DETECTION OF CITRUS CANKER IN ORANGE PLANTATION USING FLUORESCENCE SPECTROSCOPY

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ABSTRACT

Citrus canker is a serious disease, caused by *Xanthomonas axonopodis* pv. *Citri* bacteria, which infects orange trees (Citrus aurantium L.), leading to a large economic loss in the orange juice production. Brazil produces 50% of the industrialized orange juice in the world. Therefore, the early detection and control of such disease is important for Brazilian economy. However this task is very hard and so far it has been done by naked eye inspection of each tree. Our goal is to develop a new optical technique to detect citrus canker in orange trees using a field portable spectrometer unit. In this work, we will review three experiments on laser induced fluorescence spectroscopy (LIF) applied on citrus canker detection. Our results show that LIF has the potential to be applied on orange plantation with the purpose to detect citrus canker disease.

Keywords: Citrus Canker, fluorescence spectroscopy, diagnosis, laser.

INTRODUCTION

Presently, the interest in precision agriculture technologies has increased around the world, especially because such technologies have the potential to reduce the ecological impact of agriculture on the environment. In the last years, economical grow in developing countries have increased the demand for agriculture products; developed countries have increased the demand for biofuels as well. As a result, prices and demand for agriculture products have increased. Clearly, this whole situation demands new technologies and the use of precision agriculture.

However, prior to the application of new technologies, 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. They are very useful to detect the general characteristics of vegetation as well as color change [Penuelas & Filella, 1998], but they lack the specificity and the selectivity necessary to discriminate different

plant stresses. This happens because similar leaf pigment losses may be caused by different stress conditions [Valentini et al., 1994]. Furthermore, leaf color changes represent a late plant response to different stresses [Penuelas & Filella, 1998; Cerovic et al., 1999].

It is important to point out that 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 for precision agriculture.

Laser-induced fluorescence (LIF) of plants has been explored as a tool in vegetation studies in the last two decades [Buschmann & Lichtenthaler, 1998; Lichtenthaler & Miehe, 1997; Genty et al., 1989]. Due to 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 impacts of environmental plant stresses on several growth stages. For technical and safety reasons UV excitation has been preferred to visible excitation to monitor vegetation [Cerovic et al., 1999; Buschmann & Lichtenthaler, 1998]. 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 [Buschmann & Lichtenthaler, 1998]; and chlorophyll fluorescence (ChlF) in the red to near infrared region (650-800 nm) of the spectrum [Buschmann & Lichtenthaler, 1998]. The relative intensities of these two fluorescent bands obtained using UV excitation are highly sensitive to intrinsic leaf properties and environmental factors [Krause & Weis, 1991; Govindjee, 1995]. On the contrary, visible excitation induces mainly the chlorophyll fluorescence in 650-800 nm region of the spectrum.

The most important aspect of LIF is that it is a nondestructive and nonintrusive technique to the plant biochemistry, physiology and ecology. Besides, it is easy to use for many purposes in laboratory and fieldwork [Govindjee, 1995]. Studies, using chlorophyll fluorescence emission, have been successfully employed to detect mineral deficiencies, water and temperature stresses, and pathogens in plants [Buschmann & Lichtenthaler, 1998; Broglia, 1993].

Here we present a review on three experiments carried out in our laboratory which have applied LIF to citrus plants (*Citrus limonia* [L.] Osbeck). In the first experiment, we have worked with samples from commercial farms. Our goal was to determine if we were able to discriminate between health leaves and citrus canker contaminated leaves. In the second experiment, our goal was to discriminate between mechanical stress and citrus canker stress using laboratory samples. In the last experiment, our goal was to determine the detachment time effect on LIF when using field samples. In the sequence, we will present the materials and methods for each experiment, followed by the results and discussions. Finally, we will present our conclusions.

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MATERIALS AND METHODS

Fluorescence Spectroscopy System

Our fluorescence spectroscopy system is a portable unit (Spectr-Cluster, Cluster Ltd, Moscow, Russia) 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 periferical fibers; (iii) And an excitation source composed of a 10 mW diode laser at 532 nm (second harmonic of Nd:YAG).

