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Research Paper

Laser-induced fluorescence spectroscopy applied to early diagnosis of citrus Huanglongbing

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Over the last few years, citrus production has been threatened by diseases and plagues, especially Huanglongbing (HLB). Due to the long asymptomatic period, the most important and efficient tool for HLB management is early detection, which enables fast decisions to protect the farm. In this sense, a new methodology using a portable laser-induced fluorescence spectroscopy (LIFS) system and statistical tools was developed. It is capable of identifying not only symptomatic HLB leaves in the field, but also asymptomatic HLB trees and symptomatic citrus variegated chlorosis (CVC) trees. The differentiation reaches accuracy better than 90% and provides the ability of detecting an asymptomatic diseased tree 21 months before the symptoms appear, results supported by quantitative polymerase chain reaction (qPCR) analysis.

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Nomenclature

HLB	Huanglongbing
CVC	citrus variegated chlorosis
qPCR	quantitative polymerase chain reaction
FTIR	Fourier Transform Infrared Spectroscopy
LIFS	laser-induced fluorescence spectroscopy
LIFS-405	laser-induced fluorescence spectroscopy system with 405 nm excitation laser
PLSR	Partial Least Square Regression
CVR	Classification via Regression
RMSE	root mean squared error
ChlF	chlorophyll fluorescence
nm	nanometres
mW	milliwatt
mL	millilitre
Ppb	parts per billion
Ppm	parts per million
mg	milligram
mm	millimetre

1. Introduction

Brazil is the biggest orange producer in the world. The orange commercial production accounted 37% of global production in the 2011/2012 season (Ntombela & Moobi, 2013) followed by the United States with 16% of global production in the 2011/2012 season (Ntombela & Moobi, 2013). USA production has been rapidly decreasing in recent years mainly due to Huanglongbing (HLB) (Citrus: World Markets and Trade, 2014). Citrus diseases, such as Huanglongbing (HLB) or citrus greening, and citrus variegated chlorosis (CVC) have been threatening the citrus production worldwide (Bassanezi & Bassanezi, 2008; Bassanezi, Bergamin Filho, Amorim, & Gottwald, 2006). HLB is considered one of the most destructive citrus diseases over the last years (Aubert, 1992; Bové, 2006), due to its rapid spreading rate and the lack of an efficient means of control. Besides, the long latency period, between 6 months and 3 years (Gottwald, 2010), reinforces the severity of this disease. HLB is so aggressive that it can completely destroy a crop within 5 years after the appearance of the first symptoms (Chiyaka, Singer, Halbert, Morris, & van Bruggen, 2012; Gottwald, 2010).

The main symptom of HLB-infected citrus trees is the appearance of branches with yellow veins on the leaves (Chiyaka et al., 2012; Gottwald, 2010). The symptoms of HLB also appear in fruits, which have a salty bitter flavour and become small, misshapen, and discoloured with green areas (Chiyaka et al., 2012; Gottwald, 2010). To control the disease, Brazilian producers have adopted the following strategies: acquisition and cultivation of healthy seedlings, periodic insecticide spraying to reduce psyllids' population (the vector insect) and periodic visual inspection to remove symptomatic trees (Gottwald, 2010). Despite the rigorous control adopted to prevent HLB advance, the disease is spreading, and the main reason may be attributed to the fact that visual inspection is highly susceptible to human error. Moreover, a huge number of asymptomatic infected trees are being left in the orchards after visual inspection due to its long latency period (Bové, 2006; Gottwald, 2010).

The quantitative polymerase chain reaction (qPCR) technique requires hard sample preparation that involves DNA extraction and storage to DNA replication. These results are prone to errors in one of these steps, which are intrinsic flaws of this complex technique.

The causal agent of HLB is *Candidatus* Liberibacter spp. (Chiyaka et al., 2012; Gottwald, 2010; Kunta, Graça, Malik, Louzada, & Sétamou, 2014). Currently, HLB infected plants, in asymptomatic or symptomatic stage, can only be accurately detected by qPCR. This method consists of extracting bacteria's DNA from leaf samples and amplifying it, as was done by Pereira et al. (2010), i.e., the qPCR technique requires hard sample preparation. These results are prone to errors in one of the steps, DNA extraction and storage to DNA replication, which are intrinsic flaws of this complex technique. Due to the irregular bacterial distribution in the infected trees (Do Carmo Teixeira et al., 2005; Li, Levy, & Hartung, 2009; Tatineni et al., 2008), the test may result in a false negative if the bacterial concentration of the analysed sample is below the qPCR detection threshold. Furthermore, the application of this technique is impractical for large scale analyses, because it is an expensive and time-consuming procedure (Gottwald, 2010).

In order to overcome the qPCR and visual inspection drawbacks, alternative methods have been proposed. Fourier Transform Infrared Spectroscopy (FTIR) has been successfully used to distinguish healthy from various common citrus plant maladies, including HLB in asymptomatic diseased plants (Cardinali et al., 2012; Hawkins et al., 2010). Laser-induced breakdown spectroscopy has also shown great potential in classifying plant leaves to an accuracy greater than 90%, within 95% significance level (Pereira et al., 2010). Both methods, FTIR and laser-induced breakdown spectroscopy (LIBS), were evaluated with samples from a greenhouse, grown under controlled conditions. Sankaran and Ehsani (2013) applied another spectroscopy technique known as chlorophyll fluorescence (Maxwell & Johnson, 2000) that is widely used by researchers as a sensing method for early detection of diseases in crops. In this work, a commercial hand held fluorescence sensor was used to measure citrus leaves' fluorescence to diagnose HLB in both phases, asymptomatic and symptomatic. For this purpose, they used four different excitation frequencies to excite and three different fluorescence emission bands from the leaves. The samples were leaves collected from 8 different cultivars in 3 classes: HLB-symptomatic (qPCR positive), HLB-asymptomatic (qPCR positive, collected from trees that have not shown the symptom yet) and healthy (qPCR negative including leaves collected from diseased tree, but which tested negative for the bacteria in the qPCR test). The best success rate among a set of healthy and symptomatic leaves using the Bagging Decision Tree classifier was 97%. By including a set of asymptomatic samples, the accuracy decreases to 46% for the best classification (Bagging Decision Tree) and 25% for the worst (Naive Bayes).

