The use of physiological, biochemical and morpho-anatomical traits in tree breeding for improved water-use efficiency of *Quercus robur* L.

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### Abstract

**Aim of study:** In the present paper the nature and level of co-dependence between leaf functional traits and intrinsic water-use efficiency (WUE) were studied in one-year-old *Quercus robur* L. seedlings, grown in a common garden experiment under moderate drought conditions. The study was established to identify those traits that might potentially be utilized to improve leaf-level WUE, and therefore be used in breeding programmes to enhance drought adaptation of *Q. robur*.

**Area of study:** The study was carried out at the common garden site within the UNESCO Biosphere Reserve Mura-Drava-Danube.

**Material and methods:** The study involved one-year-old seedlings of eight half-sib lines of *Quercus robur* L. The use of physiological, biochemical, morphological and anatomical traits in tree breeding for improved water-use efficiency (*WUE*), is of great importance, due to both its economic and environmental impacts. The results showed that leaf stomata traits, particularly stomatal density (SD), and leaf dry mass per unit leaf area (LMA) were the most important traits, closely associated with *WUE*. Stomatal density achieved the highest score on PC1 (0.825), in which *WUE* had the highest loading (0.920), as well. SD was also included first in stepwise regression model.

**Main results:** These results demonstrate that under moderate water stress *WUE* in *Q. robur* half-sib lines were mainly the result of the plants' structural acclimation to surrounding environmental conditions.

**Keywords:** *Quercus robur* L.; half-sib line; intrinsic water-use efficiency; leaf functional traits.

**Authors’ contributions:** SS and SO conceived the ideas and designed the study. SS and MSN carried out the field measurements and collected the leaf samples. MB conducted measurements of morphological and anatomical leaf traits in the laboratory. MK, MSN and EV carried out the biochemical analyses. BK conducted the statistical analysis. SS, SO, BK and MK wrote the first version of the manuscript, and all of the authors contributed critically to the drafts and gave final approval for publication.

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### Introduction

Pedunculate oak (*Quercus robur* L.) decline has been observed in Europe during the last few decades, causing not only economic losses, but also losses of biodiversity and forest genetic resources (Čater, 2015). Recently, a number of factors have been identified that contribute to *Q. robur* decline; e.g. Thomas et al. (2002) listed the most important stress elicitors in the oak decline complex, grouping them roughly into biotic (insect larvae, bark beetles, pathogenic fungi, and microorganisms) and abiotic (air pollutants, climatic extremes and site conditions). Although the causes of oak decline are complex and probably involve...
diverse direct and indirect factors (Drobyshhev et al., 2007), drought stress has been frequently reported as the main driver of oak dieback across Europe. Indeed, Andersson et al. (2011) found that the extreme drought in southern Sweden in 1992 was the triggering factor of the oak mortality in the following years. Cochard et al. (1992) reported serious oak dieback after the severe drought that occurred in France in 1976. Furthermore, Čater (2015) evidenced increased mortality rates of oak in Slovenia after remarkably dry periods in 2003 and 2013. Similarly, the dieback of oak trees is widely present in Serbia, as well, as the consequence of the long-term decline of the Danube and the Sava rivers’ water levels (Stojnić et al., 2014). This issue is not only evident among mature stands, but it is also present at regeneration sites and is reflected in failed reforestation attempts, especially during dry years (Čater & Batič, 2006; Stojanović et al., 2015). Furthermore, climate change is predicted to negatively influence pedunculate oak ecosystems and sustainable wood production (Stojanović et al., 2014; Liović et al., 2019). Bearing this in mind, it appears that conventional breeding strategies based on selection of dominant stem quality and vigorous growth rate will have to be improved in order to address increasing needs in a rapidly changing environment (Verry, 2008).

The resistance of plants to low water availability is the consequence of four possible strategies: drought escape, drought avoidance, drought tolerance and drought recovery. Among these strategies drought avoidance and drought tolerance have been noted as major mechanisms, utilized by plant species grown under water deficit (arid) conditions (Fang & Xiong, 2015). Regarding drought avoidance, water-use efficiency (WUE) is one of the key components responsible for maintaining favorable tissue water content through morphological adjustments or physiological mechanisms (Farooq et al., 2009; Kooyers, 2015). Therefore, water-use efficiency might be considered as one of the principal components of drought adaptation in plant species. Water-use efficiency can be defined from different perspectives, depending on space scale of measurement and units of exchange being used for WUE determination (Condon et al., 2004). Moreover, it might be observed at a given point of time or over a prolonged period (Yul Yoo et al., 2009). From the physiological perspective particularly interesting is intrinsic water-use efficiency of leaf gas exchange (WUE), which can be defined as the ratio of net photosynthetic rate to stomatal conductance for water vapour. In general, as drought increases, simultaneous decline in net CO₂ assimilation rate is more evident than the reduction of stomatal conductance, leading to an increase in WUE (Pita et al., 2005; Bojović et al., 2017). Although the theoretical background behind this process is well-known, the effects of varying traits on WUE have not yet been experimentally tested in many tree species. Due to very tight relationship between leaf structure and function (i.e. leaf morphological and anatomical structure and physiological processes), the relationship between WUE and other traits is rather complex and requires a deeper analysis in order to identify those which determine WUE, to the largest extent. For example, several studies have demonstrated a coupled response of net photosynthesis and stomatal conductance of CO₂ to environmental signals (Raftoyannis et al., 2006; Gallé & Feller, 2007). Along this line, stomatal morphology and density have also been shown to contribute to the diversity of stomatal conductance, which might influence WUE, due to an aforementioned close correlation between net CO₂ assimilation rate and stomatal conductance (Franks & Farquhar, 2007). Similarly, a study conducted on Fagus crenata Blume, provenances showed a significant correlation between leaf thickness on one side, and maximum photosynthetic rate and stomatal conductance on the other (Bayramzadeh et al., 2008). Furthermore, under drought conditions the imbalance between the production of different reactive oxygen species (ROS) and scavenging capacity of antioxidant defense in the cell can lead to drought induced secondary stress, a phenomenon known as oxidative stress (Avramova et al., 2017).

