Benchmark

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A fast, nondestructive method for the detection of disease-related lesions and wounded leaves

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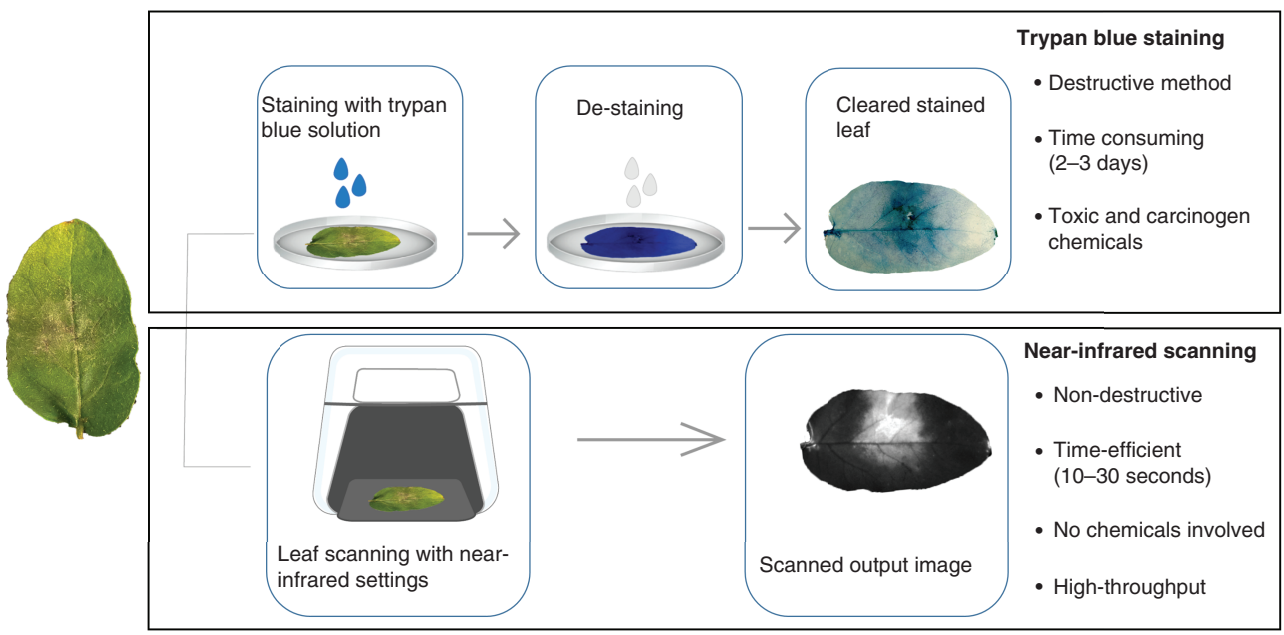
ABSTRACT

Trypan blue staining is a classic way of visualizing leaf disease and wound responses in plants, but it involves working with toxic chemicals and is time-consuming (2–3 days). Here, the investigators established near-infrared scanning with standard lab equipment as a fast and nondestructive method for the analysis of leaf injuries compared with trypan blue staining. Pathogen-inoculated and wounded leaves from potato, tomato, spinach, strawberry, and arabidopsis plants were used for proof of concept. The results showed that this newly developed protocol with near-infrared scanning gave the same results as trypan blue staining. Furthermore, a macro in FIJI was made to quantify the leaf damage. The new protocol was time-efficient, nondestructive, chemical-free and may be used for high-throughput studies.

GRAPHICAL ABSTRACT



METHOD SUMMARY

An easy and cost-effective method to quantify leaf wounding and disease lesion area without damaging the leaf was developed. Five plant species analyzed in this study provide proof of concept for the use of a common laboratory instrument, Bio-Rad ChemiDoc MP Imager (using Cy7 filter). In addition, to quantify the leaf damage, a macro in FIJI was developed.

