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Laser-induced fluorescence imaging method to monitor citrus greening disease

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ABSTRACT

The potential use of laser-induced fluorescence imaging to monitor citrus greening disease in sweet orange (*Citrus sinensis*) plants was investigated. For this purpose, an experiment using healthy plants as the control and plants of the same variety inoculated with *Candidatus* Liberibacter asiaticus (CLas) was performed to verify the fluorescence response through 8 months after bacterial inoculation. A system using laser-induced fluorescence at 473 nm of wavelength excitation was configured. Color descriptors from the collected fluorescence images were determined using the average values for red (R); green (G); blue (B); hue (H); saturation (S); value (V, or Intensity); relative red (rR); relative green (rG); relative blue (rB) and luminosity (L). The data were analyzed using a paired Student's *t*-test by means of comparison between diseased and healthy plants. The results demonstrate a potential method to identify citrus greening at an early stage of the disease (first month), prior to the onset of visual symptoms at confidence level of 95%.

1. Introduction

The detection of citrus greening or *huanglongbing* (HLB) in plants prior to the onset of visual symptoms is a challenging task. Expeditious and reliable methods are urgently needed because citrus greening contamination occurs very rapidly (Ploetz, 2007; Nava et al., 2010). The spread of disease leads to the progressive reduction of orchards, resulting in economic damage to citrus producers and related industries (Gottwald, 2010; Vojnov et al., 2010). Currently, the development of methods for early diagnosis would result in important tools for citrus greening management and control.

Recent contributions were observed at the literature and included the following techniques: X-ray fluorescence (XRF) and laser-induced breakdown spectroscopy (LIBS) combined with chemometric strategies to successfully predict the condition of orchard plants infected with *Candidatus* Liberibacter asiaticus (CLas) (Pereira and Milori, 2010). Fourier transform infrared (FTIR) spectroscopy has also been used for the diagnosis of diseased citrus plants. Sankaran et al. (2010) used mid-infrared spectroscopy, along with chemometrics, to study citrus greening infection; however, these studies did not provide early diagnosis, except for LIBS method.

Nowadays, visual inspection is one of the most applied methods to diagnose citrus greening; however, this approach is highly influenced by subjective interpretation, and diagnostic errors can be higher than 30% (Belasque Jr. et al., 2009). Additionally, plants may be infected for up to several months (between 6 and 36 months) before visual symptoms are evident (Bové, 2006). Another common method for diagnosis is to use polymerase chain reaction (PCR) to test for the bacterial deoxyribonucleic acid (DNA) (Schmittgen, 2006). The main drawback to PCR assays is the fact that these methods are time consuming and expensive (Li et al., 2007).

The potential alternatives for disease diagnosis, usually applied to monitoring processes in plants, include molecular fluorescence techniques (Valeur, 2002). Fluorescence, using blue light excitation, has been used to collect information about the loss of photosynthetic activity in leaves under stress conditions (Edner et al., 1992). Chlorophyll fluorescence can be used as an indicator of stress impacts at early growth stages (Lichtenthaler and Babani, 2000). The red chlorophyll (Chl) fluorescence band monitored is

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emitted between 690 and 800 nm, corresponding to the red and far-red spectral region (Maxwell and Johnson, 2000).

Since 2009, our research group contributed with two studies about early diagnostic of CLas infection based on mineral and organic plant composition (Pereira et al., 2010) and also, we obtained information from fluorescence images excited with ultraviolet (UV) radiation (Pereira et al., 2011).

The purpose of the present study was to complement our prior results with fluorescence images excited with UV radiation, employing laser-induced fluorescence imaging (LIFI) at a specific excitation wavelength for the diagnosis of CLas infection in sweet orange (*Citrus sinensis*). Then, a control study was also performed to compare the fluorescence response of healthy citrus plants to the response of plants inoculated with CLas bacterium, prior to the onset of visual symptoms. To study the fluorescence variations from the collected images of healthy and diseased leaves, average values for red (R); green (G); blue (B); hue (H); saturation (S); value (V, or Intensity); relative red (rR); relative green (rG); relative blue (rB) and luminosity (L) color descriptors were calculated and their influences were evaluated using a paired Student's *t*-test.

