# Stylet Penetration by *Bemisia argentifolii* (Homoptera: Aleyrodidae) into Host Leaf Tissue

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**ABSTRACT**   Silverleaf whitefly, *Bemisia argentifolii* Bellows & Perring, adults and nymphs feed on the phloem tissue of host plant leaves. Infested leaves were rapidly fixed to prevent the insects from withdrawing their mouthparts. The adult stylet bundle enters the labial groove of the labium between the first and second segments and is completely contained within the labium except during feeding. Stylet length is equal to the combined length of labial segments 2, 3, and 4. Stylet penetration can be determined by examining the position that the stylet enters the labial groove. The physical force necessary for adult stylet penetration is derived from changes in the position of the whitefly head during feeding. The head is bent over the labium, which is attached to the leaf surface, forcing the stylet bundle down the labial groove and into the host tissue. Nymphal stylet lengths were found to be longer than previously reported, and of sufficient length to reach the phloem tissue in cotton and hibiscus leaves from essentially any position on the abaxial leaf surface.

**KEY WORDS**   *Bemisia argentifolii*, whitefly, stylet, nymph, feeding

SILVERLEAF WHITEFLIES, *Bemisia argentifolii* (Bellows & Perring), feed on a wide variety of plants and cause significant economic damage to field and horticultural crops (Byrne and Bellows 1991, Bellows et al. 1994). Damage can be a direct result of feeding, transmission of viruses, or fungal growth associated with honeydew excretion. A variety of factors may influence adult whitefly host selection, feeding sites, and oviposition sites. One important factor was believed to be stylet length. Most whiteflies feed primarily on the abaxial surface of leaves, and several theories have been proposed to explain this behavior. The most common is that phloem tissue is more accessible from the underside of the leaf (Cohen et al. 1998). Other theories proposed to explain abaxial feeding include: the possibility of a thinner abaxial cuticle, the ease of stylet penetration through spongy mesophyll, which is less dense than palisade mesophyll, protection from the elements such as rain and high solar energy, reduced predation, and reduced accumulation of excreta (Lenteren et al. 1990, Chu et al. 1995). Feeding sites of whiteflies can be determined by examining salivary sheaths that are formed during stylet insertion and probing and remain intact after the adult or nymph is no longer present. Pollard (1955, 1971) believed that salivary sheaths were uncommon in whiteflies, but other researchers have described them in detail (Cohen et al. 1996a, 1996b, 1998). Of the >250 nymphs examined on intact leaves, all were seen to have salivary sheaths which extend toward vascular bundles, with 98% of the sheaths going to minor veins with two or fewer tracheary elements (Cohen et al. 1996a)

The mouth parts of whiteflies are typical for the Homoptera and have been described using light and electron microscopy (Hargreaves 1914; Pollard 1955, 1971; Forbes 1972; Domenichini 1981; Gill 1990; Byrne and Bellows 1991; Rosell et al. 1995; Weber, 1995). Mouth parts consist of the labrum, the labium and the stylets (Hargreaves 1914, Rosell et al.1995). According to Hargreaves (1914), the labium consists of three segments, with an imperfect joint dividing the middle segment. Azab et al. (1969) also described a three segmented labium in whiteflies. Gill (1990), Rosell et al. (1995), Weber (1995), and Freeman et al.(2000) all report the labium to consist of four segments. Rosell et al. 1995 indicated that the first segment appears to be an extension of the thorax.

Whitefly stylets enter the leaf by piercing the abaxial epidermal layer (Pollard 1955, Walker 1985). The penetration site may be directly through the epidermal cells or between the common wall separating adjoining epidermal cells and occasionally through the stoma (Pollard 1955; Cohen et al. 1996a, 1996b, 1998). There is evidence that some whiteflies determine the acceptability of the feeding site before, or immediately following, the initial penetration as rejection may occur before the time phloem tissue is reached (Noldus et al. 1986; Walker 1987; Byrne and Draeger 1989; Lei et al. 1998, 1999). Hunter et al. (1996) described the internal anatomy of the anterior alimentary canal of *Bemisia*, including the structure and function of precibarial and cibarial gustatory sen-

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silla, which relate to the whitefly's ability to determine the acceptability of the host.

Cohen et al. (1996a, 1996b, 1998) hypothesized that silverleaf whitefly crawlers used leaf surface cues as a means of finding phloem tissue within the range of their stylets, reported by Pollard (1955) to be 80 $\mu$m in length. The hypothesis requiring surface cues was based on the fact that whiteflies are obligate phloem feeders, and the crawlers have only a few hours to locate the phloem tissue before they starve or desiccate. Cohen et al. (1996a 1998) used whitefly feeding behavior and leaf vascularization patterns to develop a hypothesis addressing the need for surface cues to assure successful phloem feeding.

