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Citrus canker is a serious disease caused by Xanthomonas citri subsp. citri bacteria, which infects citrus plants (Citrus spp.) leading to large economic losses in citrus production worldwide. In this work, laser induced fluorescence spectroscopy (LIF) was investigated as a diagnostic technique for citrus canker disease in citrus trees at an orchard using a portable optical fiber based spectrometer. For comparison we have applied LIF to leaves contaminated with citrus canker, citrus scab, citrus variegates chlorosis, and Huanglongbing (HLB, Greening). In order to reduce the noise in the data, we collected spectra from ten leaves with visual symptoms of diseases and from five healthy leaves per plant. This procedure is carried out in order to minimize the environmental effect on the spectrum (water and nutrient supply) of each plant. Our results show that this method presents a high sensitivity (∼90%), however it does present a low specificity (∼70%) for citrus canker diagnostic. We believe that such poor performance is due to the fact that the optical fiber collects light from only a small part of the leaf. Such results may be improved using the fluorescence imaging technique on the whole leaf. © 2010 Optical Society of America

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1. Introduction

Citrus canker is a serious disease caused by Xanthomonas citri subsp. citri bacteria, which infects citrus plants (Citrus spp.) leading to large economic losses in citrus production worldwide. In Brazil, citrus canker control is done by an official governmental eradication campaign, which involves a field inspection followed by laboratory tests to confirm the disease. Therefore, early detection of such disease is important to prevent greater economic losses due contamination of new plants. However, field detection is difficult, and so far it has been done by visual inspection of each tree. Suspicious leaves from citrus plants in the field are sent to the laboratory to confirm the infection by laboratory analysis, which is a time consuming procedure [1–4]. In principle, the eradication campaign would be more effective if the analysis could be done onsite.

Recent technological advances have increased the potential applications of light–tissue interactions for the recognition of alterations in plant tissues. One of these techniques, laser induced fluorescence spectroscopy (LIF) has been investigated as a tool in plant studies over the last two decades. Its main advantages are that LIF is nondestructive and nonintrusive to the plant biochemistry, physiology, and ecology [5–7]. Besides, it is a very convenient technique to be applied either in the laboratory or in the field [8,9].

In a preliminary study [10], we have used LIF spectroscopy to investigate the changes in chlorophyll fluorescence emission due to citrus canker and citrus variegates chlorosis (CVC, caused by Xylella fastidiosa) diseases in plant leaves in the laboratory. It is important to point out that in such work the leaves were collected from commercial citrus trees and brought to the laboratory for fluorescence measurement and data processing. However, the time
between leaf detachment and LIF measurement varied from 0 up to 12 h, depending on the distance between the farms and the laboratory. Those preliminary results have shown that LIF spectroscopy is able to distinguish samples with citrus canker disease from healthy leaves. However, the samples present a large dispersion that may be due to different environmental and physiological conditions of each plant [10]. Another possibility was the influence of the detachment time, which was not investigated in that work.

In a more recent and well controlled experiment [11], we have investigated the effect of mechanical stress on the detection of citrus canker using LIF on laboratory plants. In that experiment, the dispersion in the results was much reduced since all the plants had the same water and nutrient supply conditions. Besides, the leaves were never detached from the plant. In order to investigate the effect of the detachment time, we have studied the time evolution of the chlorophyll fluorescence emission as a function of detachment time [12]. And as expected, the spectrum changes considerably in a few hours, and this must be taken into account when applying LIF on detached leaves.

In this work, we have developed a new protocol to apply LIF as a diagnostic technique for citrus canker. Using a portable optical fiber based spectrometer we have applied LIF spectroscopy to detect citrus canker and compared their spectra from citrus scab [13], citrus variegates chlorosis (CVC) [14] and HLB (Huanglongbing-HLB) [15]. In order to reduce the effect of water and nutrient supply differences between the plants we have collected spectra from ten leaves with visual symptoms of disease and from five healthy leaves per plant. To avoid any detachment time effect the measurements were performed just after the leaves were collected from the plant. Our results show that this protocol presents a high sensitivity (∼90%); however it presents a low specificity (∼70%) for citrus canker diagnostic.