In Fig. 1 we show a schematic diagram of the fluorescence spectroscopy system. It is important to point out that the backscattering signal (at the same wavelength as the excitation source) is about one thousand times more intense than the fluorescence signal. In order to observe the fluorescence signal, the system has an optical filter which reduces the backscattering signal one thousand times. In this way both signals, backscattering and fluorescence signals, present a comparable intensity. Using this system, we submitted the leaves to the fluorescence spectroscopy technique. The entire procedure was carried out under aseptic conditions. The measurements were carried out keeping the catheter probe at a distance of 2 mm from the leaf to prevent background noise and fluorescence limitations as atmospheric scattering, leaf geometry, low-power light capture, etc. This procedure also avoids any thermal effect. Nevertheless, several spectra at different laser power were taken to assured that there was not any intensity dependency.

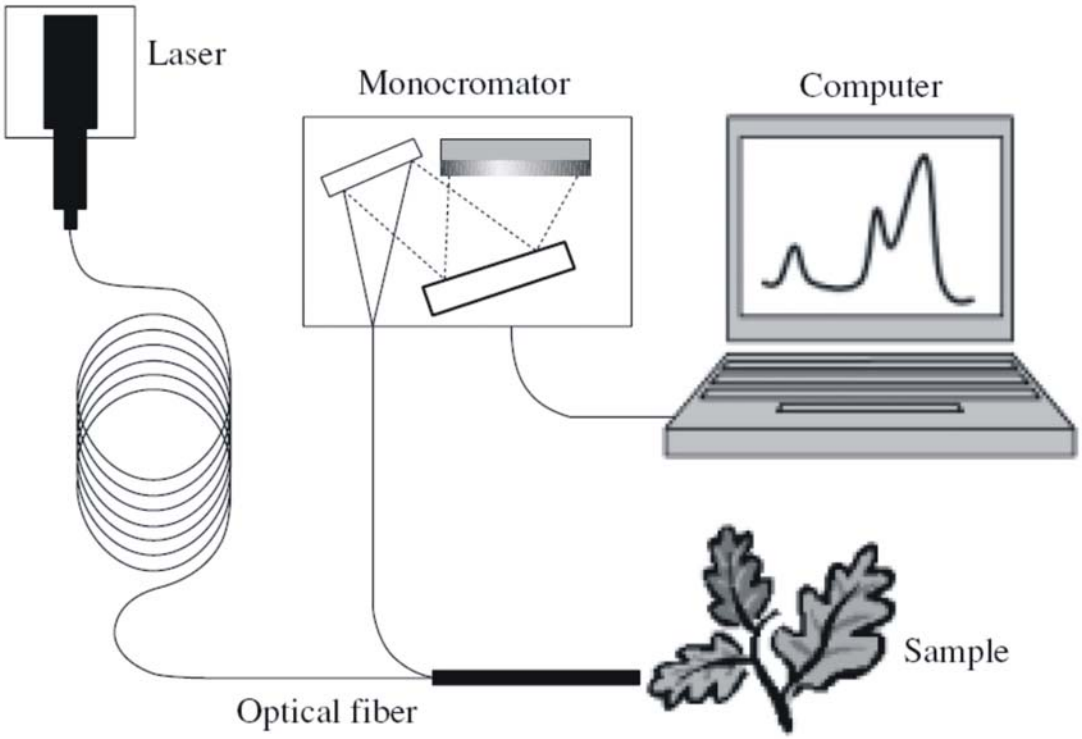

Fig. 1. Schematic diagram of the LIF system.

Fluorescence Spectrum Analysis

In Fig. 2 we show a typical fluorescence spectrum of a health leaf for excitation at 532. The fluorescence spectrum is due to the chlorophyll **a** emission [Cerovic et al, 1999]. Light in the green region excites the chlorophyll fluorescence directly while light in the UV-blue region excites the fluorescence of chlorophyll and other pigments. According to Cerovic et al. [Cerovic et al, 1999], chlorophyll fluorescence is an accurate and non-destructive 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 are three common ratios: i) Blue to red (BF/RF): defined by the ratio between the fluorescence intensity at 452nm and the fluorescence intensity at 685nm; ii) Blue to far-red (BF/FRF): It is defined by the ratio between the fluorescence intensity at 452nm and the fluorescence intensity at 735nm; iii) Red to far-red (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 532nm is limited to supply only the RF/FRF ratio [Lins et al, 2005].

