Bioimpedance Parameters as Indicators of the Physiological States of Plants *in situ*

A novel usage of the Electrical Impedance Spectroscopy technique

Elisabeth Borges, Mariana Sequeira, André F. V. Cortez, Helena Catarina Pereira, Tânia Pereira, Vânia Almeida, João Cardoso and Carlos Correia
Physics Department of the University of Coimbra
Instrumentation Center
Coimbra, Portugal
e-mail: eborgesf@gmail.com
e-mail: mariana.rodriguesequeira@gmail.com
e-mail: andre.f.cortez@gmail.com
e-mail: catawina.p@gmail.com
e-mail: taniapereira10@gmail.com
e-mail: vaniagalmeida@gmail.com
e-mail: joao.mr.cardoso@gmail.com
e-mail: correia@fis.uc.pt

Teresa M. Vasconcelos, Isabel M. Duarte and Neusa Nazaré
Escola Superior Agrária de Coimbra of the Instituto Politécnico de Coimbra
Centro de Estudos de Recursos Naturais Ambiente e Sociedade
Coimbra, Portugal
e-mail: tvasconcelos@esac.pt
e-mail: iduarte@mail.esac.pt
e-mail: neunazare@gmail.com

Abstract — **Diseases promoted by biotic or abiotic agents, characterized by a fast spreading rate and absence of symptomatology, are affecting plant species and crops of huge economic and/or forestall impact worldwide. The standard technique to diagnose diseases is the symptoms visualization by skilled personal, which is only accessible in the last stages of these diseases. As a restriction action, the plant, which is considered affected by a disease, is cut and burn along with neighbouring trees, even if these do not show evidence of the disease. Equipment and techniques able of assessing and characterizing the physiological state of plants *in vivo* and *in situ*, both in the diagnosis of diseases and also as a mean for supporting physiological studies, is clearly lacking. Herein is proposed the usage of impedance techniques to assess the physiological state of plants. Emphasis is given to the assessment of the hydric stress level of plants and its relation with the disease condition. To accomplish the study, a portable electrical impedance spectroscopy system was designed attending the biological application purpose. The procedure and the results obtained for three different species (*Pinus pinaster* Aiton, *Castanea sativa* Mill, and *Jatropha Curcas* L) of relevant economical and/or forestall interest is also presented in order to show the potential of this technique and system.**

Keywords **– plant disease; physiological state; hydric stress; biodiesel; impedance techniques**

I. INTRODUCTION

Living trees, bushes, or other types of plants, are affected by several diseases, which are caused by biological agents (for example: fungus, virus, bacteria, nematodes, insects) and/or adverse environmental conditions (promoted, for example, by: droughts, fires, extreme heat, contamination of soil, and air) [1], [2]. It may be important to assess the health state (or, in other words, the physiological state) of plants, especially when plant crops affected by such diseases and/or environmental conditions have economical and/or forestall impact [1]. Currently, there are several known diseases affecting specific crops of economic importance in certain regions of the planet [1]. For the scope of this paper and the corresponding study, it may be referred, for instance: 1) the pinewood nematode disease (PWD), affecting mostly the *Pinus pinaster* Aiton specie, which has spread worldwide with special relevance in Portugal, Japan and USA; 2) the ink disease in the chestnuts, caused by a fungus, which is affecting crops in Europe; and 3) the esca disease in the grapevines, caused by an association of fungus, which also has spread throughout the planet [1]. The major problem inherent to the referred diseases, whether they are caused by fungus, nematodes or other biotic agent (or, even not being the case, eventually by abiotic agents), is their asymptomatic behavior and fast spread [1], [2], [3]. Additionally, these diseases have no cure properly developed and commercialized to date [1], [2], [3]. The main reason is the arising of new problems due to the application of the studied solutions, mainly based on phytopharmaceutical technology, such as: soil contamination, animal and vegetal species new and unpredictable problems (such as toxicity, diseases, and extinction), among others [2], [3].

Although some instrumental prototypes were studied and patented, there is no defined methodology and accepted techniques to help to assist crops management in what concerns to the assessment of the physiological (or health) states of plants *in situ* [2], [3]. Actually, the golden standard method is the symptomatology visualization by skilled personnel [1], [2], [3]. The problem with this method is its reduced effectiveness, since the external symptoms are only able of being visually accessible during the terminal stages of the referred diseases [1], [2]. At this point, it is no longer possible to control the disease and, usually, it has already spread all over the crop, even if the symptoms are not visible in all the specimens [2]. In order to avoid the fast spreading, the plant that is considered affected, is cut and destroyed along with the neighbouring plants [2]. This preventive act poses another problem: the deforestation and the resulting economic losses arising due to the massif felling.

The characterization of physiological states of plants is also important in the perspective of marketing, consumption, and in researches for new plants' applications such as the emergent production of biodiesels [1], [4], [5]. In every case it is lacking detailed, fast enough, and robust techniques [1], [5], since the available require expensive laboratory equipment and materials, are time consuming, and hard to implement [1], [2], [6].

Hereupon, it can be said that, in general, there is a lack of equipment and systems able of evaluate and characterize the physiological state of plants, as well as a reliable and expeditious technology to allow an assessment *in situ* [1].

This overall described panorama was the main motivation for the present work [1]. Herein, the authors propose an Electrical Impedance Spectroscopy (EIS) system, developed by the team, and the usage of impedance techniques to assess the physiological states of plants *in situ* [1].

EIS has been proving efficacy and utility in a wide range of areas, from the characterization of biological tissues to living organisms [1], [7]. This passive electrical property, known as electrical impedance, is a measure of the opposition to the flow of an alternating current, which occurs when an external electric field is applied [1]. A current I crossing a section of material of impedance Z, drops the voltage V established between two given points of that section [1]. This yields the well-known generalized Ohm's law: V=IZ, where V and I are, respectively, the voltage and current complex scalars and Z the complex impedance [1]. Therefore, the result of the EIS measurements is a set of complex values (magnitude and phase), of impedance versus frequency [1].

Any biological material has its own electrical signature, whereby the physiological changes, due to diseases and nutritional or hydration levels, have direct influence in the impedance spectrum [1].

The major contributors for the biological tissues impedance are the cell membranes and the intra- and extracellular fluids [1], [7]. To electrically represent a biological tissue it is generally used a circuit that consists of a parallel arrangement between a resistor, representing the extracellular fluid, and a second arrangement connecting a resistor, simulating the intracellular fluid, and a capacitor as the membrane [1], [8], [9]. The determination of the ohmic values of the intra- and extracellular fluids, and also the capacitive value of the membrane, can be achieved by means of the theoretical Cole model [1], [10]. In this model, the achieved impedance spectrum (resistance versus reactance) is named by Cole-Cole plot [10].

The phase angle and other interrelated indexes, such as the ratio between impedance at the lowest and at the highest frequencies the system can output, Z_0/Z_∞, [7], and the ratio between impedance at the lowest frequency the system can output and 50 kHz, Z_0/Z_{50} [11], have been used to extract information about the physiological condition of biological materials. Work such the one being presented, where a deep study was carried out, the amount of data to be analyzed may hamper the use of the Cole model. The usage of the referred impedance indexes, and also the study of new ones, may be a lighter and equally valid approach.

Currently, there are instruments commercially available with high precision and resolution that can operate within a frequency range from some Hz to tens of MHz [1], [7]. However, the degradation of the excitation signal, occurring at low and high frequencies (already above 100 kHz), affects the accuracy of the measurements [1], [7]. Besides, the existing equipment is unfeasible for *in vivo* [1], [7] and in field applications, since it consists in desktop instruments, such as impedance analyzers and LCR meters [1].

It is possible to excite the sample with a current and measure a voltage or do the exact opposite [1]. The choice of the most suitable source, current or voltage, is a topic that still remains in discussion [1]. Current sources (CS) provide more controlled means of signal injection [12] and present reduced noise due to spatial variation when compared with voltage sources (VS) [1], [13]. However, due to their output impedance degradation [13], CS accuracy decreases for high frequencies [1], [14]. For this reason and since the impedance measurements are only possible when current is linear with respect to the voltage applied [9], or vice-versa, CS need expensive high-precision components [15] and a limited bandwidth operation range [1], [14], [15]. On the other hand, although EIS systems based on VS are less accurate [15], they can be built with less expensive components [15] and operate over a larger frequency range [1], [14], [15].

