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Reevaluating Near-Infrared Reflectance as a Tool for the Study of Plant Water Status in Holm Oak (*Quercus ilex* subsp. *rotundifolia*)

José Javier Peguero-Pina [1,2,*], Domingo Sancho-Knapik [1,2], Juan Pedro Ferrio [1,3], Ana López-Ballesteros [1,2], Marta Ruiz-Llata [4] and Eustaquio Gil-Pelegrín [5]

1 Departamento de Sistemas Agrícolas, Forestales y Medio Ambiente, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Avda. Montañana 930, 50059 Zaragoza, Spain; dsancho@cita-aragon.es (D.S.-K.); jpferrio@cita-aragon.es (J.P.F.); alopezb@cita-aragon.es (A.L.-B.)
2 Instituto Agroalimentario de Aragón-IA2 (CITA-Universidad de Zaragoza), 50018 Zaragoza, Spain
3 Aragón Agency for Research and Development (ARAID), 50018 Zaragoza, Spain
4 Electronics Technology Department, Carlos III University of Madrid, 28911 Leganés, Spain; mruizl@ing.uc3m.es
5 Estación Experimental de Aula Dei (EEAD), Consejo Superior de Investigaciones Científicas (CSIC), Avda. Montañana 1005, 50059 Zaragoza, Spain; gilpelegrin@eead.csic.es
* Correspondence: jjpeguero@cita-aragon.es

Abstract: Plant water status can be assessed through leaf spectral reflectance in the near-infrared (NIR), the "water bands", considering indices that include the reflectance at a band absorbed by water over and another one as reference. We have assessed i/ the accuracy of reflectance at 1450, 1599 and 1940 nm without reference bands and ii/ the potential use of leaf water content index (LWCI) for the estimation of plant water status in holm oak, the main host plant for black truffle cultivation. We demonstrated that contact measurements of leaf reflectance in the "water bands" constitute an accurate and non-invasive estimator of relative water content (RWC) in holm oak, despite the absence of a reference wavelength, probably due to the low variation in leaf thickness under dehydration. The use of a reference wavelength, which is needed for remote sensing, diminished the accuracy of RWC estimation. Contrastingly, LWCI increased the accuracy of RWC estimation as well as a reference wavelength were used. However, LWCI required the reflectance value at full turgor, diminishing its potential for implementation at field level. In conclusion, this technique would allow the continuous monitoring of the physiological state of holm oak and intelligent water control in truffle cultivation.

Keywords: holm oak; moisture stress index; leaf water content index; near-infrared reflectance; relative water content; water bands

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1. Introduction

Drylands, including areas under the Mediterranean-type climates, constitute one of the most limiting habitats for plant survival due to the negative effect of water deficit on photosynthetic activity and plant growth [1]. In this way, the quantification of water deficit through the assessment of plant water status is of paramount importance in plant physiological studies [2]. Plant water status can be described in terms of energy through the water potential (Ψ), which defines the driving force in water flow along the soil–plant–atmosphere continuum [3] and defines the biophysical limitations to this flow, including the rupture of the water column [4,5]. Moreover, plant water status can also be described in terms of volume by means of the relative water content (RWC), which is considered a direct estimation of the cell or tissue water volume respect to the maximum value at full hydration [6]. RWC is a crucial parameter for plant functioning, as it influences growth [7], cell damage [8,9] and predisposition to whole plant death [10].

Traditionally, the evaluation of plant water status has been carried out by means of invasive and destructive methods that are rather time consuming and preclude repeated measurements in the same tissue (see Sancho-Knapik et al. [11] and references therein). For this reason, the implementation of non-destructive or non-invasive techniques has been a matter of study during recent decades. In this regard, the assessment of plant water status can be carried out by evaluating the response of different physiological parameters to water stress, such as gas exchange [5,12], canopy infrared temperature [13] or changes in the spectral reflectance around the green part of the spectrum [14,15]. Alternatively, changes in Ψ and RWC can be also accurately estimated from the changes in the acoustic properties of the leaf by air-coupled broadband ultrasound spectroscopy [16–18] or from the changes in leaf reflectivity at a frequency range of microwaves (l-band at 1730 MHz) [19].

Non-destructive and real-time measurement of plant water status has been also assessed through the analysis of the spectral reflectance of leaves in the near-infrared (NIR) range. Thus, an increase in leaf reflectance in the wavelength range between 400 and 2500 nm wavelength was related with a decrease in leaf water content by Carter [20] and Carter and McCain [21]. Since then, several authors have employed different water absorption NIR bands (the so-called water bands) considering the reflectance at a band strongly absorbed by water (e.g., 1450 or 1940 nm), using a spectral region weakly absorbed by water as reference, which led to several leaf water indices [22–26]. Among them, the reflectance ratio between 1300 and 1450 nm (R_{1300}/R_{1450} index) is one of the most acknowledged indices for RWC estimation at leaf scale, although this index could be also influenced by the existence of changes in leaf thickness when plant is subjected to water stress [19,27].

However, leaf reflectance at water bands, 1450 or 1940 nm, could be unsuitable for remote sensing of plant water status at field level, as atmospheric water vapour absorbs most if not all the radiation at these wavelengths. To avoid this problem, the moisture stress index (MSI = R_{1599}/R_{820}, where R_{1599} and R_{820} are the reflectances at 1599 and 820 nm, respectively, being the latter used as reference) has been proposed as a useful tool to detect changes in plant water status at canopy level [28,29]. Other leaf water index that has been developed for remotely sensing RWC is the Leaf Water Content Index (LWCI), which originally uses the same wavelengths as MSI [30,31]. However, to the extent of our knowledge, the potential use of R_{1599} for the estimation of plant water status at leaf level to validate MSI remotely sensed at canopy level has not been explored.

In this study we evaluated and compared the reflectance values at 1450 nm (R_{1450}), 1599 nm (R_{1599}) and 1940 nm (R_{1940}) as non-destructive and non-invasive estimators of plant water status at leaf level. Specifically, the main objectives were i/ to assess the potential enhancement in the accuracy of RWC estimation when the use of reference bands is avoided, as they are also influenced by changes in leaf water content, and ii/ to evaluate the potential use of LWCI calculated with R_{1450} and R_{1940} instead of R_{1599} for RWC estimation at leaf level in holm oak (*Quercus ilex* subsp. *rotundifolia*). Holm oak is considered a paradigm of Mediterranean tree species, together with other evergreen and sclerophyllous oaks under Mediterranean-type climate in the Northern Hemisphere [32,33]. Besides the ecological importance of this species, due to the role in the landscape formation in the western Mediterranean Basin [34], its use as host plant for black truffle (*Tuber melanosporum*) cultivation constitutes a new and promising resource for many geographical areas affected by severe rural depopulation [35,36].

