High Performance Liquid Chromatography Analysis of Carbohydrates of Cotton-Phloem Sap and of Honeydew Produced by Bemisia tabaci Feeding on Cotton

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ABSTRACT
Phloem sap from cotton (Gossypium hirsutum L.) was collected from young and mature leaves by the aphid-stylet technique. Exudate was analyzed for carbohydrates by HPLC using sensitive pulsed amperometric detection. The predominant carbohydrate present (>90%) was identified as sucrose. A second, unidentified compound that was not one of the more commonly translocated sugars was detected in mature leaves. Carbohydrates in honeydew produced by the sweet-potato whitefly (Bemisia tabaci [Genn.]) feeding on cotton were sucrose, glucose, fructose, trehalulose, and a series of oligosaccharides.

A number of phloem-feeding insects have been reported to affect adversely the growth of their plant hosts (e.g. 10). The relationship between the sweet-potato whitefly, Bemisia tabaci (Genn.) (Homoptera: Aleyrodidae), and the host plant, cotton, Gossypium hirsutum L., is an important example. Whitefly populations can exceed 100 individuals/cm² of cotton-leaf surface (D.N.B., personal observation) and can extract enough phloem sap to affect directly crop yields (11). This insect also serves as a vector for at least 14 viral pathogens (2). The honeydew produced by whiteflies supports the growth of sooty mold fungi, e.g. Capnodium spp., which can lead to photosynthetic inhibition (8).

In this investigation, we utilized the aphid-stylet technique to characterize qualitatively the carbohydrate composition of phloem sap from short-staple cotton. Cotton-phloem sap has not previously been characterized, presumably because of the difficulties involved in phloem-sap extraction. We also examined the carbohydrates found in honeydew produced by B. tabaci feeding on cotton. The data obtained will improve our understanding of the carbohydrates translocated by cotton-feeding aphids.

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MATERIALS AND METHODS
Plant Material
Cotton plants, Gossypium hirsutum L., cv Delta Pine and Land Company No. 90, were cultured in potting soil under standard glasshouse conditions (sowings in January, central Arizona). Plants were watered daily and were assessed visibly to be healthy. All experiments were conducted on plants that were approximately 5 months of age.

Sap and Honeydew Collection
Phloem sap was obtained from two types of leaves: (a) young and expanding (<3 cm in length), and (b) mature and fully expanded (approximately 12 cm in length). Phloem sap from a total of three plants was analyzed. The concentration of cotton aphids, Aphis gossypii Glover, feeding on these plants was approximately three aphids/cm² of leaf (abaxial surface); these insects were used as a tool for phloem-sap extraction. Collections of phloem sap were made by severing the aphid-stylet bundles, which were inserted into the minor veins of attached leaves. Stylets were severed by a microcutter device similar to that described previously (5, 14). Phloem sap was collected from three stylets per leaf; stylet positions were proximal to the base, center, and tip of each leaf. Submicroliter droplets of pure exudate were continually collected from the severed stylets over a period of <1 h by a rounded-off capillary tube. Only pure phloem sap, from severed stylets alone (without rostra), was collected. A Tatung TUH-740 humidifier (Tatung Co. of America, Inc., Long Beach, CA) was used to prevent rapid evaporation of the exudate, which, in preliminary experiments, had resulted in the occlusion of the severed stylets.

Samples for analyses by HPLC coupled to PAD1 (below) contained approximately equal portions of exudate pooled

1 Abbreviation: PAD, pulsed amperometric detection.
from two leaves of each leaf type per plant. The collected material was stored at 4°C in 50 μL of distilled water. Samples were typically analyzed for carbohydrates within 2 h of collection; however, HPLC-PAD analyses of stored samples (3 weeks, −20°C) indicated that there was no detectable degradation.

