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RESEARCH PAPER

Air-coupled broadband ultrasonic spectroscopy as a new non-invasive and non-contact method for the determination of leaf water status

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Abstract

The implementation of non-destructive methods for the study of water changes within plant tissues and/or organs has been a target for some time in plant physiology. Recent advances in air-coupled ultrasonic spectroscopy have enabled ultrasonic waves to be applied to the on-line and real-time assessment of the water content of different materials. In this study, this technique has been applied as a non-destructive, non-invasive, non-contact, and repeatable method for the determination of water status in *Populus×euramericana* and *Prunus laurocerasus* leaves. Frequency spectra of the transmittance of ultrasounds through plant leaves reveal the presence of at least one resonance. At this resonant frequency, transmittance is at its maximum. This work demonstrates that changes in leaf relative water content (*RWC*) and water potential (Ψ) for both species can be accurately monitored by the corresponding changes in resonant frequency. The differential response found between both species may be due to the contrasting leaf structural features and the differences found in the parameters derived from the *P–V* curves. The turgor loss point has been precisely defined by this new technique, as it is derived from the lack of significant differences between the relative water content at the turgor loss point (RWC_{TLP}) obtained from *P–V* curves and ultrasonic measurements. The measurement of the turgor gradient between two different points of a naturally transpiring leaf is easily carried out with the method introduced here. Therefore, such a procedure can be an accurate tool for the study of all processes where changes in leaf water status are involved.

Key words: Drought, relative water content, turgor loss point, ultrasonic spectroscopy, water potential.

Introduction

Several destructive methods have been used to characterize plant water status, either through water potential or relative water content (Barrs, 1968; Slavik, 1974; Turner, 1981; Tyree and Jarvis, 1982). These techniques preclude repetitive measurements in a given tissue (Búrquez, 1987) and, therefore, they are not suitable for studying dynamic water changes within the same plant tissue or organ. Attempts to find a non-destructive or non-invasive technique have been a challenge during recent decades.

Thermocouple psychrometers have been successfully used for measuring plant water potential (Savage and Wiebe, 1989). However, its accuracy is highly temperature dependent (Savage *et al.*, 1983) and the need for removing the waxy cuticle from the epidermis in order to reduce the water potential equilibration time (Savage *et al.*, 1984; Wullschleger and Oosterhuis, 1987) turns this method into a destructive technique. On the other hand, leaf or canopy temperature can also be used as an indicator of water stress

Abbreviations: *f*, standardized frequency; *P–V* curves, pressure–volume curves; *RWC*, relative water content; Ψ, water potential; TLP, turgor loss point.

at different plant levels through the analysis of thermal imaging variables (Grant *et al.*, 2006*a*, *b*). Although methods such as infrared thermometry can be used for measurement at variable distances from the target (Sepulcre-Canto *et al.*, 2007), its accuracy is highly reduced by plant architecture (Grant *et al.*, 2006*a*), making this technique more suitable for use in crop science (Sepulcre-Canto *et al.*, 2007) than for accurate physiological measurements. Other authors related changes in canopy reflectance to leaf water status (Peguero-Pina *et al.*, 2008; Zarco-Tejada *et al.*, 2009). However, although these indexes are promising tools, the same concerns as for infrared thermometry can be raised. Another set of techniques are based on the continuous monitoring of turgor pressure changes as an estimator of the dynamic changes in plant water status, either controlling leaf thickness (McBurney, 1992) or by applying an external pressure. In this regard, ball tonometry is another non-destructive method that allows changes in water potential to be studied through the monitoring of cell turgor pressure (Lintilhac *et al.*, 2000; Geitmann, 2006). However, this technique can only be applied to superficial and relatively thin-walled cells, which constitutes a strong limitation for its widespread use (Lintilhac *et al.*, 2000). More recently, Zimmermann *et al.* (2008) have developed a high-precision pressure probe, which allows the online monitoring of water relations of intact leaves. Although this technique is very sensitive, versatile, and easy to handle, leaves must be clean and surface roughness must be avoided (Zimmermann *et al.*, 2008).

