

● Original Contribution

MONITORING PLANT RESPONSE TO ENVIRONMENTAL STIMULI BY ULTRASONIC SENSING OF THE LEAVES

MARIA DOLORES FARIÑAS,* DOMINGO SANCHO KNAPIK,[†] JOSE JAVIER PEGUERO PINA,[†]
 EUSTAQUIO GIL PELEGRIN,[†] and TOMÁS E. GÓMEZ ÁLVAREZ-ARENAS*

*Sensors and Ultrasonic Technologies Department, Information and Physics Technologies Institute (ITEFI), Spanish National Research Council (CSIC), Madrid, Spain; and [†]Agrifood Research and Technology Centre of Aragon (CITA), Zaragoza, Spain

(Received 12 January 2014; revised 1 April 2014; in final form 5 April 2014)

Abstract—Described here is the application of a technique based on the excitation, sensing and spectral analysis of thickness resonances of plant leaves using air-coupled and wide-band ultrasound pulses (150–900 kHz) to monitor variations in leaf properties caused by plant responses to different environmental stimuli, such as a sudden variation in light intensity (from 2000 to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$), sudden watering after a drought period, and along the diurnal cycle (3–5 days, with continuous variation in light intensity from 150 to 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and change in temperature of about 5°C). Four different widely available species, both monocots and dicots and evergreen and deciduous, with different leaf features (shape, size, thickness, flatness, vascular structure), were selected to test the technique. After a sudden decrease in light intensity, and depending on the species, there was a relative increase in the thickness resonant frequency from 8% to 12% over a 25- to 50-min period. After sudden watering, the relative increase in the resonant frequency varied from 5% to 30% and the period from 10 to 400 min. Finally, along the diurnal cycle, the measured relative variation is between 4% and 10%. **The technique revealed differences in both the amplitude of the frequency oscillations and the kinetics of the leaf response for different species and also within the same species, but for specimens grown under different conditions that present different cell structures at the tissue level.** The technique can be equally applied to the leaves of any species that present thickness resonances. (E-mail: t.gomez@ia.csic.es) © 2014 World Federation for Ultrasound in Medicine & Biology.

Key Words: Plant leaves, Water content, Drought stress, Diurnal cycles, Air-coupled ultrasound, Ultrasonic spectroscopy, Monitoring.

INTRODUCTION

The application of air-coupled ultrasound to materials characterization has advanced markedly thanks to the improvements in air-coupled transducers (see, *e.g.*, Haller et al. [1992], Hayward and Gachagan [1996], Schindel and Hutchins [1995] and Yano et al. [1987] for early contributions; Hutchins et al. [1998] for an early review; and Kelly [2004], Álvarez-Arenas [2004], Álvarez-Arenas et al. [2012], Álvarez-Arenas and Díez [2013] and Ealo et al. [2008] for later advances). Spectral analysis of thickness resonances in solid plates excited and sensed using air-coupled and wide-band ultrasonic pulses has been used before for materials characterization (see, *e.g.*, Álvarez-Arenas et al. 2002, 2010; Akseli et al. 2010; Hutchins et al. 1994; Schindel and Hutchins 1995) or as

a non-destructive testing technique (Hsu 2006; Livings et al. 2012; Schindel et al. 1996). Though plant leaves had been studied before with conventional ultrasound (Fukuhara 2002; Wilson and Dutton 2009), the use of air-coupled ultrasound and spectral analysis of the thickness resonance of leaves was first proposed by Álvarez-Arenas et al. (2009a, 2009b) and Álvarez-Arenas (2010).

Later, Sancho-Knapik et al. (2010) established a link between leaf physiologic and ultrasonic properties. They demonstrated that when the leaf relative water content (RWC) decreases, the frequency of the thickness resonance shifts toward lower values, following a sigmoid function, and that the point of inflection of this sigmoid coincides with the point of turgor loss determined from pressure–volume curves measured with a pressure chamber (Scholander et al. 1965). In Sancho-Knapik et al. (2011, 2012), a more complete analysis of thickness resonances revealed that other leaf parameters, such as damping (Q-factor) and the **elastic constant** in the thickness direction (C_{33}) can also be obtained. Comparison with

Address correspondence to: Tomás E. Gómez Álvarez-Arenas, ITEFI-CSIC, Serrano 144, 28006 Madrid, Spain. E-mail: t.gomez@ia.csic.es

cryo-scanning electron microscopy images led to the establishment of a relationship between the aforementioned parameters and the deformations that take place at the cell level.

In all these cases, the effective medium approach, considering a 1-D model, plane wave and normal incidence (Brekhovskikh 1980), provided a good representation of the measured spectral response of the first thickness resonance of plant leaves. Considering the complexity, heterogeneity and anisotropy of plant leaves, the robustness of the effective medium approach when applied to explain the measured resonances in leaves of different species is surprising (Sancho-Knapik et al. 2013). More accurate or realistic models were proposed in Álvarez-Arenas et al. (2009b) and Fariñas et al. (2013) to extract the properties of the different layers of tissue in the leaves and to measure Poisson's ratio. These models were based on a layered representation of the leaves that is closer to real leaf structure.

Yet, work remains to establish a quantitative relation between these effective ultrasonic properties and such leaf features as the anatomical properties of the different tissues, cell shape and size and cell wall composition. This is beyond the scope of this article, but as the objective of this work is to determine the possibility of using an ultrasonic technique to sense the variations in leaf properties caused by the plant's response to different environmental stimuli, it is of interest to understand the basic mechanisms that link the two types of leaf properties (ultrasonic and physiologic). At the core of this link is one of the main characteristic of biological materials: their hierarchical multiscale organization, where all levels of organization are tightly integrated. This enables plants to structurally respond with high efficiency on every hierarchical level. Hence, macroscopic properties of the plant derive mainly from the cell wall organization, making the nano- and micro-structural scales particularly relevant for biomechanical approaches (Burgert 2006). In most parenchyma tissues, the cells are densely packed together: they can be thought of as a pressurized, liquid-filled closed-cell foam (Gibson 2012). At normal or high turgor pressures, the cell walls are taut, and deformation is dominated by stretching or compression of the cell walls. The Young's modulus and strength of the parenchyma tissue are then directly proportional to its relative density. When turgor pressure decreases and the cell wall becomes less taut, it can be deformed by bending and twisting, which significantly increases cell deformability, reduces the effective cell elastic modulus and, hence, reduces the tissue effective elastic modulus. Thus, cell deformability is one of the key mechanisms that link leaf physiologic features and the properties of the spectrum of leaf thickness resonances.

In addition, there are some other mechanisms that also link the properties or the activity of the leaves with

the spectra of the first thickness resonance. (i) In addition to affecting cell deformability, as explained before, water content variations can also produce variations in leaf thickness and density. (ii) Opening and closing of stomata effectively modify boundary conditions at the leaf surface, which affects acoustic impedance. In the case of porous media, boundary conditions are expressed in terms of the interface hydraulic conditions, that is, whether fluid flow across the interface is possible or not (Deresiewicz and Skalak 1963; Gurevich and Schoenberg 1999). (iii) Cell wall elasticity (Saito et al. 2006) depends on the composition and thickness of the cell wall. It is well known that the degree of hydration can severely affect the mechanical behavior of the cell wall (Ha et al. 1997). On the other hand, living organisms have the potential to modify cell wall permeability to allow for mass transfer and signaling. Variations in cell wall permeability, as well as the presence of water molecules within the membrane, will modify cell wall elasticity.

The possibility of non-invasive, non-contact, continuous monitoring, performed in real time, is attractive to plant physiologists. Methods have been proposed for continuous monitoring of water content changes based on the measurement of leaf thickness (McBurney 1992) or measurement of the positive pressure of water inside the cells, or turgor pressure (Geitmann 2006; Lintilhac et al. 2000; Zimmermann et al. 2008 and Ehrenberger et al. 2012).

