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APPLIED OPTICS, v.47, n.11, p.1922-1926, 2008
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Detection of mechanical and disease stresses in citrus plants by fluorescence spectroscopy

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Received 27 September 2007; revised 6 December 2007; accepted 15 February 2008; posted 20 February 2008 (Doc. ID 87794); published 7 April 2008

We have investigated the detection of mechanical and disease stresses in citrus plants (Citrus limonia [L.] Osbeck) using laser-induced fluorescence spectroscopy. Due to its economic importance we have chosen to investigate the citrus canker disease, which is caused by the Xanthomonas axonopodis pv. citri bacteria. Mechanical stress was also studied because it plays an important role in the plant’s infection by such bacteria. A laser-induced fluorescence spectroscopy system, composed of a spectrometer and a 532 nm 10 mW excitation laser was used to perform fluorescence spectroscopy. The ratio of two chlorophyll fluorescence bands allows us to detect and discriminate between mechanical and disease stresses. This ability to discriminate may have an important application in the field to detect citrus canker infected trees. © 2008 Optical Society of America

OCIS codes: 170.6280, 280.1415, 300.2530.

1. Introduction

Presently, the interest in precision agricultural technologies has increased because these technologies have the potential to reduce the ecological impact of agriculture on the environment. However, prior to the application of these advances in precision agriculture, it is necessary to develop early stress detection techniques for agricultural crops. Aerial and satellite remote sensing of vegetation color and reflectance have been used with this goal in mind. They are very useful to detect the general characteristics of vegetation [1], but they lack the specificity and the selectivity necessary to discriminate different plant stresses. The reason is that similar leaf pigment losses may be caused by different stress conditions [2]. Furthermore, changes in leaf color represent a late plant response to different stresses [1,3]. The inability to discriminate between different stresses may lead to an incorrect diagnosis and to wrong management interventions with serious economic consequences. Therefore, there is a need to develop detection techniques with high specificity and selectivity.

Laser-induced fluorescence (LIF) of plants has been explored as a tool in vegetation studies in the last two decades [4–6]. Due to the high monochromatic laser spectrum, LIF may be a more accurate indicator of the physiological state of plants than other optical techniques. Therefore, its specificity and selectivity may be able to detect the impact of environmental plant stresses on several growth stages. For technical and safety reasons UV excitation has been preferred to visible excitation to monitor vegetation [3,4]. The UV excitation of green leaves induces two distinct types of fluorescence, a blue–green fluorescence (BGF) in the 400–600 nm range, which is due to several biological components [4] and chlorophyll fluorescence (ChlF) in the red to near infrared region (650–800 nm) of the spectrum [4]. The relative intensities of these two fluorescent bands obtained using UV excitation are highly sensitive to intrinsic leaf properties and environmental factors [7,8]. On the contrary, visible excitation mainly induces the ChlF in the 650–800 nm region.
of the spectrum. The most important aspect of LIF is that it is a nondestructive and noninvasive technique to the plant’s biochemistry, physiology, and ecology. Besides, it is easy to use for many purposes in the laboratory and field work [8,9]. Studies using ChIF emission have been successfully employed to detect mineral deficiencies, water and temperature stresses, and pathogens in plants [4,10–13].

In this paper, we apply LIF to citrus plants (Citrus limonia [L.] Osbeck) in the laboratory to detect mechanical stress and a pathogen infection called citrus canker caused by Xanthomonas axonopodis pv. citri bacteria. We have chosen such a plant disease due to its economic importance for many citrus production areas worldwide [14], especially for Brazil and the United States. The mechanical stress was also studied because it may facilitate the plant’s infection. Our results indicate that it is possible to detect mechanical and disease stresses and also to discriminate between them using LIF. These results may stimulate the development of a technique to be used in the field to detect citrus canker infected trees.

2. Materials and Methods

A. Mechanical and Citrus Canker Stress Models

Greenhouse plants of Citrus limonia (L. Osbeck), maintained in plastic pots, were pruned approximately 50 days before inoculation to obtain homogenized and incompletely immature (three-fourths to full expansion) leaves [15]. Inoculum of an aggressive bacterial strain of Xanthomonas axonopodis pv. citri (strain 1421) was prepared by suspending the bacteria harvested from 72 h old nutrient agar (NA) cultures in a phosphate buffer (PB). The suspension was adjusted spectrophotometrically to 10⁸ colony-forming units per milliliter of PB, and inoculum density was confirmed by plating on NA. The four treatments evaluated were composed of mechanically injured and inoculated plants in a factorial design, (a) healthy plants only mechanically stressed, (b) mechanically and diseased stressed plants, (c) diseased only stressed plants, and (d) control plants with no stress. There were ten plants per treatment. Mechanical stress was induced by passing a metallic needle (0.56 mm of diameter) entirely across the mesophyll in six points per leaf (five leaves per plant). Treatments formed by diseased plants corresponded to spray inoculation of bacterial suspension up to runoff in all leaves per plant. For comparison purposes in the health treatments the plants were sprayed with PB only. Immediately after the inoculation, humid plastic covers were placed around each plant in order to increase humidity and provide better conditions for infection.

