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Technical note: Validation of Aleppo pine transpiration rate measurements using the heat ratio method under laboratory conditions

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Abstract

Tree transpiration considerably contributes to evaporative fluxes to the atmosphere in terrestrial ecosystems. Accurate transpiration quantification promotes the knowledge of water consumption by forests and could favour an adaptive forest management, especially in a global change context. Tree transpiration can be measured by a wide range of methods, and one of the valued ones is sap flow measurements. However, species-specific validations of techniques are required. Hence the objectives of this study were to validate transpiration rate measurements by the heat ratio method (HRM) in juvenile Aleppo pine trees (*Pinus halepensis* Mill.) by using the probe misalignment correction proposed by Larsen et al. (2020). This study simultaneously recorded the transpiration rate by tree sap flow following the HRM technique (T_{HRM}) and tree water losses by load cells (T_{OBS}). These measurements were taken in combination with the environmental variables that control this process such as different vapour pressure deficit (VPD) ranges of air and the soil relative extractable water (REW). The results showed an accurate linear correspondence between T_{OBS} and the transpiration rate measurements both without and with probe misalignment correction, T_{HRM} and $T_{HRM\ MIS}$, respectively, but interestingly underestimations at high transpiration rates were observed. However, underestimations were removed when applying probe misalignment correction. $T_{HRM\ MIS}$ showed a good

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relation between the VPDxREW interaction. This study supports the notion that HRM offers accurate low values under a wide range of abiotic conditions, and is useful in isohydric species with low transpirations rates like Aleppo pine. To conclude, our results support the validation of both transpiration rate measurements by the T_{HRM} and probe misalignment correction in Aleppo pine under different environmental laboratory conditions.

1. Introduction

Global change scenarios not only predict increasing temperature and changes in precipitation patterns, but also higher drought intensity and longer duration (IPCC, 2021). Climate alterations will affect ecosystems' function and survival by promoting changes in plant water use (Lindner et al. 2010). Quantifying soil-vegetation-atmosphere water fluxes is critical for understanding both current and future ecosystem hydrology, and for providing adaptive forest management. In forest ecohydrology, transpiration is the main actual terrestrial evapotranspiration (ETa) component (Jasechko et al. 2013). On average, worldwide forests' transpiration fraction represents 64% of the total ETa water fluxes to the atmosphere (Good et al. 2015). In Mediterranean Aleppo pine forests, transpiration can contribute to 50 % ETa in an annual budget (Sabater et al. 2021). Additionally, transpiration provides plant-level information and is important in water status, leaf cooling and nutrient transport.

Tree or plant transpiration can be measured by a wide range of methods, such as lysimeters (Swanson and Whitfield, 1981; Ruiz-Yanetti et al. 2016), isotopic approaches (Scheidegger et al. 2000), atmospheric techniques (e.g. eddy covariance; Williams et al. 2004), models (Fernandes et al. 2015; Sperry et al. 2019) and sap flow measurements (Smith and Allen, 1996; del Campo et al. 2014). Sap flow methods have been used since the start of the 20th century by tracing water movement through xylem tissues (Huber, 1928). The sap flow technique is often used in woody species at the whole plant level because it presents several advantages by allowing, for example, continuous readings, it is not limited to single or a few leaf measurements, and it can be applied irrespectively of both orographic and atmospheric conditions (Forster, 2017). Of the several methods available to estimate sap flow, the most popular ones are thermal dissipation, heat pulse velocity and heat field deformation.

The heat ratio method (HRM) has been used in many articles (Vandegehuchte and Steppe, 2012) and is quite reliable for determining transpiration (Fernández et al. 2001; Williams et al. 2004). The HRM offers excellent advantages, such as: applications in the diameters of tree trunks above 25 cm (Swanson, 1994); the detection of low and reverse flows due to a rapid thermal response (Burgess et al. 2001; Marshall, 1958). The heat pulse in the HRM is discrete, unlike other techniques that use constant pulses. Due to rapid pulse diffusion when conduction and convection effects are comparable to one another, temperature rises at a point and reaches its maximum value before the centre of the heat pulse goes up to that point (Marshall, 1958).

However, the HRM presents several calculation steps to convert the measurements of temperatures before and after the heat pulse into sap flow measurements, which are associated with different uncertainties that can modify the accuracy of measurements (Looker et al. 2016). One of the main sources of uncertainty is the spacing of HRM probes in trunks. Larsen et al. (2020) proposed probe misalignment correction in Aleppo pine trunks. This approach proposes a methodology that estimates minor changes in the resulting spacing of probes compared to the theoretically prescribed one. This correction can also detect the evolution of these corrections over time by compiling the effect of growth or physiological evolution on probe positions. Applying Larsen's corrections can provide accuracy transpiration quantification and also reduce costs because probes do not need to be frequently replaced. Even though sap flow measurements are reasonably corrected when applying probe misalignment correction, the above-cited research work did not provide a validation of measurements.

Several works describe the importance of quantitative calibrations in sap flow methods for both commercial and lab-built probes to guarantee the highest quality sap flow measurements (Dix and Aubrey, 2021). In the scientific disciplines (e.g., ecohydrology) that require many probes, the cost of commercial probes can be the main disadvantage (Wiedemann et al. 2016). HRM lab-built probes can be adapted to specific plant characteristics. They are also affordable compared to commercial ones because several probes are needed for accurate monitoring and to replicate a study area (Davis et al. 2012). HRM lab-built probes have been used to measure Aleppo pine sap flow in technical works (Davis et al. 2012; Larsen et al. 2020). Davis et al. (2012) describe a guide for lab-built probes and provide accurate results in qualitative HRM calibration.

