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Contact-less, non-resonant and high-frequency ultrasonic technique: Towards a universal tool for plant leaf study

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ABSTRACT

Plant-based measurements are recognized as key methods to obtain insightful data in the field. In general, they are labor-intensive and expensive. In this context, Non-Contact Resonant Ultrasonic Spectroscopy technique (NC-RUS) emerged as a powerful alternative that enabled plant water status determination in a non-destructive, non-invasive and rapid way. However, NC-RUS is not applicable to all plant species as it depends on the possibility to excite and sense thickness resonances in the leaves. In this work, we propose and test an ultrasonic technique that can be used in all leaves, regardless of the appearance of thickness resonances. This technique is based on the contactless measurement of through transmitted airborne ultrasonic pulses in the leaves at high-frequencies and in the absence of thickness resonances, to obtain the leaf ultrasonic velocity (v_{air}). It benefits from the facts that: i) at sufficiently high frequencies (typically around 1 MHz) all leaves are non-resonant (so the technique can be applied to both resonant and non-resonant leaves), ii) the use of high-frequencies allows a greater time resolution and a further miniaturization, making possible to apply the technique to small and irregular leaves. Three different signal processing techniques were used to determine the time it takes to the ultrasonic pulse to cross the leaves (time-of-flight) from the measured signals. Two of them operate in time domain: cross-correlation, and edge detection, while the third one makes use of the Fast Fourier Transform (FFT) and operates in the frequency domain: phase-slope. If leaf thickness is also measured, ultrasound velocity can then be worked out. As ultrasound velocity is determined by density and elastic modulus, it is then closely related to water content and turgor pressure. Obtained ultrasound velocities were first validated by comparing them with those obtained by well-established and standard ultrasonic methods: water immersion transmission (v_{water}) and NC-RUS (v_{res}). The conclusions of this comparison permitted us to propose a novel methodology that combines the three signal processing techniques used to improve robustness and accuracy for the measurement of ultrasound velocity in plant leaves. It is of interest to note that a bias towards higher values of v_{air} compared to v_{res} was observed. This behavior is considered the consequence of the different influence of the leaf layered structure in these two measurements, so this feature can be further used for leaf structure analysis.

1. Introduction

The upcoming climate scenarios plus population growth require changes in the agricultural sector towards a more efficient use of water (US GAO, 2019; World Economic Forum, 2016). Improvements of irrigation techniques as well as the emergence of new ones, is due in part to the transformation of traditionally rainfed farms to irrigated ones in search of higher yields (Fernández, 2017; Zhang et al., 2021). Plant-based measurements have been suggested as a better way to optimize irrigation scheduling, since the plant integrates not only the physiological response to the available water but also soil and atmosphere status (Girona et al., 2006; Jones, 2006). However, traditional techniques for measuring the most used indices in plant water status (e.g.: relative water content or leaf and stem water potential) are either time consuming or involve a destructive and cumbersome process such as the use of the Scholander pressure chamber or psychometry (Jones, 2013).

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In this context, Non-Contact Resonant Ultrasound Spectroscopy (NC-RUS) emerged as an alternative for measuring physical properties in plant leaves among others based on their response to pressure (Zimmermann et al., 2008) or to electromagnetic waves at GHz (Sancho-Knapik et al., 2011; Sancho-Knapik et al., 2013b), or at THz (Baldacci et al., 2017; Gente and Koch, 2015; Pagano et al., 2019; Sims and Gamon, 2003) or their dielectrical properties (Zhang and Willison, 1991).

The NC-RUS technique relays on the excitation and sensing of thickness resonances in plant leaves (Álvarez-Arenas et al., 2016a,b, 2009; Sancho-Knapik et al., 2010). It was demonstrated that the leaf ultrasonic parameters extracted by NC-RUS have a close relationship with recognized leaf indicators such as relative water content or leaf water potential (Sancho-Knapik et al., 2011b; 2013b; 2016; Fariñas et al., 2021). In parallel, the development of acoustic models of the leaf helped get further information of their physical and mechanical properties and to resolve the elastic properties of different layers in the plant tissues (Álvarez-Arenas et al., 2020; Fariñas et al., 2019).

The fact that the applicability of NC-RUS depends on the possibility to excite thickness resonances in the leaves, limits the range of applicability of this technique. The establishment of the resonance condition requires the preservation of the ultrasonic wavefront inside the leave, that is, the preservation of the integrity of the phase coherence in the plane wavefront and the presence of reverberations within the leaf thickness. Any leaf feature that contributes to distort this phase coherence has the potential to affect and, in the extreme case, suppress the onset of thickness resonances. Among others, we can mention: surface roughness, non-plane-parallel surfaces and heterogeneity (examples of plant species where we didn’t find resonances are barley and *Zamioculca*). On the other hand, even when the phase coherence is preserved, a very high attenuation coefficient in the leaf may prevent the onset of the resonances when the attenuation is big enough to kill reverberations within the leaf (this is the case of some *Arabidopsis thaliana* mutants, and some tobacco and lettuce leaves). Finally, it has to be mentioned that as the parameters extraction used in the NC-RUS technique requires assumption of plane wave propagation, the use of focused beams must be avoided. This has a negative impact on the usability of this technique, as leaves smaller than the ultrasonic beam width (typically from 10 to 20 mm diameter) and leaves that do not present a similar near-flat surface cannot be measured (examples are *Olea europaea* and *Bamboo* leaves, and some *Quercus* and *Arabidopsis thaliana* leaves).

Therefore, the aim of this work is to propose and validate an ultrasonic technique that can be used for all kind of plant leaves regardless of the possibility or not to excite thickness resonances, and that can also be used independently of the size and the shape of the leaves.

The proposed technique is based on the use of high frequency ultrasonic pulses. This high frequency limit has to comply with three main requirements: i) frequency is high enough so that there are no thickness resonances in the leaves, ii) frequency is not so high so that signal to noise ratio (SNR) can be kept high enough (typically greater than 20 dB), iii) frequency is high enough so that estimation of time-of-flight error is minimized. Normally, this involves frequencies around 1 MHz. Due to the increase of the attenuation with the frequency, we have, so far, observed no leaf thickness resonances above 1 MHz, so all leaves can be considered as non-resonant in this frequency range. In addition, for frequencies above 1 MHz, ultrasound wavelength in the air is below 340 µm and it is even smaller in the leaves as ultrasound velocity use to be smaller than in air, so this provides enough resolution to measure time-of-flight with accuracy. Finally, another advantage of using high frequency signals is that for frequencies around 1 MHz it is possible to make smaller transducers (down to 5 mm diameter for the radiating surface) which permits to apply the technique to plant species with smaller and irregular leaves. Moreover, with this non-resonant technique it is possible to use focused transducers, therefore the section of the leaf measured can be even further reduced. For a concave transducer with an aperture D = 2R, the Full Width at Half Maximum of the field at focal distance F within the near field region, FWHM, is given by Álvarez-Arenas et al. (2016b):

$$FWHM = 1.4\frac{\lambda F}{2R} \tag{1}$$

Therefore, for example, for an air-coupled transducer at 1 MHz with R = 10 mm and F = 40 mm, FWHM = 0.95 mm.