Despite the large number of published studies using fluorescence sensor to control the spread of HLB, none was efficient in detecting asymptomatic diseased leaves. In the

present work, we describe a method that can successfully identify not only symptomatic leaves, but also asymptomatic diseased leaves collected from field trees. To this end, it was assumed that once infected, the metabolism, and consequently the chemical composition, of the tree alters and a asymptomatic HLB leaf may be detected with the proposed technique, even when the HLB bacteria's DNA is not detectable by the qPCR technique. The proposed method is based on an association of laser-induced fluorescence spectroscopy (LIFS) and chemometrics tools to evaluate changes in chemical compounds of leaves due to infection. The low cost of measurement, the possibility of a fast diagnosis, lack of sample preparation and high portability make this technique a good candidate to be applied as field devices. First, HLB symptomatic, HLB asymptomatic, CVC and healthy leaves were used to evaluate the classification ability of the proposed method. Secondly, to better understand the spectral differences between healthy and diseased leaves, we also evaluated the fluorescent spectral profile of some secondary metabolites, whose concentration may change during the process of infection by the pathogen. Finally, early detection of HLB was investigated by fluorescence sensing. During this study, 40 trees were monitored over two years to evaluate the diagnosis ability in asymptomatic diseased trees in the field, all of them certified with the qPCR test. It was shown that the leaf fluorescence emission was strongly affected by water stress. Thus two classifiers for diagnosis purpose were built: one for rainy and another for the dry season.

2. Materials and methods

2.1. *Leaf samples*

Only fresh citrus leaves were used in this study. Firstly, sample analyses with the LIFS system were used to verify the ability to distinguish between healthy, HLB and CVC infected leaves. These samples comprised 160 leaves collected in the field from Citrus sinensis (L.) Osbeck trees (Valencia/Swingle) divided into 4 classes: healthy, HLB asymptomatic, HLB symptomatic and CVC symptomatic leaves. The diseased samples (i.e. HLB symptomatic, HLB asymptomatic and CVC symptomatic) were taken from trees that had already shown the characteristic symptoms of the mentioned diseases. In the specific case of CVC, some symptomatic leaves had already presented the characteristics lesions but the fluorescence evaluation was not performed on it. The healthy leaves were taken from plants located in orchards with low incidence of diseases.

Secondly, in order to verify the possibility of early diagnosis of HLB, citrus trees were monitored by fluorescence sensing over a period of two years. To this end, five infected trees were eradicated from the orchard and the study was conducted considering the eight trees located around the HLB-infected tree, as shown in Fig. 1. All 40 trees selected were considered healthy at the beginning of the study, and none presented any HLB symptoms; besides they were located in a high infestation orchard and had high probability of being contaminated with the bacterium. The group of 40 trees arranged around the diseased one are referred to as the "border trees" set. Monthly, five leaves from each tree were collected and analysed over a period of two years. All leaves of the border trees were certified using qPCR analysis, following the same steps described by Pereira et al. (2010). These tests were performed at Citrus Biotechnology Laboratory at Centro APTA Citros Sylvio Moreira, after LIFS measurements.

Finally, for the development of the calibration model used to evaluate the border trees, the spectral information of three different classes of leaves was collected and measured during the same period of the border tree study. The classes are healthy, HLB asymptomatic and HLB symptomatic. All leaves belong to the same combination of canopy and rootstock cultivar as the border trees. The diseased leaves were taken from trees that already shown the characteristic visual symptoms of HLB and HLB asymptomatic leaves were collected from the same tree but from branches that did not present the visual symptoms of HLB. The healthy leaves were taken from plants with no symptoms located in orchards with low incidence of diseased trees. This leaves are referred to as "calibration set", since their classes are already attested by visual inspection. To validate the calibration model, a new group of leaves was used. Throughout 2013, leaves of the same three classes were collected monthly and measured. Hence, the classification model was constructed over 24 months of calibration set and validated with the samples collected during the third year of study.

All leaves evaluated in this study were collected from a plantation in the region of Araraquara, in the countryside of São Paulo state, Brazil. Parameters, such as weather, soil conditions, watering and fertilisation were adequate and favourable to growing the plants and the same for each tree, which was attested by Fisher Group (Citrosuco) who provided the plant material grown at Citrícola Farm. Before measurement, each leaf was cleaned with a piece of cotton wetted with distilled water and dried with dry cotton wool in order to remove any soil or dirt that could affect the LIFS spectra. After being cleaned, the leaves were kept in bags and refrigerated at 4 °C to prevent degradation. All measurements were performed with leaves *in natura* without any sample preparation. All measurements were performed in the laboratory.

2.2. *Laser-induced fluorescence spectroscopy apparatus*

For this study, a homemade portable LIFS system was used (Milori, Neto, Ferreira, Zaghi, & Venâncio, 2010). LIFS system was composed of a diode laser (Coherent – CUBE) emitting at 405 nm as excitation source, operating on continuous mode and at room temperature (23 °C). The laser output power at the optical probe exit was 35 mW. The optical probe is a bifurcated optical fibre bundle together, manufactured by Ocean Optics. It had six illumination optical fibres around one reader fibre. To attenuate the reflection of the light from the sample, a linear variable filter (LVF series from Ocean Optics) was used. It operates like a high pass filter, blocking the reflected light (~98.8%) at 405 nm and allowing the transmission (~90%) for longer wavelengths. A high sensitivity mini-spectrometer (USB2000+UV–VIS – Ocean Optics) with spectral range from 200 to 900 nm and optical resolution of 1.5 nm full width at half maximum (FWHM) was used to detect the emission fluorescence by the leaf. A dedicated notebook computer controls the

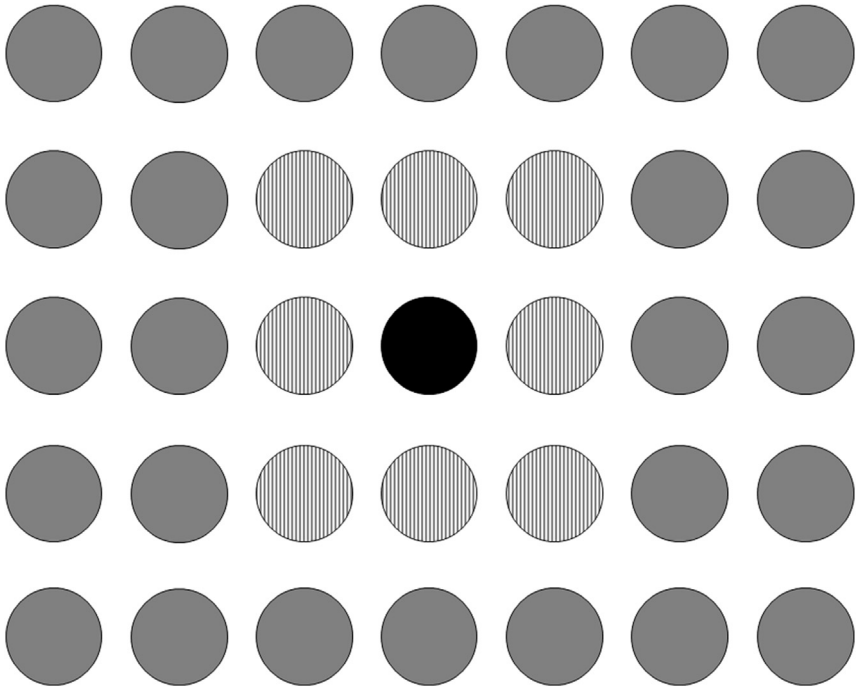

Fig. 1 – The arrangement of eight border trees (vertical pattern) around an eliminated diseased tree (black circle), located in the field. The other plants are represented by the green circles (grey circle).

