Talanta 83 (2010) 351–356

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Evaluation of the effects of *Candidatus* Liberibacter asiaticus on inoculated citrus plants using laser-induced breakdown spectroscopy (LIBS) and chemometrics tools

Fabíola Manhas Verbi Pereira[a,*], Débora Marcondes Bastos Pereira Milori[a], André Leonardo Venâncio[b], Mariana de Sá Tavares Russo[a], Polyana Kelly Martins[c], Juliana Freitas-Astúa[c,d]

[a] *Embrapa Instrumentação Agropecuária, P.O. Box 741, Zip Code 13561-206, São Carlos, SP, Brazil*
[b] *Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Departamento de Física e Matemática, Universidade de São Paulo, Av. Bandeirantes, Zip Code 14040-901, 3900 Ribeirão Preto, SP, Brazil*
[c] *Centro Apta Citros Sylvio Moreira, P.O. Box 04, Zip Code 13490-970, Cordeirópolis, SP, Brazil*
[d] *Embrapa Mandioca e Fruticultura Tropical, R. Embrapa, s/n, Zip Code 44380-000, Cruz das Almas, BA, Brazil*

ARTICLE INFO

Article history:
Received 1 July 2010
Received in revised form
16 September 2010
Accepted 18 September 2010
Available online 24 September 2010

Keywords:
Laser-induced breakdown spectroscopy
Chemometrics
Fingerprint
Plant material
Citrus greening
Huanglongbing

ABSTRACT

This study investigated the organic and inorganic constituents of healthy leaves and *Candidatus* Liberibacter asiaticus (CLas)-inoculated leaves of citrus plants. The bacteria CLas are one of the causal agents of citrus greening (or Huanglongbing) and its effect on citrus leaves was investigated using laser-induced breakdown spectroscopy (LIBS) combined with chemometrics. The information obtained from the LIBS spectra profiles with chemometrics analysis was promising for the construction of predictive models to identify healthy and infected plants. The major, macro- and microconstituents were relevant for differentiation of the sample conditions. The models were then applied to different inoculation times (from 1 to 8 months). The models were effective in the classification of 82–97% of the diseased samples with a 95% significance level. The novelty of this method was in the fingerprinting of healthy and diseased plants based on their organic and inorganic contents.

1. Introduction

Citrus greening or *huanglongbing* (HLB), is a devastating disease that has caused serious problems with citrus cultivation all over the world [1,2]. Some analytical methods based on DNA techniques were reported for the diagnosis of this disease aiming the detection of the causal agents *Candidatus* Liberibacter [3–5]. In general, most of these developed methods involve polymerase chain reaction (PCR), but other techniques for diagnostic purposes, where genetic sequences are not involved, have also been reported in the scientific literature with only minor impact [6,7].

Recently, we published a method to study the evolution of disease through profiling the metabolism of plants that could also be used with other parameters, such as variations in chemical composition [8]. In published study, the mineral constituent signals were evaluated assuming that diseases can influence the biochemical and physiological processes of plants. The analytical method employed micro-synchrotron radiation X-ray fluorescence (μSR-XRF) scanning combined with chemometrics (exploratory and classification analyses) and efficiently identified healthy and infected leaves with and without symptoms in citrus crops of the same age (approximately 40 months old). The elements with most influence in the diagnosis were K, Ca, Fe, Cu and Zn, along with a region of coherent and incoherent X-ray scatterings. The developed method used these elements' X-ray lines combined with classification tools for the construction of predictive models. These models were then utilized for determining the healthy, asymptomatic and symptomatic conditions of the plants. The results from μSR-XRF correctly classified up to 95–98% of the samples for classification data set, and the validation data correctly assigned 90% of each set samples. In both cases (classification and predictive models), the confidence level was 95%. In this case, these plants had not shown any nutrient deficiencies caused by inadequate fertilization, weather or soil condition prior to citrus greening infection [8].