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

Measurements were carried out in 16 mature leaves from 16 holm oak (*Q. ilex* subsp. *rotundifolia*) trees (one leaf per tree) first destined for truffle production located in an experimental field plot at CITA de Aragón (41.723° N, 0.809° W, Zaragoza, Spain) under Mediterranean climatic conditions (mean annual temperature 15.4 °C, total annual precipitation 298 mm). The experimental field plot is a holm oak plantation first destined for truffle production, where the total soil depth is ca. 60 cm. Holm oak trees from this plot were 25 years old and planted in 1998 using a pattern of 6 × 6 m. Trees were maintained in good health conditions and were irrigated with sprinklers (35–40 L m^{-2}) every week from April to September. Twice a year (during early winter and early summer), the experimental plot was cleaned mechanically, eliminating the presence of the herbaceous and shrubby stratum. In the early morning, 16 branches were collected from the north side of 16 trees (one branch per tree), placed in plastic bags and carried out to the laboratory. Once there, one leaf per branch was selected, and leaf petioles were re-cut under water to avoid embolism and kept immersed (avoiding the wetting of leaves) for 24 h at 4 °C until full leaf rehydration. After 24 h, leaf weight, leaf thickness and spectral reflectance parameters were individually measured at constant time intervals (10 measurements per leaf x 16 leaves = 160 measurements). Leaves were weighed and measured at different levels of relative water content (RWC), starting at full saturation (turgid weight, TW). Leaf dry weight (DW) was estimated after keeping the plant material in a stove (24 h, 70 °C). The RWC was then calculated following the expression: RWC = (FW − DW)/(TW − DW), FW being the sample fresh weight at any moment.

2.2. Leaf Thickness

Leaf thickness (µm) was determined at different levels of RWC as described in Sancho-Knapik et al. [19]. To avoid disturbances on leaf thickness measurements due to an excess of pressure over the leaf, we use an ultralow force digital contact sensor GTH10L coupled to an amplifier GT-75AP (GT Series, Keyence Corporation, Osaka, Japan). This ultra-low force sensor (having a measuring force of 0.2 N when installed facing up) applies a clamp pressure of 7 kPa, which is ten times lower than the one used by Zimmermann et al. [37] for a similar purpose.

2.3. Leaf Spectral Reflectance

Leaf reflectance at different levels of RWC was measured between 200 and 1100 nm with a visible/near-infrared spectroradiometer USB-2000 (Ocean Insight, Orlando, FL, USA) and between 900 and 2500 nm with an infrared spectroradiometer NIRQuest (Ocean Insight, Orlando, FL, USA). For these measurements, each spectroradiometer was connected to a bifurcated fiber optic cable into one end and to a tungsten halogen light source LS-1-LL (Ocean Insight, Orlando, FL, USA) into the other end. Leaf reflectance was expressed as spectral reflectance after standardization with white standard (Spectralon, Labsphere, North Sutton, NH, USA). From the recorded spectra, we considered reflectance values at 1450 nm (R_{1450}), 1599 nm (R_{1599}) and 1940 nm (R_{1940}) as estimators of leaf water content. Moreover, we calculated the ratio between the reflectance at these wavelengths and the reflectance values at 820 nm (R_{820}) as reference (R_{1450}/R_{820}, R_{1599}/R_{820} and R_{1940}/R_{820}). Finally, we also calculated the Leaf Water Content Index (LWCI) [30] as applied by Hunt and Rock [31] for 1450 nm, 1599 nm and 1940 nm (LWCI_{1450}, LWCI_{1599} and LWCI_{1940}, respectively):

$$\mathrm{LWCI}_{1450} = \frac{-\ln[1 - (R_{820} - R_{1450})]}{-\ln\left[1 - \left(R_{820} - R_{1450}^{FT}\right)\right]} \quad (1)$$

$$\mathrm{LWCI}_{1599} = \frac{-\ln[1 - (R_{820} - R_{1599})]}{-\ln\left[1 - \left(R_{820} - R_{1599}^{FT}\right)\right]} \quad (2)$$

$$\mathrm{LWCI}_{1940} = \frac{-\ln[1 - (R_{820} - R_{1940})]}{-\ln\left[1 - \left(R_{820} - R_{1940}^{FT}\right)\right]} \quad (3)$$

where R_{1450}^{FT}, R_{1599}^{FT} and R_{1940}^{FT} are the leaf reflectance values at full turgor for 1450 nm, 1599 nm and 1940 nm, respectively.

2.4. Statistical Analysis

RWC was plotted against the different spectral reflectance bands and the most suitable models (sigmoidal four parameters, linear or second-degree polynomial function) were fitted to individual data from all leaves combined (see equations in Table S1 in supplementary information). Moreover, we tested the repeatability of these associations by comparing regression lines fitted for each single leaf. For this purpose, we selected RWC values along the linear range (between 1.0 and 0.5), where indices are most sensitive. In this way, we could assess the existence of statistically significant differences at $p < 0.05$ among the leaves in the slope and intercept of the regression lines. Finally, to assess the accuracy of predictions of RWC based on the different spectral reflectance bands, we applied a cross-validation procedure. Multiple models were fitted using for calibration all possible combinations of N out of 16 leaves, with N ranging from 1 to 15 leaves. The number of models fitted ranged from 16 (for groups of 1 and 15 leaves) to 12,870 (for groups of 8 leaves). Validation statistics—coefficient of determination (R^2), root mean square error (RMSE, the standard deviation of the residuals) and mean absolute error (MAE)—were then calculated separately for each validation set, and the range and mean value for each index and number of leaves was used to define the optimal number of leaves for calibration and to compare the accuracy of model predictions based on different indices. All statistical analyses were performed in the R software environment (version 4.2.1) [38]. Cross-validation statitistics were calculated using the packages 'tidyverse' [39] and 'caret' [40] from R 4.2.1 [37]. RMSE and MAE were calculated as follows:

$$\mathrm{RMSE} = \sqrt{\frac{\sum(pred - obs)^2}{N}} \tag{4}$$

$$\mathrm{MAE} = \frac{pred - obs}{N} \tag{5}$$

3. Results

Holm oak showed a low degree of variation in leaf thickness when subjected to dehydration, only 15% between full turgor (RWC = 1) and RWC = 0.44 (Figure 1). Within this range of RWC, leaf reflectance was progressively increased throughout the near-infrared (Figure 2), showing the strong influence of leaf water content on this parameter.

