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Computer-assisted estimation of leaf damage caused by spider mites

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Abstract

The article describes a new method to assess the impact of the two-spotted spider mite, *Tetranychus urticae* Koch, on host plants. The method is based on measurement of the leaf area damaged using a computerized image analysis technique. Injured leaves were scanned on a common flat bed color scanner to obtain grey scale images. MATHEMATICA software was used for image analyzes. Leaf damage was defined as the ratio of the number of leaf image pixels in the damaged area to the total number of pixels of the entire leaf image. The precision of the proposed method was compared with two other methods based on the leaf damage index and the chlorophyll fluorescence, respectively. Results show that both the newly proposed normalized proportion of leaf area damaged (*NPLAD*) method and the leaf damage index method are sensitive enough to show significant differences in leaf damage after exposure to different densities of spider mites. The chlorophyll fluorescence method, in contrast, did not give satisfactory results. Advantages of the computer-assisted estimation of damage caused by *T. urticae* feeding are discussed.

Keywords: *Tetranychus urticae*; Acari; Tetranychidae; Herbivory; Feeding damage; Injury assessment; Computer image analysis

1. Introduction

Spider mites (Acari: Tetranychidae) are serious pests of many crops (Helle and Sabelis, 1985), and in many plant studies it is necessary to assess the extent to which mites injure a particular host plant. For example, measuring the level of feeding damage can be useful for fast estimation of pest infestation and predicting yield loss (Hussey and Parr, 1963; Tomkiewicz et al., 1993), for evaluation of host-plant resistance (Giménez-Ferrer et al., 1994; Wilson, 1994), or in studies of plant-mite interactions (Nachman and Zemek, 2002a,b).

This article details one method of measuring the leaf damage caused by feeding of the two-spotted spider mite, *Tetranychus urticae* Koch. In contrast to most herbivorous insects that directly remove a certain leaf area, spider mites penetrate the leaf surface with their stylets to a depth of 70–120 μm and suck out the cell contents (Tomczyk and Kropczyńska, 1985) while leaving the leaf area intact. Injury to both the spongy and palisade parenchyma of leaves has been observed (Mothes and Seitz, 1982). Typical symptoms of mite feeding are small, light colored punctures that, on prolonged exposure to mites, develop into irregularly shaped, white or grayish-colored spots.

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Several methods for estimating *T. urticae* feeding damage have been proposed (Hussey and Parr, 1963; De Angelis et al., 1983; Candolfi et al., 1991; Tomkiewicz et al., 1993; Iatrou et al., 1995; Nachman and Zemek, 2002a). Hussey and Parr (1963) defined the leaf damage index (*LDI*) on an ordinal scale (0–5) based on the visual assessment of leaf damage caused by spider mites. By using the *LDI*, they estimated corresponding mite population densities. This method provides a rough estimation of the leaf damage and the results are heavily affected by skills and experience of the observer making this technique largely observer dependent. De Angelis et al. (1983) and Iatrou et al. (1995) used determination of chlorophyll to assess feeding damage. This method is accurate but time-consuming and expensive. Tomkiewicz et al. (1993) converted the *LDI* defined by Hussey and Parr (1963) to the relative chlorophyll loss (chlorophyll content) of cassava using a calibration curve based on laboratory experiments. This approach allowed the conversion of the qualitative *LDI* to a quantitative variable (the chlorophyll content). Nachman and Zemek (2002a) used the average amount of chlorophyll per cm^2 of the leaf area as a measure of bean plant damage. The relationship between the *LDI* and the average chlorophyll content per cm^2 of leaf area was based on measurements of chlorophyll extract absorption using a spectrophotometer and the calibration curve defined by Tomkiewicz et al. (1993). However, methods based on assessing the chlorophyll content as a function of the damage index using a calibration curve (Tomkiewicz et al., 1993; Nachman and Zemek, 2002a) also suffer from subjective categorization of leaf injury on the index scale. Candolfi et al. (1991) used an opto-electronic image analysis system (ASBA 3, Wild Leitz AG, Switzerland) to assess feeding damage inflicted on bean leaves by *T. urticae*. The leaves were placed on a filter paper between two glass plates with a strong background light to make the feeding spots visible, and then photographed. The leaf veins on the photographs were covered with black ink so that they were not interpreted as being damaged areas. The mite damage was thus quantified by measuring the total leaf area and the leaf area destroyed by mites using the picture.