Aleppo pine (*Pinus halepensis* Mill.) is one of the most representative and extensive forest species in Mediterranean forests and covers around 25,000 km^2 (Quézel, 2000). Compared to other Mediterranean species (e.g. *Quercus* sp.,), Aleppo pine presents an isohydric strategy with a water-saver response and shows low transpiration rates (Vicente et al. 2018). Drivers of Aleppo pine transpiration vary among climatic contexts, and relative extractable water (REW) and vapour pressure deficit (VPD) are the most important ones (Chirino et al. 2015; Larsen 2021). Accurate Aleppo pine transpiration measurements and their drivers are needed to understand the integration of soil-plant-atmosphere water fluxes and to, hence, plan hydrological and climatological studies and forest ecosystem research.

Due to large covered areas and the relevance of this species, Aleppo pine transpiration quantifications are key for the ecohydrology knowledge in the Mediterranean Basin and its forest management. This study tries to offer a response to the need to perform calibration on sap flow probes in an isohydric water-saver species (i.e. low transpiration rates) by providing high-quality sap flow data (Dix and Aubrey, 2021). This study aims to: i) validate the Aleppo pine transpiration rates measured by the HRM technique under different laboratory environmental conditions compared to load cells as a direct method to measure the plant transpiration rate; ii) test the probe misalignment correction proposed by Larsen et al. (2020) under different environmental conditions. An auxiliary objective is to assess the environmental drivers of Aleppo pine transpiration under laboratory conditions. Aleppo pines were submitted to different environmental conditions, such as VPD and REW, as drivers of transpiration oscillations and transpiration rates, determined by two different techniques HRM and load cells. The hypotheses were: i) the high transpiration values measured by the HRM technique globally underestimated high sap flow rates specifically, while low sap flow rates were measured with higher accuracy due to the rapid thermal response of the HRM method; ii) transpiration in a wide range of values measured by the HRM technique and corrected through the methodology proposed in Larsen et al. (2020) presented higher accuracy measurements and smaller bias errors than without probe misalignment correction; iii) VPD and REW conditioned transpiration by promoting stomatal closure under high evaporative demand and/or water scarcity conditions. However, the interaction of the two variables was expected to be the main factor in Aleppo pine transpiration.

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2. Material and methods

2.1. Plant material

The experiment was conducted in the Plant Experimentation Unit of the University of Alicante (Spain) over 57 days in spring 2019. Three juvenile Aleppo pine individuals were used for this experiment. Individuals were grown in pots filled with mixed substrate with peat, compost and coconut fibre. Soil volume was measured as the geometric volume delimited by each pot. For the soil apparent density measurements, the weight and volume of soil samples were calculated under saturated conditions. Then soil sample were dried for three days at 60 ºC. The resulting dry weights were divided by the initial volume to obtain soil apparent density. After the experiment, the three pines were cut and separated into leaves and woody components (trunk and branches), which were weighed directly after cutting to obtain the fresh individual weight. Then they were dried for seven days at 60 ºC and were finally weighed to obtain the dry weight. Sap wood water content (m_c) was measured following Eq. (1), where w_f and wd were the fresh and oven-dried weight of the trunks sample, respectively (Marshall, 1958).

$$mc = \frac{(wf - wd)}{wd} \tag{1}$$

Basic wood density (p_b) was measured as the dry weight divided by the green volume (Marshall, 1958). The green volume was measured by the water displacement of four wooden cubes per tree (approx. 20x20x20 mm). Cubes were dried for seven days at 60 ºC and weighed to obtain dry weight measurements. The sap wood area was measured as the area occupied by exudates of water and sap after cutting the trunk at the breast height. Before the experiment, a calibration analysis between the known weights and the weight measured by load cells was performed to test and adjust linearity in the load cells (Table 1).

2.2. Experimental setup

Aleppo pine transpiration was measured using two simultaneous, but different and independent, techniques: the heat ratio method by means of sap flow probes (T_{HRM}) and the weighing of water losses by means of load cells (lysimeter technique, T_{OBS}). Sap flow and water losses were two different approximations followed to estimate the transpiration rate per tree (T, kg h^{-1}). Pots were wrapped in film and plastic bags to avoid evaporation loss directly from soil. The pine individuals were located in the Plant Experimentation Unit a week before the experiment started for them to acclimatise to the experimental conditions. The limitation of having only three individuals was overcome by including a long experimental period (57 days) using half-hourly measurements.

2.3. Heat ratio method

The heat ratio method (HRM) is described in detail in Burgess et al. (2001), and the principle of measurements is found in Williams et al. (2004). The HRM employs temperature probes inserted into the active xylem at equal distances (0.6 cm) down- and upstream from the heat probe. More details of the lab-built construction of the HRM probes are found in Sect. Appendix A and Appendix B.

For a given tree, sap velocity (V, cm h^{-1}) was obtained following Eq. (2), (6) and (7) described in Burgess et al. (2001). Then the transpiration rate (T_{HRM}, kg h^{-1}) was upscaled by multiplying each sap wood area per tree (Table 1).