The final goal is to obtain, using this high frequency and non-resonant technique, leaf ultrasonic parameters that are related with leaf water content and turgor pressure (Fariñas et al., 2013; Sancho-Knapik et al., 2011). In this work, we focus on the measurement of the ultrasound velocity in the leaf as we know that ultrasound velocity is related with the elastic modulus in the leaf thickness direction according to Eq. (2):

$$v = \sqrt{\frac{M}{\rho}} \tag{2}$$

where M is the elastic modulus and ρ the leaf density. It is well known that effective leaf modulus is strongly dependent on the water content through the turgor potential. Previous works showed the potential of the ultrasonic velocity as a fair estimator of leaf water status (Álvarez-Arenas et al., 2020; Fariñas et al., 2013).

The utility of this proposal depends on some limiting factors related to the use of higher frequencies: the increment in the ultrasonic attenuation with frequency in the leaves is expected to have a negative impact on the signal to noise ratio (SNR), the sensors’ sensitivity decreases at higher frequencies (this also has a negative impact on the SNR), and the inherent reduction of ultrasonic wavelength gives rise to an increasing sensitivity of the ultrasonic wave to smaller leaf irregularities and imperfections that may increase the variability of the measurement and may compromise the accuracy of the time-of-flight estimation. Practice tells us that, in general, we can measure leaves as far as the magnitude of their transmission coefficient is above −80 dB, this can be used to set the upper limit for the usable frequency bandwidth.

2. Material and methods

2.1. Materials

2.1.1. Plant material and preparation

Up to 21 leaves from 11 evergreen plant species (see Table 1) were collected, during winter 2021–2022, to ensure a broad representation of different shapes, thicknesses and other features that may influence their ultrasonic response. The selected samples were taken early in the

Table 1
Thickness range, frequency range of the first resonance appearance and the adequate high-frequency range considered in time-of-flight measurements for each species in the study.

Species	Thickness (µm)	1st Resonant frequency (MHz)	High frequency range for tof measurements (MHz)
Epipremnum aureum	225–250	0.21–0.23	0.4–0.8
Coffea arabica	225–245	0.27–0.30	0.4–0.8
Persea americana	160–180	0.55–0.6	0.8–1.3
Hedera helix	300–340	0.25–0.30	0.7–1.0
Ligustrum lucidum	235–325	0.2–0.28	0.6–1.2
Buxus sempervirens	310–330	0.41–0.45	0.6–1.3
Arbutus unedo	430–446	0.27–0.29	0.6–1.2
Trifolium	310–350	0.35–0.40	0.6–1.2
Pelargonim grandiflorum	450–680	0.12–0.13	0.4–0.8
Chlorophytum comosum	368	–	0.3–0.7
Nerium oleander	340–400	0.29–0.38	0.6–1.2

morning and subsequently introduced in plastic containers with water in order to ensure a water–vapor saturated atmosphere. Once in the laboratory, the petioles were partially cut before placed in water. In order to ensure full hydration, leaves were kept 24 h at 5 °C.

2.1.2. Ultrasonic equipment

For the contactless measurements, three pairs of wide band air-coupled ultrasonic piezoelectric transducers were used. All of them were designed and built at CSIC lab (Álvarez-Arenas, 2013, 2004). Their central frequencies were 0.25, 0.65 and 1.2 MHz, peak sensitivities of −25, −30 and –32 dB and active area diameters of 20, 15 and 10 mm, respectively. Each pair of sensors were embedded in a u-shaped holder that kept transmitter and receiver facing each other at a fixed distance. The holder had a slot specifically designed to place the leaf and easily enable to take measurements at normal incidence.

For measurements in water, a pair of commercial 1 MHz transducers (A392S; Olympus, Houston, TX, USA) were embedded aligned in a 110 × 56 mm water tank with a leaf holder in between.

A commercial pulser/receiver, P/R, (5077PR; Olympus, Houston, TX, USA) was used. Excitation signal is a negative semicycle of square wave tuned to the transducers centre frequency. The repetition rate (pulse repetition frequency, PRF) was set to 1 kHz. The receiver transducer was connected to the receiver stage of the P/R: the analogical signal was low-pass filtered (10 MHz cut-off frequency) and amplified. The flatness of the amplifier in the frequency range of interest (0.2–2.0 MHz) were verified both in terms of amplitude and phase, as non-amplitude flatness may affect time-of-flight estimations using the cross-correlation method, and the non-phase flatness may affect the time-of-flight estimations by using the phase spectra. We verified the absence of any jitter in the received signal, as this may affect the estimated time-of-flight, and then averaged and stored using an oscilloscope (DPO7054; Tektronix, WA, USA) with the impedance set at 1 MΩ and controlled through Matlab (Mathworks, Inc., Natick, USA). The oscilloscope was triggered using the trigger signal output provided by the P/R and an edge detection trigger method, so this improved signal stability and enabled an absolute time reference that made possible to compare signals obtained with and without leaf in between the transducers.

2.2. Methodology and experimental procedures

The experimental setup for the time-of-flight measurement in air is described on section 2.1.2. A 200 V-amplitude semi-cycle of square waved tuned to transducers center frequency was used to drive the air-coupled sensors. The received signals were amplified 40 dB, digitized at 20 MS/s with 8 bit (vertical resolution) and averaged 16 samples before acquired. A blank measurement was taken before placing each leaf in between the transducers with the purpose of using it as reference. Then, the leaf was measured in a point situated in the intercostal panel midway between the midrib and the margin, avoiding secondary veins when possible.

The transmission coefficient was first measured for all leaves in the whole frequency range available. This permitted to determine the presence of resonances and the frequency range where the measurements of the time-of-flight will be performed with the proposed technique. This frequency range correspond to frequencies high enough so that no resonances appear and low enough to avoid a large loss that compromise the SNR (typically above −80 dB).

When there is no leaf between transmitter and receiver the time it takes the ultrasonic pulse to travel from transmitter (Tx) to receiver (Rx) is given by:

$$t_1 = \frac{D}{v_0} \tag{3}$$

where D is the separation between transmitter and receiver and v_0 is the ultrasound velocity in the medium (water or air).

When the leaf is placed between transmitter and receiver, a small fraction of the fluid in the travelling path from Tx to Rx is replaced by the leaf. Now, the time to get from Tx to Rx is given by:

$$t_2 = t^{fluid} + t^{leaf} \tag{4}$$

where, t^{fluid} is the time travelling in the fluid between Tx and Rx and t^{leaf} is the time travelling in the leaf. t^{fluid} is given by:

$$t^{fluid} = \frac{D-h}{v_0} \tag{5}$$

where h is the thickness of the leaf (in this case was measured independently using a micrometer (Mitutoyo ± 1 µm, Tokio, Japan)).