KEYWORDS:

image processing • leaf damage • lesions • phenotyping • plant disease • potato • *Phytophthora*

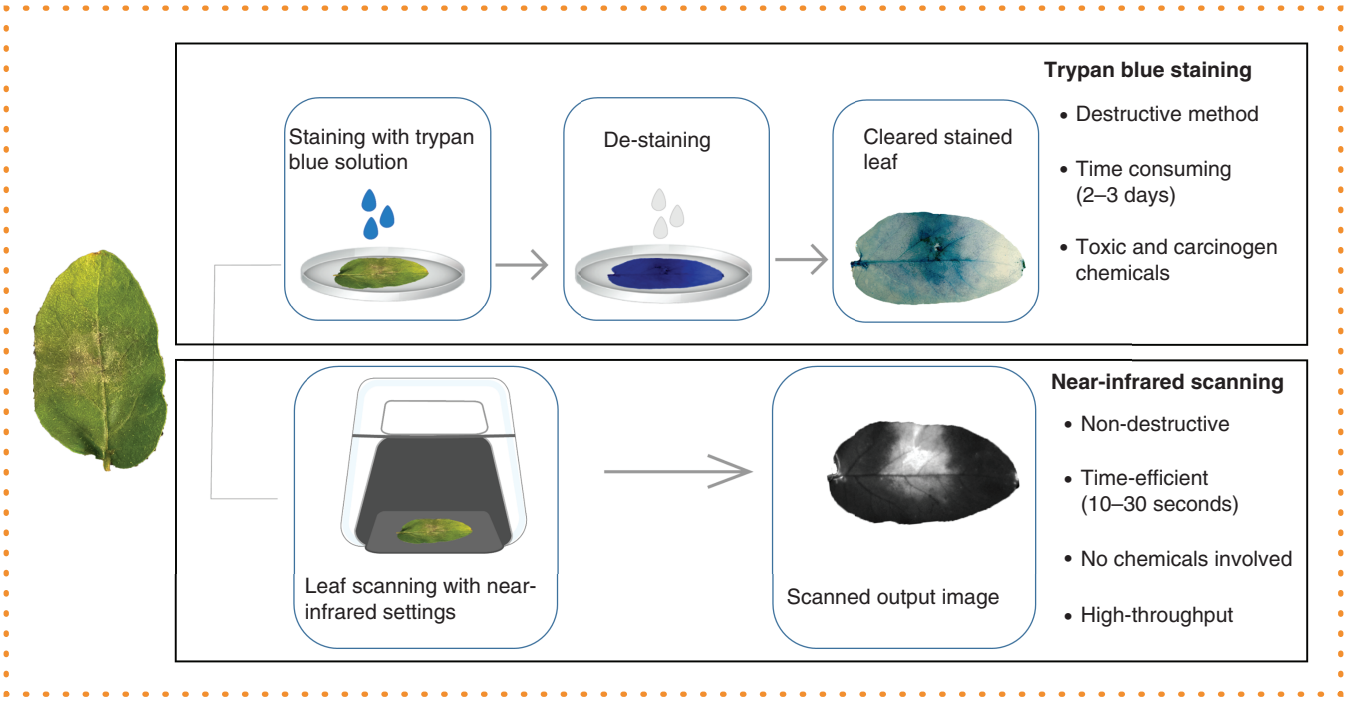


Figure 1. Comparison between the traditional trypan blue staining method and a newly established infrared scanning protocol. Trypan blue staining takes 2–3 days to obtain the results, and involves toxic and carcinogenic chemicals. Infected/damaged leaves are placed into the trypan blue solution overnight and replaced with destaining solution after 1–2 days. With the newly developed near-infrared scanning method, a leaf is placed into the scanner, the image is produced within 10–30 seconds and the leaf can be analyzed further.

Plants encounter many biotic and abiotic stressors in the environment [1]. Leaf wounding can be caused by both biotic and abiotic stress. Many pathogens penetrate the plant tissues using wounded leaf tissue or natural entry points. For example, in the case of lettuce leaves, *Escherichia coli* can penetrate via injured spots on the plant [2]. Therefore, it is important to be able to detect leaf wounding and pathogen attack using easy and affordable measures.