Our understanding of whitefly stylet penetration and stylet movement within plant tissues has been incomplete. Using rapid methods of killing and fixing whitefly adults and nymphs as they feed on host leaves, we have obtained more accurate measurements of stylet lengths and gained new insights into the mechanism of stylet insertion. More accurate measurements of stylet length permit us to determine the ability of the whitefly to reach phloem tissue at various depths in host leaves.

The objectives of this study were: to document stylet length of adults and nymphs, to show how the stylet bundle moves through the labium and penetrates the host leaf, to determine the depth of stylet penetration, and to evaluate the hypothesis that stylet length is a limiting factor for successful feeding of whitefly nymphs and adults.

## Materials and Methods

All whiteflies used in this study were silverleaf whiteflies, *B. argentifolii*, and are referred to simply as whiteflies in this paper. Whiteflies were obtained from the USDA-ARS Western Cotton Research Laboratory, Phoenix, AZ. Insects were maintained on either cotton (*Gossypium hirsutum* L.) or hibiscus (*Hibiscus rosa-sinensis* L.) plants in rearing cages at the USDA-ARS Biosciences Research Laboratory, Fargo, ND. Colony cages were placed within a walk-in environmental chamber, equipped with 485 W high-pressure sodium lamps on a 15-h light cycle at 28°C and 9-h dark cycle at 22°C.

Both cotton and hibiscus leaves infested with adult and nymphal whiteflies were prepared for scanning electron microscopy by fixation in 2,2-dimethoxypropane (DMP) acidified by the addition of one drop of chilled 12.1N HCl to 100 ml. Leaves infested with feeding adults and nymphs were placed in dry ice-cooled DMP, which killed the insects before they could retract their stylets. Acidified DMP reacts with water in the tissue, forming methanol and acetone, resulting in an extremely rapid dehydration (Bjerke et al. 1979). Samples were rinsed several times in absolute ethanol and critical point dried in a Tousimis autosamdri critical point drier model 810, using $CO_2$ as the transitional fluid. Leaves, with adult whiteflies and nymphs still attached, were mounted on aluminum stubs, coated with Au/Pd (60:40) in a Balzers SCD 030

sputter coater. Specimens were examined and photographed with a JEOL JSM 6300 scanning electron microscope. Before sputter coating, some adults and nymphs were carefully lifted from the leaves, pulling the stylets out of the salivary sheaths. These specimens were mounted, sputter coated, and examined as described above.

For light microscopy, cotton and hibiscus leaves infested with whitefly nymphs and adults were cleared and examined. First-instar nymphs (crawlers) were removed from hibiscus leaves and placed on sweet potato (*Ipomoea batatas* L.) leaves, which had not been previously exposed to whiteflies. These crawlers were allowed to feed and molt before fixation and clearing. Leaves were cleared using several techniques, including placing them in boiling 95% ethanol, hot 1N sodium hydroxide, or by following the procedure of Cohen et al. (1998). Once the cytoplasm was cleared from the leaf, the leaf tissue was stained with 0.2% acid fuchsin in equal parts of 95% ethanol and glacial acetic acid (Backus et al. 1988). The tracheary elements of the xylem were readily visible following this staining procedure. It was possible to identify probe sites on the epidermal layer of the leaves and identify salivary sheaths within the mesophyll parenchyma after the tissue had been cleared and stained. Nymphs, both on and off the leaves, were cleared in the same manner. Portions of the same leaves were also fixed in formalin - glacial acetic acid - ethyl alcohol (FAA), embedded in paraffin, serial sectioned on a rotary microtome and stained with Conants quadruple stain according to the methods of Johansen (1940). Both cleared and sectioned specimens were examined and photographed using an Olympus BH2 compound light microscope.

## Results

Adult feeding sites were common along the margins of the epidermal cells (Figs. 1 and 3) but also occurred in the middle of the epidermal cell. Where possible, scanning electron microscopy was used to examine both the surface view and a sectional view of a penetration site along the margin of the cell wall. In 80 such samples, we found that all of the stylets penetrated directly through the cytoplasm of the epidermal cell (Fig. 3) and not through the common wall between cells. Of the serial sections prepared for light microscopy, only four of 40 observed penetrations, appeared to pass through the wall between cells; the others all went through the cytoplasm of the epidermal cell. Of the 300 penetration sites observed on cotton leaves, using scanning electron microscopy, only one stylet was found to be inserted through a stoma. The tip of the labium appeared to be glued or anchored to the leaf surface before or at the time the stylet bundle penetrated the leaf (Fig. 2). Anchoring material can be seen in scanning electron micrographs even after the stylets are withdrawn (Fig. 1).