2. Materials and methods

2.1. Citrus plants sampling

Plant material from 5-month-old Valencia sweet orange [*C. sinensis* (L.) Osbeck] grafted onto Citrumelo Swingle [*Citrus paradisi* Macfad. cv. Duncan X *Poncirus trifoliata* (L.) Raf.] rootstock was evaluated. A total of 120 plants were tested. It is important to mention that the chosen scion-rootstock combination used for this investigation is commonly found in groves around the world.

Sixty plants were bud-inoculated with CLas. Two bud woods (each one with 2–3 cm long) were taken from three-year-old sweet orange plants exhibiting typical symptoms of citrus greening and PCR positive for CLas, and inoculated onto the plant samples. One month after CLas inoculation, monthly laboratory assessments begun. Confirmation of the citrus greening disease was also checked monthly using real-time quantitative PCR (RT-qPCR). DNA extraction was performed from the same plants using a method described by Murray and Thompson (1980). The detailed procedure of DNA extraction and RT-qPCR assays was done as described by Pereira et al. (2010). The PCR analyses were performed only on midrib and petioles. These sixty plants were named diseased.

The remaining healthy sixty plants (no CLas inoculation) were used as control samples, without CLas inoculation. Then, they were designated healthy. Three leaves from each plant were considered to be a representative sampling of the entire plant and were collected at a specific position in relation to the third set of leaves downward from the apical part of the plants. Leaves were collected on the same day that images were to be acquired. Before image acquisition, all leaves were cleaned using a piece of cotton wetted with deionized water and then dried in air. The study was performed over 8 months, from July 2009 to March 2010. The images and PCR tests from the 6th month after inoculation were not recorded due to problems with the experimental configuration setup.

2.2. LIFI system

For these experiments, a laboratory system was configured for performing laser-induced fluorescence imaging. A diode-pumped solid-state blue laser system (DPSSL-473-50, Roithner LaserTechnik, Vienna, Austria) at 473 nm was used for fluorescence excitation of the leaf samples. This wavelength is resonant with chlorophyll absorption. The resulting fluorescence image was recorded using a charge-coupled device (CCD) digital camera with eight megapixels resolution (DSC-F828, Sony, Tokyo, Japan). The camera lens (Carl Zeiss Vario-Sonnar® T∗, Zeiss, Gottingen, Germany) was equipped with a modulation transfer function (MTF) and also is T∗ coated to suppress reflexes and to obtain better reproduction of the image colors. The backscattered laser radiation was passed through a colored band filter (03FCG083, Melles Griot Photonics Components Group, Carlsbad, USA). Some steps were necessary for application of laser-induced fluorescence imaging system described as follow.

The images were acquired using 5 mW of laser power stabilized with 1.10 A current after the laser turned on. The zoom amplification was 3.6×. A diagram of the LIFI system setup is shown in Fig. 1. A backside leaf was fixed using adhesive tape (3M do Brasil, Sumaré, Brazil) on a dark metallic holder. In addition, the reflectance phenomenon was avoided using a dark chamber as described in Fig. 1. The room was also kept dark during image acquisition, and the recording time was approximately 2 s. The dimensions of each image were 3264 × 2448 pixels (width × height), and the bit intensity was 24. The vertical and horizontal resolution was 72 dots per inch (dpi). The flash function was deactivated. The focal distance of the CCD digital camera was 13 mm.

2.3. Data treatment

For all experiments, one image per backside leaf was obtained on the region marked with the white circle as shown in Fig. 1. For data treatment, a 200 × 200 pixels region that displays high homogeneity of color under blue excitations was manually cropped from each entire image. This information was verified using the average values for color descriptors according to Santos et al., in press. The computed color descriptors were: R, G, B, H, S, V or I, rR, rG, rB and L.