acquisition spectra. An electromechanical optical shutter was used to control exposure time of the sample to the laser light. This ensures that the measurement would be carried out in a steady-state level of fluorescence, avoiding the Kautsky effect influence. The shutter was also controlled by the computer during the acquisition process. For each measurement, the CCD sensor was adjusted for an integration time of 60 ms and 20 scans per sample. Software was developed to control the measurement process, to perform the pre-treatment of signal and to analyse the spectra. For simplicity, this system will be referred to as laser-induced fluorescence spectroscopy system with 405 nm excitation laser (LIFS-405) and a schematic diagram of the experimental set-up is shown in Fig. 2.

All measurements with the LIFS-405 system were performed on the abaxial leaf surface, on the right side of the midrib next to the petiole. This choice were made due to the presence of a cuticle layer on the adaxial side that acts as protection of the leaf against UV radiation (Yeats & Rose, 2013), a region close to the excitation light used in LIFS-405. Thus, the measurements protocol adopted in this work positioned the optical probe on the abaxial surface, always in the same region in order to standardise the analyses. Only when there was a lesion on the measurements region of the leaf, as a symptom of an advanced stage of CVC disease, was the protocol to deviate the probe to a region next to the lesion and never to measure it.

2.3. *Secondary metabolites analysis: 3D fluorescence*

In order to understand the leaves' fluorescence spectra, 3 metabolites: coumarin umbelliferone, flavonoid hesperidin, and flavonoid naringin were analysed. Fluorescence measurements were taken on a Perkin-Elmer LS 50B luminescence spectrophotometer and processed using Origin Pro software version 9.0. Three-dimensional spectra were acquired in increments of 20 nm excitation wavelengths, from 200 to 500 nm, while the scanning emission spectra range was from 300 to 900 nm. This procedure generated 50 scans for 3D processing.

In order to compare and analyse the spectral profile and peak relationship, the spectral baseline was corrected by subtracting the lowest intensity, and the resulting spectrum was normalised by area. All treatments were performed using free Scilab 5.3 software.

The metabolites were prepared in aqueous solutions. Water was chosen as solvent because it does not emit fluorescence or change the emission properties of the secondary metabolites. Moreover, water is also found in leaves. The solutions were prepared at different concentrations according to the maximum dilution of each compound in deionised water and below the saturation limit of the detector. For the coumarin umbelliferone, 1 mg was diluted into a 500 mL volumetric flask and it was necessary to use an ultrasound bath to achieve dissolution. Then, through a pipette, from the prior solution, other dilutions were made in order to obtain a final solution of 10 ppb. For flavonoids, 4 mg and 2 mg of hesperidin and naringin, respectively, were diluted in 100 mL volumetric flasks to obtain 40 ppm and 20 ppm solutions, respectively. The prepared solutions were kept isolated from electromagnetic radiation to avoid any degradation of the material.

2.4. *Spectral treatment and classification method*

Initially, the pre-processing spectral treatment was conducted as described below. First, the average of 20 scans per leaf was

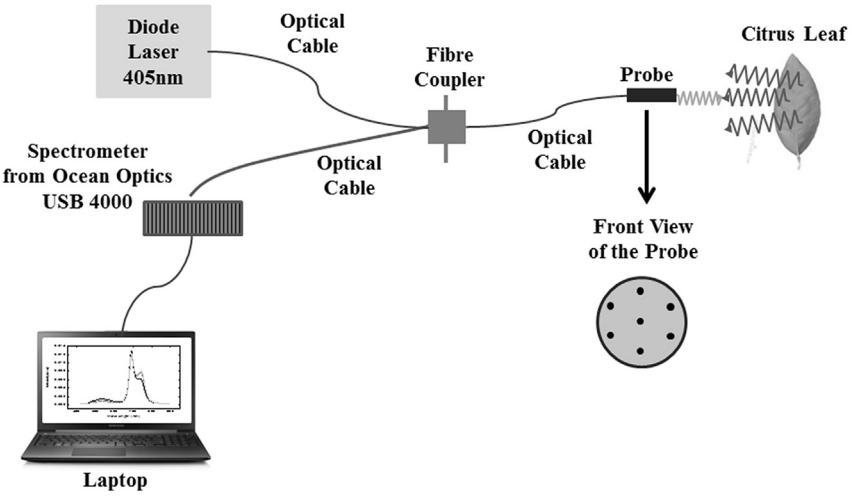

Fig. 2 – Experimental set-up of LIFS-405 developed by the Optic and Laser laboratory of Embrapa Instrumentation. This system is composed of a diode laser emitting at 405 nm, an optical shutter, and a tight bundle of 7 optical fibres to conduct the excitation light to the leaf. A high sensitivity spectrometer (USB4000-Ocean Optics) and a linear silicon charge coupled device (CCD) array simultaneously detected the light emitted by the leaf. A dedicated notebook computer controls the acquisition process and analyses the spectra.

evaluated. All the fluorescence leaf spectra gathered with the LIFS-405 had the spectral baseline corrected to remove optical and electronical offset, and then normalised by the area under the curve in order to emphasise only spectral profile differences and enable the comparison between the spectra.

Secondly, 1593 relative intensities values data of the fluorescence emission spectra, one for each emitted wavelength, were used as input attributes for training and validating the classifiers. These data were exported to WEKA software (Hall et al., 2009) for a classifier construction combining the Classification via Regression (CVR) and the Partial Least Square Regression (PLSR), as described by Frank, Wang, Inglis, Holmes, and Witten (1998) and used by Cardinali et al. (2012). PLSR is a method widely used in chemometrics (Wold, Sjöström, & Eriksson, 2001) for evaluating the concentration of chemical compounds in samples. This kind of regression is characterised by finding a linear transformation in the predictor variables, i.e. the spectral points, that provides the best correlation with the response variables, as described by Wold et al. (2001).

