An Inexpensive, Accurate Method for Measuring Leaf Area and Defoliation Through Digital Image Analysis

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ABSTRACT We report a protocol using a common desk-top scanner and public domain software for measuring existing leaf area and leaf area removed as a result of herbivory. We compared the accuracy and precision of this method to that of a standard leaf area meter. Both methods were used to measure metal disks of a known area, the area of soybean (*Glycine max* L.) leaves, and the area removed by simulating leaf feeding with a hole-punch. We varied the amount of injury across a low, medium, and high degree of simulated feeding. The mean area of 10 cm^2 and 50 cm^2 metal disks was more accurately estimated with the leaf area meter than the desk-top scanner. Leaf area estimates from both methods were highly correlated. The desk-top scanner accurately estimated the leaf area removed from the low, medium, or high degree of simulated leaf feeding. However, the leaf area meter overestimated low levels of simulated feeding injury. Though measuring a leaf's surface area with a desk-top scanner requires two steps (creating a digital image file and calculating the area represented by that image), the overall time required to measure leaf injury is shorter than with a leaf area meter. This relatively simple and inexpensive method of estimating leaf area and feeding damage has advantages in certain experimental situations where a prefeeding measurement of the leaf is impossible or undesirable, or when small amounts of feeding occur.

KEY WORDS leaf area, herbivory, video image analysis, soybean

MANY BIOLOGICAL STUDIES require measurement of leaf area and defoliation. Measuring leaf area removed as a result of insect herbivory can be useful for evaluating host–plant resistance (Jansky et al. 1999), pesticide activity (Gonzalez et al. 1992, Hoy and Hall 1993, Wheeler and Isman 2001), and plant-insect interactions (Peterson et al. 1993, Hammond et al. 2000). Prior studies measuring herbivory (leaf area removed) have used visual estimates (Stotz et al. 2000), hand tracings of injured leaves (Hoy and Hall 1993), or a comparison of treated leaves to an appropriate control (Gonzalez et al. 1992, Wheeler and Isman 2001). The latter methods use a leaf area meter or a sampling grid to estimate leaf area. Though useful, each of these methods has significant drawbacks; leaf area meters are expensive and measuring leaf area by hand with a sampling grid is time consuming.

Recently, digital cameras have been used to measure infection by plant pathogens (Lindow and Webb 1983), insect feeding (Alchanatis et al. 2000, Su and Messenger 2000), and to capture insect images (Mitchell and Lasswell 2000). James and Newcombe (2000) used the Adobe Photoshop (version 3.0, Mountain View, CA) software package to measure leaf feeding of *Phratora califonrica* Brown (Coleoptera: Chrysomelidae) on hybrid poplars (*Populus* spp.). These estimates of leaf feeding were used to train personnel to visually estimate percentage of damage in leaf samples. Using public domain software (Scion Image) and a digital-imaging system, Wheeler and Isman (2001) measured area of untreated and treated leaf discs. We have developed a related protocol using a common desk-top scanner and public domain software to measure existing leaf area and area removed by herbivory. We validated this method by comparing it with a standard leaf area meter.

Materials and Methods

Creating a Digital Image of a Leaf. Individual leaves or objects of known area were scanned into a digital format using a Hewlett–Packard ScanJet 6200C desk-top scanner (Hewlett–Packard, Cupertino, CA) with HP Precision Scan Pro (version 1.1, images scanned at 150 dpi) software. The scanner and the software were operating on a Dell Optiplex GX1 computer with a Pentium III processor with 128 MB RAM. An object was placed on the scanner; the lid of the scanner was closed, and a preliminary scan was made using the preview feature of the software. The preliminary image was converted from color to grayscale (selected from the output type menu). The highlight and shadow levels within the exposure adjustment (selected from the tools menu) were manipulated to

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create a black image on a white background. The final version was saved as a TIFF file without LZW compression. Formats that involve compression (GIF, JPEG, and TIFF with LZW compression) are not compatible with the image analysis software.

We used public domain software (Image 4.0.2 for Windows, National Institutes of Health, Bethesda, MD) to measure the surface area of objects in a digital format. This program is available for Apple and Windows operating systems, and as of 14 May, 2002, could be downloaded at the following addresses: Apple (http://rsb.info.nih.gov/nih-image/) and Windows (http://www.scioncorp.com).

We opened the TIFF file to be analyzed within the Image software, and using the set scale option (selected from the analyze menu), we selected a unit (cm) to convert pixels to a unit of measurement. We included a standard of known dimensions (a ruler) within the image for calibrating the pixel conversion. Once the units had been selected, the grayscale-image was adjusted so that the image was composed of only black and white. Within the map box (selected from the Windows menu), a threshold option was selected to convert all color values to either a one (black) or zero (white). The image could then be selected with the magic wand (selected from the tool box menu). A black region was selected with the magic wand. The software moved to the right of the spot until the margin of the region was reached and the entire contiguous region was outlined. With the object outlined, the surface area was calculated by selecting the measure option (selected from the analyze menu).

