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LEAF PENETRATION PATTERN OF AULACASPIS TUBERCULARIS (HEMIPTERA: DIASPIDIDAE) STYLET IN MANGO

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ABSTRACT

Mango (Mangifera indica L.) is an intensively cultivated fruit in Mexico, the leading exporter of this product in the world. One important limiting factor in mango production is the white mango scale (Aulacaspis tubercularis Newstead; Hemiptera: Diaspididae). White mango scale infestation causes irreversible leaf yellowing and death, and it lowers fruit quality below export requirements. Feeding mechanisms of these diaspidids have not been extensively studied; no histological studies on this subject are known. We histologically analyzed leaf tissues penetrated by the stylet bundle (SB) of white mango scale females, in order to follow the SB cellular path, to observe the extent of visually-detectable leaf cell damage, and to determine if this insect feeds on phloem sap. Mango plants of cv. 'Manila' were artificially infested with this insect in the laboratory. Histological slices from infested leaves were processed for microscope observation of the intact SB. Through this process a complete SB could not be observed, so its full accurate length could not be reported. However, the SB length was estimated at 3 mm, i.e., 3 times the total length of the female body length, which was 1 mm. The SB path inside the leaf was mostly intracellular through the mesophyll, but it also pierced lignified xylem cells and nutrient-rich phloem tissues in the vascular bundles. Thick red masses were formed along the SB path, possibly containing phenolic compounds. Cell lysis or collapse was not observed in the SB-injured leaf tissue.

Key Words: mango, white mango scale, stylet bundle, mesophyll, leaf vascular bundles

RESUMEN

El mango (Mangifera indica L.) es uno de los principales cultivos frutícolas en México, el primer exportador mundial de este producto. Una limitante de su producción es la escama blanca del mango (Aulacaspis tubercularis Newstead; Hemiptera: Diaspididae), cuyas hojas infestadas cambian de verde pálido a amarillo y al final se necrosan, y la infestación demerita la calidad de los frutos de modo que no se pueden exportar. La alimentación de los diaspídidos no se ha estudiado extensamente y en la literatura revisada no se encontraron reportes histológicos sobre el tema. En este estudio se hizo un análisis histológico de hojas penetradas por el haz de estiletes (HE) de la hembra de escama blanca del mango, para determinar su ruta celular, detectar daños celulares en la hoja, y determinar si el insecto se alimenta de savia del floema. Plantas de mango var. ‘Manila’ fueron infestadas con este insecto en laboratorio. Cortes histológicos de porciones de hojas infestadas se procesaron para observar el HE intacto al microscopio. No se encontró el HE completo hasta su punta, por lo que no se pudo determinar con precisión su longitud total. No obstante, se pudo estimar que al menos miden 3 mm, el triple de la longitud de la hembra (1 mm). La penetración del HE es predominantemente intracelular a través del mesófilo, pero también puede atravesar células lignificadas del xilema y tejidos del floema ricos en nutrientes en los haces vasculares. El HE encuentra tejidos vasculares ricos en nutrientes, pero no permanece ahí sino que atravesía por completo el haz vascular, incluyendo las fibras que lo envuelven, para continuar atravesando el mesófilo. En la ruta de penetración del HE se observaron unas zonas engrosadas y rojas, posiblemente constituidas de material fenólico. No se observó lisis ni colapso celular en el tejido vegetal atravesado por el HE.

Palabras Clave: mango, escama blanca del mango, haz de estiletes, mesófilo, haces vasculares
Mango (*Mangifera indica* L.; Sapindales: Anacardiaceae) is one of the main cultivated fruits in Mexico, with 184,768 ha cultivated, 1,536,654 tonnes produced (SIAP 2011), and 275,366 tonnes exported. These statistics place the country as the world’s leading mango exporter (FAO 2011). Mexico possesses physiographic characteristics and climate that permit optimal mango development, but pest infestations can severely impact yield and fruit quality (SENASICA 2009) and restrict profitability. The white mango scale (*Aulacaspis tubercularis* Newstead; Hemiptera: Diaspididae) is economically the second most important mango pest in Mexico, right after fruit flies (*Anastrepha* sp.; Diptera: Tephritidae) the most severe pest (SENASICA 2009). This pest appeared in the state of Nayarit in 2003 (Urias-Lopez et al. 2010) from an undocumented origin, and spread to the states of Guerrero and Michoacán in later years (SENASICA 2009). The white mango scale is a sessile armored scale, and this group of scales includes some of the most damaging and invasive pests in world agriculture (Miller & Davidson 2005; Andersen et al. 2010; Rehmat et al. 2011). These insects can insert a chitinous tube in any aerial plant part. This tube is composed of 4 styles, 2 maxillae and 2 mandibles, that interlock to form 2 canals, one for saliva (sali- vary canal) and one for sucking food (food canal). The tube is known as the stylet bundle (SB). These insects generally feed on parenchymal cells (Heriot 1934) or vascular bundle tissue (Sadof & Neal 1993) and inject toxic saliva (Peña et al. 2009; Rehmat et al. 2011).