Transpiration was also improved by applying the probe misalignment correction described in Larsen et al. (2020) ($T_{HRM\ MIS}$, kg h^{-1}). In this case, Eq. (2) in Burgess et al. (2001) was not applied. Instead, a modification of Eq. (8) described in Larsen et al. (2020) was applied to calculate two different sap velocities: V_1 and V_2. For V_1 calculations it was assumed that the distance between the heat and the downstream temperature probe (X_1) underwent misalignment (different to 0.6 cm), while the distance between the heater and upstream probe (X_2) was fixed to -0.6 cm. The opposite was assumed to calculate the other sap velocity (V_2). To evaluate X_1 and X_2, it was necessary to achieve zero-flow conditions, which were identified as night episodes when null-weight variations were recorded by the load cells per tree. Four events of zero-flow conditions were selected during the study period. Events were clearly detected at night (23:00-2:30), when the vapour pressure deficit came close to zero. An average of the half-hour temperature readings per event was performed and Eq. (6) in Larsen et al. (2020) was applied. Finally, the average of the resulting X_1 and X_2 values for each of the zero-flow events (Table D1) were used in Eq. (8) in Larsen et al. (2020) to obtain V_1 and V_2. This means that the resulting value described in Table D1 replaced the slope and intercept described in Eq. (8) in Larsen et al. (2020). Thus, the modification herein applied did not take into account temporary probe misalignment behaviour because no temporal dependence of X_1 and X_2 was observed among the selected events. V_1 and V_2 were averaged ($\overline{V}$). Then $\overline{V}$ was applied to Eq. (8) detailed in Burgess et al., (2001), and it was upscaled to transpiration by multiplying per sap wood area.

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Table 1. Features associated with each pine individual.

	Pine number 1	Pine number 2	Pine number 3
Soil volume (m^3)	0.061	0.061	0.060
Soil apparent density (g cm^{-3})	0.133	0.149	0.163
Fresh individual weight (kg)	12.85	9.87	6.28
Dry individual weight (kg)	8.8	6.4	4.2
Percentage of dried-leave weight over the dry individual weight (%)	18	24	30
Sap wood water content (m_c, dimensionless)	0.42	0.58	0.60
Basic wood density wood (p_b, kg m^{-3})	0.50	0.53	0.49
Sap wood area (cm^2)	22.05	15.55	12.86
Absolute error of load cell (g)	23	27	58
Load cell model	GRUPO EPELSA, Model MV2, Emax 500 Kg	GRUPO EPELSA, Model MV2, Emax 500 Kg	GRUPO EPELSA, Model LC2, Emax 100 Kg

2.4. Load cells design and environmental measurements

Load cells were sampled on a 10 minute basis. Due to the inherent data scattering found in this output type, a moving average was used over each weight time series. To estimate water losses, the difference between the current and previous averaged weight values was used.

Soil water content (SWC) was indirectly calculated using the weighing data recorded by the load cells, soil field capacity and apparent density measured in the laboratory, and by adding each water volume during the irrigation pulses. The following equation was applied to obtain relative extractable water (REW) (Granier, 1987), where SWC_i was the instantaneous value of SWC. SWC_{max} and SWC_{min} were the maximum and minimum values of the data series, respectively.

$$REW = \frac{SWC_i - SWC_{min}}{SWC_{max} - SWC_{min}} \quad (2)$$

The air vapour pressure deficit of the atmosphere (VPD, kPa) was calculated from the temperature and relative humidity values (Allen et al. 1998).

Actual vapour pressure (ea) = $0.6108^{(17.27 \text{ x Temp}) / \text{Temp} + 273.3}$

Saturation vapour pressure (es) = ea x $\frac{RH}{100}$

Vapor pressure déficit (VPD)= es-ea (3)

Air temperature (Temp, ºC) and relative humidity (RH, %) were recorded by an EE08-PFT1V11E605/T48 High-Precision Miniature T/H Sensor. Global solar radiation (W m^{-2}) was recorded by an Apogee Instruments Ltd. SP-110-C: Self-Powered Pyranometer Sensor.

All records were recorded by a datalogger (Campbell Scientific CR800 Series Datalogger) associated with a multiplexor (Campbell Sci model AM16/32B Relay Analogue Multiplexer) and connected to a 12 V battery (Lead Acid Battery, RS Pro, 12 V 55 Ah, AGM). All the variables were measured on a 10-minute basis, except for T_{HRM}, whose resolution was half-hourly.

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2.5. Data analysis

The obtained T_{OBS} and T_{HRM} data did not follow normal distributions, but strongly skewed distributions towards values below 0.02 kg h^{-1}. Two data filters were used to achieve normal distributions to then perform coherent statistical analyses. Firstly, a radiation filter threshold was defined as 5 W m^{-2} to select only the data corresponding to the diurnal conditions. Secondly, random selection was employed to attain enough data values at different bins that defined normal distribution. To validate the heat ratio transpiration measurements, a linear model was fitted in R (R Core Team 2021) by taking T_{OBS} as the dependent variable, and T_{HRM} and $T_{HRM\ MIS}$ as the independent variables. The root mean squares of the models were fitted by the 'rmse' function of the hydroGOF R Package (Zambrano-Bigiarini, 2020).