The generated classifier associates the nominal classes (healthy, CVC, HLB symptomatic and HLB asymptomatic), with numbers. This association is done through a binarization process, in which for the reference class the value attributed is 1 and for the other classes the value attributed is 0 (Frank et al., 1998). To start the process, it is necessary to split the entire leaf spectra set of data into calibrating (or training) set, to adjust the regression model, and test set, to validate the generated model. The main purpose of PLSR is to construct a linear model for each element of the test, as mentioned, and the model returns adjusted values between 0 and 1. The higher the value is to one, the greater is the similarity between the test spectrum and the reference class. The same procedure is repeated for all classes using the same calibrating and testing set. For each sample tested, it is possible to evaluate the probability prediction for each class, which is proportional to the value returned by the regression models.

In order to validate the classification model generated, the cross-validation method was performed (Wold et al., 2001). This method separates the data set into n folders or groups, of which n-1 are used for training the classifier and one is used for testing. Then, the folder that was separated for the test is returned back into the dataset and another folder is withdrawn to carry out the same procedure. This iteration process was repeated until all groups have been tested. For further generalisation of the model, it is possible to repeat the cross-validation procedure by randomising the samples of each group. Each cross-validation procedure leads to different results, and its employment ensures that the accuracy would not be based due on a particular partition of training and test sets. Thus, performing k executions of cross validation, at the end of the process, it is possible to obtain kn results.

The classification of the spectra set was evaluated by 10 executions of 10-fold stratified cross-validation. The same procedure was repeated by varying the number of PLS components while maintaining the same sub-sets of each cross-validation run for a comparison of accuracy. All the best components numbers were compared by the Student t-test with statistical confidence interval of 95% according to the success rate and the root mean squared error (RMSE). The success rate was obtained by the ratio of the number of correctly classified samples and the total number of samples. Then, the component number was chosen based on the highest success rate, the lowest RMSE and the fewest components possible with a significant statistical difference.

Finally, because of the classification model, it was possible to mount a confusion matrix. The columns of the confusion matrix show how the leaves were classified and the rows show the nominal class. Thus, the main diagonal shows the correctness of the model. The values represented in the confusion matrix correspond to the rights and wrongs obtained in the classification of the input class leaf.

3. Results and discussion

The results are presented in three subsections: (i) Classifier performance evaluation among the difference of the four classes: healthy, HLB-asymptomatic, HLB-symptomatic, and CVC-symptomatic; (ii) comparison between LIFS spectra of leaves and secondary metabolites; and (iii) seasonal classifiers evaluation for monitoring the border trees over two years.

3.1. *Classifier induced with Partial Least Square Regression*

Any chemical change in citrus plants caused by a stress can be identified by leaf fluorescence; these fluorescence data were used as input attributes to construct the induced classifiers, with the aim of better characterising the classes of leaves. This evaluation was performed by several runs of cross-validation to determine the optimum number of components responsible for the best classification results. The highest success rates in correctly classify the leaves classes were achieved using 13–20 components as shown on Fig. 3. Within this optimal components number interval, no statistical significant difference was observed according to the Student's t-test with statistical confidence interval of 95%. Therefore, 13 components were chosen based on the lowest classification error and the fewest components possible.

Table 1 shows the confusion matrix obtained by the constructed classifier, combining CVR and PLSR, using 10 executions of 10-fold stratified cross-validation with 13 components to correctly classify the 4 nominal classes: healthy, CVC symptomatic, HLB asymptomatic, and HLB symptomatic. The confusion matrix had an average success rate of 90%, with the healthy class showing the highest success rate. As expected due to the similarities, some confusion was observed between healthy class and HLB asymptomatic, totalling 9% of misclassified samples in these classes. Surprisingly, no confusion was observed between HLB symptomatic and CVC symptomatic with healthy leaves; the technique does not misclassify an infected plant as healthy. In addition, the confusion between the CVC and HLB leaves was less than 18%, corroborating the potential of the technique to distinguish diseases as well. The confusion between HLB symptomatic and HLB asymptomatic may not be considered an error, because both means HLB positive but only differ by advance stage of the disease in the plant. As shown in Table 1, the classifier identified HLB symptomatic and HLB asymptomatic with success rates of 95% and 87.25%, respectively.

3.2. *Laser-induced fluorescence spectroscopy system with 405 nm excitation laser spectral differences*

The typical spectrum for each class obtained with the LIFS-405 system is shown in Fig. 4. According to this figure, two distinct fluorescence bands are observed: a blue–green emission, between 400 and 600 nm, and a red to near infrared emission, between 650 and 800 nm. Both emission bands play an important role in the construction of the induced classifier presented in the previous subsection. The blue–green emission band is strongly associated with the presence of secondary metabolites such as ferulic acid, flavonoids, coumarin and quercetin. These metabolites are usually associated with the defence mechanism of citrus plants. The second emission band is the characteristic emission of chlorophyll fluorescence (ChlF) (Buschmann, 2007; Lichtenthaler, 1990; Lichtenthaler & Miehe, 1997; Lichtenthaler & Rinderle, 1988; Papageorgiou, 1975).

Aiming at understanding the blue–green fluorescence on the LIFS-405 spectrum, a study was conducted in collaboration with the Natural Products Laboratory of Federal University of São Carlos. In this study, the fluorescence emissions of 3 common secondary metabolites found in citrus leaves were evaluated. These three metabolites (umbelliferone (coumarim), hesperidin (flavonoid) and naringin (flavonoid)) are usually involved in the process of the plant's reaction to disease (Kawaii et al., 2000; Stanley & Jurd, 1971).

A 3D fluorescence emission of umbelliferone, hesperidin and naringin in aqueous solution is shown in Fig. 5(a–c), respectively. The black lines represent the contours and the colours represent the emission intensity. The peaks and