Adult stylets average 217 ± 4 $\mu$m in length ($n = 40$). The portion of the stylet bundle extended beyond the tip of the labium represents the portion of the total
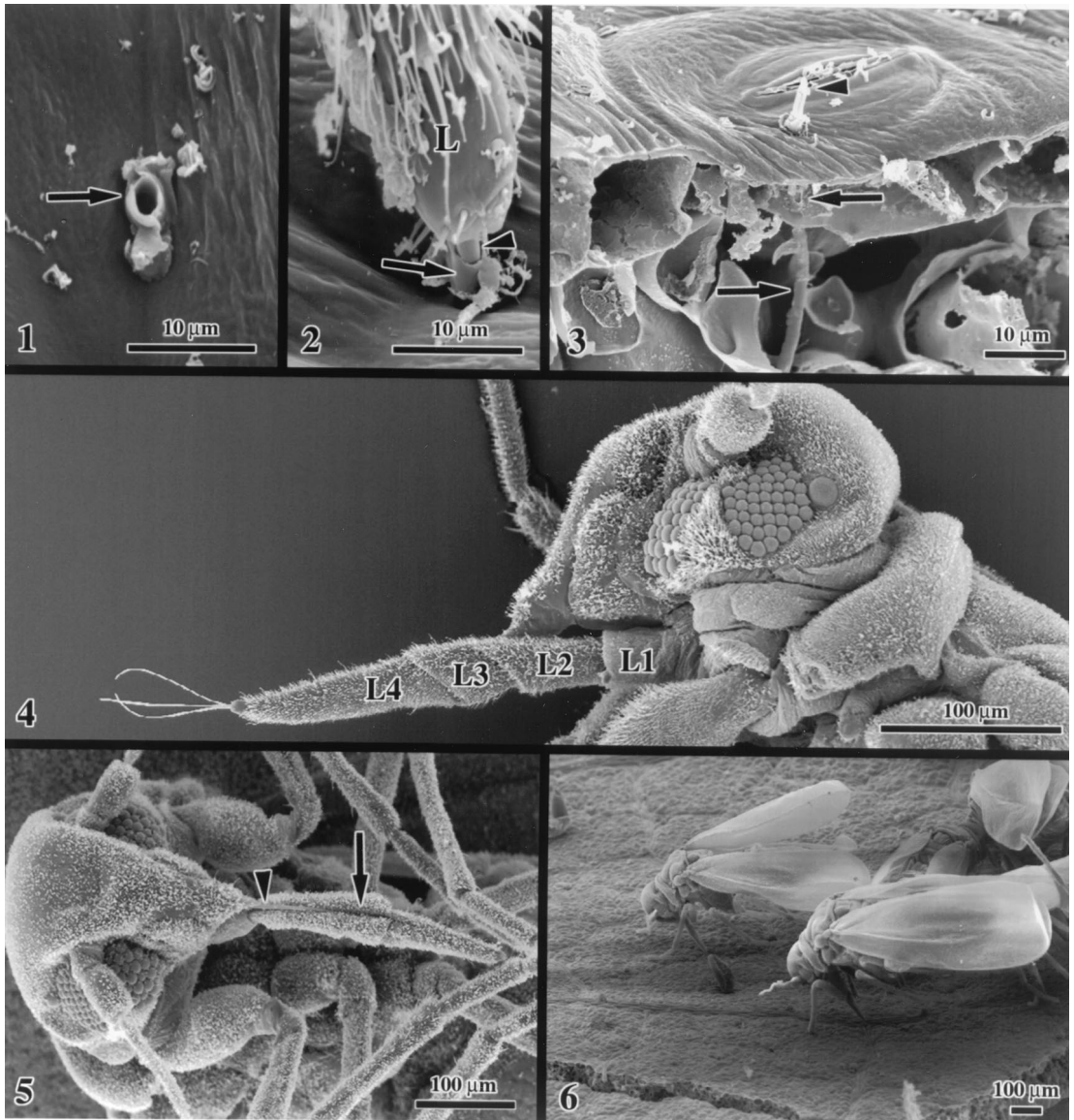
**Figs. 1–6.** (1) Whitefly probe site along the common wall between two epidermal cells. Glue-like material (arrow) is evident even after the whitefly has been removed from the leaf. (2) Details of the labial tip (L), stylet bundle (arrow head), and glue-like material (arrow) on the abaxial epidermis of a cotton leaf. (3) Fractured section through an abaxial epidermal layer, which shows the stylets (arrow head) penetrating along the margins between epidermal cells and the salivary sheath (arrows) within the epidermal cell, exiting into the intercellular space of the mesophyll. (4) Lateral view of the head of a whitefly showing the four segments of the labium (L1-L4). The distance from the junction of labial segments 1 and 2 to the position of the head along the labial groove is equal to the length of the stylet extended past the tip of the labium (Table 1). (5) Frontal view of the whitefly head showing the labial groove (arrow) along the top of the labium and the insertion point (arrow head) of the stylet bundle. (6) Adult whiteflies, in the feeding position, on an abaxial leaf surface of a host plant.