To test the environmental drivers of $T_{HRM\ MIS}$ under laboratory conditions, a linear mixed-effects model (nlme package) was fitted in R (R Core Team 2021) with $T_{HRM\ MIS}$ as the dependent variable, VPD, REW and their interaction (VPDxREW) as the fixed effects factors, and Aleppo pine individuals were fitted as a random effect. Both VPD and REW were the continuous variables. Global solar radiation was not included because it correlated highly with VPD (cor.test > 0.8). Marginal and conditional R^2 were calculated using the piecewiseSEM R package (Lefcheck, 2016) to assess the proportion of variance explained by the fixed and by both the fixed and random effects (Nakagawa and Schielzeth, 2013).

3. Results

3.1. Comparison of the transpiration rate using the heat ratio method technique and load cells

The coefficients of determination showed a linear relation between the transpiration measured by T_{HRM} and load cells, T_{OBS} (Fig. 1a and 1c), with good prediction ability (R^2> 0.5; Table 2). The linear relations showed slopes lower than 1, which imply an underestimation in the absolute values (Fig. 1a). However, accuracy was high at low rates, but accuracy was low and provided transpiration underestimations at high rates (Fig. 1a, Fig. D1a, c, e). The biggest difference between the method measurements (T_{OBS} - T_{HRM} or absolute error) that represented the greatest underestimations were 0.11 kg h^{-1}, 0.09 kg h^{-1} and 0.15 kg h^{-1} for pine number 1, pine number 2 and pine number 3, respectively (Fig. D1a, c, e). The T_{HRM} values around zero were different among pine individuals, even

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though they presented negative values, especially in pine number 1 (Fig. 1a).

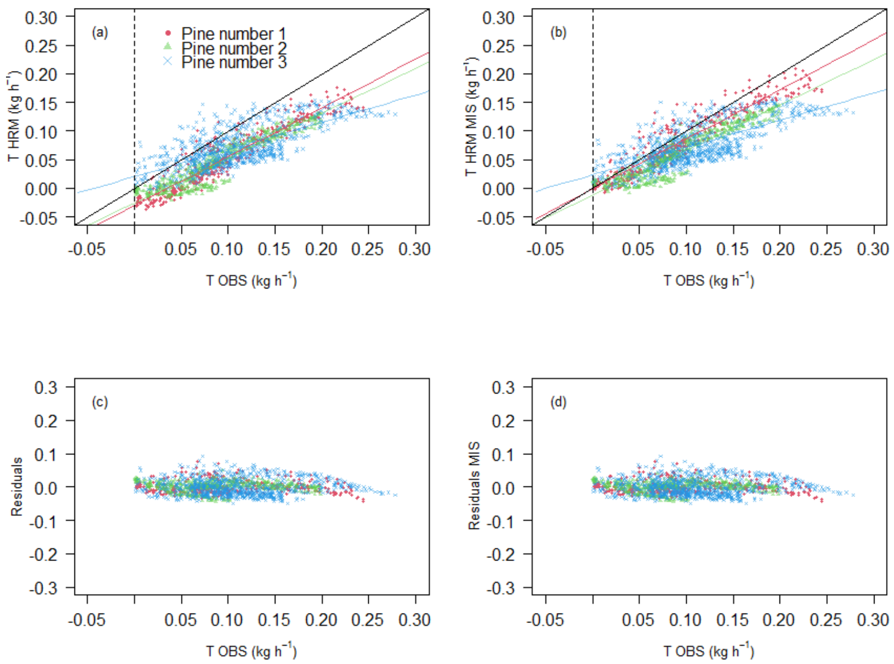

Figure 1. (a) Linear regression of the transpiration rates measured by load cells (T_{OBS}) vs. those measured by heat ratio method probes (T_{HRM}). (b) Linear regression of T_{OBS} vs. T_{HRM} rectified by the probe misalignment correction proposed in Larsen et al. (2020) ($T_{HRM\ MIS}$). (c) Residuals of the models (Table 2) vs. T_{OBS}. (d) Residuals of the models using misalignment correction data (Table 3) vs. T_{OBS}. The black line shows the curve defined by the 1:1 simulated regression. The dashed line depicts the 0 kg h^{-1} T_{OBS} values.

Table 2. Summary statistics of the linear model of the transpiration rate measured by the heat ratio method probes (T_{HRM}, kg h^{-1}) according to the transpiration rate measured by load cells (T_{OBS}, kg h^{-1}).

	Pine number 1	Pine number 2	Pine number3
Intercept	-0.030 ± 0.002	-0.026 ± 0.002	0.020 ± 0.002
T_{OBS}	0.853 ± 0.016	0.785 ± 0.014	0.475 ± 0.017
R^2	0.88	0.86	0.50
p-value	< 2.2e-16	< 2.2e-16	< 2.2e-16

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When the probe misalignment correction proposed by Larsen et al. (2020) was applied ($T_{HRM\ MIS}$), the ability of the regressed model prediction was also high (Fig. 1b and 1d, Table 3). The slope and intercept between $T_{HRM\ MIS}$ and T_{OBS} came closer to 1 and 0, respectively (Table 3), which promoted high accuracy along transpiration rates. $T_{HRM\ MIS}$ presented lower root mean squares values than the T_{HRM} models, which means more accurate measurements when probe misalignment correction was applied (Table 4a and 4b). There were fewer negative $T_{HRM\ MIS}$ values than negative T_{HRM} values, and the transpiration *versus* T_{OBS} relations were much more homogenous between pines for the particular case of the misalignment correction technique than for the method without correction (Fig. 1a and Fig. 1b). Pine number 1 presented the highest probe misalignment value, while pine number 3 had the lowest (Table D1). Likewise, the highest and lowest root mean squares values were for pine number 1 and pine number 3, respectively (Table 4b). Despite the fact that pine number 3 obtained the lowest misalignment value, it also had the least accurate load cell, with absolute errors above double that for the other load cells (Table 1). This fact was taken as a less accurate comparative result than in the other pines.

