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PAPER

Fluorescence images combined to statistic test for fingerprinting of citrus plants after bacterial infection

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The citrus greening (or *huanglongbing*) disease has caused serious problems in citrus crops around the world. An early diagnostic method to detect this malady is needed due to the rapid dissemination of *Candidatus Liberibacter asiaticus* (CLAs) in the field. This analytical study investigated the fluorescence responses of leaves from healthy citrus plants and those inoculated with CLAs by images from a stereomicroscope and also evaluated their potential for the early diagnosis of the infection caused by this bacterium. The plants were measured monthly, and the evolution of the bacteria on inoculated plants was monitored by real-time quantitative polymerase chain reaction (RT-qPCR) amplification of CLAs sequences. A statistical method was used to analyse the data. The selection of variables from histograms of colours (colourgrams) of the images was optimized using a paired Student's *t*-test. The intensity of counts for green colours from images of fluorescence had clearly minor variations for healthy plants than diseased ones. The darker green colours were the indicators of healthy plants and the light colours for the diseased. The method of fluorescence images is novel for fingerprinting healthy and diseased plants and provides an alternative to the current method represented by PCR and visual inspection. A new, non-subjective pattern of analysis and a non-destructive method has been introduced that can minimize the time and costs of analyses.

1. Introduction

The bacterium *Candidatus Liberibacter asiaticus* (CLAs) is one of the causal agents of citrus greening, also known as *huanglongbing*. This disease is responsible for serious problems in citrus crops around the world, and up to now there is not a wealth of diagnostic options.^{1,2} A search for new and early diagnostic methods to map citrus diseases is required, and the need is becoming more acute due to the rapid dissemination of CLAs in the field.³

Polymerase chain reaction (PCR), based on DNA amplifications of bacteria, has become the most applied method for diagnosis of citrus greening.⁴ Another approach, based on the evaluation of mineral constituents, was previously studied and is suitable for understanding the effects of the citrus greening disease on citrus crops.^{5,6}

In the literature, studies using fluorescence images generated by leaves of citrus plants were utilised as a method of monitoring processes in plants.^{7,8} Alterations of chemical and physical properties intrinsic to the leaf, and stress caused by environmental factors or even diseases can be monitored by molecular fluorescence responses.^{9,10} Different wavelengths of excitation can be related to several pieces of information, for example, UV excitation of green leaves leads to two distinct types of fluorescence: (i) a blue-green fluorescence (BGF) from 400 to 600 nm, which is due to some constituents such as lignin and ferulic acid¹¹ and (ii) a chlorophyll fluorescence (ChlF) where relative high bands ranged in 690 and 740 nm, equivalent to red and far-red regions of the spectrum, that were associated with the chlorophyll content of the leaves.^{12,13}

Images of fluorescence from microscopy have been used to investigate the distribution of phenolic compounds in plant cells and tissues. Various classes of these compounds can also be

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visualized by auto-fluorescence using appropriate conditions of excitation.¹¹

In this case, the main purpose of the present study was to observe the response of the citrus plants to inoculation with CLas bacteria. For these investigations, two sets of plants were studied: healthy plants and plants from the same variety but inoculated with CLas. To study the colour variations from fluorescence images, histograms of colours, also known as colourgrams, were calculated. These are defined as plots of the distribution of colour frequencies, where the abscissa axis represents the variables, for example Red (R), Green (G), Blue (B), Hue (H), Saturation (S), Value (V), Luminosity (L), and other scales of colours; the ordinate axis characterizes the counts for each colour. The histograms of colours can be calculated from the image without any pre-processing¹⁴ and the values obtained for each scale of colour is recorded based on equations already reported.¹⁵

The potential information from colour evaluations of biological materials such as leaves was reported using digital image analysis for croton leaf variegation.¹⁶ Studies dedicated to early diagnosis of macronutrients deficiency were performed in legumes applying H, S, I (equal to value) and L*a*b* colour systems and also digital images.¹⁷

The final objective of this study was to propose an analytical method employing fluorescence images associated with colourgrams and to demonstrate their potential for the study of the infection effects caused by CLas in sweet orange. The data of histograms of colours were evaluated using a statistical method.

