

Photosynthetic insights into winter-green leaves in *Quercus pubescens* Willd. seedlings

 Martin Bobinac¹,  Nikola Šušić^{2,*},  Mirjana Šijačić-Nikolić¹,  Ivona Kerkez Janković¹ and  Sonja Veljović-Jovanović²

¹University of Belgrade, Faculty of Forestry, Department of Forestry and Nature Protection, Kneza Višeslava 1, 11030 Belgrade, Serbia

²University of Belgrade, Institute for Multidisciplinary Research, Department of Life Sciences, Kneza Višeslava 1, 11030 Belgrade, Serbia

*Corresponding author: nikola.susic@imsi.bg.ac.rs

Received: April 17, 2024; Revised: May 22, 2024; Accepted: May 24, 2024; Published online: June 13, 2024

Abstract: *Quercus pubescens* Willd. is a deciduous species that can retain leaves in the winter either as dead, standing leaves (marcescence), or as living, winter-green leaves. The retention of green leaves through winter is rare in continental areas. Winter-green leaves were observed on one-year-old seedlings of *Q. pubescens* in the winter of 2020 in the nursery of the Faculty of Forestry that lasted until the end of April 2021. The photosynthetic activity of photosystem II was measured using the rapid light curve method based on modulated pulse chlorophyll fluorescence. We assessed the potential photosynthetic activity of the leaves across the range of physiological stages: winter-green leaves retained from the previous growing season and on the leaves from the first, second, and third growth flush during the growing season. Photosystem II of winter-green leaves attained ~50-60% of the maximal photosynthetic activity obtained in spring and summer leaves, respectively. Climate data indicated that winter-green leaves exhibited frost resistance in the winter of 2020/2021, as their photosynthetic activity was preserved despite 33 days with sub-zero temperatures. The rapid light curve method also revealed the gradual acclimation of seedlings on a flush level, with leaves from the third flush best able to use available light under high temperatures and insolation efficiently. The results of rapid light use as an indicator of seedling acclimation are discussed. Some remarks on the possible practical significance of the winter-green leaves phenomenon (as in genetic selection) are highlighted.

Keywords: frost and light acclimation; leaf senescence; *Quercus pubescens* Willd. — downy oak; PSII activity; winter-green leaves

INTRODUCTION

In temperate deciduous species, leaves are formed at the beginning of the growing season and usually shed before the winter (and sometimes during the growing season). The shoot elongation and leaf emergence occur in determinate (such as the case of flush growth) or indeterminate (succeeding) modes of shoot growth, as reviewed by Kikuzawa and Lechowicz [1]. Leaf senescence occurs in the fall when day length and temperature decrease [2]. Once the nutrients are salvaged and translocated from the senescing leaves, the leaves die and decay [3] and are removed from the stem by natural abscission [2]. However, senescence may fail (“aborted senescence”), leaving withered, dead leaves throughout the winter in a leaf pattern known as marcescence [4]. The timing of leaf senescence depends on physiological,

genetic, and environmental factors [3] that influence the longevity of the leaves throughout seasons. In some cases, such as in certain *Populus* clones [5,6], *Rhamnus cathartica* L. [7], or *Alnus cordata* (Loisel.) Duby [8], the trees can retain green leaves until late fall. Less commonly, the leaves of temperate deciduous trees are retained throughout the winter and remain green and physiologically active until the beginning of the next growing season. This has been reported in *Q. lobata* Née [9,10] and blackberry (*Rubus fruticosus* s.l.) [11]. Winter-green leaves can also be found in mutants of different species [3].

Changing environmental conditions may extend the growing seasons of *Q. pubescens* [12]. Also, aborted senescence, marcescent leaf habit [4,13], and retention of green leaves may occur if the winters are mild