This ultrasonic technique has potential for application to attached naturally transpiring leaves because of its non-invasive, non-contact character and its capability to operate in real time to measure rapid variations; however, so far, it has only been applied in the laboratory with detached leaves. The purpose of this work is to test this technique in the continuous recording of attached leaves, and to determine its capability and sensitivity as a tool for the study of the dynamic changes that occur in plant leaves in response to different environmental stimuli. Therefore, the objective is not to fully study the response of a given species, but to provide, as proof of concept, a first test of the applicability of this technique. Toward this end, a few species with different types of leaves and representing adaptative solutions to different environments were selected. With respect to environmental stimuli, we selected three representative cases: (i) sudden changes in light intensity, (ii) sudden watering and (iii) diurnal cycle of solar radiation (diurnal/circadian cycle). In this article, and as a first approach, we focus only on the variations in resonance frequency, f_{res} . This parameter is closely correlate with other leaf parameters; is easy to measure, robust and straightforward; and does not require any model assumption as it is derived directly from the measurements. More complete analysis of the resonance spectra will be the topic of future works.

METHODS

Plant material: description and properties

Two dicotyledonous species (the evergreen *Hibiscus rosa-sinensis* and the deciduous *Vitis vinifera*) and two monocotyledonous species (*Epipremnum aureum* and *Dracaena marginata*) were studied. They were selected to test the applicability of the technique in different species (monocot/dicot, evergreen/deciduous) with different kinds of leaves (thickness, density, shape, size, elasticity vascular system distribution, *etc.*) (see Table 1) that live in different environments and have developed quite different adaptive solutions. These plants are also used differently. *Hibiscus rosa-sinensis* is used mainly for ornamental applications, as are *E. aureum* and *D. marginata*, but the latter two species are also used as air cleaners because of their ability to remove some indoor pollutants. Finally, *V. vinifera* is used for grapes and wine production. To facilitate the reproducibility of the results, we also selected species available worldwide that can be grown in pots. All of the plants used were planted in soil-filled pots (pot volumes of 2, 2, 5 and 1 L, respectively). Two different *E. aureum* plants were used for the experiments. These were genetically identical and obtained by vegetative reproduction, but one was grown for 12 months without exposure to direct sunlight (I), and the other was grown for 12 months under direct sunlight (II). It is well known that several features of plant form, physiology and resource allocation vary with the level of irradiance to which plants are acclimated and/or ecologically restricted (Givnish 1988). In this case, the leaves of these two plants clearly differed in terms of thickness, color and elasticity, as reported before for other species (Onoda *et al.* 2008). Differences in leaf tissue morphology resulting from different types of exposure to sunlight were investigated further in cryo-scanning electron microscopy images of the cross-section fractures (see Fig. 1). Both leaves have similar adaxial and abaxial epidermis; however, the leaf grown without direct sunlight (Fig. 1a) had an almost negligible palisade parenchyma, with one discontinuous row of small cells (25–50 μm) and a highly porous spongy mesophyll that occupied almost the whole leaf. On the other hand, the leaf grown with direct sunlight (Fig. 1b) had a well-defined palisade parenchyma with a close-packed row of relatively larger cells (50–75 μm). As an initial characterization of these materials, 10 different leaves of each species were ultrasonically characterized at full turgor from analysis of the magnitude and phase spectra of the first thickness resonance in the transmission coefficient using the procedure proposed by Álvarez-Arenas *et al.* (2009a). For *V. vinifera* and *E. aureum* leaves, such measurements had already been reported by Fariñas *et al.* (2013). For the other two species studied here, some representative measurements and theoretical fits are illustrated in Figure 2. Averaged

leaf parameters so obtained are summarized in Table 1. The lowest ultrasonic velocity and the largest value of $\alpha_0/f_{\text{res}}^{\text{M}}$ were observed in the *E. aureum* (I) leaves. These results are consistent with the highly porous structure and loose packing of these cells, as observed in Figure 1a, with a reduced palisade parenchyma and a large spongy mesophyll. Finally, and with the purpose of completing this initial characterization, leaves were cut and measured as they dried using both the ultrasonic technique and the pressure chamber method, according to the procedures described by Sancho-Knapik *et al.* (2010, 2011). Variations in leaf water potential and first thickness resonance, f_{res} , with RWC were measured. It was thus possible to determine the turgor loss point (TLP) and to obtain RWC, water potential and relative variation in f_{res} , $(f_{\text{res}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$, at the TLP, where $f_{\text{res}}^{\text{M}}$ is the resonant frequency at full turgor. Results are summarized in Table 2.

Experimental setup and equipment

The experimental setup used to monitor the variations in ultrasonic properties of plant leaves in response to environmental stimuli included the following elements.

Air-coupled ultrasonic system for measuring the ultrasonic properties of leaves. Two pairs of air-coupled transducers were used. They are wide-band transducers developed, designed and built at the Spanish National Research Council and have center frequencies of 250 and 650 kHz, frequency bands of 0.15–0.35 and 0.35–0.95 MHz, peak sensitivities of –25 and –30 dB, electrical impedances between 100 and 200 Ω and active area diameters of 15 and 20 mm, respectively (see Álvarez-Arenas 2004 for further details). Transducer responses in both the time and frequency domains are illustrated in Figure 3. Transducers were embedded in a U-shaped holder that maintained them facing each other at distances of 30–50 mm. The holder also had a slot in which leaves could be easily positioned between the transducers for measurements. This holder provides the necessary robustness for the system so that it can be easily manipulated without affecting the integrity of the signal. Leaves are located approximately at the middle point and at normal incidence (see Álvarez-Arenas 2013 for further details). Figure 4 is a photograph of the ultrasonic sensors attached to a *V. vinifera* leaf. A commercial pulser/receiver (5077PR, Olympus, Houston, TX, USA) was used to drive the transmitter transducer (200-V-amplitude semi-cycle of square wave tuned to the transducer center frequency) and to amplify and filter the electrical signal provided by the receiver transducer (up to 40 dB and low pass filtered: 10 MHz). The signal was then sent to a digital oscilloscope (TDS5054, Tektronix, Beaverton, OR, USA) with the impedance set at 1 M Ω and the

Table 1. Averaged leaf properties and their standard variations

Species	$f_{\text{res}}^{\text{M}}$ (kHz) ($\pm 15\%$)	Velocity (m/s) ($\pm 5\%$)	$\alpha_0/f_{\text{res}}^{\text{M}}$ (Np/(m·kHz)) ($\pm 7\%$)	Density (kg/m ³) ($\pm 5\%$)	Thickness (μm) ($\pm 15\%$)	C_{33} (MPa) ($\pm 7\%$)
<i>Epipremnum aureum</i> (I)	245	165	3.5	860	330	23
<i>Epipremnum aureum</i> (II)	260	200	2.3	915	390	37
<i>Hibiscus rosa-sinensis</i>	275	210	2.8	930	380	40
<i>Dracaena marginata</i>	645	320	1.7	890	250	92
<i>Vitis vinifera</i>	540	215	4.1	760	190	35

bandwidth set at 20 MHz and averaged (between 80 and 120 samples). Samples were digitized at 2 and 5 MS/s, for measurements in the 250- and 650-kHz bands, respectively, and at 8 bit (amplitude). The result was then transferred to the oscilloscope PC for further signal analysis. First, a rectangular time window was applied to the transmitted waveform to filter out the reverberations within the air cavities. The signal was padded with zeroes up to 4K to increase frequency resolution, and then the Fourier transform was extracted using the fast Fourier Transform (FFT) algorithm. Real and imaginary parts of the FFT are used to compute the magnitude and phase spectra of the transmitted signal. Further calculations to obtain the frequency location of the maximum transmission (resonant frequency) or the Q-factor of the resonance were performed in MATLAB (The MathWorks, Natick, MA, USA) (see [Sancho-Knapik et al. 2012](#)). Given these conditions for the analogue-to-digital conversion, **the precision in the determination** of the resonant frequency is determined by the discretization in frequency, which is 488 and 1220 Hz in the 250- and 650-kHz frequency bands, respectively. Hence, precision in the determination of the relative variation in the resonant frequency is in the range 0.3%–0.8%. Repeatability of the measurement of the resonant frequency is <0.8%.