The distribution of residuals when a regression analysis based on T_{OBS} was performed was constant along T_{HRM} and $T_{HRM\ MIS}$ (Fig. 1c and 1d). The maximum underestimations (T_{OBS} - $T_{HRM\ MIS}$) per individual were 0.08 kg h^{-1}, 0.08 kg h^{-1} and 0.15 kg h^{-1} for pine number 1, pine number 2 and pine number 3, respectively (Fig. A3b, d, f).

Table 3. Summary statistics of the linear model of transpiration rates measured by the heat ratio method probes corrected by the probe misalignment correction proposed by Larsen et al. (2020) ($T_{HRM\ MIS}$, kg h^{-1}) according to the transpiration rate measured by load cells (T_{OBS}, kg h^{-1}).

	Pine number 1	Pine number 2	Pine number 3
Intercept	-0.002 ± 0.002	-0.012 ± 0.001	0.023 ± 0.002
$T_{HRM\ OBS}$	0.871 ± 0.017	0.790 ± 0.014	0.476 ± 0.017
R^2	0.88	0.87	0.50
p-value	< 2.2e-16	< 2.2e-16	< 2.2e-16

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Table 4. Root mean square of the regression residuals and differences between the transpiration values determined by the different methods: load cells (T_{OBS}) and the heat ratio method (T_{HRM} and $T_{HRM\ MIS}$). Lower root mean squares values represent a more accurate relation between the variables T_{HRM} vs. T_{OBS} and $T_{HRM\ MIS}$ vs. T_{OBS}. Letters are associated with the panels in Fig. 1.

	Pine number 1	Pine number 2	Pine number 3
(a) T_{HRM} vs. T_{OBS}	0.049	0.049	0.053
(b) $T_{HRM\ MIS}$ vs. T_{OBS}	0.025	0.037	0.051
(c) Residuals vs. T_{OBS}	0.11	0.10	0.13
(d) Residuals $_{MIS}$ vs. T_{OBS}	0.11	0.10	0.13

3.2 Effect of environmental conditions on the transpiration response

The half-hourly REWs ranged from 0 to 1 (dimensionless), while the hourly VPD attained minimum and maximum values of 0.27 kPa and 2.82 kPa, respectively. The $T_{HRM\ MIS}$ values ranged from -0.01±0.01 kg h^{-1} to 0.17±0.01 kg h^{-1} (Fig. 2). Model predictors included a positive interaction between VPD and REW. The model showed good predictive ability for the environmental conditions (R^2 marginal of 0.61; Table 5). Model predictors included a positive interaction between VPD and REW (Table 5). Global solar radiation was not included because it correlated highly to VPD (cor.test > 0.8). The VPDxREW interaction resulted in simple patterns in the variation of both $T_{HRM\ MIS}$. Higher $T_{HRM\ MIS}$ values were predicted under the conditions according to both the high REW and high VPD values (Fig. 2).

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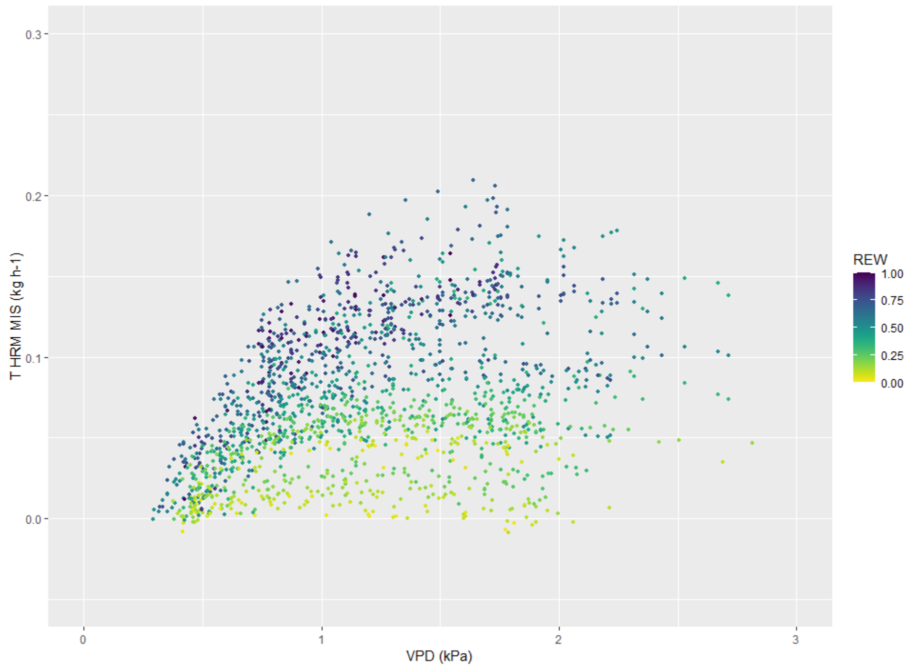

Figure 2. Representation of the response of the transpiration rate measured by the heat ratio method probes corrected by Larsen's misalignment probes corrections ($T_{HRM\ MIS}$) to the environmental variables interaction (VPDxREW, vapour pressure deficit x relative extractable water). REW was represented as a gradient scale colour, where purple depicts wet conditions and yellow dry conditions. The figure is a graphical explanation of the models in Table 5.