The developed EIS system, presented also in this paper, is able to perform AC scans within a selectable frequency range [1] and it can drive either a current or a voltage signal to excite a biological sample *in situ* or *in vivo*. It also implements the phase sensitive detection (PSD) method, although the one used has a novel implication, which is presented in further section. The instrumentation was designed to be cost-effective and usable in several applications [1]. The Table I resumes the main characteristics of the developed EIS system.

This paper will present the most relevant studies obtained for three different plant species: pine *(Pinus pinaster* Aiton), cheastnut (*Castanea sativa* Mill) and *Jatropha Curcas* L. The choice of the plant species under study was substantiated by the economic and/or forestalls relevance they have. Chestnuts and pines have a crucial economic impact in our country and are currently affected by uncontrolled diseases: the nematode disease, in the case of pine trees, and the ink disease, in the case of chestnut trees. *Jatropha curcas*, by other hand, is a tropical species, which seeds are being studied and used for biodiesel production. The available equipment to assess seeds quality is mainly constituted by heavy and expensive laboratorial instruments, which required methodology is time consuming and usually implies the usage of expensive materials/reagents. Accordingly, a true physiological assessment, based on a reliable technique, may help to assist the ongoing studies and contribute for the advance of its state of the art.

The assessment of the hydric stress (HS) level and its relation with the disease condition was the main physiological state being focused on this work [1].

The HS refers the internal hydration condition of a plant and it is one of the most relevant parameters to assess physiological states [5]. This parameter takes special significance in the assessment of diseases, since water absorption by the plant is one of the physiological processes firstly and strongly affected during a biotic or abiotic disease condition [5].

The following sections of this paper are: 1) *System Design*, where the developed EIS system is presented in detail; 2) *Assessment of the HS Level*, which presents the method and the results obtained for the HS assessment for the three studied species; 3) *Study of Disease Condition*, which presents the method and the results obtained for the study of the nematode disease in *Pinus pinaster* specie; 4) *Discussion*, where results are explained with more detail; and 5) *Conclusions*, where the main obtained results is resumed.

TABLE I. SUMMARY OF SPECIFICATIONS OF THE EIS SYSTEM

	Range	
Parameter	***Current Mode***	***Voltage Mode***
Measuring method	2 electrodes	
Frequency	1 kHz to 1 MHz	
Signal amplitude	25 uA	4.6 V
Impedance magnitude	2.5 kΩ to 100 kΩ	1.5 kΩ to 2.2 MΩ
Impedance phase	-π rad to π rad	-π rad to π rad
Mean absolute magnitude error	1675.45 Ω	709.37 Ω
Mean absolute phase error	2.45 %	2.06 %
Mean distortion	0.29 %	0.48 %
Mean SNR	117.0 dB	118.8 dB
Calibration	Automatically calibrated by software	

II. SYSTEM DESIGN

In order to study the plants' bioimpedance behaviour and extract conclusions from their physiological states, an EIS system was designed and built attending the requirements of portability, reliability, fast data assessment, and accessible methodology.

A. *General Description*

The developed EIS system employs three main modules: signal conditioning unit, acquisition system (PicoScope® 3205A) and a laptop for data processing (Matlab® based software) [1] (see Figure 1).

The digital oscilloscope PicoScope® 3205A synthesizes and provides the excitation AC signal to the conditioning unit (ADC function) [1]. It also digitizes both excitation and induction signals at high sampling rates (12.5 MSps) and transfers data to the computer via USB where it is stored [1].

The signal conditioning unit receives the excitation AC signal, coming from the PicoScope®, and amplifies it to be applied, through an electrode, to the sample under study [1]. The induced AC signal is collected by a second electrode, which redirects it to the conditioning unit, where it is amplified. Both excitation and induced signals are conduced to the PicoScope® to be digitized [1].

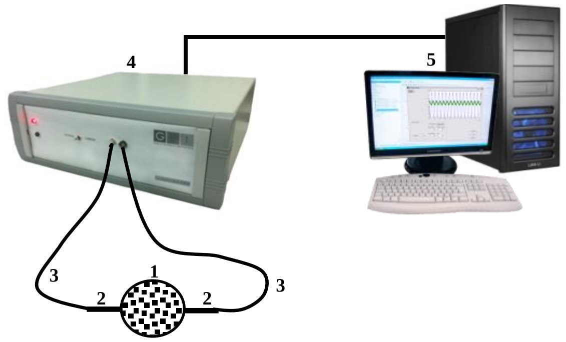

Figure 1. Schematics of the EIS system (OEM version) – 1) Biologic sample; 2) electrodes; 3) short coaxial cables; 4) EIS system conditioning unit and acquisition system, with the Picoscope®3205A incorporated; 5) laptop/PC.

The EIS system is able to generate both voltage and current signals. An external switch allows the user to select one of these two sources of excitation leads to optimal data. The specifications of both excitation modes are described below.

B. *Design Specifications*

Already studied by Seoane, Bragós and Lindecrantz, 2006 [16], the current mode circuit employs the current-feedback amplifier AD844 in a non-inverting ac-coupled CS configuration (see Figure 2) [1].

During the impedance measurements, the dc-blocking capacitor, between the source and the electrode, tends to charge due to residual DC currents [1], [15]. For this reason, the transimpedance output of the AD844 easily reaches the saturation [1]. To overcome this problem, it was implemented a DC feedback configuration, which maintains the dc voltage at the output close to 0V, without compromising the output impedance of the source [1]. Therefore, the output current is maintained almost constant over a wide range of frequencies [1].

The high speed voltage-feedback amplifier LM7171 is employed in the voltage mode circuit (see Figure 2) [1]. Although it behaves like a current-feedback amplifier due to its high slew rate, wide unit-gain bandwidth and low current consumption, it can be applied in all traditional voltage-feedback amplifier configurations, as the one used [1]. These characteristics allow maintaining an almost constant voltage output over a wide range of frequencies [1].

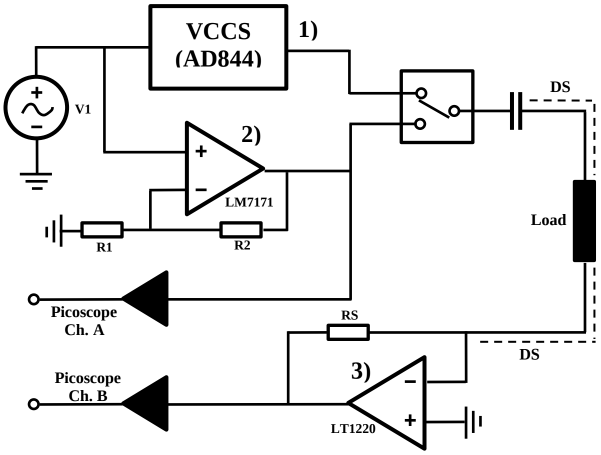

Figure 2. Schematic of the EIS system conditioning unit - 1) AC current source; 2) AC voltage source; 3) current/voltage sense.

The high speed operational amplifier, LT1220 (see Figure 2), senses the current or voltage signals from voltage or current excitation modes, respectively [1]. It performs input with reduced offset voltage and it is able of driving large capacitive loads [1].

The value of the gain, for both current and voltage excitation sources, can be changed in order to extend the range of impedance magnitude [1]. The transductance gain of the LT1220 is currently set to 5.1 kΩ [1]. This value actually defines the gain of the system, which is taken into account for the impedance data algorithmic calculation. This means that, since the gain values are known and also the amplitude of the AC excitation signal, V_1, from the PicoScope® (see Figure 2), the EIS system is calibrated automatically by software [1].

C. PSD Method

To assess the impedance phase shift it is implemented a digital Phase Sensitive Detection (PSD) method with a novel implication. As stated in the literature, the PSD method is a quadrature demodulation technique that implements a coherent phase demodulation of two reference (matched in phase and quadrature) signals [17]. It is also known that this method is preferable over others especially when signals are affected by noise [17].

The signal from the Picoscope® that corresponds to the current, VI=Bsin(ωt+φ2), is set as the reference signal. Since the phase of the signal VI is not controlled, it is easily understandable that it does not necessarily contain a null phase. This statement remains valid whether VI is used to excite the sample, in the current mode, or whether it corresponds to the current passing through the sample, in the voltage mode. The signal from the Picoscope® that corresponds to the voltage, VV=A sin(ωt+φ1), also contains a non-null phase. Both amplitudes, A and B, are also different from each other and none equals to 1.