B. Fluorescence Spectroscopy System

Our fluorescence spectroscopy system is a portable unit (Spectr-Cluster, Cluster Ltd., Moscow, Russia), and it is composed of, (i) one spectrometer, which operates in the 350–850 nm range, (ii) one Y-shaped fiber, which delivers the laser light through one central fiber and collects the fluorescence from the tissue using six other fibers, and (iii) an excitation source at 532 nm (second harmonic of an Nd:YAG laser pumped by a diode laser) with a total power on the order of 10 mW. We should point out that ideally it would be better to perform LIF using UV as well as visible excitation in order to obtain as much information as possible about leaf conditions. However, our long term goal is to produce a portable and affordable device for field work. Since a UV laser source is much more expensive than one at 532 nm, we have decided to work only with one at 532 nm. The measurements were carried out keeping the catheter probe at a distance of 2 mm above the leaf to prevent direct contact and therefore any thermal effect. Nevertheless, we took several spectra at different laser powers, and we observed that the spectrum did not present any intensity dependency, assuring no thermal effect on the incident spot.

Evaluations were made on the day of the mechanical and disease stress inducement procedures before their application (0), and 6, 13, 20, 27, 33, 42, 49, 55, and 60 days after. All the measurements were performed on attached leaves, and we have used three leaves from each plant, and in each leaf three spectra were taken. For the healthy leaves the probe was placed ~3 mm from the midrib, and for the healthy plants that were only mechanically stressed the catheter probe was placed on the side of the needle-induced injury. For the diseased plant, the probe was placed between the apparently healthy tissue (green appearance) and the necrotic or yellow parts, which correspond to the citrus canker symptoms. The probe area was ~1 mm × 1 mm. We should point out that the signal from the backscattering is approximately a thousand times more intense than the one from the inelastic scattering. To simplify the analyses we have used an optical filter to reduce it 1000 times, in such way that both signals have about the same intensity. Using this system the leaves were submitted to the fluorescence spectroscopy technique under aseptic conditions. The control group showed no changes.

3. Fluorescence Spectrum Analysis

In Fig. 1 we show a typical fluorescence spectrum for excitation at 532 nm for a control leaf with no stress. The fluorescence spectrum is due to the chlorophyll a emission [4]. To simplify the analysis we normalize the fluorescence spectrum by the backscattering of the spectrum obtained at the excitation wavelength peak. Light in the green region excites the ChIF directly while light in the UV-blue region excites the fluorescence of chlorophyll and other pigments. According to Cerovic et al. [4], ChIF is an accurate and nondestructive probe of photosynthetic efficiency, which can reflect the impacts of different physiological and environmental factors on a plant. In the literature, it is very common to use different fluorescence ratios to detect different stresses. There

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are three common ratios, (i) blue to red [blue fluorescence/red fluorescence (BF/RF)], defined by the ratio between the fluorescence intensity at 452 nm and the fluorescence intensity at 685 nm, (ii) blue to far-red [blue fluorescence/far-red fluorescence (BF/FRF)], defined by the ratio between the fluorescence intensity at 452 nm and the fluorescence intensity at 735 nm, and (iii) red to far-red [red fluorescence/far-red fluorescence (RF/FRF)], defined by the ratio between the fluorescence intensity at 685 nm and the fluorescence intensity at 735 nm. It depends only on the chlorophyll content. The BF/RF and BF/FRF ratios can only be obtained using UV excitation. Therefore, the laser-induced fluorescence at 532 nm is limited to supplying only the RF/FRF ratio [16].

However, as pointed out by Cerovic et al. [4], the ChlF band maxima at 685 and 735 nm may shift due to environmental conditions. In fact, we have observed such effects in our spectra, and as a result it introduces a variation on the RF/FRF ratio. To avoid this effect we have used a figure of merit (FM) approach, which we have defined as

$$FM = \frac{\int_{680}^{712} I(\lambda) d\lambda}{\int_{712}^{750} I(\lambda) d\lambda},$$

where the FM is the ratio of two integrations of the spectrum $[I(\lambda)]$ at different wavelength ranges (680–712 and 712–750 nm). Its advantage is that even if the fluorescence band maxima shift, such effects will be minimized after the integration. We have used such a procedure to detect citrus canker in a recent paper [17].

4. Results and Discussion

In Fig. 2, we present the FR/FRF ratio [Fig. 2(a)] and FM [Fig. 2(b)] as a function of time after inoculation. Each point is an average over all leaves and plants (a total of 90 spectra). The error bars were taken as the variance of all measurements. We can observe that the overall behavior of the FR/FRF ratio is similar to the FM for all treatments; however the FM presents small error bars. As pointed out before, we believe that the integration reduces the effect of peak shifts, and therefore it reduces the variance of the results. For this reason, we would rather use the FM for our discussion.