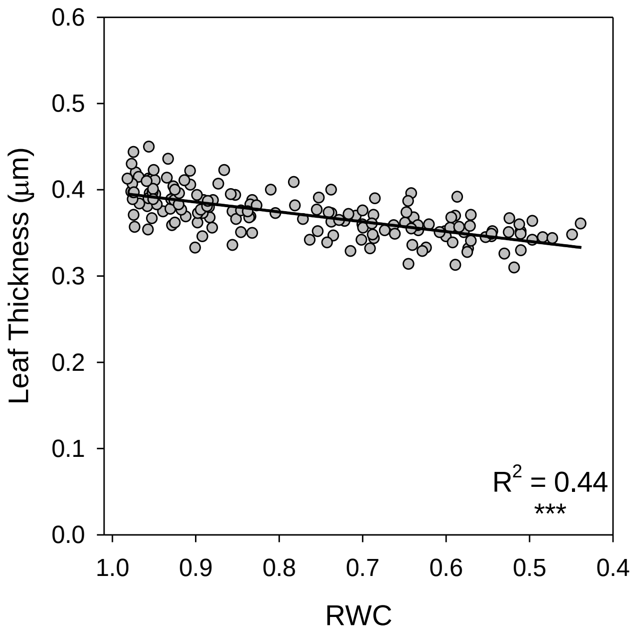

Figure 1. Relationship between relative water content (RWC) and leaf thickness for the measured holm oak leaves. Black line represents the linear regression between both variables ($n = 160$), *** means a p-value < 0.001, and R^2 represents the coefficient of determination.

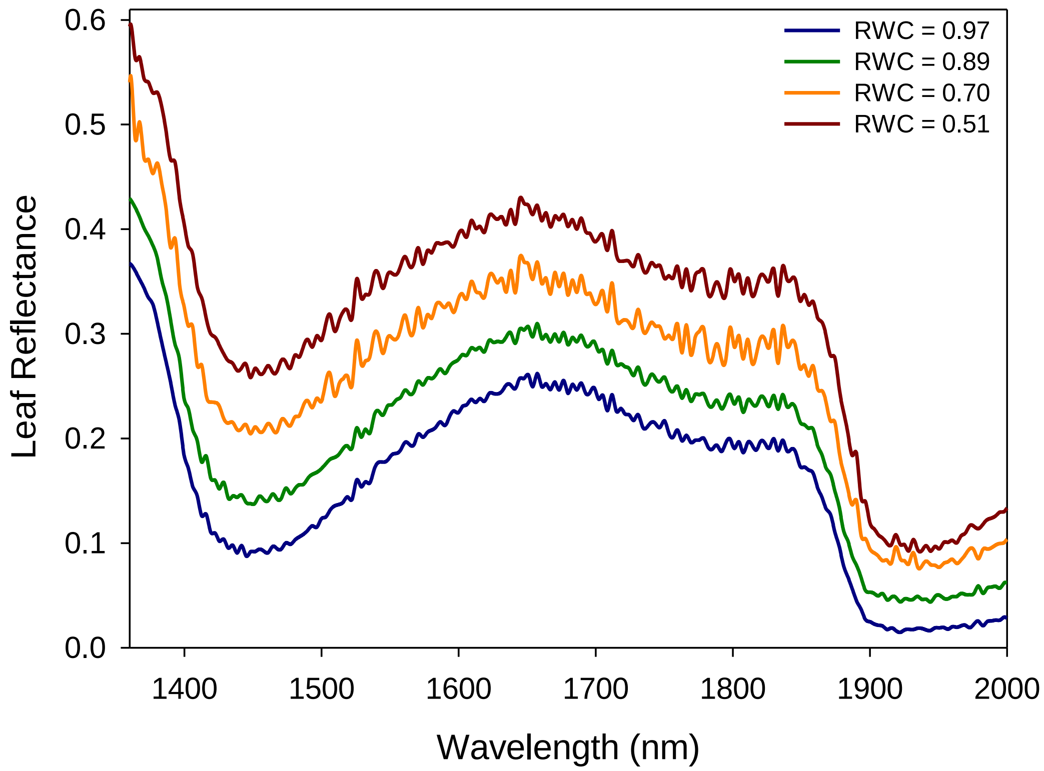

Figure 2. Leaf reflectance between 1350 and 2000 nm for a single leaf of holm oak with different levels of relative water content (RWC).

The relationships between RWC and leaf reflectance at the three wavelengths considered (R_{1450}, R_{1599} and R_{1940}) are presented in Figure 3. We fitted sigmoidal models to the data because it explained the variability of the measured data better than a linear model. The coefficient of determination (R^2) obtained for R_{1450} (R^2 = 0.96, $p < 0.0001$) was higher than those obtained for R_{1599} (R^2 = 0.94, $p < 0.0001$) and R_{1940} (R^2 = 0.91, $p < 0.0001$) (Figure 3, Table 1).

Table 1. Statistical parameters of the relationships between the relative water content (RWC) and the different spectral reflectance indices considered. *n* is the number of data points observed; F ratio is the ratio of the variance explained by a factor to the unexplained variance; R^2 is the r-squared; S.E. of Est. is the standard error of the estimation.

Reflectance Index	*n*	F Ratio	*p*-Value	R^2	S.E. of Est.
R_{1450}	160	1112	<0.0001	0.96	0.0129
R_{1599}	160	766	<0.0001	0.94	0.0139
R_{1940}	160	557	<0.0001	0.91	0.0091
R_{1450}/R_{820}	160	1200	<0.0001	0.88	0.0259
R_{1599}/R_{820}	160	301	<0.0001	0.66	0.0343
R_{1940}/R_{820}	160	833	<0.0001	0.84	0.0164
$LWCI_{1450}$	160	1213	<0.0001	0.96	0.0273
$LWCI_{1599}$	160	1191	<0.0001	0.94	0.0345
$LWCI_{1940}$	160	718	<0.0001	0.93	0.0194