The following block diagram, shown in Figure 3, supports the mathematics implicit in this novel PSD method.

Along with the mathematical demonstration (not presented in this paper due to its extension), the developed PSD algorithm was tested with Matlab® for several phases and amplitudes, without the theoretical requirements (i.e., ensure that the reference signal has null phase at the origin and that its amplitude equals to 1 [17]). For all the tests it was showed an always corrected phase shift assessment, when compared to the results obtained for a reference signal with the theoretical characteristics (see Figure 4).

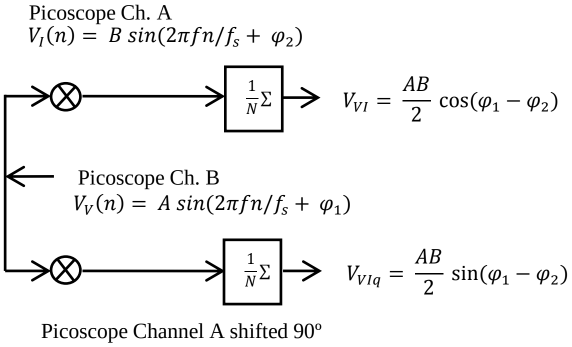

Figure 3. Schematic of Phase-Sensitive Demodulator implemented in the developed EIS system.

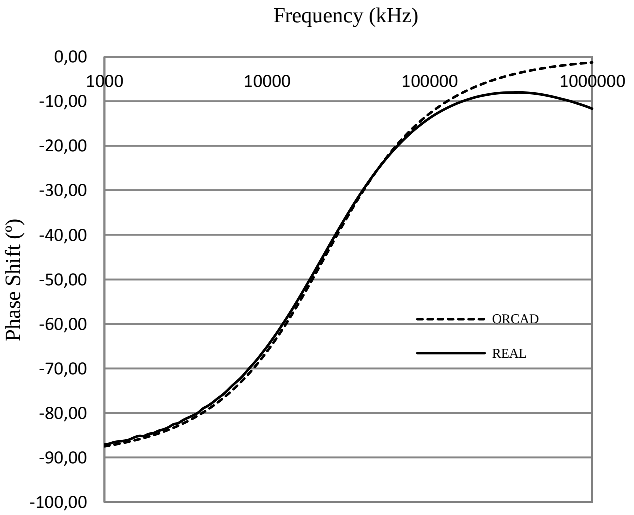

Figure 4. Comparison between impedance phase of a real data and Cadence® simulated data for a RC circuit. The deviation that occurs between the graphics, at high frequencies, is due to the influence of stray capacitances (see section below).

D. Electrodes and Cables

The developed EIS system employs two beryllium cooper gold platted needles as electrodes, each with around 1.02 mm in diameter [1]. In order to reduce the dispersion of the surface current density flow [1], [10] and also to reduce damage on the biologic sample [1], the electrodes are inserted so lightly as possible (1 to 5 mm deep depending on the thickness of the cork).

The electrodes are connected to the acquisition unit through coaxial cables. The employment of this type of cables is justified by the necessity to obtain an optimized signal-to-noise ratio [1]. However, coaxial cables introduce high equivalent parasitic capacitances, which promote phase shift errors, especially at high frequencies, during the bioimpedance measurements [1]. For this reason, the employed RG174 RF coaxial cables (capacitance of 100 pF.m-1) are as short as possible (around 15 cm). It was also implemented the driven shield technique to the coaxial cables, which permits to partially cancel the capacitive effect, that otherwise is generated between the internal and the external conductors, by putting both at the same voltage [18]. Reductions in the capacitive effect of 20.4%, in the current mode, and around 35.8%, in the voltage mode, at the highest frequencies are verified. Figure 5 depicts the capacitive effect reduction by the usage of the driven shield technique.

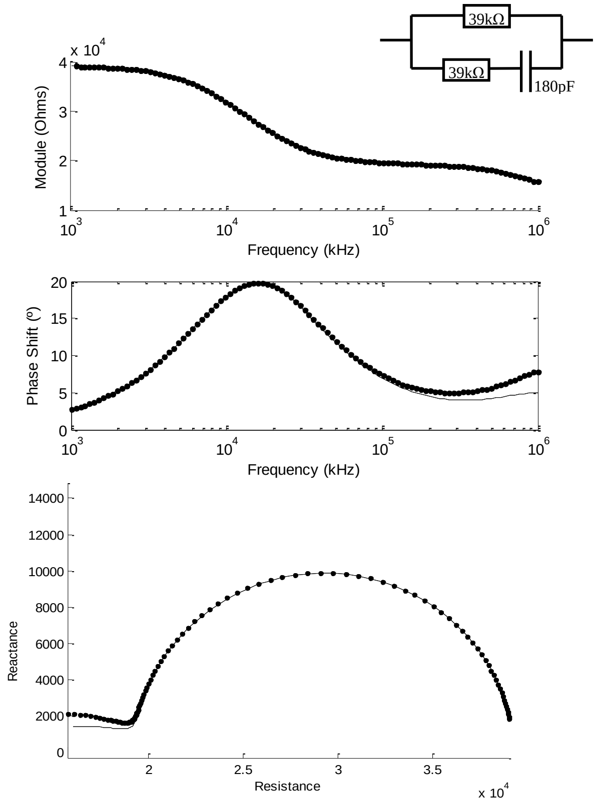

Figure 5. Bode and Cole-Cole diagram showing the reduction of cables capacitive effect by the application of the driven shield technique. The voltage mode excitation was used to analyze the circuit at the right top. The reduction is more noticeable at high frequencies where the capacitive effects have more influence.

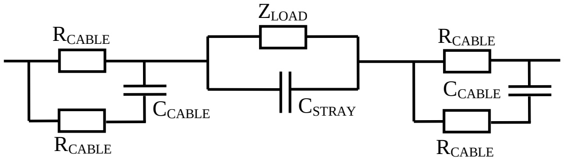

Figure 6. Equivalent electric circuit of all parasitic elements affecting impedance measurements of a load, Z_{LOAD}. The effect of the stray capacitances from cables, C_{CABLE}, is minimized by the driven shield. Other stray capacitance effect, C_{STRAY}, due primarily to the phase shift of amplifiers, can be minimized by software.

The phase shift occurred due to capacitive effects is not only promoted by cables, but also by the amplifiers [1]. The cumulative effect of the phase shift errors outcomes in an inflexion point in the impedance spectra that occurs at high frequencies (see Figure 5) [1].

This type of error affects not only the phase at high frequencies, but also the impedance magnitude, which presents a typical decline shown at the Bode diagrams (see Figure 5) [1]. In the developed EIS system, the slight decline of the impedance magnitude is due to the loss of the product gain-bandwidth of the LT1220 for high frequencies [1].

The equivalent electric circuit of such a system foresees this capacitive effect behavior, which is represented through an extra capacitor, called stray capacitance, placed in parallel with the load in analysis [1]. It is considered that stray capacitances induce systematic errors since their effect is present in all measurements. Although the results are not greatly affected by the influence of stray capacitances, it is opportune to be aware of the real equivalent circuit (see Figure 6). By this way, it is possible to consider and/or discount the effect of all parasitic elements where justified.

III. ASSESSMENT OF HYDRIC STRESS LEVEL

The HS level was one of the studied impedance parameters due to its significance, as explained in Section I. Below are presented the used materials and methods, along with the main results.

A. Materials and Methods

For assessing EIS profiles and studying the HS level the following specimens were used: 1) eight young healthy pine trees (*Pinus pinaster* Aiton), with about 0.8 meters tall and 1 to 2 centimeters in diameter; 2) eight young healthy chestnuts trees (*Castanea sativa* Mill), with about 0.5 meters tall and 1 to 2 centimeters in diameter; and 3) eight young healthy *Jatropha Curcas* L. trees, with about 0.2 meters tall and 3 to 4 centimeters in diameter [1].

The populations of different plant species were kept under controlled environment conditions – temperature (27 – 30 ºC), luminosity, soil content and watering – in order to reduce the quantity of variables that may change the EIS profiles [1].

A portable EIS system version was employed to perform the EIS measurements. The electrodes were placed in the trunk of each tree, in a diametric position, and about 20 cm above the soil, in the case of the pine and chestnut trees, and about 10 cm above de soil, in the case of *Jatropha curcas* (since these specimens were shorter in height) [1]. It was used the voltage mode of excitation [1], since the achieved impedance signals were cleaner for this mode (i.e., optimal signal-to-noise ratio due to better current signals amplitudes). A frequency range between 1 kHz and 1 MHz was selected to accomplish the EIS analysis [1], since this frequency range has been shown to be sufficiently wide for studying these species.