However, as pointed out by Cerovic et al. [Cerovic et al, 1999], the chlorophyll fluorescence 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. In order to avoid this effect we have used a figure of merit (FM_1) approach, which we have defined as:

$$FM_1 = \frac{\int_{680}^{712} I(\lambda)d\lambda}{\int_{712}^{750} I(\lambda)d\lambda} \qquad (1)$$

where the FM_1 is the ratio of two integration of the spectrum I((λ) at different wavelength ranges (680-712nm by 712-750nm). In order to investigate the citrus canker we have also used another figure of merit (FM_2), which we have defined as:

$$FM_2 = \frac{\int_{680}^{800} I(\lambda)d\lambda}{\int_{547}^{620} I(\lambda)d\lambda} \qquad (2)$$

where the FM_2 is the ratio of two integration of the spectrum I((λ) at different wavelength ranges (680-800nm by 547-620nm). FM_1 correlates the peaks of chlorophyll fluorescence emission and FM_2 correlates all chlorophyll fluorescence emission with the leaf fluorescence emitted in the yellow-orange spectral region (non chlorophyll emission). We should point out that we have used such a procedure to detect citrus canker in a recent paper [Marcassa et al., 2006]. However, the definition of the figure of merit used here is an inverse order of our initial work [Marcassa et al., 2006]. This is due to the fact that definition of FM_1, used here, is more reliable for citrus canker detection than FM_2.

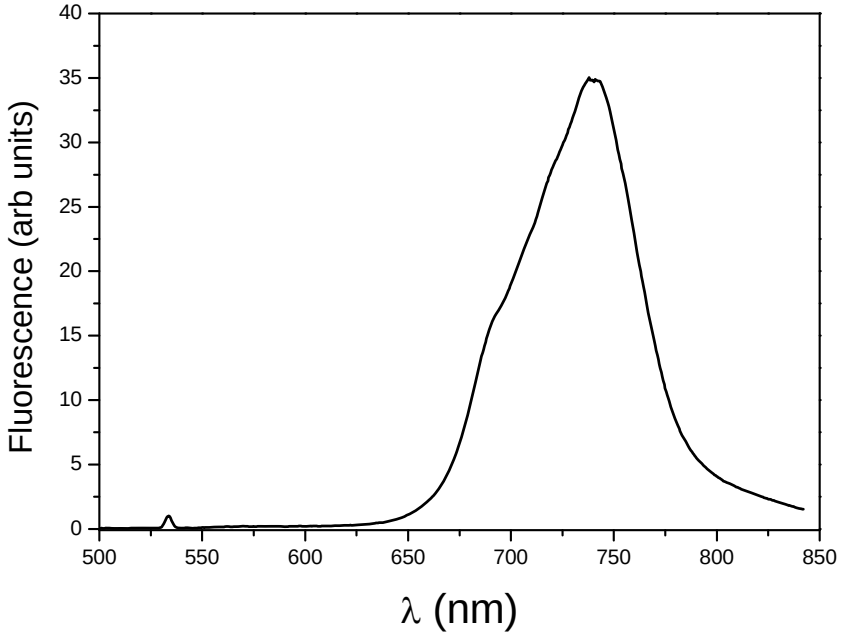

Fig. 2 – Typical fluorescence spectrum of orange leaf for excitation at 532nm

Experiment 1 – Detection of Citrus canker using field samples

About 500 orange leaves, presenting visual symptoms of citrus canker, were selected from several commercial farms around São Carlos city for this experiment. The control leaves (about 500) were collected from healthy plants in the same farms. For comparison, leaves contaminated with Citrus Variegates Chlorosis (caused by *Xylella fastidiosa* bacteria) were also collected [Vauterin et al.,1995; Simpson et al., 2000]. This is important because both diseases present very similar visual symptoms. All samples were brought to the laboratory to avoid the influence of environmental factors on the measurements. Nevertheless, in order to discriminate between these two diseases, traditional laboratorial tests were carried out after the fluorescence measurement.

Experiment 2 –Detection of Mechanical and citrus canker stresses

Greenhouse plants of Citrus limonia ([L.] Osbeck), maintained in plastic pots, were pruned approximately fifty days before inoculation to obtain homogenized and incompletely immature (three-fourths to full expansion) leaves [Graham & Leite Jr., 2004]. Inoculum of an aggressive bacterial strain of Xanthomonas axonopodis pv. citri was prepared by suspending the bacteria harvested from 72-hours-old nutrient agar (NA) cultures in phosphate buffer (PB). The suspension was adjusted spectrophotometrically to 10^8 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; 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 cross 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 to provide better conditions for infection.