Researchers commonly rely on the manual measurement of infection lesions during plant–pathogen interaction studies to record the data. Measurement of the plant lesion is time-consuming and prone to human errors, which can reduce accuracy. There is also a chance of missing small differences in the infection site if the leaf tissue color is similar to that of healthy tissue. Furthermore, manual measurements are often done by either measuring mean lesion diameter or by recording the length and width of the lesion and calculating the area via the geometric shape that closely relates to the specific shape of the lesion. Because of the irregular shape of lesions, manual measurement may result in a less accurate outcome. To overcome some of the limitations of direct visual monitoring and to obtain a clear picture of inoculated or wounded tissue, trypan blue staining is used [3].

Trypan blue is an azo dye and is widely used to stain dead tissue. In plant science, it has been used to visualize disease lesion area and wounded leaves [4]. However, as shown in Figure 1, trypan blue staining requires 2–3 days and involves working with toxic chemicals. Additionally, the laborious protocol makes it difficult to work with a large number of samples. Furthermore, after staining with trypan blue, leaf samples are destroyed and cannot be used for further studies, such as the extraction of DNA to quantify pathogen biomass. A fast nontoxic method to quantify leaf injury that is as accurate as the trypan blue staining method is needed.

Here, the authors described a new method of quantifying leaf disease and wounded area with a commonly used instrument in molecular biology laboratories, namely the Bio-Rad ChemiDoc MP Imager (model number: 12003154). This equipment is widely used for the visualization of, for example, protein gels and blots. In addition to the use of visible light (wavelengths between 400 and 700 nm), the imager also allows the detection of far-red and near-infrared fluorescence and chemiluminescence. The ChemiDoc MP Imager has a set of filters that control the different spectral ranges of excitation and emission. In this new methodology, the Cy7 filter was used with the low-light fluorescence application for near-infrared fluorescence detection. The Cy7 filter is installed in Bio-Rad ChemiDoc MP Imager by default and can be upgraded into an older version on ChemiDoc scanners. The Cy7 filter allows transmission between 755 and 777 nm. The focus area was chosen in the scanner settings according to the size of the leaf and lesion. Cy7 was selected and a 5-s exposure was typically used to record the images. Exposure settings occasionally needed to be adjusted depending on the type of leaf. Once the focus area and manual exposure were selected, the same settings were used to process all the samples from the same experiment. In total, it took 5–30 s to acquire the image with the scanner in a.JPG or.TIFF format.