Plant damage can also be estimated indirectly by a chlorophyll fluorescence method that provides information on plant stress arising from mite feeding injuries (Iatrou et al., 1995; Buschmann et al., 1996). This method is based on the observation that plants react to a wide range of environmental stresses by reducing the ratio of variable fluorescence (F_v) to maximum fluorescence (F_m), indicating a reduced efficiency of photosystem II phytochemistry (Bolhàr-Nordenkampf et al., 1989). Iatrou et al. (1995) measured chlorophyll fluorescence in situ with this method on intact bean plants with various densities of spider mites using a portable fluorometer.

Most of the methods discussed above, however, either provide only a rough estimate of leaf injury, or require specialized and expensive equipment and high labor costs for processing samples and conducting analyses. In this article we describe an inexpensive, simple, and accurate method to measure leaf damage by spider mites based on computer image analysis. We provide a MATHEMATICA code for the analysis.

2. Materials and methods

2.1. Experimental settings

We used the bean plant, *Phaseolus vulgaris* L., var. Katka as our model host plant. Plants were grown in plastic pots in a greenhouse using standard soil medium. No pesticides or fertilizers were applied. Artificial light was provided with metal-halide lamps to ensure sufficient light intensity and long day photoperiod. All plants were of approximately the same age (between two and three weeks).

Damage inflicted to leaves by spider mites was measured on leaf discs of 1.9 cm diameter cut from leaves at the second node. The experiment was arranged in a randomized block design with three experimental treatments per block. Leaf discs used within one block were taken from the same bean leaf to account for between-leaf variability and were randomly assigned to the following treatments: 0 (control), 20 and 40 adult spider mite females per disc. Each treatment was replicated ten times with a new plant used for each replication. Spider mites were placed on leaf discs that were laid on pieces of plastic foam in a Petri dish (diameter 15 cm) filled with water. Leaf discs were oriented ventral-side-up as spider mites predominately feed on the lower leaf surface (Tomczyk and Kropczyńska, 1985). Petri dishes with leaf discs were then placed in a climate controlled box with constant temperature (25 °C) and photoperiod (LD 18:6 h) for five days. After this period, leaf discs were gently cleaned of mites, their eggs and webbing using a fine brush.

2.2. Image analysis

To obtain digital images, leaf discs were scanned individually with a Phantom 636 CX flat bed color scanner (Microtek) and Microtek Scan Wizard 2.53 driver (Phantom Scanner Software) using CorelSCAN 8 software. Parameter settings were set to default values when using the scanning hardware with the exception that we used the 'millions of colors' setting and a resolution of 300 dpi. Software settings were monochromatic photo, grey scale (8 bits), and resolution 300 dpi. No post-scanning editing (e.g. picture brightness, picture contrast, intensity of picture, sharpness of picture, tool for removing of dustiness and scratch and picture noise reduction) was performed.

Digital images were analyzed using MATHEMATICA 4.0 (Wolfram, 1999), a common algebraic computer program. According to our methodology, each pixel within a leaf-disc image is characterized by its grey level, which is a number ranging from 0 (black), to 255 (white). Using a pre-defined threshold for hue of grey color, we then identify pixels representing the area damaged by spider mites. The proportion of leaf area damaged (*PLAD*) is then defined as a fraction of the number of pixels in the damaged area to the total number of pixels of the entire leaf image. The *PLAD* therefore varies between 0 (no feeding damage) and 1 (leaf completely damaged). Thus, as mite feeding damage increases (increasing leaf brightness), we expect an increase in the *PLAD*.

We processed the image data as follows. First, it was necessary to define the number of pixels of the whole leaf disc. For this we removed the background pixels (white paper) from the scanned image. To do this, we defined an upper threshold ($UT = 200$ units of the grey scale) and replaced all grey scale values higher than 200 with the value 255 (white color). The value for the upper threshold (*UT*) depends on the properties of the material used for the background when scanning leaves and must be chosen by the operator. In our case we chose $UT = 200$, which sufficiently separated the grayish scale of the white background paper from the leaf. To separate the damaged area of the leaf from the rest of the leaf we defined a lower threshold (*LT*). The area containing pixels within the range from *LT* to *UT* values characterizes the damaged part of the leaf disc. While the upper threshold was the same for all replicates, the lower threshold was leaf-dependent because there was certain variability in color between individual leaf discs. The lower threshold (*LT*) was determined by visual inspection on the computer screen for each replicate (consisting of the two treatments and control in our case) and the value that fitted best was selected. We used only three color levels for image data visualization on the computer screen: black was used for undamaged areas, grey for damaged areas and white for the background. This coloring facilitated visual comparison of leaf disc images on the screen.

