Citrus leafminer (Lepidoptera: Gracillariidae) in lime: Assessment of leaf damage and effects on photosynthesis

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Studies were conducted to quantify leaf area damage to 'Tahiti' lime by citrus leafminer (CLM) (Phyllocnistis citrella Stainton) and to relate leaf damage to larval density and the length of time mining per leaf. Visual estimates of leaf damage were similar among five evaluators and were positively correlated with image analysis determinations. The number of CLM larvae per leaf and the number of days of mining were positively correlated with visual estimates of leaf damage and negatively correlated with net photosynthesis. Leaf damage by CLM was negatively correlated with net photosynthesis of potted trees and trees in an orchard. The data indicate that visual estimation is an accurate and rapid technique to assess leaf damage by CLM, and that leaf area damage and reductions in net photosynthesis due to CLM are related to the number of larvae per leaf as well as to mining duration. © 1997 Elsevier Science Ltd

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Citrus leafminer (CLM), Phyllocnistis citrella Stainton (Lepidoptera: Gracillariidae: Phyllocnistinae) attacks all species of citrus as well as some related species in the family Rutaceae (Heppner, 1993). The first observation of CLM in the United States was in May 1993 in lime orchards in southern Florida. Since then, CLM has spread throughout Florida and infestations have been observed in orchards of all commercial citrus species (Knapp et al., 1995).

Adult CLM oviposit on young leaves and destroy the epidermis by mining tissues adjacent to the leaf surfaces (Knapp et al., 1995). Leaf mines are usually on the abaxial surface but can also be found on the adaxial surface resulting in a typical injury characterized by twisted galleries and the epidermis appearing as a silvery film (Pandey and Pandey, 1964). This eventually results in necrotic tissue, leaf curling, and often abscission of the infested tissue (Heppner, 1993). The effect of CLM damage on growth and productivity of citrus has not been clearly defined (Binglin and Mingdu, 1996; Knapp et al., 1995). Thus, the percentage of the annual leaf flushes that needs to be protected from CLM damage, thereby preventing economic reduction in tree growth and yield, has not been determined (Knapp et al., 1995).

Several methods have been used to quantify leaf damage caused by various leaf miner species. Through mechanical defoliation of tomatoes, Keularts (1980) simulated Liriomyza sativae Blanchard mining injury and related defoliation to yield reduction. However, the relationship between mechanical defoliation and the number of L. sativae mines or mined area was not demonstrated. Maier (1983) multiplied the number of mines by the average area per mine and divided the product by the total leaf area, to determine damage from apple blotch miner. In recent studies with CLM, there was a poor correlation between the number of mines and percent leaf damage (Peña and Schaffer, unpublished). This was presumably due to the large variability in larval size and instars in a single leaf. To determine insect density treatment levels, it is necessary to accurately measure damage and to quantify the physiological capacity of a plant species to withstand injury (Sances, Wyman and Ting, 1979). Many leaf miner species have been shown to reduce net photosynthesis of their host plants due to leaf tissue damage (Welter, 1989). In all studies of leaf damage by CLM, leaf injury has been determined by visually estimating the percentage of leaf area damaged (Knapp et al., 1995; Peña, 1994; Mingdu and Shuxin, 1989). To quantify leaf damage, the accuracy of these visual estimates needs to be determined. Comparison of visual estimates with less subjective methods of damage assessment, such as image analysis, may provide an indicator of the accuracy and variability of the visual assessment. The purposes of this study were to compare visual leaf damage estimates from CLM on lime with image analysis estimates, and to relate the number of CLM larvae per leaf and mining duration to leaf damage and net photosynthesis.
Materials and methods

Comparison of visual leaf damage estimates to image analysis estimates

Two young shoots were sampled from the periphery of each of 21 mature 'Tahiti' lime (Citrus × Tahiti) trees in an orchard at the University of Florida, Tropical Research and Education Center, Homestead on 28 July 1995. Shoots were individually placed in plastic bags and immediately brought to the laboratory. Two leaves located at one third distance from the apical leaf of each shoot were removed from each flush and the percentage of area damaged per leaf surface (abaxial and adaxial) was individually assessed on 42 leaves (84 leaf surfaces, 42 adaxial and 42 abaxial) by five evaluators. CLM damage was defined as the visual percentage of the leaf area mined by each larva. Means and standard deviations of visual damage estimates were calculated and means compared among evaluators by analysis of variance. Using the same leaves that were used for estimating damage visually, the percentage of leaf area damaged by CLM was measured for each leaf surface individually by the same five evaluators using a video image analysis system (Bioscan Optimas version 4.0, Bioscan Incorporated, Edmonds, WA) connected to a 486 personal computer. First, to preserve leaf surface images, each leaf surface was recorded with a video camera (Model 1 RGB, Microimage Video System, Boyertown, PA) with a Nikon 18 mm f: 3 : 5 Nikkor lens, displayed on a video monitor and the image was stored on a floppy disk. These stored images were then manipulated independently by each evaluator with the software features to improve contrast and retouch (or paint) the CLM's path of damage so that it clearly stood out from the rest of the leaf surface (Figure 1). Comparisons between visual and image analysis determinations were conducted independently for adaxial and abaxial leaf surfaces since each stored image represented only one surface. To determine total leaf damage, the percentage of damage to the abaxial and adaxial surfaces were summed and divided by two. The percentage of area damaged per leaf surface was determined with a feature of the image analysis software that separates, measures and calculates areas of interest. The damaged area for this leaf surface was determined to be 12% using the image analysis software.