Table 5. Summary statistics of the linear mixed-effects models of the transpiration measured by heat ratio method (T_{HRM}) according to the environmental variables. Pine individuals are fitted as a random factor. Abbreviations: VPD: vapour pressure deficit, REW: relative extractable water. The cross indicates the VPDxREW interaction. Asterisk (*) depicts a p-value lower than 0.05.

$T_{HRM\ MIS}$	Fixed effects	Random effects (pine individuals)
Residual error	-	0.014
Intercept	0.023 ± 0.009*	0.025
VPD	-0.006 ± 0.003*	
REW	0.014 ± 0.007*	
VPDxREW	0.094 ± 0.005*	
R^2 Marginal	0.61	
R^2 Conditional	0.70	

4. Discussion

A good linear relation appeared between the transpiration rate measurements by following the HRM technique (T_{HRM} and $T_{HRM\ MIS}$, with and without probe misalignment correction, respectively) and load cells (T_{OBS}). At a transpiration rate lower than 0.10 kg h^{-1}, the transpiration measurements showed the highest accuracy, which agrees with the theory of the heat ratio method (HRM), having a rapid thermal response time (Burgess et al. 2001; Burgess and Dawson, 2008). Therefore, our results support the usefulness of the HRM in Aleppo pine transpiration rates because this method is capable of acquiring accurate data when this species drastically reduces transpiration under Mediterranean summer conditions. For other species, this method has been reported as useful for measurements taken under night conditions (Forster, 2017). Interestingly, the measurements in this study showed qualitatively lower accuracy at transpiration rates above 0.10 kg h^{-1}. This fact has also been reported in previous reviews (Burgess and Dawson, 2008) in commercials methods (Fuch et al. 2017) and in the method validation for other species (Bleby et al. 2004). However, heat pulse methods present lower underestimations across sap flow methods (Steppe et al. 2010).

The results herein obtained support the validation of the HRM in Aleppo pine. A sporadic temperature ratio measurement must be converted into a complete tree transpiration rate by a few equations that combine wood- and tree-specific parameters. For this reason, the accuracy of transpiration rates can be explained by the experimental individual-specific measurements of the variables involved in transformations: sap wood water content (m_c), basic wood density (p_b) and sap wood area. Some reports about transpiration estimations in *Pinus* sp. using the HRM technique apply the same value for m_c and p_b for all the individuals (del Campo et al. 2014; Larsen et al. 2020), while other articles apply different values per individual (Alvarado-Barrientos et al. 2013; Eliades et al. 2018). In this study, individual-specific measurements were obtained and applied as transpiration rate conversions. Contrary, if the mean value of the three individuals for m_c and p_b had been applied, the resulting maximum underestimations (T_{OBS} - T_{HRM}) would have increased, with values of 0.25 kg h^{-1}, 0.20 kg $h^{-1}$and 0.30 kg h^{-1} for pine number 1, pine number 2 and pine number 3, respectively.

Some reports describe uncertainties in transpiration measurements associated with sap wood area (Hatton et al. 1992), with m_c (López-Bernal et al. 2014; Vandegehuchte and

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Steppe 2012; Vergeynst et al. 2014), and also with both m_c and p_b (Swanson, 1983), and their relationship with thermal diffusivity (Looker et al. 2016). Contrarily to the thermal diffusivity measurements based on m_c and p_b (Burgess et al. 2001; Vandegehuchte and Steppe 2012), the thermal diffusivity originally noted by Marshall (1958) was herein applied ($2.5x10^{-3}$ cm^2 s^{-1}). This value is an intermediate between the thermal diffusivity of water and dry wood ($1.4x10^{-3}$ cm^2 s^{-1} and $4.0x10^{-3}$ cm^2 s^{-1}, respectively). Similar thermal diffusivity values appear in angiosperm when applying Burgess et al. (2001) measurements. This scenario suggests that employing the thermal diffusivity proposed by Marshall (1958) does not imply major errors in transpiration measurements.

Once probes are inserted into trunks, it is difficult to assess how accurate probe alignment is. To gain maximum accuracy, the validation of the probe misalignment correction introduced by Larsen et al. (2020) as a modification of the former proposed by Burgess et al. (2001) presents the highest accuracy compared with the HRM technique without correction. Probe misalignment correction Larsen et al. (2020) not only provides higher transpiration rate quantification accuracy, but also shifts negative or approximately zero transpiration rates among other values. Due to the water-saver Aleppo pine strategy, the accuracy of the low transpiration rates of this technique ensures good transpiration rate measurements under high evaporative demand and water scarcity conditions. When applying correction, the underestimation at flows above 0.10 kg h^{-1} decreased, which led to higher accuracy in the transpiration rate estimations under wetter and colder conditions. Consequently, the improvement made while taking transpiration rate measurements when applying correction promoted better estimations along the seasonal variability of Mediterranean areas. However, magnitude varied depending on the degree of probe misalignment. The error variability in the magnitude of the transpiration rate has been shown in previous reports (Sun et al. 2012; Rubilar et al. 2017). The larger the probe misalignment, the greater the correction of the T_{HRM} values, and the smaller the probe misalignment, the lesser the correction of T_{HRM} values. For this reason, the results in this paper support the application of probe misalignment correction in all the sampled individuals.