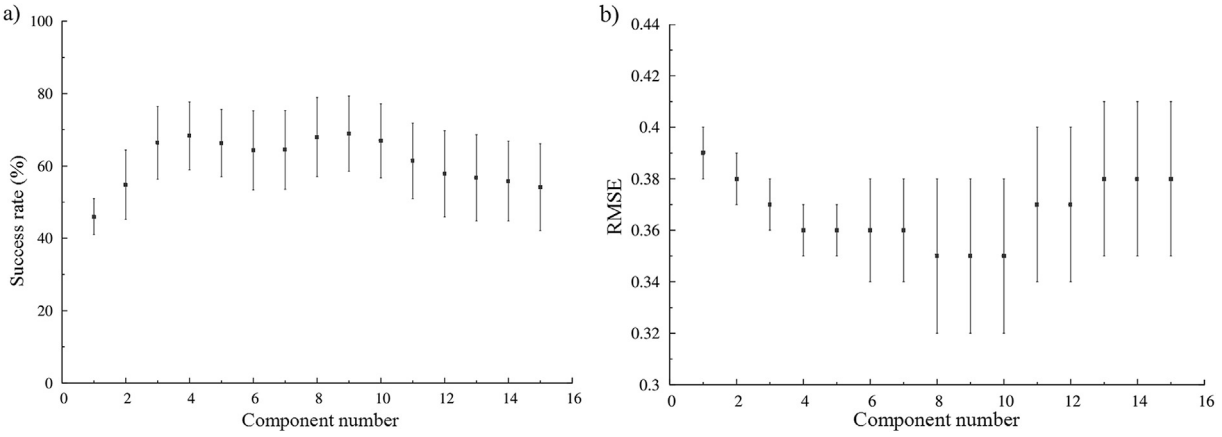

Fig. 3 – Evaluation of the best number of components to execute the classifier induced via PLS regression in function of the (a) success rate and (b) RMSE, employing the LIFS-405 spectra data as an input attribute. By correlating both graphics and comparing them statistically, the best component number to work with for diagnosis purpose is 13.

Table 1 – Confusion matrix obtained by the classifier, constructed combining the Classification via Regression (CVR) and the Partial Least Square Regression (PLSR), through 10 executions of 10-fold stratified cross-validation. The columns refer to the predictions of the classifier, and the lines refer to the nominal classes of the samples.

Nominal class	Classified as			
	CVC	Healthy	HLB asymptomatic	HLB symptomatic
CVC	**82.75**	0	12.25	5
Healthy	0	**96.5**	3.5	0
HLB asymptomatic	7.25	5.5	**86.75**	0.5
HLB symptomatic	5	0	1.75	**93.25**

Bold shows the number of correct classified instances.

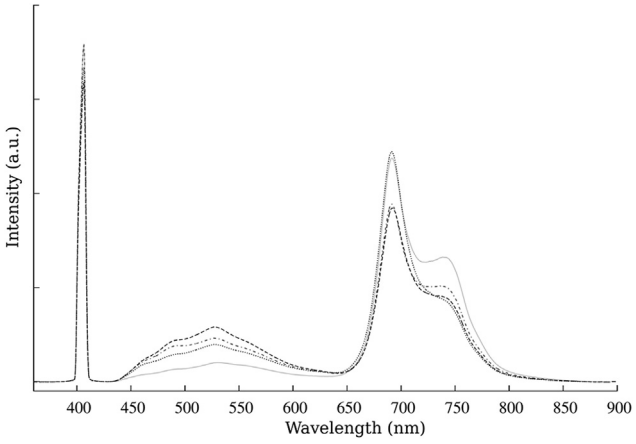

Fig. 4 – Typical spectra obtained with the laser-induced fluorescence technique with an excitation light of 405 nm. In these spectra, two different bands are observed that correspond to the blue–green fluorescence, from 410 to 630 nm, and the chlorophyll fluorescence in the red to near infrared region, from 650 to 800 nm. It is also possible to observe the spectral differences between the different classes: healthy (solid line), HLB asymptomatic (dash dot line), HLB symptomatic (dot line) and CVC (dash line).

diagonal bands with highest intensities are due to the excitation lamp and second order diffraction.

Umbelliferone fluoresced mainly between 425 and 500 nm (Fig. 5a), and its maximum intensity occurred at 320 nm excitation and 460 nm emission wavelength (1 ppb). In other regions, the fluorescence intensity was very low. With higher concentration (2 ppm), umbelliferone also fluoresced with excitation at 405 nm (Fig. 6). In this concentration, the 3D sweep was not used because the emission intensity at 320 nm saturated the equipment.

The umbelliferone emission fluorescence, shown in Figs. 5a and 6, coincides with the blue–green emission observed in Fig. 4 and may possibly contribute to the leaf fluorescence emission. The other compounds (Fig. 5b and c), on the other hand, show no significant fluorescence emission intensity between 300 and 500 nm, and hence cannot explain the leaf profile in Fig. 4.

In Fig. 4, considerable differences between healthy and disease leaves were found at 690 nm and 740 nm, mostly due to chlorophyll-a emission. ChlF is a well-known technique to measure photosynthetic activity in plants, and can be used to evaluate the plant's health, or stress factors (Agati, 1998; Buschmann & Lichtenthaler, 1998). The incident wavelength light on the leaves is 405 nm and is readily absorbed by the superficial part of leaf cells (Cerovic, Samson, Morales, Tremblay, & Moya, 1999; Lichtenthaler & Rinderle, 1988). Most of the ChlF emitted travels just a short distance inside the leaf epidermis before the fluorescence emitted inside the leaf is re-absorbed by chlorophyll *in situ* (Cerovic et al., 1999; Lichtenthaler & Rinderle, 1988). Therefore, high chlorophyll concentration leads to smaller emission intensity in the band 690 nm, but higher emission intensity at 740 nm due to the re-absorption effect. Otherwise, when the chlorophyll concentration is low, as occurred in the stressed plant, the emission intensity at 690 nm increases, because of reduced re-absorption, and results in a diminishing emission at 740 nm. This behaviour indicates that the chlorophyll emission spectra may be used to distinguish healthy from diseased leaves, as in Fig. 4.

3.3. *Monitoring trees in the field: a study of early diagnosis*

The main proposal of this study was to evaluate the LIFS-405 system as a tool for early diagnosis of HLB in a crop. Border trees were monitored monthly with the LIFS-405 system between March 2011 and March 2013. Five leaves from each border tree were analysed with the optical system. In the beginning of the study (December 2011), leaves from all border tress were analysed using the qPCR.

It is well known that the fluorescence spectroscopy technique in citrus leaves is very sensitive to water stress conditions (Cerovic et al., 1999; Marcassa et al., 2006; Kancheva et al., 2008). In this way, the calibration set were divided into two groups: leaves collected in the dry season and leaves collected in the rainy season. In order to demonstrate the tendency for clustering of the classes according to the season, first a principal component analysis (PCA) was evaluated. Figure 7a and b displays the score plot for two principal components (PC), PC1 × PC2, in rainy and dry season respectively. In both graphs, it is possible to infer that the data are composed of two main groups; 1) healthy and HLB asymptomatic; and 2) HLB symptomatic leaves. There is a superposition between HLB asymptomatic and healthy classes in both graphs. However, when comparing the PCA plot among the classes in two different seasons, the confusion between healthy and asymptomatic HLB for the rainy leaves group is