The variability among evaluators was determined for visual and image analysis determinations of leaf damage. Visual determinations of leaf area damage were compared to image analysis determinations by linear regression analysis. The slopes of the regression lines for each evaluator were compared by a test for homogeneity of slopes. In addition, coefficients of variability were compared between visual and image analysis estimates of leaf damage.

Effect of larval density per leaf and mining duration on leaf damage and photosynthesis

Two-year-old 'Tahiti' lime trees, which were propagated by air-layering, were obtained from a commercial nursery and re-potted in a commercial media (Pro-Mix, Premier Brands Inc., Red Hill, PA) in 3-liter polyethylene pots and placed in a glasshouse. Trees were 30 months old, they were defoliated to induce production of new vegetative shoots. An experiment to test the effect of larval density per leaf and mining duration on leaf damage and net photosynthesis was conducted in November 1995 and repeated in July 1996. Young leaves (two-three days old), were exposed to > 200 CLM adults for 24 h in an infested glasshouse. Adult CLM were fed apple juice dispensed through cotton wicks. Trees were removed from the infested glasshouse 1 day after exposure and the presence of CLM eggs was verified with a hand lens on all leaves.

Trees were moved to a net-covered cage to exclude pests other than CLM. Average temperatures in the cage were 31.6 and 34.1°C in November 1995 and July 1996 and minimum night temperatures in the glasshouse were 8.3 and 19.4°C in November 1995 and July 1996, respectively. Trees were spaced 0.5 m apart within the cage. Each day, leaves were examined with a hand lens and larvae were removed from the leaf or left undisturbed to obtain larval densities of 0, 1, 2, 3, 4, or 5 CLM per leaf for 1, 2, 3, 4, or 5 days. Thus, the experimental design was a factorial arrangement with six CLM densities and five mining durations as the main effects. There was a total of 125 leaves (5 densities × durations × 5 replications) on 10 trees used in November 1995, and 250 leaves (5 densities × 5 durations × 10 replications) on four trees used in July 1996. After 5 days, the majority of the larvae pupated.
After all larvae on a leaf pupated, no additional CLM came in contact with the leaf.

Eight weeks after all larvae pupated, when leaves were fully matured and hardened, the percentage of leaf area damaged was visually estimated for leaves in each treatment, and net photosynthesis was measured on each leaf that had been infested. Net photosynthesis was determined with a LCA-3 portable gas exchange system with a PLC-3B leaf cuvette (Analytical Development Corporation, Hoddesdon, Herts., England) as described by Larson, Schaffer and Davies (1991). Photosynthesis was measured on 6.25 cm² in the center mid-vein area of each leaf. After photosynthetic determinations, the total leaf area was measured for each leaf with LiCor LI-3000 leaf area meter (LiCor, Inc., Lincoln, NE) and the actual leaf area damaged was determined by multiplying the total leaf area by the fraction of the leaf area that was damaged.

Effect of CLM damage on net photosynthesis in an orchard

To determine the relationship between CLM damage and net photosynthesis for mature trees in an orchard, two separate field experiments were conducted in September 1995. Leaves representing a wide range (0-70%) of leaf area damage from CLM were tagged on mature ‘Tahiti’ lime trees in an orchard at the University of Florida, Tropical Research and Education Center, Homestead. The total number of leaves tagged was 41 in the first experiment and 30 in the second experiment. For each leaf, the percentage of damaged leaf area was visually estimated and net photosynthesis was determined as previously described. The relationship between leaf area damage and net photosynthesis was described by Larson, Schaffer and Davies (1991). Slopes of the linear regression lines (visual leaf damage estimates versus image analyses estimates for each evaluator) were compared by individual t-tests of all possible pairs of slopes using SAS software.