Among the non-destructive methods, the use of ultrasonic waves to determine the water content of a plant was first used by Torii *et al.* (1988). They found a strong correlation between ultrasonic velocity through the stem and leaf water status on *Nicotiana tabacum*. In fact, it is well known that the propagation of ultrasound waves in a partially saturated porous solid strongly depends on the water content (Santos *et al.*, 1990). Some of the disadvantages of this technique are that: (i) contact between the transducers and the stem is required, (ii) only one value of the frequency could be registered; and (iii) thin-layered samples could not be measured. Since then, the use of ultrasonic propagation through a plant organ has been developed to analyse leaf structural features (Fukuhara *et al.*, 2006). However, as far as is known, it has not been used for monitoring changes in leaf water status when there is no contact between the transducers and the sample, mostly due to the common use of water as the medium for the propagation of the signal.

An ultrasonic spectroscopy, or broadband pulse technique, was first presented by Sachse and Pao (1978) for the measurement of the phase velocity of ultrasounds in dispersive materials based on the analysis of the the phase spectrum from which phase and group velocity of longitudinal waves can be obtained. At the same time, Haines *et al.* (1978) applied the broadband ultrasonic spectroscopy to thin-layered media. In this case, plate samples were immersed in water and the transmitted broadband ultrasonic pulses were measured. Later, this technique was extended to measure attenuation and velocity of shear waves (Kline, 1984; Wu, 1996). Afterwards, Pialucha *et al.* (1989) determined the phase velocity in dispersive solids from measurements in plate samples based on the amplitude instead of on the phase spectrum. Such developments allowed the implementation of a technique called 'Broadband ultrasonic spectroscopy', which is based on frequency–domain analysis, by using the Fourier transform, of broadband pulses transmitted or reflected through a sample. As a result, frequency, velocity, and attenuation of ultrasonic waves in materials can be measured. The development of new broadband ultrasonic emitters and receivers, able to transmit and receive ultrasonic signals to and from the air or any gas (air-coupled transducers) has made it possible to apply the broadband ultrasonic spectroscopic method to the measurement of materials without immersion of the sample in water, or the use of any coupling fluid to attach the transducer to the surface of the material. This opened the way to investigate by ultrasonic means, materials like paper, microporous membranes, aerogels or wood that cannot be wetted to carry out the measurements (Gómez Álvarez-Arenas, 2003*a*).

Compared with other ultrasonic techniques, broadband ultrasonic spectroscopy can be applied to dispersive materials and thin samples. In addition, unlike other conventional ultrasonic techniques, air-coupling requires neither any contact with the sample nor use of coupling fluids, so no contamination is introduced, and very rapid inspection is possible as well as an on-line and real-time assessment of the status of the sample. Air-coupled ultrasonic spectroscopy provides information not only from one of the surfaces of the membrane, but also about the whole volume. The back surface can also be used for precise time-delay measurements, even when pulses overlap in the time-domain and when strong dispersion takes place (Gómez Álvarez-Arenas, 2003*a*).

Gómez Álvarez-Arenas (2003*b*) found that the propagation of ultrasound waves in a partially saturated porous solid strongly depends on the water content. He applied broadband ultrasonic spectroscopy to several uniform membrane filters, finding a differential response signal in the transfer function of each membrane depending on the water content. Any variation of the water content may modify the density and the stiffness of the porous solid, hence, changing the ultrasound velocity. On the other hand, variation of the water content may also alter the presence and/or conformation of voids which has a strong influence on the scattering of ultrasounds and, as a consequence, on the attenuation coefficient. Consequently, our hypothesis is that ultrasonic waves could be a promising tool to determine leaf water content, in spite of the fact that propagation of ultrasounds in leaves is expected to be strongly dispersive due to its complex structure that comprises solid, fluid, and gas phases. Therefore, the main objective of this study is to apply the air-coupled broadband ultrasonic spectroscopy to leaves with different structural features, as a non-destructive, non-invasive, and non-contact method, relating ultrasonic parameters with leaf water status.