Since the leaf is a layered composite material (whose layers may comprise, at least: epidermis, palisade parenchyma and spongy parenchyma), then the time it takes the ultrasonic signal to cross the leaf is given by:

$$t^{leaf} = \sum_{i=1}^{N} \frac{h_i}{v_i} \tag{6}$$

where i denotes each layer in the leaf, N is the total number of layers, h_i is the thickness of each layer and v_i the velocity in each layer. Hence, we can define and averaged ultrasound velocity in the leaf v_{leaf} that is obtained from:

$$v_{leaf} = \frac{h}{t^{leaf}} \tag{7}$$

where h is the thickness of the leaf. That is:

$$v_{leaf} = \sum_{i=1}^{N} h_i \Big/ \sum_{i=1}^{N} \frac{h_i}{v_i} = 1 \Big/ \sum_{i=1}^{N} \frac{h_i/h}{v_i} \tag{8}$$

hence, t_2 is given by:

$$t_2 = \frac{D-h}{v_0} + \frac{h}{v_{leaf}} \tag{9}$$

and, v_{leaf} is obtained from:

$$v_{leaf} = h \Big/ \left((t_2 - t_1) + \frac{h}{v_0} \right) \tag{10}$$

where, as explained above, v_{leaf} is the averaged ultrasound velocity in the leaf and $\Delta t = t_2 - t_1$ is obtained from the difference in time-of-flight between the signals received with and without the leaf in between the transducers.

That is, calculation of the ultrasonic velocity in the leaf only depends on the leaf thickness, the velocity in the outer medium (air) and the differences of time-of-flight obtained with and without leaf between Tx and Rx, that is: Δt.

It is interesting to note that the averaged velocity in this case is different to the averaged velocity obtained in the NC-RUS technique. In the latter case, the resonance condition appears when the wavelength in the leaf equals *h/2*. However, effective wavelength (λ_{eff}) is obtained from:

$$\lambda_{eff} = \frac{h}{f} \sum_{i}^{N} \frac{v_i}{h_i} \tag{11}$$

consequently, the way mean velocity is obtained in this case is different compared to the previous one, thus it can be expected that different values are obtained for the leaf mean velocity if we use a non-resonant or a resonant technique. For the case of an isotropic layer both values are expected to be equal, but the larger the difference between the layers, the larger the difference in the estimated velocities.

The time-of-flight determination can be obtained by standard experimental techniques and procedures. Factors such as pulse distortion, pulse attenuation or low signal-to-noise ratio (SNR) can make it difficult to obtain this parameter in a reliable and repeatable way. For this reason, in order to estimate the differences in time-of-flight (Δt Eq.

(10)), we used three different signal processing algorithms well described in literature, namely: cross-correlation, phase spectrum (or phase-slope) and edge detection (Hull et al., 1984; Papadakis, 1976; Truell et al., 1969). Although these methods are well-known and widely applied, each of them has particular assumptions that must be taken into account for the present study.

The cross-correlation method assumes that the pulse distortion between the two pulses to be compared is negligible so that Δt can be estimated from the time shift required to obtain the maximum overlap (between the two pulses). Consequently, the applicability of this method in plant leaves may be compromised since a significant level of signal distortion is common in plant leaves. On the one hand, ultrasonic attenuation in the leaves increases with the frequency so that leaves behave acoustically as a low-pass filter, causing the loss of the high frequency components of the incident pulse and, hence, its distortion. Depending on the thickness of the leaf, the ultrasound attenuation in the leaf and its increase with the frequency, this distortion may be become large enough to make difficult or, even, questionable the use of the cross-correlation technique. On the other hand, heterogeneities in the leaves as thickness changes, presence of scatterers and interfaces and internal discontinuities also have an impact on signal distortion.

The phase spectrum method calculates the Fourier transform of the received signals with and without leaf in between the transducers and uses this information to obtain Δt. As phase $\phi(\omega)$ is given by ωt where ω is the angular frequency and t the time, then $\Delta\phi(\omega) = \omega\Delta t(\omega)$; and $\Delta\phi$ is the difference between the phase spectra obtained with and without leaf in between the transducers. It is worthwhile to note that in this case it is possible to measure a time-of-flight that is frequency dependent ($\Delta t(\omega)$), that is, it is possible to obtain the variation of the velocity with the frequency, what we call dispersion. It is interesting that this is one of the sources of pulse distortion and that the phase spectrum method can then be used to calculate ultrasonic velocity in dispersive materials. In the case when the variation of $\Delta\phi(\omega)$ is linear with the frequency, then this means that $\Delta\phi(\omega) = \omega\Delta t$, namely, Δt is constant and it can be obtained from the slope of $\Delta\phi(\omega)$ versus ω. In this case, the main source of error in the determination of Δt comes from the error in the estimation of the slope of $\Delta\phi$ vs ω and this comes from the SNR and the effective bandwidth where phase spectra measurements where performed: the narrower the bandwidth is, the larger the error becomes.

Finally, the edge detection method or amplitude threshold method establishes a threshold and determines the time of arrival of the pulse as the time the signal amplitude crosses this threshold. This involves signal normalization and the determination of such threshold. This method, though simple is not easy to implement in a robust way as results can be very dependent on the threshold imposed and on the difference in SNR between the two signals to be compared. To avoid this problem, the double threshold method also called as the window sliding method, uses a window of N samples which is shifted in time along the signal (Li et al., 2014). At each step, the number of samples in the window N exceeding the threshold is obtained. If this number exceeds the second threshold, then the time-of-flight is obtained. Moreover, in the case of the presence of significant pulse distortion it is questionable if this way to calculate Δt results in a meaningful estimation of wave velocity.

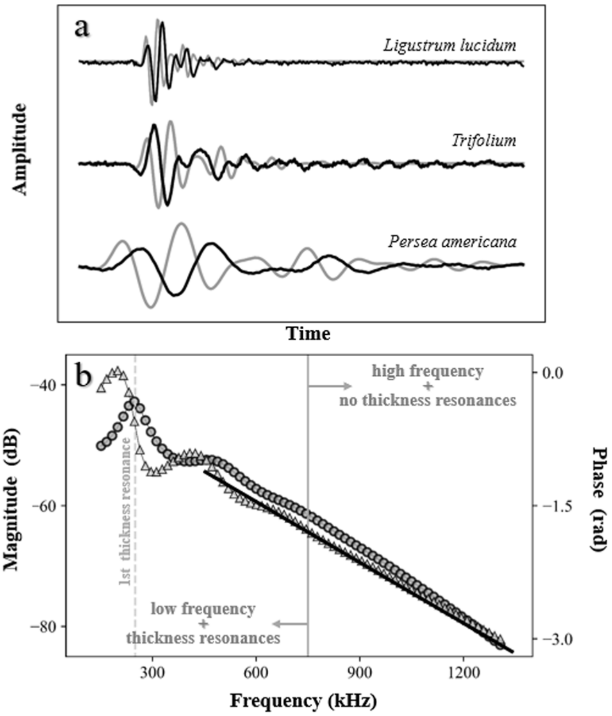

Fig. 1. a) Normalized temporal signals (solid black) with its corresponding reference in air (solid grey) of: *Ligustrum lucidum*, *Trifolium*, and *Persea americana*; b) Magnitude (dark grey dots) and phase (light grey triangles) spectrum of the transmission coefficient in a *Nerium oleander* leaf. The solid black line represents the linear fitting of the phase (phase-slope method).