Routine acquisitions took place between 11 a.m. and 1 p.m. since it was already verified in previous studies that at this time period the trees' impedance is lower and presents less variation (see Figure 7, for example) [1].

To study the HS level variation, EIS monitoring was performed over 8 months, for one individual at a time, and for each plant species [1]. After understanding the variation of the EIS profile of regularly watered plants, the specimen being monitored was kept without watering during a period of a time (usually 3 to 4 weeks). When the HS effects were visible through external plant morphology symptoms, the specimen was regularly watered again to avoid the dead of the biologic material. In the case of the studied pines, the visualization of external symptoms was only possible near the time the specimen was about to die (which precluded the recovery of some individuals). For this reason, the variation of the water abstinence time period (WAP) and the plant response to it, measured by the EIS system, was one of the main focuses of this study.

B. *Results*

Several impedance parameters were determined for each impedance spectrum, however only the main indexes, such as the index $Z_1/Z_{50,}$ will be presented as results, due to paper space limitations and also because this is a parameter already studied in the literature [1]. The index 1 is used instead of the index 0, as stated in the Introduction section, which corresponds to the lowest frequency analyzed by the system (1 kHz) [1].

The time continuous measurements allowed revealing a daily oscillation of the EIS profiles [1]. To verify this behaviour it was assessed the R_1/R_{50} (R representing the magnitude module) for a period of 6 days [1]. A Fast Fourier Transform was determined for this ratio just to confirm that the rhythmic signal had a period of exactly 24 h [1]. A clear frequency value of 11.57 µHz, which corresponds to a frequency of 24 h, was founded [1]. This rhythmic signal was also perceptible for other studied indexes, although they are not presented in the paper.

The higher values of the ratio R_1/R_{50} correspond to the night period, where the luminance and temperature are lower in relation to the day period [1]. Furthermore, these studies and previous ones revealed that the variation of the impedance values is lower during the period where the illumination was higher (between 11 a.m. and 1p.m.).

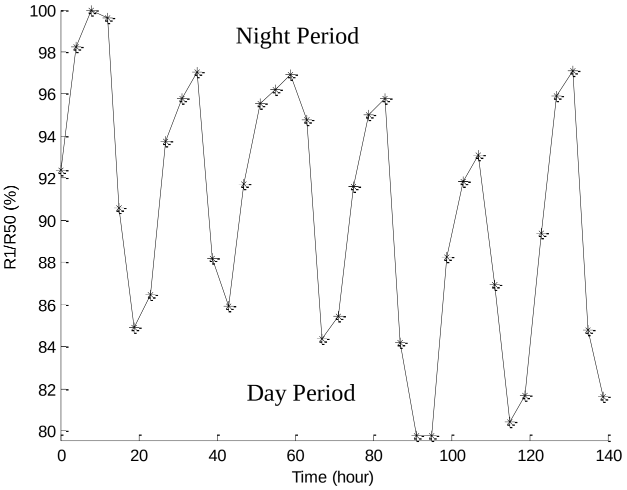

Figure 7. Variation of the R_1/R_{50} ratio during the monitoring of a healthy pine tree with regular watering. The impedance values show a daily oscillation that is characteristic of the studied trees.

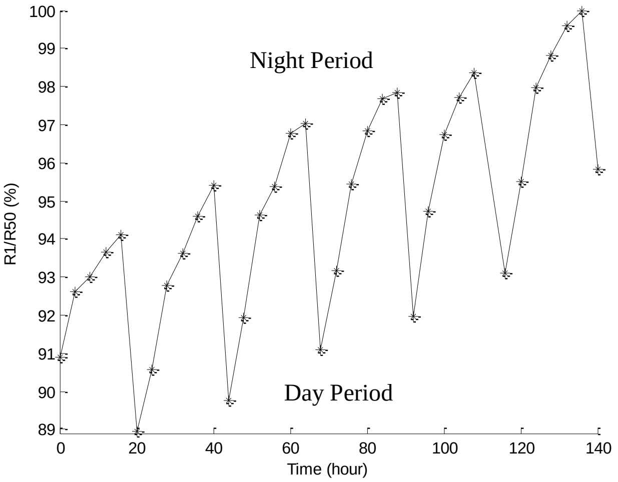

Figure 8. Variation of the R_1/R_{50} ratio during the monitoring of a healthy chestnut without watering. The impedance values show a daily oscillation that is characteristic of the studied trees. The successive higher values of each cycle are due to the incrinsing level of HS.

Data obtained for the EIS monitoring and the study of the HS level, revealed a consistent behavior for all the studied species: the R_1/R_{50} ratio tends to increase with higher values of HS [1]. After introducing regular watering, the same parameter progressively tended to the typical values of hydrated trees [1]. This effect is much more visible in the chestnuts, i.e., the variation of impedance parameters due to HS is higher and faster for the chestnuts, when compared with pines [1].

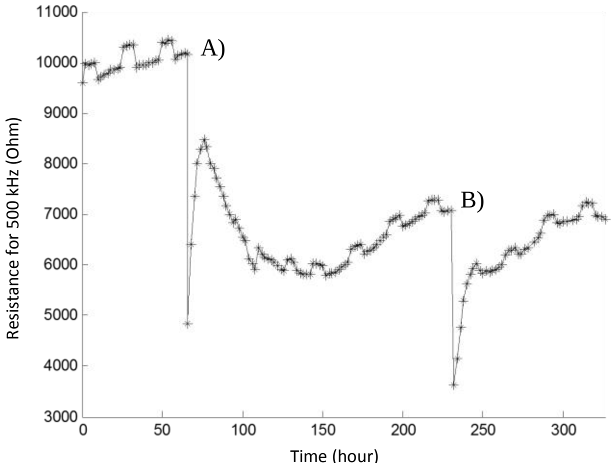

Figure 9. Variation of the R_{500} index during the monitoring of a healthy *Jatropha curcas* (12 days). The initial WAP was 6 months, period after which the EIS measurements started. The plant was watered at the 4th and 9th days of monitoring, points A) and B) respectively. It is visible the immediate response to watering and also an immediate rise of resistance after the normal impedance values restoration.

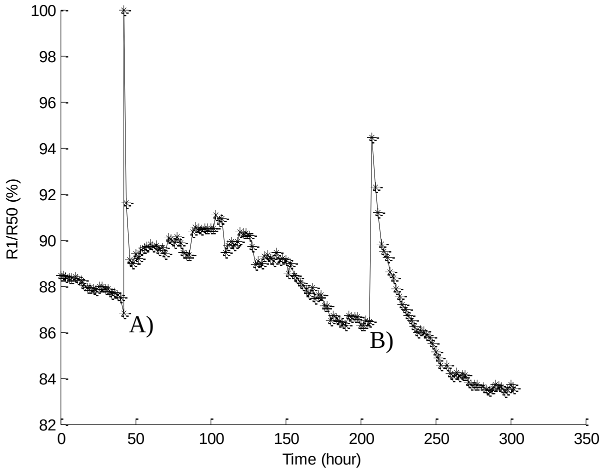

Figure 10. Variation of the R_1/R_{50} index during the monitoring of a healthy *Jatropha curcas* (12 days). The initial WAP was 6 months, period after which the EIS measurements started. The plant was watered at the 4th and 9th days of monitoring, points A) and B) respectively. It is visible the immediate response to watering and also an immediate rise of resistance after the normal impedance values restoration.

In relation to *Jatropha curcas* it was observed a totally different behaviour. To achieve measurable and significant levels of HS it was necessary to leave the plant without watering during months (5 months at least). After watering, the response of the plant was instantaneous, even faster than the chestnut response. However, depending on the HS level, the establishment of the normal hydration values may take 1 to 2 days or, otherwise, it may be practically immediate (see Figure 9). Once the HS levels were established, impedance values start to rise also immediately.

The rate of impedance augmentation is faster for the first weeks (2 to 3 weeks) when the plant is without watering and then tends to stabilize towards a value, which is maintained during months. The achieved different results, in relation to pine and chestnut trees, could be explained due the existence of latex vessels in the *Jatropha curcas* specie [1]. Figure 9 shows the particular behaviour of *Jatropha curcas* for the index R_{500} (resistance at a frequency of 500 kHz).