The search for new diagnostic methods is critical, and the current technology (PCR) has several limitations [7]. These limitations include unequal distribution of pathogens in host plants, low pathogen concentration and the presence of PCR inhibitors in

* Corresponding author. Tel.: +55 16 2107 2877; fax: +55 16 2107 2902.
E-mail address: fmverbi@uol.com.br (F.M.V. Pereira).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.09.021

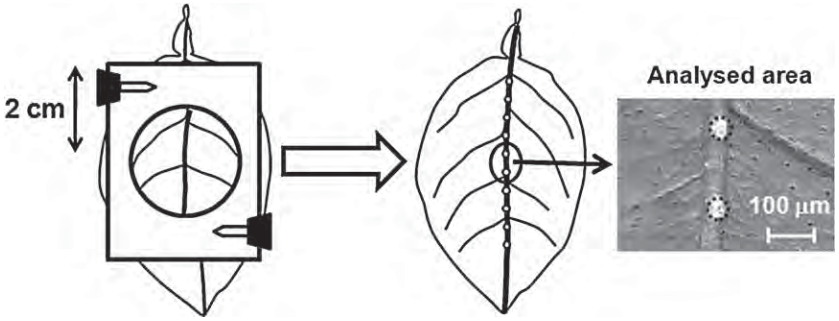

Fig. 1. Diagram of the points of the leaves scanned by LIBS.

citrus extracts can complicate the analysis [9]. In addition, the PCR application is restricted when the plants exhibit certain symptoms indicative of the disease [2].

In addition, the determination of only Fe, Mn and Zn can lead to misinterpretations of nutritional deficiency or inadequate fertilization for plants instead of citrus greening disease. One of the effects of citrus greening in the leaves, known as chlorosis, resembles those induced by Fe, Mn and Zn deficiencies [2].

One alternative to PCR-based methods is to evaluate the elements in the matrix of plants as a possible fingerprint for healthy and diseased states. Laser-induced breakdown spectroscopy (LIBS) is a potential spectral technique for this purpose, because it is capable of scanning samples in different positions, has a wide spectral range, allows both organic and mineral characterization and does not require pre-treatment (like acid digestion) of the sample, thereby improving the analytical frequency and cost [10].

Moreover, another useful advantage of LIBS for this study was the detection of electromagnetic radiation generated after excitation of the atoms, ions or molecules present in the sample matrix. In this case, the analytical signal of the excited species was represented by one or more lines of emission in the collected spectrum. The axes of the abscissa and ordinate corresponded to the wavelength range and intensity of the lines, respectively [11–13].

The objective of this study was to investigate the effects of *Candidatus* Liberibacter *asiaticus* (CLas) on inoculated citrus plants from a greenhouse using the mineral and organic signal profiles of leaves as a fingerprint. The sample spectra were obtained by LIBS, and the data were analyzed by chemometrics strategies. Furthermore, the evolution of the bacteria on the inoculated plants was monitored by real-time quantitative-PCR (RT-qPCR) [14] amplification of CLas sequences.

2. Experimental

2.1. Source of inoculum and plant material

The experimental plants used were 5-month-old Valencia sweet oranges [*Citrus sinensis* (L.) Osbeck] grafted onto Citrumelo Swingle [*Citrus paradisi* Macfad. cv. Duncan X *Poncirus trifoliata* (L.) Raf.] rootstock. All plants used in the study were initially healthy. One hundred plants were inoculated with two bud woods (each 2–3 cm long) taken from 3-year-old sweet orange plants exhibiting typical symptoms of citrus greening and were positive for CLas. Along with the CLas, the bud woods also carried a mild strain of *Citrus tristeza virus* (CTV) routinely used for pre-immunization in Brazil. Therefore, the healthy control plants (100 plants in total) were also grafted with bud woods containing the same CTV strain found in the wild in Brazil. All of the experimental plants were grown in 4 L plastic bags containing Plantmax citrus substrate (Eucatex, São Paulo, Brazil) and maintained under greenhouse conditions with temperatures not exceeding 30 °C during the entire experiment. The total of 200 plants were automatically irrigated every day and fertilized when necessary.

2.2. Experimental procedure

The plants were divided into two categories of equal size, healthy (60 samples) and inoculated (60 samples), and each plant was represented by three leaves. The first category was kept as control samples. The excess number of plants grown described in the previous section (100 of each state, healthy and inoculated) was a guarantee against possible loss or problems during the inoculation procedure. After 1 month of inoculation, LIBS measurements of the samples were acquired monthly thereafter. The collection of the three leaves was done on the third set of leaves, situated down from the apical part of the plant. The leaves were taken 1 day before the beginning of each experiment and stored at 4 °C inside dark plastic bags. Each leaf was cleaned with the aid of a piece of cotton wetted with deionized water and then dried in the air.