Results and discussion

Variability of visual damage estimates

The relationship between visual and image analysis estimates of leaf damage were the same for abaxial and adaxial surfaces (no interaction, \( P > 0.05 \)). Therefore, data were analyzed using leaf surfaces as replications (number of replications = 84 per evaluator) rather than whole leaves. There was no significant difference in visual estimation or image analysis determination of leaf damage among evaluators (Table 1). Visual estimation of leaf damage was relatively consistent among the five evaluators as indicated by the small standard deviations for each leaf surface evaluated. The means of the visual damage estimates were higher than those of image analysis determinations for each evaluator. The mean difference between the two estimation methods, for an individual leaf, ranged from 15% (evaluator 4) to as high as 32% (evaluator 3) (data not shown). This is consistent with reports that visual estimations of leaf area damage tend to grossly overestimate damage caused by insect feeding (Kogan and Turnipseed, 1980). The coefficient of variability was not significantly different between visual and image analysis determinations of leaf damage (t-tests, \( P > 0.14 \)). However, the mean coefficient of variability tended to be higher for the visual estimates (40.9 ± 39.9) compared to the image analysis (31.8 ± 39.4) determinations. The variability observed for the image analysis determinations may be the result of some subjectivity as the operator needed to manually enhance the color of the damaged areas by tracing over them so that they could be easily distinguished from the rest of the leaf.

There was a strong linear relationship between the visual estimates of leaf area damaged and damage determined by image analysis for each evaluator (Figure 2). Slopes of the regression lines were homogeneous (\( P > 0.05 \)) for all but one evaluator. This may have been because that evaluator was less experienced at visually estimating leaf damage by CLM than the other four evaluators. Data from all homogenous slopes were pooled and pooled data were analyzed by regression analysis (Figure 3). A linear relationship

<table>
<thead>
<tr>
<th>Evaluator</th>
<th>Visually estimated leaf damage (%)</th>
<th>Image analysis estimates of leaf damage (%)</th>
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<tr>
<td>1</td>
<td>25.8</td>
<td>21.1</td>
</tr>
<tr>
<td>2</td>
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<tr>
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<td>21.9</td>
</tr>
<tr>
<td>5</td>
<td>26.4</td>
<td>21.1</td>
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ANOVA statistics

\( P = 0.16 \)

\( F = 1.67 \)

\( df, df \) error 4,411

\( 0.49 \)

\( 0.86 \)

\( 4,412 \)
Effect of larval density per leaf and mining duration on leaf damage and photosynthesis

The number of days mining a leaf and the number of CLM larvae per leaf both significantly affected the percentage of leaf area damaged (Figure 4). As the number of larvae increased and the number of days mining increased, there was a significant increase in percentage of leaf area damaged (Figure 4). The percentage of leaf damage due to increased larval density was negligible in one experiment (Figure 4a), whereas in the second experiment, larval density had a much greater effect on leaf damage (Figure 4b). However, even when larval density had a large effect on leaf damage, the effect was not as great as would have been expected. This may have been due to feeding competition among larvae within a leaf, particularly at

image analysis took 3–5 min per leaf, whereas visual estimation was done in less than 1 min per leaf. Thus, in the field, visual estimates are more practical, particularly when a large number of leaves are being sampled.

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The number of days mining and the number of larvae per leaf were negatively correlated with net photosynthesis (Figure 5). However, the coefficient of determination for the response surface was not very high ($r^2 = 0.30$ and 0.37 in 1995 and 1996, respectively; $P < 0.001$). Stepwise multiple regression analysis indicated that most of the variability in net photosynthesis was due to the number of days mining (partial $r^2 = 0.29$ and 0.34 in 1995 and 1996, respectively; $P < 0.001$), and relatively little variability in net photosynthesis was due to the number of CLM larvae per leaf (partial $r^2 = 0.01$ and 0.07 in 1995 and 1996, respectively, $P < 0.001$). A similar negative correlation between leafminer density and net photosynthesis was observed for *Buculatrix pyrivorella* Kuroko, a lepidopterous leaf miner of peach (Fujie, 1982). However, for *B. pyrivorella*, the coefficient of determination ($r^2 = 0.67$) for cumulative leafminer density versus net photosynthesis was higher than that observed for CLM. The relative low coefficients of determination observed for CLM may have been due to the fact that some mines contained living tissue. Therefore, although a particular portion of the leaf was considered damaged, it still was photosynthetically active, albeit at a reduced rate compared to healthy tissue. For *Phyllocnorycter blancardella* (F), a lepidopterous leaf mining species of apple, reductions in net photosynthesis were attributed to increased mesophyll resistance and decreased chlorophyll concentrations (Proctor et al., 1982). We did not measure chlorophyll concentration of 'Tahiti' lime leaves. However, mined areas were often chlorotic or necrotic, indicating that reduced net photosynthesis was due, at least in part, to a reduction in chlorophyll content.