Evaluations were made on the day of mechanical and disease stress inducement procedure 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 about 3 mm from the midrib, and for the healthy plants only mechanically stressed the catheter probe was placed on the side of the needle-induced injury. For 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 control group showed no changes.

Experiment 3: Effect of leaf detachment time

Forty health orange leaves were selected from a commercial farm near São Carlos city and brought to the laboratory for the fluorescence measurement. During the whole experiment, they were stored in the dark at 24 ºC under 100% relative humidity. The detachment time was varied from 0 to 12 hours in one hour steps. The laser probe was placed about 3 mm from the midrib leaf and always on the same spot of each leaf during the whole experiment.

RESULTS AND DISCUSSION

Initially, we present the results on the detection of citrus canker using field samples. The Fig. 3a shows a graph of FM_2 as a function of FM_1, for healthy (open circles) and citrus canker contaminated leaves (open triangles) respectively. It is possible to observe clearly that the contaminated leaves are concentrated in a region of high FM_1 and low FM_2. In fact, most of the contaminated leaves are in the region where $FM_1 > 0.8$ and $FM_2 < 100$. On the other hand, the healthy leaves are dispersed in a broader range. We believe that this dispersion may reflect the fact that the samples were collected in several different fields, which may have different conditions of water and nutrient supply.