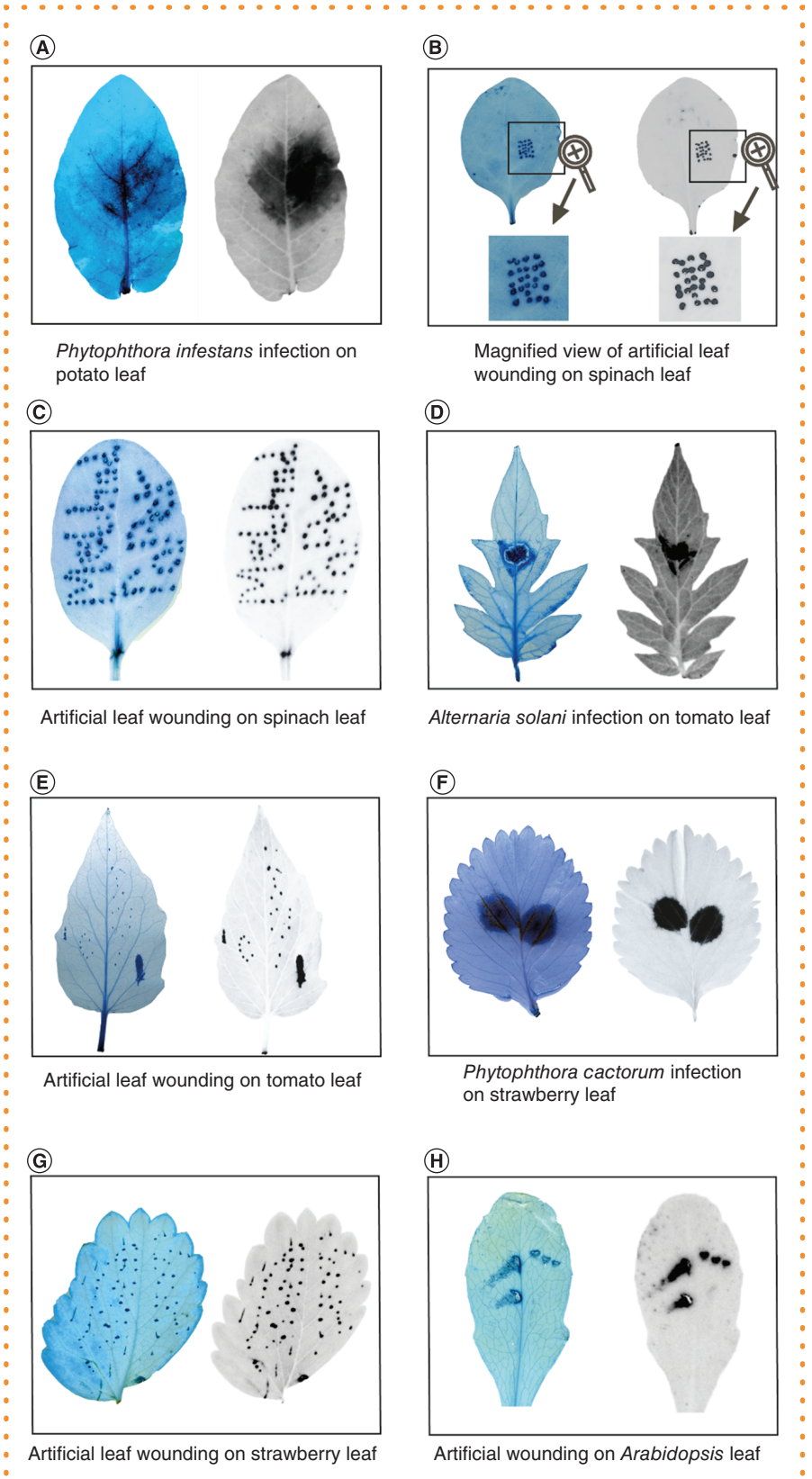


Figure 2. Examples of plant leaves stained with trypan blue (blue) and scanned with the new near-infrared scanning protocol (black and white). In each square, on the left side is a leaf stained with trypan blue; on the right side is an image acquired with a Bio-Rad scanner using Cy7 settings. **(A)** *Phytophthora infestans* infection on a potato leaf. **(B & C)** Artificial leaf wounding on spinach leaves. **(D)** *Alternaria solani* infection on a tomato leaf. **(E)** Artificial leaf wounding on a tomato leaf. **(F)** *Phytophthora cactorum* infection on a strawberry leaf. **(G)** Artificial leaf wounding on a strawberry leaf. **(H)** Artificial wounding on an arabidopsis leaf.