A severe white scale infestation may retard mango growth in the nursery. White mango scale infested young trees are most vulnerable to excessive leaf loss and twig death, especially during the dry season (drought) (Cunningham 1991; Daniel & Joubert 2009; Rehmat et al. 2011). Infestations on fruits cause external lesions and pink spots (Cunningham 1991), which decrease their quality and make them unacceptable for export (Cunningham 1991; Daniel & Joubert 2009). Infested leaf areas turn from pale green to yellow and finally become necrotic (Cunningham 1991; Miller & Davidson 2005). According to Hill et al. (2011) the feeding behavior of diaspidids has not been extensively studied, and there are no reports on histological studies of the white mango scale SB path in the mango leaf.

This study analyzed mango leaf tissues punctured by the female white mango scale SB at the histological level, to observe the penetration path and visually-detectable plant cell damage along the path. An additional objective was to learn if this scale feeds on phloem sap as other hemipteras do.

**MATERIALS AND METHODS**

In a controlled-environment growth chamber, colonies of white mango scale were bred on graft-ed plants of the mango ‘Manila’ variety. Healthy, pest-free leaves were inoculated by direct contact with infested leaves collected in an orchard located in Huejutla, state of Hidalgo. Once pest propagation was successful, colonized leaves by live females were collected. These leaves were cut into 20 sections, approximately 6.25 mm² in size, for the anatomical study.

Leaf tissue preparation (fixing, dehydrating, clearing, staining and mounting) followed the techniques of López et al. (2005). Leaf samples were fixed in FAA (formaldehyde (10%), glacial acetic acid (5%), ethanol 96% (50%) and distilled water (35%) for 24 h). After fixing, samples were washed in running water for 15 min and dehydrated in ascending ethyl alcohol (10, 30, 50, 70, 80, 96 and 100%, v/v) dilutions for 4 h periods. After dehydration, samples were washed, first with a 1:1 mixture of 100% alcohol: xylene for 4 h, and then with xylene for 3 h. Once clarified, leaf tissues were placed in melted paraffin (Paraplast® Plus Sigma®) at 60 °C for 24 h to be embedded. From each paraffin embedded sample 260 to 280 successive slices, 9 µm thick, were cut. Samples were cut transversely and tangentially to the leaf using a Spencer 820® rotary microtome. Slices were mounted on glass slides and stained with Safranin O (Sigma-Aldrich®) and fast green (Sigma®). Coverslips were placed with synthetic resin (Golden Bell®).

Slices were examined one by one under a Carl Zeiss® photomicroscope III until SB segments were located and photographed with a digital microscopy camera PaxCam 3®. Micrographs of live females with SB inserted in the mango leaf were also taken by a Carl Zeiss® (Tessovar) stereoscopic microscope fitted with a PaxCam 3® camera. Based on these micrographs, the SB penetration path in the leaf tissue was digitally reconstructed using The Gimp® (v. 2.8.4) software. A tridimensional model was digitally assembled from the histological cuts with the software Blender® (v. 2.63).

Intact SB observations of the mango leaf were possible after a bleaching treatment. Thus ten 6.25 mm² leaf tissue samples (each one infested with one white scale female) were washed with commercial sodium hypochlorite (4-6% of NaOCl), as used by Valero & Durant (2001), Castellaro et al. (2007) and González & Briones-Salas (2012). Washed samples were placed in 70% alcohol for 48 h, then washed with distilled water for 15 min. After the water wash, samples were transferred to another Petri dish with 10 mL of commercial NaOCl for approximately 2 h until totally bleached. Sample bleaching was verified with an American Optical Company® (Model 571) stereoscopic microscope. Bleached samples were then placed in distilled water for 24 h to eliminate sodium hypochlorite from the leaf tissue. After this period, samples were dehydrated once with...
70% ethanol, once with 90% ethanol and twice with 100% ethanol for 5 h periods. Samples were then clarified with xylene for 2 h, and mounted on slides and coverslips with Canada Balsam. Observation and photography of the leaf tissues were done by a Carl Zeiss® Photomicroscope III fitted with a PaxCam 3® digital camera. The camera was focused on the stylet bundle (SB), but the photos varied in the focal point. Digital assembly of the images from the SB penetration route in the leaf tissue was accomplished by The Gimp® (v. 2.8.4) software.