2.4. Color descriptors evaluation

Evaluations of ten color descriptors from fluorescence images were performed using a paired Student's *t*-test (Christian, 1994). In this case, using a total of 2559 images of both healthy and diseased plants, the average values for R, G, B, H, S, V, rR, rG, rB and L were calculated and compared in order to identify the most discriminatory descriptor. MatLab 2007R was used for the calculation of *t*-values. A *t*-value was obtained for each descriptor (total of 10) from the obtained images. The paired Student's *t*-test was per-

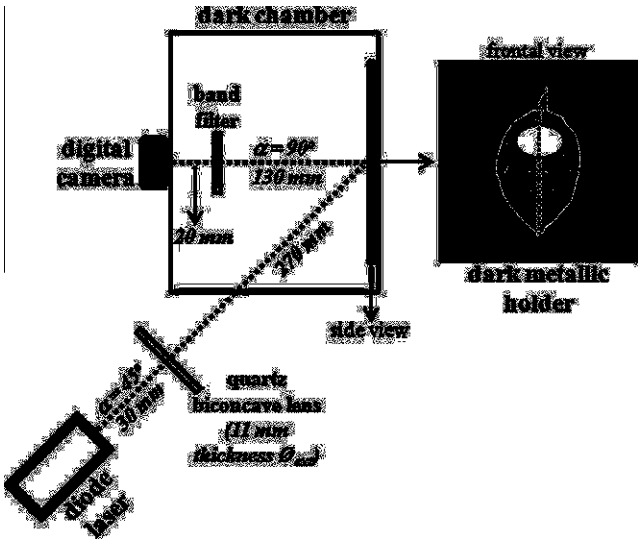

Fig. 1. Diagram of the laser-induced fluorescence imaging (LIFI) system.

Table 1
Paired Student's *t*-values calculated from average color descriptors for fluorescence images of healthy and diseased leaves. The *t*-values marked with an asterisk are higher than the t_{crit}.

Month	*n*	D.F.	*t*-Values for color descriptors										t_{crit}, $P < 0.05$	% (+CLas)
			R	G	B	H	S	V	rR	rG	rB	L		
1st	408	406	2.657*	8.481*	1.381	3.290*	6.656*	2.657*	7.103*	5.808*	0.616	3.090*	1.966	<5
2nd	345	343	0.697	2.659*	1.646	0.251	0.952	0.697	1.883	1.368	2.260*	0.747	1.967	Around 20
3rd	351	349	1.547	2.161*	1.280	0.342	1.523	1.547	2.383*	0.622	1.041	1.650	1.967	
4th	345	343	3.371*	1.734	2.689*	1.348	2.233*	3.371*	0.701	2.320*	2.516*	3.171*	1.967	Around 70
5th	354	352	0.155	3.282*	0.584	1.758	3.506*	0.155	1.976*	3.477*	1.161	0.150	1.967	
7th	411	409	0.606	6.611*	1.374	9.961*	3.844*	0.606	5.992*	8.170*	3.779*	1.840	1.966	
8th	345	343	0.110	6.208*	1.736	3.027*	1.335	0.110	6.512*	5.588*	2.964*	1.625	1.967	>80

formed and the calculated *t*-value was compared with the tabulated one (t_{crit}), at confidence interval of 95%.

3. Results

The information obtained from the average values for green (G) descriptor provided enough data as fingerprint of the fluorescence variations to determine the condition of the plants. The remarkable result was the ability to diagnose diseased plants only after 1 month of inoculation. The discrimination was also verified for subsequent months and can be checked with the *t*-values for up to following months after bacterial infection, as shown in Table 1. It is important to mention that the data size was different according to the availability of the plant material.

From Table 1, it is also possible to observe significative *t*-values for other variables, but the green demonstrated differences for most months. The *t* value for this descriptor were higher after the first month, decreased between the second to fourth months and increased again between the fifth and eighth months as shown in Table 1. The fourth month was the only period where the differences between healthy and diseased were not significative at confidence level of 95% using the green descriptor. At fourth month, the average values for blue descriptor were important to observe differences among the conditions of the plants and the *t*-value was equal to 2.689, (t_{crit} = 1.967, D.F. = 343, $P < 0.05$).

Results from our RT-qPCR assays can only provide positive diagnosis at four months after inoculation for most of plants (70%, shown in Table 1). The range of values in percentage for CLas-positive of each tested set between one and eighth months, using RT-qPCR was lower than 5% for first month, around 20% for second and third months, approximately 70% for fourth, fifth and seventh months and higher than 80% for last month, also shown in Table 1.