2.3. Parameters of LIBS measurements

The measurements were performed with a LIBS2500 spectrometer (Ocean Optics, Dunedin, USA). This system had a Q-switched 1064 nm Nd:YAG laser operating at a 50 mJ maximum power energy and a 10 Hz frame rate. The system of detection had seven high-resolution fiber optic spectrometers, each one with a 2048 element linear silicon CCD array. The setup configuration used a fixed Q-switch delay time of 48.5 μs, an energy of laser pulse of 50 mJ and a delay between scans averaging 1 ms. All of the tests were carried out in the air, and the distance from the sample to the collecting optics was approximately 7 mm. The spectra were acquired from 189 to 966 nm with an approximate optical resolution of 0.1 nm. Each raw leaf was fixed between two samplers made of Al (4.5 cm × 3.5 cm) with a circular orifice of 2.5 cm in diameter, using the help of two binder clips (Fig. 1). The measurement positions on the leaves were marked with manual X and Y stages. As shown in Fig. 1, the scanning was executed on the midrib (central vein) of the backside of the leaves. Each spectrum was obtained by one laser shot per scanned point only in this region of the leaves. A total of 10 spectra were recorded per scan of each leaf. The distances between the scanned points were random while trying to scan the full longitudinal part of the midrib. Using the described conditions, the laser did not cross the leaf, while the diameter of the abraded point was approximately 50 μm also shown in Fig. 1.

2.4. Data set treatment

The software Pirouette 4.0 rev.2 (Infometrix, Inc., Bothell, USA) was used for transformation and pre-processing of the LIBS spectra. The applied tools of chemometrics, principal component analysis

(PCA) and soft independent modeling of class analogy (SIMCA) were performed with the same software.

2.5. Sampling and DNA extraction

Plant sampling and DNA extraction began 1 month after inoculation. The same leaves measured by LIBS were also used for DNA extraction on a monthly basis. Total DNA was extracted from 100 mg (fresh weight) of midribs and petioles using the cetyltrimethyl ammonium bromide (CTAB) method described by Murray and Thompson [15]. The DNA was eluted in 60 μL of elution buffer (1:10 of Tris–EDTA 1 mol L^{-1}, 20 μg μL^{-1} of RNAse A), and the concentration and quality were analyzed with agarose gel electrophoresis and a NanoDrop™ 8000 (Thermo Fisher Scientific, Wilmington, Denmark). All DNA were then standardized to a concentration of 10 ng μL^{-1}.

2.6. RT-qPCR assay

The primers and probes used for the RT-qPCR assay were designed by Coletta-Filho et al. [16] using Primer Express software (version 2.0, Applied Biosystems, Foster City, U.S.A.) and were synthesized by the same company. The primer sequences (AS-84F 5′-TCACCGGCAGTCCCTATAAAAGT-3′ and AS-180R 5′-GGGTTAAGTCCCGCAACGA-3′) and probe (AS-NED-MGB-111T 5′-ACATCTAGGTAAAAACC-3′) were based on the 16S rDNA sequence of CLas (GenBank AY919311). The optimized RT-qPCR assay required 0.8 μmol L^{-1} of each primer (forward and reverse), 0.2 μmol L^{-1} of the probe for CLas, 1× TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 4 μL of standardized DNA template (10 ng μL^{-1}), 1× Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems) as an internal control for normalization of the amount of total DNA in each reaction and autoclaved Milli-Q water to a final volume of 20 μL. The fast amplification protocol was 20 s at 95 °C followed by 40 cycles of amplification (3 s at 95 °C and 30 s at 60 °C). The amplification, data acquisition and data analysis were done with the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems) using the sequence detection software (version 1.4). Each run was comprised of two replicates for the tested DNA (inoculated plants), the CLas-negative (healthy plants), the CLas-positive (positive controls by conventional PCR) and a non-template control (NTC).