Materials and methods

The broadband ultrasonic spectroscopy technique: experimental set-up

The broadband magnitude ultrasonic spectroscopy technique is described and schematically depicted in Gómez Álvarez-Arenas (2003*a*). Briefly, the experimental set-up (Fig. 1) consists of two pairs of specially designed air-coupled piezoelectric transducers; in this case with a centre frequency of 0.75 MHz, a working frequency range of 0.3–1.2 MHz, and with a radiating diameter area of 20 mm (Gómez Álvarez-Arenas, 2004). The active element is a disc made of piezoelectric fibres embedded in an epoxy matrix, poled in the thickness direction and with metalized surfaces (sputtered gold). These transducers are positioned facing each other at a distance of 2 cm. It should be noted that previous studies showed that the distance between the transducers had no influence on the signal obtained. A high voltage (100–400 V) square semicycle (duration 0.67 μs) is applied to the transmitter transducer which converts this electrical signal into an ultrasonic pulse and launches it into the air. The receiver transducer collects this signal and converts it into an electrical one, which is then amplified (up to 59 dB) and filtered (low-pass filter at 10 MHz). Eventually, an oscilloscope digitized it, averaged the number of waveforms to reduce the high frequency noise (typically up to 100 waveforms), performed Fast Fourier Transforms (FFT), both magnitude and phase, and transfered the data to a computer for storage.

The experimental procedure of the ultrasonic technique is as follows. First, transmission from a transmitter is directly measured into the receiver. This provides a calibration of the system, in particular, this permits the frequency-band response to be normalized, both magnitude $-I_0(\omega)$- and phase $-\varphi_0(\omega)$-, of the whole system: transducers, electrical generator, and electrical receiver. Then the leaf is held with supports for a few seconds between the transducers at normal incidence. No contact between the leaf and the transducers is necessary.

When the ultrasounds impact normally on the leaf surface, part of the energy is reflected back while the rest is transmitted, propagates through the leaf, and reaches the back surface. Then part of the energy is reflected back while the rest is transmitted through the interface and received at the receiver transducer after travelling through an air gap. This process is repeated for each of the multiple internal reflections in the leaf until the attenuation of the ultrasounds in the material produces the decay of this reverberation. The data measured in this case are both magnitude $-I(\omega)$- and phase $-\varphi(\omega)$-spectra, obtaining the magnitude [$T=I(\omega)-I_0(\omega)$] and the phase [$\Phi=\varphi(\omega)-\varphi_0(\omega)$] of the transmittance. The use of magnitude and phase spectra simultaneously to obtain the thickness of the leaf, its density, and the velocity and the attenuation of the ultrasounds in it, is described in more detail by Gómez Álvarez-Arenas *et al.* (2009).

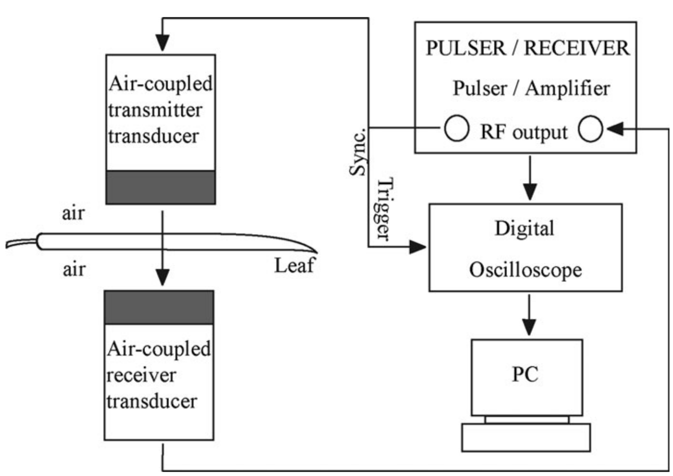

Fig. 1. Schematic representation of the experimental set-up.

Plant material and experimental conditions

In order to apply the broadband ultrasonic spectroscopy technique to species with very different leaf structural features, measurements were carried out in mature leaves collected from two different species, i.e. *Populus×euramericana* (Dode.) Guinier and *Prunus laurocerasus* L. (Table 1). For each species, branches were collected from a single tree; they were kept under water in order to avoid embolism and the leaves were taken with the whole petiole. Thereafter, leaves were rapidly introduced to plastic containers with water in order to ensure a water-vapour saturated atmosphere. After 24 h, weight, ultrasonic parameters, and water potential were individually measured per leaf at constant time intervals until values close to –3 MPa for *P.×euramericana* and close to –5 MPa for *P. laurocerasus* were reached. Leaves were subsequently dried in an oven and weighed when completely dry.