Populations of sweet-potato whitefly (Bemisia tabaci) were cultured as previously described (3), except G. hirsutum was the host plant. Whitefly honeydew was collected individually from eight plants by placing 100 adult whiteflies on the abaxial surface of fully expanded leaves in clip cages made from 5.0-cm diameter Petri dishes. Honeydew droplets were allowed to fall to the bottom part of the Petri dishes for 48 h; the samples were then stored at −70°C until carbohydrate analysis. Previous studies (3) indicated no postcollection compositional changes within the collection period used.

HPLC Analysis of Carbohydrates

Carbohydrates of honeydew were solubilized from Petri plates in 2.0-mL HPLC-grade water (prepared from glass-distilled water, using ion-exchange cartridges; Norganic, Waters) and applied to polyethylene columns containing 1 mL each of Dowex 50 W (H+ form) and Amberlite IRA-68 (acetate form). Petri dishes were washed with an additional 3.0 mL of water, and washes were applied to the columns. Neutral substances (sugars) were eluted with 5 mL of water and evaporated to dryness under vacuum at 40°C. The dry residue was redissolved in 1 to 2 mL of HPLC-grade water and then passed through a 0.45-μm membrane filter for particulate removal. Samples were injected into an isocratic HPLC system (LKB 2150 pump; Pharmacia-LKB, Piscataway, NJ) by a Rheodyne 7010 injector with a 20-μL sample loop. The flow rate was 0.6 mL/min; degassed HPLC-grade water was the mobile phase. Sugar separation was by a Bio-Rad HPX-87C stainless steel column (300 mm × 7.8 mm i.d.) maintained at 85°C with a Bio-Rad column heater (Bio-Rad Laboratories, Richmond, CA). A guard column at room temperature was used between the injector and analytical column. Detection was via refractive index (Knauer, model 298). A Hewlett-Packard 3390A integrator was used for peak area quantification and retention time determination.

Because of the small quantities of phloem-sap collected, carbohydrates of phloem sap were separated by a sensitive HPLC-PAD method (pulsed amperometric detector limit, <10 pmol/injection) similar to that described by Rocklin and Pohl (12). Samples were injected directly into a HPLC-PAD system (Spectrophysics SP8700 pump, San Jose, CA), by a Rheodyne 7125 injector with a 100-μL sample loop (Rheodyne Inc., Cotati, CA). The system was operated isocratically at 1.0 mL/min with degassed 0.15 N NaOH as the mobile phase. Separation of the sugars was achieved by a Dionex CarboPac PA-1 analytical column (250 mm x 4 mm i.d.; Dionex Corp., Sunnydale, CA) maintained at 25°C. A Spectrophysics SP4290 integrator was used for peak area quantification and retention time determination.

Carbohydrates of phloem sap were identified by cochromatography with authentic D-sugars. Honeydew carbohydrates were identified by a comparison of the retention times of honeydew sugars with those of sugar standards or by cochromatography. Carbohydrate standards were from commercial sources, primarily Sigma, except verbascose, which was a gift from Dr. P. Kerr (DuPont). Trehalulose, for which no standard was available, was identified by NMR as reported earlier (1). Ion-exchange resins were from Sigma.

RESULTS AND DISCUSSION

Initial attempts to collect cotton-phloem sap using the convenient method described by King and Zeevaart (9) were unsuccessful. These authors excised petioles under a solution of EDTA. As a chelating agent, EDTA prevents the formation of callose over sieve-tube plates, allowing the sap to flow into solution. However, with cotton, HPLC profiles of soluble sugars obtained by this method were complex (e.g. >10 peaks detected per sample), and the reducing sugars, glucose and fructose, typically represented greater than 20% of the total area, indicating contamination. It seems likely that the contaminating sugars came from damaged or ruptured cells, or perhaps resulted from metabolism of sucrose.

We employed the more laborious aphid-stylet technique to obtain pure phloem sap from cotton, which has not previously been characterized. Results obtained from the HPLC-PAD separation of carbohydrates from collected exudate are shown in Figure 1. We have determined that for cotton, as with most plants studied, the major sugar translocated is sucrose. Sugar standards (panel A) were selected based on their accepted occurrence in phloem sap of various plants (for a compilation of phloem transport sugars, see ref. 17). Carbohydrate profiles of phloem sap from expanding leaves (panel B) and those from fully expanded leaf material (panel C) were similar in that sucrose was identified (panel D) as the predominant sugar (>90%, area basis). However, a second peak (retention time, 3.0 min) was always detected in phloem sap from fully expanded leaves, but this peak was either severalfold less abundant or not detected in replicate samples of phloem-sap collected from expanding leaf material.