System of conventional sensors to monitor variations in plant environment. Four different sensors were

employed to measure air and soil temperature, soil tension and light intensity. For soil tension measurements, a Watermark 200SS-V sensor (Irrometer, Riverside, CA, USA) was used. It consists of a pair of highly corrosion-resistant electrodes that are embedded within a granular matrix. A current was applied to obtain a resistance value. The sensor correlated the resistance to soil water tension. With respect to light intensity, a photosynthetically active radiation sensor was used. As photosynthesis is driven by the number of photons in this band, the intensity was measured in number of photons (in mol) per second and per unit surface ($\mu\text{mol m}^{-2} \text{s}^{-1}$). The photosynthetic photon flux (PPF) sensor (SQ-200-5, Apogee Instruments, Logan, UT, USA) was used for this purpose. According to the manufacturer, its sensitivity is 2.0 mV per $\mu\text{mol m}^{-2} \text{s}^{-1}$ with a calibration factor of 0.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ per mV, and measurement repeatability is <1%. In all the measurements reported in this article, the source of light was the sun. All of these sensors were connected to a data logger (FieldLogger FL, NOVUS, Porto Alegre, Rio Grande do Sul, Brazil). This is a microprocessor-based data acquisition and recorder that can handle analogue inputs and can operate as a remote terminal unit linked to a PC for on-line recording or as a stand-alone data logger with real-time clock and graph capabilities. In this case, it was used as a stand-alone data logger. It has eight channels that accept

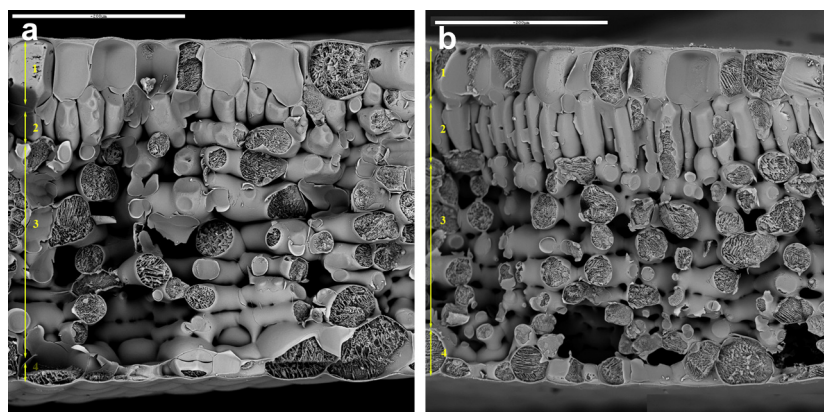


Fig. 1. Cryo-scanning electron microscopy (SEM) images of the cross sections of two leaves of *Epipremnum aureum*. (1) Adaxial epidermis. (2) Palisade parenchyma. (3) Spongy mesophyll. (4) Abaxial epidermis. (a) Grown without direct sunlight (*Epipremnum aureum* I). (b) Grown with direct sunlight (*Epipremnum aureum* II). Bar = 200 μm .

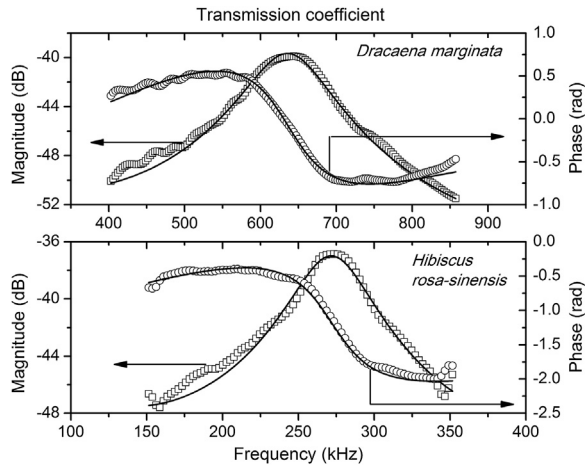


Fig. 2. Measured magnitude (\square) and phase (\circ) spectra of the first thickness resonance in the transmission coefficients for *Dracaena marginata* and *Hibiscus rosa-sinensis* leaves. The solid line represents the theoretical fit according to the one-layer model at normal incidence (Álvarez-Arenas 2010).

different input sensors. The clock was synchronized with the clock of the CPU of the oscilloscope PC which controlled the acquisition of ultrasonic data.

Non-ultrasonic techniques to measure other leaf properties. Stomatal conductance, g_s . Stomata are small pores on the leaf surface that are responsible for taking in and expelling CO_2 and moisture from and to the outside air, respectively. g_s is the measure of the rate of passage of CO_2 entering, or water vapor exiting, through the stomata of a leaf and is usually expressed in $\text{mmol m}^{-2} \text{s}^{-1}$. g_s , or its inverse, stomatal resistance, is directly related to the boundary layer resistance of the leaf and the absolute concentration gradient of water vapor from the leaf to the atmosphere and it is under direct biological control of the leaf through the use of guard cells, which surround the stomatal pore and have the ability to open or close the pores depending on environmental conditions and plant needs. A SC-1 Leaf Porometer, (Decagon Devices, Pullman, WA, USA) was used for

Table 2. Averaged leaf properties obtained at the turgor loss point obtained with the ultrasonic technique and the pressure chamber method

Species	RWC	Turgor loss point	
		Water potential (MPa)	$(f_{\text{res}}^{\text{TLP}} - f_{\text{res}}^{\text{M}}) / f_{\text{res}}^{\text{M}}$ (%)
<i>Epipremnum aureum</i>	0.96	−0.52	10
<i>Hibiscus rosa-sinensis</i>	0.93		23
<i>Dracena marginata</i>	0.94		22
<i>Vitis vinifera</i>	0.9	−1.99	13

RWC = relative water content; TLP = turgor loss point.

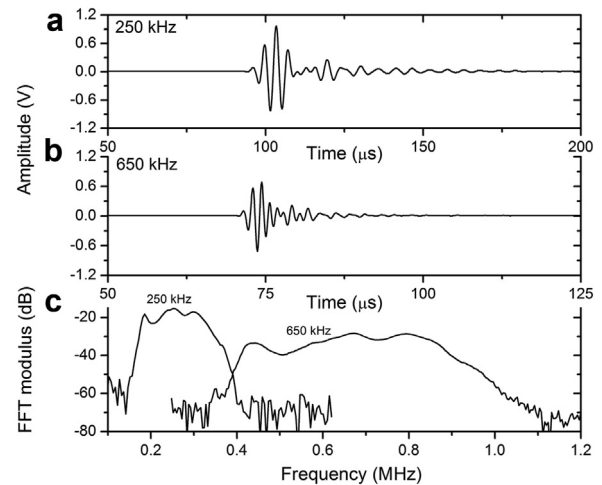


Fig. 3. Response of the two pairs of transducers (center frequency: 250 and 650 kHz) in the time and frequency (fast Fourier transform [FFT]) domains. Transmitter–receiver separation was 33 mm (250 kHz) and 24 mm (650 kHz). Pulser amplitude was set to 200 V, and receiver gain, to 0 dB.

these measurements. According to the manufacturer, its accuracy is within 10% of the measured value.

Water potential, ψ (MPa). The pressure chamber method was used to determine leaf water potential. Basically the method consists of increasing the pressure around a leaf within a pressure chamber, while the cut end of the petiole remains outside the chamber and, as a consequence, at atmospheric pressure. The applied pressure needed to force the water reaching the cut end equals the original tension of the water in the leaf, that is, leaf water potential (Slavík 1974).