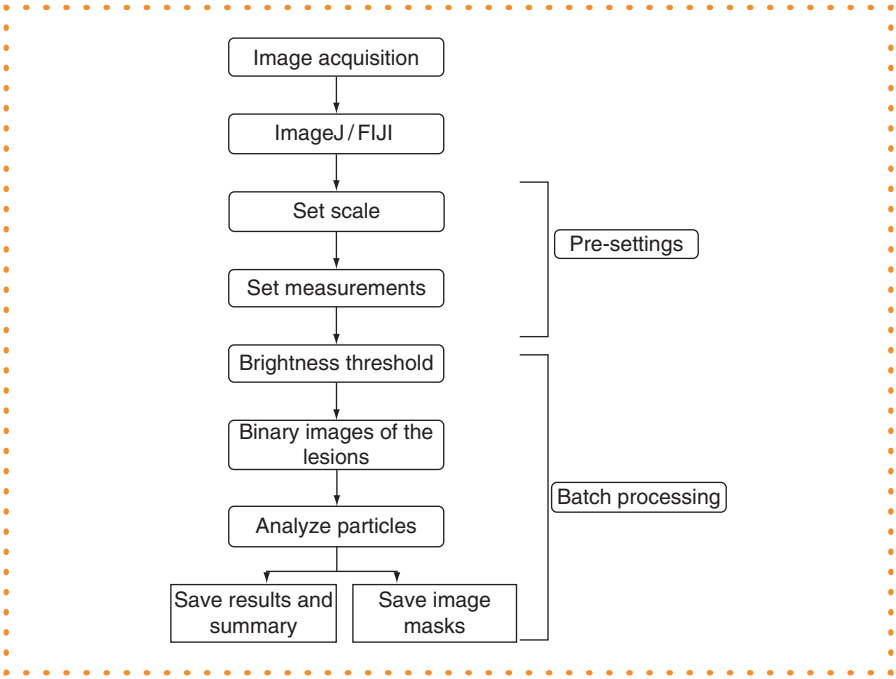


Figure 3. Workflow of image quantification in FIJI. A macro code in FIJI, given in the supplementary material, was run for batch analysis of the scanned images and for quantification of the lesion area.

After the leaves were scanned, they were subjected to trypan blue staining as described by Bach-Pages and Preston [5] with minor modifications, for comparison. Leaves were placed in a petri plate with trypan blue solution, boiled for 5 min, and left overnight on a rotary shaker. The following day, the solution was replaced with destaining solution (chloral hydrate or ethanol), and the samples were left on the shaker overnight. On the third day, the destaining solution was replaced with 50% glycerol, and images were taken with an Epson perfection V750 pro scanner.

To evaluate the efficiency of the near infrared-technique, several plant species were used, namely, potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), spinach (*Spinacia oleracea*), strawberry (*Fragaria* × *ananassa Duch*.) and arabidopsis (Arabidopsis thaliana), with plant–pathogen interactions and artificial leaf wounding (Figure 2). For plant–pathogen interactions, potato leaves infected with *Phytophthora infestans* (inoculated as described in Kieu *et al.* [6]) were used, and analyzed five days post inoculation. *P. infestans* is the causal agent of late blight disease, one of the most devastating diseases of potato [7]. Symptoms of late blight start as small brown or black irregular lesions and often look water-soaked. Due to its irregular pattern of infection, it can be difficult to directly quantify the disease lesion. Other examples included in this study were *Alternaria solani* (early blight) infection on tomato leaves (inoculated as described by Chaerani *et al.* [8]) and strawberry leaves infected with *Phytophthora cactorum* (crown rot; inoculated as described by Iqbal *et al.* [9]). Mechanical wounding was done with a scalpel blade and needle. Potato, tomato, spinach, strawberry, and arabidopsis leaves were used for mechanical wounding. Leaves were analyzed 6–12 hours after wounding. All examples showed the same results using near-infrared scanning or trypan blue staining (Figure 2 & Supplementary Figure 1).

Next, potato leaflets inoculated with *P. infestans* were used to quantify the disease lesions with the new method and to compare the results with manual measurements. Manual measurements were carried out either by measuring the diameter of the lesion on fresh material two times with a ruler or by quantifying disease area from RGB images in FIJI. After manual measurements, the same leaves were placed inside the Bio-Rad ChemiDoc MP Imager (Figure 4A). Users can adjust the optical zoom in Bio-Rad ChemiDoc MP depending on the average size of the leaflets. Scanned images were analyzed in FIJI (Fiji Is Just ImageJ, version 1.53c), an open-source software for image analysis that is extensively used in biological sciences [10]. A macro function was written in FIJI for quantifying leaf disease or wound lesion area and other morphology parameters of multiple scanned images from the Bio-Rad ChemiDoc MP Imager (Figure 3 & Supplementary File 1). Before running the macro function, the scale and parameters to be measured are set manually. The scale of the images obtained from the Bio-Rad ChemiDoc MP Imager can be adjusted manually if the user prefers to work with a unit of length (i.e., cm or inches) instead of pixels. In FIJI, the scale is adjusted at *Analyze* > *Set Scale*, where the user can set a conversion of pixels to the unit of length of choice. To find how many pixels compose, for example, 1 cm, the user can take an image of a ruler with the Bio-Rad ChemiDoc MP Imager, use the line function to draw a line over 1 cm on the ruler, and adjust the scale at *Analyze* > *Set Scale*. The distance in pixels of the line is given, the known distance should be set as 1, and the unit of length should be set according to the user's choice. With the scale already set, the user can close the example image and run the FIJI macro. Further information about FIJI