We also measured the amount of leaf area removed. While the leaf was selected, the damaged area was highlighted after inverting the colors of the image (inverse selected from the edit menu). The amount of leaf area removed was measured from this inverted image within the boundaries of the original selection.

Experimental Design and Data Analysis. We compared the accuracy and precision of leaf area estimates from the desk-top scanner with estimates from a LI-COR LI-3000 leaf area meter (LI-COR, Lincoln, NE). Three separate tests were conducted using metal disks of a known area, a single soybean leaf, and multiple soybean leaves.

In our first test, metal disks were used to evaluate the precision and accuracy of both methods to estimate a known area (10 cm^2 or 50 cm^2 disk). Each disk was scanned five separate times with the leaf area meter and the desk-top scanner. The location of the disk on the desk-top scanner and leaf area meter was adjusted with each scan. Descriptive statistics were used to compare the accuracy (mean) and precision (standard error of the mean, SEM) of each method. We used Student's t-test to determine if the areas estimated by either method differed from the actual area of the metal disks.

In our second test, we compared the leaf area meter with the desk-top scanner to estimate leaf area and leaf area removed from a single soybean leaf. A single soybean (*Glycine max* L., Asgrow 'Ag2201') leaf was harvested, and the petiole was removed from a plant in the late reproductive stage grown in a greenhouse. The middle leaflet was taken from the third most distal trifoliate. The uninjured leaf was damaged with 1, 5, 20, and eventually 40 nonoverlapping holes (0.13 cm^2) from a metal hole-punch. All holes were made within the boundaries of the leaf. At each level of injury (including the uninjured condition), the leaf was scanned five times, with its position on the desk-top scanner and leaf area meter adjusted with each scan.

In a third test, we used multiple soybean leaves that ranged in size as a more practical test of the accuracy of the desk-top scanner to measure leaf and hole area. Unlike the previous test, only a single scan was made with the desk-top scanner. However, five scans were made with the leaf area meter. Leaves that had a minimal amount of preexisting damage were randomly assigned to four treatment groups of five leaves each: no damage, 1 hole per leaf, 5 holes per leaf, or 25 holes per leaf, and leaves were damaged as before.

In each test, the means and SEM of leaf area and leaf area removed were calculated. In the second test, because the true area of the individual leaf was not known, we used paired t-tests to determine if the difference between the leaf area estimated from both methods differed from zero. Student's t-tests analyzed whether the difference between the estimated leaf area removed by either method differed from the actual area removed by the hole punch. In the second test, we also conducted a correlation analysis on the leaf area as measured by both methods (SAS Institute 1996).

Time Required to Measure Leaf and Damage Area. We measured the total time to scan a single leaf five-times with the leaf area meter, per the method described above. Five different leaves were measured in this way and a mean time calculated. For the desk-top scanner, we measured the time to convert five sets of images with five leaves each to a single digital image. We then measured the time to calculate leaf area and injury (leaf area removed) from these digital files. All time estimates assumed that a user was familiar with the software and hardware and that the hardware was on long enough to begin working immediately (i.e., "warmed-up").

Results

The mean surface area of a 10 cm^2 and 50 cm^2 metal disk was more closely estimated with the leaf area meter than the desk-top scanner. For both disks, the mean surface area estimated by the leaf area meter was

Table 1. Mean surface area ± SEM of standard metal disks as measured by two methods

Disk area, cm^2	Leaf area meter			Desk-top scanner		
	Area, cm^2	t	P	Area, cm^2	t	P
10	10.04 ± 0.02	1.65	0.17	10.17 ± 0.02	7.61	0.002
50	50.05 ± 0.04	1.26	0.28	50.33 ± 0.08	4.00	0.02

Student's t-tests compared difference of estimated disk area to actual disk area (df = 4) for each method.

Table 2. Mean surface area ± SEM of a single soybean leaf before and after simulated herbivory as measured by two methods

Object	Leaf area meter	Desk-top scanner	t^a	P
Undamaged leaf	29.74 ± 0.02	29.54 ± 0.01	9.00	0.0008
Damaged leaf, holes				
1	29.36 ± 0.02	29.41 ± 0.01	2.44	0.07
5	28.54 ± 0.02	28.54 ± 0.02	0.21	0.84
20	25.82 ± 0.03	25.61 ± 0.02	9.95	0.0006
40	22.84 ± 0.03	22.31 ± 0.04	13.00	0.0002

$n = 5$ measurements of the same leaf
[a] Paired *t*-tests compared difference of estimated leaf area of each method to zero (df = 1).

not significantly different from the actual surface area (Table 1). The surface area estimated by the desk-top scanner was significantly different from the actual area of both disks. The desk-top scanner slightly overestimated the surface area.