Leaf relative water content. A precision balance was used to weigh the leaves. In addition, an oven was used to dry them (80°C for 48 h) to obtain the dry



Fig. 4. Photograph of the transducers attached to one of the leaves of a *Vitis vinifera* plant.

Table 3. Averaged leaf properties and their standard variations measured at two different PPF levels

Species	Direct sunlight (PPF: 1900–2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$)		Darkness (PPF: <150 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	
	g_s ($\text{mmol m}^{-2} \text{s}^{-1}$)	Ψ (MPa)	g_s ($\text{mmol m}^{-2} \text{s}^{-1}$)	Ψ (MPa)
<i>Hibiscus rosa-sinensis</i>	85 \pm 30	−0.7 \pm 0.13	0	−0.34 \pm 0.11
<i>Epipremnum aureum</i> (I)	14 \pm 5	−0.37 \pm 0.11	0	−0.24 \pm 0.05
<i>Epipremnum aureum</i> (II)	45 \pm 9		0	
<i>Vitis vinifera</i>	40 \pm 8		0	
<i>Dracaena marginata</i>	41 \pm 10		0	

PPF = photosynthetic photon flux.

mass. RWC was obtained from the weight, the weight at full turgor and the dry mass according to the procedure described by [Sancho-Knapik et al. \(2011\)](#).

Experimental procedures

Three different kinds of experiments were performed to test the capability of the ultrasonic system to measure and monitor variations in leaf properties caused by variations in environmental conditions or by the action of external stimuli: (i) variations in leaf properties after a sudden and drastic reduction of the level of light intensity; (ii) variations in leaf properties after a sudden irrigation event following a forced drought period; (iii) variations in leaf properties caused by changes in plant activity along the diurnal cycle (variations in light intensity, temperature, moisture, etc.).

To study the influence of light intensity, the experiments were carried out during the period May–September 2013 in Madrid. In all cases, the source of light was the sun. Light intensity was measured with the PPF sensor Apogee SQ-200-5. Plants were placed at a laboratory

window looking to the west, so direct sunlight reached the plants between 14:00 and 18:00 h. The day before the measurements, the pots were amply watered. The next day, at about 15:00, after 1 h of direct sunlight exposure (PPF between 1800 and 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$), we measured g_s and ψ . Then, the light intensity was drastically reduced (down to PPF <150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) by closing the blinds. The ultrasonic system monitored the variation in f_{res} , by measuring the transmitted ultrasonic signal through the leaves every 10 s and extracting the FFT. The relative variation in f_{res} was measured until it reached a constant value, which usually occurred after 30–40 min. At that point, we measured g_s and ψ again. This experiment was repeated between three and five times in five different leaves.

To study the influence of watering the plant after a forced drought period, the pots were not watered for several days, depending on the plant and capacity of the pot. In some cases, we also used the ultrasonic system to determine the evolution of leaf ultrasonic properties with the drought. The day the measurements were performed, pots were amply watered early in the morning. The response of the plant leaves was monitored by the ultrasonic system: leaf thickness resonance was measured every 5 s, until a stationary state was reached, which occurs between 30 min and several hours depending on the plant and its initial state. During this time, the light intensity was kept constant (<150 $\mu\text{mol m}^{-2} \text{s}^{-1}$), as was the air temperature. For each plant, this experiment was repeated between four and five times in different levels in five different leaves.

To study the variation in leaf properties caused by changes in plant activity along the diurnal cycle, some initial tests were performed during the summer of 2012 by monitoring *E. aureum* leaves for 5- to 10-day periods. A more systematic study was performed during the period May–August 2013, in cycles of 3–4 days under stable (anticyclone) weather conditions. This is clearly a more complex case, compared with the two previous ones, and comprises multiple stimuli and a complex response of the plant at different scales ([Huang et al. 2012](#)). The measurements were performed in Madrid, and the plants were placed at a laboratory window

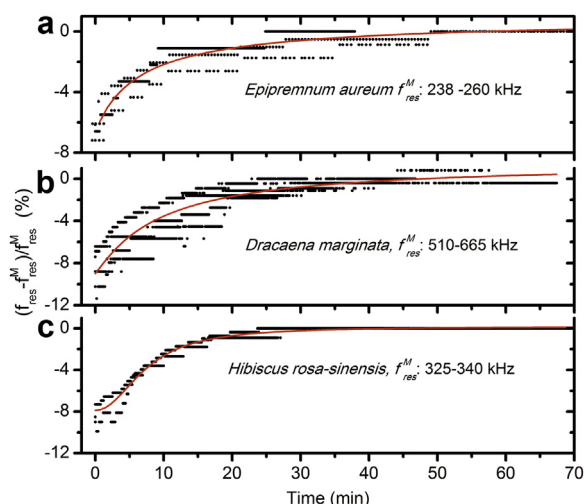


Fig. 5. Relative variation in thickness resonance, $(f_{\text{res}}(t) - f_{\text{res}}^M) / f_{\text{res}}^M$, with elapsed time after changing the light intensity (at time $t = 0$) from 1800–2000 to <150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. (a) *Epipremnum aureum*. (b) *Dracaena marginata*. (c) *Hibiscus rosa-sinensis*.

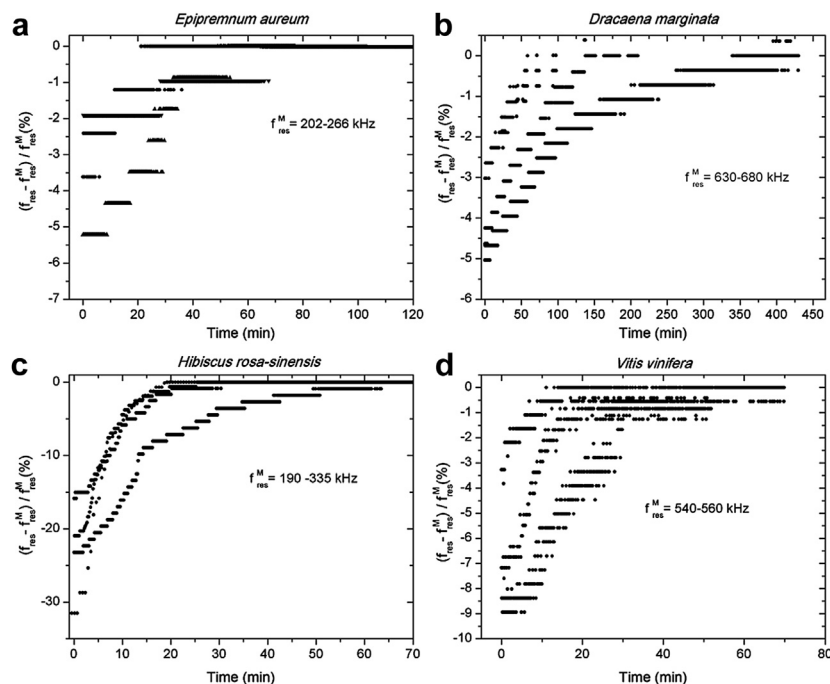


Fig. 6. Relative variation in thickness resonance, $(f_{\text{res}}(t) - f_{\text{res}}^M) / f_{\text{res}}^M$, with the elapsed time after the plant is watered (at time = 0). (a) *Epipremnum aureum*. (b) *Dracaena marginata*. (c) *Hibiscus rosa-sinensis*. (d) *Vitis vinifera*.

looking to the west, so direct sunlight reached the plants at intervals between 14:00 and 18:00 h. Air and soil temperature, soil tension and photosynthetically active radiation were measured and stored in the autonomous data logger (Novus Field Logger) every 10 min. Ultrasonic measurements were controlled with the oscilloscope PC and taken every 10 min as well. The night before the monitoring started, the pots were amply watered. The next day, we started measurements early in the morning, and no additional water was supplied during the 3–4 days of monitoring. This procedure was repeated between three and five times for each plant in five different leaves.