Some examples are shown in Fig. 1. Fig. 1a shows some signals in the time domain to illustrate the effect of pulse distortion. Pulse distortion is very reduced for *Ligustrum lucidum* leaves, noticeable for *Trifolium* leaves and very large for *Persea americana* leaves. Fig. 1b shows magnitude and phase spectra of the transmission coefficient measured in *Nerium oleander* leaves. In general, the leaf attenuation increases with frequency, reducing the bandwidth available which could lead to slope estimation error. In the case shown in Fig. 1b, it is possible to measure leaf response up to 1.3 MHz, which corresponds to the limit loss of −80 dB. Thickness resonances are limited to frequencies below 0.7 MHz, so it is clear that it is possible to measure these leaves, in the absence of resonances, in the frequency range 0.7–1.3 MHz. In this frequency range, variation of the phase spectrum differences versus frequency is linear and the slope provides the difference in time-of-flight needed to work out velocity. As mentioned above, when the reduction of this frequency range becomes significant, the accuracy of the phase-slope method can be questionable, this may happen when the range of thickness resonance frequencies extend to higher frequencies and the losses increase faster with frequency.

Another example is shown in Fig. 2. In this case, we show the signal in the time domain received with (grey) and without (black) a *Pelargonium grandiflorum* leaf (*Geranium*) in between the transducers (Fig. 2a), using the pair of air-coupled sensors centered at 0.65 MHz. The signal received with the leaf in between the transducers arrives later because the ultrasound velocity in the leaf is lower than the ultrasound velocity in the air. The signal distortion is evident and this is the result of the increase of the attenuation with the frequency: leaf operates as a low pass filter and reduces the frequency band of the pulse, increasing the ringing. Then the two signals are aligned by using the Δt values obtained from the cross-correlation ($\Delta t = 2.5$ μs; Fig. 2b), the phase-slope ($\Delta t = 2.7$ μs; Fig. 2c) and the edge detection ($\Delta t = 1.8$ μs; Fig. 2d) methods. It can be clearly seen the differences in how these three methods align both signals as well as the differences in the estimation of Δt.

Fig. 3 complements this example and shows the magnitude and phase spectrum. Measurements in the frequency range 0.15–0.35 MHz were obtained with the pair of transducers centered at 0.25 MHz, and measurements in 0.4–0.85 MHz were obtained with the pair of transducers centered at 0.65 MHz. It is clear, that the lower frequency range (0.15–0.35 MHz) presents two thickness resonances. The first one is slightly out of the band, and must be located around 0.1–0.12 MHz, while the second order is located at 0.25 MHz. For the frequency range 0.4–0.85 MHz, the leaf response is free of resonances. Therefore, this is the frequency range selected for the out-of-resonance measurements. Losses increase continuously and in a very significant way from −50 to −90 dB (this explains the observed pulse distortion, the narrowing of the band of the signal transmitted through the leaf, its shift towards lower

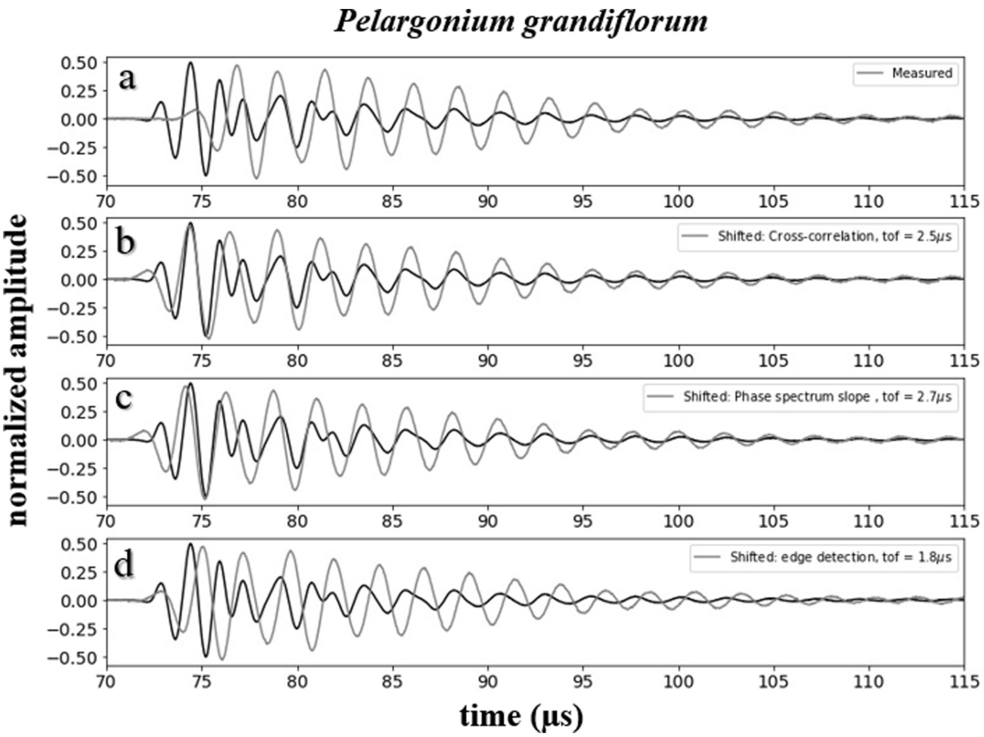

Fig. 2. Measurements taken at 0.65 MHz center frequency displayed in the time domain on *Pelargonium grandiflorum* leaves: solid grey lines show signals received after passing through the leaves and solid black lines show the references: a) signals showing the real delay measured between reference (only propagated through air) and *Pelargonium grandiflorum* leaf; Measurements and references are aligned after the following methods for time-of-flight calculation: b) cross-correlation; c) phase-slope; d) edge detection.

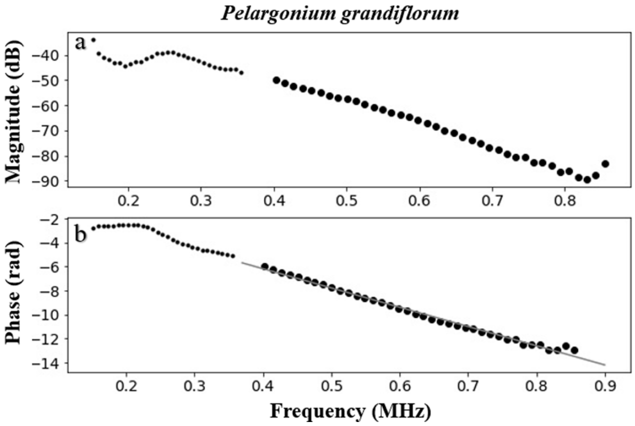

Fig. 3. Transmission coefficient spectrum of *Pelargonium grandiflorum* leaf a) magnitude; and b) phase. The solid grey line represents the linear correlation between phase and frequency.

frequencies and the longer ringing, see Fig. 2) and phase variation is linear with the frequency, so this permits to calculate Δt from the slope of $\Delta\phi$ vs ω. The grey solid line in the phase spectrum corresponds to the linear regression.