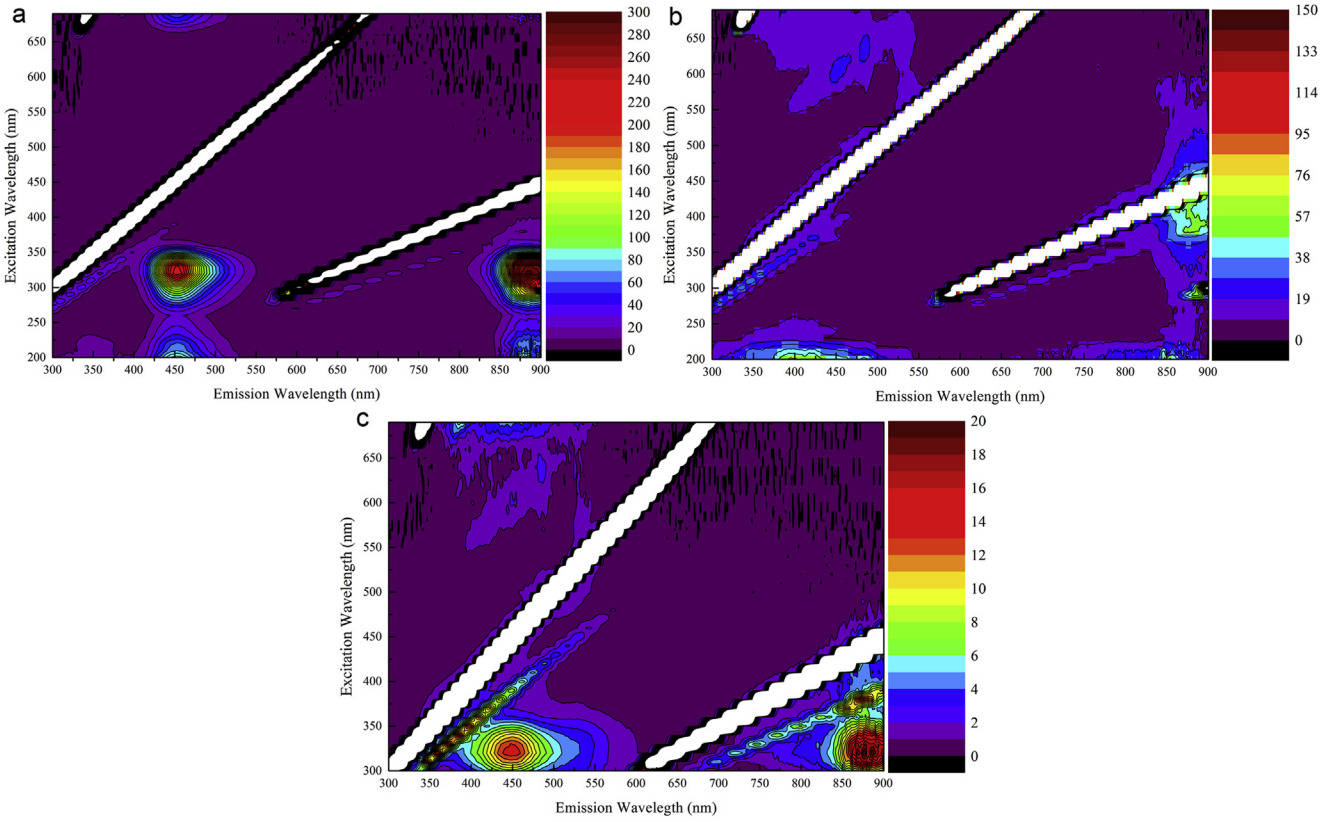

Fig. 5 – 3D plot for the aqueous solution of (a) the coumarin umbelliferone, at 10 ppb, in which it is possible to observe that the maximum intensity occurs at an emission wavelength of 450 nm, with an excitation wavelength of 325 nm; (b) the flavonoid hesperidin, at 40 ppm, for which the maximum emission wavelength was 400 nm with an excitation wavelength of 200 nm; and (c) the flavonoid naringin, 20 ppm concentration, in which the maximum emission occurred at 425 nm with excitation at 325 nm.

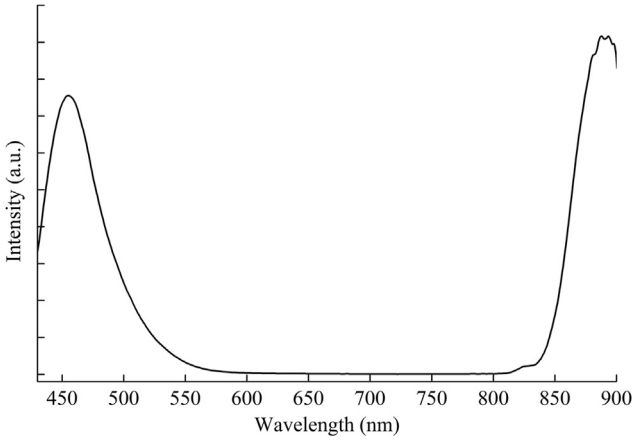

Fig. 6 – Fluorescence emission spectra of the coumarin umbelliferone, at 2 ppm, obtained with an excitation wavelength of 405 nm. It is possible to observe an emission peak at 450 nm. The second peak corresponds to the first-order diffraction.

higher than the observed within dry season group, probably due to the great recovery capability of the plant along this season. In other words, the plant is no longer subjected a drought stress and get the ability to recover itself. Additionally, in the dry season leaves group, besides the overlapping between healthy and HLB asymptomatic, it is possible to observe that the clusters centre of each one are slightly displaced suggesting the use of seasonal classifiers for improving the correct diagnosis.

Thus, seasonal classifiers were constructed with the calibration set, labelled in two group levels of rain and temperature at the farm in the period of study (Fig. 8). Usually, in Brazil, the dry season extends from April to September, while the rainy season extends from October to March. The rainy season classifier accounted for 900 leaves in total, equally divided into 3 classes: healthy, HLB symptomatic and HLB asymptomatic. The dry season classifier had a total of 1290 leaves, with 430 in each class. For the construction of both classifiers, only samples that better characterised each class were used. For evaluation of both classifiers the cross-validation was executed and the samples were trained separately for each season. All incorrectly classified samples were removed to avoid future confusion and to make the classes features more consistent. The number of samples per class was always maintained balanced: an important step for not favouring the class with greater number of samples, and thus better characterised, during the classification process.