Targeting specific functional traits to improve WUE, as well as understanding the relative contribution of these characteristics to it should be one of the principal goals in the genetic improvement of Q. robur. Therefore, the objective of the present research was to assess the relevance of various functional traits as indicators of WUE. We hypothesized that application of various multivariate statistical methods (i.e. principal component analysis, stepwise regression analysis and path coefficient analysis) will enable the identification of leaf functional traits that could serve as indicators of Q. robur adaptation to drought, as well as for evaluation of the nature and magnitude of the relationship between physiological, biochemical and morpho-anatomical characteristics, on one side, and WUE, on the other.

Material and methods

Plant material and experimental design

The study involved one-year-old seedlings of eight half-sib lines of Quercus robur L. originating from the UNESCO Biosphere Reserve Mura-Drava-Danube. Acorns used for the progeny trial establishment were collected from eight dominant mother trees in October.
2015 and were sown in seedbeds at the beginning of November 2015. The seed was sown manually to the depth of approximately 2 cm below soil surface. The progeny trial was founded at the Experimental Estate of the Institute of Lowland Forestry and Environment (45°17’ N; 19°53’ E). The trial was established in a randomized complete block design, with three replications. In total, 150 seed were sown per each half-sib line (i.e. 50 seed per single replication) with 30 × 30 cm spacing. Total area of experimental plot was 110 m².

The climate at this site is temperate continental with an average annual temperature of 11.4°C and total annual precipitation of 647 mm (Fig. 1). The records were obtained from the weather station, “Rimski Šančevi” (45°20’ N; 19°51’ E). The soil at the trial site belongs to a type of undeveloped alluvial soil (fluvisol), sandy loam form, with a dominant fraction of fine sand (63.5%) and silt+clay fraction in a lower concentration (34.7%) (Krstić et al., 2014).

In the present study 18 parameters were analyzed, which might be roughly classified into physiological, biochemical, and morpho-anatomical (Table 1). The field measurements of leaf gas exchange and leaf chlorophyll content, as well as the collection of plant material for biochemical, morphological and anatomical analyses were performed between the 5th and 7th of August 2016. Before the research was started, the seedlings were neither irrigated nor fertilized, but only were subjected to ambient climate conditions. Soil moisture expressed as soil water potential (MPa) was measured automatically at soil depth of 15-20 cm (the zone of seedlings’ root system) at 30-minute intervals, using common gypsum blocks (Delmhorst Inc., USA) connected to a data-logger. Prior to the measurements, soil water potential ranged between -0.984 and -1.203 MPa, which, according to Lui et al. (2010), can be characterized as “moderate drought”.

All analyses were performed on ten seedlings per single half-sib line, on insect and disease-free leaves. In total, 80 sample leaves were used for the measurements of leaf gas exchange parameters. On the last day of field work, the same leaves were collected for the biochemical analyses. Morphological and anatomical traits were studied on three leaves per plant.

**Physiological analyses**

Measurements of net photosynthesis (A), stomatal conductance (gₜ), intercellular CO₂ concentration (Cᵢ) and transpiration rate (E) were performed using the portable photosynthesis device ADC BioScientific Ltd. LCpro+. All measurements were conducted on sunny days between 09:00 and 11:00 a.m., on fully expanded and hardened sun-leaves. The measurements were made under constant light conditions (1000 μmol m⁻² s⁻¹) and CO₂ concentrations (350 μmol mol⁻¹), whereas the temperature and humidity inside of chamber were taken ambient from the atmosphere (Topić et al., 2012). Intrinsic water-use efficiency (WUE) was calculated as the ratio of net assimilation rate to stomatal conductance (A gᵢ⁻¹) (Flexas et al., 2013).

Leaf chlorophyll content (Chl) was estimated with a SPAD-502 meter (Minolta Inc., Osaka, Japan). Five replications per a single leaf were made. The obtained SPAD values were converted to chlorophyll content (μg cm⁻²) following Cerovic et al. (2012):

\[
\text{Chl} = (99 \times \text{SPAD value}) \times (144 - \text{SPAD value})^{-1}
\]  

**Biochemical analyses**

About 200 mg of freeze-dried powdered plant material was macerated with 70% ethanol (in water) in a ratio of 1:10 (w/v) and then vigorously shaken and centrifuged at 10000 rpm for 15 min at 4°C. The supernatant was used for determination of radical scavenger capacity (RSC) against NO, DPPH and ABTS radicals, as well as for the determination of the total phenolic content (TPC) and the amount of total flavonoids.