Another example, *Persea americana* leaf, is shown in Fig. 4 and this case shows some relevant differences compared to the previous one. Fig. 4 shows the signal received with (grey) and without (black) *Persea americana* leaf in between the transducers (Fig. 4a), using the pair of transducers centered at 1.2 MHz. The signal received with the leaf in between the transducers arrives slightly later because the ultrasound velocity in the leaf is lower than the ultrasound velocity in the air. The signal distortion is not so evident as in the previous case, which suggests that the increment of attenuation with the frequency is more reduced in this case. The two signals (with and without leaf) are aligned by using the Δt values obtained from the cross-correlation ($\Delta t = 0.4$ µs; Fig. 4b), the phase spectrum slope ($\Delta t = 0.2$ µs; Fig. 4c) and the edge detection methods ($\Delta t = 0.0$ µs; Fig. 4d). Even in this case, with a more reduced signal distortion, it can be clearly seen the differences in how these three methods align both signals as well as the differences in the estimation of Δt.

Fig. 5 complements this example and shows the magnitude and phase spectrum. Measurements in the frequency range 0.4–0.85 MHz were obtained with the pair of transducers centered at 0.65 MHz, and measurements in 0.9–1.5 MHz were obtained with the pair of transducers centered at 1.2 MHz. It is clear, that the lower frequency range (0.4–0.8 MHz) presents one thickness resonance, though more attenuated than in previous example, but still clearly visible. For the frequency range 0.9–1.5 MHz, the leaf response is free of resonances. Therefore, this is the frequency range selected for the out-of-resonance measurements. Losses increase continuously from −55 to −65 dB (which is a much more reduced variation than in previous case: −50 to −90 dB), this explains the more reduced pulse distortion observed in this case. Nonetheless the effect of SNR reduction can be observed at frequencies over 1.3 MHz. In addition, phase variation is linear with the frequency, so this permits to calculate Δt from the slope of $\Delta\phi$ vs ω. The grey solid line in the phase spectrum correspond to the linear regression.

In addition, and in order to validate the results obtained with the proposed technique, the same leaf samples were measured using two rather conventional ultrasonic methods: through transmission in water immersion and NC-RUS. The only purpose of using these techniques is for comparison and validation. Water immersion technique must be used carefully as the water can interact with the leaf and modify its properties. Therefore, it can be used with the required precautions as a validation tool with fully hydrated leaves, as revealed by the fact that the application of this technique apart from this situation is very limited (Fukuhara, 2002; Fukuhara et al., 2005; Torii et al., 1988). Water immersion measurements were taken following same procedure described above for air with the equipment specifically outlined in 2.1.2.

On the other hand, as explained in the introduction, NC-RUS cannot be used with leaves that do not resonate, as is the motivation of this work. For the comparison of the presented technique, some leaves have been selected that resonate at low frequencies, while at high frequencies do not as a consequence of the increase of the attenuation with the frequency. This special case allows the comparison in the same leaf of

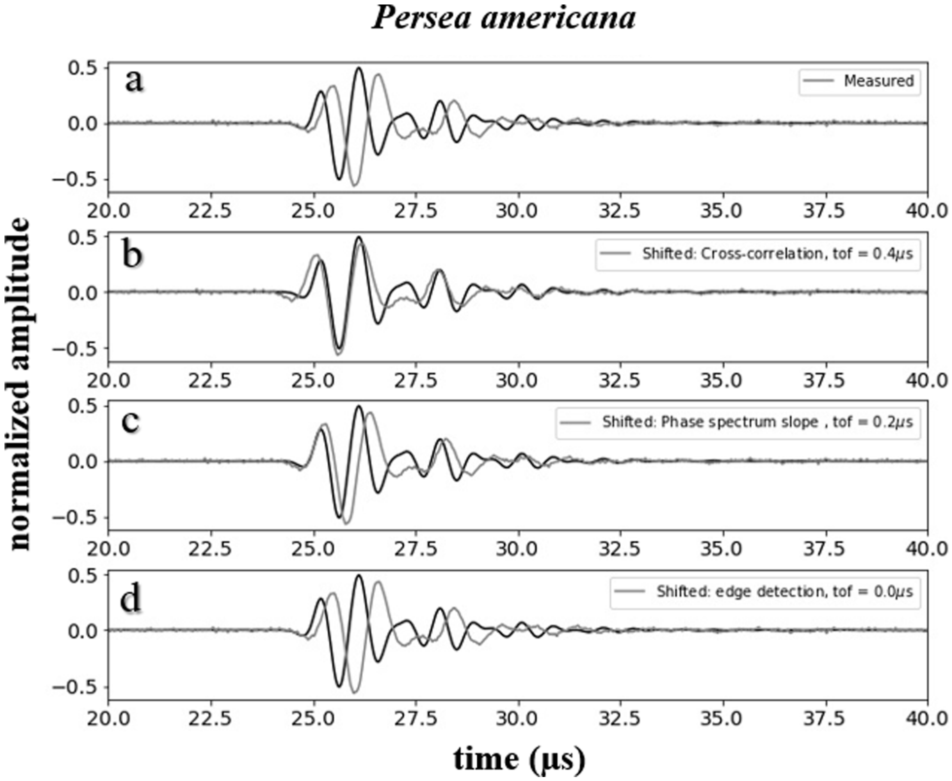

Fig. 4. Measurements taken at 1.2 MHz center frequency displayed in time domain on *Persea americana* leaves: solid grey lines show signals received after passing through the leaves and solid black lines show the references: a) measurement with the delay with respect to the reference as a consequence of the ultrasonic velocity in the *Persea americana* leaf below the propagation velocity of the air; measurements and references are aligned after the following methods for time-of-flight calculation: b) cross-correlation; c) phase-slope; d) edge detection.

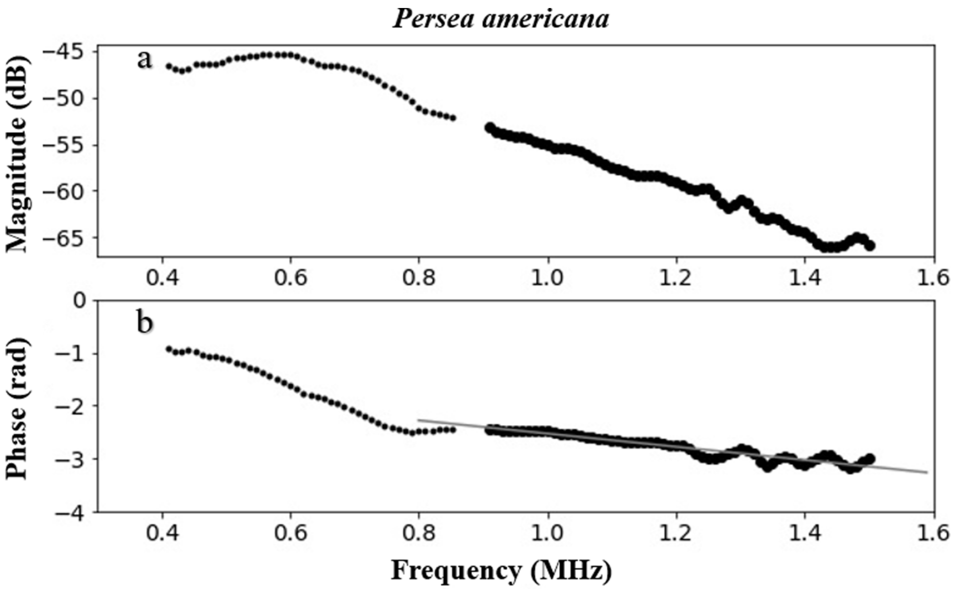

Fig. 5. Transmission coefficient spectrum of Persea americana leaf a) magnitude; and b) phase. The solid grey line represents the linear correlation between phase and frequency.

the measurements obtained from NC-RUS in the low frequency range, and time-of-flight in air in the high frequency range (see Fig. 1b).