RESULTS AND DISCUSSION

In general, it is observed that the frequency of the first-order thickness resonance of plant leaves changes when the plant is subjected to the different stimuli studied here. The actual behavior depends on the type of stimuli, the specific properties of the leaves of this species, the environmental conditions under which the plant was grown and the actual state of the leaf and the plant itself. The species studied here were chosen to reflect different plant types (monocot/dicot, evergreen/deciduous) having leaves with different properties (thickness, density, shape, size, elasticity vascular system distribution, tissue physiology, *etc.*) and found in different environments in which they have developed quite different adaptative solutions.

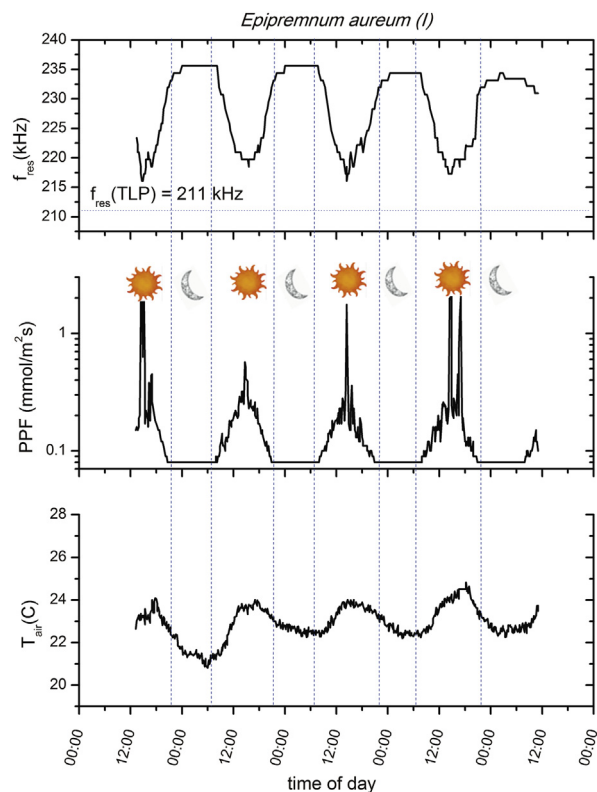


Fig. 7. Evolution of the thickness resonant frequency of an *Epipremnum aureum* (I) leaf over 4 days. Photosynthetic photon flux (PPF) and ambient temperature measurements are also shown.

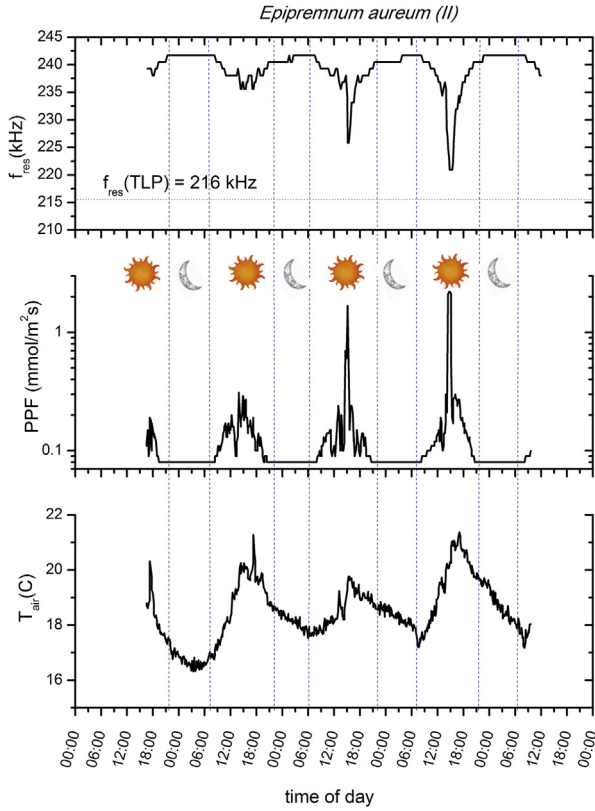


Fig. 8. Evolution of the thickness resonant frequency of an *Epipremnum aureum* (II) leaf over 3.5 days. Photosynthetic photon flux (PPF) and ambient temperature measurements are also shown.

It is expected that the technique can be equally applied to any other species in which thickness resonances of the leaves can be measured, though the actual behavior will depend on the specific features of the species and the plant under study.

Ultrasonic monitoring of plant response to light intensity reduction

Variations in g_s and ψ with light intensity reduction are summarized in Table 3. Figure 5 illustrates the relative variation in resonant frequency with elapsed time, $(f_{\text{res}}(t) - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$, where $f_{\text{res}}^{\text{M}}$ is the maximum value of the leaf resonant frequency that is obtained when the plant is in darkness ($f_{\text{res}}^{\text{M}} = f_{\text{res}}^{\text{dark}}$), and $t = 0$ corresponds to the time when light intensity was reduced. Data in Table 3 indicate that the reduction in light intensity induces stomatal closure and an increase in ψ . This decrease in transpiration, mediated by stomatal closure, shifts the balance between water supply and water evaporation and increases leaf RWC and turgor pressure. Concerning leaf ultrasonic properties, Figure 5 illustrates that f_{res} increases in all cases, and the maximum value is achieved when the plant is placed in the dark. As explained before, this

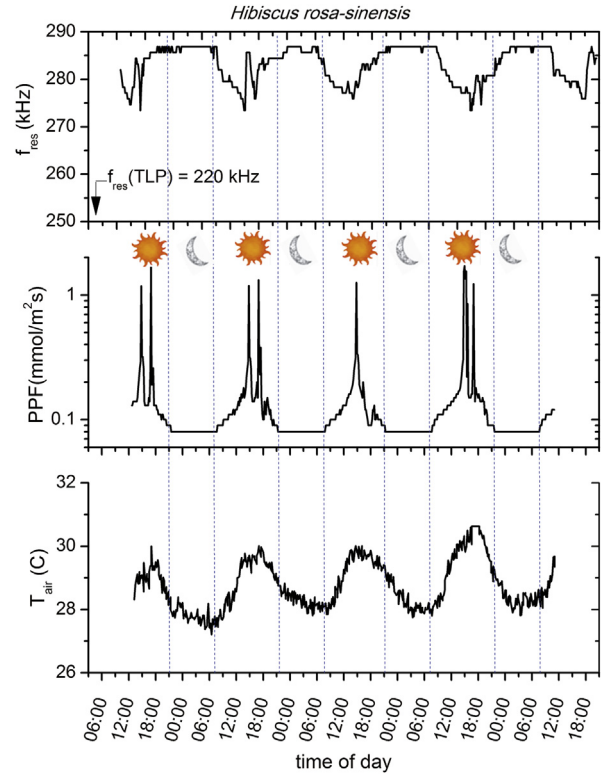


Fig. 9. Evolution of the thickness resonant frequency of a *Hibiscus rosa-sinensis* leaf over 4 days. Photosynthetic photon flux (PPF) and ambient temperature measurements are also shown.

shift of f_{res} toward higher values is produced by an increase in the ultrasound velocity in the leaf, which is the result of a stiffening of the tissues caused by the increase in turgor pressure. The variation in f_{res} with time can be estimated by the sigmoid function (also illustrated in Fig. 5).

$$\frac{f_{\text{res}}(t) - f_{\text{res}}^{\text{dark}}}{f_{\text{res}}^{\text{dark}}} = \frac{A - 1}{1 + (t/t_0)^p}$$

where t is the elapsed time, t_0 is the time at which the point of inflection of the sigmoid is located, p determines how fast is the sigmoid transition from the lower to the upper bounds and $A = f_{\text{res}}^{\text{light}}/f_{\text{res}}^{\text{dark}}$, where $f_{\text{res}}^{\text{light}}$ is the minimum value of the resonant frequency (measured at $t = 0$). A sigmoid is selected because this law is well fitted to describe a transition between two states (as is the case here) and provides a quantification of the variation rate or the location of the point of inflection.