3. Results and discussion

3.1. RT-qPCR results

The importance of PCR for this study was to verify the efficiency of bacterial inoculation on the plants. The RT-qPCR assays identified positive results for CLas infection, as the amount of DNA increased from month to month, after the inoculation of the plants. The rate of positive diagnostics was shown in Fig. 2. The values in percentage, were, 4, 20, 22, 70, 66, 67 and 82, from the first to the eighth months. The LIBS and PCR tests from the sixth month after inoculation were not recorded due to problems with the experimental set up. It is important to mention that the inoculated samples with CLas-negative, from beginning of the experiments, were also considered diseased condition in the next sections.

3.2. LIBS data treatment

A total of 2560 leaves were analyzed, considering healthy (1273) and inoculated (1287) plants. In the eighth month, the bacteria DNA were detected in 82% of the diseased leaves. Data information using only measurements of the midrib did not make a significant difference in the results of citrus greening evaluations, which was in agreement with prior studies with μSR-XRF [8]. Therefore, only this region was utilized for LIBS measurements to avoid the laser boring through the leaves. In addition, the midrib diameter from the leaves backside is bigger than that observed from the leaves front side. This characteristic permitted a better laser focalization for the midrib leaf scanning. The measurements were executed directly on the leaves, *i.e.*, without any pre-treatment of the samples. However, the intrinsic characteristics of the leaves, such as matrix effect and thickness, could reduce the intensity of the element lines or increase the noise in the spectrum. Therefore, it was necessary to extend the potential of the LIBS information using chemometrics tools with the goal of extracting maximal information.

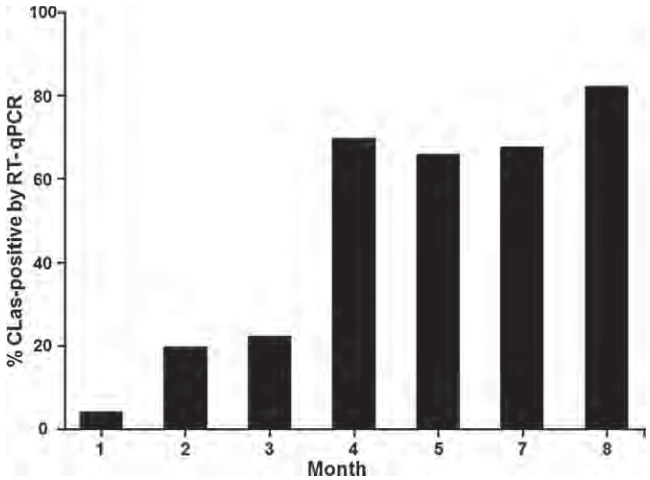

Fig. 2. Monthly percentage values of positive diagnostics for CLas bacteria using RT-qPCR.

The resulting LIBS spectra had a high resolution of approximately 0.1 nm and the number of variables for this configuration was 13,746, as shown in Fig. 3a. As a result of the large amount of data, data analysis was limited due to slow processing. Therefore, the data were pre-treated computing the average among 10 by 10 independent variables. After this step, the resolution changed from 0.1 nm to 1 nm and the number of variables of the spectrum showed in Fig. 3a was reduced to 1375, as indicated in Fig. 3b. This procedure was useful not only in reducing the number of variables but also in reducing the noise in the spectra. Using this procedure, no difficulties were encountered in future data interpretation.

The average deviations among the signals of replicate measurements were 35 and 38 counts, for healthy and diseased leaves, respectively.

Another possible limitation with the data was related to the baseline of the spectra. In the spectrum shown in Fig. 3a, the differences in the baselines at the beginning of each spectrometer, which are marked from 1 to 7, can be seen. The range of each spectrometer used in these experiments, in nanometers, was 188.84–292.43 (1); 286.25–383.49 (2); 377.22–506.98 (3); 501.33–618.15 (4); 612.06–716.07 (5); 710.23–800.14 (6) and 793.49–966.08 (7). These differences in the offset spectra could then cause misinterpretations. To overcome these limitations, the base 10 logarithm transform and mean-centering were applied as pre-processing strategies. The advantage of the logarithm transform was the emphasis of small data values relative to higher ones. To perform the mean-centered pre-processing of spectral variables, the mean was subtracted from each data point to produce a mean-centered matrix that resulted in the value of the mean being equal to zero for all variables [17].