[14]. This phenomenon is primarily reported for *Q. pubescens* on warm sites in the Mediterranean region [15,16]. Multiple leaf flushing adds to the phenological plasticity of oaks. Shoot elongation and leaf expansion occur rapidly during each flush growth, alternating with bud development to form a rhythmic pattern [17]. The number of flushes varies, but oaks may exhibit several growth flushes in a growing season [18,19]. Observations of flush-level acclimation offer more detailed insights into morphological and physiological responses in oak seedlings, enhancing our understanding of their phenotypical plasticity [20,21]. Leaf chemistry can also vary considerably within different flushes [22]. As the total growth of oak seedlings is directly related to multi-flush growth, the trait is helpful in designing regeneration systems tailored to incentivize optimal offspring growth [18,23]. Multi-flush growth is also important in morphological studies for species identification [24] or inflorescence morphology on adult trees [25]. A recent study [26] revealed that multi-flush growth has a role in defense priming against herbivores by conveying the priming memory from older to most recent flushes. In this context, we chose the flush-level approach in our study, as it is recommended for morphological and physiological comparisons using samples from different flushes [21,27]. The study aimed to compare the photosynthetic capacity of winter-green leaves with that of spring and summer leaves from flushes developed in the following growing season. This was done to assess the acclimation of the photosynthetic capacity of oak seedlings across different growth flushes throughout the growing seasons. The working hypothesis was that the photosynthetic capacity of winter-green leaves is impaired compared to spring and summer leaves. The rapid light curve (RLC) method was used, as it is widely applied in greenhouse and field conditions to assess the photosynthetic activity of plants [e.g. 28-30]. In varying light environments, employing RLC offers a rapid assessment of photosynthetic capacity. This assessment considers the leaf's capacity to withstand short-term light variations and reflects its immediate light exposure history [31-33]. Thus, it is advantageous in fieldwork with light-adapted plants.

MATERIALS AND METHODS

Site conditions

The research was conducted on downy oak (*Quercus pubescens* sensu lato) seedlings with winter-green leaves retained from the 2020 growing season in the nursery of the Faculty of Forestry of the University of Belgrade (44°46'57.2"N, 20°25'31.6"E) in open air conditions. Winter-green leaves were also observed in 2022 and 2023. The soil in the nursery is loam to clay loam with a pH in KCl between 7.14 and 7.24. The basic climate parameters for 2020, 2021, and 2022 were calculated using data from the Republic Hydrometeorological Institute of Serbia for Belgrade meteorological station and compared to the 1991-2020 averages [34]. The mean temperature in 2020, 2021, and 2022 was 13.7-14.5°C and higher by 4-10% than that of the 1991-2020 average. Precipitation was 654.3, 795.3, and 639.7 mm in 2020, 2021, and 2022, respectively. Total precipitation in 2020 and 2022 was lower by 6-8% compared to the 1991-2020 average, while in 2021, it was 14% higher. Total insolation in the growing seasons of 2020, 2021, and 2022 was higher by 12.1-16.1% compared to the 1991-2020 average (Supplementary Fig. S1). The mean daily relative humidity (%) during measurement campaigns was 77% (20/04/2021), 62% (28/05/2021), and 51-55% (14 and 15/09/2021).

Plant material

Seedlings were selected from the generative progeny test established in October 2019 from 20 maternal trees originating from the Košutnjak Forest. Acorns were collected from the local population of the zonal association *Quercetum cerridis-virgilianae* Jov. et. Vuk. 77 [35]. Watering and weeding were carried out periodically. One-year-old seedlings with winter-green leaves originated from one half-sib line (mother tree coordinates: 44°46'31.6"N 20°25'28.0"E). The phenomenon was also observed in the offspring of other half-sib lines (Supplementary Fig. S2); however, winter-green leaves were not recorded in all the seedlings within a single family (Supplementary Fig. S3). We used seven normally developed seedlings with fully formed and undamaged winter-green leaves. Measurements were taken for each plant using three fully developed leaves located near the terminal bud during the following physiological

stages. (Supplementary Fig. S4): winter-green leaves on 20/04/2021; first growth flush on 28/05/2021 and second and third growth flush on 14/09 and 15/09/2021. Thus, the total sample consisted of 21 leaves for each leaf set. The third flush was an exception, with six out of seven experimental plants displaying growth during this phase, resulting in a sample of 18 leaves (81 leaves analyzed in total). One limitation is that we could not determine whether the winter-green leaves originated from the first flush or from subsequent flushes during the 2020 growing season.

Chlorophyll fluorescence measurements

The photosynthetic activity of photosystem II (PSII) was calculated using the rapid light curves measurements of the modulate pulse chlorophyll fluorescence with a portable chlorophyll fluorimeter Junior PAM (Gademann Instruments GmbH, Würzburg, Germany).