2. Experimental

2.1 Plant material and sampling

The experimental set of plants used in the study consisted of five-month-old Valencia sweet orange [*Citrus sinensis* (L.) Osbeck] cuttings grafted onto Citrumelo Swingle [*Citrus paradisi* Macfad. cv. Duncan X *Poncirus trifoliata* (L.) Raf.] rootstock. All plants were healthy before the inoculation with CLas. The procedure of infection was performed with two bud woods (each 2 to 3 cm long) taken from three-year-old sweet orange plants that were positive for CLas and exhibited typical symptoms. The experimental plants were grown in 4 L plastic bags containing Plantmax citrus substrate (Eucatex, São Paulo, Brazil) and maintained under greenhouse conditions with a controlled temperature of approximately 30 °C during the whole experiment. The irrigation of the plants was automatically done every day and fertilization was made when necessary.

The analyses were performed on two categories of plants of equal size: healthy (60 plants) and inoculated (60 plants). The healthy plants were kept as control samples. Three leaves were taken from each plant. The samples (leaves) were collected at a specific location in relation to the third set of leaves positioned downward from the apical part of the plant. The leaves were collected on the same day that fluorescence images were to be acquired. Before the image acquisition, each leaf was cleaned with the aid of a piece of cotton wetted with deionized water and then dried in air.

After one month of citrus plants inoculation with CLas, monthly laboratory experiments were started. The final duration of the study was 8 months (from July 2009 to March 2010).

2.2 DNA extraction and real-time quantitative PCR assays

DNA extraction was performed by sampling from the same described plants using a method described by Murray and Thompson.¹⁸ Briefly, total DNA was extracted from 100 mg (fresh weight) of midribs and petioles using cetyltrimethyl ammonium bromide (CTAB). The DNA was eluted in 60 µL of elution buffer (1 : 10 of Tris-EDTA 1 mol L⁻¹, 20 µg µL⁻¹ of RNase A), and its concentration and quality were analyzed with agarose gel electrophoresis and NanoDrop™ 8000 (Thermo Fisher Scientific, Wilmington, USA). The final concentration of all DNA was standardized to a concentration of 10 ng µL⁻¹.

The primers and probes used for the real-time quantitative PCR (RT-qPCR) assay were constructed by Coletta-Filho *et al.*¹⁹ using the Primer Express software (version 2.0; Applied Biosystems, Foster City, USA) and were synthesized by the same manufacturer. The primer sequences (AS-84F 5'-TCACCGG-CAGTCCCTATAAAAGT-3' and AS-180R 5'-GGGTTAAGTCCCGCAACGA-3') and probe (AS-NED-MGB-111T 5'-ACATCTAGGTAAAAACC-3') were based on the 16S rDNA sequence of CLas (GenBank AY919311). The optimized RT-qPCR assay required 0.8 µmol L⁻¹ of each primer (forward and reverse), 0.2 µmol L⁻¹ of the probe for CLas, 1× TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 4 µL of standardized DNA template (10 ng µL⁻¹), 1× Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems) as an internal control for normalization of the amount of total DNA in each reaction and autoclaved Milli-Q water to a final volume of 20 µL. The fast amplification protocol ran for 20 s at 95 °C followed by 40 cycles of amplification (3 s at 95 °C and 30 s at 60 °C). The amplification, data acquisition and data analysis were done with the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems) using the sequence detection software (version 1.4). Each run was comprised of two replicates for the tested DNA (inoculated plants), the CLas-negative (healthy plants), the CLas-positive (positive controls by conventional PCR) and a non-template control (NTC).

2.3 System of fluorescence image induced by ultraviolet radiation

One necessary adjustment for fluorescence microscope imaging was a 1.17 s exposure time on a digital camera for UV excitation using a SteREO Lumar.V12 (Zeiss, Göttingen, Germany) stereomicroscope. The UV excitation was done using a combination of a mercury-vapour short-arc lamp and a bandpass optical filter resulting in a band with maximum at 365 nm and bandwidth around 20 nm. For emission, it was used a longpass glass colour filter with cut-off position at 400 nm and 90% of transmittance. The linear set of the software Axio Vision release 4.6.3 (Zeiss) was used, and its parameters were -0.50 brightness, 1.00 contrast, and 1.00 gamma with 5% in best fit. A digital camera (AxioCamMRc5, Zeiss) with 5 M pixels of resolution was coupled to the stereomicroscope. The focus was within 14 and 15 mm with a 12.0 mm field. The zoom amplification was 30×. The resolution of the image was 1292 × 968 pixels (width × height), and the intensity of bits was 24. The area of luminous incidence was 0.3 × 1.0 mm. Two laminas (Bioslide, CAT no. 7102, California, USA) fixed by binder clips were utilized to