Neither the leaf area meter nor the desk-top scanner consistently overestimated the area of the single soybean leaf (Table 2), when compared with each other. Only the estimated leaf area of the damaged leaf with five holes was clearly similar for both methods. Though there may have been a marginal difference ($P = 0.07$) between the methods when the leaf was damaged with one hole, all other estimates of this leaf's area, undamaged or damaged, were significantly different between the two methods. In general, where significant differences occurred, estimates made from the leaf area meter were greater than those of the desk-top scanner. Estimates of leaf area from the desk-top scanner were generally less variable than estimates from the leaf area meter. For this individual leaf measured multiple times, the desk-top scanner more closely estimated the actual leaf area removed, and the leaf area meter consistently overestimated the area removed (Table 3).

Leaf area estimates from both methods were significantly correlated (Fig. 1, $n = 20$, $r = 0.99$, df = 1, $P < 0.0001$) for leaves ranging in size from 8.11 cm^2 to 76.42 cm^2. No significant divergence from this pattern was evident at any leaf size. When estimating a constant level of simulated herbivory to multiple leaves, the leaf area meter's estimate of herbivory simulated by a single hole was significantly greater than the actual value (Table 4, $t = 6.09$, df = 1, $P = 0.004$). Unlike the previous test, where a single leaf was scanned multiple times with the desk-top scanner and a mean area calculated, in this test many leaves were scanned only once. As in the first test, the one-hole level of injury was accurately estimated by the desk-top scanner. Also, the desk-top scanner accurately estimated the simulated herbivory from 5 and 25 holes. The SEM of measurements taken with the leaf area meter was consistently higher than those of the desk-top scanner.

The average time to measure the surface area and injury area of a set of five leaves with the desk-top scanner and Image software was 3:25 ± 0:03 (min:s), which included creation of the digital file and measuring each leaf within that file. To measure the area of those same five soybean leaves using the leaf area meter, the average time was 6:10 ± 0:10, almost twice the time needed with the desk-top scanner.

Discussion

Measuring the surface area of a metal disk, the leaf area meter was more accurate and had greater precision then the desk-top scanner (Table 1). Measuring the area of a single leaf, we did not observe a consistent trend of one method overestimating area relative to the other (Table 2). We observed greater precision in measurements made with the desk-top scanner, as seen in the consistently lower SEM values than those from the leaf area meter. A high degree of correlation existed between the two methods in estimating leaf area (Fig. 1). So, although our first test indicated superior performance of the leaf area meter, the desk-top scanner performed as well, if not better, in the latter two tests involving single and multiple soybean leaves. Two differences in the construction of the desk-top scanner versus the leaf area meter may be responsible for the difference in performance between a hard, flat object like the metal disks and a leaf with varying thickness. The desk-top scanner's cover may provide better horizontal compression. Keeping the leaf static and moving the scanning optics, unlike the leaf area meter that moves the object across a static optic, may also assist measuring leaf area, as well as estimating herbivory.

The desk-top scanner performance surpassed that of the leaf area meter in estimating simulated herbivory. In our second test with increasing levels of injury to a single leaf, the leaf area meter was unable to accurately estimate the amount of leaf area removed (Table 3). However, the desk-top scanner accurately estimated the amount of leaf area removed in two of the four levels of simulated herbivory. In the

Table 3. Mean area (± SEM) removed from a single soybean leaf by simulated herbivory as measured by two methods

Damaged leaf, holes	Leaf area removed (cm^2)	Leaf area meter			Desk-top scanner		
		Area, cm^2	t^a	P	Area, cm^2	t^a	P
1	0.13	0.38 ± 0.02	14.3	0.0001	0.13 ± 0.00	0.0	1.0
5	0.65	1.20 ± 0.03	36.3	<0.0001	0.67 ± 0.002	6.5	0.003
20	2.60	3.92 ± 0.03	50.3	<0.0001	2.62 ± 0.007	3.5	0.02
40	5.20	6.90 ± 0.03	67.6	<0.0001	5.20 ± 0.01	0.0	1.0

$n = 5$ measurements of the same leaf
[a] Student *t*-tests compared difference of estimated hole area to actual hole area (df = 4) for each method