2. Materials and Methods

A. Fluorescence Spectroscopy System and LIF Procedure

Our fluorescence spectroscopy system is a portable unit (Spectr-Cluster, Cluster Ltd., Moscow, Russia), which was already described in detail in Refs. [10–12]. Briefly, it is composed of (i) one spectrometer, which operates from 350 nm up to 850 nm; (ii) one Y-shaped fiber, which delivers the laser light through one central fiber and collects the fluorescence from the leaf using six peripheral fibers; and (iii) an excitation source composed of a 10 mW solid state laser at 532 nm (the second harmonic of a Nd:YAG laser). The system also includes an optical filter, which reduces the backscattering signal approximately 1000 times. This allows us to obtain comparable intensity for the backscattering and fluorescence signal.

The spectra were collected from ten leaves with visual symptoms of disease and from five healthy leaves per plant. And in order to avoid any detachment time effect the LIF measurements were performed in the first five minutes after the leaves were collected from the plant. The measurements were performed by placing the optical fiber probe at a fixed distance of 2 mm above the leaf to avoid any thermal effect [11]. For healthy leaves the optical fiber probe was placed about 3 mm from the midrib. For the citrus canker, citrus scab, and CVC symptomatic leaves the fiber probe was placed between the apparently healthy tissue (green appearance) and the necrotic or yellow parts of the leaf. For HLB symptomatic leaves, the fiber probe was placed on mottle symptoms at the leaf. Each leaf spectrum is an average of 150 fluorescence measurements at the same region in the leaf. The entire procedure was carried out under aseptic conditions and took about 1 min per leaf.

B. Experimental Samples

In the experiment, 224 citrus trees were evaluated in six farms from four counties in São Paulo State, Brazil. The leaf samples came from plants of Citrus reticulata (Blanco), Citrus sinensis (L. Osbeck), Citrus aurantiﬁolia (Swingle), Citrus latifolia (Tanaka), Citrus limonía (L. Osbeck), and C. sinensis x C. reticulata hybrid that presented disease symptoms of citrus canker, citrus scab, CVC, and HLB. A total group of 2240 symptomatic leaves and 1120 healthy leaves were collected. After LIF measurements onsite, the leaves were transported in closed styrofoam boxes to laboratory for disease diagnostics using traditional tests [1–4]. Citrus canker and citrus scab diseased samples were differentiated by isolation of Xanthomonas bacterium pathogen in nutrient agar (NA) media [1]. CVC and HLB diseased samples were diagnostic by PCR protocols. The results were used to compose statistical tests for experimental data analysis.

3. Fluorescence Spectrum Analysis

As described in the literature [5], there are several fluorescence ratios that have been used to detect different plant stress. In this work, we are using an excitation source at 532 nm, therefore we are limited to obtaining only the red to far-red ratio (RF/FRF), which is defined by the ratio between the chlorophyll fluorescence intensity at 685 nm and the fluorescence intensity at 735 nm. However, as pointed out by Ćerovic et al. [5], the chlorophyll fluorescence band maxima at 685 and 735 nm may shift due to environmental conditions, leading to changes in the RF/FRF ratio. In fact, we have observed such effects in our previous work [11], and as a result we proposed a variant of the RF/FRF ratio, which is the figure of merit (FM). In the present work, we will use the RF/FRF ratio as well as FM ratio, which is defined as

$$FM = \frac{\int_{685}^{712} I(\lambda) d\lambda}{\int_{712}^{735} I(\lambda) d\lambda}$$

(1)
where \( I(\lambda) \) is the fluorescence signal as a function of wavelength \( (\lambda) \) and \( FM \) is the ratio of two integrals of \( I(\lambda) \), one in the range of 680–712 nm and another in the range of 712–750 nm. We will also use the ratio between the chlorophyll fluorescence and the backscattering signal, which is defined as

\[
FM_R = \frac{\int_{680}^{712} I(\lambda) d\lambda}{\int_{712}^{750} I(\lambda) d\lambda},
\]

where \( FM_R \) is the ratio of two integration of \( I(\lambda) \) at different wavelength ranges (680–800 nm by 520–540 nm). This parameter correlates all chlorophyll fluorescence emission with the backscattering signal. It should be pointed that the backscattering signal contains information on the reflectance properties of leaf surface, and it has been used as a tool for investigation of biotic and disease plant stress [5,16,17], including for citrus plants [18–20]. Unfortunately, the relative intensity of the backscattering and fluorescence signals, detected in our system, depends strongly on the optical filter used. Therefore, the ratio between fluorescence and reflectance obtained in this work are particular to the present experimental setup.