In order to test the repeatability of the technique, two different experiments were made in *P.×euramericana*. On the one hand, the same leaf location was measured ten times in a row at six different relative water content (*RWC*) values. On the other hand, one leaf at ten different locations was measured ten times in a row on and at six different *RWC* values.

P–V analysis

P–V relationships were determined following the free-transpiration method described in previous studies (Dreyer *et al.*, 1990; Corcuera *et al.*, 2002; Brodribb and Holbrook, 2003). The water-relations parameters analysed were: leaf water potential at the turgor loss point, Ψ_{TLP}; maximum bulk modulus of elasticity, ε_{max}; maximum turgor; and the relative water content at the turgor loss point, RWC_{TLP}. Relative water content (*RWC*) was calculated as a ratio of the difference between leaf fresh weight minus leaf dry weight and the difference between leaf saturated weight minus leaf dry weight.

Ultrasonic parameters measurements

The ultrasonic parameter directly measured was the transmitted pulse in the time domain. The Fourier transformation enabled the magnitude and phase to be obtained giving rise to a transmittance (*T*) versus frequency curve for each output. The value of the frequency associated with the maximum *T* at the peak curve was

Table 1. Leaf phenology and leaf morphological parameters [thickness, length, maximum width and leaf mass area (LMA)] for *Populus×euramericana* and *Prunus laurocerasus*

Data are expressed as mean ±SE of five leaves for each species studied.

	Populus×euramericana	***Prunus laurocerasus***
Phenology	Deciduous	Evergreen
Thickness (μm)	284±12	531±8
Length (cm)	11.8±0.6	11.6±0.2
Maximum width (cm)	13.8±0.3	3.9±0.1
LMA (mg cm^{-2})	13.88±0.08	19.32±0.22

compared to the leaf water potential (Ψ, –MPa) and to the *RWC*. In order to contrast the values obtained for both species, frequency values were standardized by means of dividing each single value between the maximum value obtained at RWC=1.00 for each leaf studied.

Statistical analysis

The relationships between standardized frequency (*f*) and *RWC* were adjusted to a cubic function ($RWC=y_0+af+bf^2+cf^3$) for each leaf of both species. The turgor loss point was calculated as the inflexion point of each regression equation. On the other hand, a Student's *t* test was used to compare (i) the parameters obtained from *P–V* analysis between both species and (ii) the RWC_{TLP} obtained from *P–V* analysis and those obtained from ultrasonic measurements. All statistical analyses were carried out using SAS version 8.0 (SAS, Cary, NC, USA).

Results

Table 2 shows the parameters derived from the pressure–volume curves for both species. It should be noted that *Prunus laurocerasus* showed higher values of Ψ_{TLP}, ε_{max}, and Π_0 than *Populus×euramericana* (P <0.05). However, the difference found in RWC_{TLP} between both species was negligible.

In Fig. 2, the relationship between frequency and transmittance for a single leaf of *P.×euramericana* and *P. laurocerasus* at different levels of *RWC* are represented. A drop in the maximum values of transmittance when leaf *RWC* decreased was observed for both species, as long as there was a movement of the whole curve to lower values of frequency. It is remarkable that, not only changes on the values of frequency and transmittance of the peak were observed, but that changes on curve shape also occurred. While *RWC* decreased, the signal of transmittance gained more noise and the shape of the curve got wider at a constant vertical distance from the peak. In this way, the curves at 0.80 and 0.85 of *RWC* for *P.×euramericana* and at 0.87 and 0.83 of *RWC* for *P. laurocerasus* showed more dispersion and they were wider than curves obtained for higher values of *RWC* (Fig. 2).

Figure 3 shows the relationships between the mean values of *f* and *RWC* for the leaves of *P.×euramericana* and *P. laurocerasus*. It should be noted that the differences found in the frequency values measured for both species (Fig. 2) made the use of the standardized frequency (*f*) necessary in order to compare them. The relationship between *f* and *RWC* was adjusted to a cubic function (R^2=0.99, P <0.0001 for both species), which is characterized by the existence of an inflexion point. In our experiment, the inflexion point determines a change in the evolution of *f* as *RWC* decreases. This point can be compared with the intersection point of the two-phase linear equation for the relationship between leaf water potential and *RWC* found by Brodribb and Holbrook (2003). These authors considered that the intersecting point can be associated with the turgor loss point, such as for the inflexion point in the *f–RWC* relationships. For *P.×euramericana*, the RWC_{TLP} found from the *f–RWC* relationships was 0.89±0.01, whereas it was 0.88±0.01 from the analysis of *P–V* curves. For *P. laurocerasus*, the RWC_{TLP} found from the *f–RWC* relationships and from