Another group of extracts were prepared by mixing 100 mg of fresh plant material with 0 mL of 50 mM K-phosphate buffer (pH 7.0) and then centrifuged at 15000 g for 10 min at 4°C. After that, the supernatant was separated and used for determination of ferric reducing antioxidant power (FRAP) and total soluble proteins. All spectrophotometric measurements were performed on a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA).

Radical scavenger capacity (RSC) against 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical was performed according to the method of Arnao (2000).
Working probes consisted of 10 µl of leaf extract and 250 µl of 0.004 % (w/v) solution of DPPH which was previously prepared in ethanol (95 %). The reaction mixture was shaken vigorously and the absorbance of remaining DPPH was measured at 520 nm after 30 min. DPPH radical scavenging capacity (RSC) was expressed in percentage (%) and calculated by following equation:

\[
\text{RSC} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where: \(A_{\text{control}}\) is the absorbance of the DPPH reagent without extract and \(A_{\text{sample}}\) is the absorbance of DPPH solution in presence of the sample.

Ethanolic extracts were also estimated to their radical scavenging capacity against another commercial chromophore, 2.2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS\(^\bullet+\)) according to method described by Miller & Rice-Evans (1997). Radical scavenger capacity against ABTS\(^\bullet+\) was measured at 730 nm by mixing 10 µl of ethanolic extract and 250 µl of ABTS solution and percentage of ABTS inhibition was calculated according to the same formula as for the DPPH.

The NO radical scavenging capacity was estimated by using Griess Illosvory diazotization reaction, according to the method given by Hensley et al. (2003). Working probes consisted of 10 µl of ethanolic extract and 60 µl of 10 mM sodium nitroprusside that was dissolved in phosphate buffered saline (PBS). The probes were incubated under light at room temperature for 150 minutes. After incubation, 120 µl of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, 2.5% H\(_3\)PO\(_4\)) was added to the probes and absorbance was measured at 577 nm. The NO RSC (%) was calculated according to the previously given equation (2), where \(A_{\text{control}}\) was the absorbance of probes without the extract and \(A_{\text{sample}}\) is the absorbance of the reaction mixture with the sample.

Determination of flavonoids was performed by using the aluminum chloride colorimetric method with slight modifications (Chang et al., 2002). Briefly, working probes consisted of 30 µl of the ethanolic extract, 90 µl of ethanol, 6 µl 1.0 M NaCl, 6 µl 0.75 M AlCl\(_3\), 150 µl of water and after an incubation of 30 minutes at room temperature, the absorbances of the probes were measured at 415 nm. The amount of total flavonoids was calculated by using calibration curve constructed with quercetine as a standard (10-250 µg) and results were expressed as quercetine equivalents in milligrams per gram of dry weight (mg QE g\(^{-1}\) DW).

Total phenolic content (TPC) was estimated by application of method given by Kim et al. (2003). Probes were made by mixing 25 µl of ethanolic extract and...
with 125 μl of four-time diluted Folin–Ciocalteu reagent (FCR) in water and 100 μl 7.5 % Na₂CO₃. The absorbance of the probes was recorded at 760 nm. The standard curve was formed by using a range of different concentrations of gallic acid as a calibrator and the results were expressed as milligram of gallic acid equivalents per g of dry weight (mg GAE g⁻¹ DW).

Ferric reducing antioxidant power (FRAP) assay was performed as described by Benzie & Strain (1996). Quantity of 10 μl of extract was allowed to react with 225 μl of acidic FRAP reagent and the absorbance of dark blue ferrous complex (Fe²⁺-(TPTZ))₃⁻ was measured at 593 nm. FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6) and 10 mM TPTZ solution (2.4.6-tripyridyl-s-triazine) and 20 mM FeCl₃, 6 H₂O in ratio 10:1:1. The standard curve was constructed by using trolox as a calibrator and reducing capacity of extracts was expressed as μmoles of Trolox equivalent per gram DW sample (μmol TE g⁻¹ DW).

Soluble protein content was determined following the Bradford method (1976) and expressed as mg protein per g of dry weight (mg g⁻¹ DW). Bovine serum albumin (BSA) was used as a standard to form calibration curve.

Leaf anatomical and morphological analyses

Stomatal density per mm² (SD), stomata guard cell length (LS) and width (WS) were determined from leaf prints made following the protocols of Kardel et al. (2010) and Stojnić et al. (2015a). The samples were taken from the fully developed and sun-exposed leaves from the spring flushes (Stojnić et al., 2015a).