The photochemical activity of PSII was calculated using WinControl software (v3.29; Heinz Walz GmbH, Effeltrich, Germany) and the photochemical quantum yield of PSII ($Y(II)$). For the RLCs, 12 PAR (actinic) intensities were used, namely 25, 45, 65, 90, 125, 190, 285, 420, 625, 820, 1150, and 1500 $\mu\text{mol cm}^{-2} \text{s}^{-1}$. At each PAR intensity, a saturation pulse of 10,000 $\mu\text{mol cm}^{-2} \text{s}^{-1}$ lasting 0.8 seconds was applied. The light duration of each actinic light was 10 s, thus providing information on the photochemical activity of photosystem II. At each actinic level, quantum yield [$Y(II)$] and electron transport rate (ETR) were calculated. As the leaves were light-adapted, the quantum yield was calculated in WinControl software according to Genty et al. [36]:

$$Y(II) = (F_M' - F')/F_M' \quad (1)$$

The relative electron transport rate (ETR) was calculated as follows:

$$\text{ETR} = \text{PAR} \times \text{ETR-Factor} \times P_{\text{PS2}}/P_{\text{PS1}} \times Y(II) \quad (2)$$

According to the equipment manual, the WinControl-3 software default value for the ETR-Factor is 0.84, while the PPS2/PPS1 (photons absorbed by PS II relative to photons absorbed by photosynthetic pigments) default is 0.5.

Statistical analysis

To test for differences in the mean values of photosynthetic activity in winter-green leaves of *Q. pubescens* seedlings, we used nonparametric statistics (Kruskal-Wallis test) after verifying that the assumptions for parametric tests (normal distributions and equal variances) were not met. Statistica 6.0 computer software (Stat Soft, Inc.), Statgraphic Plus 5.0 (Statistical Graphics Corporation, USA), and Adinsoft XLSTAT 2017 software (free trial version) were used for statistical analysis.

RESULTS

Despite 33 frost days recorded during the winter of 2020/2021 (Supplementary Figure S1), the winter-green leaves maintained their green leaf area, showing early signs of senescence and/or frost damage on only a few leaves. (Fig. 1). Winter-green leaves exhibited 63, 61, and 47% of the maximal photosynthetic activity (ETR) of leaves measured on the first, second, and third growth flush in the 2021 growing season, respectively. Quantum yield values indicated that winter-green leaves utilized 30-40% of the absorbed photons for a photochemical reaction in the low light range (25-420 $\mu\text{mol cm}^{-2} \text{s}^{-1}$). Spring and summer leaf flushes behaved similarly in this range, with a quantum yield between 36 and 57%. As PAR levels increased, quantum yield decreased in all leaf sets, being lowest in winter-green leaves (0.12) and highest in third flush leaves (0.26) (Fig. 2). Quantum efficiency of PSII expressed as the initial slope of the RLC (Fig.

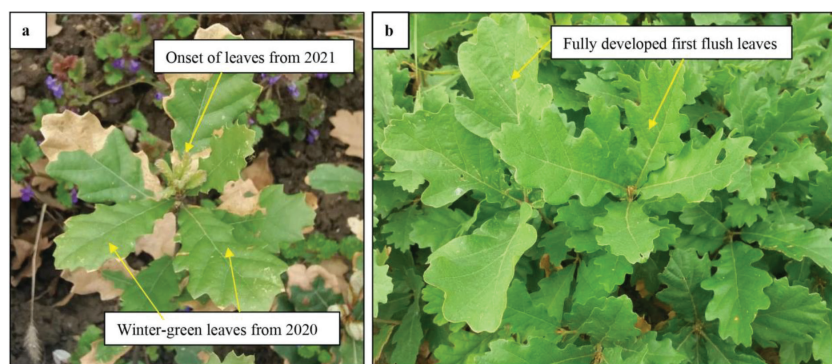


Fig. 1. a – Winter-green leaves from the 2020 growing season and the onset of leaves from the 2021 growing season on 1-year-old downy oak (*Quercus pubescens* Willd.) seedling on 20/04/2021; **b** – first flush leaves (28/05/2021).