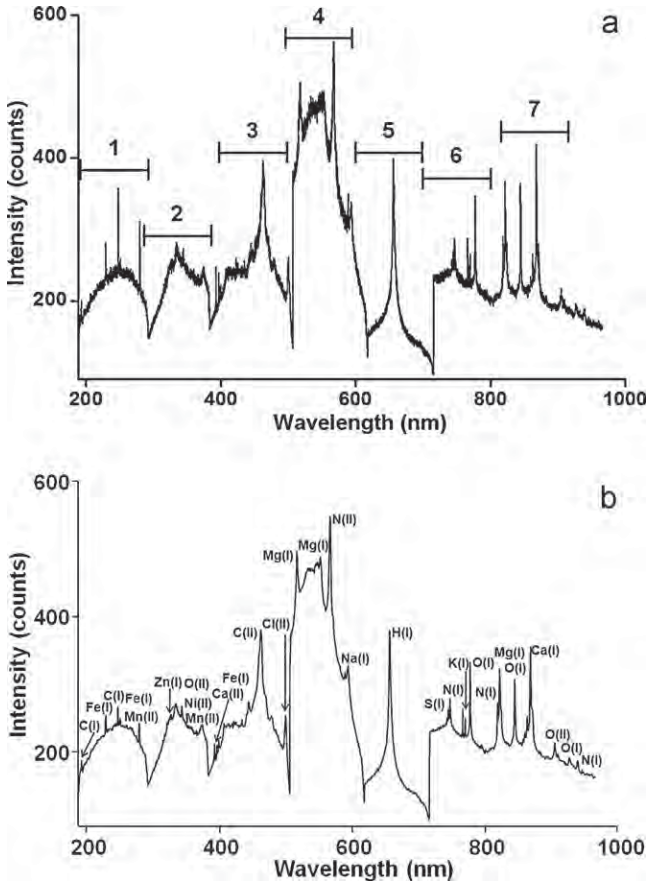

Fig. 3. Original spectrum of a healthy leaf as measured by LIBS (a) and transformed spectrum computed from the average among ten by ten independent variables (b).

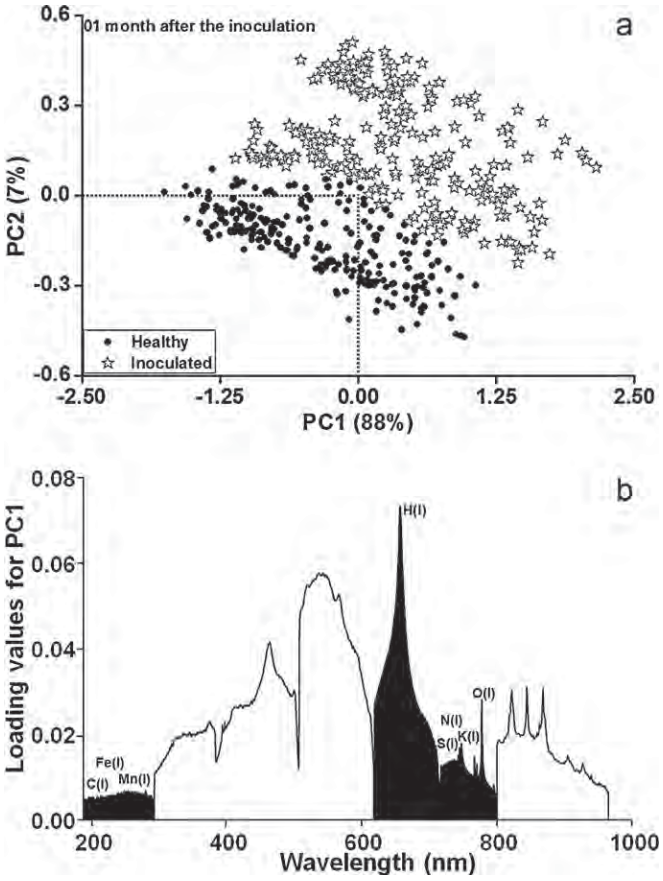

Fig. 4. Score (a) and loading plots (b) for the data matrix, 1 month after inoculation, containing 510 spectra and 1375 variables.