The morphological analyses were conducted on the leaves from which stomata imprints were previously taken. The leaf area (LA) was measured with an ADC Bioscientific Ltd. AM300 Portable Leaf Area Meter. Afterwards, the same leaves were dried at 70°C for 72 hours and leaf dry mass was determined. Leaf dry mass per unit area (LMA) was calculated using the formula after Reich et al. (1992) and expressed in mg cm⁻²:

\[ \text{LMA} = \text{DM} \times \text{LA}^{-1} \] (3)

Statistical analysis

The data were analyzed in the software Statistica 13 (TIBCO Software Inc., 2017) and R 3.3.2. for Windows (R Core Team, 2017). For each dependent variable, Shapiro–Wilk’s and Levene’s tests were performed to analyze normality of distribution and homogeneity of variances, respectively (Sánchez-Gómez et al., 2013). Variables: LA, Ci, E, QE and FRAP were log-transformed, while RSC NO, RSC DPPH and RSC ABTS were arcsine-transformed in order to meet the assumptions of normality and homoscedasticity. Analysis of variance (ANOVA) and Tukey HSD (honestly significant difference) test (α=0.05) were provided for each trait to verify the significance of differences between half-sib lines.

Relationships between characters were analyzed using three multivariate statistical methods: principal component analysis, stepwise forward regression analysis and path coefficient analysis (Thurstone, 1969; Dewey & Lu, 1959; Li, 1975; Kovacevic et al., 2008; Kovacevic, 2014). Principal component analysis was used in order to group the examined original variables according to their codependence. For this purpose the first five principal components were selected according to Kaiser’s criterion; i.e. components with eigenvalues higher than 1. Considering the absence of correlation between principal components, characteristics with the highest correlation (loading) within the same principal component are supposed to be correlated, therefore being included in the same group. The rotation of the first five principal components was performed by application of the Varimax method, in order to maximize the variability of loadings of the examined characteristics within the selected principal components. Considering multicollinearity within groups, stepwise regression analysis was performed in order to form a model that would consist of few variables, but still describe the most of WUE variability. The variables were included in the model within stepwise procedure in such an order to achieve the highest possible increment of coefficient of determination (Wonnacott & Wonnacott, 1981). Traits A and gₛ were excluded from these analyses since the WUE, is calculated directly from them, and, therefore, is highly correlated with these traits. Variables selected by stepwise regression analysis entered path co-efficient analysis in order to determine their direct and indirect effect on WUEᵢ.

Results

We found significant difference in WUEᵢ between Q. robur half-sib lines, although the results evidenced a narrow range of WUE variation (7.8 μmol mol⁻¹). The highest mean value of WUEᵢ was observed in half-sib line B05, whereas the lowest was recorded in half-sib line B17. In addition, the analysis of variance showed that most of the studied traits varied significantly between Q. robur half-sib lines. The highest variability was observed for physiological traits (except for chlorophyll content), while among biochemical and morphoanatomical characteristics, significant differences were observed only for certain parameters (TPC, TFC, TSP, RSC DPPH, LMA, SD and LS) (Table 2). Based on the
Table 2. Tukey HSD test and One-way ANOVA analysis for examined parameters of Quercus robur L. half-sib lines. Values followed by different letters indicate significant differences between half-sib lines based on one-way ANOVA.

| Parameter | B02   | B05   | B06   | B09   | B11   |
|-----------|-------|-------|-------|-------|-------|
| LA        | 28.52 | a     | 32.05 | a     | 28.77 | a     | 28.80 | a     | 27.78 | a     |
| LMA       | 6.42  | bc    | 6.50  | abc   | 6.72  | abc   | 5.97  | c     | 6.83  | ab    |
| Cᵢ        | 260.1 | ab    | 242.3 | c     | 244.5 | bc    | 233.1 | a     | 269.6 | a     |
| E         | 6.64  | a     | 7.12  | a     | 6.42  | ab    | 6.89  | a     | 5.64  | c     |
| gs        | 0.318 | a     | 0.259 | c     | 0.219 | d     | 0.296 | ab    | 0.261 | c     |
| A         | 10.7  | a     | 10.0  | ab    | 8.4   | c     | 10.2  | a     | 9.6   | a     |
| WUEi      | 34.1  | abc   | 38.8  | a     | 32.8  | ab    | 34.9  | abc   | 37.8  | ab    |
| Chl       | 29.7  | b     | 31.2  | ab    | 28.9  | b     | 34.2  | ab    | 32.7  | ab    |
| TPC       | 331.5 | a     | 264.3 | b     | 252.0 | e     | 204.6 | d     | 229.7 | bc    |
| TFC       | 110.6 | a     | 90.6  | ab    | 69.4  | bc    | 66.6  | cd    | 87.3  | abc   |
| FRAP      | 34.0  | ab    | 36.0  | abc   | 31.5  | c     | 32.1  | c     | 47.1  | a     |
| BSA       | 69.0  | ab    | 81.5  | a     | 66.1  | ab    | 55.3  | b     | 77.0  | a     |
| RSC NO    | 74.7  | ab    | 74.3  | abc   | 66.9  | abc   | 65.5  | bc    | 72.1  | abc   |
| RSC DPPH  | 55.3  | bc    | 61.1  | ab    | 59.7  | ab    | 71.0  | a     | 65.8  | ab    |
| RSC ABTS  | 49.8  | ab    | 46.7  | ab    | 40.1  | ab    | 39.8  | b     | 45.2  | ab    |
| SD        | 297   | ab    | 360   | abc   | 367   | ab    | 276   | c     | 414   | a     |
| LS        | 23.5  | ab    | 23.3  | abc   | 22.9  | cd    | 24.3  | a     | 22.2  | d     |
| WS        | 16.8  | ab    | 17.0  | a     | 16.5  | ab    | 17.3  | a     | 16.1  | b     |