In relation to the R_1/R_{50} index – just to compare with the previous two cases – it tends to decrease for higher values of HS level and, after introducing regular watering, the ratio abruptly assumes the typical values (see Figure 10) [1].

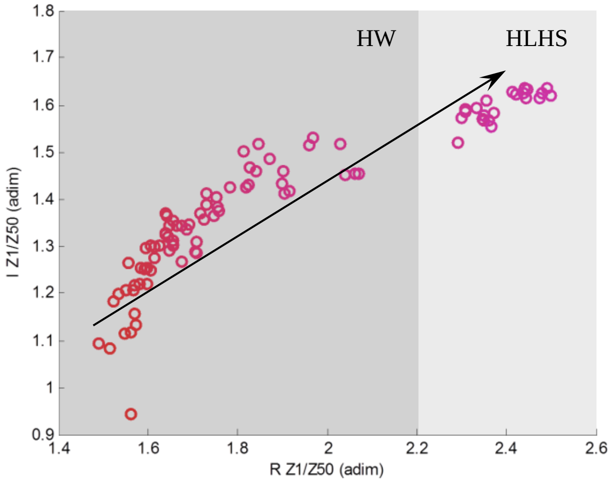

Figure 11. Evolution of the Z_1/Z_{50} ratio, in a real vs imaginary plot, during the monitoring of a healthy chestnut tree while kept without watering. The arrow indicates the direction of the Z_1/Z_{50} ratio evolution. The impedance values of the regions HW (healthy and watered) and HLHS (high level of HS) correspond to the indicated physiological states.

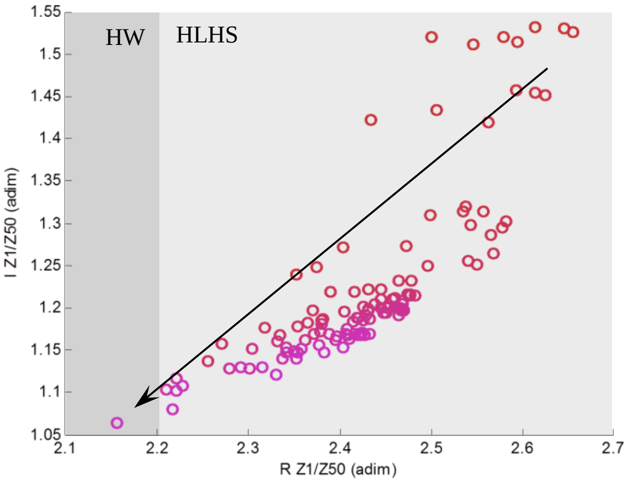

Figure 12. Evolution of the Z_1/Z_{50} ratio, in a real vs imaginary plot, during the monitoring of a healthy chestnut, with high level of HS, after intruducing regular watering. The arrow indicates the direction of the Z_1/Z_{50} ratio evolution. The impedance values of the regions HW (healthy and watered) and HLHS (high level of HS) correspond to the indicated physiological states.

In order to consider the variation of the total impedance, and not only the variation of its real part (resistance), there were studied other impedance indexes, such as the Z_1/Z_{50} index, presented in this paper.

Besides the monitoring of one plant of each group/specie, there were also performed single acquisitions to the seven remaining plants, which were kept under regular conditions (including watering).

The calculation of the mean HS values of these healthy and watered trees, allowed the determination of trees with higher HS levels and also tress with diseases (see Section IV). This information together with the graphical representation of this specific index, Z_1/Z_{50}, allows the easy identification of regions that corresponds to different plant physiological conditions.

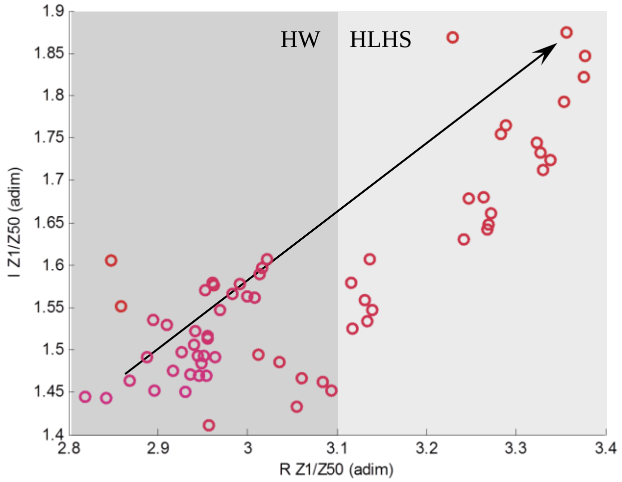

Figure 13. Evolution of the Z_1/Z_{50} ratio, in a real vs imaginary plot, during the monitoring of a healthy *Jatropha curcas* tree, with high level of HS, after intruducing regular watering. The arrow indicates the direction of the Z_1/Z_{50} ratio evolution. The impedance values of the regions HW (healthy and watered) and HLHS (high level of HS) correspond to the indicated physiological states.

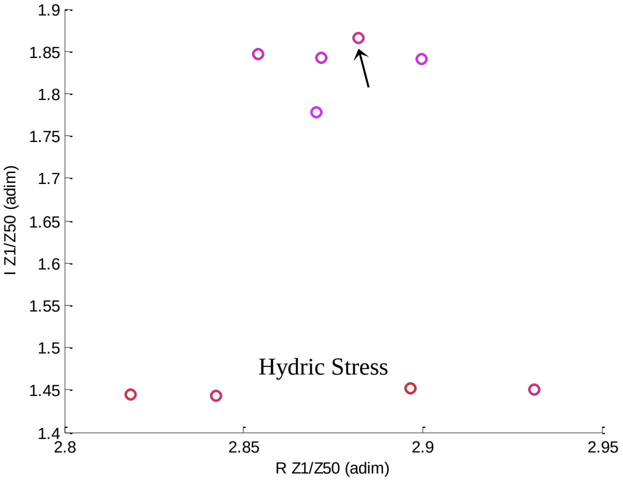

Figure 14. Example of the abrupt evolution of the Z_1/Z_{50} ratio, in a real vs imaginary plot, during the monitoring of a healthy *Jatropha curcas* tree after watering. The arrow indicates the first Z_1/Z_{50} value after watering.

The previous figures do not include the region that corresponds to the disease physiological condition, since this was not the purpose of the *Jatropha curcas* study. As it will be shown in the following section, the impedance values for this condition are much higher (at least for the imaginary part of the impedance) whereby they are out of the range of the presented plots.

IV. STUDY OF DISEASE CONDITION

In order to understand the EIS behaviour and the physiological states variations for a plant affected with a disease, it was performed a study with pines where some individuals were inoculated with nematodes. Below, the study undergone is explained, along with the main obtained results.

A. Materials and Methods

To perform this study, there were used twenty four pine trees (*Pinus pinaster* Aiton), with about 2.5 meters tall and 2 to 3 centimetres in diameter [1]. Pine trees were placed at a greenhouse, in vases, and under controlled water environment [1]. Temperature, humidity and luminosity were also controlled. In order to keep a difference of the hydric stress level, half of the tree population was less watered (2 minutes per day, ~ 66.67 mL/day) when compared with the other half (5 minutes per day, ~133.37 mL/day) [1].

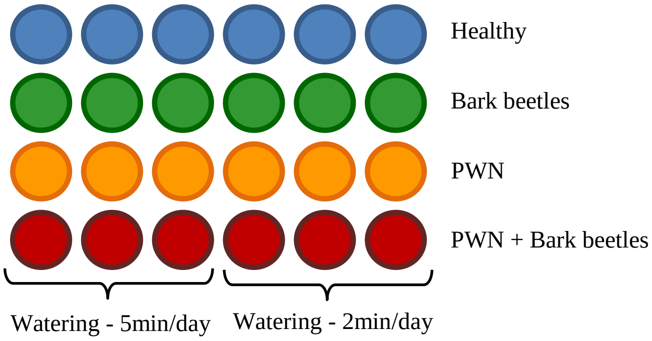

Figure 15. Schematic of the distribution of the sub-population groups of pines at the greenhouse. Each group of three pines has different conditions.