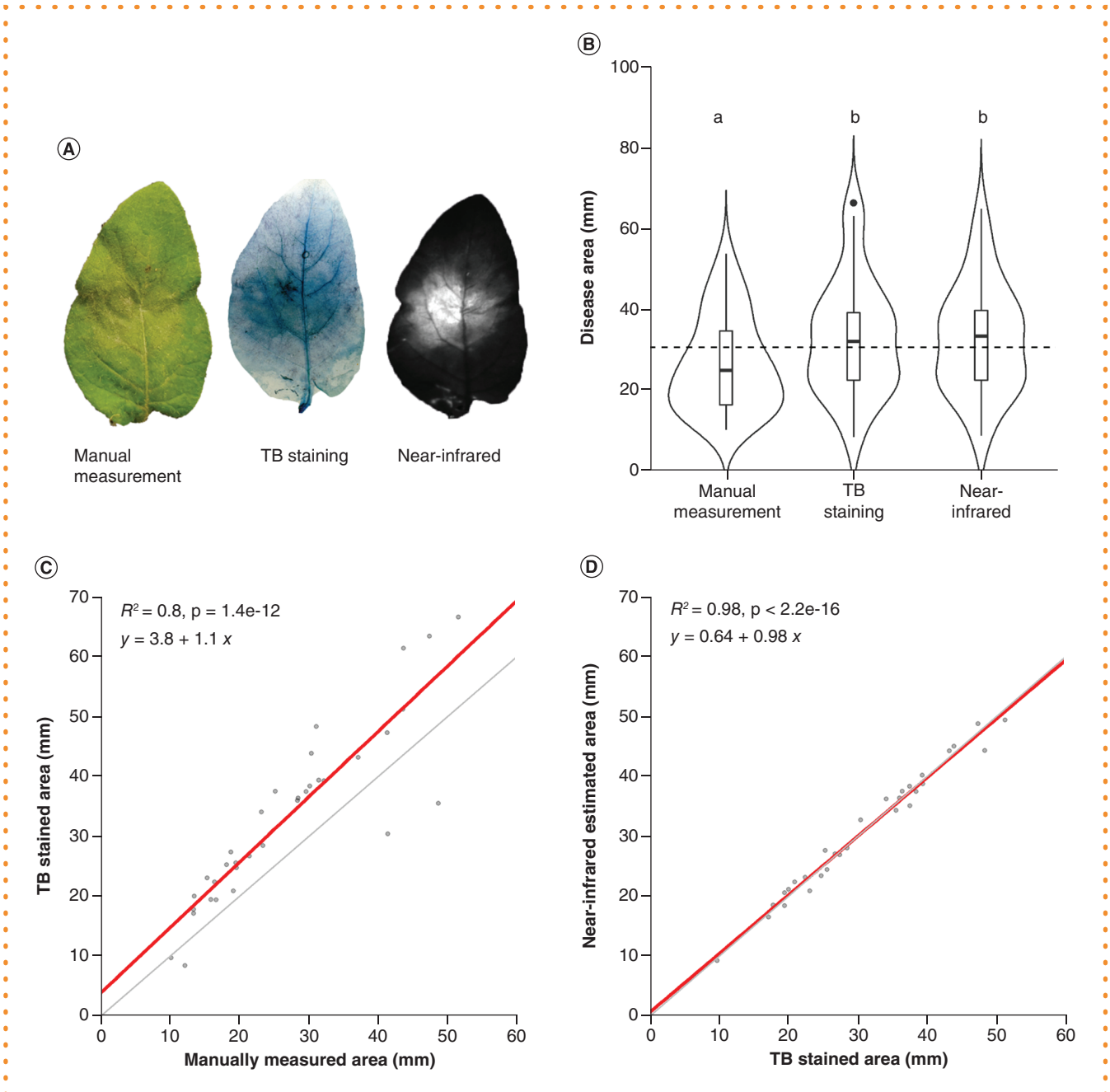


Figure 4. Validation of near-infrared scanning method for detection of *Phytophthora infestans* disease area on potato leaves, using two commonly used methods as reference. (A) *P. infestans*-infected potato leaf, evaluated using manual measurement, trypan blue staining and the near-infrared scanning method. **(B)** Differences in the diseased area detected using three methods were assessed with analysis of variance (Anova; n = 34), followed by Tukey *post-hoc* test. Different letters in the violin plot indicate significant differences ($p < 0.05$) between methods. Regression analyses of **(C)** manually measured or **(D)** trypan blue stained area, with near-infrared estimated diseased area (mm). The regression line (red) shows the result of either $y_{Near\text{-}infrared} = \beta_0 + \beta_1 x_{Manual}$ or $y_{Near\text{-}infrared} = \beta_0 + \beta_1 x_{Trypan\ Blue\ staining}$ model (red); line with slope 1 and intercept 0 (gray) are shown.

macro script steps provided in Supplementary Material 1. With the presettings already adjusted, the macro will allow the user to select an input folder, where the leaf images will be stored, and an output folder, where the lesion binary images and analysis results will be stored. During processing, the lesion area is identified through a brightness threshold, converting the picture to a binary image. Once all images are processed, all results are stored as an Excel file (.csv format) in the output folder.

By comparing the different methods of lesion quantification, we found no significant difference between trypan blue staining and the near-infrared scanning method; moreover, we observed that manual measurements, either by a ruler or by free-selection on FIJI, were

significantly different than both the trypan blue-stained scanned leaflets and the near-infrared scanning method (Figure 4B & Supplementary Figure 2). These results highlight the difficulties of defining the lesion sizes by the naked eye or by relying on formulas that calculate the area of specific geometrical shapes as a proxy to the actual infection area. As expected, measurements done directly by hand on fresh material resulted in smaller area values, as the edges of