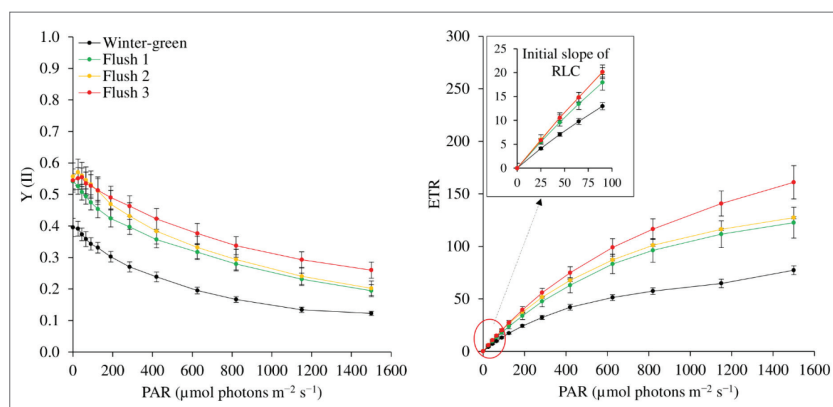


Fig. 2. Rapid light curves at four different physiological stages of seedling development: winter-green leaves retained from winter 2020, measured in April 2021; leaves from the first spring flush (Flush 1) and leaves from the second and third flushes developed during summer 2021 (Flush 2 and Flush 3). The results represent average values (\pm standard error of the mean) of quantum yield of Photosystem II (Y) and electron transport rate (ETR) based on 21 leaves from 7 plants for each of the physiological stages except for Flush 3 where 18 leaves from 6 plants were used.

2) showed a decrease in winter-green leaves by around 25% compared to other leaf flushes.

The differences for Y(II) and ETR between the winter-green leaves and the leaves of the first, second, and third growth flushes were statistically significant at all levels of PAR (Tables 1 and 2). Therefore, the working hypothesis can be accepted, i.e., that the photosynthetic capacity of the winter-green leaves was impaired compared to that of the spring and summer leaves. At most PAR levels, there were no significant differences in leaf photosynthetic activity between the different growth

Table 1. Statistical testing of photochemical quantum yield of Photosystem II between different leaf flushes based on the Kruskal-Wallis test

Stages PAR $\mu\text{mol cm}^{-2} \text{s}^{-1}$	WG	F1	F2	F3	Kruskal Wallis test $df = 3$	Significance
	n = 21	n = 21	n = 21	n = 18		
0	^A 0.40 (0.11)	0.54 (0.11)	0.56 (0.15)	0.55 (0.13)	^C $K = 20.18$	P<0.05
	^B 0.40 [0.17]a	0.56 [0.17]b	0.63 [0.20]b	0.60 [0.11]b		
25	0.39 (0.09)	0.53 (0.11)	0.57 (0.12)	0.55 (0.10)	$K = 23.85$	P<0.05
	0.38 [0.09]a	0.56 [0.14]b	0.62 [0.12]b	0.60 [0.10]b		
45	0.37 (0.08)	0.51 (0.12)	0.56 (0.12)	0.56 (0.11)	$K = 27.02$	P<0.05
	0.37 [0.10]a	0.57 [0.14]b	0.61 [0.12]b	0.60 [0.06]b		
65	0.36 (0.08)	0.49 (0.12)	0.54 (0.12)	0.54 (0.13)	$K = 24.31$	P<0.05
	0.35 [0.12]a	0.54 [0.23]b	0.60 [0.13]b	0.59 [0.06]b		
90	0.34 (0.07)	0.47 (0.12)	0.53 (0.13)	0.53 (0.13)	$K = 24.77$	P<0.05
	0.33 [0.09]a	0.51 [0.21]b	0.59 [0.11]b	0.58 [0.05]b		
125	0.33 (0.06)	0.45 (0.13)	0.51 (0.13)	0.52 (0.13)	$K = 22.34$	P<0.05
	0.33 [0.10]a	0.50 [0.23]b	0.56 [0.13]b	0.57 [0.05]b		
190	0.30 (0.06)	0.42 (0.13)	0.47 (0.14)	0.50 (0.13)	$K = 22.48$	P<0.05
	0.30 [0.09]a	0.46 [0.22]b	0.52 [0.15]b	0.55 [0.06]b		
285	0.27 (0.06)	0.40 (0.11)	0.43 (0.13)	0.47 (0.11)	$K = 26.99$	P <0.05
	0.27 [0.09]a	0.43 [0.18]b	0.51 [0.06]bc	0.51 [0.06]c		
420	0.24 (0.05)	0.36 (0.11)	0.38 (0.13)	0.43 (0.11)	$K = 24.54$	P<0.05
	0.24 [0.09]a	0.38 [0.15]b	0.41 [0.19]b	0.46 [0.07]b		
625	0.19 (0.05)	0.32 (0.09)	0.33 (0.12)	0.38 (0.11)	$K = 28.21$	P<0.05
	0.19 [0.06]a	0.32 [0.14]b	0.34 [0.18]b	0.42 [0.08]b		
820	0.17 (0.03)	0.28 (0.08)	0.29 (0.11)	0.34 (0.10)	$K = 29.38$	P<0.05
	0.17 [0.04]a	0.28 [0.11]b	0.30 [0.16]b	0.36 [0.09]b		
1150	0.13 (0.03)	0.23 (0.07)	0.24 (0.09)	0.30 (0.09)	$K = 32.84$	P<0.05
	0.13 [0.04]a	0.23 [0.10]b	0.24 [0.13]bc	0.31 [0.10]c		
1500	0.12 (0.03)	0.19 (0.07)	0.20 (0.08)	0.26 (0.09)	$K = 27.84$	P<0.05
	0.12 [0.03]a	0.20 [0.06]b	0.20 [0.11]b	0.27 [0.11]b		