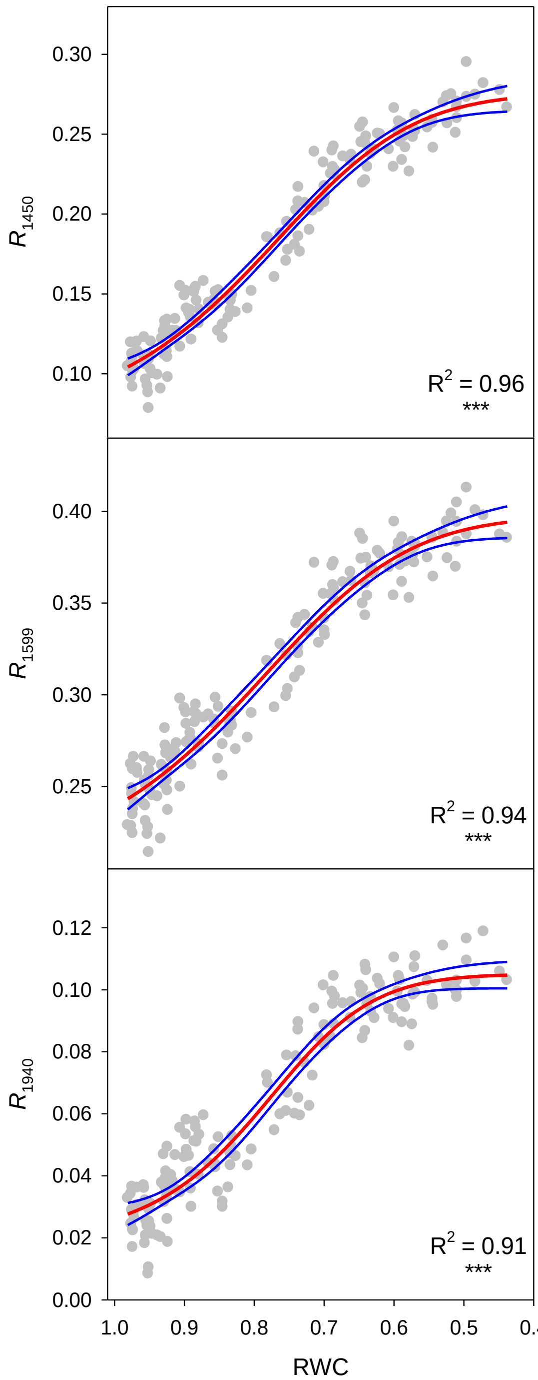

Figure 3. Relationships between the relative water content (RWC) and R_{1450} (**upper panel**), R_{1599} (**medium panel**) and R_{1940} (**lower panel**) for holm oak leaves. Red lines represent the sigmoidal regression models fitted between variables (n = 160). Blue lines represent the 95% confidence interval for the regression. *** means a p-value < 0.001, and R^2 represents the coefficient of determination.

Figure 4 shows the ratio between the reflectance at these wavelengths and the reflectance values at 820 nm (R_{820}) as reference (R_{1450}/R_{820}, R_{1599}/R_{820} and R_{1940}/R_{820}). In this case, the associations were adjusted to linear models and all correlations were statistically significant at $p < 0.0001$ (Table 1). However, it should be noted that the coefficients of determination (R^2) were lower (Figure 4, Table 1) than those obtained without using the reference band (Figure 3, Table 1).

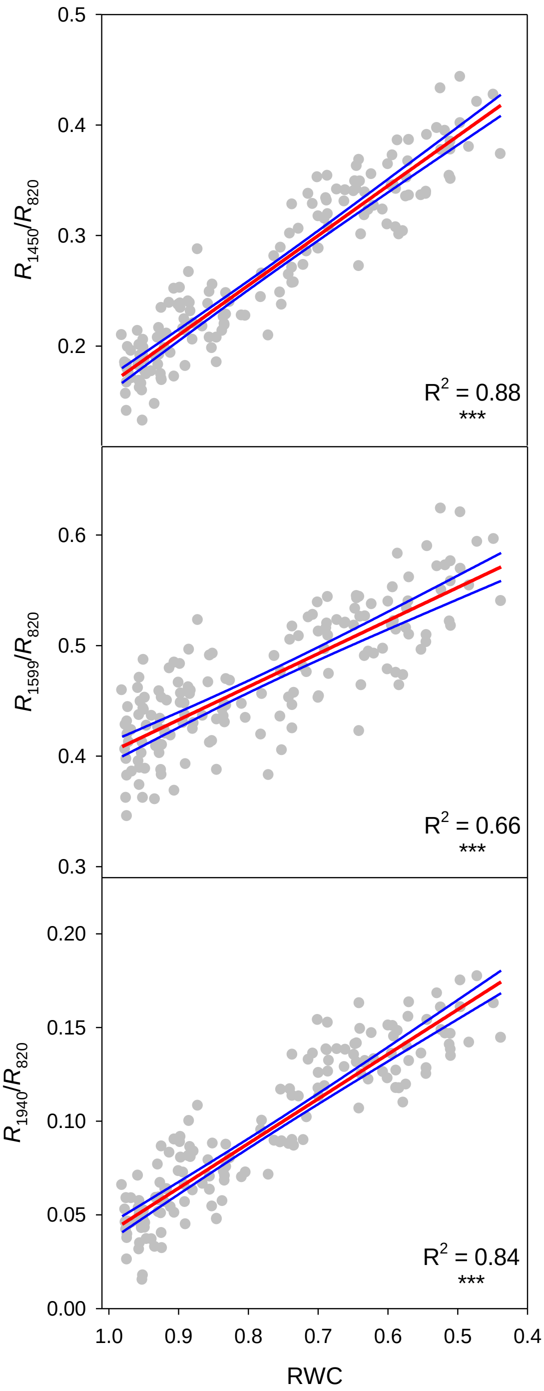

Figure 4. Relationships between the relative water content (RWC) and R_{1450}/R_{820} (**upper panel**), R_{1599}/R_{820} (**medium panel**) and R_{1940}/R_{820} (**lower panel**) for holm oak leaves. Red lines represent the lineal regression between variables (n = 160). Blue lines represent the 95% confidence intervals for the regression. *** means a p-value < 0.001, and R^2 represents the coefficient of determination.

The association between RWC and the indices $LWCI_{1450}$, $LWCI_{1599}$ and $LWCI_{1940}$ was best described using non-linear models (Figure 5), which yielded similar results to those obtained only using the reflectance values without any reference band (Figure 3). Thus, the relationship between RWC and $LWCI_{1450}$ ($R^2 = 0.96$, $p < 0.0001$) and between RWC and $LWCI_{1940}$ ($R^2 = 0.93$, $p < 0.0001$) were adjusted to sigmoidal models, whereas the relationship between RWC and $LWCI_{1599}$ was adjusted to a second-degree polynomial function ($R^2 = 0.94$, $p < 0.0001$) (Figure 5, Table 1).