the lesions are hard to define with the naked eye. This is particularly true for a pathogen such as *P. infestans*, with hemibiotrophic behavior, as the pathogen invades adjacent cells without any obvious initial symptoms. Nonetheless, the manual measurements still correlated well with the measurements done on the trypan blue-stained leaflets (R^2 = 0.8; Figure 4C), and with the near-infrared method (R^2 = 0.81; Supplementary Figure 2). By comparing the trypan blue staining method to the new proposed quantification method (Figure 4D), we found a high correlation among the measurements (R^2 = 0.98). This implies that the newly developed scanning technique could be used to quantify disease- and wound-related lesions, rather than using the trypan blue staining method.

In summary, we established an easy and affordable method (assuming that Bio-Rad ChemiDoc MP Imager is available in the laboratory) to quantify leaf wounding and lesion area without damaging the leaf. This newly developed method is robust and correlates well with traditional trypan blue staining. The protocol does not require any staining procedure, which saves time and allows further processing of the leaf material after scanning. Moreover, this method may potentially reduce errors from manual measurement inaccuracies or from underestimation when applying set formulas for calculating the lesion area. The scanning method also results in binary images of the leaves and lesions, which can be readily used in scientific publications. Finally, this method also allows the batch processing of many images at a time.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2021-0045

Author contributions

All authors contributed to this project. MAZ, EA and RV contributed to the conception and design of the study. MAZ designed and performed all experiments and collected image data. MS wrote the Macro program in FIJI, performed image analysis and helped in discussion and to describe the program. MAZ and EA wrote the manuscript. All authors contributed to the manuscript, as well as read and approved the submitted version.

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Financial & competing interests disclosure

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