RESEARCH ARTICLE

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_i) 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_i, 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 *Q. robur*. Eighteen leaf parameters were analyzed; i.e. physiological, biochemical, morphological and anatomical. The data were processed using multivariate statistical methods: a) principal component analysis, b) stepwise regression analysis, and c) path coefficient analysis.

Main results: 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_i. Stomatal density achieved the highest score on PC1 (0.825), in which WUE_i had the highest loading (0.920), as well. *SD* was also included first in stepwise regression model.

Research highlights: These results demonstrate that under moderate water stress WUE_i 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 (Drobyshev *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 remarkedly 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 (Verryn, 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$_i$*), 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 CO_2 assimilation rate is more evident than the reduction of stomatal conductance, leading to an increase in *WUE$_i$* (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$_i$* 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$_i$* and other traits is rather complex and requires a deeper analysis in order to identify those which determine *WUE$_i$* to the largest extent. For example, several studies have demonstrated a coupled response of net photosynthesis and stomatal conductance of CO_2 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$_i$* due to an aforementioned close correlation between net CO_2 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$_i$*, 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$_i$*. 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$_i$*, 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 (g_s), intercellular CO_2 concentration (C_i) 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 CO_2 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_i) was calculated as the ratio of net assimilation rate to stomatal conductance ($A\,g_s^{-1}$) (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):

$$Chl = (99 \times SPAD\ value)(144 - SPAD\ value)^{-1} \quad (1)$$

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 2 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).
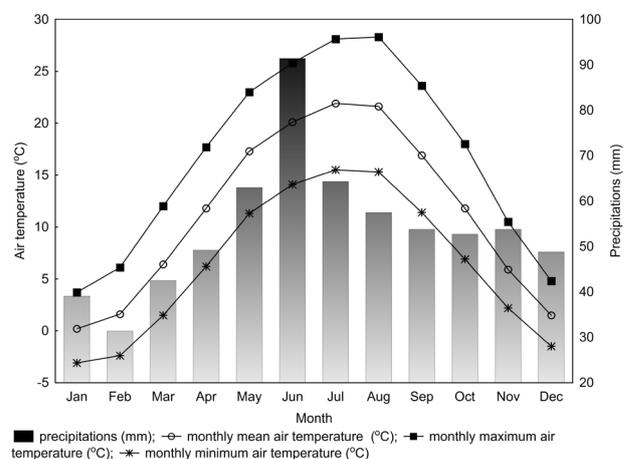
Figure 1. Climate diagram for the weather station ,Rimski Šančevi" (norm period: 1981–2010).

Table 1. List of study parameters

Parameter	Acronym	Unit
Physiological		
Intercellular CO_2 concentration	Ci	$\mu mol \ mol^{-1}$
Transpiration	E	$mmol \ m^{-2} \ s^{-1}$
Stomatal conductance	g_s	$mol \ m^{-2} \ s^{-1}$
Net photosynthesis	A	$\mu mol \ m^{-2} \ s^{-1}$
Intrinsic water-use efficiency	WUE_i	$\mu mol \ mol^{-1}$
Chlorophyll content	Chl	$\mu g \ cm^{-2}$
Biochemical		
Total phenolic content	TPC	$mg \ GA \ g^{-1}$
Total flavonoid content	TFC	$mg \ QE \ g^{-1}$
Ferric reducing antioxidant power	FRAP	$\mu mol \ TE/g$
Total soluble protein content	TSP	$mg \ BSA \ g^{-1}$
Radical scavenger capacity against $ABTS^{\bullet+}$	RSC ABTS	%
Radical scavenger capacity against NO radical	RSC NO	%
Radical scavenger capacity against DPPH free radical	RSC DPPH	%
Anatomical and morphological		
Stomatal density	SD	number per mm^2
Length of stomata guard cell	LS	μm
Width of stomata guard cell	WS	μm
Leaf area	LA	cm^2
Leaf dry mass per unit of leaf area	LMA	$mg \ cm^2$

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:

$$RSC = ((A_{control} - A_{sample}) / A_{control}) \times 100 \quad (2)$$

where: $A_{control}$ is the absorbance of the DPPH reagent without extract and A_{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_3PO_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 $A_{control}$ was the absorbance of probes without the extract and A_{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 NaCH_3COO and 6 µl 0.75 M $AlCl_3$ and 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

with 125 μl of four-time diluted Folin–Ciocalteu reagent (FCR) in water and 100 μl 7.5 % Na_2CO_3. 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 $(Fe^{2+}-(TPTZ)_2)^{2-}$ 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 $FeCl_3$ 6 H_2O 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 mm^2 (*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*:

$$LMA = DM\ LA^{-1} \qquad (3)$$

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, cha-racteristics with the highest correlation (loading) wit-hin 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 prinipal components. Considering multi-colinearity 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_i variability. The variables were included in the model within stepwise procedure in such an order to achieve the highest possible increament of coefficient of determination (Wonnacott & Wonnacott, 1981). Traits *A* and g_s were excluded from these analyses since the WUE_i 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 WUE_i.