3. Results

3.1. Initial characterization

Table 1 shows the measured species, the leaf thicknesses measured range, the frequency range for the appearance of the first thickness resonance and the frequency range where the high-frequency non-resonant measurements were performed. As thickness resonances have to be avoided in the high frequency non-resonant technique, this frequency range depends on the frequency where resonances appear. In addition, the upper limit for the frequency range depends on the attenuation in the leaves. For example, for *Epipremnum aureum* leaves first resonance appears between 210 and 230 kHz and then, high frequency measurements were taken in the frequency range 0.4–0.8 MHz. For *Buxus sempervirens*, resonances appear at 419–450 kHz and high-frequency measurements were performed at 0.6–1.3 MHz.

3.2. Validation of time-of-flight measurements in air (non-resonant): Comparison with water immersion measurements

Fig. 6 shows ultrasound propagation velocities measured in 21 leaves using the time-of-flight method in air (v_{air}) and in water (v_{water}). Velocities obtained with the three different signal processing methods to determine the difference in time-of-flight are shown together with the resultant linear fittings of each method. The species outliers and deviations vary across different methods for time-of-flight determination. The edge detection method achieved the best performance ($R^2 = 0.96$) and the slope of the correlation is closer to the ideal case where $\mathrm{v_{air}} = \mathrm{v_{water}}$ ($\mathrm{v_{air}} = 0.98\mathrm{v_{water}}$). Though for leaves with higher velocity some outliers appeared as is the case of *Trifolium* ($\mathrm{v_{air}} = 500$ m/s) and *Chlorophytum comosum* ($\mathrm{v_{air}} = 475$ m/s). The trend observed in these outliers is stronger for the phase-slope method (*Trifolium*: $\mathrm{v_{air}} = 700$ m/s; *Chlorophytum comosum*: $\mathrm{v_{air}} = 501$ m/s) and consequently the linear fitting further deviates from ideal ($\mathrm{v_{air}} = 1.07\mathrm{v_{water}}$), even though a fair correlation was obtained ($R^2 = 0.93$). Similarly, the cross-correlation method presents good correlation ($R^2 = 0.95$) nonetheless, its robustness is higher since the occurrence of outliers is weaker, as for example *Nerium oleander*: $\mathrm{v_{water}} = 425$ m/s; $\mathrm{v_{air}} = 297$ m/s or *Persea americana leaves*: $\mathrm{v_{water}} = [388, 380]$ m/s.; $\mathrm{v_{air}} = [192, 182]$ m/s. These outliers result in a slope below 1 in the linear fitting obtained ($\mathrm{v_{air}} = 0.83\mathrm{v_{water}}$), behavior that is closer to the expected bias between air and water ultrasonic velocities.

Fig. 7 shows the correlation between $\mathrm{v_{air}}$ and $\mathrm{v_{water}}$ calculated as the mean time-of-flight using the cross-correlation, phase-slope and edge detection methods after removing the outliers. This approach excels in performance ($R^2 = 0.97$) and improves the robustness since the appearance of outliers is attenuated. The slope of the linear fitting is lower than 1 ($\mathrm{v_{air}} = 0.92\mathrm{v_{water}}$), pointing that the velocities in water tend to be higher than in air. This trend was previously observed in literature (Álvarez-Arenas et al., 2009), and may be due to the interaction between the water as coupling medium and the plant tissues (e.g., overhydration may exist), which further supports the idea that the use of water coupling techniques must be avoided in general.

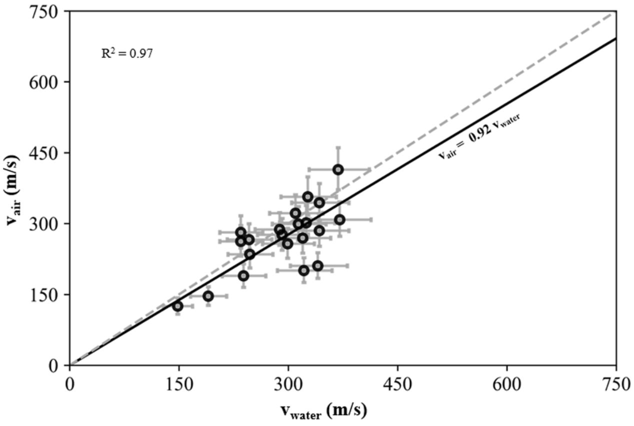

Fig. 7. Mean and variability of plant leaf velocity measured by the contactless high-frequency time-of-flight technique ($\mathrm{v_{air}}$), obtained as the mean of the cross-correlation, phase-slope and edge detection methods after removing the outlier, plotted against water immersion velocity ($\mathrm{v_{water}}$). The dashed grey line represents the ideal case in which $\mathrm{v_{air}} = \mathrm{v_{water}}$ and the solid black line represents the linear correlation.

3.3. Validation of time-of-flight measurements in air (non-resonant): Comparison with NC-RUS

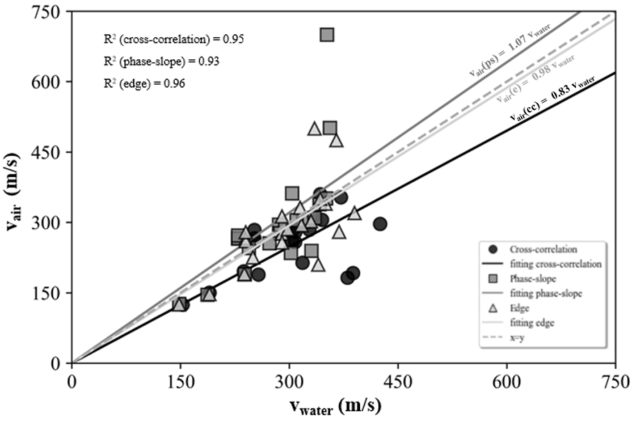

Fig. 6. Ultrasonic velocity in the leaves measured in through transmission water-coupled ($\mathrm{v_{water}}$) and air-coupled ($\mathrm{v_{air}}$) using three different methods for time-of-flight determination: cross-correlation (black dots), phase-slope (dark-grey squares) and edge detection (light-grey triangles) with the corresponding linear fittings for each case represented as same-colored solid lines. The grey dashed line represents the ideal case in which the velocities obtained with both techniques are identical.