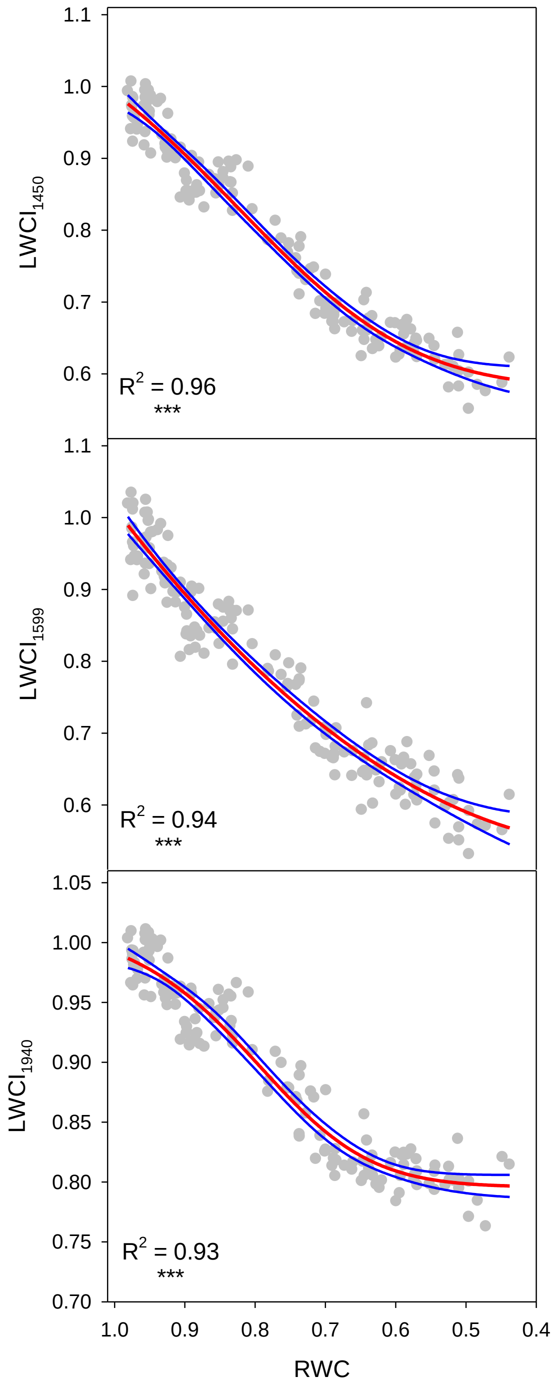

Figure 5. Relationships between the relative water content (RWC) and $LWCI_{1450}$ (**upper panel**), $LWCI_{1599}$ (**medium panel**) and $LWCI_{1940}$ (**lower panel**) for holm oak leaves. Red lines represent the sigmoidal (upper and lower panels) and second-degree polynomial (medium panel) regressions between parameters (n = 160). Blue lines represent the 95% confidence intervals for the regression. *** means a p-value < 0.001, and R^2 represents the coefficient of determination.

Table 2 shows the statistical comparison among the linear relationships obtained between RWC (values between 1.0 and 0.5) and the different spectral reflectance indices, fitted for each measured leaf. The results indicated that, in all cases, the slopes were not statistically different at $p < 0.05$ among the different measured leaves (Table 2). Regarding the intercepts, they were statistically different at $p < 0.05$ among the different measured leaves when the water absorption bands (R_{1450}, R_{1599} and R_{1940}) were used without considering a reference band or using the simple ratio with R_{820} as reference (R_{1450}/R_{820}, R_{1599}/R_{820}

and R_{1940}/R_{820}) (Table 2). By contrast, the intercepts for $LWCI_{1450}$, $LWCI_{1599}$ and $LWCI_{1940}$ were not statistically different at $p < 0.05$ among the different measured leaves (Table 2).

Table 2. Statistical parameters of the comparison of intercepts and slopes for the linear relationships between the relative water content (RWC, values between 1.0 and 0.5) and the different spectral reflectance indices for each measured leaf. *n* is the number of leaves; F ratio is the ratio of the variance explained by a factor to the unexplained variance.

Reflectance Index	*n*	F Ratio Slope	*p*-Value Slope	F Ratio Intercept	*p*-Value Intercept
R_{1450}	16	0.36	0.9856	3.04	0.0004
R_{1599}	16	0.42	0.9703	4.67	0.0000
R_{1940}	16	0.33	0.9910	3.35	0.0001
R_{1450}/R_{820}	16	0.74	0.7358	4.77	0.0000
R_{1599}/R_{820}	16	1.26	0.2406	4.94	0.0000
R_{1940}/R_{820}	16	0.61	0.8643	4.87	0.0000
$LWCI_{1450}$	16	0.36	0.9867	0.78	0.6938
$LWCI_{1599}$	16	0.32	0.9925	0.65	0.8267
$LWCI_{1940}$	16	0.54	0.9102	1.18	0.2949

In general, RMSE and MAE showed the sharpest decrease when the number of leaves used for calibration increased from one to four, reaching a plateau from around five–six leaves (Figure 6 and Table S2 in supplementary information). Indices based on a simple ratio (R_{1450}/R_{820}, R_{1599}/R_{820} and R_{1940}/R_{820}) showed the highest RMSE and MAE values and were the most sensitive to the number of leaves used for calibration. Single-band indices (R_{1450}, R_{1599} and R_{1940}) were more sensitive to the number of leaves used for calibration than LWCI indices ($LWCI_{1450}$, $LWCI_{1599}$ and $LWCI_{1940}$), but reached similarly low RMSE and MAE for calibration sets including five or more leaves. Within each index formulation (single band, simple ratio or LWCI), indices including the 1450 band showed the best performance (e.g., RMSE= 4.0%; MAE= 3.3% for R_{1450}; see Table 3).

Table 3. Summary validation statistics for the predictive linear models built for each of the indices tested. For each statistic, mean and range (between brackets) of the 8008 models fitted using all possible combinations of six leaves for calibration, leaving the rest of leaves for validation. N cal and N val, number of observations included in the calibration and validation sets, respectively; R^2, RMSE and MAE, coefficient of determination, root mean square error and mean absolute error for the validation set.

Index	N cal	N val	R^2	RMSE	MAE
R_{1450}	60	100	0.94 [0.93 0.96]	4.0 [3.4 4.9]	3.3 [2.8 3.8]
R_{1599}	60	100	0.92 [0.90 0.94]	4.8 [3.9 6.1]	3.8 [3.1 4.9]
R_{1940}	60	100	0.89 [0.87 0.91]	5.6 [4.7 6.6]	4.6 [4.0 5.4]
R_{1450}/R_{820}	60	100	0.89 [0.86 0.91]	5.7 [4.8 7.2]	4.6 [4.0 6.2]
R_{1599}/R_{820}	60	100	0.67 [0.60 0.75]	9.7 [8.3 13.5]	7.8 [6.5 11.0]
R_{1940}/R_{820}	60	100	0.84 [0.81 0.89]	6.7 [5.5 8.2]	5.4 [4.4 7.0]
$LWCI_{1450}$	60	100	0.94 [0.93 0.96]	4.0 [3.3 4.5]	3.2 [2.8 3.5]
$LWCI_{1599}$	60	100	0.92 [0.91 0.94]	4.6 [3.9 5.2]	3.7 [3.2 4.1]
$LWCI_{1940}$	60	100	0.90 [0.87 0.93]	5.4 [4.4 6.2]	4.2 [3.7 4.7]