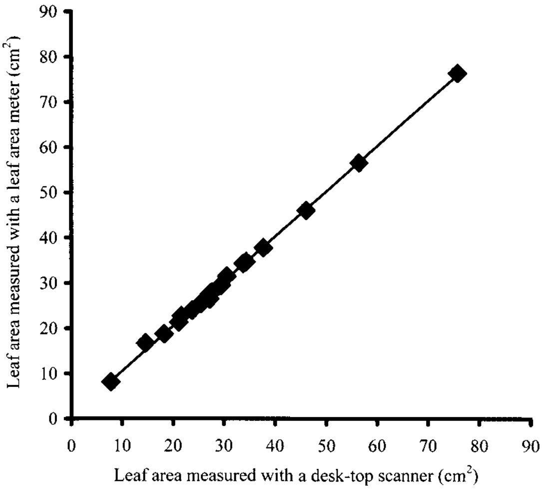

Fig. 1. Relationship between leaf area (cm^2) measured with a LI-COR 3000 leaf area meter compared with leaf area measured from a digital image created with a Hewlett–Packard ScanJet 6200C desk-top scanner and analyzed using the NIH Image software. The line drawn represents the linear regression ($y = 1.0x + 0.5$, $R^2 = 0.99$, df = 1, 19; $P < 0.001$).

final test (Table 4), the leaf area meter was unable to accurately estimate the injury produced by a single hole punch (0.13 cm^2). The leaf area meter was able to accurately estimate the 0.65 cm^2 and 3.25 cm^2 level of injury (Table 4). However, the precision of these measurements made by the leaf area meter was less than that of the desk-top scanner.

Improvement in any given methodology often involves selecting from two of the three following attributes; quality, expediency, and cost. We have demonstrated that a desk-top scanner can accurately and precisely measure leaf area and area removed by herbivory. We have also shown that this method is faster and possibly easier to use than a leaf area meter when the goal is measuring defoliation. To measure defoliation with a leaf area meter, the leaf area before herbivory must be estimated. Given certain experimental protocols and depending upon the feeding behavior of the insect, this is not always possible or convenient. However, the desk-top scanner method does not require such an initial measurement. Also, the

Table 4. Mean area removed (± SEM) from multiple soybean leaves by simulated herbivory as measured by two methods

No. holes	Leaf area removed, cm^2	Area cm^2: Leaf area meter	Area cm^2: Desk-top scanner	Mean difference cm^2
1	0.13	0.29 ± 0.03[a]	0.16 ± 0.02 ns	0.13 ± 0.05[b]
5	0.65	0.77 ± 0.07 ns	0.67 ± 0.01 ns	0.09 ± 0.07 ns
25	3.25	4.82 ± 1.24 ns	3.15 ± 0.09 ns	1.67 ± 1.24 ns

n = 5 leaves

[a] Paired t-test between actual and estimated leaf area (t = 6.09, df = 1; P = 0.004)

[b] Paired t-test between estimated hole-area from both methods (t = 2.68, df = 1; P = 0.06)

20 cm by 30 cm dimension of the desk-top scanner allows for multiple leaves to be scanned. More than one leaf can be included in the digital image file, further saving time when area measurements are made.

Difficulties in accurate measurement of feeding can arise when feeding occurs at the leaf edge and the magic wand selection tool cannot follow the original leaf margin. By connecting the edges of this break with the line drawing tool, an estimate can still be made, though it may underestimate total injury. To avoid this potential disadvantage of using the desk-top scanner, we have modified our feeding assays to prevent such an underestimate by presenting test subjects with leaf tissue of a square shape (O'Neal et al. 2002). Using leaves cut to this shape allowed us to connect edges disrupted by leaf feeding. We should also point out that the leaf feeding measured by O'Neal et al. (2002) did not involve the complete consumption of the entire tissue at a feeding site. By adjusting the exposure of the scanner, we were able to distinguish the areas where feeding occurred. The last issue of cost is much clearer. Desk-top and portable leaf area meters range in cost from \$2000 to \$3000, respectively. Though prices can vary, a desk-top scanner (HP ScanJet 5300C) typically costs \$200-\$300 or less.

Digital image analysis has been used in a variety of novel methods for applied agricultural research. A more complex methodology for measuring insect feeding was developed by Alchanatis et al. (2000) using a video-image system. Their methodology allowed for real-time measures of feeding behavior by coupling digital video images with a personal computer to measure the amount and timing of leaf feeding.

A closely related technique to that presented here was developed by Su and Messenger (2000), who used digitized images to estimated termite consumption of wood. Su and Messenger were able to measure twice the area of the HP Scanjet 6200C by using a set focal distance with a digital camera. They converted these pictures to a black-and-white digital image before measuring the surface area (SigmaScan 2.0, Jandel Corporation 1995) and observed a significant relationship between wood weight loss and surface area removed. Though not explored here, the protocol of Su and Messenger (2000) suggests that leaf area and defoliation could be measured in a nondestructive manner by replacing the desk-top scanner with a digital camera.

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