Effect of leaf damage by CLM on photosynthesis of potted trees

For potted trees, the percentage of leaf area damaged was inversely related to net photosynthesis (Figure 6, $r^2 = 0.35$ and 0.56 in 1995 and 1996, respectively; $P < 0.001$). Several leaves with leaf damage greater than 35% began to respire, as indicated by a negative net photosynthetic rate. The relatively low coefficient of determination between leaf area damaged by CLM and net photosynthesis may have been due to differences in photosynthetic responses to mining between mined and non-mined portions of the leaf. Johnson et al. (1983) observed that mined portions of tomato leaves infested with *L. sativae* as well as non-mined areas adjacent to mines, exhibited decreased photosynthetic rates. Thus, a low level of mining activity by *L. sativae* resulted in considerable reductions in net photosynthesis. Martens and Trumble (1987) observed that palisade mesophyll cells of lima beans that were destroyed by mining by *Liriomyza trifolii* Burgess were replaced with photosynthetically active cells.

Citrus leafminers mine both the adaxial and abaxial surface of the leaf. When damage to the abaxial and adaxial leaf surfaces were analyzed independently, there was no significant effect or interaction between leaf surface area damaged and net photosynthesis ($P < 0.05$). Previous studies found that *L. trifolii*, which mines only the palisade mesophyll, caused a greater reduction of net photosynthesis than *Liriomyza*

the third instar, when the larvae are largest and the greatest amount of mining activity occurs. Stepwise multiple regression analysis indicated that the percentage of damage per leaf was more highly correlated with the number of days mining (partial $r^2 = 0.61$ and 0.47 in 1995 and 1996, respectively; $P < 0.001$) than the number of larvae per leaf (partial $r^2 = 0.03$ and 0.15 in 1995 and 1996, respectively; $P < 0.001$).

There are different reports in the literature on the relationship between leaf surface damage by CLM and the number of miners. Knapp et al. (1995) reported 40–50% of the leaf area may be damaged when more than three larvae are found in a single *Citrus* leaf. Sponagel and Diaz (1994) found that 1.62 larvae per leaf caused 12.7% damage to a single lime leaf, whereas 2.20 larvae could cause 26.7% damage to *Citrus sinensis* leaves. Our results were similar to the above-mentioned results, however, they demonstrated that mining-days are a better indicator of leaf area damaged than the number of miners per leaf. Therefore, cumulated leaf mining days may be the best method to correlate CLM with damage symptoms.
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**Figure 6.** Effect of the percentage of leaf area damaged from citrus leafminers (*Phyllocnis citrella* Stainton) on net photosynthesis of 'Tahiti' lime trees in (a) November 1995 and (b) July 1996. The regression line for (a) November 1995 is represented by: $y = 5.34 + 0.49x$, $r^2 = 0.35$, and for (b) July 1996 by: $y = 2.73 + 0.56\exp(-x/55.63)$, $r^2 = 0.56$, $P < 0.001$.

**Figure 7.** Effect of the percentage of leaf area damaged from citrus leafminers (*Phyllocnis citrella* Stainton) on net photosynthesis of 'Tahiti' lime trees in an orchard. The regression line is represented by: $y = -0.166\exp(-x/66.143)$, $r^2 = 0.36$, $P < 0.001$.

*huicobrensis* Blanchard, which mines only the spongy mesophyll on the abaxial surface (Parrella et al., 1985).

**Effect of leaf damage by CLM on photosynthesis of trees in an orchard**

The regression models relating the percentage of leaf area damaged to net photosynthesis were the same for both orchard experiments. Therefore, data are shown only for the first orchard experiment (Figure 7). Similar to observations of potted trees, net photosynthesis in an orchard was negatively correlated with the percentage of leaf area damaged. The coefficients for trees in the orchard ($r^2 = 0.36$ and $0.34$ for orchard experiments 1 and 2, respectively; $P < 0.001$) were generally similar to those observed for trees in the glasshouse. Mingdu and Siau (1989) reported that damage levels above 30% reduced net photosynthesis. However, in an orchard the effects of insect herbivory on photosynthesis need to be examined on a whole-canopy level to account for photosynthetic compensation by non-infested leaves as well as other factors such as tree growth and crop load (Francesconi et al., 1996).

Our studies demonstrated that leaf damage to citrus by CLM larvae can be accurately assessed by visual estimation. The percentage of leaf area damaged by CLM and subsequent reductions in net photosynthesis are highly correlated with mining duration and, to a lesser extent, the number of CLM larvar per leaf. Also, on a leaf area basis, net photosynthesis is significantly reduced by CLM. However, in order to use reductions in net photosynthesis for establishing damage thresholds for CLM, effects of CLM on photosynthesis need to be investigated on a whole-canopy basis.

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