Table 2. Parameters derived from the pressure–volume curves for *Populus×euramericana* and *Prunus laurocerasus*: water potential at the turgor loss point (Ψ_{TLP}, –MPa), relative water content at the turgor loss point (RWC_{TLP}), maximum bulk modulus of elasticity (ε_{max}, –MPa), and maximum turgor (–MPa)

Data are expressed as mean ±SE. Different letters indicate significant differences at P < 0.05.

	Populus×euramericana	*Prunus laurocerasus*
Ψ_{TLP} (–MPa)	1.72±0.11 a	2.66±0.21 b
RWC_{TLP}	0.88±0.01 a	0.89±0.01 a
ε_{max} (–MPa)	11.34±0.63 a	17.53±0.87 b
Maximum turgor (–MPa)	1.54±0.04 a	2.72±0.11 b

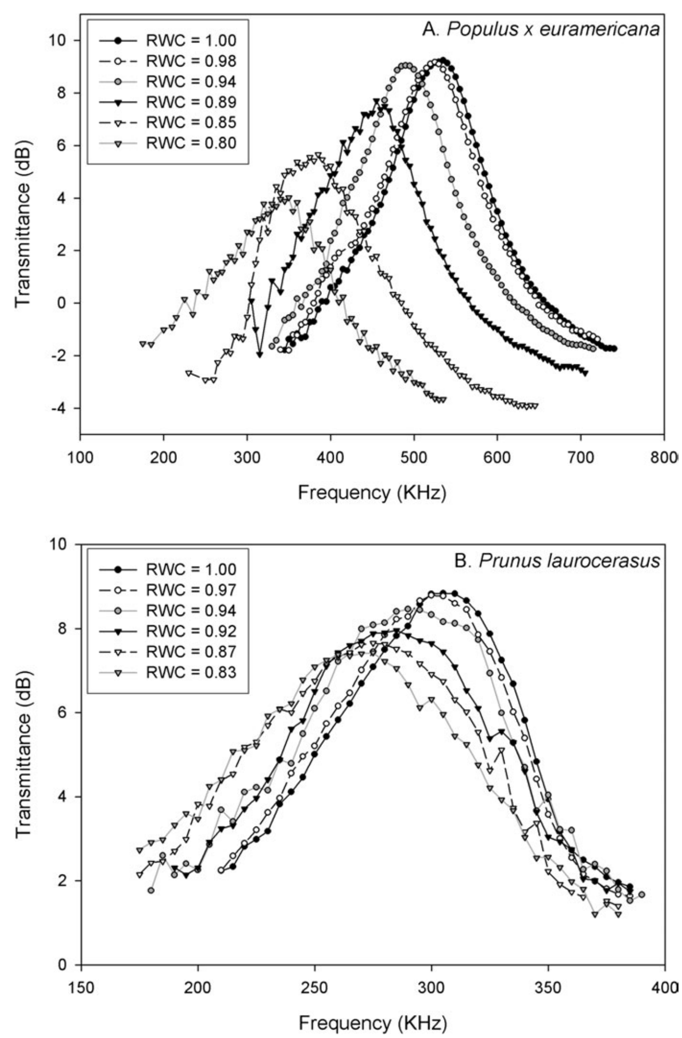

Fig. 2. Relationship between the frequency (KHz) and transmittance (dB) for (A) *Populus×euramericana* and (B) *Prunus laurocerasus* for a single leaf of each species at different levels of relative water content (*RWC*).

the analysis of P–V curves were 0.89±0.01. However, the differences in RWC_{TLP} values obtained from both techniques (P–V curves and ultrasonic measurements) were not statistically significant at P <0.05.

In Fig. 4, the relationships between the mean values of f and Ψ for the leaves of *P.×euramericana* and *P. laurocerasus* is represented. The relationship between f and Ψ was also adjusted to a cubic function (R^2=0.99, P <0.0001 for both species). Although the relationship for *P.×euramericana* could also be adjusted to a linear correlation equation (R^2=0.97, P <0.0001), we have considered that the cubic function reflected better the relationship between f and Ψ for this species.