The number of pixels with values between the *LT* and *UT* (A) quantified the area damaged by *T. urticae* while the number of pixels with grey scale lower than *UT* (B) quantified the size of the whole leaf disc. The proportion of the leaf area damaged was then defined as $PLAD = A/B$. Since some pixels of control leaf discs were also marked as damaged, we adjusted the obtained values by application of the formula:

$$NPLAD = \frac{A_T/B_T - A_C/B_C}{1 - A_C/B_C} \qquad (0 \leq NPLAD \leq 1) \tag{1}$$

where *NPLAD* is the normalized proportion of leaf area damaged, *T* and *C* are treatment and control, respectively. This method of correction for false positive damaged cells corresponds to Abbott's formula (Abbott, 1925).

2.3. Comparison with other methods

The proposed method was compared with the *LDI* (Hussey and Parr, 1963). For this purpose, the *LDI* was estimated for each leaf disc by visual inspection using an ordinal scale from 0 (no damage) to 5 (completely destroyed) (Hussey and Parr, 1963; Tomkiewicz et al., 1993; Nachman and Zemek, 2002a). Half-indices were used to increase resolution of the method. The experimenter handled leaf discs in random order and did not know the mite density used for a particular disc.

In addition, separate experiments were conducted to estimate stress in *T. urticae*-injured leaf discs by the chlorophyll fluorescence method (Iatrou et al., 1995). The parameters – initial fluorescence F_0, maximum fluorescence F_m, and variable fluorescence F_v ($F_v = F_m - F_0$) – were measured using a Closed FluorCam desktop imaging fluorometer (Photon Systems Instruments, Brno, Czech Republic) described elsewhere (Nedbal et al., 2000). Two measurements were carried out on each leaf disc: one before spider mites were introduced, and one after 3 days of mite feeding.

Densities of *T. urticae* were 0 (control), 20 and 40 adult females per disc. Fluorescence parameters were measured on the lower leaf surface because it is a more sensitive indicator of damage caused by mite feeding than the upper leaf surface (Iatrou et al., 1995).

2.4. Statistical analyses

Statistical evaluation of differences between the *NPLAD* values of scanned leaf discs and differences between estimated *LDI* values were performed using Wilcoxon signed rank tests for paired (dependent) samples (Siegel and Castellan, 1988). We also conducted a Spearman correlation analysis (Siegel and Castellan, 1988) of the leaf damage measured by both methods. A model describing the relationship between *NPLAD* and *LDI* was fitted by non-linear regression. The computations were done using the PROC UNIVARIATE, PROC CORR and PROC NLIN functions in SAS/STAT (SAS Institute, 2000).

3. Results

The leaf area damaged by *T. urticae* feeding was easily recognized as lighter spots in scanned images of leaf discs (Fig. 1 left). Differences between treatments were evident. Grey pixels in new 3-color images indicating damaged area (Fig. 1 right) showed good correspondence with the damaged area seen in the original images. Only a few pixels of control leaf discs were also marked as damaged (max. 2%, see white spots in Fig. 1A, right). The estimated *LT* values for all replicates were in the range 120–135 grey scale units (average 129 units).

The estimated damage level expressed as the *NPLAD* ranged from 0.1240 to 0.3707 and from 0.2677 to 0.4973 in treatments with 20 and 40 spider mites per disc, respectively. Fig. 2 shows that the mean *NPLAD* increased 1.5-fold on going from 20 to 40 spider mites. Statistical analysis revealed significant differences between *NPLAD* of leaf discs with 20 mites versus leaf discs with 40 spider mites (Wilcoxon signed rank test, $n = 10$, $P_{\text{two-tailed}} = 0.0020$). Time required to assess *NPLAD* values, including scanning of a leaf disc and image data processing, did not exceed 5 min per value after some experience with the software.