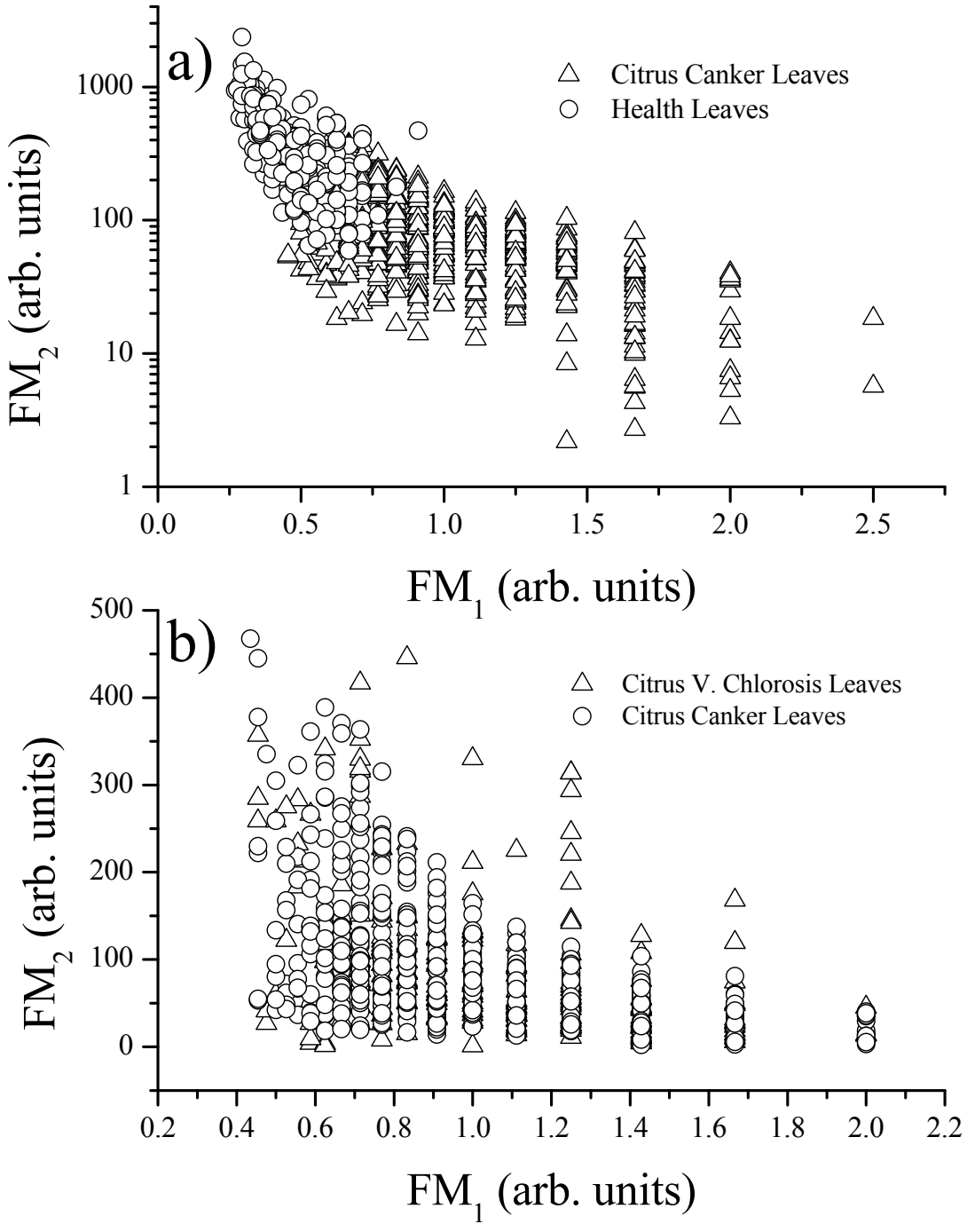

Fig. 3. a) FM_2 as a function of FM_1 for healthy leaves (open circles) and citrus canker contaminated leaves (open triangles). b) FM_2 as a function of FM_1 for citrus canker contaminated leaves (open circles) and citrus variegates chlorosis (open triangles).

We also compared the results obtained for citrus canker contaminated leaves (open triangles) with citrus variegates chlorosis contaminated leaves (open circles) in Fig. 3b. The results show that both diseases present very similar visual symptoms and fluorescence spectrum characteristics as well. Therefore, because the points are very much overlapped, it is not possible to observe a clear discrimination between the two diseases. This may indicate that both diseases produce similar pigment losses, which modifies the chlorophyll fluorescence in the same way. This is an issue that must be investigated in future experiments.

In the second experiment, we investigate the detection of mechanical and citrus canker stresses on leaves using LIF. In Fig. 4, we present FM_1 as a function of time after inoculation. Each point is an average over all leaves and plants (a total of 90 spectra) and the error bars is the variance of all measurements. As expected, the control did not present any appreciable changes in FM_1 during the whole experiment. The healthy leaves only mechanically stressed present a sudden rise in the FM_1, but after 40 days they did recover and the resulting FM_1 was about the same as the control. This behavior can be easily understood, since the mechanical stress cannot alter the chlorophyll fluorescence indefinitely in the leaf. In fact, we have noticed that the healthy leaves mechanically stressed only presented tissue necrosis on the wound site after 1-2 days without any visual signal of chlorophyll loss. The mechanically and diseased-stressed leaves present an increase of FM_1 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 any more for the leaf situation. We should point out that the overall increase in FM_1 for the diseased stressed leaves is consistent with chlorophyll loss [Cerovic et al., 1999].

There are two of our observations accordingly with previous reports in the literature. The first one is that the mechanical stress presents a much faster response than the disease stress. It occurs for the reason that mechanical and plant pathogen-induced disease are both stress conditions to the plant, but there are important differences between these two processes [Hildmann et al., 1992]. While mechanical injuries become stressful for a short time period, minutes or hours only, the disease processes induced by plant pathogens can take days or months to develop in the same plant organ or tissue [Taiz & Zeiger, 2002; Wick et al., 2003].

Nevertheless, two other parameters interfere in the development of the stress; one is the time necessary for the plant to recognize the presence of the stress factor; and the other is the time for the plant to mobilize its defense apparatus. Generally, strong stress processes are recognized faster by the plant [Wick et al., 2003]. That is the case for mechanical factors when compared with plant pathogen-induced diseases, which are less aggressive and take longer time to affect, and be recognized, by the host [Wick et al., 2003; Agrios, 1997; Goodman et al., 1986]. These finding are consistent with our results.