Ultrasonic monitoring of plant response to watering

Variation in $(f_{\text{res}}(t) - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ after a sudden irrigation following a forced drought period is illustrated in Figure 6. Unlike the previous experiment, this variation is now affected by two different mechanisms. On the one hand,

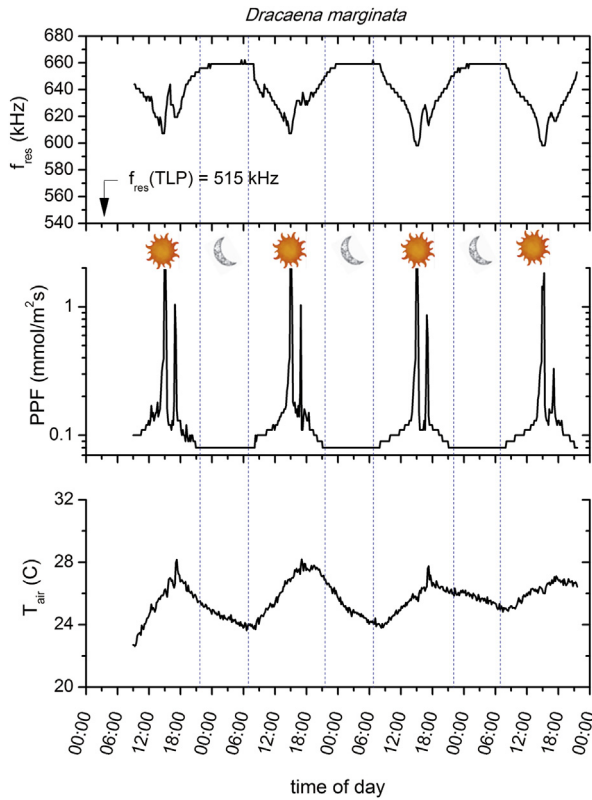


Fig. 10. Evolution of the thickness resonant frequency of a *Dracaena marginata* leaf over 3.5 days. Photosynthetic photon flux (PPF) and ambient temperature measurements are also shown.

the total ranges of variation, $(f_{\text{res}}(t=0) - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$, is determined mainly by leaf tissue properties; in particular, by how much the cell wall tautness can change with leaf RWC. On the other hand, the kinetics of the process is determined by the velocity of the water transport from the soil to the leaves. This depends on many factors such as the permeability of the cell wall, the plant vascular system, the distance between the leaf and the roots, the integrity of the vascular system and the transport mechanisms in the actual water path that connects the leaf and the roots. In all cases, a shift in f_{res} toward higher frequencies is observed. This is the expected result considering that the RWC of the leaves and, consequently, the tautness of the cell walls are expected to increase after watering. Clear differences between species can be observed in the range and rate of variation of f_{res} . In addition, if we compare $(f_{\text{res}}(t=0) - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ in Figure 6 with the value of $(f_{\text{res}}^{\text{TLP}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ (measured at TLP, see Table 2), we observe two different types of behaviors. For *E. aureum* and *D. marginata*, $(f_{\text{res}}(t=0) - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ is considerably smaller than $(f_{\text{res}}^{\text{TLP}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$, whereas for *H. rosa-sinensis* and *V. vinifera*, both magnitudes are similar. The explanation of these differences may well correspond to the different plant strategies used to cope with water shortage:

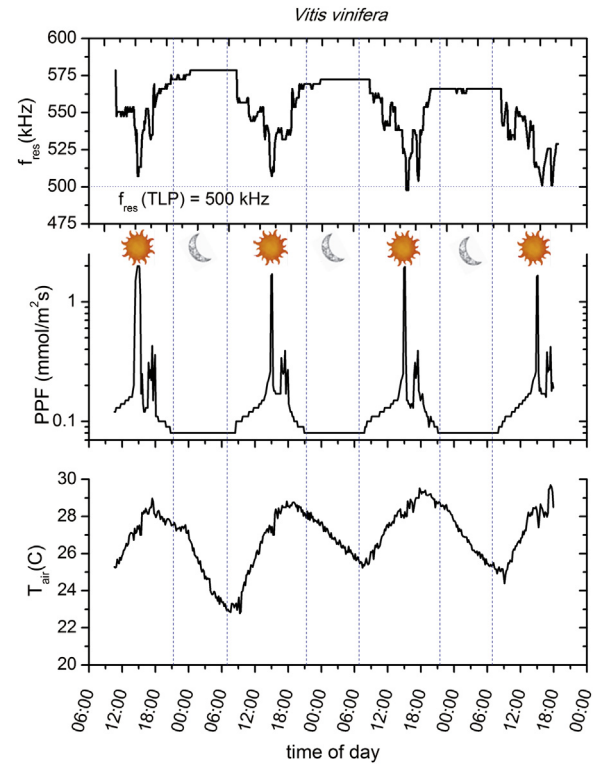


Fig. 11. Evolution of the thickness resonant frequency of a *Vitis vinifera* leaf over 3.5 days. Photosynthetic photon flux (PPF) and ambient temperature measurements are also shown.

Although some species can reduce leaf RWC close to the value for TLP, some others, with a more “conservative strategy,” limit the water loss well before reaching TLP. More data are required to draw any conclusion in this sense, but the present study reveals the potential of the technique for this application.

Unlike previous experiments, and according to the two different mechanisms that determine the kinetics of this process (cell wall elasticity and vascular system), large variations between species and between different leaves of the same species in the time needed to reach $f_{\text{res}}^{\text{M}}$ are observed. Two different factors determine these variations. The first corresponds to variations in the initial point (*i.e.*, differences in how dry the plant was at $t = 0$). The second corresponds to variation in the vascular system caused by the possible appearance of different levels of embolisms produced by the forced drought that can limit, at least initially, the capacity to transport water and/or the velocity with which the water is transported.

Ultrasonic monitoring of variations in the plant along the diurnal cycle

Measured variations in leaf properties (resonant frequency, ambient temperature and light intensity)

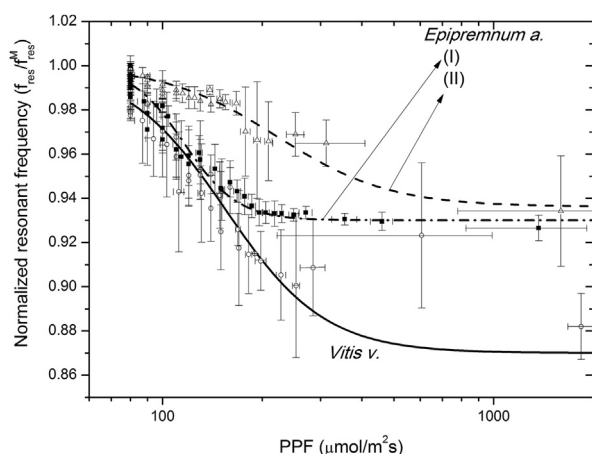


Fig. 12. Variation in normalized frequency, $f_{\text{res}}(\text{PPF})/f_{\text{res}}^{\text{M}}$, with the photosynthetic photon flux (PPF). Experimental averaged data: ■, *Epipremnum aureum* (I); △, *Epipremnum aureum* (II); ○, *Vitis vinifera* and fit to a sigmoid.