LDI values estimated by visual inspection ranged from 1 to 3 and from 1.5 to 3.5 in treatments with 20 and 40 spider mites per disc, respectively. The mean *LDI* increased about 1.4-fold on going from 20 to 40 spider mites (Fig. 2). Differences between the *LDI* of leaf discs with 20 mites versus leaf discs with 40 spider mites were also statistically significant, but the *P* value was higher than that obtained for the comparison of *NPLAD* values (Wilcoxon signed rank test, $n = 10$, $P_{\text{two-tailed}} = 0.0078$). Therefore, the *NPLAD* parameter can be considered as a more sensitive indicator of *T. urticae* feeding damage than *LDI*, and hence the proposed method should be able to discern smaller differences in injury. On the other hand, the estimation of *LDI* values was faster compared to *NPLAD* values, taking just a few seconds for an experienced observer.

A Spearman correlation analysis showed that *LDI* and *NPLAD* were positively correlated ($r_s = 0.4793$; $n = 20$; $P = 0.0325$). Since *LDI* is bound to be 0 and 5 for *NPLAD* being equal to 0 and 1, respectively, the simplest relationship satisfying these constraints is a straight line given as

$$LDI = 5 \cdot NPLAD \tag{2}$$

However, to allow for a non-linear relationship between the two measures, the model

$$LDI = 5(1 - (1 - NPLAD)^b) \tag{3}$$

where b is a positive parameter, was also fitted to data. Eq. (3) reduces to Eq. (2) if $b = 1$, whereas a b value significantly different from unity would indicate non-linearity. The total unexplained variation in data was computed as the sum of squared deviations between the observed values of *LDI* and the predicted values according to the null model (Eq. (2)), whereas the residual variation was calculated as the sum of squared deviations between the observed *LDI* values and the predicted values based on Eq. (3). When Eq. (3) was fitted to data by means of PROC NLIN in SAS, b was found to be 1.9894 (SE = 0.1949), which is significantly different from unity ($t_{19} = 5.072$; $P < 0.001$). Hence, the curvilinear relationship shown in Fig. 3 is a significantly better fit than a straight line passing through (0,0) and (1,5). The unexplained variation declined from 28.454 when $b = 1$ to 9.286 when $b = 1.9894$, implying that the non-linear model was capable of explaining 67.4% of the total variation in the observed values of *LDI*.