RESULTS

We frequently observed that the exposed non-penetrating SB had roughly the same length as the female body (Fig. 1). Successive histological slices from infested mango leaves followed the SB penetration path through the leaf from the piercing site on the leaf epidermis. Upon entering the spongy mesophyll, the SB changed direction several times, including oblique (Fig. 2A) and parallel orientations in relation to the leaf surface (Fig. 2B).

Through 2 digital reconstructions (Figs. 3A and 3B), each one made from 13 successive histological slices (117 µm in total thickness), we found that in mango leaves the SB was able to traverse through the palisade and spongy parenchyma and through vascular bundles until it reached the opposite epidermis where the path was lost. From 4 successive slices (Fig. 4) across a leaf vascular bundle, which corresponds to one section (Fig. 3B), a three dimensional reconstruction (Fig. 5) was created with Blender (v. 2.63). This reconstruction showed that most of the penetration by the SB was intracellular, because pericellular paths were not clearly observed. These slices also showed that the SB not only pierced primary cell walls, but also the lignified secondary walls of the xylem.

Samples in this study did not contain the SB tip, thus complicating the measurement of its total length. However, by assembling the non-penetrated and penetrated SB sections observed after bleaching with NaOCl (Fig. 6a), SB length was estimated at 3 mm, i.e., 3 times the length of the female body of 1 mm. The bleaching technique confirmed the capability of the SB to penetrate all leaf tissues, and that most of the pathway was located in the spongy and palisade parenchyma. Pierced mango leaves showed several dark and reddish masses on the path of the SB and in adjacent cells (Fig. 7). However, the piercing of cell by the SB caused neither lysis nor collapse in the affected and surrounding cells.

DISCUSSION

Histological evidence indicates that after penetrating the coriaceous cuticle and epidermis, the white mango scale SB explores the interior of mango leaf tissue, including vascular bundles, by changing its path yet maintaining most of its pathway in the mesophyll. The regulation of these directional changes cannot be determined with this research, but it seems to differ from other hemipteran species in which SB movements have been attributed to mechanoreceptors in the maxillae and mandibles that serve to cause the SBs to avoid mechanical resistance in leaf tissues (Smith 1985). Our results are similar to those reported by Washington & Walker (1990) for the California red scale (Aonidiella aurantii) (Hemiptera: Diaspididae) SB when penetrating

![Fig. 1. (I) White mango scale female in a ventral view, with a portion of its stylet bundle before penetration of the leaf of mango variety ‘Manila’. A = Rostrum, B = Point of insertion of the stylet bundle into the leaf. (II) White mango scale stylet bundle perpendicularly traversing the cuticle of the leaf’s adaxial surface. SB = Stylet bundle, Ep = Epidermis, PP = Palisade parenchyma, A = Rostrum, B = Insertion point of the stylet bundle in the foliar lamina.](https://bioone.org/doi/abs/10.1653/0015-4609(2014)97[70%]2.0.CO;2)
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citrus leaves tissues. The California red scale SB moves perpendicularly into the palisade parenchyma and then obliquely or parallel to the spongy parenchyma, keeping more than 85% of its length in both parenchyma tissues. Our observations closely match those of Heriot (1934) that scale stylets usually penetrate slowly because of their long length.

White mango scale SB is able to pierce cell walls, even the lignified secondary walls of the xylem, in exploring along an intracellular route, probably because pericellular pathways do not yield food while intracellular paths do. This scale is then one of few armoured scale species that are capable of piercing lignified plant cells walls like fibers and the xylem vessels in vascular bundles. Leaf vascular bundle penetration by SB has been previously reported in only 2 species of scales (Heriot 1934; Washington & Walker 1990).

Intracellular penetration by SBs was observed by Sadof & Neal (1993) for *Unaspis euonymi* (Comstock) (Hemiptera: Diaspididae) feeding on *Euonymus fortunei* (L.) and *Euonymus japonica* (L.) leaves, but only through the mesophyll. In kiwi (*Actinidia chinensis* L.) infested by scales *Hemiberlesia rapax* (Comstock) and *H. lataniae* (Signoret) (Hemiptera: Diaspididae), Hill et al. (2011) found SB penetrating the epidermis and parenchyma of the stem bark, without reaching the fiber layer below the parenchyma or the phloem below the fibers. According to Washington & Walker (1990), the California red scale SB avoids the lignified xylem cells in citrus leaves.

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**Fig. 2.** (A) Transversal slice of mango leaf (9 µm thick) which shows a stylet bundle fragment of a white mango scale female penetrating the mesophyll in an oblique direction, and (B) Tangential slice of mango leaf (9 µm thick) which shows another fragment of stylet bundle penetrating the spongy mesophyll. SB = Stylet bundle, PP = Palisade parenchyma, SP = Spongy parenchyma.