improve flatness for leaves and then to enhance the focus of the image obtained by stereomicroscope. The laminas were clear glass, with unground edges, 25.4×76.2 mm (width $\times$ high), 1.0–1.2 mm thick. The leaves were positioned between the laminas, and this material did not show alterations of fluorescence radiation. The room was kept dark during image acquisition. The recording time for each image was approximately 2 s. According to our tests, the applied parameters did not modify the fluorescence properties of the leaves. The image regions extended from the central area of the leaves, out from the midrib region (Fig. 1). This procedure was repeated for all samples during the whole experimental period.

2.4 Generation of data and chemometric tools

A total of 2184 histograms were computed using a routine performed in MatLab 2007R (The MathWorks Inc., Natick, USA) using as parameters the equations for colour scale calculation.^{14,15}

The scale of the colour histogram was composed of 2560 colour variables: 1–256 corresponded to Red (R), 257–512 Green (G), 513–768 Blue (B), 769–1024 Luminosity (L), 1025–1280 relative Red (rR), 1281–1536 relative Green (rG), 1537–1792 relative Blue (rB), 1793–2048 Hue (H), 2049–2304 Saturation (S) and 2305–2560 Intensity (I) (or value).

3. Results and discussion

3.1 Preliminary investigations

One of the challenges of this study was to establish the appropriate area of the original images to comprise the dataset. The desirable characteristics for each image were good representation and uniformity. The size of the files was another concern considering that the large quantity of data could be the limiting factor for the throughput of evaluations. Two adjusted parameters were performed to account for the data features: the first one was to manually select one area (300×300 pixels) from the original image (1298×968 pixels) for all images; another adjusted parameter was the exposure time on a digital camera to

obtain the best quality image, avoiding overexposure. Each representative region of the image of 300×300 pixels displayed a high homogeneity of colours under UV excitation.

A paired Student's *t*-test²⁰ was performed to evaluate the images for changes in the colour scale. The intention was to identify which colour presented the highest differences between images from healthy to diseased plants. Then, the area of each colour scale from the colourgram was the parameter for these interpretations. In summary, the calculated areas of each colour from healthy and diseased leaves in a given period were systematically compared by a Student's paired *t*-test. A template using Microsoft Office Excel 2007 (Microsoft™) was optimized for the calculation. A *t*-value was obtained for each colour of all acquired images and the months were examined separately. From this information, the colour with the highest *t*-values was the green for most images and months. The parameters for *t*-test were obtained using a confidence interval of 95%. The procedure of selection of the variables was summarized in Fig. 1.

Then, using only the green colour areas data, it was possible to discriminate fluorescence images for diseased and healthy leaves. Using the green colour area results we calculated the mean, standard deviation and median for healthy and diseased leaves. It is important to remark that the samples also showed differences based on the age of plants. The evaluations were then separately performed for each period. Examples of fluorescence images excited by ultraviolet radiation, generated by the stereomicroscope are presented in Fig. 2. The inscribed colourgrams in the images showed the variations between the green colours and the plots are on the same scale for better comparisons. Another observation for the exemplified fluorescence images in Fig. 2 was related to the intensity of counts for green colours. In the case of diseased plants, the green colour counts ranged 3488 to 5875 (equivalent to 68% of variation); otherwise, healthy plants had minor variations within 7565 to 9218 (22%).

3.2 Evaluation of green colour areas

Fig. 2 shows some representative images from healthy (left column) and diseased (right column) leaves. From these images it

Fig. 1 Pictorial summary of selection of the variables by paired Student's *t*-test.

