MICROSTRUCTURE OF FEEDING IN NYMPHAL *BEMISIA ARGENTIFOLII* IN COTTON AND CANTALOUPE

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Abstract

Studies using confocal and light microscopy revealed that feeding structures of 145 attached *B. argentifolii* nymphs (2nd-4th instar) always reached vascular tissue in cotton and cantaloupe leaves. Analysis of stained and cleared leaves permitted detailed examination of the course of intact feeding structures (salivary sheaths) from the plant's under surface to the target tissue. Nearly every salivary sheath made complex turns and contained several branches that could be considered mistakes in the course of locating target veins. Only minor veins were found to be the targets of the whitefly nymphs. The position of minor veins were invariably associated with elongated surface cells. Although they were in contact with or close to elongated epidermal cells, all attached nymphs made mistakes in their progress towards the target bundles. Confocal microscopy revealed that the specific targets within the veins were apparently always phloem cells. This technique also showed that the sheaths often wrapped around spongy parenchyma cells on their course to veins. Once within the bundle, the sheaths often wrapped around the xylem elements and seemed always to terminate in phloem elements. Very often a single sheath that reached a minor vein would branch at the bundle into two or more (sometimes four or five) sheaths.

Introduction

Over the past five years, the silverleaf whitefly *Bemisia argentifolii* Bellows and Perring (formerly known as strain B of *Bemisia tabaci*) has been reported as the cause of enormous damage to crops throughout the world (Inayatullah, 1985; Bellows et al., 1994; Byrne and Bellows, 1991). Its resistance to several pesticides and its capability of colonizing hundreds of species of crop plants have been well-documented, and its ability to develop vast populations (in excess of 10 billion per acre (Cohen et al., 1993)) thwarts efforts to control this insect by conventional means. The damage caused by *B. argentifolii* has been attributed to materials that the insects inject into the plant, depletion of the plant's sap, contamination of plant surfaces by the honeydew excreted by the insects and inoculation of the plant with pathogens (Byrne and Bellows, 1990). Despite the vast impact of this pest due to its feeding activities, there remain important gaps in our understanding of the feeding mechanisms of *B. argentifolii*.

Pollard (1955), Blua and Toscano (1994), Cohen et al. (1993) and Cohen and Hendrix (1994) raised questions about vascular tissue as the sole feeding target of *B. tabaci* and *B. argentifolii*. Using various techniques, including histochemistry and biochemical analysis, these authors found that whiteflies occasionally feed on tissues other than those found in veins. However, Cohen et al. (1996) found that when feeding intact nymphs and their feeding sites are studied with light microscopy using clearing and staining with no microtomy, all feeding sites include complete salivary sheaths that begin at the labium of the nymph and terminate in a vein. The same authors showed that in sectioned leaf tissue, about 30% of all salivary sheaths appeared to terminate in sites other than veins. This indicated that the Histological procedures caused artifacts that clouded interpretation of whitefly feeding. Several questions and unresolved issues remain after these studies. First, what is the course of the stylets after epidermal penetration? Do they penetrate spongy parenchyma cells or circumvent them? Is the course to the veins direct, indicating sensing of the target; or is the search for veins random? Finally, once the stylets reach the vein, do they make immediate penetration and remain intact; or do they show evidence of more complex searching. Using whole tissue that was cleared and stained so that 3-dimensional fields could be observed, we used electron, light and confocal microscopy to answer these questions.

Materials and Methods

Plants used: Cantaloupe (*Cucumis melo cantalupensis*) and cotton (*Gossipium hirsutum*) were grown in greenhouses during July and August of 1994 and 1995) in greenhouses in Phoenix, AZ. Insects: When cantaloupe plants reached the 10 fully expanded leaf stage and cotton reached the 6 leaf stage, plants were transferred into another greenhouse that was highly infested with *B. argentifolii*. Voucher specimens are being kept at the Western Cotton Research Laboratory, USDA, ARS, Phoenix, AZ. Fully expanded leaves that were infested with at least 10 second to forth instar nymphs were studied.

Leaves that were to be examined by histological treatment were prepared according to slight modifications of the technique described by Berlyn & Miksche (1976). The leaves were fixed in FAA (0.5 ml formalin: 0.5 ml glacial acetic acid: 9 ml 50% EtOH) for 20 h. We dehydrated fixed specimens in an alcohol series from 50-100%, cleared in xylene and infiltrated with Paraplast^{R}. We made 10µm

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sections of leaf tissues and stained them either with the saffranin/fast green method of Pollard (1955) or with a periodic acid method (Berlyn & Miksche, 1976). Sections were placed for 5 min sequentially in each of the following solutions serially: xylene, 100%, 95%, 50%, 25% EtOH, distilled H_2O, periodic acid, H_2O, Schiff's reagent, H_2O, sodium metabisulfate, H_2O. Sections were then re-desiccated in an alcohol series as described above and returned to xylene before being mounted on slides.

