Patterns of Starch Accumulation in Alfalfa Subsequent to Potato Leafhopper (Homoptera: Cicadellidae) Injury

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ABSTRACT Patterns of starch accumulation in alfalfa, Medicago sativa L., were studied in response to injury caused by potato leafhopper, Empoasca fabae (Harris). Using image analysis of iodine-stained leaf tissues, we compared lightness scores of leaves from leafhopper-injured and healthy plants sampled at early morning and mid-afternoon. In addition, we related lightness scores to standard chemical analyses for starch and sugar. The lightness scores were significantly related to starch concentrations but not to sugar concentrations. In the morning samples, all fully developed and developing leaves above the site of feeding indicated higher starch concentrations than comparable leaves on healthy plants. In contrast, all leaves from mid-afternoon samples and leaves below the site of feeding from morning samples did not significantly differ between injured and healthy plants. The observation that the lack of starch degradation during the night occurs in all tissues distal to the feeding site, and especially in developing leaves, suggests that the mechanism of starch accumulation is not just feedback from phloem blockage caused by leafhopper feeding but also a change in starch degradation in all chloroplasts without regard to source-sink status.

KEY WORDS Empoasca fabae, starch accumulation, sap feeding

Carbon metabolism in plants is a complex cycle with many components (Hopkins and Hünner 2004). Beginning in the chloroplasts, carbon dioxide is fixed via photosynthesis into the primary photosynthetic product triose-P. Typically, part of the triose-P leaves the chloroplasts and is converted into sucrose, which is then transported from the leaf through the phloem to drive various metabolic processes. Translocation through phloem tissue allows for the distribution of sucrose throughout the plant. The remainder of triose-P is either used immediately in the leaf or is stored in the form of starch to be used during periods of darkness (Warlaw and Passioura 1976). When sap-feeding insects feed on vascular tissues of their host plant, they prevent normal resource acquisition by the plant (Raven 1983). Of greater economic importance are those sap-feeding species that cause additional direct injury associated with feeding, leading to structural and functional changes in the vascular tissues and effects on carbon metabolism (Ecalle Zhou and Backus 1999).

Potato leafhopper, Empoasca fabae (Harris), is such a direct-damaging feeder. It is a major pest of alfalfa, Medicago sativa L., as well as numerous other crops (Ball 1919, Lamp et al. 1994). The leafhopper feeds on alfalfa by repeated penetration of narrow stylets into the vascular tissue through which plant material is ingested and saliva is injected (Backus and Hunter 1989, Kabrick and Backus 1990). Research has demonstrated that through a combination of mechanical and salivary stimuli, potato leafhopper feeding enhances a wound response in alfalfa that results in hypertrophy of cambial cells, collapse of the phloem cells, and generation of xylem cells, along with a number of other structural changes (Ecalle and Backus 1995a, b). When this occurs, photoassimilates transported through the phloem build up around the injured site (Johnson 1934; Hibbs et al. 1964; Nielsen et al. 1990, 1999), and rates of photosynthesis are reduced (Womack 1984, Flinn et al. 1990). Thus, leafhopper feeding initiates a cascade of changes in alfalfa, and ultimately, the injury is expressed as "hopperburn," a characteristic yellowing of leaves (Ball 1919) as well as delayed plant maturity, reduced nutritive components, stunted growth, and reduced yields (Kindler et al. 1973, Hower 1989, Hutchins and Pedigo 1989).

Currently, the cascade of plant physiological changes subsequent to vascular injury by potato leafhopper that leads to hopperburn is unclear. One way to provide such information is to monitor the pattern of accumulation of carbohydrates in the plant. Starch is a stable form of carbohydrate in alfalfa and is therefore a suitable way to measure the carbohydrate flux. In a healthy plant, the maximum rate of photosynthesis is faster than translocation and therefore excess photoassimilates are stored during the day as starch granules in the chloroplasts (Warlaw and Passioura 1976). At night, the starch is converted to sucrose and transported through the phloem to various parts of the plant.
As a consequence, the starch granules are smallest in the morning because most of the carbohydrate has been transported out of the leaves during the night. By late afternoon, the granules are enlarged after accumulating photoassimilates throughout the day.