Fig. 2 Fluorescence images for different periods of evaluation for healthy (left side) and diseased (right side) plants.

is observed that the healthy ones are darker than the diseased, using as parameter the green colour scale positioned below the images in Fig. 2. The profiles of the histograms for the green

colour (inscribed in images) of diseased citrus show clear alterations of the areas mainly for images on Fig. 2h and j. Through these results, the darker green colours were the indicators of healthy plants and the light colours for the diseased ones.

Fig. 3 shows a box plot for the whole period of the presented study and the first month presented a remarkable differentiation between the healthy and diseased leaves using green colour areas. Additional information observed in Fig. 3 is the mean (represented by black squares), median (horizontal line inside the boxes) and an interval that retains 95% of the samples: $\text{mean} \pm 1.96\text{SD}$ (SD, standard deviation). The periods with more differences verified by the t -test after the inoculation were 1, 3, 5, 7 and 8 months. Significant differences were not verified for months 2 and 4, when the t -test was applied (noted by asterisk in Fig. 3). This happened because it is possible that some secondary metabolites were at lower concentrations level in these specific periods (months 2 and 4). But, the most important information was the fact that even in the first month of inoculation, differences between healthy and diseased leaves were observed using the proposed method. This difference was also reproducible at months 3, 5, 7 and 8.

The prior symptoms of citrus greening such as blotchy yellow regions were noted by visual inspection only from the third month of inoculation. It is important to mention, after 8 months of experiments some diseased plants presented leaves with visual health characteristics. The images from the sixth month after inoculation were not recorded because a break in testing was necessary, due to problems with the experimental setup.

According to our results, the RT-qPCR assays can only provide positive diagnosis for 70% of the diseased samples after four months of inoculation, as also shown in Fig. 3. After the first month, the number of leaves positively diagnosed for CLAs by RT-qPCR was 4%. By the fifth and seventh months, 66 and 67% positive diagnosis was observed, respectively and by the eighth month, 82% diagnosis was achieved on these analyzed leaves. This diagnosis rate is due to either the uneven distribution of the

Fig. 3 Box plot with data for 2184 fluorescence images and their respective green colour areas, considering different periods after inoculation with *Candidatus Liberibacter asiaticus*. Asterisk represents the month with no significant difference with the paired Student's t -test, at confidence interval of 95% ($t_{\text{tab}} = 1.968$, $\text{DF} = 310$, $P < 0.05$).

bacteria in the plants²¹ or the low concentration of the pathogen. PCR requires the presence of high concentrations of the bacteria in the sample. Studies using PCR-tests associated with transmission electron microscopy (TEM) about early events in citrus greening were carried out by Folimonova and Achor.²² They tested sweet orange and grapefruit plants from a greenhouse and observed that the detection of the disease by PCR can take a period within 3–6 months and depends on the leaves age and also on the disease stage.

The proposed analytical method is based on the stress caused by the pathogen CLas on the citrus plants and the information obtained here shows consistent diagnosis up to the fifth to eighth month. Novel information was established by the molecular fluorescence images as useful potential for the fingerprinting of diseased leaves with citrus greening.

Some improvements in the presented method are necessary for future applications, as the selection of images size can be automatically done using a computer routine to automate and improve the speed of the method, but the utility of the information in the fluorescence images was established.

4. Conclusions

The analytical method described in this paper was able to potentially diagnose the condition of healthy and diseased citrus leaves up to the fifth month after the infection with CLas. The verified variations from the first to fourth months have to be more investigated. The noteworthy advantages of the method were analyses without chemical pre-treatment of the leaves and low cost of image acquisition compared with the existing methods. Fluorescence images can be better exploited as an alternative to the current diagnostic method (PCR). This system employs a consolidated technique for plant evaluation, introduces a new pattern of analysis and uses a non-destructive method that, in many cases, can minimize the time and cost of analysis and can reveal more information than visual inspection.

The main advantage of the use of fluorescence images is that they can be used as indicators to discriminate healthy and diseased samples. The obtained results improve the feasibility of early diagnosis of citrus greening. Thus, one example of the application of this selection process is to generate a library of standard images (or histograms for the green colour) of the fluorescence for diseased citrus plants, or even healthy ones. In addition, the developed analytical method can be extended to other matrices and diseases. The goal is to use this kind of tool to minimize the presence of inoculums in the orchards controlling the spread of disease.

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