stylet length used in a specific feeding event (Fig. 4). As there is no mechanism to withdraw the stylet bundle into the head, the stylet bundle is completely contained within the labium when the adult whitefly is not feeding (Fig. 5). The stylet bundle enters the labial groove between the first and second segments in nonfeeding adults and extends to the tip of the labium.

The total stylet length can be determined by measuring the combined lengths of labial segments 2, 3, and 4 (Figs. 4 and 5). The distance from the junction of segments 1 and 2 to the position of the head along the labial groove (Fig. 4) is equal to the length of the stylet bundle that is extended into the leaf. The portion of the stylet bundle extending past the tip of the labium
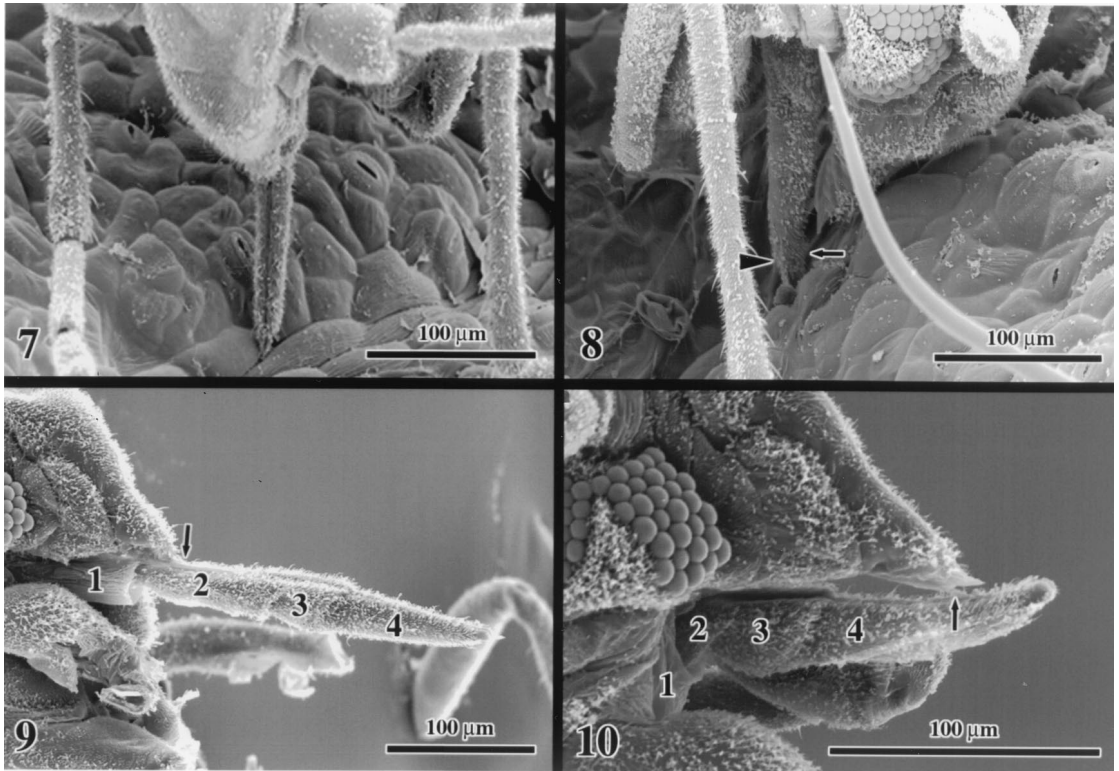
**Figs. 7–10.** (7) Adult whitefly in the feeding position. Note that the head is lowered to the degree that the stylet bundle enters at approximately the midpoint of the labium. This position would extend about half the length of the stylet bundle into the leaf. (8) Side view of a feeding whitefly with the stylet bundle (arrow) entering the labial groove near the tip of the labium (arrow head). Almost the entire length of the stylet has penetrated the host leaf. (9) Whitefly with the stylet bundle almost completely enclosed in the labial groove. Note the appearance of the labial segments (1–4), especially the length and morphology of the first segment. The arrow marks the point where the stylet enters the labial groove. (10) A whitefly that had the stylet bundle completely extended into a host leaf at the time it was fixed and removed. The first labial segment is considerably shorter than those found with less of the stylet extended into the leaf. The arrow marks the point where the stylet enters the labial groove.