In a plant injured by potato leafhopper, researchers have hypothesized that the blockage of phloem causes excess photoassimilates in the leaves to inhibit the net transport of additional sucrose out of the leaf tissue (i.e., feedback inhibition) (Johnson 1938, Hibbs et al. 1964). This process is associated then with further conversion of sucrose to starch for storage in the granules. Therefore, although granules enlarge throughout the day similarly in healthy and injured plants, at night the breakdown of starch and transport out as sucrose is expected to occur at a much lower rate in injured plants. Thus, injured plants are expected to contain much larger granules (and more starch) in the morning than are healthy plants.

Our experiments sought to compare these hypothesized patterns of starch accumulation in alfalfa leaves from leafhopper-injured and healthy plants, to gain an understanding of the mechanisms of physiological changes resulting from leafhopper feeding. Previous studies have proven that a buildup of carbohydrates above the site of injury does occur (e.g., Hibbs et al. 1964); however, no distribution pattern in leaves was determined. We expected that more starch is present in leaves on injured plants compared with healthy plants, especially in the morning. Iodine stain, which stains starch blue-black, was used to investigate the patterns of starch accumulation subsequent to potato leafhopper injury.

**Materials and Methods**

Two experiments were conducted. First, we compared the pattern of starch accumulation in individual leaves in leafhopper-injured and healthy alfalfa plants. During this experiment, we observed that developing leaf tissue of injured plants also was stained by iodine, suggesting the surprising result that starch accumulated in these energy-starved tissues. We performed a second experiment to verify this observation on developing leaf tissue to provide supporting evidence for the pattern of staining from the first experiment and to compare the staining procedure to traditional starch and sugar chemical analyses.

Potato leafhoppers were derived from a culture originating from adults collected from an alfalfa field in Washington County, Maryland, and maintained on broad bean, *Vicia faba* L. Before each experiment, adults were placed in a cage with ‘WL323’ alfalfa for oviposition. Nymphs that hatched from these eggs and that had fed on alfalfa throughout development were used for the study. Specimens of *E. fabae* from studies are maintained at the Department of Entomology (University of Maryland).

Alfalfa plants were grown in a greenhouse from a susceptible clone of ‘Ranger’ and were used for experiments after 14 d of growth. In both experiments, injury was induced in a similar manner. One stem was chosen from each plant, and 2.5-cm² plastic cage was used to enclose an area of stem below the uppermost three leaves. Two fourth to fifth instars of potato leafhopper were inserted into half of the cages and allowed to feed for 2.5 d. The other half remained empty to serve as controls. Plants were destructively sampled at two times, one shortly before sunrise (6:30 a.m.), and one late in the afternoon (3:00 p.m.). Plants were cut close to soil for analysis.

**Experiment 1.** A completely randomized design was used to compare plants of two treatments, injured and healthy, sampled at two different times, with 10 plants for each treatment and sampling time combination. Plants grown in the greenhouse were moved to a growth chamber 2 wk before the experiment. The chamber was set at 20°C at night and 25°C during the day with a photoperiod of 14:10 (L:D) h. Plants received 120 μmol/m²/s photosynthetically active radiation during the day.

At each sampling time, stems were cut, and cages and nymphs were removed. Sampled stems were then tagged, submerged in 80% ethanol, and boiled for 90 min to remove all pigment. One stem was removed in a randomly determined sequence (the remainder staying in cool ethanol) and dipped in iodine stain (8 g of KI, 400 ml of H₂O, and 0.8 g of I₂) for 45 s. The time interval ended with a plunge into a beaker of water to dilute the dye enough to halt the dying process. The stem was then spread on a glass plate so that all leaves were flat, and a digital analysis system (CIAS-400, CID, Inc., Vancouver, WA) was used to capture an image of each leaf on a light table. This process was repeated for each stem.

All images were converted to black and white, and lightness of each leaf on the stem was rated with a computer generated grayscale, which assigned values to each pixel of the image from 1 (maximum pigment) to 256 (lack of pigment). Background (nonleaf) pixels with values of 200–256 were deleted. These values of background pixels were determined by manually scanning values from each picture. A lightness score was computed for each leaf as the average pixel value for all leaf pixels. Scores were computed using the first four leaves above and the first two leaves below the caged internode. However, only the first three leaves above and the first leaf below the internode provided sufficient replication for statistical analysis.