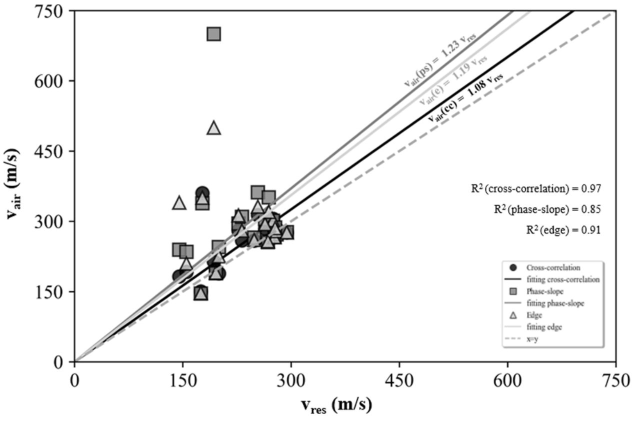

Fig. 8. Ultrasonic velocity in the leaves measured by NC-RUS ($\mathrm{v_{res}}$) and air-coupled high-frequency technique ($\mathrm{v_{air}}$) using three different methods for time-of-flight determination: cross-correlation (black dots), phase-slope (dark-grey squares) and edge detection (light-grey triangles) with the corresponding linear fittings for each case represented as same-colored solid lines. The grey dashed line represents the ideal case in which the velocities obtained with both techniques are identical.

Fig. 8 shows ultrasound propagation velocities measured in 19 leaves using the time-of-flight method in air (v_{air}) and the NC-RUS (v_{res}). Velocities obtained with the three signal processing methods already used in 3.2. section to determine the difference in time-of-flight, are shown together with their resultant linear fittings. Cross-correlation achieved the best performance ($R^2 = 0.97$) and the slope of the linear fitting is the closest to the ideal case where $\mathrm{v_{air}} = \mathrm{v_{res}}$ ($\mathrm{v_{air}} = 1.08\mathrm{v_{res}}$). It is worth highlight the *Trifolium* ($\mathrm{v_{res}} = 177$ m/s; $\mathrm{v_{air}} = 360$ m/s) which constitutes the main outlier with this method. The performance using edge-detection is still good ($R^2 = 0.91$), but the slope of the correlation deviates from ideal case ($\mathrm{v_{air}} = 1.19\mathrm{v_{res}}$) as the appearance of outliers is stronger (*Trifolium:* $\mathrm{v_{res}} = [193, 177]$ m/s; $\mathrm{v_{air}} = [500, 350]$ m/s or *Persea americana*: $\mathrm{v_{res}} = 145$ m/s; $\mathrm{v_{air}} = 340$ m/s). The lower performance was obtained using the phase-slope method ($R^2 = 0.85$; $\mathrm{v_{air}} = 1.23\mathrm{v_{res}}$).

Finally, Fig. 9 shows $\mathrm{v_{air}}$ which is obtained from the average of the three methods used to calculate time-of-flight after removing outliers, against NC-RUS. The linear correlation in this case is similar to the one obtained using exclusively the cross-correlation method ($R^2 = 0.96$). The slope of the linear fitting is greater than 1 as expected but deviates slightly further than in the cross-correlation case ($\mathrm{v_{air}} = 1.13\mathrm{v_{res}}$). Main

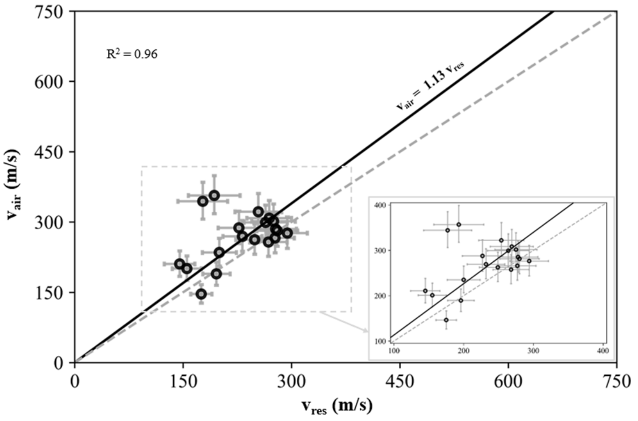

Fig. 9. Mean and variability of plant leaf velocity measured by the contactless high-frequency time-of-flight technique (v_{air}), obtained as the mean of the cross-correlation, phase-slope and edge detection methods after removing the outlier plotted against velocity measured by NC-RUS (v_{res}).

outliers appear at low v_{res} velocities such as *Trifolium* ($v_{res} = [177, 193]$ m/s; $v_{air} = [344, 357]$ m/s) and *Pelargonium grandiflorum* ($v_{res} = 175$ m/s; $v_{air} = 147$ m/s) leaves.

According to the latter results, the proposed method for time-of-flight estimation averaging cross-correlation, phase-slope and edge detection approaches after removing outliers, performs similarly to applying cross-correlation alone.

3.4. Final results

Table 2 summarizes the obtained velocities using the standards: water immersion (v_{water}) and NC-RUS (v_{res}), as well as the velocities obtained with the proposed technique in air at high-frequencies estimating the time-of-flight by using the average value from cross-correlation, phase-slope and edge detection, removing outliers (v_{air}).

In general, these values confirm the general trends appointed in the previous sections. Firstly, as it can be observed, generally v_{water} is greater than v_{air} as the interaction of the coupling water with the porous leaf can produce an overhydration. Secondly, v_{air} is greater than $v_{res.}$ This is consequence of the different origin of the estimated velocities using both methods: while v_{air} depends on the time difference between the calibration without leaf and the measurement, v_{res} is calculated at the resonance condition ($\lambda = h/2$) (see section 2.2.). According to this, we can interpret the difference between these velocities as an estimation of the degree of anisotropy in the leaf structure. In these terms, we may think that the mesophyll in the case of *Epipremnum aureum*, *Ligustrum lucidum* or *Arbutus unedo* is less heterogeneous than in *Persea americana* or *Hedera helix*. Lastly, intraspecific variations are slightly lower using v_{air} than v_{water} and v_{res}. However, in order to draw any conclusion in this respect, further work should be carried out focused on a systematic selection of the leaves under study.

Table 2
Velocity values for each leaf measured in water immersion, air high-frequency and NC-RUS. Measurement error is about 5%.

Species	v_{water} (m/s)	v_{air} (m/s)	v_{res} (m/s)
Epipremnum aureum	238	190	196
Coffea arabica	247	235	200
Persea americana	321	201	155
	340	211	145
Pelargonim grandiflorum	190	147	175
	148	126	–
Hedera helix	310	322	254
	325	302	275
	288	288	227
	314	299	264
Ligustrum lucidum	246	266	277
	235	263	249
	234	281	280
Arbutus unedo	299	258	268
	291	276	294
	342	285	278
Trifolium	342	344	177
	319	269	232
Chlorophytum comosum	368	414	–
Nerium oleander	370	308	269
Buxus sempervirens	327	269	232

4. Discussion

Ultrasound velocity has been obtained in leaves of 11 different species by using the proposed high-frequency technique in through transmission mode to measure time-of-flight in absence of thickness resonances. Measurements performed show that it is possible to obtain transmitted signals in air through the plant leaves at frequencies around 1 MHz with a SNR good enough to estimate time-of-flight in spite of the large attenuation in leaves, in the air and the relatively lower transducers sensitivity (Fig. 1).