of 40 adult whiteflies removed from cotton leaves ranged in length from 43 to 151 $\mu$m with an average of 68 $\pm$ 4 $\mu$m.

The mechanical forces necessary for inserting and withdrawing the stylets appear to result from changes in the adult whiteflies head position during feeding. After the tip of the labium is anchored to the leaf surface, the adult adopts a feeding position (Fig. 6) and lowers its head pushing the stylet bundle down the labial groove (Figs. 7 and 8) and into the host leaf. The entire length of the stylet bundle can be inserted into the leaf in this manner. In the initial stage of feeding the head is positioned so that the stylet bundle enters the labial groove near the junction of segments 1 and 2 (Figs. 7 and 9). As the depth of penetration increases the stylet slides down the labial groove and at the maximum penetration depth, the stylets enter the labial groove near the tip of the labium (Figs. 8 and 10). As the head is lowered, moving the stylet bundle down the labial groove, there is a change in shape of the first labial segment. When the stylet is completely within the labium, the nonfeeding position, the first labial

segment appears elongated, has ridges running along the long axis and is devoid of the setae that characterize the other three segments (Fig. 9). When the stylet is extended to the maximum length (Figs. 8 and 10), the first labial segment appears to collapse or fold into the thorax (Fig. 10). We did not note changes in the length of the other three labial segments. When the stylets are maximally extended, segment 2, however, may be partially obscured within the folds of the collapsed first segment. Accurate measurements of the labium length are difficult to obtain and can be significantly affected by the viewing angle. Mandibular and maxillary protractor muscles probably play a major role in directional movements of the stylets and may also play a limited role in stylet advancement, however, the physical force required for insertion or withdrawal of the stylets depend on the changes in position of the head in relation to the anchored labium.