The repeatability of the technique was checked with *P.×euramericana* leaves. On the one hand, the value of the frequency measured in the same leaf location remained fairly constant and the differences were not statistically significant at P <0.01, for the six RWC values considered (data not shown). On the other hand, the differences found in the frequency values measured for the same leaf at ten different locations were not statistically significant at P <0.05, for the six RWC values considered (data not shown).

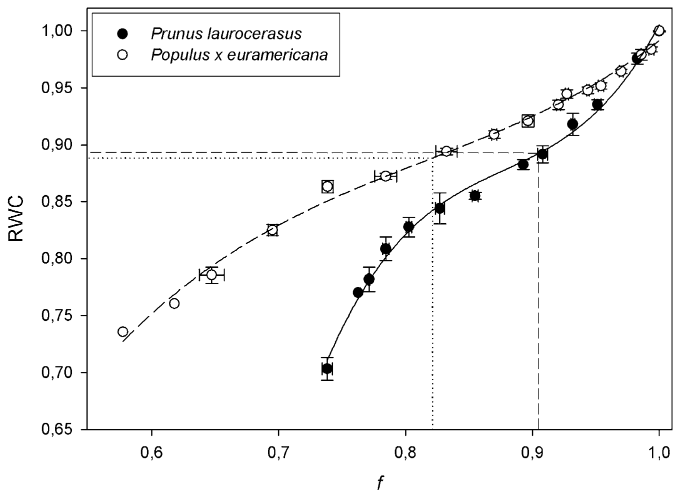

Fig. 3. Relationship between standardized frequency (f) and relative water content (RWC) for *Populus×euramericana* and *Prunus laurocerasus*. Data are expressed as mean ±SE of ten leaves for each species studied.

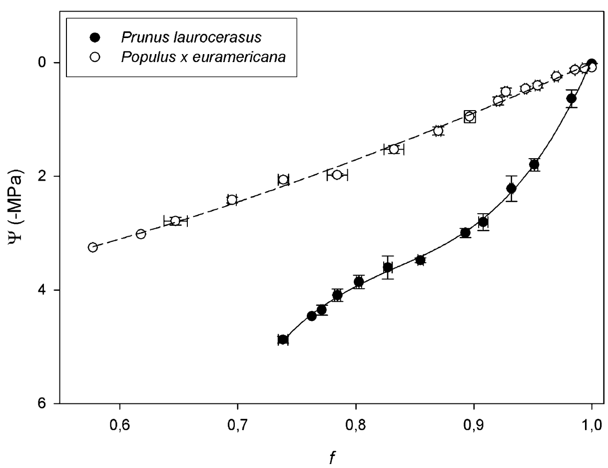

Fig. 4. Relationship between standardized frequency (f) and water potential (–MPa) for *Populus×euramericana* and *Prunus laurocerasus*. Data are expressed as mean ±SE of ten leaves for each species studied.

Discussion

In this study, the broadband ultrasonic spectroscopy technique has been proven as a non-destructive, non-invasive, non-contact, and reproducible method for the dynamic determination of leaf water status. The procedure is based on the excitation of thickness resonances on the leaves, specifically, changes in the frequency at the maximum transmittance (leaf resonant condition) have been revealed as an optimum indicator of changes in RWC of leaves with contrasting structural features. The frequency–transmittance output curves were surprisingly well explained by an ultrasonic model, assuming the medium as continuous and non dispersive, specifically those with higher values of RWC (Fig. 2), in spite of the high complexity and heterogeneity of the structure of leaves. However, when the frequency values of *P.×euramericana* and *P. laurocerasus* were compared, they were very different in range (Fig. 2), which may be related to the differences found in leaf structural parameters between both species (Table 1). The strong relationship found between RWC and the frequency associated with the maximum transmittance is mainly due to the mechanical softening of the leaf as it loses water. This produces a reduction in the velocity of ultrasounds that eventually leads to the observed reduction of the resonant frequency of the leaf.