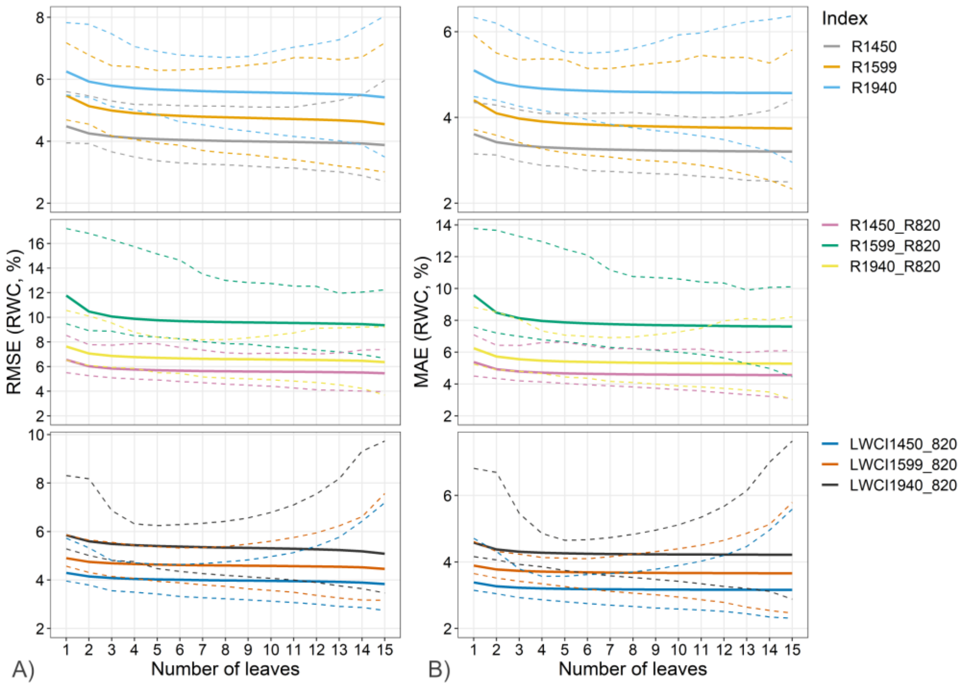

Figure 6. Minimum, maximum and mean of Root Mean Square Error (RMSE, (**A**)) and Mean Absolute Error (MAE, (**B**)) for the set of independent samples used for validation, as a function of the number of leaves used for calibration (1–15). Values calculated across models fitted using all possible combinations of N leaves. For ease of comparison, spectral indices are grouped according to their formulation (single band, simple ratio or LWCI).

4. Discussion

In this study we have shown that leaf reflectance in the "water bands" can constitute an accurate, non-destructive and non-invasive estimator of plant water status in holm oak. Specifically, the leaf reflectance at the three wavelengths considered (1450, 1599 and 1940 nm) displayed a strong association with RWC despite the absence of a reference wavelength (Figure 3, Table 1). This fact reflected the high accuracy of this technique to detect changes in RWC above different physiological thresholds for holm oak (e.g., the turgor loss point or the catastrophic hydraulic failure due to drought-induced xylem embolism) [34]. Our results contrast with those obtained by Sancho-Knapik et al. [19], who found an absence of changes in R_{1300}/R_{1450} index at high leaf RWC values for *Populus* × *euramericana* leaves. These authors stated that this fact could be explained by the marked changed in leaf thickness displayed by this species at high RWC values, as leaf reflectance in the "water bands" is also influenced by the total amount of water within the leaves, which is strongly influenced by leaf thickness [27]. Thus, changes in leaf thickness may offset the changes in near-infrared reflectance values due to leaf water losses, preventing its use for the estimation of plant water status at high RWC values [19]. However, we have evidenced that the percentage of variation in leaf thickness was very low for holm oak when subjected to water stress, i.e., only 15% throughout all the range of RWC analyzed (Figure 1), which implied that changes in leaf water content were the main factor explaining the changes in leaf reflectance in the "water bands" (Figure 2). Thus, this experimental procedure may be suitable for contact measurements of plant water status in this species, even when the use of a reference band is avoided.

However, contact measurements as employed in the present study do not allow the continuous monitoring and real-time tracking of water status of the whole plant. To do this, it is necessary to implement the remote sensing of plant water status at canopy or field level and, consequently, several issues should be considered. First, it is well known that canopy reflectance depends on the absolute amount of incident light, so it is necessary the use of a reference wavelength weakly absorbed by water to standardize and compare the measurements (e.g., 820 nm). In this study, we have assessed if the use of this reference wavelength may enhance the accuracy of "water bands" (R_{1450}/R_{820}, R_{1599}/R_{820} and R_{1940}/R_{820}) when compared with R_{1450}, R_{1599} and R_{1940} at leaf level. However, contrary to expectations, the accuracy was strongly diminished when the ratio of reflectances was considered, especially for R_{1599}/R_{820} (Figures 3 and 4, Table 1). This fact could be explained by the existence of a certain degree of absorption by water at 820 nm. A second problem arises when considering the measurement of 1450 or 1940 nm at canopy level, as the atmospheric water vapor shows high absorptance at these bands. For these reasons, satellites and drones cannot acquire measurements at these wavelengths, despite these wavelengths yielding much higher correlation coefficients than 1599 nm when using R_{820} as reference (Figure 4, Table 1), and detection of changes in plant water status at canopy level has been carried out by means of the moisture stress index (MSI = R_{1599}/R_{820}).

Alternatively, we propose the application of LWCI as a way for improving the accuracy of the estimation of plant water status in holm oak, and a reference wavelength can be also used. In this sense, we have shown that the coefficients of determination obtained with LWCI were very similar to those obtained using the reflectance values without reference band (Figures 3 and 5, Table 1). Thus, it must be highlighted the enhancement in the accuracy of RWC estimation of $LWCI_{1599}$ when compared with R_{1599}/R_{820}. Moreover, when considering RWC values above 0.5, the slopes and the intercepts of the lineal relationships were not statistically different at $p < 0.05$ for LWCI among the different measured leaves (Table 2), which reinforces the robustness and repeatability of these indices for predicting changes in RWC of holm oak. Hence, $LWCI_{1599}$ could be considered a promising tool for detection of changes in plant water status at canopy level. However, it must be noted that the application of LWCI requires the reflectance value at full turgor, which could constitute a disadvantage for its implementation in satellites or drones.

Our results confirm that leaf reflectance in the "water bands" is an accurate tool to monitor changes in water status of holm oak. As stated in the Introduction, this species is the main host plant for black truffle producing systems, which are mainly located in areas with low rainfall and degraded soils, so irrigation is a basic management practice to ensure continuous and homogeneous truffle harvest [41,42]. Therefore, the validation of leaf reflectance in the "water bands" for the evaluation of water status of holm in truffle orchards would allow the development of non-destructive, accurate and easy to interpret decision-making tools that enable the acquisition of real-time and accurate information about when irrigation is needed, minimizing subsequent intervention in plantations.

5. Conclusions

This study demonstrated that leaf reflectance in the "water bands" is an accurate tool to monitor changes in water status of holm oak, even when the use of a reference band is avoided. According to our results, for measurements at the leaf level, predictive models based on single bands, and particularly on the 1450 nm band, allow robust predictions when a sufficient number of leaves (5–6) are used for calibration. However, the use of a reference wavelength for remote sensing measurements (such as in MSI) diminished the accuracy of the detection of changes in RWC in holm oak. Alternatively, the application of LWCI improved the accuracy of the estimation of plant water status in holm oak, and a reference wavelength can be also used, reaching accuracies comparable to single-band models. However, this index required the reflectance value at full turgor, which could constitute a disadvantage for its implementation in satellites or drones. In conclusion, this technique would allow the continuous monitoring and real-time tracking of the physiological state of holm oak and intelligent water control in truffle cultivation. For this purpose, the use of remote sensing data obtained by satellite or UAV from natural holm oak stands or truffle plantations under different degree of water deficit would be very useful for the validation of this technique at the field level, including leaves of different age, morphology and composition. Moreover, this technique may be also suitable for other plant species with a low variation in leaf thickness when subjected to dehydration.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14091825/s1, Table S1: Models and equations used for fitting the individual data for each of the indices tested; Table S2: Summary validation statistics for the predictive linear models built for each of the indices tested.