Using the ten diseased leaves we calculated the average values of \( FM \), \( FM_R \), and \( RF/FRF \) for each plant. Average values of \( FM \), \( FM_R \), and \( RF/FRF \) were also obtained for the healthy leaves for each plant. In order to reduce the effect of water and nutrient supply differences between the plants, we divided each parameter of the diseased leaves by the same parameter of the healthy leaves. This allows us to create normalized parameters, for example \( FM_n = FM_{\text{disease}}/FM_{\text{healthy}} \), which must minimize the influence of environmental conditions, as already demonstrated in Ref. [21].

4. Results and Discussion

The plants were separated in four groups: (a) citrus canker, (b) citrus scab, (c) CVC, and (d) HLB. In each group, we calculate the average value (Av), standard deviation (SD) considering the number of plants (N) of all normalized parameters (\( FM_n \), \( FM_{Rn} \), and \( RF/FRF_n \)) for each citrus disease, which are shown in Table 1. Those results allow us to point out a few observations: (a) the \( FM_n \) and \( RF/FRF_n \) parameters present the smallest standard deviation for citrus canker, approximately 13% for \( FM_n \) and 25% for \( RF/FRF_n \). This is similar to the other diseases as well; (b) the figure of merit approach presents a more discriminating result than the \( RF/FRF_n \) ratio only, as we have already observed; c) the direct comparison between citrus canker and the other diseases is not an easy task, since each disease has a different number of samples; (d) the citrus canker and HLB disease present very similar parameters, which does not allow us to discriminate between them by using the normalized parameters presented in Table 1.

Visual symptoms of citrus canker disease are completely different from HLB disease, since the latter does not present necrotic parts on the leaf, and therefore we will exclude HLB from our analyses. Some of the visual symptoms of citrus canker may be confused with other citrus diseases, specially with citrus scab. We should also point out that the management of citrus scab and CVC does not involve eradication.

For this reason, we propose a diagnostic procedure for citrus canker using LIF that will be either positive or negative for citrus canker. It is not our goal to diagnose either citrus scab or CVC. And we hope that the citrus scab and CVC samples will be classified as negative for citrus canker disease. The diagnostic procedure starts by analyzing \( FM_n \). We considered each sample as negative for citrus canker if either \( FM_n < 1.11 \) or \( FM_n \geq 1.82 \), and it will be positive if \( 1.25 \leq FM_n < 1.82 \). If \( 1.11 \leq FM_n < 1.25 \), then we must analyze \( FM_{Rn} \). It will be negative if \( FM_{Rn} \geq 1.2 \) and positive if \( FM_{Rn} \leq 0.9 \). If \( 1.11 \leq FM_n < 1.25 \) and \( 0.9 < FM_{Rn} < 1.2 \), then we must analyze \( RF/FRF_n \). The sample will negative if \( RF/FRF_n \leq 1.35 \) and positive for \( RF/FRF_n > 1.35 \). We should point out that \( FM_n \) and \( RF/FRF_n \) are very related to each other, however in the present work we were unable to determine an upper value of \( RF/FRF_n \) for positive citrus canker leaves. This may be due to the fact that \( RF/FRF_n \) presents a larger variance than the \( FM_n \) parameter [11]. This procedure is described in Fig. 1, which presents a fluxogram of the tests applied on the parameter values. Other procedures were ana-

### Table 1. Average Value (Av), Standard Deviation (SD), and Number of Plant (N) of All Normalized Parameters (\( FM_n \), \( FM_{Rn} \), and \( RF/FRF_n \)) for Each Citrus Diseased Sample