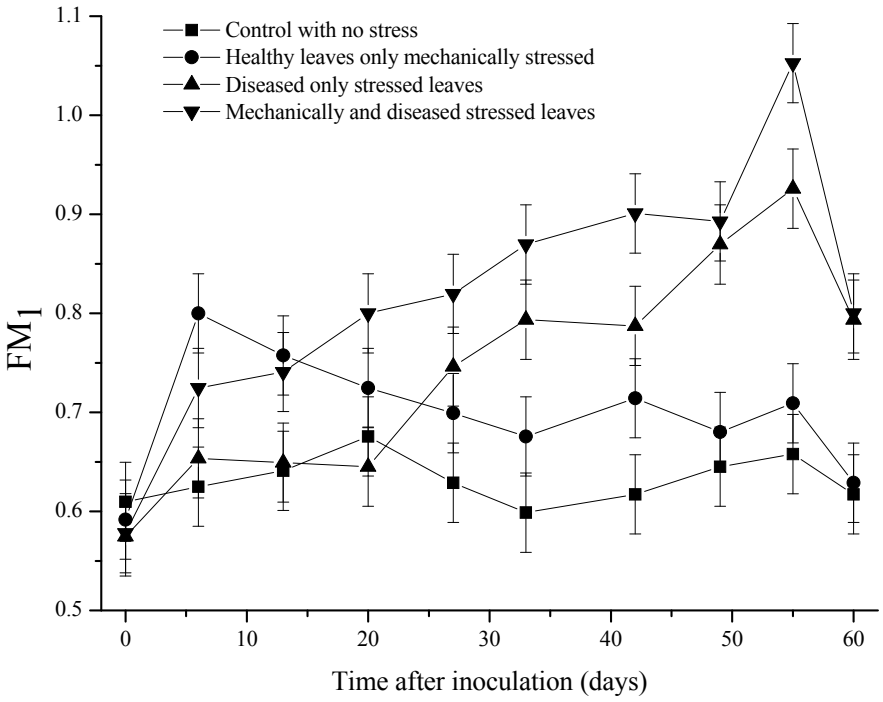

Fig..4. FM_1 as a function of time after inoculation: Health leaves without mechanical stress (control) (■); Health leaves with mechanical stress (●); Citrus canker infected leaves without mechanical stress (▲); Citrus canker infected leaves with mechanical stress (▼).

Our second important observation is that the FM_1 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 present work is formed by a plant pathogen that enters in its host (citrus plants) by natural openings (stomata) or open wounds [Goto, 1990; Gottwald & Graham, 1990]. As showed before by Vernière and co-workers [Vernière et al., 2003], 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 in the host tissues. Therefore, this is consistent with our observations. We should point out also that the discrimination using LIF between healthy leaves only mechanically stressed and diseased stressed leaves (without mechanical stress) was possible for almost the entirely period of experimentation. Nevertheless, the discrimination between the mechanically stressed leaves (health and inoculated) was possible only after 20 days post inoculation.

The two experiments, discussed so far, allow us to conclude that the use of laboratory plants leads to a better citrus canker detection than using the samples from the commercial farms. There are two major differences between these two samples. First, the farm samples present a larger dispersion on the conditions of

water and nutrient supply than the laboratory plants. Second, the farm samples were detached from the plant and the LIF measurement was at different detachment times, in fact this parameter varied from 3 up to 12 hours, depending on the distance between the farms and the laboratory. And it is not clear how this parameter would affect our previous results; however it is expected some effect since there will be a biotic stress for the leaf.

Then, the third experiment was proposed to respond this question and its results are presented in Fig. 5. There, the behavior of the figures of merit FM_1 and FM_2 are presented as a function of detachment time. Each point is an average over all leaves, and the error bars are the variance of all measurements.

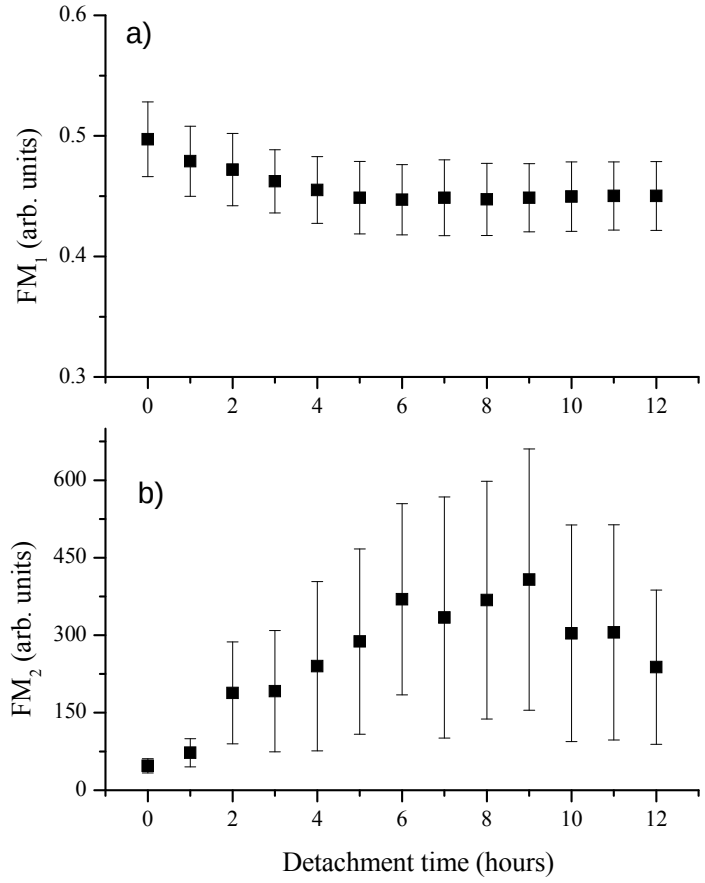

Fig. 5. a) FM_1 and b) FM_2 parameters as a function of detachment time, respectively.