Three main types of sugars are known to be translocated in the phloem: (a) sucrose, (b) raffinose-series oligosaccharides (raffinose, stachyose, verbascose, ajugose), and (c) polyols (mannitol, sorbitol). In an effort to identify compounds, exudate from mature leaves was cochromatographed with standards for the above sugars (except ajugose, which is transported with sucrose and raffinose-series oligosaccharides [17]). However, none of the sugars had a retention time of 3.0 min (Fig. 1A, D; retention times for the oligosaccharides raffinose, stachyose, and verbascose were 14.3, 15.4, and 19.7 min, respectively). In fact, no sugar that we examined, including those unlikely to be found in phloem sap (arabinose, fructose, galactose, glucose, maltose, mannose, ribose, trehalose, xylose), comigrated with the unidentified compound (data not shown).

The HPLC-PAD method used for the analysis of exudate carbohydrates relies on the presence of hydroxyl groups for detection. Thus, if present at sufficient levels, the amino acids, hydroxy-proline, serine, threonine, tyrosine, in addition to carbohydrates, could be detected in phloem sap. However, cochromatography experiments demonstrated that the retention times of these amino acids (hydroxy-proline and serine, 4.5; threonine, 3.3; tyrosine, >30 min) did not correspond to...
CARBOHYDRATES OF COTTON PHLOEM AND OF WHITEFLY HONEYDEW

Figure 1. HPLC-PAD chromatograms of phloem-sap carbohydrates. For details of HPLC separation procedures, see "Materials and Methods." A, Sugar standards, 0.16 nmol each: 1, myo-inositol; 2, dulcitol and sorbitol; 3, mannitol; 4, sucrose. B, Exudate from young, expanding leaf material (<3 cm in length); i, injection peak. C, Exudate from mature, fully expanded leaf material (approximately 12 cm in length). Arrow indicates the elution position of the unknown compound. D, Sugar standards (0.16 nmol each) cochromatographed with exudate from mature leaf material (cf. panels A, C).

Table I. Carbohydrate Composition of Honeydew Produced by Bemisia tabaci Feeding on Cotton Host Plants

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Total Soluble Carbohydrate %</th>
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<tbody>
<tr>
<td>Oligosaccharides</td>
<td>29.5 ± 5.9</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.1 ± 1.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.3 ± 1.8</td>
</tr>
<tr>
<td>Fructose</td>
<td>11.7 ± 1.1</td>
</tr>
<tr>
<td>Trehalulose</td>
<td>43.1 ± 6.2</td>
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</tbody>
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Honeydew was collected from 100 whiteflies per fully expanded leaf. Sugars were identified by comparison with standards. Total soluble carbohydrate was based on the sum of the peak areas as determined by integration. A total of eight plants were examined. Values are means ± ses (n = 8).
the levels of sucrose are much lower in honeydew relative to phloem sap (3) (Table I, Fig. 1). The observation that whiteflies feeding on pumpkin, which translocates raffinose-series sugars (3, 6), produced nearly fivefold less trehalulose (3), supports this idea.

Currently, we are conducting research to determine if isomaltulose synthase (EC 5.4.99.10), the enzyme presumably responsible for trehalulose biosynthesis, is present in whitefly mycetocytes. The adaptive significance of altering the disaccharide linkage of sucrose to form trehalulose is unclear. It may possibly be that bacteria contained in whitefly mycetocytes, under conditions in which sucrose is limiting, sequester the reduced carbon in an alternative form (trehalulose), which is unavailable to the host insect. A similar mechanism has been identified in Pseudomonas (15).

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LITERATURE CITED