All leaves collected in 2013 were used as a validation set for the seasonal classifiers. They were also divided into two

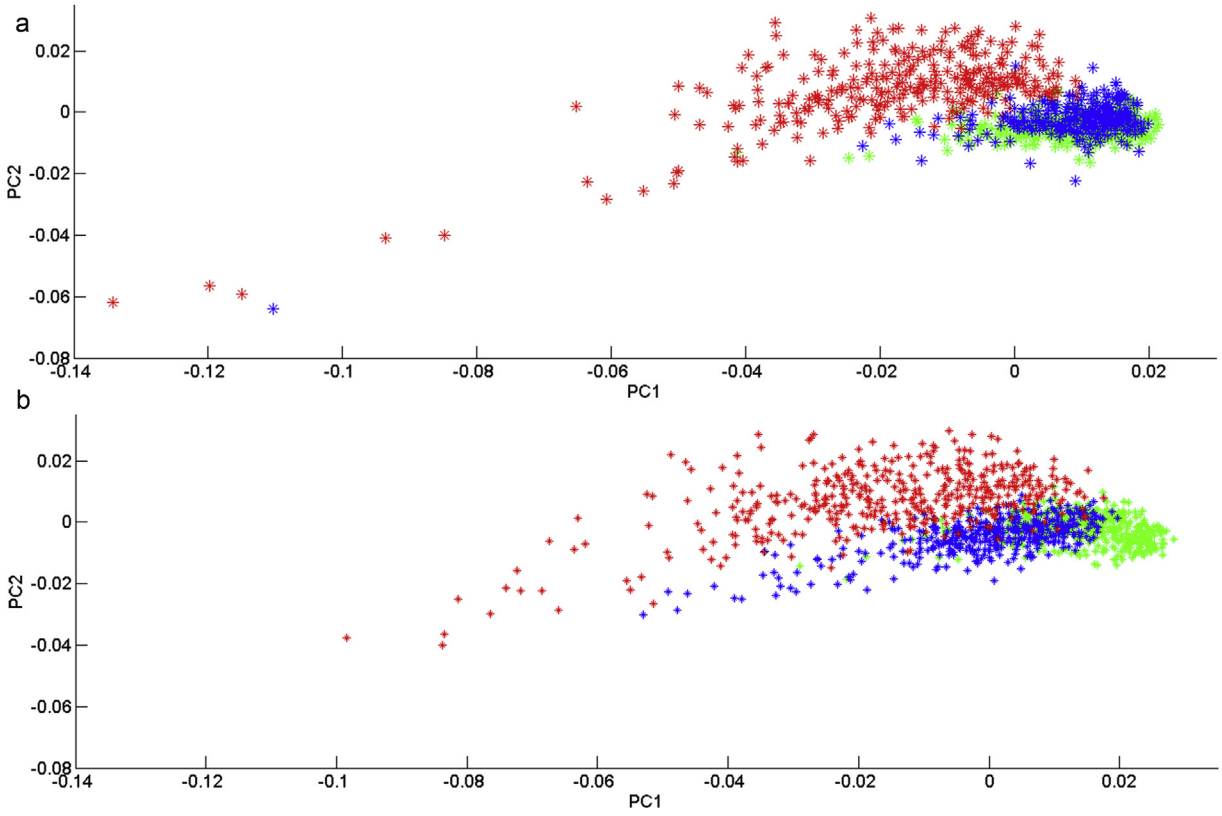

Fig. 7 – 3D PCA score plot from the calibration set employed in the seasonal classifiers: (a) rainy and (b) dry. It is possible to observe the separation groups of the three classes involved: healthy (green symbol), HLB asymptomatic (blue symbol), HLB symptomatic (red symbol).

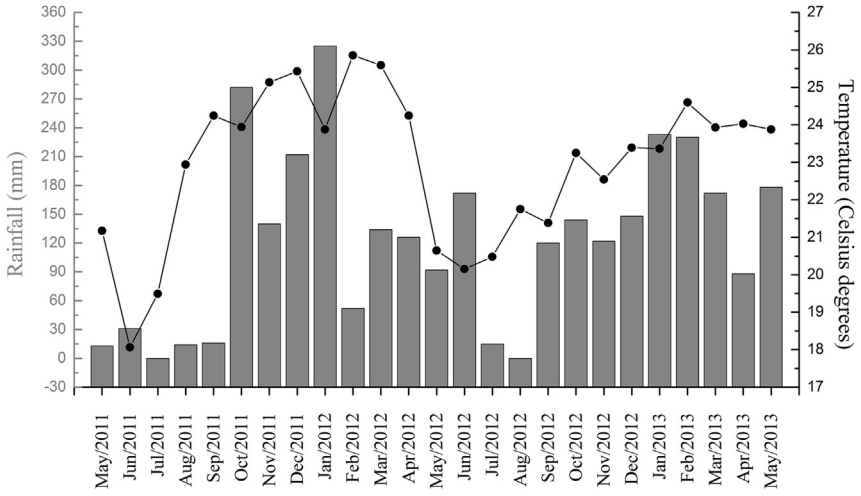

Fig. 8 – Rainfall and temperature values in the field. These data were provided by the farm. The rainy and dry months used to create the season classifier were based on the rainfall (column graphic) and temperature (circles and line) values.

groups: one from rainy and another from dry months, and were tested with the corresponding classifier as shown in Table 2. A success rate of 90% was achieved for the rainy seasonal classifier, and 85% for the dry seasonal classifier. Particularly, when the rainy seasonal classifier was assessed to classify dry seasonal leaves, the success rate dropped to 56%. Similarly, the success rate of the dry seasonal classifiers dropped to 66% in classifying rainy seasonal leaves.

After validating with the samples from 2013, the seasonal classifiers were employed to diagnose the border trees as

Table 2 – Confusion matrices obtained for seasonal classifiers validation. For this procedure, we used samples collected in a different year and separated seasonally.

Nominal class	Classified as		
	Healthy	HLB symptomatic	HLB asymptomatic
Confusion matrix: validation of rainy classifier (300 leaves)			
Healthy	**83%**	5%	12%
HLB symptomatic	4%	**92%**	4%
HLB asymptomatic	10%	5%	**85%**
Confusion matrix: validation of dry classifier (225 leaves)			
Healthy	**82.7%**	1.3%	16%
HLB symptomatic	1.3%	**98.7%**	0
HLB asymptomatic	25.3%	1.3%	**73.3%**

healthy or HLB-infected. A tree was considered infected with HLB if at least 3 of the 5 leaves evaluated were classified as diseased by seasonal classifiers. Following this criterion, 38 plants (95%) were diagnosed as infected by the LIFS system before May 2013. Among all monitored border trees, visual inspection only diagnosed one as diseased in March 2013. The same tree had been identified as infected since July 2011 by the seasonal classifiers, i.e. 21 months before symptoms appeared. In addition, the LIFS system classified all 5 leaves of this plant as diseased in March 2013. On the other hand, for qPCR technique, the diagnosis of the same tree was negative for the presence of the bacteria that causes HLB in December 2011.