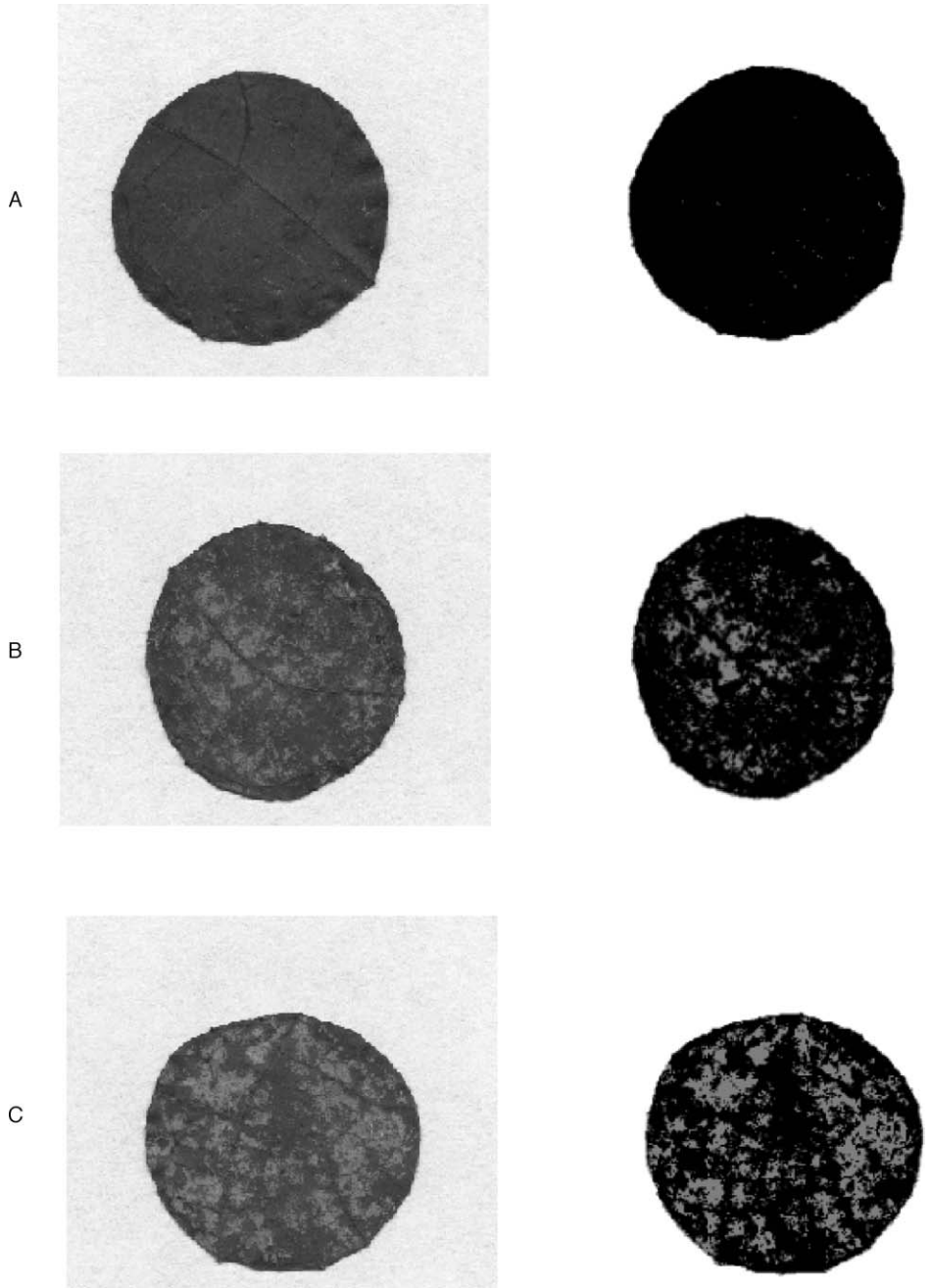

Fig. 1. Scanned images of leaf discs (one replication block; left) used for leaf damage assessment. Image data (right) were processed to display area damaged (grey), area not affected by *T. urticae* feeding (black) and the background area (white). A - control leaf disc (no mites), B - leaf disc with 20 spider mites and C - leaf disc with 40 spider mites.

Chlorophyll fluorescence measurements revealed that the ratio F_v/F_m in leaf discs without spider mites increased during the experimental period (Fig. 4). Similarly, the ratio was higher in leaf discs damaged by spider mites compared to initial values but lower than in control discs at the end of the experiment, indicating higher stress in mite-infested discs. However, no differences in F_v/F_m ratio between discs with 20 and 40 *T. urticae* were found (Fig. 4). Measurement of chlorophyll fluorescence took approximately 5 min per sample.

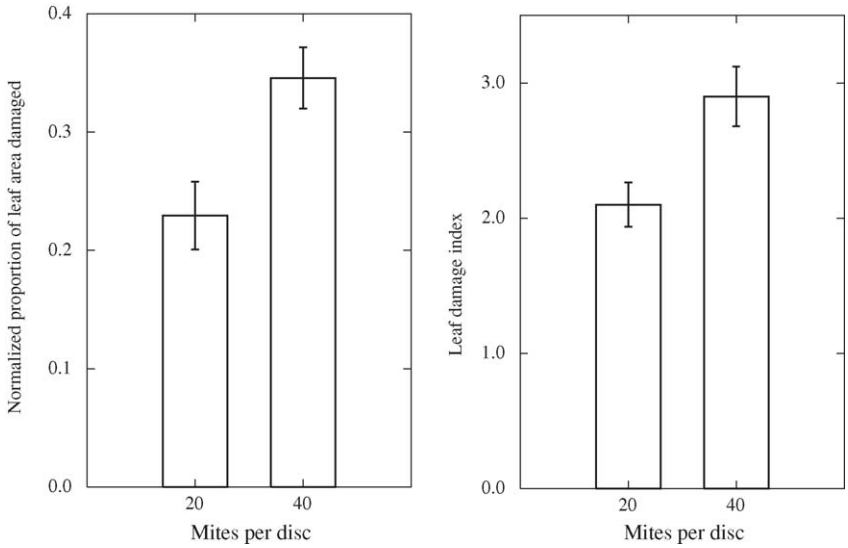

Fig. 2. The effect of mite density on normalized proportion of leaf area damaged (*NPLAD*, left) and leaf damage index (*LDI*, right). Bars show means ±SE.