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References

1. Peguero-Pina, J.J.; Vilagrosa, A.; Alonso-Forn, D.; Ferrio, J.P.; Sancho-Knapik, D.; Gil-Pelegrín, E. Living in Drylands: Functional Adaptations of Trees and Shrubs to Cope with High Temperatures and Water Scarcity. *Forests* **2020**, *11*, 1028. [CrossRef]
2. Jones, H.G. Monitoring plant and soil water status: Established and novel methods revisited and their relevance to studies of drought tolerance. *J. Exp. Bot.* **2007**, *58*, 119–130. [CrossRef]
3. Passioura, J.B. Water in the Soil-Plant-Atmosphere Continuum. In *Physiological Plant Ecology II. Encyclopedia of Plant Physiology*; Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H., Eds.; Springer: Berlin/Heidelberg, Germany, 1982; Volume 12/B, pp. 5–33. [CrossRef]
4. Sperry, J.S.; Stiller, V.; Hacke, U.G. Xylem Hydraulics and the Soil–Plant–Atmosphere Continuum: Opportunities and Unresolved Issues. *Agron. J.* **2003**, *95*, 1362–1370. [CrossRef]
5. Peguero-Pina, J.J.; Mendoza-Herrer, Ó.; Gil-Pelegrín, E.; Sancho-Knapik, D. Cavitation Limits the Recovery of Gas Exchange after Severe Drought Stress in Holm Oak (*Quercus ilex* L.). *Forests* **2018**, *9*, 443. [CrossRef]
6. Canny, M.J.; Huang, C.X. Leaf water content and palisade cell size. *New Phytol.* **2006**, *170*, 75–85. [CrossRef]
7. Teulat, B.; Monneveux, P.; Wery, J.; Borries, C.; Souyris, I.; Charrier, A. Relationships between Relative Water Content and Growth Parameters Under Water Stress in Barley: A QTL Study. *New Phytol.* **1997**, *137*, 99–107. [CrossRef]
8. Sancho-Knapik, D.; Gómez Álvarez-Arenas, T.; Peguero-Pina, J.J.; Fernández, V.; Gil-Pelegrín, E. Relationship between ultrasonic properties and structural changes in the mesophyll during leaf dehydration. *J. Exp. Bot.* **2011**, *62*, 3637–3645. [CrossRef] [PubMed]
9. Scoffoni, C.; Vuong, C.; Diep, S.; Cochard, H.; Sack, L. Leaf Shrinkage with Dehydration: Coordination with Hydraulic Vulnerability and Drought Tolerance. *Plant Physiol.* **2014**, *164*, 1772–1788. [CrossRef] [PubMed]
10. Sapes, G.; Sala, A. Relative water content consistently predicts drought mortality risk in seedling populations with different morphology, physiology and times to death. *Plant Cell Environ.* **2021**, *44*, 3322–3335. [CrossRef] [PubMed]
11. Sancho-Knapik, D.; Gómez Álvarez-Arenas, T.; Peguero-Pina, J.J.; Gil-Pelegrín, E. Air-coupled broadband ultrasonic spectroscopy as a new non-invasive and non-contact method for the determination of leaf water status. *J. Exp. Bot.* **2010**, *61*, 1385–1391. [CrossRef]
12. Alonso-Forn, D.; Peguero-Pina, J.J.; Ferrio, J.P.; Mencuccini, M.; Mendoza-Herrer, Ó.; Sancho-Knapik, D.; Gil-Pelegrín, E. Contrasting functional strategies following severe drought in two Mediterranean oaks with different leaf habit: *Quercus faginea* and *Quercus ilex* subsp. *rotundifolia*. *Tree Physiol.* **2021**, *41*, 371–387. [CrossRef]
13. Sancho-Knapik, D.; Mendoza-Herrer, Ó.; Alonso-Forn, D.; Saz, M.A.; Martín-Sánchez, R.; dos Santos Silva, J.V.; Ogee, J.; Peguero-Pina, J.J.; Gil-Pelegrín, E.; Ferrio, J.P. Vapor pressure deficit constrains transpiration and photosynthesis in holm oak: A comparison of three methods during summer drought. *Agric. For. Meteorol.* **2022**, *327*, 109218. [CrossRef]
14. Peguero-Pina, J.J.; Morales, F.; Flexas, J.; Gil-Pelegrín, E.; Moya, I. Photochemistry, remotely sensed physiological reflectance index and de-epoxidation state of the xanthophyll cycle in *Quercus coccifera* under intense drought. *Oecologia* **2008**, *156*, 1–11. [CrossRef] [PubMed]