Table 2. Continued

| Parameter | B13   | B15   | B17   | F-test |
|-----------|-------|-------|-------|--------|
| LA        | 27.27 | a     | 28.12 | a     | 31.54 | a     | 0.44  |        |
| LMA       | 6.32  | cd    | 6.51  | abc   | 6.94  | a     | 3.20  | **     |
| Cᵢ        | 240.7 | c     | 259.2 | ab    | 272.5 | a     | 6.41  | **     |
| E         | 6.96  | a     | 5.93  | bc    | 5.84  | bc    | 5.16  | **     |
| gs        | 0.308 | ab    | 0.283 | bc    | 0.289 | abc   | 8.15  | **     |
| A         | 10.3  | a     | 10.0  | ab    | 8.8   | bc    | 3.54  | **     |
| WUEi      | 33.5  | bc    | 35.5  | abc   | 31.0  | c     | 2.15  | *      |
| Chl       | 31.4  | ab    | 32.6  | ab    | 31.6  | ab    | 1.34  |        |
| TPC       | 326.8 | a     | 215.9 | cd    | 325.4 | a     | 12.96 | **     |
| TFC       | 94.2  | ab    | 60.9  | d     | 92.5  | ab    | 3.45  | **     |
| FRAP      | 43.7  | ab    | 43.0  | ab    | 39.2  | abc   | 2.20  |        |
| BSA       | 82.3  | a     | 70.7  | ab    | 81.3  | a     | 2.59  | *      |
| RSC NO    | 75.3  | a     | 66.6  | abc   | 65.1  | c     | 1.85  |        |
| RSC DPPH  | 61.0  | ab    | 67.5  | a     | 46.1  | c     | 3.40  | **     |
| RSC ABTS  | 50.8  | a     | 42.3  | ab    | 39.8  | b     | 1.42  |        |
| SD        | 307   | cd    | 338   | bcd   | 330   | bcd   | 5.15  | **     |
| LS        | 23.0  | bc    | 23.3  | abcd  | 24.0  | ab    | 3.00  | *      |
| WS        | 16.5  | ab    | 17.1  | a     | 17.1  | a     | 1.91  |        |

Results of principal component analysis, the first five principal components (PCs), accounting for 95.7% of the total variance, were selected (Table 3). The first three principal components explained 71% of total variation among the studied half-sib lines; e.g. PC1 accounted for 20.2% of total variance,
Table 3. Eigenvalues and contribution to the total variability for the first five principal components (PCs), rotated by Varimax method and correlations between original variables and the first five PCs (loadings) for examined *Quercus robur* L. half-sib lines.

| Variable | PC2 | PC3 | PC1 | PC4 | PC5 |
|----------|-----|-----|-----|-----|-----|
| LA       | -0.069 | -0.009 | -0.002 | -0.160 | 0.979 |
| LMA      | 0.028 | 0.869 | 0.284 | -0.306 | 0.260 |
| C_i      | 0.081 | 0.925 | -0.061 | -0.069 | -0.044 |
| E        | 0.299 | -0.915 | -0.101 | -0.144 | 0.168 |
| WUEi     | -0.130 | -0.299 | 0.920 | 0.025 | 0.134 |
| Chl      | -0.237 | -0.025 | -0.202 | 0.910 | -0.033 |
| TPC      | 0.679 | 0.185 | -0.450 | -0.533 | 0.059 |
| TFC      | 0.844 | 0.130 | -0.105 | -0.370 | 0.089 |
| FRAP     | 0.477 | 0.556 | 0.173 | 0.591 | -0.220 |
| TSP      | 0.731 | 0.486 | 0.099 | 0.060 | 0.387 |
| RSC NO   | 0.916 | -0.219 | 0.267 | -0.042 | -0.129 |
| RSC DPPH | -0.296 | -0.475 | 0.399 | 0.629 | -0.356 |
| RSC ABTS | 0.923 | -0.214 | 0.082 | -0.034 | -0.252 |
| SD       | 0.004 | 0.536 | 0.825 | 0.024 | 0.172 |
| LS       | -0.349 | -0.317 | -0.818 | 0.015 | 0.309 |
| WS       | -0.396 | -0.333 | -0.641 | 0.172 | 0.414 |

Eigenvalue 4.167 3.951 3.234 2.176 1.789
% of total variance 0.260 0.247 0.202 0.136 0.112
Cumulative percentage 0.260 0.507 0.710 0.846 0.957

*) The highest loadings of particular original variable are underlined.