Three different signal processing techniques has been used to obtain differences in the time-of-flight (cross-correlation, edge detection and phase-slope) (Figs. 2-5). In addition, and to validate the obtained results, two conventional ultrasonic techniques have also been used to estimate time-of-flight and velocity in the same leaves (water immersion and NC-RUS).

The comparison of the proposed technique with the velocity measurements in water (Fig. 6) reveals that the edge detection method achieved the best performance ($R^2 = 0.96$) and the slope of the correlation is closer to the ideal case where $v_{air} = v_{water}$. Though for leaves with higher velocity some outliers appeared as is the case of *Trifolium* ($v_{air} = 500$ m/s) and *Chlorophytum comosum* ($v_{air} = 475$ m/s). The trend observed in these outliers is stronger for the phase-slope method (*Trifolium:* $v_{air} = 700$ m/s; *Chlorophytum comosum:* $v_{air} = 501$ m/s), even though a fair correlation was obtained ($R^2 = 0.93$). Similarly, the cross-correlation method presents good correlation ($R^2 = 0.95$) nonetheless, its robustness is higher since the occurrence of outliers is weaker. Fig. 7 shows the comparison of the estimated ultrasound velocity by taking the average of the values provided by the three different signal processing techniques and taking out outliers. This approach excels in performance ($R^2 = 0.97$) and improves the robustness since the appearance of outliers is attenuated. In addition, this procedure involves no extra experimental work or any additional measurements as the three methods operate on the same measured signals. It can also be appreciated that the slope of the linear fitting is lower than 1, pointing out to the fact that ultrasound velocities measured in water tends to be higher than those measured in air. This trend was observed before by Álvarez-Arenas et al. (2009), and can be due to the interaction between the water as coupling medium and the plant tissues in the leaf, supporting the idea of the complexity that water immersion techniques involve in porous materials.

Fig. 8 shows the comparison of the ultrasonic velocities obtained in air at high-frequency applying cross-correlation, phase-slope and edge detection. In this case, cross-correlation excels in performance ($R^2 = 0.97$) as the contribution of the outliers is weaker than with the other two procedures. In addition, Fig. 9 presents the result of applying the proposed method that takes the average of the cross-correlation, phase-slope and edge detection removing the outlier against the velocity using the NC-RUS technique. In this case, correlation is good ($R^2 = 0.96$) and the slope of the linear fitting is higher than 1, revealing the bias of ultrasonic velocities obtained by the time-of-flight method (v_{air}) towards larger values than v_{res} (see Table 2).

We conclude that any of the three signal processing methods can be used to estimate time-of-flight, though it was confirmed that the potential outliers would be consequence of different source of errors: in the current case, edge detection and phase-slope seemed to be sensitive to similar issues since their outliers happened in the same species while cross-correlation outliers appeared in different leaves. Therefore, we proposed a method to combine these three estimators to produce a more robust and accurate procedure. It is worthwhile noting that the combination of different techniques for time-of-flight estimation to improve accuracy, robustness or to increase range of applicability has been already used in other fields, for example in range sensing (see for example, Kredba and Holada, 2017; Saad et al., 2011).

Furthermore, the trend showed in v_{air} values to be greater than the ones obtained from NC-RUS could be explained based on previous works where it was demonstrated that in layered tissues the resonance spectra is distorted so that the velocity estimated from the first thickness resonance presents a bias towards lower values (Álvarez-Arenas et al., 2018, 2020). Accordingly, this information can be used as an indicator of the degree of anisotropy between the tissues within the leaf (palisade parenchyma and spongy mesophyll). Based on this assumption, in cases where v_{res} and v_{air} are similar such as *Arbutus unedo*, *Ligustrum lucidum* or *Epipremnum aureum,* they would correspond to plant species whose forming tissues are acoustically similar. On the contrary, in cases where v_{res} and v_{air} differ, such as *Trifolium, Hedera helix* or *Persea americana,* they would correspond acoustically to more heterogeneous tissues. Although from the experiments performed, we cannot conclude to what extent these appreciations are due to inter-species or intra-species traits. Further studies involving a systematic selection of plant species should be carried out in order to obtain advanced conclusions in this regard.

5. Conclusions

The velocity of ultrasound propagation along the thickness direction in plant leaves can be obtained at high frequencies (i.e., frequencies high enough to avoid thickness resonances, if present) by measuring time-of-flight of ultrasonic pulses in the leaf by using wideband airborne pulses and through transmission configuration. Typically, this requires working at frequencies higher than 0.6 MHz. Results obtained over the last 15 years working with ultrasound in plant leaves revealed that using state of the art ultrasonic air-coupled technology (as that used in this work), it is possible to transmit ultrasonic pulses through plant leaves up to values of transmission coefficient losses in the leaf as low as −80 dB. Depending on the leaves, this level of losses may correspond to frequencies around 1.5 MHz. This means, that the typical frequency range for ultrasound velocity estimation using the non-resonant technique to measure time-of-flight corresponds to 0.7–1.5 MHz. This is the typical frequency window that permits to avoid thickness resonances (if present) and poor SNR.

This work is focused on the estimation of the ultrasound velocity in the leaves as we know that this depends on the leaf elastic modulus who strongly depends on water content and turgor potential (Sancho-Knapik et al., 2011; Sancho-Knapik et al., 2013a). The most robust assessment of the ultrasonic velocity is based on the calculation of the mean time-of-flight obtained by applying cross-correlation, phase-slope and edge detection algorithms after removing outliers. The results obtained have been verified with other standard techniques such as water immersion and NC-RUS.

Measurements show that there is a trend to estimate higher velocities applying this air-coupled time-of-flight technique than using NC-RUS. This behavior can be explained by the dissimilar contribution of the different layers within the leaf: the ultrasonic response of palisade parenchyma and spongy mesophyll does not interact in the same way when the measurements are taken in resonance as in through transmission at higher frequencies (Álvarez-Arenas et al., 2018, 2020). This bias in velocity can be used to estimate the degree of anisotropy between palisade parenchyma and spongy mesophyll tissues within the leaf.

Future work will explore the further potential of using this air-coupled high frequency non-resonant technique to measure variations in the ultrasound velocity with the modification in the leaf water content, to measure other ultrasonic parameters (like the attenuation coefficient) and to reduce the leaf section (below 2 mm^2) where measurements are taken (by reducing the transducer aperture and by using focused transducers). This will allow not only the use of this technique in species with smaller or more irregular leaves but also will make possible to measure gradients of properties in the same leaf.

CRediT authorship contribution statement

María Dolores Fariñas: Investigation, Funding acquisition, Conceptualization, Writing – original draft. **Domingo Sancho-Knapik:** Writing – review & editing. **José Javier Peguero-Pina:** Writing – review & editing. **Eustaquio Gil-Pelegrín:** Validation, Conceptualization, Writing – review & editing. **Tomás E. Gómez Álvarez-Arenas:** Investigation, Validation, Methodology, Funding acquisition, Conceptualization, Writing – original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: M.D. Farinas reports financial support was provided by Spain Ministry of Science and Innovation.

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