15. Sancho-Knapik, D.; Mendoza-Herrer, Ó.; Gil-Pelegrín, E.; Peguero-Pina, J.J. Chl Fluorescence Parameters and Leaf Reflectance Indices Allow Monitoring Changes in the Physiological Status of *Quercus ilex* L. under Progressive Water Deficit. *Forests* **2018**, *9*, 400. [CrossRef]
16. Sancho-Knapik, D.; Medrano, H.; Peguero-Pina, J.J.; Mencuccini, M.; Fariñas, M.D.; Álvarez-Arenas, T.G.; Gil-Pelegrín, E. The Application of Leaf Ultrasonic Resonance to *Vitis vinifera* L. Suggests the Existence of a Diurnal Osmotic Adjustment Subjected to Photosynthesis. *Front. Plant Sci.* **2016**, *7*, 1601. [CrossRef] [PubMed]
17. Álvarez-Arenas, T.E.G.; Sancho-Knapik, D.; Peguero-Pina, J.J.; Gil-Pelegrín, E. Surface Density of the Spongy and Palisade Parenchyma Layers of Leaves Extracted from Wideband Ultrasonic Resonance Spectra. *Front. Plant Sci.* **2020**, *11*, 695. [CrossRef] [PubMed]
18. Fariñas, M.D.; Sancho-Knapik, D.; Peguero-Pina, J.J.; Gil-Pelegrín, E.; Gómez Álvarez-Arenas, T.E. Contact-less, non-resonant and high-frequency ultrasonic technique: Towards a universal tool for plant leaf study. *Comput. Electron. Agric.* **2022**, *199*, 107160. [CrossRef]
19. Sancho-Knapik, D.; Gismero, J.; Asensio, A.; Peguero-Pina, J.J.; Fernández, V.; Álvarez-Arenas, T.E.G.; Gil-Pelegrín, E. Microwave L-band (1730 MHz) accurately estimates the relative water content in poplar leaves. A comparison with a near infrared water index (R_{1300}/R_{1450}). *Agric. For. Meteorol.* **2011**, *151*, 827–832. [CrossRef]
20. Carter, G.A. Primary and secondary effects of water content on the spectral reflectance of leaves. *Am. J. Bot.* **1991**, *78*, 916–924. [CrossRef]
21. Carter, G.A.; McCain, D.G. Relationship of leaf spectral reflectance to chloroplast water content determined using NMR microscopy. *Remote Sens. Environ.* **1993**, *46*, 305–310. [CrossRef]
22. Peñuelas, J.; Filella, I.; Biel, C.; Serrano, L.; Save, R. The reflectance at the 950–970 nm region as indicator of plant water status. *Int. J. Remote Sens.* **1993**, *14*, 1887–1905. [CrossRef]
23. Sims, D.A.; Gamon, J.A. Estimation of vegetation water content and photosynthetic tissue area from spectral reflectance: A comparison of indices based on liquid water and chlorophyll absorption features. *Remote Sens. Environ.* **2003**, *84*, 526–537. [CrossRef]
24. Seelig, H.D.; Adams, W.W.; Hoehn, A.; Stodieck, L.S.; Klaus, D.M.; Emery, W.J. Extraneous variables and their influence on reflectance-based measurements of leaf water content. *Irrigat. Sci.* **2008**, *26*, 407–414. [CrossRef]
25. Seelig, H.D.; Hoehn, A.; Stodieck, L.S.; Klaus, D.M.; Adams, W.W.; Emery, W.J. The assessment of leaf water content using leaf reflectance ratios in the visible, near-, and short-wave-infrared. *Int. J. Remote Sens.* **2008**, *29*, 3701–3713. [CrossRef]
26. Wu, C.; Niu, Z.; Tang, Q.; Huang, W. Predicting vegetation water content in wheat using normalized difference water indices derived from ground measurements. *J. Plant Res.* **2009**, *122*, 317–326. [CrossRef] [PubMed]
27. Seelig, H.D.; Hoehn, A.; Stodieck, L.S.; Klaus, D.M.; Adams, W.W., III; Emery, W.J. Plant water parameters and the remote sensing R_{1300}/R_{1450} leaf water index: Controlled condition dynamics during the development of water deficit stress. *Irrigat. Sci.* **2009**, *27*, 357–365. [CrossRef]
28. Rock, B.N.; Vogelmann, J.E.; Williams, D.L.; Vogelmann, A.F.; Hoshizaki, T. Remote detection of forest damage. *Bioscience* **1986**, *36*, 439–445. [CrossRef]
29. Watt, M.S.; Leonardo, E.M.C.; Estarija, H.J.C.; Massam, P.; de Silva, D.; O'Neill, R.; Lane, D.; McDougal, R.; Buddenbaum, H.; Zarco-Tejada, P.J. Long-term effects of water stress on hyperspectral remote sensing indicators in young radiata pine. *For. Ecol. Manag.* **2021**, *502*, 119707. [CrossRef]
30. Hunt, E.R.; Rock, B.N.; Nobel, P.S. Measurement of leaf relative water content by infrared reflectance. *Remote Sens. Environ.* **1987**, *22*, 429–435. [CrossRef]
31. Hunt, E.R.; Rock, B.N. Detection of changes in leaf water content using Near- and Middle-Infrared reflectances. *Remote Sens. Environ.* **1989**, *30*, 43–54. [CrossRef]
32. Gil-Pelegrín, E.; Saz, M.A.; Cuadrat, J.M.; Peguero-Pina, J.J.; Sancho-Knapik, D. Oaks under Mediterranean type climates: Functional response to summer aridity. In *Oaks Physiological Ecology. Exploring the Functional Diversity of Genus Quercus L.*; Gil-Pelegrín, E., Peguero-Pina, J.J., Sancho-Knapik, D., Eds.; Tree Physiology, Volume 7; Springer: Berlin/Heidelberg, Germany, 2017; pp. 137–193. [CrossRef]
33. Peguero-Pina, J.J.; Sisó, S.; Flexas, J.; Galmés, J.; García-Nogales, A.; Niinemets, Ü.; Sancho-Knapik, D.; Saz, M.Á.; Gil-Pelegrín, E. Cell-level anatomical characteristics explain high mesophyll conductance and photosynthetic capacity in sclerophyllous Mediterranean oaks. *New Phytol.* **2017**, *214*, 585–596. [CrossRef] [PubMed]
34. Peguero-Pina, J.J.; Sancho-Knapik, D.; Barrón, E.; Camarero, J.J.; Vilagrosa, A.; Gil-Pelegrín, E. Morphological and physiological divergences within *Quercus ilex* support the existence of different ecotypes depending on climatic dryness. *Ann. Bot.* **2014**, *114*, 301–313. [CrossRef]
35. Oliach, D.; Vidale, E.; Brenko, A.; Marois, O.; Andrighetto, N.; Stara, K.; Martínez de Aragón, J.; Colinas, C.; Bonet, J.A. Truffle Market Evolution: An Application of the Delphi Method. *Forests* **2021**, *12*, 1174. [CrossRef]
36. Mauri, P.; Manzanera, J. Protocol of Somatic Embryogenesis: Holm Oak (*Quercus ilex* L.). In *Protocol for Somatic Embryogenesis in Woody Plants*; Jain, S.M., Gupta, P.K., Eds.; Forestry Sciences, Volume 77; Springer: Berlin/Heidelberg, Germany, 2005; pp. 469–482. [CrossRef]

37. Zimmermann, D.; Reuss, R.; Westhoff, M.; Gebner, P.; Bauer, W.; Bamberg, E.; Bentrup, F.W.; Zimmermann, U. A novel, non-invasive, online-monitoring, versatile and easy plant-based probe for measuring leaf water status. *J. Exp. Bot.* **2008**, *59*, 3157–3167. [CrossRef] [PubMed]
38. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2022; Available online: https://www.R-project.org/ (accessed on 5 September 2023).
39. Wickham, H.; Averick, M.; Bryan, J.; Chang, W.; McGowan, L.D.; François, R.; Grolemund, G.; Hayes, A.; Henry, L.; Hester, J.; et al. Welcome to the tidyverse. *J. Open Source Softw.* **2019**, *4*, 1686. [CrossRef]
40. Kuhn, M. Building Predictive Models in R Using the caret Package. *J. Stat. Softw.* **2008**, *28*, 1–26. [CrossRef]
41. Büntgen, U.; Egli, S.; Schneider, L.; von Arx, G.; Rigling, A.; Camarero, J.J.; Sangüesa-Barreda, G.; Fischer, C.R.; Oliach, D.; Bonet, J.A.; et al. Long-term irrigation effects on Spanish holm oak growth and its black truffle symbiont. *Agric. Ecosyst. Environ.* **2015**, *202*, 148–159. [CrossRef]
42. Piñuela, Y.; Alday, J.G.; Oliach, D.; Castaño, C.; Bolaño, F.; Colinas, C.; Bonet, J.A. White mulch and irrigation increase black truffle soil mycelium when competing with summer truffle in young truffle orchards. *Mycorrhiza* **2021**, *31*, 371–382. [CrossRef] [PubMed]