caused by variations in plant activity along the diurnal cycle (time of day) are illustrated in Figures 7–11. The expected value of f_{res} at the TLP is also illustrated. In general, the variation in the thickness resonant frequency of the leaves with the time of day follows the variations in light intensity (PPF). The sensitivity of f_{res} to PPF varies between different species, but in all cases it is observed that the minimum value of f_{res} is obtained at the maximum value of PPF, which, in our experimental setup, was achieved between 1:00 and 6:00 PM. The maximum relative variations in thickness resonance $(f_{\text{res}}^{\text{min}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ are for *E. aureum* (I), $5 \pm 1\%$; for *E. aureum* (II), $7.5 \pm 1\%$; for *H. rosa-sinensis*, $5.2 \pm 1\%$; for *D. marginata*, $8.5 \pm 1\%$; and for *V. vinifera*, $12.5 \pm 1\%$. These variations are similar to those in Figure 5, so it seems that the main factor affecting the thickness resonances of the leaves is light intensity. If we compare $(f_{\text{res}}^{\text{min}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ with $(f_{\text{res}}^{\text{TLP}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ as we did before, we observe two different types of behavior. For *E. aureum*, *D. marginata* and *H. rosa-sinensis*, the former is considerably smaller, whereas for *V. vinifera*, both magnitudes are similar. In addition, in *E. aureum* (I), *D. marginata* and *V. vinifera*, it is possible to observe a slight reduction in the maximum value of the resonant frequency reached

during the night as the number of days of monitoring increases. This same phenomenon was observed during the longer monitoring periods in the summer of 2012 in *E. aureum* leaves. This can be attributed to the progressive decay in soil water content. Finally, there are also significant variations in the sensitivity of $(f_{\text{res}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ to variations in light intensity. While for some species a small increase in PPF produces significant variation in $(f_{\text{res}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$, other species seem to have a light intensity threshold and changes in $(f_{\text{res}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ are negligible for light variations below this threshold. Leaves of the former type also exhibit a saturation of $(f_{\text{res}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$, and once the maximum variation is achieved, this value remains constant regardless of a possible further increase in light intensity. Some of these features can be observed in Figure 12, which illustrates the variation in $f_{\text{res}}(\text{PPF})/f_{\text{res}}^{\text{M}}$ with light intensity (PPF) for some cases; in addition the following sigmoid function is fitted into the experimental data:

$$\frac{f_{\text{res}}(\text{PPF})}{f_{\text{res}}^{\text{M}}} = \frac{f_{\text{res}}^{\text{min}}}{f_{\text{res}}^{\text{M}}} + \frac{1 - f_{\text{res}}^{\text{min}}/f_{\text{res}}^{\text{M}}}{1 + (\text{PPF}/\text{PPF}_0)^p}$$

Variation in f_{res} with PPF takes place between two bonds: an upper bound, $f_{\text{res}}^{\text{M}}$ (corresponding to the f_{res} value at dawn), and a lower bound, $f_{\text{res}}^{\text{min}}$ (corresponding to f_{res} at maximum sunlight exposition), where PPF_0 is the point of inflection of the curve $f_{\text{res}}(\text{PPF})$, and the parameter p determines the velocity of the transition between these two states. Obtained values of these parameters are shown in Table 4. Measurements indicate that both p and PPF_0 depend on both the plant species and the environmental conditions under which the leaves were grown. In this sense, the differences between *E. aureum* (I) and (II) leaves are marked. The plant grown without direct sunlight (I) responded faster to light intensity, and a slight increase in light intensity produced a significant decrease in resonant frequency; moreover, for PPF values as low as $200 \mu\text{mol}/\text{m}^2 \cdot \text{s}$, the leaf response has already reached a saturation value. On the contrary, for *E. aureum* (II), the plant grown under direct sunlight, the resonant frequency was much less sensitive to changes in light intensity below $200 \mu\text{mol}/\text{m}^2 \cdot \text{s}$ and did not reach a saturation value until $800 \mu\text{mol}/\text{m}^2 \cdot \text{s}$.

Table 4. Parameters of the sigmoid fit in Figure 12

Species	Photosynthetic photon flux at the point of inflection ($\mu\text{mol}/\text{m}^2 \cdot \text{s}$)	p	Photosynthetic photon flux at frequency response saturation (99% of total variation) ($\mu\text{mol}/\text{m}^2 \cdot \text{s}$)
<i>Epipremnum aureum</i>			
(I)	116	2.54	165
(II)	221	5.41	446
<i>Vitis vinifera</i>	154	2.89	390

CONCLUSIONS

The work reported here indicates that the air-coupled and wide-band ultrasonic technique described is able to detect, in a non-destructive, non-invasive, non-contact, rapid manner, variations in leaf thickness resonances caused by the response of the plant to different environmental stimuli. Mechanisms that link modification of the main features of leaf thickness resonances with the variation in leaf parameters and plant activity have been proposed.

Decreasing light intensity (from 2000 to $150 \mu\text{mol m}^{-2} \text{s}^{-1}$) produces an increase in the leaf thickness resonant frequency (between 8% and 12%, depending on the species). This is explained by the reduction in plant transpiration, the closure of stomata and the concomitant shift in water equilibrium in the leaves. Watering the plant after a period of forced drought produces an increase in the thickness resonant frequency (5%–30% depending on the species and the plant) which is attributed to the increase in cell turgor pressure resulting from the increase in the RWC of the leaves. The kinetics of this phenomenon depends on the water transport channels between the soil and the leaves, and the time span varies between 10 and 400 min. These variations can be attributed to differences in the length, efficiency or integrity of these channels. Observed variations in the resonant frequency along the diurnal cycle (4%–10%) largely reflect the variations in light intensity. In addition, the kinetics of this response and the presence of saturation levels seem to depend on the mechanisms these plants use to adapt to the sunlight intensity available.

Finally, we observed that compared with the variation at the TLP, $(f_{\text{res}}^{\text{TLP}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$, the variation in $(f_{\text{res}} - f_{\text{res}}^{\text{M}})/f_{\text{res}}^{\text{M}}$ differs significantly depending on the stimuli and the species. Although in some cases we observed fluctuations in the resonant frequency much smaller than the variation observed at the TLP, in other cases, these variations are similar. This may reflect differences between different species and different individuals in dealing with the environmental stimuli. Clearly, further work and experimental evidence are needed, but these results reveal the potential of this technique as a tool that can provide meaningful data.

Acknowledgments—Authors acknowledge funding from the Spanish Ministry for Economy and Competitiveness, through Project DPI2011-22438, and a Botin Foundation grant given to M. D. Fariñas.