Nymphs feed during all four instars (Fig. 11). The first nymphal stage (crawlers) have functional legs and are capable of locomotion (Fig. 12), whereas the
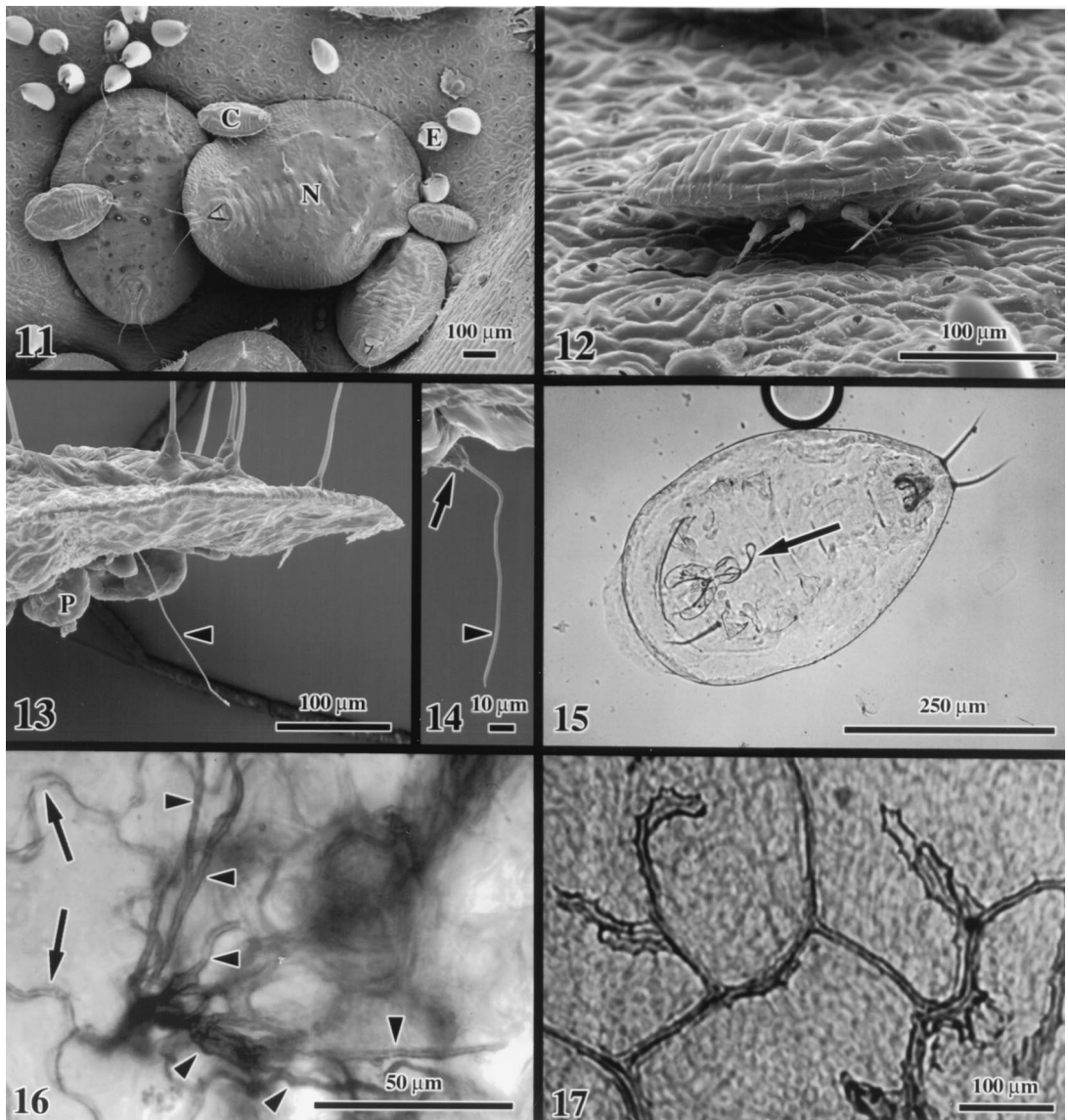
**Figs. 11–17.** (11) Whitefly nymphs on the abaxial surface of a cotton leaf. Note the difference in size of the eggs (E), crawler (C) and fourth instar nymph (N). (12) Crawler on the abaxial surface of a cotton leaf. Once the crawler begins to feed it becomes flattened against the leaf surface. (13) Fourth instar nymph removed from a leaf following fixation. Note the 200 $\mu$m stylet (arrow head) and the pads (P) which are characteristic of the fourth instar whiteflies. (14) Rostrum (arrow) and stylet bundle (arrow head) of a whitefly nymph removed from a leaf following fixation. (15) Cleared third instar nymph showing a portion of the stylet (arrow) folded or looped within the body. (16) Cleared sweet potato leaf showing highly branched salivary sheaths (arrow heads) formed by a single whitefly crawler. The high degree of salivary sheath branching clearly demonstrates that the nymph is able to at least partially withdraw and reinsert its stylet bundle. The epidermal cell walls are also visible (arrows). (17) Cleared, stained cotton leaf showing the pattern of vascular tissue. Superimposing the image of the cleared crawler (Fig. 15) over the image of the cleared cotton leaf (Fig. 17) shows that the crawler can reach the phloem tissue from essentially any position on the abaxial leaf surface with stylets that range from 110 to 140 $\mu$m in length.

next three nymphal stages have only pads (Fig. 13). Once the crawlers begin to feed, they settle down and become flattened and appressed to the leaf surface. The rostrum of the nymph is short (Fig. 14), with sensilla on its tip. The stylet bundle slides through a groove similar to that found on the labium of the adult. Serrations or ridges were found on the mandibular stylets similar to those described on the adults. Nymphs increase significantly in size with each molt (Fig. 11), and although the stylets are longer with each new stage they do not lengthen in direct proportion to the increase in body length (Table 1). There was a

Table 1. Stylet and body lengths (mean ± SEM) for *B. argentifolii* nymphs removed from leaves

| Nymphal instar | $n$ | Body | | Stylet | |
|---|---|---|---|---|---|
| | | Length, $\mu$m | | Length, $\mu$m | Range, $\mu$m |
| First | 10 | $264.8 \pm 9.4^a$ | | $113.8 \pm 4.2^a$ | 110–140 |
| Second | 10 | $331.5 \pm 7.1^b$ | | $135.0 \pm 3.5^b$ | 124–160 |
| Third | 10 | $459.6 \pm 10.8^c$ | | $141.9 \pm 5.4^c$ | 120–171 |
| Fourth | 10 | $709.5 \pm 21.0^d$ | | $158.7 \pm 7.5^d$ | 130–200 |

Means within a column followed by different letters are significantly different ($P < 0.05$; $t$-test).