An example spectrum, and the detected lines for one healthy leaf, is shown in Fig. 3b. Six micronutrients signals were detected in the tested citrus plants: Fe, Zn, Ni, Mn, Cl and Na. Lines for three major constituents, C, H and O, and five macronutrients, Ca, N, K, Mg and S, were also verified. The spectral lines information of each element was obtained from National of Standards and Technology (NIST) atomic spectra database [18].

3.3. Analysis of citrus leaves by LIBS

Using preliminary information, it was not possible to observe any differences in condition when all of the samples (from first to eighth month) were put together in the same PCA model. One of the interesting results from this model was that the samples from the beginning of the experiment showed differences based on the age of the plants, month to month. The models were then applied to each period, separately, and the results were as follows. The entire spectrum was used for data analysis. Some sets of samples were found to be more representative of the healthy and inoculated differences, and these were utilized for the models. A model for each month is also interesting because the age of the plants is a well controlled parameter.

The model performed by PCA for the first month demonstrated two well-defined clusters, one for healthy plants and another for inoculated plants, with PC1 and PC2 accounting for 95% of explained variance (Fig. 4a). Fig. 4b shows the loading values and the elemental contribution for healthy samples in black. From left to right in Fig. 4b, these regions were C(I) (193.027 and 247.856 nm), Fe(I) (229.817 and 251.428 nm), Mn(I) (279.482 and 279.827 nm), H(I) (656.279 nm), S(I) (744.335 nm), N(I) (746.831 nm), K(I) (766.490 and 769.896 nm) and O(I) (777.417 nm).These macronutrients are important in this early stage of development for growth and regulation processes in the plants [19], as shown in Table 1. From these initial results, it was possible to verify alterations in the metabolism of the plants as a consequence of inoculation, even in the asymptomatic stage. The highest loading values through principal components were utilized as criteria to select the important elements showed in Table 1. This information was evaluated together with the clusters of the score plots.

After 3 months, the observed symptoms during this period were leaves with characteristic signs of citrus greening, such as blotchy yellow regions. For this data set, other additional

Table 1
Important variables for discrimination of state of healthy from PCA analyses.

Period of measurements		1st month	3rd month	5th month	8th month
Major constituents	C	R	R	N	R
	H	R	R	R	R
	O	R	R	N	R
Macronutrients	Ca	N	R	N	N
	K	R	R	N	N
	Mg	N	R	R	N
	N	R	R	R	N
	S	R	R	N	N
Micronutrients	Cl	N	R	N	N
	Fe	R	R	N	R
	Mn	R	R	N	R
	Na	N	R	R	N
	Ni	N	R	N	R
	Zn	N	R	N	R

R = relevant, N = negligible.

Table 2
Results for the classification and validation data sets using SIMCA.

Training data set (393 spectra)					Validation data set (115 spectra)				
Condition	Healthy	Inoculated	No match	Correct predictions (%)	Condition	Healthy	Inoculated	No match	Correct predictions (%)
1st month									
Healthy	175	28[a]	0	86	Healthy	55	2[a]	0	96
Inoculated	5[a]	185	0	97	Inoculated	0	58	0	100
Training data set (708 spectra)					Validation data set (75 spectra)				
Condition	Healthy	Inoculated	No match	Correct predictions (%)	Condition	Healthy	Inoculated	No match	Correct predictions (%)
3rd month									
Healthy	304	42[a]	1	88	Healthy	25	0[a]	1	96
Inoculated	13[a]	348	0	96	Inoculated	0[a]	49	0	100
Training data set (836 spectra)					Validation data set (93 spectra)				
Condition	Healthy	Inoculated	No match	Correct predictions (%)	Condition	Healthy	Inoculated	No match	Correct predictions (%)
5th month									
Healthy	373	62[a]	2	85	Healthy	42	0[a]	1	98
Inoculated	69[a]	327	3	82	Inoculated	3[a]	46	1	92
Training data set (813 spectra)					Validation data set (87 spectra)				
Condition	Healthy	Inoculated	No match	Correct predictions (%)	Condition	Healthy	Inoculated	No match	Correct predictions (%)
8th month									
Healthy	349	58[a]	5	85	Healthy	28	7[a]	3	74
Inoculated	63[a]	336	2	84	Inoculated	4[a]	43	2	88