Leaves that were used for whole-mount analysis of veins were decolorized with 95% EtOH, then cleared with lactic acid, glycerol, water (LGW) (1:1:1). The clearing procedure took 25 min in an autoclave at 120°C, 15 psi. Leaves to be used for scanning electron microscopy (SEM) were collected from the field during times of peak whitefly infestation (mid-September). They were placed in an acidified solution of 2,2-dimethoxypropane (DMP) for 1 h then in absolute ethanol before critical point drying, cutting and sputter coating. Transverse sections of leaves were fixed to stubs and examined by SEM.

Whole-mounts of cotton leaves that were to be used for measurements of stained stylet sheaths were first placed in McBride's solution (Backus et al., 1988) for 10 to 20 h, until salivary sheaths were deeply stained. Sections were then destained in 95% EtOH for 15-30 min, and then cleared in LGW and autoclaved as described above. Sections that retained too much stain after this procedure were either further destained in 95% EtOH or placed in fresh LGW and re-autoclaved. Specimens were mounted in LGW for microscope observations. Mounted sections were placed under a Wild Makkroscop equipped with a video camera, video recorder and monitor. Images magnified 256 x were projected on the monitor screen where they could be traced manually on clear acetate sheets. Lengths of veins were measured with flexible tubing calibrated in µm with a stage micrometer. Other specimens that were analyzed and photographed with a compound microscope were examined at 400 x, and measurements were made with an ocular micrometer calibrated with a stage micrometer to the nearest 10 µm. Means and standard errors of the vein length of each host plant species were computed. Measurements were made on cotton leaves of the distance from the labium at the point of leaf contact to the point of attachment on the vein in 200 specimens from a total of 25 whole-mounts.

Evaluation of "mistakes" made in stylet course was made by counting the number of sheath tracks that went in a direction other than one that would lead directly to the vein. Only instances were counted where there was ultimately a correct choice made ending in successful connection with the vein.

We examined 100 stained specimens (cleared nymphs in intact feeding position on leaves) with light microscopy at 400 x to determine placement of mouthparts in relationship to plant structures. Veins were classified as single-stranded, double-stranded, triple-stranded (minor veins) and four-or-more stranded, according to the number of xylem bundles that were noted. We further examined 45 cleared and stained nymphs with the aid of confocal microscopy with a Leitz (get information from Dennis M.) confocal microscope with laser light exciting at 545 nm. We used 50, 100, 200 and 400 x to study the placement of sheaths throughout the plant material, recording images at 0.5, 1.0, 2.0 or 5 µm intervals from the point of contact of the insect's labium and the plant surface to the vein region beyond the reach of the salivary sheath. We used the Leitz system's "movie" and 3-dimensional reconstruction programs to view composite images of all focal planes containing feeding structures.

Results

All attached nymphs had sheaths that went to veins. Many nymphs were touching the elongated surface cells that always corresponded to bundles within the plants, but sometimes these nymphs were attached to another vein. The courses of the salivary sheaths were always indirect and often tortuous and multi-branched. The sheaths were never seen to penetrate mesophyll cells (spongy parenchyma or palisade cells), and the sheaths rarely reached beyond the above side of the veins.

The 3-dimensional reconstructions and "composite movies" made it evident that the very complex twisting and folding of the sheaths in x, y, and z planes renders it impossible to fully interpret the path of the salivary sheaths in 2-dimensional histological sections. At least 83% of all sheaths, somewhere in their course to a target vein, made a turn or a branch of the salivary sheath that was more than 90° away from the final point of contact between the sheath and the vein. We called this misdirected turns "mistakes" that ended in successful feeding. We found many other sheaths that were neither attached to veins nor to nymphs, and since we could not differentiate abandoned adult-feeding sites and unsuccessful nymphal attempts to locate a vein, we did not further consider sites that did not have attached nymphs.

Through the use of confocal "optical sectioning" of the feeding sites and associated plant tissue, we were able to make detailed studies of the behavior of the salivary sheaths in relationship to veins. First, no stylet penetration took place in xylem elements. Second, the sheaths often went around the veins in more than 300° arcs. Almost always, the sheaths went between vein cells until they terminated in a phloem element. In 41 of the 45 sheaths that we followed with confocal microscopy, we found multiple branches, each terminating in a separate phloem element. Although the stylets themselves did not stain and fluoresce, they could often be seen as shadows within the fluorescing sheaths, evidently from having quenched fluorescence from the part of the sheath on the opposite side of the light.

Although cantaloupe and cotton leaves have different vein structure from one-another, we found no overt differences in the salivary sheath microstructure in the two plant species.