The main purpose of this study was to assess the disease physiological state condition of the pines for the specific case of the PWD. As stated in the Introduction section, this disease is caused by the pinewood nematode (*Bursaphelenchus xylophilus* Nickle), PWN, which is housed in the tracheas of the *T. destruens* Wollaston, a bark beetle. Before the PWN arrival, the bark beetles coexisted with pines without causing tree damage. To prove the harmlessness of this pest, in terms of HS, and also to have a control population group, both PWN and the bark beetles were inoculated in separated groups of pines. Six pines were inoculated with PWN, other 6 pines were inoculated with bark beetles, other 6 pines were inoculated both with PWN and bark beetles, while the remaining 6 were kept healthy [1]. The pines of each sub-group were positioned at the greenhouse in such a way that half of them, i.e. three pines for each sub-group, were regularly watered (5 min/day) while the other half was less watered (2 min/day) [1] (see Figure 15 for a better understanding).

The pines inoculated with bark beetles were covered with a Lusatril tissue to avoid beetles escape [1]. In each tree there were placed 15 insects, which were collected immediately after the emergence phase [1].

The method used to inoculate the PWN was somehow innovative [1]. Three 2 x 2 cm rectangle of cork were removed at different locations of the trunks and, the exposed phloem, was erased with a scalpel in order to improve the adhesion of the nematodes [1]. In each incision it was placed a 0.05 mL of a PWN suspension, in a total of 6000 nematodes per tree [1]. After the inoculation, each removed rectangle of cork was fixed in the respective place and wrapped with plastic tape [1].

The EIS measurements started seventy days after the inoculations [1]. At this period, pines inoculated with PWN presented a decay rounding the 40% and certain symptoms of the disease were already visually accessible [1]. Two of the healthy pines died (decay of 100%) due to hydric stress, and all the remaining trees appeared healthy [1].

There were taken two EIS measurements per tree between 11 a.m. and 1 p.m. The portable version of the EIS system was used with a frequency range from 1 kHz to 1 MHz was applied in the voltage mode [1]. The electrodes were placed in the trunk of each tree in a diametric position, about 30 cm above the soil [1].

The trunk of each pine tree, inoculated with the PWN, was cut in three different regions in order to relate the EIS data with the stage of the disease and the number of nematodes per section [1]. The cuts were performed: a) immediately below the inoculation incision (180 cm above the soil); b) 30 cm above the soil (where EIS measurements took place); and c) in the middle of the previous two cuts (approximately 80 cm above the soil) [1].

After these measurements, two healthy pines were monitored independently by two EIS portable systems [1]. After a week of monitoring, both pines were inoculated with PWN and the measurement continued during 7 more weeks [1]. The purpose of this last experiment was to study the variation of the EIS profiles during the pine decay due to the PWD [1].

B. *Results*

Several impedance parameters were assessed in order to study the physiological states of the trees [1]. Z_1/Z_{50} was the impedance parameter that showed the best results [1].

Similar values of Z_1/Z_{50} were obtained for both healthy pines and pines inoculated with bark beetles, suggesting that the beetles' action does not affect the physiological states of pines [1], at least in terms of the HS levels variation. In fact, it was expected a similarity between both EIS profiles since it is known that these insects does not damage the inner structure of the trees.

The pines inoculated with nematodes and those inoculated both with nematodes and bark beetles presented also similar values for the Z_1/Z_{50} index, but different from those obtained for the previous sub-groups [1]. In relation to the previous sub-populations, the values are characterized by a higher dispersion in terms of reactance (see Figure 16) [1]. Besides, it is observed a clear relation between the number of nematodes and the reactance dispersion for the Z_1/Z_{50} parameter: the higher the number of nematodes is, the higher the reactance value of Z_1/Z_{50} becomes (see Figure 17) [1].

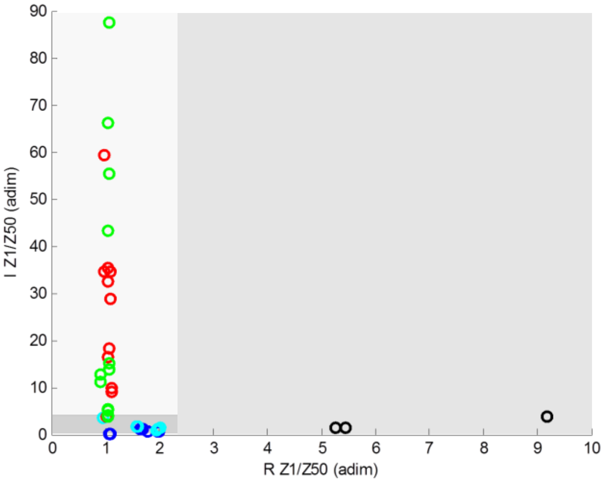

Figure 16. Values of the impedance parameter Z_1/Z_{50} for each of the 24 pine trees. Note that two values are represented for each pine. The impedance values of the regions HW (healthy and watered), HLHS (high level of HS) and D (disease) correspond to the indicated physiological states.

TABLE II. NUMBER OF NEMATODES IN THE TRUNKS OF PINE TREES PER CUT SECTION

Tree	Cut Section	Number of nematodes in 0,05 mL
1	***a***	1
	b	0
	c	133
2	***a***	0
	b	43
	c	1
3	***a***	0
	b	0
	c	112
4	***a***	4
	b	20
	c	0
5	***a***	0
	b	17
	c	0
6	***a***	0
	b	0
	c	14

In fact, the counting of nematodes in the several cut sections revealed that the concentration of nematodes was higher in the cut sections b) and c) for the pines less watered (pines 1, 2 and 3) – see Table II [1]. The concentration of nematodes in the lower parts of the trunks was much higher for the pines with less watering than for those with regular watering (pines 4, 5 and 6), since nematodes tend to move toward watered regions along the trunk [1]. For this reason, the referred relation was more perceptible for the pines with lower watering, than for those receiving more watering (see Figure 18), since the accumulation of nematodes in section c (see Table II) was higher for the first group [1].

The dispersion in terms of resistance is not significant when compared with values from pines in other physiological conditions (see Figure 16).

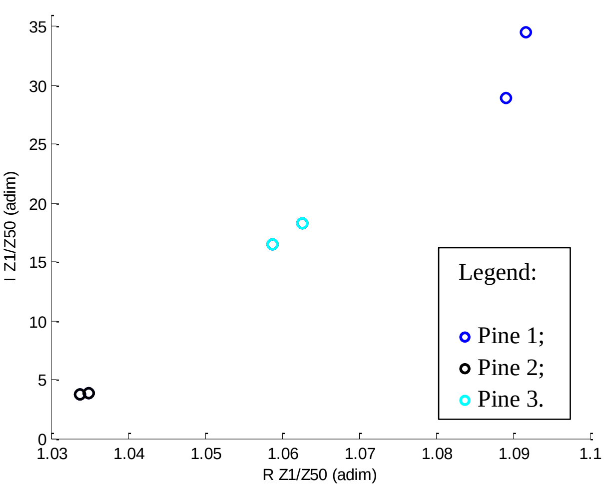

Figure 17. Values of the impedance parameter Z_1/Z_{50} for the pines inoculated with nematodes and with low watering (pines 1, 2 and 3 from the Table II). Note that there are represented two values for each pine.

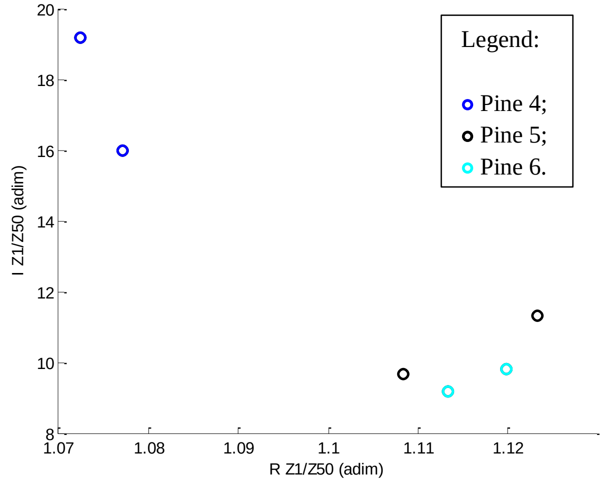

Figure 18. Values of the impedance parameter Z_1/Z_{50} for the pines inoculated with nematodes and with regular watering (pines 4, 5 and 6 from the Table II). Since the number of nematodes is low in these pines, the linear relation between them and the Z_1/Z_{50} values is not perceptible. Note that there are represented two values for each pine.

The pines that died due to HS (decay of 100%) were also studied and the Z_1/Z_{50} parameter presented the highest resistance values, in relation to all the other pines [1].