168% increase in body length from the crawler to the fourth instar but only a 39% increase in the average length of the stylet. Stylet lengths were determined from nymphs removed from leaves during the feeding process. Stylet length may be even longer than reported here, as a portion of the stylet may remain coiled within the body of the nymph (Fig. 15) if the total stylet length is not required to reach phloem tissue.

The branching characteristic of salivary sheaths produced by both adults and nymphs provides clear evidence that the stylets can be partially withdrawn and reinserted. The highly branched salivary sheaths, produced by nymphs transferred as crawlers from hibiscus leaves to sweet potato leaves, demonstrates that the stylet can at least be partially withdrawn and reinserted several times during the feeding process (Fig. 16). Cast stylets were found in all exuviae, documenting that new stylets are formed with each molt and must be inserted into the plant tissue in order for the new instar to feed.

Superimposing micrographs of cleared crawlers (Fig. 15) over micrographs of cleared, stained, cotton leaves (Fig. 17) shows the number of small vascular bundles accessible to both nymphs and adults with average stylet lengths. In cotton leaves examined, the distance from the abaxial epidermal surface to the phloem ranged from 53 to 107 $\mu$m, whereas the length of the nymphal stylets ranged from 113 to over 200 $\mu$m. Therefore, it is possible for any nymphal instar to reach phloem tissue from almost any point on the abaxial leaf surface.

## Discussion

There are frequently slight depressions between the walls separating epidermal cells, and we commonly observed whitefly probe sites in these areas. The placement of the labium in these depressions may help to stabilize it during penetration and feeding. Probe sites were also found through the center of the tangential walls. Regardless of location of the penetration sites, there was always material present that appeared to anchor or 'cement' the tip of the labium to the cell surface. This salivary material appears to be similar to that of the pea aphid (Nault and Gyrisco 1966). Regardless of the position of the penetration sites the stylets almost always entered the outer epidermal wall, transversed the cell cytoplasm and exited through the inner epidermal wall into the intercellular spaces of the mesophyll. It is difficult, or impossible, to determine the path of the stylets past the outer epidermal wall without clearing and staining the salivary sheaths, examining serial sections, or having the capability of seeing the epidermal wall and cell lumen in the same field of view. In only four of 120 penetration sites examined in detail, did the stylets appear to penetrate the common wall between cells. With no evidence to suggest an enzymatic digestion of cell walls, we conclude that the stylets take the path of least resistance by penetrating the outer epidermal wall, pass through the cytoplasm, and exit through the inner epidermal wall into the intercellular space of the mesophyll.

Cleared, stained leaves showed many probe sites without accompanying salivary sheaths indicating that the whitefly probed the surface but abandoned the site without extending the stylet bundle into the mesophyll. The complex course of the stylet to the phloem tissue, as documented by the branched and curved nature of the salivary sheath, suggests that the path is not predetermined and that there may be considerable trial and error in reaching a feeding site. Each dead-end branch indicated that the stylet was partially pulled back, and a new direction was selected. We also, observed numerous sites on both cotton and hibiscus leaves where the anchoring material was apparent, indicating that a probe was initiated but a salivary sheath was never developed. Several researchers have indicated that adult whiteflies make a decision regarding the acceptability of a host long before the stylets reach the phloem tissue of the leaf (Noldus et al. 1986, Walker 1987, Janssen et al. 1989). Walker (1987) found that the bayberry whitefly (*Parabemisia myricae* Kuwana) made a decision regarding the acceptability of lemon leaves before penetration of the thick, mature leaf cuticle, and Janssen et al. (1989) found that rejection may occur when the stylets reach into the epidermis or just beyond. Hunter et al. (1996) described the location and number of sensilla in the precibarial and cibarial sensory organs in *B. tabaci* and suggested that the whitefly may use these organs to make a decision regarding the acceptability of a host long before the stylets reach the phloem. Thus, host acceptability may be made by sampling the leaf cuticle, the material below the cuticle, or by sampling the contents of the epidermal cell. If whitefly penetrations are through the common wall between epidermal cells, without disrupting the symplast of either cell, and then through the intercellular spaces or apoplast between cells, a feeding decision might depend solely on extracytoplasmic substances. Probing and feeding profiles in whiteflies were determined to be due to plant factors at all tissue levels according to Lei et al. (1998).