Discussion

The most conspicuous facts to emerge from this study are that 1) all attached nymphs had salivary sheaths terminating in veins; 2) the targeted veins were all minor veins; 3) most of the salivary sheath is in the air space in the leaf, with only 5-10% of the sheath length passing through epidermal cells; 4) the paths of the salivary sheaths were almost always very sinuous and complex, indicating undirected search for target veins; 5) the sheaths branched often once they reached the veins, with individual branches going to separate phloem elements; 6) the target tissues always appeared to be phloem elements.

Although several earlier studies (Pollard, 1955; Blua and Toscano, 1994; and Cohen and Hendrix) indicated that *B. argentifolii* might feed adventitiously on non-vascular tissue the present study and a recent one by Cohen et al. (1996) present evidence that all attached nymphs were feeding on minor veins. The present study goes further to show that the 45 sheaths that were examined with confocal microscopy all led to phloem elements; and furthermore, none of the sheaths was seen to penetrate mesophyll cells. All of these sheaths were followed for their entire length from the whitefly's labium to the vein, so there was no chance of missing (due to sectioning) a branch of a sheath that may have strayed into a mesophyll cell.

The observation that *B. argentifolii* nymphs always feed on minor veins (described by Cohen et al., 1996 as having 1-3 xylem elements), is partially explained by our observations that the larger veins had sclerenchymous tissues associated with them, evidently making them harder to penetrate. We found several stained salivary sheaths that were associated with larger veins, but we never observed them to penetrate the sclerenchymous sleeves of these bundles, and we never found a nymph attached. Such sheaths are assumed to have been produced either by adults that probed and departed, or they were evidences of unsuccessful efforts at feeding crawlers (1st instar nymphs) that abandoned the feeding site. Such feeding on minor veins by *B. argentifolii* is in marked contrast with feeding by aphids whose preferred targets are larger veins (Gibson, 1972).

When examining salivary sheath placement in plant tissue with transverse sections of leaves as subjects (Cohen et al., 1996; Walker, 1985; Pollard, 1955), it is often difficult to see the positioning of the salivary sheaths in relationship to the plant cells. It appears from many sections that we have examined that the sheaths either go through mesophyll cells or through intralamellar matrices to get to their destination. However, the use of whole, cleared tissue that has been cleared and examined by confocal microscopy allows a much more precise interpretation. We found with these techniques that an extremely small portion of the sheath material is placed within the symplast, and the vast majority of the sheath is in the air space between cells.

This fact is very important in terms of potential application of host plant resistance strategies such as those suggested by Puri et al., 1985, Rao et al., 1993; Wilson et al., 1993; Cohen et al., 1996 and Dreyer and Campbell, 1987. The suggested strategies include selection of plant varieties with less available or unapparent veins and selection of plants that have resistant intercellular matrix material. However, it is clear from our observations that since most of the salivary sheath of *B. argentifolii* is outside of cells, and most penetration of epidermis is through cells rather than between them, resistance mechanisms based on changes in the biochemistry of the pectins or other interlamellar matrices would be useless.

The fact that there are so many twists and turns in the sheath materials found in the spongy parenchyma suggests that *B. argentifolii* has some difficulty locating appropriate bundle targets. This raises the question of how the nymph gets information that helps it make corrections to find feeding targets. A better understanding of this aspect of behavior would certainly give us a stronger basis for selecting or modifying host plants more suited to baffling the whiteflies' location tactics.

Similarly, the observation that nearly all nymphs had sheaths that branched at the vein is intriguing for several reasons. It is known that phloem elements remain alive and capable of conducting sap for only a few hours to a few days (Mauseth, 1988). The branching sheaths raise the question of whether the branching is in response to the plant's natural shutting-down of a given bundle that is co-incidentally being used by the nymph or that the feeding activities of the nymph triggered a pre-mature shut-down of that phloem element forcing the whitefly to withdraw and seek a new phloem element.

In summary, we have found several details in the feeding habits of *B. argentifolii* that could not be observed except in intact leaf tissue. Use of confocal microscopy allowed us to reconstruct 3-dimensional views of the feeding sites in relation to plant tissues that have not been sectioned. This has allowed us to observe the extremely complex nature of the feeding structures, the convolutions and branchings of the salivary sheaths, especially after the vein has been reached. These techniques have allowed us to confirm that successful feeding is always terminated in veins, specifically in phloem elements. These feeding details offer a basis for developing whitefly-resistant plants, and they also present some constraints on previously suggested approaches to host plant resistance ways of controlling *B. argentifolii*.

References

1. Byrne, D.N. & T.S. Bellows. 1991. Whitefly biology. Annual Review of Entomology. 36: 431-457.

2. Backus, E.A., W.B. Hunter & C.N. Arne. 1988. Technique for staining leafhopper (Homoptera: Cicadellidae) salivary sheaths and eggs within unsectioned plant tissue. Journal of Economic Entomology. 81: 1819-1823.