REFERENCES

- Akseli I, Dey D, Cetinkaya C. Mechanical property characterization of bilayered tablets using nondestructive air-coupled acoustics. *AAPS PharmSciTech* 2010;11:90–102.
- Álvarez-Arenas TEG, Montero FR, Moner-Girona M, Roig A, Molins E. Viscoelasticity of silica aerogels at ultrasonic frequencies. *Appl Phys Lett* 2002;81:1198–1200.
- Álvarez-Arenas TEG. Acoustic impedance matching of piezoelectric transducers to the air. *IEEE Trans Ultrason Ferroelectr Freq Control* 2004;51:624–633.
- Álvarez-Arenas TEG. Simultaneous determination of the ultrasound velocity and the thickness of solid plates from the analysis of thickness resonances using air-coupled ultrasound. *Ultrasonics* 2010;50:104–109.
- Álvarez-Arenas TEG. Air-coupled piezoelectric transducers with active polypropylene foam matching layers. *Sensors* 2013;13:5996–6013.
- Álvarez-Arenas TEG, Calás H, Cuello JE, Fernández AR, Muñoz M. Noncontact ultrasonic spectroscopy applied to the study of polypropylene ferroelectrets. *J Appl Phys* 2010;108:074110.
- Álvarez-Arenas TEG, Díez L. Novel impedance matching materials and strategies for air-coupled piezoelectric transducers. In: *Proceedings, 2013 IEEE Sensors, Baltimore, Maryland, USA, 3–6 November 2013*. New York: IEEE; 2013.
- Álvarez-Arenas TEG, Sancho-Knapik D, Peguero-Pina JJ, Gil-Pelegrin E. Noncontact and noninvasive study of plant leaves using air-coupled ultrasounds. *Appl Phys Lett* 2009a;95:193702.
- Álvarez-Arenas TEG, Sancho-Knapik D, Peguero-Pina JJ, Gil-Pelegrin E. Determination of plant leaves water status using air-coupled ultrasounds. In: *Proceedings, 2009 IEEE International Ultrasonics Symposium, Rome, Italy, 20–23 September 2009*. New York: IEEE; 2009b. p. 771–774.
- Álvarez-Arenas TEG, Shrout TR, Zhang SJ, Lee HJ. Air-coupled transducers based on 1–3 connectivity single crystal transducers. In: *Proceedings, 2012 IEEE International Ultrasonics Symposium, Dresden, Germany, 7–10 October 2012*. New York: IEEE; 2012. p. 2230–2233.
- Brekhovskikh LM. *Waves in layered media*. 2nd ed. New York: Academic Press; 1980.
- Burgert I. Exploring the micromechanical design of plant cell walls. *Am J Bot* 2006;93:1391–1401.
- Deresiewicz H, Skalak R. On uniqueness in dynamic poroelasticity. *Bull Seism Soc Am* 1963;53:783–788.
- Ealo JL, Camacho J, Fritsch C, Seco F, Roa J. A fabrication procedure for airborne ultrasonic phased arrays based on cellular electromechanical film. In: *Proceedings, 2008 IEEE Ultrasonics Symposium, Beijing, China, 2–5 November 2008*. New York: IEEE; 2008. p. 891–894.
- Ehrenberger W, Rüger S, Rodríguez-Domínguez CM, Díaz-Espejo A, Fernández JE, Moreno J, Zimmermann D, Sukhorukov VL, Zimmermann U. Leaf patch clamp pressure probe measurements on olive leaves in a nearly turgorless state. *Plant Biol* 2012;14:666–674.
- Fariñas MD, Sancho-Knapik D, Peguero-Pina JJ, Gil-Pelegrin E, Gomez Álvarez-Arenas TEG. Shear waves in vegetal tissues at ultrasonic frequencies. *Appl Phys Lett* 2013;102:103702.
- Fukuhara M. Acoustic characteristics of botanical leaves using ultrasonic transmission waves. *Plant Sci* 2002;162:521–528.
- Geitmann A. Experimental approaches used to quantify physical parameters at cellular and subcellular levels. *Am J Bot* 2006;93:1380–1390.
- Gibson L. The hierarchical structure and mechanics of plant materials. *J R Soc Interface* 2012;9:2749–2766.
- Givnish TJ. Adaptation to sun and shade: A whole-plant perspective. *Aust J Plant Physiol* 1988;15:63–92.
- Gurevich B, Schoenberg M. Interface conditions for Biot's equations of poroelasticity. *J Acoust Soc Am* 1999;105:2585–2589.
- Ha MA, Apperley DC, Jarvis MC. Molecular rigidity in dry and hydrated onion cell walls. *Plant Physiol* 1997;115:593–598.
- Haller MI, Edward L, Khuri-Yakub BT. 1–3 Composites for ultrasonic air transducers. In: . New York: IEEE; 1992. p. 937–939.
- Hayward G, Gachagan A. An evaluation of 1–3 connectivity composite transducers for air-coupled ultrasonic applications. *J Acoust Soc Am* 1996;99:2148–2157.
- Hsu DK. Nondestructive testing using air-borne ultrasound. *Ultrasonics* 2006;44:1019–1024.
- Huang W, Pérez-García P, Pokhilko A, Millar AJ, Antoshechkin I, Riechmann JL, Mas P. Mapping the core of the *Arabidopsis* circadian clock defines the network structure of the oscillator. *Science* 2012;336:75–79.
- Hutchins DA, Schindel DW, Bashford AG, Wright WMD. Advances in ultrasonic electrostatic transduction. *Ultrasonics* 1998;36:1–6.

- Hutchins DA, Wright WM, Schindel DW. Ultrasonic measurements in polymeric materials using air-coupled capacitance transducers. *J Acoust Soc Am* 1994;96:1634–1642.
- Kelly SP, Hayward G, Álvarez-Arenas TEG. Characterization and assessment of an integrated matching layer for air-coupled ultrasonic applications. *IEEE Trans Ultrason Ferroelectr Freq Control* 2004;51:1314–1323.
- Lintilhac PM, Wei C, Tanguay JJ, Outwater JO. Ball tonometry: A rapid, non-destructive method for measuring cell turgor pressure in thin-walled plant cells. *J Plant Growth Regul* 2000;19:90–97.
- Livingston RA, Dayal V, Barnard DJ, Hsu DK. Flaw investigation in a multi-layered, multi-material composite: Using air-coupled ultrasonic resonance imaging. *AIP Conf Proc* 2012;1430:1176–1183.
- McBurney T. The relationship between leaf thickness and plant water potential. *J Exp Bot* 1992;43:327–335.
- Onoda Y, Schievig F, Anten NPR. Effects of light and nutrient availability on leaf mechanical properties of *Plantago major*: A conceptual approach. *Ann Bot* 2008;101:727–736.
- Saito T, Soga K, Hoson T, Terashima I. The bulk elastic modulus and the reversible properties of cell walls in developing *Quercus* leaves. *Plant Cell Physiol* 2006;47:715–725.
- Sancho-Knapik D, Álvarez-Arenas TEG, Peguero-Pina JJ, Fernández V, Gil-Pelegrín E. Relationship between ultrasonic properties and structural changes in the mesophyll during leaf dehydration. *J Exp Bot* 2011;62:3637–3645.
- Sancho-Knapik D, Álvarez-Arenas TEG, Peguero-Pina JJ, Gil-Pelegrín E. Air-coupled broadband ultrasonic spectroscopy as a new non-invasive and non-contact method for the determination of leaf water status. *J Exp Bot* 2010;61:1385–1391.
- Sancho-Knapik D, Calás H, Peguero-Pina JJ, Ramos Fernández A, Gil-Pelegrín E, Álvarez-Arenas TEG. Air-coupled ultrasonic resonant spectroscopy for the study of the relationship between plant leaves' elasticity and their water content. *IEEE Trans Ultrason Ferroelectr Freq Control* 2012;59:319–325.
- Sancho-Knapik D, Peguero-Pina JJ, Fariñas MD, Álvarez-Arenas TEG, Gil-Pelegrín E. Ultrasonic spectroscopy allows a rapid determination of the relative water content at the turgor loss point: a comparison with pressure-volume curves in 13 woody species. *Tree Physiol* 2013;33:695–700.
- Schindel DW, Hutchins DA. Through-thickness characterization of solids by wideband air-coupled ultrasound. *Ultrasonics* 1995;33:11–17.
- Schindel DW, Hutchins DA, Zou L, Sayer M. The design and characterization of micromachined air-coupled capacitance transducers. *IEEE Trans Ultrason Ferroelectr Freq Control* 1995;42:42–50.
- Schindel DW, Hutchins DA, Grandia WA. Capacitive and piezoelectric air-coupled transducers for resonant ultrasonic inspection. *Ultrasonics* 1996;34:621–627.
- Scholander PF, Bradstreet ED, Hemmingsen EA, Hammel HT. Sap pressure in vascular plants negative hydrostatic pressure can be measured in plants. *Science* 1965;148:339–346.
- Slavík B. Methods of studying plant water relations. Berlin: Springer; 1974.
- Wilson PS, Dunton KH. Laboratory investigation of the acoustic response of seagrass tissue in the frequency band 0.5–2.5 kHz. *J Acoust Soc Am* 2009;125:1951–1959.
- Yano T, Tone M, Fukumoto A. Range finding and surface characterization using high-frequency air transducers. *IEEE Trans Ultrason Ferroelectr Freq Control* 1987;34:232–236.
- Zimmermann D, Reuss R, Westhoff M, Geßner P, Bauer W, Bamberg E, Bentrup FW, Zimmermann U. A novel, noninvasive, online-monitoring, versatile and easy plant-based probe for measuring leaf water status. *J Exp Bot* 2008;59:3157–3167.