4. Discussion

The differences in the images were very tenuous and more accurate investigations were necessary, as shown in Fig. 2. This same figure also shows the color descriptors information for first month (range and mean ± standard deviation). Over the testing period, the fluorescence response varied. These variations cannot be evaluated by visual interpretation only because it could be mislead. In this case, the average values for ten color descriptors were fundamental to show the differences between the healthy and diseased samples. These values were also important for data clarification because even in the first month, differences between diseased and healthy leaves were detected. This is a relevant feature since visual inspection of the images cannot detect differences among them. In addition, the colors observed by human eyes are a superposition of three color descriptors, R, G and B (Gonzalez and Woods, 2008). The presented method in this paper decomposes the images in ten different variables (R, G, B, H, S, V, rR, rG, rB and L).

The average values for green descriptor was significant different for most months. The only exception was at the fourth month, where the average values for blue descriptor was the discriminatory color. This question must be better investigated, because it may be associated to distinct types of secondary metabolites considering other periods.

The variations of the range of values in percentage for CLas-positive were due to some probable factors (i) the uneven distribution of the bacterium in the plants (Li et al., 2009) or (ii) the low concentration of the pathogen. The key point of this proposed analytical method is to diagnose the disease based on the stress caused by CLas infection on the plants and the variations on fluorescence responses that this stress can cause. The principal limitation of PCR for the diagnosis of citrus greening is that method requires the presence of the bacterium in the sample.

The monthly percentage values of positive diagnostics for CLas obtained by RT-qPCR represent the qualitative results for this bacterium presence. They were obtained using 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).

It is important to emphasize that it was possible to observe, using visual inspection, the first signs of the disease only five months after inoculation. This visual inspection for diagnosis of

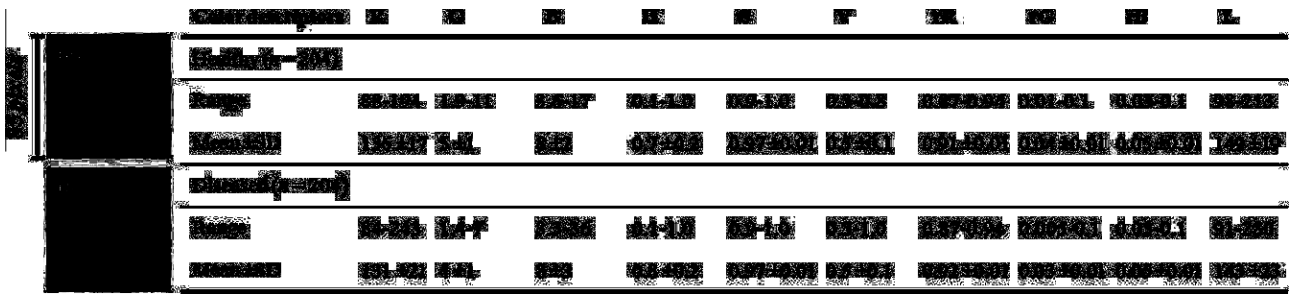

Fig. 2. Laser-induced fluorescence images excited at 473 nm for (a) healthy – without inoculation and (b) diseased citrus leaves after first month of bacterium inoculation (reduced 2.35 times from original size).

field crops is unachievable due to the great number of plants; or can be easily misinterpreted as nutritional deficiency in the plants instead of citrus greening disease.

Therefore, LIFI technique allowed an accurate early diagnosis for first month after inoculation. The advantage of LIFI, when compared with RT-qPCR, is low-cost analysis and, this technique shows high potential to be applied in large scale and field crops due to the fastness of the measurements. This approach can be considered a feasible method for citrus greening diagnosis considering that the images were acquired without any pre-treatment of the leaves and the diagnostic were reproducible for all months.

In this way, the fluorescence images, at 473 nm of excitation wavelength, demonstrate a promising method for citrus greening diagnosis from the first month of infection and this information was reproducible for other tested periods. The paired Student's *t*-test reveals applicable information from the fluorescence images for identification of the disease.

5. Conclusions

In conclusion, the acquisition of fluorescence images in association with average values for color descriptors and paired Student's *t*-test described in this paper can be used for the monitoring of citrus greening. The advantages of this method include the analysis without chemical pre-treatment of the leaves, elimination of the subjective nature associated with the visual inspection method and its potential use as an alternative to PCR methods.

The developed method achieved to diagnose citrus greening from the first month after the CLas inoculation. The direct application of the analytical method developed here is as an early diagnostic test that could be used to effectively control the spread of disease.

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