**Experiment 2.** Using 16 plants, an experiment was performed with the same treatment and sampling time combinations applied as a completely randomized design. However, the experiment was conducted in the greenhouse to simulate more natural light levels (increasing during the day to a maximum of ~300 μmol/m²/s). In addition, four stems from each plant were prepared as described above, instead of just one. One of the four stems was analyzed using iodine stain as described above, although instead of sampling all leaves above and below injury, only the leaf directly above and the leaf directly below the site of injury were sampled. In addition, developing leaves...
from the growing tip were sampled. Lightness scores were determined as described above.

The tissues of the remaining three stems were combined for chemical analysis. Leaves were separated into those directly above injury, those below injury, and new growth (i.e., developing leaves). Each tissue sample was immediately put in liquid nitrogen and stored at −80°C to avoid degradation. After lyophilization and grinding, two to four samples of a similar treatment were combined to form a new sample of 25–50 mg. Samples were combined because each individual sample did not carry enough mass on its own. One milliliter of 80% ethanol was then added to each new sample followed by 15 s of vortexing and 10 min of centrifugation at 14,000 rpm. Supernatant was then removed. The cycle of ethanol addition, vortexing, and centrifugation was repeated three to six times. Supernatants were combined into a single tube for use in sugar analysis.

For starch analysis, 500 μl of high-performance liquid chromatography-grade water was added to the pellet. After warming in boiling water for 10 min, 400 μl of 200 mM acetate buffer (pH 5.0) and 100 μl of enzyme solution (100 U of amyloglucosidase [product A3514, Sigma, St. Louis, MO], 4000 U of α-amylase [product A0273, Sigma], and 10 ml of 200 mM sodium acetate buffer) also were added. Tubes were next vortexed and set to incubate for 24 h at 55°C. Glucose [TRINDER] reagent was added to identify glucose amount. After vortexing and 1 h in a 37°C hot water bath, absorbance was read at 505 nm on a spectrophotometer. A standard curve was constructed from 0 to 200 μg of glucose. Starch concentration was calculated as glucose concentration multiplied by 0.9 to account for the mass difference between glucose and the anhydroglucose that comprises starch.

For sugar analysis, an aliquot was taken from the supernatant, mixed with an anthrone solution (Koehler 1952), and then read at 625 nm on a spectrophotometer. A standard curve was constructed from 0 to 200 μg of glucose.

**Statistical Analysis.** Analysis of variance (ANOVA) was performed on the data from both experiments with the MIXED procedure (SAS Institute 1997). To account for correlation among the residuals, various covariance structures were modeled and Akaike information criterion was used to ascertain which of the covariance structures fit best (Littell et al. 1996). Data collected in the morning and in the afternoon were analyzed separately for both experiments. For experiment 1, we tested for the fixed effects of leaf position (first [A1], second [A2], and third [A3] leaf above the internode, and the first [B1] leaf below the internode) and leafhopper injury (no leafhopper injury or injured) on the lightness scores. For experiment 2, we tested for the fixed effects of leaf position (first leaf above the internode, first leaf below the internode, and new growth) and leafhopper injury (as described above) on the lightness scores. For both experiments, plant served as a random effect, and leaf position was nested within plant and leafhopper injury. We compared the scores in the comparable leaf of injured plants to the same leaf of healthy plants and made pairwise comparisons for significant differences (α = 0.05) by using Fisher’s least significant difference test. Only meaningful comparisons were made. For the chemical analyses completed for plant tissues during experiment 2, we used PROC GLM to regress concentrations against lightness scores, by using plant tissue as a covariate. In addition, we analyzed for nonparametric correlations among starch concentration, sugar concentration, and lightness scores using the Spearman rank correlation test.

**Results**

**Experiment 1.** Leaves sampled in the morning stained darker (=lower lightness score) on injured plants (mean score 146 ± 17, n = 42) than on healthy plants (mean 162 ± 12, n = 36), indicating elevated starch levels on injured plants. Leaves sampled in the afternoon stained darker than the morning samples, although scores for injured and healthy leaves were similar (mean score for leaves on injured plants 136 ± 12, n = 45; mean for leaves on healthy plants 137 ± 12, n = 47). In addition, we observed (but did not digitize scores) that stems and developing leaves distal to the point of injury also stained darker.