The anatomy of the adult whitefly head has been described in detail (Rosell et al.1995, Weber 1995, Hunter et al. 1996), and their descriptions suggest that it is not possible for the stylets to be withdrawn into the head cavity. The stylets enter the labial groove between the first and second segment and are essen-

tially equal in length to labial segments 2, 3, and 4. If the labium is a constant length, the question is how does the insect penetrate and extend the stylet into the leaf. Our description of the movement of the stylets through the labial groove as the adult whitefly head changes position over the anchored labium can account for both penetration and extension. The stylets can be extended into the leaf to a depth or extent equal to the total length of the stylet.

We found adult whitefly stylets to be much longer than the distances from the epidermal layers to the phloem in the host plants examined. However, it must be kept in mind that previously published data documented that the course to the phloem is not direct (Cohen et al. 1996a). The length of the extended stylets of insects removed from the leaves may not represent the length actually required for feeding as we did not determine if these insects had actually reached the phloem.

Weber (1931) described a shortening of the labium in the greenhouse whitefly during the feeding process. Labial shortening during feeding has also been reported for aphids (Forbes 1977). The current study found no evidence that labial segments 2, 3, and 4 of whiteflies shortened, nor was shortening of these segments required for the stylets to reach the phloem from the abaxial surface of cotton or hibiscus leaves. The impression that labial segments 2–4 shorten during the feeding process may be due to the difficulty in seeing and measuring the entire labium as the whitefly feeds.

The adult stylets are more than long enough to reach the phloem from either the abaxial or adaxial epidermal surface of the host plants examined. Thus, the theory that whiteflies must feed from the abaxial surface of cotton leaves if they are to reach the phloem does not appear to be valid. There is a preference for feeding from the abaxial surface, but this can no longer be explained on the basis of stylet length. One or more of the other criteria, such as reduced predation, protection from rain and solar energy, or the ability to stay free of honeydew may explain the preference for abaxial feeding.

It is very difficult to determine the portion of the nymph stylet used in feeding as the rostrum and stylet insertion points are out of sight below the nymph. The complex branching of nymph salivary sheaths, however, clearly show that the stylets can be partially withdrawn and then reinserted during feeding. Stylets found in all exuviae document that they are withdrawn before molting and new stylets must, therefore, be inserted after the molt. Lei et al. (1996) concluded that greenhouse whiteflies feeding on cucumber spent most of their time with the stylets inserted within a sieve element but also noted that the stylets were removed during molting and remained in the exuviae.

We found nymph stylet lengths for all instars to be longer than those previously reported. Pollard (1955) noted that first-instar nymphs for *B. tabaci* had stylets 80 $\mu$m long compared with the 120 $\mu$m we measured for *B. argentifolii*. Longer stylets at all stages require a new interpretation of the feeding limitations previ-

ously proposed. In young expanding leaves, the phloem is relatively close to the abaxial surface. During leaf maturation, mesophyll parenchyma cells enlarge and the leaves become thicker resulting in an increase in the distance from the epidermis to the phloem tissue. Whitefly nymphs undergo several molts on cotton leaves, and with each molt the new stylets are longer than in the previous stage of development. The increase in stylet length is more than adequate to continue reaching the phloem tissue in cotton leaves. Fourth instar nymphs may have stylets 200 $\mu$m or longer, more than long enough to reach the phloem in cotton leaves located on the apical 15 nodes of the plant (Chu et al. 2000). Earlier reports (Cohen et al. 1996a, 1996b) suggested that crawlers with short stylets (80 $\mu$m) required some type of surface cue to locate the position of phloem tissue. Surface cues may exist and may be involved in selecting feeding sites but they are not required because of short stylet lengths. This is evident if a comparison is made between the depth of phloem tissue in the top 20 nodes of field grown cotton cultivars that range from 53 to 127 $\mu$m (Chu et al. 2000) with the average stylet lengths of whitefly nymphs (crawlers, 113 $\mu$m; second instar, 135 $\mu$m; third instar, 142 $\mu$m; fourth instar, 158 $\mu$m). Nymph stylet lengths are difficult to determine, and the lengths reported above were measured after the insects were removed from the leaves.

Cleared cotton leaves show numerous small veins within the areoles that can support whitefly feeding. Many of the smaller veins are not associated with elongated abaxial epidermal cells, previously considered to provide clues to the location of veins (Cohen 1996a, 1996b, 1998). When an image of a cleared nymph is superimposed over the image of a cleared leaf, at the same magnification, it is clear that the nymph stylet is long enough to reach the phloem tissue from essentially any point on the abaxial leaf surface of either cotton or hibiscus leaves without requiring a surface cue.

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