[a] Incorrect predictions.

elements were included for classification as healthy, including Fe(I) (229.817, 251.428 and 399.739 nm), C(I) (247.856 nm), Mn(II) (270.845 and 344.199 nm), Mn(I) (279.827 nm), Zn(I) (328.233 nm), Ni(II) (334.924 nm), Ca(II) (393.366 and 396.847 nm), C(II) (426.726 nm), Cl(II) (478.132 nm), Mg(I) (516.732, 517.268, 552.840 and 821.303 nm), N(II) (567.602 nm), Na(I) (588.995 and 589.592 nm), H(I) (656.279 nm), S(I) (744.335 nm), K(I) (766.490 and 769.896 nm), O(I) (777.417 and 844.636 nm), N(I) (818.802 nm), Ca(I) (863.395 nm) and O(II) (868.609 nm), as shown in Table 1. These micronutrients were more important during the later stage of development, as they contributed to the structure of cellular components [19]. The other elements in the spectrum were important in the classification of leaves as diseased.After 5 months, the most important region of the spectra for the classification of healthy samples was represented by Mg(I) (516.732, 517.268 and 552.840 nm), N(II) (567.602 nm), Na(I) (588.995 and 589.592 nm) and H(I) (656.279 nm). The last measurements were done after 8 months and the most important elements for separation of the healthy scores were Fe(I) (229.817, 251.428 and 399.739 nm), C(I) (247.856 nm), Zn(I) (328.233 nm), O(II) (328.747 nm), Ni(II) (334.924 nm), Mn(II) (344.199 nm) and H(I) (656.279 nm).

These results were also very interesting because, at this stage, some diseased leaves were symptomatic and the elements Fe, Zn and Mn were important for this discrimination according to our research using μSR-XRF [8].

The results of the PCA models are summarized in Table 1. As can be seen, the line for H was constant over the four different intervals. The healthy samples had a tendency to be associated with higher organic composition. In the third month, the presence of micronutrients, such as Mn, Cl and Na, was an evidence of a new process in the healthy plants. In the last period of measurements, the counts for Zn were decreased by 76% after 8 months for some diseased plants. At this stage, the change in Zn was mainly related to yellowing of the leaves. These results emphasized that the mature plants had different chemical characteristics, which was indicated by variations of their constituents (Table 1).

3.4. Potentialities of classification models

The potential of the LIBS spectra in the identification of plants infected by citrus greening was estimated by means of four constructed models for classification using the chemometrics method SIMCA [20] across the whole spectral region. This method was chosen based on the good results obtained during previous research using μSR-XRF [8]. The best pre-processing of the variables for all SIMCA classification models was the same as that done for the PCA analyses. All models were optimized using three principal components, for each class (healthy and inoculated) with explained variance from 96% to 99%. The samples that constituted the data sets were selected in a random fashion with the help of PCA models and were considered modeled by the SIMCA method.

From the model constructed for the first month of tests, it was possible to verify accurate predictions for the classification and validation sets, as shown in Table 2. The third month model presented a better prediction for the classification of the data set than the model for the first period. A total of two samples, one for each model of classification and validation, were not matched in any category. In other words, according to the models they were not healthy neither diseased.

In the fifth month, five samples did not correspond to any class: two for the healthy category and three for the diseased category. In the validation data set, one spectrum for each condition was not matched.

The data sets for the eighth month demonstrated correct predictions up to 74–88% for validation data set (Table 2). These values were considered adequate, but the number of unmatched samples had increased. The distribution of the total unmatched data was seven for the classification model and five for the validation one (Table 2). For the classification, five healthy samples were not included in any category, and three were not included in the validation set.

Interesting results were obtained during the later periods (from fifth to eighth, months), where the values of the correct predictions were lower than those observed from the early periods, even

though they were considered statistically good (correct predictions higher than 70%).

4. Conclusions

The method described here used the controlled procedure of inoculation, data from LIBS measurements and chemometrics tools to effectively investigated nuances in the mineral and organic compositions of diseased and healthy plants.

The major, macro- and micronutrients showed monthly variations that were useful for the differentiation of healthy plants from those plants that were inoculated, but in an asymptomatic stage.