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Citrus Canker</th>
<th>Citrus Scab</th>
<th>HLB</th>
<th>CVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>( FM_n )</td>
<td>Av 1.5 SD 0.2 N 108</td>
<td>Av 1.1 SD 0.2 N 63</td>
<td>Av 1.6 SD 0.2 N 26</td>
<td>Av 2.0 SD 0.3 N 27</td>
</tr>
<tr>
<td>( FM_{Rn} )</td>
<td>Av 0.9 SD 0.3 N 108</td>
<td>Av 1.0 SD 0.4 N 63</td>
<td>Av 0.9 SD 0.8 N 26</td>
<td>Av 0.8 SD 0.5 N 27</td>
</tr>
<tr>
<td>( RF/FRF_n )</td>
<td>Av 2.0 SD 0.5 N 108</td>
<td>Av 1.3 SD 0.4 N 63</td>
<td>Av 2.1 SD 0.5 N 26</td>
<td>Av 3.0 SD 0.65 N 27</td>
</tr>
</tbody>
</table>

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lyzed as well, but the one used here presented the best results.

In the sequence, a binary classification test (diagnostic test) of diagnostic procedure outcome versus the citrus canker diagnostic from the laboratory test was performed. The results are presented in Tables 2 and 3. Table 2 presents the number of true positives (TPs), true negatives (TNs), false positives (FPs), and false negatives (FNs) of the test. Table 3 shows the statistical values of sensitivity (S), specificity (S'), positive predictive value (PPV), negative predictive value (NPV), prevalence (P), accuracy (A), likelihood-ratio positive (LRP), and likelihood-ratio negative (LRN). We should point out that the procedure and values presented in Fig. 1 were the ones that also presented the best results for the diagnostic, by maximizing the TPs and minimizing the FPs.

The proposed diagnostic procedure presents a good sensitivity (∼90%) but with a low specificity (∼70%) for citrus canker disease. In order to understand such result, we analyzed in more detail FMn. Most of the samples, approximately 80%, that were classified as TP present 1.25 ≤ FMn ≤ 1.82. Approximately 70% of the ones classified as TN present either FMn < 1.11 or FMn < 1.82. We should also mention that 82% of the CVC samples presented FMn ≥ 1.82 and that 50% of the citrus scab presented FMn < 1.11. The other parameters do not have much information on the leaf condition. We should also point out that such samples also presented a low standard deviation in FMn (from 5% to 15%). On the other hand, the FP or FN samples present a larger standard deviation in FMn, which varies from 25% to 35%. We believe this standard deviation is consequently the principal cause of FPs and FNs and low specificity. A possible source for this large variation is the fact that the optical fiber is placed in only a very small part of the diseased leaf. We have placed it between the apparently healthy tissue (green appearance) and the necrotic or yellow parts of the leaf, the fiber only collects light from a small portion, since its area is smaller than 1 mm². One possible solution would be to increase the number of leaves per plant or take spectra from several places on a single leaf; both procedures would decrease the standard deviation, but that would be time consuming. A more viable solution is fluorescence spectroscopy imaging to a more accurate diagnostic.

We should point out that in fact the main limitation of the proposed technique is the large number of FP results. Since the management for positive plant is its eradication and also the eradication of all plants in at least 30 m radius, that would lead to the eradication of a minimum 100 healthy plants and therefore a large economic loss. Although, we have not included the HLB disease in our analysis, the technique presents some potential for the detection of HLB, or other citrus or plant diseases as well. In principle, if a plant does not present any visual symptom of citrus canker but it does present average parameters similar to citrus canker we may be able to diagnose HLB disease. However, this must be investigated in more detail.

5. Conclusion

In summary, we have proposed a procedure to apply LIF as a detection tool for citrus canker using a portable optical fiber based spectrometer in the field. The diagnostic procedure was applied to citrus canker, citrus scab and CVC. The procedure presents a good sensitivity (∼90%) but with a low specificity (∼70%) for citrus canker. Such poor results were associated with the small collection area of the optical fiber probe. To improve its performance fluorescence spectroscopy imaging must be applied. At the moment, we are developing such instrumentation to perform such a technique in the field.

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References


![Fig. 1. Fluxogram of the diagnostic procedure. Tests of figure of merit values focusing the citrus canker detection are shown.](image-url)