As expected, the control did not present any appreciable changes in the FM during the whole experiment. The healthy leaves that were only mechanically stressed presented a sudden rise in the FM, but after 40 days they did recover, and the resulting FM was approximately the same as in the control. This behavior can be easily understood, since the mechanical stress cannot alter the ChlF indefinitely in the leaf. In fact, we have noticed that the healthy leaves that were mechanically stressed only presented tissue necrosis on the wound site after 1 to 2 days without any visual signal of chlorophyll loss. The mechanically and diseased stressed leaves presented an increase of the FM faster than the diseased only leaves. This occurs because the mechanical stress facilitates the installation of the bacteria in the plant in the early stages of the infection process. The mechanically and diseased stressed leaves presented visual disease symptoms (tissue necrosis and chlorophyll loss) between 4 and 13 days after inoculation and the diseased only leaves between 13 and 30 days. At the end of the experiment, there were no major differences between these samples, because the disease stage was so advanced that the initial mechanical stress did not matter anymore for the leaf situation. We should point out that the overall increase in the FM for the diseased stressed leaves is consistent with chlorophyll loss [4].

There are also two of our observations that are in full agreement with previous reports in literature, which have used different techniques. The first one

![Fig. 1. Typical fluorescence spectrum of an orange leaf for excitation at 532 nm.](image)

![Fig. 2. (a) FR/FRF ratio and (b) FM as a function of time after inoculation for citrus plants (Citrus limonia [L.] Osbeck). Healthy (solid square) leaves without mechanical stress (control), (solid circle) health leaves with mechanical stress, (solid triangle) citrus canker infected leaves without mechanical stress, (solid inverted triangle) citrus canker infected leaves with mechanical stress.](image)
is that the mechanical stress presents a much faster response than the disease stress. To understand this we need to consider that mechanical and plant pathogen-induced diseases are both stress conditions to the plant, but there are important differences between these two processes [18]. On the one hand, most of the environmental factors, such as mechanical injuries, become stressful for a short time period, only minutes or hours [19,20]. On the other hand, disease processes induced by plant pathogens can take days or months to develop in the same plant organ or tissue.

Both mechanical and plant pathogen-induced stresses are recognized by affected plants [21], and there are two important parameters that determine whether stress processes will take place and how much they will develop [22]. One is the plant recognition time, which is the time necessary for the plant to recognize the presence of the stress factor, and the other is the defense plant response time, which is the time for the plant to mobilize its defense apparatus. Generally, strong stress processes are recognized faster by the plant [20]. That is the case for mechanical factors when compared with plant pathogen-induced diseases, which are less aggressive and take a longer time to affect, and be recognized by, the host [20,22,23]. These findings are consistent with our results.

Our second important observation is that the FM for citrus canker infected leaves with mechanical stress drops faster than the contaminated leaves without mechanical stress. We should point out that the citrus canker stress model used in this paper is formed by a plant pathogen that enters in its host (citrus plants) by natural openings (stomata) or open wounds [24,25]. As shown by Vernière et al. [26], the presence of any kind of wound on the aerial surface of the citrus tissues can exacerbate the citrus canker infection. Higher amounts of the bacteria in the host surface induce faster symptoms, and the presence of wounds on the citrus plants’ surfaces promotes the same effect, because higher amounts of bacterial cells can enter directly into the host tissues. Therefore, this is consistent with our observations. We should also point out that the discrimination using LIF between healthy leaves that were only mechanically stressed and diseased stressed leaves (without mechanical stress) was possible for almost the entire period of experimentation. Nevertheless, the discrimination between the mechanically stressed leaves (health and inoculated) was possible after only 20 days postinoculation.

5. Conclusion

In this paper we have shown that laser-induced fluorescence spectroscopy can be used for the detection of mechanical and disease stresses. We were able to detect both stresses and discriminate between them during most of the experimental period. To develop an all-optical technique for an accurate detection of citrus canker in the field, we must maximize our discrimination ability as shown in [17], and therefore, this technique should not be applied to leaves with recent mechanical stress. However, in principle LIF spectroscopy presents a potential use as a detection method for plant stresses in citrus canker infected trees in field conditions. The ability of LIF to discriminate different diseases in orange plants still remains to be determined; however experiments to test it are being carried out in the field at the present moment.

We acknowledge financial support from Fapesp (Fundação de Amparo à Pesquisa do Estado de São Paulo)—Programa (Center on Optics and Photonics) and Conselho Nacional de Pesquisas (CNPq) (Programa Rhae). We thank Eduardo Duarte Faria for technical help in this experiment.

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