whereas PC2 and PC3 accounted for 26% and 24.7%, respectively. The rest of the components (PC4 and PC5) varied to a lesser extent (together: 23.8% of total variance). Similar contribution of the selected principal components to the total variance suggests similar contribution of analogue groups of characteristics to discrimination of the examined half-sib lines. The highest correlation with PC1 corresponded to *WUE* (0.920), and those variables related to leaf anatomical traits (i.e. stomatal size and density): *SD* (0.825), *LS* (-0.818) and *WS* (-0.641) (Table 3). Variables with the highest loadings with PC2 were mostly biochemical parameters: *RSC NO* (0.916), *RSC ABTS* (0.923), *TFC* (0.844), *TSP* (0.731) and *TPC* (0.679). A relatively high loading with PC2 was also determined for the *FRAP* (0.477). The variables with the highest scores on PC3 were the gas exchange parameters *C_i* (0.925) and *E* (0.915), as well as *LMA* (0.869). The second highest loading of *WUE* was with PC3 (0.299), therefore PC1 and PC3 were selected for the visual presentation of relationships of examined traits with *WUE* (Fig. 2). *WUE* and six other traits were the closest to the circle, meaning that most of their variation was explained by PC1 and PC3. Likewise, the angles between vectors of *WUE* and *LMA*, *C_i* and *E* were more orthogonal than the angles between *WUE* and *SD*, *LS* and *WS*, confirming the results of grouping based on loadings with principal components presented in Table 3.

Stepwise regression analysis was used to determine the traits contributing for the majority of total half-sib-lines-level *WUE* variability. The results revealed that almost the entire variation in leaf *WUE* was attributed to six variables (*SD*, *LMA*, *FRAP*, *Chl*, *WS* and *C_i*) (Table 4). Stepwise regression model with *SD* and *LMA* achieved high coefficient of determination (80.4%), which highlights the relevance of these traits in the explanation of total leaf *WUE* variation. Since these two traits belong to different PCA groups, it demonstrates the ability of this statistical method to select properties that best describe the dependent variable, with a minimum of collinearity between them.

According to path coefficient analysis performed on traits selected by stepwise regression analysis, standardized path coefficients for all selected traits were significantly different from zero. Thus, it could be considered that direct effect of every selected variable on *WUE* was statistically significant (Table 5). Among these traits, *SD* and *LMA* achieved the highest direct effects on *WUE*, but no significant indirect effect through any other selected trait. Other parameters, selected by stepwise regression analysis, had both significant direct and indirect effects.
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Discussion

Considering the negative effects of drought on *Q. robur* forests in Europe, as well as the projected scenarios concerning the impact of climate change on this species, it might be concluded that the plant’s economy of water consumption will be of primary importance for oak survival. For this reason, there is a widespread opinion that the adaptive capacity of trees toward drought should take priority over high timber quality in order to preserve the diversity of forest genetic resources (Kelleher *et al.*, 2015). Since water-use efficiency has been seen as the most important component of drought adaptation in plants (de Almeida Silva *et al.*, 2012), certain authors believe that breeding of plants characterized by high water-use efficiency is one of the key objectives of sustainable, climate-smart forestry (Flexas *et al.*, 2013). Indeed, the trees with higher water-use efficiency show a specific water-saving strategy that allows them to avoid large water losses and/or maintain higher assimilation rates than trees with lower water-use efficiency, which could have an impact on their competition for growth and survival, especially in drought prone environments (Mészáros *et al.*, 2007; Brendel *et al.*, 2008).

The presence of statistically significant differences between *Q. robur* half-sib lines in terms of majority of examined leaf functional traits is presumably the result of their divergent genetic make-up. In general, leaf physiological traits showed the highest variability between half-sib lines, with only *Chl* showing no statistical differences between them. A possible explanation of this phenomenon might be a low heritability of leaf chlorophyll content, as already documented by Roussel *et al.* (2009) for *Q. robur* genotypes.

The results further showed that although the majority of examined characteristics did not achieve important direct effect on *WUE*$_i$, the valuable information of the relationship between morpho-anatomical traits and *WUE*$_i$ was observed by PCA. We evidenced that all of the examined leaf stomatal traits were in the same PCA group (PC3) with *WUE*$_i$, suggesting that these characteristics can be good indicators to be used in the selection for water-use efficient *Q. robur* genotypes. Moreover, according to stepwise regression analysis and path coefficient analysis, stomatal density achieved the strongest direct effect on variation of *WUE*$_i$. Stomata size and density have a key role in the regulation of gas exchange between the leaves and the atmosphere. Controlling water flux inside plants, stomata also modulate a balance between water loss and CO$_2$ uptake.

### Table 4. Results of forward stepwise regression analysis for *WUE*$_i$ as dependent variable in examined *Quercus robur* L. half-sib lines

| Step | Included independent variable | Change of coefficient of determination ($R^2$) | F-value | p-level (to include) |
|------|-------------------------------|-----------------------------------------------|--------|---------------------|
| 1    | SD*                          | 0.388                                          | 3.798  | 0.099               |
| 2    | LMA                          | 0.416                                          | 10.586 | 0.023               |
| 3    | FRAP                         | 0.140                                          | 9.884  | 0.035               |
| 4    | Chl                          | 0.027                                          | 2.739  | 0.196               |
| 5    | WS                           | 0.028                                          | 41.116 | 0.023               |
| 6    | *C*$_i$                      | 0.001                                          | 292.605| 0.037               |