3. Bellows, T.S., T.M. Perring R.J. Gill & D.H. Headrick. 1994. Description of a species of *Bemisia* (Homoptera: Aleyrodidae). Annals of the Entomological Society of America 87: 195-206.

4. Berlyn, G.P. & J.P. Miksche. 1976. Botanical microtechnique and cytochemistry. Iowa State University Press, Ames, Iowa.

5. Blua, M.J. & N.C. Toscano. 1994. *Bemisia argentifolii* (Homoptera: Aleyrodidae) Development and honeydew production as a function of cotton nitrogen status. Environmental Entomology. 23: 316-321.

6. Chu, C.C., T.J. Henneberry & A.C. Cohen. 1995. Silverleaf whitefly (Homoptera: Aleyrodidae): Host preference and factors affecting oviposition and feeding site preference. Environmental Entomology In Press.

7. Cohen, A.C., C.M. Newman & D.L. Hendrix. 1993. Histochemical studies of feeding sites of *Bemisia tabaci* on cotton leaves, . In: D.J. Herber & D.A. Richter (Eds.). Proceedings, Beltwide Cotton Conferences, 9-16 February, National Cotton Council of America, Memphis, TN. p. 959.

8. Cohen, A.C. & D.L. Hendrix. 1994. Demonstration and preliminary characterization of α-amylase in the sweetpotato whitefly, *Bemisia tabaci* (Aleyrodidae: Homoptera). Comparative Biochemistry & Physiology. 109B, 593-601.

9. Cohen, A.C., T.J. Henneberry and C.C. Chu. 1996. Geometric... Entomologia experimentalis et applicata. (In Press)

10. Dreyer, D.L. & B.C. Campbell. 1987. Chemical basis of host-plant resistance to aphids. Plant, Cell and Environment. 10: 353-361.

11. Gibson, R.W. 1972. The distribution of aphids on potato leaves in relation to vein size. Entomologia experimentalis et applicata . 15: 213-223.

12. Inayatullah, C. M.A. Ghani & A. Ghaffer. 1985. Cotton whitefly, *Bemisia tabaci* (Genn.) and its control. Lahore, Punjab, Directorate of Agricultural Information. 90 pp.

13. Lenteren, J.C. van & L.P.J.J. Noldus. 1990. Whitefly--plant relationships: behavioral and ecological aspects. In: Whiteflies: their Bionomics, Pest Status and Management. (Ed.) D. Gerling. Intercept, Andover, UK. pp. 47-89.

14. Mauseth, J.D. 1988. Plant Anatomy. The Benjamin/Cummings Publishing Company, Inc. Menlo Park, CA. 560 pp.

15. Pollard, D.G. 1955. Feeding habits of the cotton whitefly *Bemisia tabaci* Genn. (Homoptera: Aleyrodidae). Annals of Applied Biology. 43: 664-671.

16. Puri, S.N. A.S. Ansingkar, V.N. Ajankar, R.C. Lavekar, G.D. Butler, Jr. & T.J. Henneberry. 1993. Effect of cotton leaf morphology on incidence of *Bemisia tabaci* Genn. on cotton. Journal of Applied Zoological Research. 4: 41-44.

17. Rao, N. Venugopal, A.S. Reddy, R. Ankaiah, Y.N. Rao & S.M. Khasim. 1990. Incidence of whitefly (*Bemisia tabaci*) in relation to leaf characters of upland cotton (*Gossypium hirsutum*). Indian Journal of Agricultural Sciences 60: 619-624.

18. Reese, J.C., D.C. Margolies, E.A. Backus, S. Noyes, P. Bramel-Cox & A.G.O. Dixon. 1994. History, Development, and Application of AC Electronic Feeding Monitors. M.M. Ellsbury, E.A. Backus, & D.L. Ullman, Eds. Proceedings Thomas Say Publications in Entomology. pp. 52-72.

19. Walker, G. 1985. Stylet penetration by the bayberry whitefly, as affected by leaf age in lemon, *Citrus limon*. Entomologia experimentalis et applicata. 39: 115:121.

20. Walker, G.P. & T.M. Perring. 1994. Feeding and oviposition behavior of whiteflies (Homoptera: Aleyrodidae) interpreted from AC electronic feeding monitor waveforms. Annals of the Entomological Society of America 87: 363-374.

21. Wilson, F.D., H.M. Flint, B.R. Stapp and N.J. Parks. 1993. Evaluation of cultivars, germplasm lines, and species of *Gossypium* for resistance to Biotype "B" of sweetpotato whitefly (Homoptera: Aleyrodidae). Journal of Environmental Entomology. 86: 1857-1862.