The monitored healthy pines were watered at different rates: one with low watering (2 min/day) and another with regular watering (5 min/day) [1]. After one week of monitoring both were inoculated with PWN [1]. As Figure 19 a) shows, it was again observed dispersion in reactance for the Z_1/Z_{50} index [1]. The reactance values, and consequently this dispersion, were successively higher as time was passing and the disease was evolving [1]. The higher values of reactance were achieved for the pine with less watering [1].

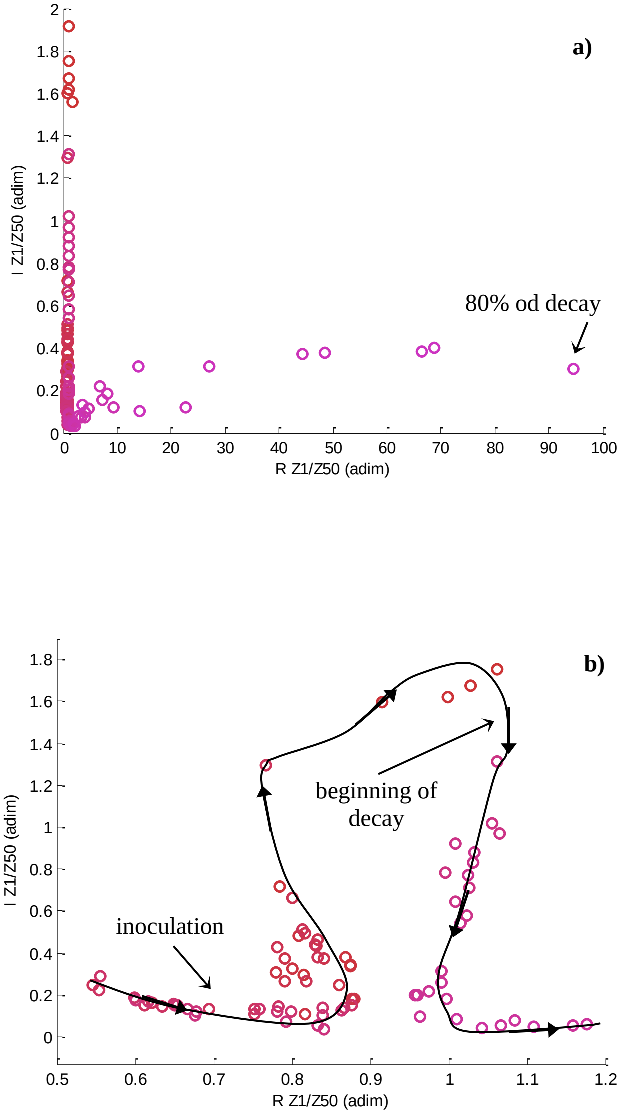

Figure 19. a) Evolution of the Z_1/Z_{50} during the monitoring of a pine, which was inoculated with nematodes during the measurements. b) Closer view from the Z_1/Z_{50} evolution, showing a hysteresis-like behaviour.

These results corroborate the previous ones: it was expected that the number of nematodes increase in the below part of the trunk for the pines with less watering and, consequently, to observe a higher rising of the reactance for the Z_1/Z_{50} parameter [1]. After the 6th week, pines start to decay strongly and it was observed a relevant decrease of the reactance and a significant increase of the resistance for the same parameter – see Figure 19 a). The higher values of resistance were achieved for the pine with less watering, and also in a shorter period of time [1]. At the end of the monitoring, the decay of the pines, evaluated by an expertise, was about 80 % for the pine with regular watering and 100 % for the pine with less watering [1].

From the Figure 19 b), which shows a closer view of the Z_1/Z_{50} index evolution during the monitoring, it is observed that the path that corresponds to the rising of the population of nematodes is different from the path followed during the period of decay [1]. For this reason, it is possible to affirm that PWD evolution has a hysteresis-like behavior [1].

It is worth noting that some impedance parameters may be preferable than others, depending on the physiological condition that is being studied. To evaluate the disease condition, the Z_1/Z_{50} index, allowed showing the great reactance increase that is characteristic of these diseases (since the cellular membranes are affected, which promotes a decrease of membranes capacitor effect) – see Figure 19.

Other parameters, such as R_1/R_{50}, which shown to be useful in the previous study, may not be the most adequate to study this specific case of disease condition (see Figure 20).

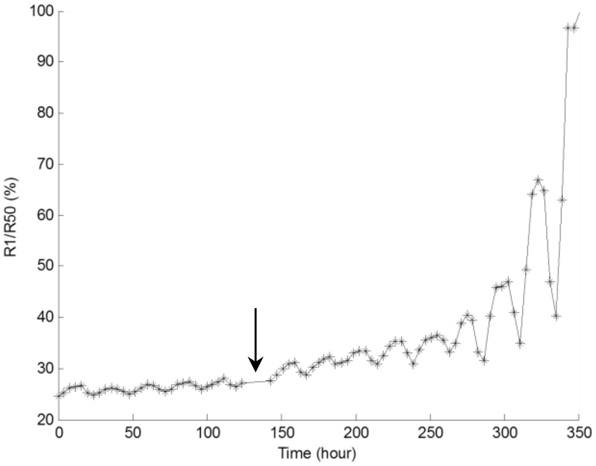

Figure 20. Evolution of the R_1/R_{50} during the monitoring of a pine, which was inoculated with nematodes during the measurements. It is possible to observe the daily oscilation and the increase of reactance for higher levels of HS as time was passing and the disease was evolving. The arrow indicates the graph bellow were some data is missing due to a system crash during the monitoring.

V. DISCUSSION

Assessing physiological states of plants, using impedance techniques, implies the knowledge of the typical EIS profiles of the species under study, i.e., the EIS profiles for healthy individuals under controlled environment conditions [1]. For this reason, the studies presented herein required an exhaustive EIS assessment and monitoring, performed over months, and extensive data analysis [1].

The greatest difficulty inherent in this type of studies is the purchase and maintenance of the plant specimens, which in addition to being time consuming, is extremely expensive. Besides, some of the performed tests required the death of the specimen under study, which worsens the referred problem. For this reason, a true statistical analysis, which may be required to achieve reliable results, was not possible to accomplish. However, the results presented herein constitute an innovative approach to assess physiological conditions of plants, giving plausible and interesting clues to solve the problem of the lack of techniques, and also methods, in this specific area.

The biological application study aimed at discriminating between different physiological states of three plant species, which have economical/forestall relevance: pine *(Pinus pinaster* Aiton), cheastnut (*Castanea sativa* Mill) and *Jatropha Curcas* L. [1] Attention was given to the assessment of the HS level and the disease condition.

To accomplish this task, an EIS system able to perform AC sweeps over a sufficient large frequency range, was developed. The design of the EIS system took into account the robustness, efficiency and celerity of the bioimpedance analysis [1]. The portability, adaptability to different biological applications and the implementation of easily accessible and affordable components were also considered aspects [1]. The system versatility is another advantage [1]. It allows the user to choose the settings of the analysis that best fit a specific application, such as: frequency limits, number of intervals of the scan and type of signal excitation (voltage or current) [1]. Besides, a driven shield technique is applied in order to overcome problems inherent to stray capacitive effects from cables [1]. The maximum phase shift reduction estimated was about 20.4 % for the current excitation mode and about 35.8% for the voltage mode [1]. The system also implements a PSD method with a novel implication to determine the impedance values, which shown to be effective.

The great amount of data, typical in these types of studies, imposes a problem to the use of theoretical models; such is the case of the Cole model. For this reason, the approach followed in this work was the research of bioimpedance indexes that better expressed specific physiological conditions. Although, a specific bioimpidance index does not require a true impedance analysis, i.e., a frequency scan, the method used has proven the necessity of a spectroscopy analysis, since the indexes relations and their correlation to the physiological conditions may be better expressed for some frequencies than others.

The obtained results suggest that the implemented method may constitute a first innovative approach for the assessment of physiological states of plants and to the early diagnosis of plant diseases [1]. The consistency of the results obtained for the three studied species reveals the transversality of the method [1].

Two of the most interesting bioimpedance indexes are the ratio Z_1/Z_{50} and also the ratio R_1/R_{50}, which were studied and presented in this paper.

EIS profiles showed a consistent behaviour whit the HS level of the three studied species [1]. The Z_1/Z_{50} impedance parameter presents increased values of both reactance and resistance when the hydric stress is high, for the pines and chestnuts [1]. In the case of the *Jatropha curcas*, this parameter presented decreased values of both reactance and resistance when the HS was high [1]. This inverse behaviour may be explained due to the presence of latex vessels in this species, which may function as water reservoirs [1].