*Independent variables: SD - stomatal density, LMA - leaf dry mass per unit of leaf area, FRAP - ferric reducing antioxidant power, Chl - chlorophyll content, WS - width of stomata guard cell, *C*$_i$ - intercellular CO$_2$ concentration.
thus determining water-use efficiency (Holland & Richardson, 2009). Previous studies have demonstrated that stomatal conductance largely depends on stomatal aperture characteristics, which in turn determine maximum theoretical conductance, as well as the speed of stomata response to environmental signals (Dow et al., 2014). Aasamaa et al. (2001) believe that length of the stomatal pore plays the most important role in the determination of stomatal conductance in trees grown under different water supplies. Similar results (i.e. stomatal length was positively correlated with $g_s$) were also reported by Ohsumi et al. (2007). These results have been confirmed by our study as well, since a positive correlation between $g_s$ and $LS$ (data not shown) was observed. However, guard cell size also affects the speed of stomata response to environmental factors. Hetherington & Woodward (2003) stated that larger stomata often exhibit slower responses in comparison to the smaller ones, showing a greater potential for hydraulic dysfunction under water stress. Likewise, plants characterized with higher $g_s$ generally exhibit lower water-use efficiency (Lawson & Blatt, 2014). In the present study we observed a negative correlation between $WUE_i$ and $LS$, and positive between $WUE_i$ and $SD$, suggesting that selection for water-use efficient half-sib lines should be oriented toward those with more numerous and smaller sized stomata. Such approach would be in accordance with the results of Aasamaa et al. (2001), who demonstrated a clear negative correlation pattern between the length of the stomatal pore and sensitivity to increasing drought in six forest tree species.

Although stomatal size and density define stomatal conductance, stomatal density is negatively correlated with stomatal length, in general (Pearce et al., 2006; Pyakurel & Wang, 2014), that has also been confirmed by our research. Concerning relationship between $SD$ and $g_s$, the opposite results have been reported. While Pearce et al., (2006) reported that stomatal density is positively correlated with $g_s$ in riparian poplar species, a number of experiments showed that the relationship between stomatal density and stomatal conductance does not seem to be straightforward (Jones, 1997; Ilgin & Caglar, 2009; Lawson & Blatt, 2014). Likewise, in contrast to stomata length, recent studies have demonstrated high sensitivity of stomatal density to different climate and environmental conditions (Stojnić et al., 2015a,b). According to Aasamaa et al., (2001) smaller stomata respond faster to environmental signals than larger stomata, so the combination of smaller stomata and high stomatal density might provide greater plant capacity to rapidly adjust stomatal conductance and optimize gas exchange.

Besides the notable influence of $SD$ on $WUE_i$, the second strongest direct effect on the variation of $WUE_i$ was achieved by $LMA$, as demonstrated by the path coefficient analysis. In contrast, $LA$ was grouped in

| Variables through which the indirect effect was achieved | SD | LMA | FRAP | Chl | WS | Ci |
|---------------------------------------------------------|----|-----|------|-----|----|----|
| SD                                                      -  | 1.273 | 0.711* | -0.262 | -1.115* | 0.762* |
| LMA                                                    -0.941 | -    | -0.405* | 0.491* | 0.545* | -1.004* |
| FRAP                                                   -0.074 | -0.057 | -    | -0.061 | 0.082 | -0.084* |
| Chl                                                    0.058 | 0.147 | -0.130 | -    | -0.117 | 0.030 |
| WS                                                     -0.186 | -0.123 | -0.132 | 0.088 | -  | -0.074 |
| Ci                                                     0.034 | 0.061 | 0.036 | -0.006 | -0.020 | -  |
PC5 group, apart from others, indicating poor effect on WUE, variability. Recent studies have evidenced that LMA is linked with WUE, mainly through the tight correlation with both A and g s (Hassiotou et al., 2010). Indeed, structural leaf changes related to variation in LMA usually correspond to alterations in photosynthetic capacity, thereby affecting WUE. High LMA is related to the formation of thicker leaves and dense mesophyll tissues as the consequence of large number of tightly packed cells with more lignified cell walls (Niinemets, 2001). Such leaf structure implies changes in maximum photosynthetic rate as the result of longer paths for water diffusion from stomata to chloroplast, as well as higher chloroplast shading in the interior of the leaf (Novriyanti et al., 2012; Read et al., 2014). Due to increased water diffusion resistance in mesophyll as the consequence of water path through the intercellular spaces to stomata, high LMA is frequently associated with reduced CO2 diffusion, and therefore, a lower g s (Muir et al., 2017). Nevertheless, despite low stomatal conductance values, a high LMA might positively affect both water-use efficiency and photosynthetic capacity in plants growing under stress conditions, due to the increased number of chloroplasts in the mesophyll tissues (Bresson et al., 2011; Stojnić et al., 2015b). In addition, compact mesophyll tissue (i.e. high LMA) is also shown to cause greater leaf resistance to the water diffusion in the process of transpiration (Novriyanti et al., 2012). This study also confirms these results, since we found that LMA and E were highly negatively correlated, having their highest loadings on PC3 (0.869 and -0.915, respectively).

