phloem, xy: Xylem. Scale bars = 100 μm

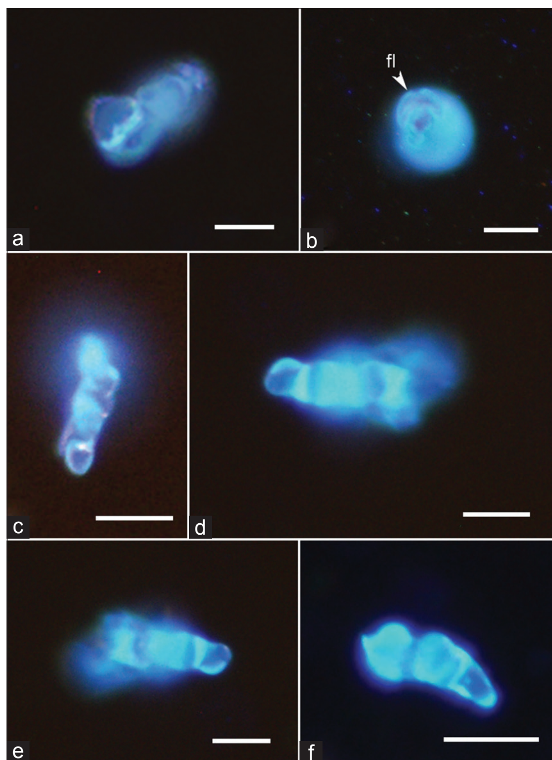

Figure 3: Blue autofluorescence of salivary sheaths deposited by *Diaphorina citri* adults through membrane and exposed to 14 h light/day for 1 day (a and b), 2 weeks (c and d), 4 weeks (e), and 7 weeks (f). Fl: Flange; Scale bars = 20 μm

7 days postfeeding by the psyllids and that branched sheaths declined faster than nonbranched ones, whereas the number of stubby unbranched sheaths actually increased 7 days postfeeding. Although these results are difficult to reconcile (especially the increase in the number of stubby sheaths postfeeding), the authors indicated that the persistence of stylet tracks was inversely correlated with leaf maturity and suggested that *D. citri* sheaths were being dissolved by plant sap enzymes. Yang *et al*.[15] also speculated that stylet tracks would disappear as rapidly in tissues of other host plants of *D. citri*. Using epifluorescence microscopy of cross sections in citron leaves, we showed here that the salivary sheaths of *D. citri* in these leaves only start showing signs of degradation 3–4 weeks postfeeding of the psyllids that apparently complete degradation takes place in 5–6 weeks, which was true in both healthy and CLas-infected leaves. This time range is more consistent with previous reports that salivary sheaths of the beet leafhopper persisted for 20–30 days,[14] whereas the much thicker sheaths of the larger glassy-winged sharpshooter leafhopper[21] persisted up to 80 days postdeposition by these insects into the host plant.[11,12]

We further showed that degradation of *D. citri* salivary sheaths starts at the flange and continues gradually inward and that the inner parts of the sheaths in or near the phloem were still apparently intact 5–6 weeks postdeposition by the psyllids. Since these sheaths are mostly intercellular within the mesophyll tissues and mostly intracellular in the phloem,[8,9] it is possible that they are being degraded by modifying enzymes from the extracellular matrix in the mesophyll tissue.

We also showed that our results are not due to "fading" of the autofluorescence of the salivary sheaths with time, since *D. citri* sheaths deposited through membranes did not fade up to 7 weeks following their deposition by the psyllids [Figure 3]. However, we cannot exclude the possibility of interaction with extracellular matrix that could be quenching the autofluorescence of these salivary sheaths with time. However, this seem unlikely since Yang *et al*.,[15] using light microscopy techniques (without autofluorescence), reported that *D. citri* salivary sheaths start dissipating 2 weeks earlier than what our results are showing here (1 week vs. 3 weeks).

Finally, we show that degradation of these salivary sheaths was not associated with fungi or bacterial accumulations, which suggests that it might be due to enzymatic activities in the leaf

tissues of host plants, especially in the mesophyll. The primary structure of hemipteran stylet sheaths was recently reported to consist of highly branched glucose polysaccharides apparently held together by proteins that can be rapidly degraded by specific digestive enzymes that reduce the structured sheaths to a liquid-like substance.[24] Our results in comparison with previous reports[11,12,14,15] suggest that the degradation speed of hemipteran salivary sheaths might be variable depending on the insect species, on host plant species, maturity, and other physiological conditions, as well as on various environmental conditions. These factors must be taken into consideration if the salivary sheaths/tracks will be used to study feeding behavior, host preference, or host resistance for hemipteran insects.

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Conflicts of interest

There are no conflicts of interest.

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