Despite not only the main focus of this study being the validation of the HRM technique for water-saver isohydric species with low transpiration rates, but also probe misalignment correction in this technique, it is interesting to report that the VPDxREW interaction was the main environmental driver of Aleppo pine transpiration under laboratory conditions. The importance of VPD and REW in evaporative fluxes has been shown separately in several articles about Mediterranean vegetation (Chirino et al. 2011; Manrique et. al 2017). However, very few reports are available about the interaction between VPD and soil water content (Grossiord et al. 2020). Aleppo pine is extensive in Mediterranean regions which, according to climate change projections, are one of the world regions where air temperature and extreme drought are expected to increase, and precipitation patterns are predicted to change (Lionello and Scarascia, 2018). These predictions will directly increase VPD and modify REW, which may have impacts on vegetation. In fact, a rising temperature and changes in precipitation regimes have already been related to dieback and forest decay processes worldwide (Allen et al. 2015; Hartmann et al. 2018). Future research should investigate if the same interaction occurs under field conditions compared to those studies that have shown simpler drivers.

In relation to technical issues, the cost of plant material, time and equipment is high in calibration experiments, and frequently it is a limiting factor. Besides, transport logistics and performing calibration with increasing tree size are challenges (Dix and Aubrey, 2021). In this study, plant material only contained three juvenile Aleppo pine individuals and the equipment for measuring water losses contained two types of load cells with distinct resolutions. Nevertheless, the confidence in the results herein presented is strengthened by the large temporal scale and the wide range of environmental conditions. The ranges of environmental conditions (VPD and REW) used in this paper is consistent with the range observed in fieldwork studies. Although it is true that similar environmental conditions to those of a Mediterranean summer (high evaporative demand and scarce water availability) were not simulated, under our conditions Aleppo pine induces stomatal closure and transpiration rates drastically drop.

5. Conclusions

It should be noted that the results reported in this paper are associated with Aleppo pine species, which is an isohydric water-saver species with low transpiration rates well adapted to drylands. Thus, the conclusions are related to this functional plant ecological response and a specific method (HRM technique). However, this paper provides not only a validation of transpiration rate measurements following the theory and transformation described in Burgess et al. (2001), but also the first validation of the probe misalignment correction proposed by Larsen et al. (2020). The results highlighted the importance of measuring specific individual physiological properties (m_c, p_b, sap wood area) to reduce

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errors in transpiration measurements, and suggested corrections to obtain accurate absolute quantifications of transpiration rates in species with a functional plant strategy based on low transpiration rates like those in drylands and Mediterranean areas.

Appendices

Appendix A. Lab-building of the heat ratio method probes

Heat ratio probes were designed as described by Burgess et al. 2001 and were built following the original design detailed by Davis et al. (2012). The sap wood depth of the Aleppo pine individuals was shallower than the sap wood depth of the aged individuals, which are normally measured in the field (Fig. A2b). Thus, the longitude of the needle and their associated material were halved. The heat ratio method (HRM) requires three probes: a heater and two thermocouples. Thermocouples were constituted by 25 cm constantan (TFCC-005-100) and a type E junction of 25 cm chromium (TFCH-005-100). The constantan (0.3 cm) and chromium wires (2.3 cm) were welded together and placed firstly inside a glass tube (0.1 cm x 2.3 cm, MODEL), and secondly inside a needle (0.13 cm x 2.3 cm, STERICAN Hypodermic Needles). The heater was made of a 20 cm constantan wire of (TFCC-005-100) and a 4 cm-long aluminium wire. They were welded together (0.5 cm). The constantan wire was coiled in 2 cm of aluminium wire and was placed inside a needle of 2.3 cm. A combination resistance of 7.9 ohms (TE connectivity, Series ER58, 7 W Axial, ±5 %: 4.7 Ω + 2.2 Ω 1 Ω) was added to the ends of the wire to obtain a total resistance of about ~14.5 ohms.

All the HRM probes were inserted into a horizontal plane above the soil surface at breast height. The heater and thermocouples were placed in a vertical position and the symmetry was aligned (Fig. A1a). The distal part of each sensor was enclosed in a box (FEMATEL 100x100x55 mm) (Fig. A1b). The HRM probes were placed on the west side of the trunk to avoid the maximum global solar radiation from the east side due to the Plant Experimental Unit's features. All the equipment was wrapped with reflective insulation to protect it from solar radiation (NOMAREFLEX) (Fig. A2a).

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Figure A1. (a) Vertically and symmetry position of heater and thermocouples. Heater was represented in black colour. Thermocouples were represented in red and blue colour, and they were located at 0.6 cm upstream and downstream of the heater. (b) Protecting box with the distal part of each sensor, resistances, and connectors.

Figure A2. (a) All heat ratio method probes equipment were wrapped with a reflective insulation to protect it from solar radiation. (b) Exemplification of needle deep in a cross-section trunk.