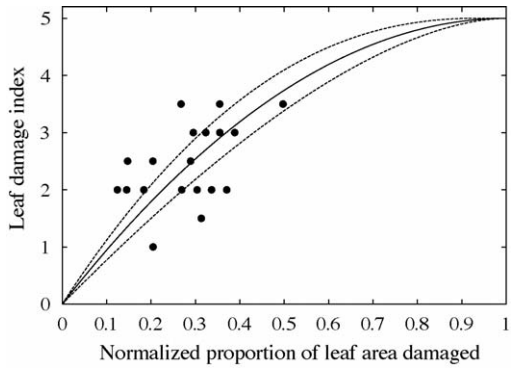

Fig. 3. The relationship between the normalized proportion of leaf area damaged (*NPLAD*) and the leaf damage index (*LDI*). The full line represents the fit of the Eq. (3) to data with $b = 1.9894$ (SE $= 0.1949$), while the dashed lines show the 95% confidence limits about predicted line. Dots are observed values.

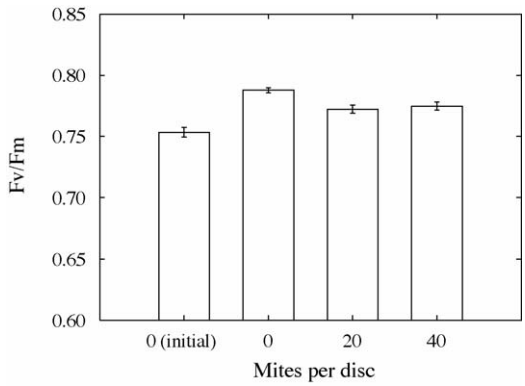

Fig. 4. The effect of mite density on the ratio of variable to maximum fluorescence, F_v/F_m. Bars indicate means with ±SE as vertical lines. The leftmost bar shows the mean of all freshly-cut discs; other bars show measurements after 3 days.

4. Discussion

Our results show that both the newly proposed normalized proportion of leaf area damaged (*NPLAD*) method and the widely used leaf damage index (*LDI*) method are sensitive enough to show significant differences in leaf damage after exposure to different densities of spider mites. However, the *NPLAD* method is likely to be more precise than the *LDI*. Although it is possible to convert the qualitative *LDI* index to a quantitative variable (e.g. chlorophyll content) using a calibration curve (Tomkiewicz et al., 1993; Nachman and Zemek, 2002a), the estimates are also affected by subjective visual categorization of leaf damage. In contrast, the *NPLAD* is expressed on a quantitative scale and minimizes subjectivity associated with the *LDI* (i.e., researcher skills and experience).

Correlation analysis revealed a significant positive relationship between the *NPLAD* and the *LDI*, as expected. The non-linear model showed a significantly better fit than a straight line passing through (0,0) and (1,5). This finding accords with the curvilinear relationship found between the *LDI* and the amount of chlorophyll per cm^2 leaf area (Nachman and Zemek, 2002a). Concave shape of the fitted curve may have several reasons. For example, while the computer counts individual pixels, when examined visually one can only roughly estimate the percentage of the whole feeding area, within which there are still undamaged cells, which can lead to overestimation of the *LDI* as compared to the *NPLAD*.

The chlorophyll fluorescence method did not give satisfactory results as no differences in damage between the two mite densities were found. Although this method, developed primarily for plant physiology research, can also be used to measure leaf damage (Iatrou et al., 1995; Buschmann et al., 1996), it is sensitive to physiological changes not directly caused by spider mite feeding, such as water stress or toxins. It is almost impossible to separate the influence of feeding from the other effects. Iatrou et al. (1995) found only a slight change of F_v/F_m between various densities of *T. urticae*. Moreover, the method was validated using whole intact plants, whereas in our experiments detached leaves also suffered from mechanical damage to the plant tissue, which induces stress and influences photosynthesis. Another disadvantage of this method is that measurements of chlorophyll fluorescence require special and expensive equipment (fluorometer).