Results

We found significant difference in WUE_i between *Q. robur* half-sib lines, although the results evidenced a narrow range of WUE_i variation (7.8 μmol mol^{-1}). The highest mean value of WUE_i 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. The parameter acronyms are defined in Material and Methods

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	bcd	6.50	abc	6.72	abc	5.97	d	6.83	ab
C_i	260.1	ab	242.3	c	244.5	bc	233.1	c	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	ab
WUEi	34.1	abc	38.8	a	38.2	ab	34.9	abc	37.8	ab
Chl	29.7	b	31.2	ab	28.9	b	34.2	a	32.7	ab
TPC	331.5	a	264.3	b	252.0	bc	204.6	d	229.7	bcd
TFC	110.6	a	90.6	ab	69.4	bcd	66.6	cd	87.3	abc
FRAP	34.0	bc	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	de	360	abc	367	ab	276	e	414	a
LS	23.5	abc	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	**
C_i	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	cde	338	bcd	330	bcde	5.15	**
LS	23.0	bcd	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 exmined half-sib lines. The highest correlation with PC1 corresponded to WUE_i (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_i was with PC3 (0.299), therefore PC1 and PC3 were selected for the visual presentation of relationships of examined traits with WUE_i (Fig. 2). WUE_i 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_i and *LMA*, C_i and *E* were more orthogonal than the angles between WUE_i 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_i variability. The results revealed that almost the entire variation in leaf WUE_i 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_i 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, stan-dardized 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_i was statistically significant (Table 5). Among these traits, *SD* and *LMA* achieved the highest direct effects on WUE_i, but no significant indirect effect through any other selected trait. Other parameters, selected by stepwise regression analysis, had both significant direct and indirect effects
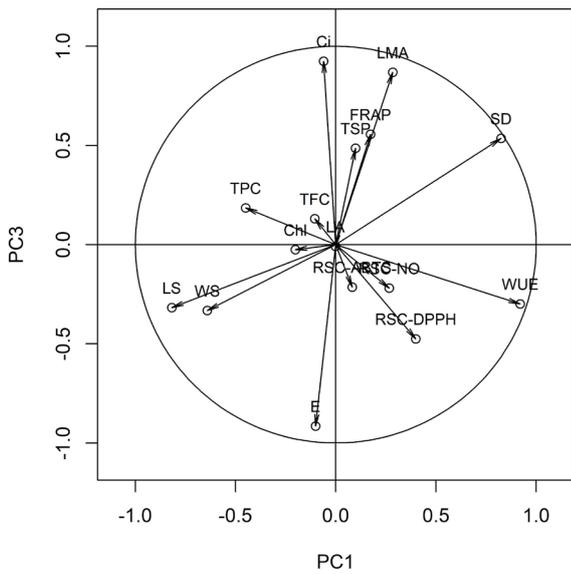
Figure 2. Loadings of original characteristics with the first and third principal components gained after Varimax rotation of the first five principal components.

through *SD* and *LMA*. The complex linkage on dominant direct and indirect effects of the examined traits on leaf-level WUE_i of *Q. robur* half-sib lines is given in the Table 6.

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 independe 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.

Table 5. Results for standardized path coefficients for WUE_i as dependent variable in examined *Quercus robur* L. half-sib lines

	Standardized path coefficient (p)	Standard error for p (SEp)	F-test	
SD	1.731	0.003	269881.137	**
LMA	-1.280	0.005	76681.902	**
FRAP	-0.180	0.002	5574.180	**
Chl	-0.382	0.003	22934.096	**
WS	0.288	0.003	12454.925	**
C_i	0.078	0.003	585.209	*

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

Table 6. Indirect effects of the examined traits on leaf-level WUE_i of *Quercus robur L.* half-sib lines

Variables through which the indirect effect was achieved	Indirect effect of selected variables					
	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
C_i	0.034	0.061	0.036	-0.006	-0.020	-

PC5 group, apart from others, indicating poor effect on WUE_i variability. Recent studies have evidenced that LMA is linked with WUE_i 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_i. 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 enhanced water path through the intercellular spaces to stomata, high LMA is frequently associated with reduced CO_2 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_i 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 isoferulic 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_i, 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 CO_2 (C_i) caused by ABA-induced stomatal closure during severe drought, and therefore decreasing CO_2 availability for photosynthesis (Chaves *et al.,* 2003; Sánchez-Rodríguez *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_i. 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 *Q. 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 as 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 *Q. 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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