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Appendix B. List of the materials to construct and install the heat ratio method probes

- Thermocouple Extension. (EXPP-E-20-1000 T/C).
- Heater Extension. H05V-F. Two-wire cable. 5 mm^2.
- Adapter USB/RS232 (Digitus).
- Relay (Relay SSR Crydom DRA1-CMX60D10).
- Glacier Flex (500 FR).
- Anticondensation adhesive tape.
- Aluminium repair tape.10 m x 50 mm. Argent.
- Gel accumulator (SET 2 ACUM 400GR N1).
- Sub-mini w/window purple (SMPW-E-M).
- Heat-shrink tube. Thermocouple: 6.4/3.2 mm, 3.2/1.5 mm and 2.4/1.5 mm. Heater: 6.0/3.0 mm

Appendix C. Experimental setup

The three pines were placed inside a module (6.4x16x4.5 m, width x length x height). The module structure was made of hot-dip galvanised steel. A system of aluminium profiles was placed on exterior walls. The roof was made of industrial-quality glass (4-2 mm). The front and sides glasses were double-glazed (4-2 mm), and the interior glasses were single-glazed (4-2 mm). The extractor and fog system controlled the laboratory conditions. Extractors were a monoblock of galvanised steel, with reversible flat or curved blade propellers, automatic blind, polypropylene front protection, motor: IP-55 protection, tension III/230/440 V-50 HZ. The fog system worked at high pressure (60 kg/cm^2). The system contained special high-pressure misting nozzles and an HP FFL high-pressure nose with a non-drip valve to ensure that 90 % of droplets would be less than 10 microns in size. The fog system was close to pine number 2 and at the same distance from pine numbers 1 and 3. Pines were arranged in a straight line at the start of the module. Pine number 1 was 1.5 m from pine number 2, and 3 m from pine number 3. The global solar radiation recorder and the air humidity and temperature recorder were 2 m in a straight line from pine number 2. Pines were ~3 m high.

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Appendix D. Supplementary results

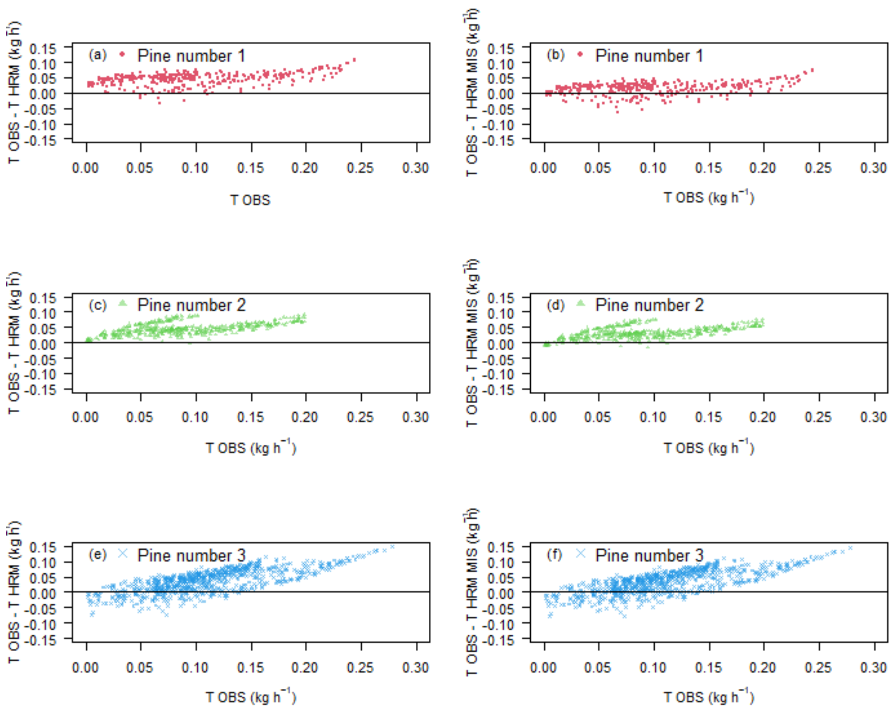

Figure D1. (a), (c) and (e) Relationship between the transpiration difference between methods: load cells and heat ratio method probes ($T_{OBS} - T_{HRM}$) vs. T_{OBS}. (b), (d), and (f) Relationship between the transpiration difference between methods: load cells and heat ratio method probes applying the probe misalignment correction proposed by Larsen et al. (2020) ($T_{OBS} - T_{HRM\ MIS}$) vs. T_{OBS}.

Table D1. Mean and standard deviation of temperature probes distance respect the heater probe and the corresponding root mean square. The correct distance between the temperature and heater probes was 0.6 cm. The closest distance values to 0.6 cm indicate the higher precision in probes insertion, therefore a minor correction in transpiration rates values.

	Pine number 1	Pine number 2	Pine number 3
X_1 (cm)	0.69 ± 0.02	0.65 ± 0.01	0.62 ± 0.01
X_2 (cm)	-0.49 ± 0.02	-0.55 ± 0.01	-0.58 ± 0.01
Root mean square of the distance to value	0.078	0.050	0.020

Code availability

The code that supports the findings of this study are available from the corresponding author upon reasonable request (sabaterblasco@gmail.com).

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request (sabaterblasco@gmail.com).

Author contribution

AV and JB conceptualised the study. AMS made the sensors under the supervision of JAV and JB. AMS implemented the sensors and was responsible for the data processing and the laboratory work. JAV wrote the script for the datalogger and was responsible for the technical aspect of the study. AMS, AV and JAV worked on the data analysis. AMS and JAV prepared the paper with inputs from all the co-authors. All authors worked in the preparation and revision of the published work.

Competing interests

The authors declare that they have no conflict of interests.

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