Since most of the studies were focused to explain the effects of drought on either metabolic and transcriptomic profiles or specific enzyme activities in different species (Mittler et al., 2006), this paper kept track on the relationship between WUE, and radical scavenger activity assessed by the biochemical assays. Recently, several studies have evidenced that biochemical biomarkers might be efficiently used as the indicators of environmental stress effects on the physiology of seedlings (Popović M et al., 2016; Schiop et al., 2015). Likewise, Popović B et al. (2016) reported increased biosynthesis of specific phenolic compounds, such as myricetin, chrysin, kaempferol and isoflavanolic acid etc., as well as increased activity of biosynthetic enzyme (PAL-phenylalanin ammonium lyase) under induced drought stress in poplar clones. Finally, Štajner et al. (2013) demonstrated increased percentage of radical scavenger activity against DPPH radical and increased FRAP value under drought conditions during summer months in beech species, as well as in different melliferous tree species, such as Fraxinus sp. and Robinia pseudoacacia (Štajner et al., 2011). However, our results evidenced that the examined biochemical characteristics, presumably related to tolerance of oxidative stress, did not have considerable significance in conditions of moderate drought. Indeed, only FRAP was selected by stepwise regression analysis and, according to the results of path coefficient analysis, showed significant effect on the WUE, both directly and indirectly through SD and LMA.

Several studies have evidenced that under moderate drought conditions carbon assimilation in plants decline mostly due to stomatal closure (Flexas & Medrano 2002; Stojnić et al., 2016). In contrast, under conditions of low water availability photosynthetic rate is also co-limited by non-stomatal features including electron transport rate and photochemistry (Petridis et al., 2012). Severe drought may lead to imbalance between antioxidant defenses and the increased level of reactive oxygen species (ROS), therefore causing an oxidative stress in plants (Štajner et al., 2011). Namely, the decline in the internal CO2 (C) caused by ABA-induced stomatal closure during severe drought, and therefore decreasing CO2 availability for photosynthesis (Chaves et al., 2003; Sánchez-Rodriguez et al., 2010), prevent regeneration of NADP+ (i.e. main electron acceptor) by the Calvin cycle leading to enhanced electron leakage to molecular oxygen in chloroplasts, which, as a consequence, has an increased generation of different ROS (Nahar et al. 2018). Under stress conditions, the absorbed light energy may not be entirely used for photosynthesis, therefore causing its repression - photoinhibition (Petridis et al., 2012). To avoid such damage, plants tend to increase their antioxidant capacity by producing increased amounts of various compounds with antioxidant properties in order to suppress oxidative stress. Increased antioxidant and reducing capacity (FRAP value) of extracts in sense of increased concentration of compounds with high antioxidant properties could have preventing and mitigating effects towards these oxidative injuries of PSII.

On the other hand, grouping of the examined traits by principal component analysis suggested that none of the examined biochemical characteristics, as well as chlorophyll content were grouped by PC1 or PC3, which had the highest loadings with WUE. Most of them were allocated in PC2 group, while FRAP and RSC DPPH were together with Chl in the PC4 group. Similar results have been reported by Ghasemzadeh & Jaafar (2011), who observed lack of correlation between water-use efficiency, on one side, and total phenolics and flavonoids, on the other. It seems that drought conditions at the timepoint of sampling were still mild enough to trigger oxidative stress and cause major metabolic disturbances, which could affect antioxidant properties of the whole leaf extract, so these correlations were neglectable.
Conclusion

The results of our study demonstrated that under moderate water stress $WUE_i$ was mainly the result of the plants’ structural acclimation to surrounding environmental conditions. We found that in such conditions stomatal density and leaf dry mass per unit of leaf area have achieved significant and dominant direct effect on $WUE_i$ in Quercus robur half-sib lines. Stomatal density achieved the highest score on PC1, in which $WUE_i$ had the highest loading. Likewise, $SD$ was the first to be included in the stepwise regression model, having the highest direct effect on $WUE_i$, and, therefore, indicating that stomatal regulation could be considered a dominant contributor to the regulation of $WUE_i$, in the conditions of moderate drought. In addition, significant direct effect of $LMA$ on $WUE_i$ suggests that leaf structure strongly affected the water diffusion inside mesophyll tissues and related physiological processes of leaf gas exchange.

On the other hand, none of the examined biochemical traits were grouped by any of the principal components that had the highest loadings with $WUE_i$, suggesting their poor effect on $WUE_i$ in conditions of moderate drought. Only FRAP was selected by stepwise regression analysis, achieving poor direct effect on variation of $WUE_i$.

Taking into account the foregoing discussion, we believe that selection based on identification and quantification of the genetic correlations between $WUE_i$ and structural leaf traits should have a vital place in future breeding programmes oriented towards improvement of $WUE_i$, especially in the conditions of moderate water deficit. Therefore, high intrinsic water-use efficiency of Quercus robur species can possibly be achieved by indirect selection of breeding material via $SD$ and $LMA$, the traits that have the highest direct effect on $WUE_i$. Similar approaches have already been recommended by other authors for certain crop species (Hui et al., 2008; Golparvar & Karimi, 2012; Wu & Bao, 2012).

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