An ANOVA of the three leaves above and the one leaf below the site of injury also showed a significant difference between healthy and injured leaves in the degree of stain for starch (Table 1; Fig. 1). Specifically, in the morning samples, leaves above the point of vascular injury in leafhopper-exposed stems were significantly darker than those leaves of healthy stems (A1, P = 0.01; A2, P = 0.02; A3, P = 0.01). In contrast, the leaf below injury (B1) did not differ significantly between injured and healthy stems (P = 0.23). In the afternoon samples, lightness scores did not differ between injured and healthy stems for any of the leaves above or below injury (A1, P = 0.39; A2, P = 0.19; A3, P = 0.53; B1, P = 0.58).

**Experiment 2.** The iodine stain observations were similar to observations of experiment 1 with significant treatment differences (lightness scores from injured versus healthy stems, Table 2). In the early morning samples, the leaf above the point of injury again stained darker (i.e., lower lightness scores) than leaves from healthy stems (Fig. 2, P = 0.004). In the afternoon samples, scores were similar in both healthy and

<table>
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<tr>
<th>Time of day</th>
<th>Source</th>
<th>NDF</th>
<th>DDF</th>
<th>F value</th>
<th>Probability</th>
</tr>
</thead>
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<tr>
<td>a.m. Leaf</td>
<td>3</td>
<td>34.3</td>
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<td>0.32</td>
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<tr>
<td>Treatment</td>
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<td>20.2</td>
<td>14.5</td>
<td>0.001</td>
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<td>1.2</td>
<td>0.33</td>
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<tr>
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</table>

DDF, denominator degrees of freedom; NDF, numerator degrees of freedom; Trt, treatment.
injured plants \( (\text{Fig. 2, } P = 0.09) \). Again, leaves below injury at both sampling times showed no differences in scores \( (P = 0.50 \text{ for a.m., } P = 0.59 \text{ for p.m.}) \). Developing leaves showed a pattern similar to the A1 leaf; specifically, developing leaves from injured stems sampled in the morning had lower scores in comparison with developing leaves from healthy plants \( (P = 0.04) \), whereas afternoon samples showed no such differences \( (P = 0.41) \). The significant interaction between injury and leaf type in the early morning \( (P = 0.03) \) demonstrates the different injury response of developing leaves and the leaf above the cage \( (\text{i.e., greater starch concentration with injury}) \) in comparison with the leaf below the cage \( (\text{no difference in starch concentration with injury}) \).

Starch analysis revealed a pattern compatible with those found using iodine stain: higher concentrations of starch were negatively correlated with the lightness score \( (\text{Spearman coefficient } -0.62, P = 0.03) \). Sugar was not significantly correlated with the lightness scores \( (\text{coefficient } -0.45, P = 0.14) \), but sugar was positively correlated with starch concentration \( (\text{coefficient } 0.69, P = 0.01) \). The regression analysis, using leaf type as a covariate, demonstrated a significant relationship between the lightness score and starch concentration \( (\text{Fig. 3; } F = 17.8; \text{df} = 3, 11; P = 0.0007; r^2 = 0.87) \).

**Discussion**

Previous research has clearly shown that potato leafhopper injury to stem tissue causes reduced phloem translocation of photoassimilates \( (\text{Nielsen et al. 1990, 1999}) \) and that carbohydrate levels (including starch) increase on leaves of injured plants \( (\text{Johnson 1938, Hibbs et al. 1964}) \). Our results from both experiments confirm that starch concentration of leaf tissue increases above the site of leafhopper injury compared with healthy plants, although the increase is

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**Table 2.** ANOVA (PROC MIXED) results for lightness scores from iodine staining for starch for early morning and midafternoon samples, experiment 2