The relationship between f and RWC was characterized by the existence of an inflexion point. This point could be related to the intersecting point found by Brodribb and Holbrook (2003) for the relationship between Ψ and RWC, which has been associated with the turgor loss point. The turgor loss point has been considered to be a threshold for many physiological processes (Brodribb and Holbrook, 2003; Thomas *et al.*, 2006; Mitchell *et al.*, 2008). In our study, there were no statistically significant differences between RWC_{TLP} derived from the analysis of P–V isotherms and those obtained from the relationship between f and RWC for both species. Therefore, the turgor loss point could be estimated through the calculation of the inflexion point of the f–RWC relationship. However, the data presented are preliminary and need be confirmed in future attempts by incorporating results otained from a larger range of plant species and tissues.

The decrease in the frequency associated with the maximum transmittance (thickness resonance of the leaf) before the turgor loss point can be explained by the mechanical softening of the leaf. This softening overcomes the effect of density reduction due to the loss of water and gives rise to a decrease in the velocity of ultrasounds in the leaf. The slope of the relationship between f and RWC during this phase is clearly higher in *P. laurocerasus* because the loss of turgor in this species is sharper than in *P.*×*euramericana*. This fact can be explained by the higher value of ε_{max} recorded for *P. laurocerasus*. A simple mathematical model to explain this behaviour can be developed, assuming the leaf cells as a compact arrangement of fluid droplets, each one surrounded by an elastic membrane (or fluid-filled microballoons). Compressibility of such an arrangement, which is determined by the deformation of such cells at the point of contact with others, is used to determine the overall compressibility of granular media (Duran, 2000). It is proposed that compressibility increased when leaf water potential became more negative and the tension decreased, which would bring about a reduction in the frequency associated with the maximum transmittance.

The turgor loss point (associated with the inflexion point) determines a change in the evolution of f as RWC decreases (Fig. 3). This change coincided with a strong increase in the noise of the transmittance signal (Fig. 2). This new phase deserves a different physical explanation than the former one. It is proposed that an increment in the leaf internal heterogeneity may underlie the change in the acoustic properties of the leaf, which are associated with an increase in signal attenuation. The higher irregularity in the acoustic pathway could be related to an increment in the gas-filled spaces in the internal tissues. The cavitation of minor veins has been proposed as a common phenomenon during water stress (Trifilo *et al.*, 2003), which could explain the formation of gas-filled obstacles to acoustic propagation. However, the changes in frequency during the dehydration process remained quite progressive, although cavitation is a sharper process that may have a sudden change in the acoustic signal. Another mechanism that could explain the increment in the gas-filled spaces is the lateral shrinkage of mesophyll cells, as shown by cryoscanning observation of dehydrated leaf tissues (McBurney, 1992). However, we consider that a full explanation of the structural changes experimented by the leaf during dehydration deserves further investigation, in order to obtain a definitive mechanistic explanation.

In spite of this and from a methodological point of view, the broadband ultrasonic technique proves to be a promising new method for the determination of the turgor loss point, as the inflexion point of the relationship between the standardized frequency and RWC during drying, in a non-contact and non-destructive way. The advantage of this procedure, as compared to the classical analysis of the P–V isotherms (Corcuera *et al.*, 2002) is that it is outstanding in terms of simplicity and time consumption. Moreover, when the frequency is standardized, the accuracy of this new technique seems to be dependent on the natural heterogeneity existing in a set of leaves, as it is shown for the P–V isotherms method. However, nowadays, this method cannot be applied to species with leaves smaller that the radiating area of the transducer. To solve this problem, transducers with a radiating diameter area lower than 20 mm are now being developed.

The ability for registering rapid changes in turgor values, under conditions of free leaf transpiration, may constitute a tool of paramount importance for the study of dynamic processes associated with this variable. Thomas *et al.* (2006) suggested that the cell pressure probe is the only available method for the direct measurement of turgor in plant cells, due to the possible artefacts related to the use of isopiestic psychrometers. The changes in frequency along the positive turgor phase can be effectively used as an indicator of the changes experienced by the leaf tissues examined by the waves. Although a direct estimation of a single cell cannot be achieved, the smaller the area exposed to the ultrasonic waves, which can be optically modified, the bigger the spatial resolution of the method. The measurement of the turgor gradient between two different points of the leaf blade, in order to relate such a gradient with water transport within an attached, naturally transpiring leaf can easily be carried out with the method presented here.

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