The FM_1 parameter (fig. 5a) presents a small and well behaved time dependence, varying only about 10% from t=0 to t=4 hours, and then it stabilizes. On the other hand, the FM_2 parameter (fig.5b) presents a very strong dependence on the detachment time. It basically starts at FM_2 (t=0 hours) =50 and then increased almost linear up to 9 hours, reaching a maximum (almost FM_2 (t=9 hours) ≈ 400). Beyond this point, it starts to decrease again. This means that in a

couple of hours such parameter can change almost one order of magnitude. We should point out that the data also presents a large dispersion.

For sure the observed results are related with chemical and biological changes after the detachment of the leaves. There are several processes in the leaf that could account for such changes. It is well known that the detachment effect on plant leaves depends on the plant species. However, in general it is observed a decrease of chlorophyll, RNA, protein, sugar, and water contents. Besides, there is also an ion unbalance in the leaf. All these factors result on the reduction of the photosystem II activity in the photosynthesis [Lawlor, 2001]. These effects are clearly a senescence process pattern accelerated by the leaf detachment and by the storage in the dark. We should point out that in our previous work the leaves were kept in the dark as well after collection at the fields [Marcassa et al., 2006].

The acceleration of the senescence process due to leaf detachment and storage in the dark is almost immediate, as observed by Wardley et al. [Wardley et al., 1984]. One could expect that such effect would be due to the degradation of the ratio chloroplasts per plant cell. But that is not the case in the beginning of the senescence process. In fact, the constituents of the chloroplasts, cytoplasm and contents of organelles, are degraded in the early stage of the senescence process. Of course, as the process evolves the ratio chloroplasts/plant cell also declines [Wardley et al., 1984]. All these effects are almost immediate; however normal visual symptoms of accelerated senescence, as curling and loss of turgor, are usually only observed beyond few hours after detachment. However, their appearance may depend on several different leaf parameters.

We believe that our measurements are detecting some of these processes. However, our goal in this experiment is not to identify which process is more important for the observed results. But it is to discuss the implications on the first experiment. In that experiment, we have observed that health leaves presented a FM_2 parameter varying from 100 up to 1600; and FM_1 parameter varying from 0.2 up to 0.7 respectively. Since those measurements were performed at several different detachment time (varying from 0 to 12 hours), we can conclude that the dispersion observed in FM_2 in those results could be due, at least partially, to the detachment time effect. On the other hand, because FM_1 presents weaker time dependence, we believe that its dispersion could be related to different conditions of water and nutrients supply of the leaves. However, this remains to be investigated.

CONCLUSION

To summarize, in this work we have applied fluorescence spectroscopy on orange leaves in order to develop an optical detection technique for citrus canker diagnosis. The results from the first experiment have shown that LIF technique is able to discriminate between healthy leaves and citrus canker contaminated leaves. However, it is unable to distinguish between the Citrus Canker and the Citrus Variegates Chlorosis. One possible explanation for such observation is that this is due to the fact that both diseases cause a similar loss of pigments in the leaves. However, more experiments are required on this issue.

In the second experiment, we were able to detect stresses, the mechanical and the citrus canker disease. It was also possible to discriminate between them during most of the experimentation period. We can also conclude that in order to develop an all-optical technique for an accurate detection of citrus canker in the field, we must maximize our discrimination ability, and therefore, this technique should not be applied to leaves with recent mechanical stress.

For the last experiment, we can conclude that if we want to avoid dispersions due to detachment time effect in the application of LIF for the detection of diseases in citrus leaves, we should perform the measurements onsite. We also concluded that the detachment time effect may be responsible for the dispersion of the first experiment results.

The application of LIF spectroscopy technique on citrus canker detection still present some limitations, which must be overcome before it can become practical. Nevertheless, it potential is vast, and requires further investigation, especially on field conditions.

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