The evolution of the Z_1/Z_{50} impedance parameter may be used to predict risky HS levels of a plant.

However, as it was shown, the HS level of a plant exerts much more influence over the real part of the impedance than over its imaginary part. Actually, the phase shift alterations due to progressively higher levels of HS are almost inexistent. For this reason, the R_1/R_{50} or other interrelated resistance (real part of the impedance) indexes may constitute preferable approaches to study the HS level and its use as a risk predictor.

In addition to the study of the HS level and its influence in the plant physiological condition, it was studied the disease physiological condition for the pines, were inoculations with PWN and bark beetles were performed.

The pines inoculated with PWN presented Z_1/Z_{50} ratio with high values of reactance, what suggests that current flows preferably trough the cytosol [1]. In fact, the action of the nematodes inside the tree may destroy cell membranes, which means that membranes capacitor effect becomes less significant in the impedance measurement [1].

It was also shown a relation between the number of nematodes and the Z_1/Z_{50} index: the increasing of nematodes number is proportional to the reactance ratio augmentation [1].

On the other hand, the action of bark beetles seems not to interfere, at least in measurable terms, in the level of HS of pines, since the values obtained were similar to those obtained for healthy pines [1].

The index Z_1/Z_{50} for healthy trees with high levels of HS (decays above 80) presented high values of resistance, due to water loss [1]. High resistance values were also obtained for the trees in advanced stages of the disease [1]. This means, that for this specific case, the method is not able to distinguish between healthy trees and trees with the PWD [1]. However, it is known that the advanced stages of the PWD induce high levels of hydric stress, which allows to infer that, in practical terms, the situation is exactly the same, i.e., the tree presents high probability to die [1]. On the other hand, in the stages where the method is able to distinguish between trees with the PWD and healthy trees, and since the decay was determined to round the 40%, the diagnosis could help to assist pine management [1]. If a cure is available, at these stages, a treatment could be administrated and reverse the disease evolution [1].

The implemented method has allowed identifying three clear regions on the Z_1/Z_{50} graph that corresponds to: healthy and watered trees (HW), tress with high level of HS (HLHS) and trees with disease (D). Although, the high levels of HS, obtained by the R_1/R_{50} analysis, may drive the tree to dead or, in other words, the levels of HS may be used as a risk indicator, the use of several indicators and biompedance indexes is necessary to perform a reliable study (at least these two: R_1/R_{50} and Z_1/Z_{50}). In fact, the studied disease indicated a clear region characterized by higher levels of Z_1/Z_{50} reactance and normal levels of Z_1/Z_0 resistance, where the levels of HS based on the R_1/R_{50} ratio seemed normal. Besides, as stated, the final stages of the disease presented impedance similar values to those obtained for trees with HS levels. What is worth adding is that it may be possible to interfere, with a cure for example, during the evolution of the disease when the Z_1/Z_{50} graph shows a D physiological condition. If the graph shows a HLHS condition the tree is probably about to die either it has a disease promoted by biologic agents, such the PWN, or not.

In order to summarize the discussion, the method studied and presented herein, based on bioimpedance indexes, may constitute a first innovative approach to the assessment of the main physiological conditions (healthy state, HS level and disease), since the referred indexes may be used as risk predictors.

VI. CONCLUSION

The developed EIS system showed to be a robust, reliable and easy to implement equipment in the assessment of the physiological states of living plants. Its main advantages are: portability, since it allows *in vivo* and *in situ* measurements; adaptability to different biological samples; and versatility, since it is possible to adjust the parameters of the acquisition according to the sample under study.

The method studied and presented herein, based on bioimpedance indexes, such as Z_1/Z_{50} and R_1/R_{50}, allowed to determine three distinct physiological states: healthy and watered plants, plants with high level of HS and plants with disease.

Although a real statistical analysis is missing, the method presented in this paper may constitute an interesting solution for the assessment of physiological states of plants, since the bioimpedance indexes may easily be implemented as risk predictors, which may help to assist forest and crops management and also physiological plant studies.

ACKNOWLEDGMENT

We acknowledge support from Fundação para a Ciência e Tecnologia, FCT (scholarship SFRH/BD/61522/2009).

REFERENCES

[1] E. Borges, M. Sequeira, A. F. V. Cortez, H. C. Pereira, T. Pereira, V. Almeida, *et al*, "Assessment of Physiological States of Plants in situ – An Innovative Approach to the use of Electrical Impedance Spectroscopy", BIOTECHNO 2013, pp. 1–8, March 2013.

[2] L. Martins, J. Castro, W. Macedo, C. Marques, and c. Abreu, "Assessment of the spread of chestnut ink disease using remote sensing and geostatistical methods," Eur J Plant Pathol, vol. 119, pp. 159–164, April 2007.

[3] CABI and EPPO, "Data sheets on quarantine pests – *Bursaphelenchus xylophilus*," EPPO quarantine pests, contract 90/399003.

[4] W. Parawira, "Biodiesel production from *Jatropha curcas*: a review," Scientific Research and Essays, vol. 5(14), pp. 1796–1808, July 2010.

[5] T. Repo, G. Zhang, A. Ryyppo, and R. Rikala, "The electrical impedance spectroscopy of scots Pine (*Pinus sylvestris* L.) shoots in relation to cold acclimation," Journal of Experimental Botany, vol. 51 (353), pp. 2095–2107, December 2000.

[6] A. Pirzad, M. K. Shakiba, S. Zehtab-Salmasi, S. A. Mohammadi, R. Darvishzadeh, and A. Samadi, "Effect of water stress on leaf relative water content, chlorophyll, proline and soluble carbohydrates in *Matricaria chamomilla* L.," Journal of Medicinal Plants Research, vol. 5(12), pp. 2483–2488, June 2011.

[7] L. Callegaro, "The metrology of electrical impedance at high frequency: a review," Meas. Sci. Techno, vol. 20, 022002, February 2009.

[8] U. G. Kyle, I. Bosaeus, A. D. De Lorenzo, P. Deurenberg, M. Elia, J. M. Gómez, *et al*, "Bioelectrical impedance analysis – part I: review of principles and methods," Clinical Nutrition, vol. 23(5), pp. 1226–1243, October 2004.

[9] U. Pliquett, "Bioimpedance: a review for food processing," Food Engineering Reviews, vol. 2(2), pp. 74–94, June 2010.

[10] S. Grimnes and O. G. Martinsen, "Bioimpedance and bioelectricity basics," 2nd Edition, Academic Press of Elsevier, 2008.

[11] T. Hayashi, M. Iwamoto, and K. Kawashima, "Identification of irradiated potatoes by impedance measurements," Biosci Biotechnol Biochem, vol. 56(12), pp. 1929–1932, December 1992.

[12] M. Rafiei-Naeini, P. Wright, and H. McCann, "Low-noise measurement for electrical impedance tomography," IFMBE Proceedings, vol. 17(10), pp. 324–327, 2007.

[13] A. S. Ross, G. J. Saulnier, J. C. Newell, and D. Isaacson, "Current source design for electrical impedance tomography," Physiol Meas, vol. 24(2), pp. 509–516, May 2003.

[14] P. J. Yoo, D. H. Lee, T. I. Oh, and E. J.Woo, "Wideband bio-impedance spectroscopy using voltage source and tetra-polar electrode configuration," Journal of Physics, vol. 224(1), pp. 224-228, 2010.

[15] G. J. Saulnier, A. S. Ross, and N. Liu, "A high-precision voltage source for EIT," Physiol Meas, vol. 27(5), pp. 221–236, May 2006.

[16] F. Seoane, R. Bragós, and K. Lindecrantz, "Current source for multifrequency broadband electrical impedance spectroscopy systems – a novel approach," Proceedings IEEE Eng Med Biol Soc, vol. 1, pp. 5121–5125, August 2006.

[17] C. He, L. Zhang, B. Liu, Z. Xu, and Z. Zhang, "A Digital Phase-sensitive Detector for Electrical Impedance Tomography", IEEE proceedings, 2008.

[18] T. Yamamoto, Y. Oomura, H. Nishino, S. Aou, and Y. Nakano, "Driven shield for multi-barrel electrode," Brain Research Bulletin, vol. 14(1), pp. 103-104, January 1985.