<table>
<thead>
<tr>
<th>Time of day</th>
<th>Source</th>
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<th>DDF</th>
<th>F value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
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<td>0.04</td>
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<tr>
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<td>0.02</td>
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<tr>
<td>Leaf×Trt</td>
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<td>13</td>
<td>4.8</td>
<td>0.03</td>
<td></td>
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<tr>
<td>p.m. Leaf</td>
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<td>11.5</td>
<td>6.3</td>
<td>0.01</td>
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<tr>
<td>Treatment</td>
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<td>8.2</td>
<td>2.5</td>
<td>0.15</td>
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<tr>
<td>Leaf×Trt</td>
<td>2</td>
<td>11.5</td>
<td>0.6</td>
<td>0.57</td>
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</tbody>
</table>

DDF, denominator degrees of freedom; NDF, numerator degrees of freedom; Trt, treatment.
significant only in the morning. One hypothesis for the accumulation of starches subsequent to injury is because sugar transport through the phloem is restricted, which ultimately inhibits the transport of sugars from the leaf (e.g., feedback inhibition; Hibbs et al. 1964).

However, in addition to the possibility of feedback inhibition, other mechanisms are suggested by the significantly greater accumulation of starch in developing leaves of injured stems in comparison with developing leaves of healthy stems (experiment 2). New growth represents a sink for carbohydrate metabolism in which the demand for carbon and energy is high, and thus starch levels should remain low in spite of blockage of translocation by injury at a distant location on the plant stem. Experiments have demonstrated that expanding alfalfa leaves use recently accumulated photosynthate in leaves, rather than carbohydrates transported from the roots (Hendershot and Volenec 1989). Although it has long been thought that starch accumulation in plants is governed by source-sink relationships at the tissue level (i.e., the site of photosynthesis as the source of carbon and energy responds to the need of assimilate-using organs such as storage and reproductive tissues), more recent evidence suggests that starch synthesis in the chloroplast, sucrose synthesis in the cytosol, and triose-P movement between the chloroplast and cytosol are in delicate balance (Hopkins and Hünner 2004). Thus, leafhopper injury may impact cellular processes by some mechanism in addition to that caused by feedback inhibition.

Focusing on the site of potato leafhopper feeding, Ecale Zhou and Backus (1999) demonstrated that feeding results in increased levels of starch synthesis in the vicinity of phloem injury. They showed that injury results in the formation of starch granules in cortical parenchyma and suggested that large amounts of carbon are being shunted from phloem into nearby parenchyma storage, where repair of blocked sieve cells proceeds. Phloem bypasses are formed between 4 and 8 d after injury to reopen the blockage. In contrast, our study examined areas both near to and distant from the site of injury. Our research extends their findings in that we observed that starch accumulation occurs throughout all tissues distal to the feeding site. In addition, we observed that injury is associated with a lack of degradation of starch during the night and that the injury does not affect the maximum accumulation of starch in the afternoon.

In addition to starch accumulation, injury by potato leafhopper has been associated with a decrease in rates of photosynthesis (Womack 1984, Flinn et al. 1990, Lamp 1997). Starch in the chloroplasts may cause a decrease in photosynthesis through a variety of mechanisms (Herold 1980). First, the accumulation of starch could directly affect photosynthesis by distorting thylakoid membranes. Second, large granules could interfere with light adsorption. Last, increased starch could increase triose-P, resulting in a decrease in CO2 concentration. Thus, starch accumulation may be responsible for reduced rates of photosynthesis in injured plants. A number of techniques (e.g., chlorophyll fluorescence, internal CO2 assimilation curves, enzyme activity) are available to identify specific mechanisms (Hopkins and Hünner 2004).

Another hypothesis to explain the leafhopper-induced decrease in rates of photosynthesis is currently being investigated in our laboratory. Although other researchers have suggested that phloem disruption by leafhopper injury was the primary cause of reduced photosynthesis rates (Flinn et al. 1990), we now know that injury causes reduced gas flow through the stomata, limiting the flow of CO2 to chloroplasts (Lamp et al. 2004). This function related to the xylem also may be associated with other delicately balanced molecular functions, such as starch–triose-P–sucrose interactions. Our observations of starch accumulation in developing leaf tissue are compatible with this hypothesis.
Reduced photosynthesis and starch accumulation are two of many physiological responses of alfalfa associated with hopperburn and reduced crop yield. A better understanding of these physiological responses is an important step in the ability to develop alfalfa cultivars that are tolerant to sap-feeding insects, thereby protecting the crop from damage.

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