Thirteen plants were considered positive for the bacteria infection by qPCR, which for 10 of these plants the LIFS system also diagnosed them as HLB infected, but some months before. In particular, two of them were diagnosed as HLB-infected in the same month of the reference technique test, presenting all the 5 leaves classified as diseased by the LIFS-405 system. Just in one case, the proposed technique was not successful in diagnosing the HLB before qPCR, i.e., attesting to the fact that 3 or more leaves as diseased. On that way, the number of infected trees detected by the reference technique, approximately 33%, was less than those diagnosed by LIFS-405 technique, which corresponds to 75%.

For the 26 remaining trees, the qPCR results were inconclusive. With the fluorescence technique, 17 plants were diagnosed as diseased since 2011; the other six plants were considered infected since 2012 and one tree was HLB-infected since 2013. Only two are still considered healthy in the field. Aware of this, additional measurements are being carried out in order to corroborate this study and to obtain better results, reinforcing the potential of the LIFS technique in diagnose diseases in citrus crops.

Due to long period, the monitoring border trees study showed that the latency stage of the disease could be much greater than two years, corroborating to the few months to one or more year estimation done for the asymptomatic phase of HLB (Gottwald, 2010). It is important to note that the fluorescence features obtained from asymptomatic plants show changes in their chemical profile within a year, depending on climate conditions, especially when subjected to water stress. Because of such seasonal behaviour, the accuracy of early diagnosis with LIFS systems may change, just as observed by visual inspection, in which, during a year, sometimes a large number of symptomatic trees was identified as symptomatic in the field, and other months, the visual inspection did not detect even a single plant. Thus, the plant had a high capacity of rehabilitation due to abiotic variations, masking any chemical changes that the disease may be causing. Therefore, when climate factors are included in the analysis, higher classification accuracy was achieved and early diagnoses were successfully obtained with the LIFS-405 system.

An infestation curve of the border trees orchard is presented in Fig. 9. One may see that when the LIFS-405 is used as a sensor scout, the detection growth rate of HLB infestation is faster than that obtained by visual inspection in the orchard, in which growth rate is very smooth in the beginning and suddenly several trees present the symptom when HLB is spread in the field. Thus, it does not reveal the real state of the plantation. Figure 9 also shows that in the first 8 months of 2011, about 75% of the border trees were detected as HLB positive while the visual inspection was not able to detect even a single tree. The first HLB tree identified by visual inspection was about 22 months after the beginning of the border trees monitoring. This graphic shows the economic importance for the productive sector to include such rapid monitoring for HLB, once it is possible to scan quickly an entire field and allow for fast detection of the disease. This technique is interesting due to its high potential to produce field devices, associated with fast analysis, a lack of sample preparation, and a low cost measurement method.

4. Conclusions

The main results of this work present a new measurement protocol using the portable LIFS-405 system and statistical tools as a new diagnosis apparatus for HLB capable of identifying not only symptomatic plants in the crop, but also asymptomatic HLB trees in the field. The diagnoses were performed through alteration in the optical properties of leaves due to diseases. In this way, the LIFS-405 fluorescence system, combined with statistical analyses, was demonstrated to be an excellent alternative tool for early disease identification in field. Analysis of the induced classifiers was based on the entire set of leaf fluorescence data as input, which is very efficient for the correct diagnosis of HLB, even in asymptomatic stage. The system may also distinguish among four types of leaves: healthy, HLB-asymptomatic, HLB-symptomatic and CVC symptomatic with classification accuracy greater than 90%. The research findings indicate that the diagnosis success is a consequence of the overall fluorescence response of the leaf due to multiple fluorophores composition, whose concentration depends on healthy state of the plant, not only on the concentration of a specific fluorophore. The 3D fluorescence data shown in this work supported the LIFS-405 spectral answer.

Over 3 years of the border trees monitoring, it was observed that the chemical profile of the leaves depends on the climate condition. Due to seasonal behaviour of the tree, the abiotic

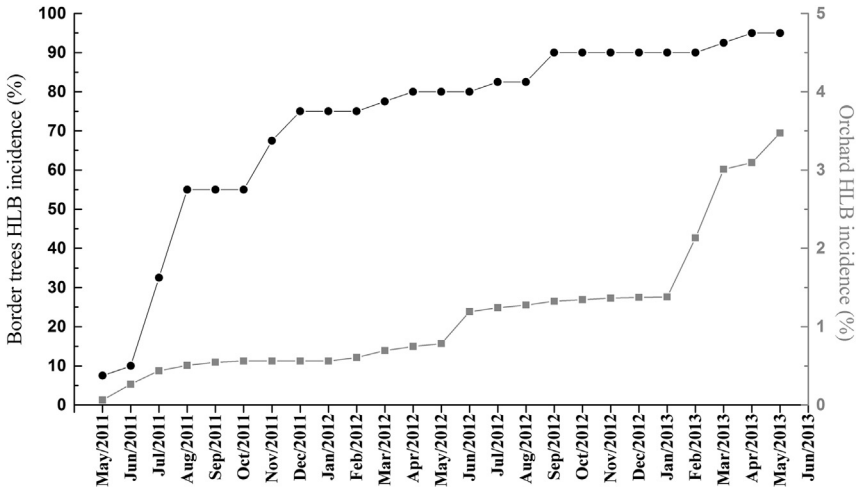

Fig. 9 – Evolution of HLB incidence on the border trees attended and diagnosed by LIFS-405 system (circles) compared to the HLB incidence curve with respect to the entire orchard studied, and whose diagnosis was made by visual inspection in the same period (squares).

stress increases the expression of symptoms in diseased trees so that the number of trees, which present symptoms of HLB, changes along the year. Thus, it is possible to infer a high capacity of rehabilitation by the plant in some periods of the year, which may camouflage any chemical changes that the disease may be causing. Therefore, to achieve higher classification accuracy, it was important to build a seasonal classifier. When climate factors are included in the analysis, early diagnoses were successfully carried out with LIFS-405 system 21 months before the symptoms appeared. Contrary to expectations, through the analyses of fluorescence spectra, the asymptomatic phase of HLB could last more than 6–12 months.

Despite qPCR being a good and efficient test, its success in obtaining the right diagnosis depends on finding *Ca*Las DNA in the samples. Due to the non-homogeneous *Ca*Las distribution inside the plant, the probability of diagnosing an infected asymptomatic tree with qPCR is very low compared to the LIFS-405 system, which detects the chemical alteration caused by the infection. Along these lines, it is important to highlight that effective management to control HLB inevitably involves systems that can perform, effectively, early diseases diagnosis in the field, and can allow infestation mapping construction, which enables the producer to assess strategies to avoid the spread of the disease in the crop.

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