The plants, independent of the condition (healthy or diseased), showed differences based on the age, month to month. Then, the models were performed for each period, separately. The applied models for monthly periods would be useful in the fingerprinting of healthy and diseased plants considering that the age of the plants is usually controlled and knowable.

The LIBS associated with SIMCA was useful to detect diseased samples from first month of inoculation with 97% of correct predictions (classification model) over 4% to current method PCR.

This study successfully demonstrated a new promising method for analyzing the effects of CLas bacteria on inoculated citrus plants and the consequences of the infection on nutrient composition.

Acknowledgements

We are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the fellowship awarded to F. M. V. Pereira (Process 2007/08618-2), to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the fellowship awarded to A. L. Venâncio, to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Process 578576/2008-2) and to Grupo Fischer - Divisão Citrosuco for providing the plant material.

References

[1] A.A. Vojnov, A.M. Amaral, J.M. Dow, A.P. Castagnaro, M.R. Marano, Appl. Microbiol. Biotechnol. 87 (2010) 467.
[2] J.M. Bové, J. Plant Pathol. 88 (2006) 7.
[3] D.C. Teixeira, C. Saillard, C. Couture, E.C. Martins, N.A. Wulff, S. Eveillard-Jagoueix, P.T. Yamamoto, A.J. Ayres, J.M. Bové, Mol. Cell. Probes 22 (2008) 139.
[4] Z. Wang, Y. Yin, H. Hu, Q. Yuan, G. Peng, Y. Xia, Plant Pathol. 55 (2006) 630.
[5] S. Jagoueix, J.M. Bove, M. Garnier, Int. J. Syst. Bacteriol. 47 (1997) 224.
[6] J.-S. Kim, U.S. Sagaram, J.K. Burns, J.-L. Li, N. Wang, Phytopathology 99 (2009) 50.
[7] S. Sankaran, A. Mishra, R. Ehsani, C. Davis, Comput. Electron. Agric. 72 (2010) 1.
[8] F.M.V. Pereira, D.M.B.P. Milori, J. Anal. Atom. Spectrom. 25 (2010) 351.
[9] W. Li, L. Levy, J.S. Hartung, Phytopathology 99 (2009) 139.
[10] L.C. Trevizan, D. Santos Jr., R.E. Samad, N.D. Vieira Jr., C.S. Nomura, L.C. Nunes, I.A. Rufini, F.J. Krug, Spectrochim. Acta Part B 63 (2008) 1151.
[11] C. Pasquini, J. Cortez, L.M.C. Silva, F.B. Gonzaga, J. Braz. Chem. Soc. 18 (2007) 463.
[12] M. Galiová, J. Kaiser, K. Novotný, O. Samek, L. Reale, R. Malina, K. Páleníková, M. Liška, V. Čudek, V. Kanický, V. Otruba, A. Poma, A. Tucci, Spectrochim. Acta Part B 62 (2007) 1597.
[13] J.W.B. Braga, L.C. Trevizan, L.C. Nunes, I.A. Rufini, D. Santos Jr., F.J. Krug, Spectrochim. Acta Part B 65 (2010) 66.
[14] T.D. Schmittgen, in: M.T. Dorak (Ed.), Real-time PCR (BIOS Advanced Methods), Taylor and Francis, New York, 2006, p. 127.
[15] M. Murray, W.F. Thompson, Nucleic Acids Res. 8 (1980) 4321.
[16] H.D. Coletta-Filho, E.F. Carlos, K.C.S. Alves, M.A.R. Pereira, R.L. Boscariol-Camargo, A.A. Souza, M.A. Machado, Eur. J. Plant Pathol. 126 (2010) 53.
[17] P. Geladi, Spectrochim. Acta Part B 58 (2003) 767.
[18] NIST Atomic Spectra Database (version 4), [Online]. Available: http://physics.nist.gov/asd3, National Institute of Standards and Technology, Gaithersburg, MD, USA.
[19] B.B. Buchanan, W. Gruissem, R.L. Jones, Biochemistry & Molecular Biology of Plants, American Society of Plant Physiologists, Rockville, 2002.
[20] M.A. Sharaf, D.L. Illman, B.R. Kowalski, Chemometrics, Wiley, New York, 1986.