The *NPLAD* assessment is a simple and relatively inexpensive method because it does not require any special equipment beyond usual computer software and hardware. It is not as fast as the *LDI* estimation, but the time required to measure the *NPLAD* may be further reduced by using a faster computer, scanning more leaf samples at the same time and using standard computer packages (e.g., MATHEMATICA) to automate processing of many scanned images. In this way it should be possible to assess *NPLAD* in several tens of samples per hour. The advantage of using MATHEMATICA is that it has several predefined utilities for image analysis, and can be readily used without developing new software. Other standard packages such as Maple or Matlab can also be used for similar purposes. Moreover, scanned images can be stored on digital media (e.g., on CD or DVD) for later analysis. It is also possible to calculate absolute values of damaged area by incorporating calibration procedures and simple modification of the algorithm. When using a digital camera to obtain leaf images (instead of a scanner) the proposed method can also be applied for measurement of leaf damage *in situ* on intact plants without disturbing the mites on the leaves.

5. Conclusions

We conclude that the precision of the *NPLAD* method is sufficiently high to recognize differences between various leaf damage levels caused by *T. urticae* feeding on bean plants. The method provides a reliable tool for leaf damage evaluation for use in both applied and experimental ecology. We believe that our methodological approach can be used also in a wider context, such as with other host plants or pest species (e.g. eriophyoid mites, thrips and aphids) that cause similar feeding damage as spider mites do. Presumably it may also be used to analyze characteristics outside the visible spectrum (something the *LDI* method cannot do) if digital images are obtained by means of an infra red camera.

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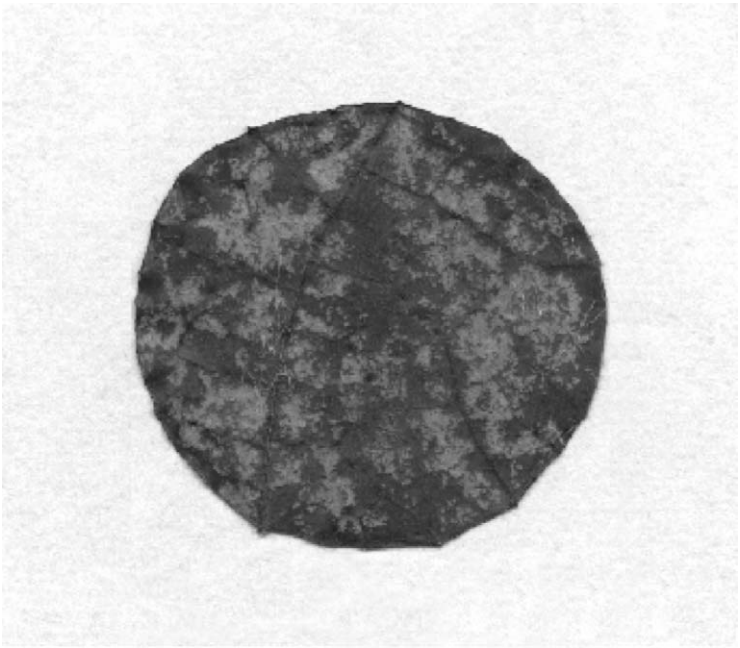
Appendix A. Listing of the MATHEMATICA code

(* Sets the path to the directory that contains the scanned image(s) *)

```
SetDirectory["C:/"];
```

(* Reads and shows the scanned image*)

```
image=Import["z2_5_300.tif"];
Show[image];
```



(* Reads dimensions of the scanned image *)

```
imagearray = newimagearray = imageprocessed=image[[1,1]];
dim = Dimensions[imagearray];
```

(* Definitions of various thresholds for analysis. These values depend on the image and should be set by the experimenter. LT: lower threshold level, UT: upper threshold level, WHITE: definition of white color, BLACK: definition of black color, GRAY: definition of gray color for damaged areas *)

```
LT = 120;
UT = 200;
WHITE = 255;
BLACK = 4;
GRAY = 150;
```

(* Visualization of the damaged area of the leaf using two shades only. Black/gray areas denote undamaged/damaged parts of the leaf. *)

```
Do[Which[imagearray[[i, j]] >UT,newimagearray[[i, j]]=WHITE,
imagearray[[i, j]] ≤ LT,newimagearray[[i, j]]=BLACK,
UT ≥ imagearray[[i, j]] ≥ LT,newimagearray[[i, j]]=GRAY],
```

```
{i, 1, dim[[1]]}, {j, 1, dim[[2]]}];
image[[1,1]]=newimagearray;
Show[image];
```

```
<< Statistics`DataManipulation`;
```