**Fig. 3.** Two digital reconstructions (A and B), both from 13 histological slices (117 µm total thickness) of the route of penetration of white mango scale stylet bundle in mango leaves. SB = Stylet bundle, Ep = Epidermis, PP = Palisade parenchyma, VB = Vascular bundle.
Since the SB tip of white mango scale did not remain in the phloem for feeding on its nutritious sap, but rather continued on its exploration of the mesophyll, we propose that this insect does not feed solely on phloem sap, as other sucking species do, such as the greenbug (*Schizaphis*...
graminum (Rond.) (Hemiptera: Aphididae) feeding on wheat (*Triticum aestivum* L.) (Goussain et al. 2005) and the aphid *Greenidea ficicola* Takahashi (Hemiptera: Sternorrhyncha: Aphididae) feeding on guava (*Psidium guajava* L.) (David et al. 2009). The SB of these aphid species penetrates plant cells mostly by a peripheral path. Therefore, the white mango scale possibly feeds on the me-

Fig. 5. Lateral view (a) and a 45° view (b) of the tridimensional model of four histological slices each one 9 µm thick (corresponding to Figure 4), which displays the intracellular penetration of a white mango scale stylet bundle in mango leaf vascular tissue. The cells of the primary wall, in gray, and the cells of the secondary wall (lignified), in dark gray. A = Spongy parenchyma, B = Xylem, C = Phloem, D = Fibers.

Fig. 6. Mango leaf cleared with sodium hypochlorite, which shows the intact stylet bundle of a white mango scale female across 41 high resolution photographs in which the stylet bundle is digitally colored black. A = Insertion site of stylet bundle in the leaf’s adaxial surface, B = Last foliar field with evidence of the stylet bundle, C = Micrograph that shows a portion of stylet bundle.
sophyll cellular juice while its SB passes through these cells.

Despite the long SB pathway of the white mango scale, no cell lysis (neither in pierced nor in surrounding cells) was detected in the leaf tissues. In fact no cell volume reduction was detected due to cell lysis, as it would happen if the insect had sucked empty the pierced cells, unless the sucking was so slow that water from surrounding leaf cells could refill the pierced cell and thus prevent cell collapse. Losses of cell turgor and volume appear in mesophyll cells of plants subjected to drought stress conditions (Steudler et al. 1977). With the techniques used in this study we could not accurately identify the mechanism that prevents cell lysis. However, in natural infestations, the colonies of nymphs cause leaf chlorosis and even necrotic spots, perhaps because these colonies consist of groups of at least 49 individuals (Urías-López et al. 2010), and all of them simultaneously penetrate a small area of the leaf surface.

Histological observations in this study support the hypothesis that these scales prefer parenchymal tissues for feeding. Likewise Sadof & Neal (1993) found that 62% of U. euonymi SB tips were located in the palisade parenchyma of Euonymus fortunei (L.) and E. japonica (L.) leaves. In the scale species Hemiberlesia rapax and H. lataniae, Hill et al. (2011) also found that SBs were located only in the parenchyma of the stem bark of kiwi, and they inferred that these scales obtain nutrients from this plant tissue.

The thick red masses observed in the pathway of the white mango scale SB and neighboring plant cells after staining with safranin were presumed to be phenolic deposits, since Zamora-Magdaleno et al. (2001) demonstrated that this stain conferred a dark red label to the phenolic deposits accumulated in cellular walls and intercellular spaces of avocado fruit (Persea americana Mill.) by infection from Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. These authors confirmed this observation by a nitrogen reaction for total polyphenols. In our study, the presence of such phenolic accumulations did not cause the collapse either of the affected or the surrounding cells. Hill et al. (2011) also found that some of the injured cells of the kiwi stem were filled with dark-colored phenolic material, although most of these cells showed signs of lysis or collapse after 4 weeks.

**Conclusions**

The stylet bundle of the white mango scale female is very long (aprox. 3 mm), roughly 3 times the length of the insect itself, though it was not possible to observe the SB tip. From the SB path, we concluded that the female feeds mainly on the contents of the mesophyll cells as it pierces them, and without causing their collapse. However, to verify if the stylet bundle sucks these cells empty, it is necessary to analyze consecutive pierced leaf cells by transmission electron microscopy. The exploration pathway of the SB through the leaf tissues is mostly intracellular, and the SB is capable of piercing the lignified cell walls of vascular bundles. Along the penetration route, some thick reddish masses were observed, which we attributed to the accumulation of phenolic material.
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