(* Vector c contains three values:

c[[1]] is the number of pixels with shades lower than LT,
c[[2]] is the number of pixels with shades equal or above LT and below UT,
c[[3]] is the number of pixels with shade values larger or equal to UT *)

```
c=RangeCounts[Flatten[imagearray],{LT,UT}];
```

(* Calculation of the PLAD *)

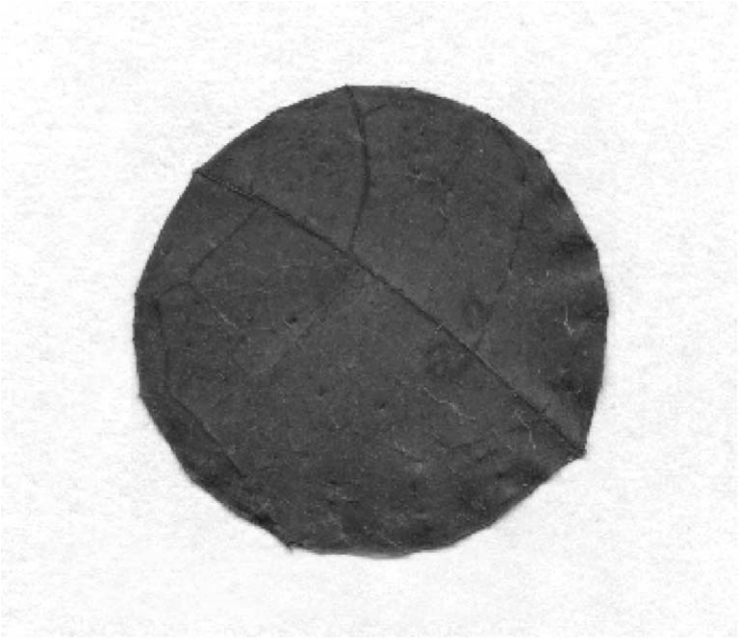
```
Print["PLAD=",PLAD =c[[2]]/(c[[1]] + c[[2]])//N];
```

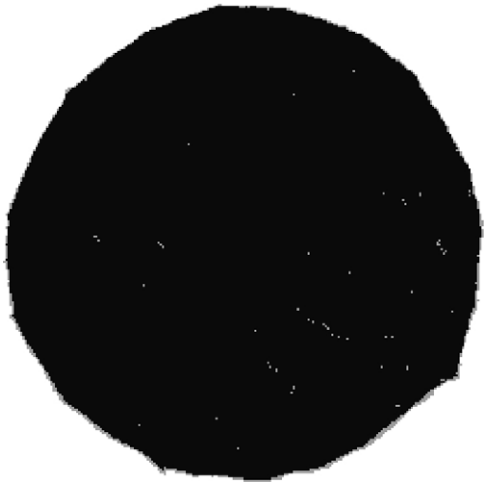
PLAD = 0.307132

(* Computation of the Abbot formula. This requires a control leaf disc. All the calculations are the same as above. *)

```
imagecontrol = Import["k5_300.tif"];
Show[imagecontrol];
imagecontrolarray =
newimagecontrolarray=imagecontrol[[1,1]];
dim = Dimensions[imagecontrolarray];
Do[Which[imagecontrolarray[[i, j]] >UT,
newimagecontrolarray[[i, j]]=WHITE,
imagecontrolarray[[i, j]] ≤ LT,
newimagecontrolarray[[i, j]]=BLACK,
UT ≥ imagecontrolarray[[i, j]] ≥ LT,
newimagecontrolarray[[i, j]]=GRAY],{i, 1, dim[[1]]},
{j, 1, dim[[2]]}];
```

```
imagecontrol[[1, 1]]=newimagecontrolarray;
Show[imagecontrol];
ccontrol = RangeCounts[Flatten[imagecontrolarray],{LT,UT}];
```





(* Calculation of the PLAD for the control. This value should be either equal, or close to zero. *)

```
Print["PLADcontrol=",
PLADcontrol = ccontrol[[2]]/(ccontrol[[1]] + ccontrol[[2]])//
N;
```

PLADcontrol= 0.0168574

(* Calculation of the NPLAD (the Abbot formula)*)

$$\texttt{Print}\left[\texttt{"NPLAD="},\texttt{NPLAD} = \frac{\texttt{PLAD} - \texttt{PLADcontrol}}{1 - \texttt{PLADcontrol}}//N\right];$$

NPLAD = 0.295252

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