WG – winter-green leaves; F1, F2 and F3 – leaves from the first, second and third growth flush (a spurt of growth) developed during the growing season; A, mean values \pm SD; B, median values [interquartile range]; C, K values, Kruskal-Wallis test was applied to examine differences among medians; df = degree of freedom. Medians in a row without a common superscript letter differ, as analyzed by Steel-Dwass-Critchlow-Fligner procedure after Kruskal-Wallis test (P<0.05); n, sample size (number of leaves).

flushes in the 2021 growing season, although the third flush leaves tended to perform a higher photochemical activity of PSII at higher PAR levels (Fig. 2). However, statistical analysis suggests that only at PAR levels 285 and 1150 were there significant differences of Y and ETR between the third and the first growth flush (Tables 1 and 2).

DISCUSSION

The leaves of *Q. pubescens* from the 2020 growing season exhibited prolonged longevity, maintaining photosynthetic activity throughout the winter until the onset of the next growing season. The photosynthetic capacity of winter-green leaves was reduced compared to leaves that developed in the subsequent growing season. The

winter-green leaves of *Rubus fruticosus* s.l. also exhibited relatively low photosynthetic activity, indicating adaptation to sub-zero temperatures [11]. We noticed the same pattern in *Q. pubescens*, as the number of days with sub-zero temperatures in the winter of 2020/2021 was considerable (33 days). Longer and stronger frosts are needed for the initiation of senescence processes in *Q. pubescens*, as demonstrated by Holland et al. [14] in comparisons with *Q. frainetto* Ten., *Q. cerris* L., and *Q. robur* L. Our results imply that *Q. pubescens* seedlings can retain winter-green leaves and utilize winter days with positive temperatures and higher total insolation, thus extending their functional longevity (as defined in [37]). The species showed considerable ecological plasticity, altering its leaf habit under favorable conditions. The subsequent flushes that emerged during the

Table 2. Statistical testing of electron transport rate (ETR) between different leaf flushes based on the Kruskal-Wallis test

Stages PAR $\mu\text{mol cm}^{-2} \text{s}^{-1}$	WG n = 21	F1 n = 21	F2 n = 21	F3 n = 18	Kruskal Wallis test df = 3	Significance
25	^A 4.11 (0.96)	5.5 (1.18)	6.00 (1.30)	5.80 (1.33)	^C K = 24.36	P<0.05
	^B 4.00 [1.00]a	5.90 [1.40]b	6.60 [1.20]b	6.35 [1.05]b		
45	7.06 (1.57)	9.60 (2.24)	10.58 (2.32)	10.56 (2.04)	K= 26.99	P<0.05
	7.00 [1.90]a	10.80 [2.60]b	11.60 [2.30]b	11.35 [1.12]b		
65	9.79 (2.29)	13.46 (3.12)	14.87 (3.42)	14.76 (3.59)	K= 24.27	P<0.05
	9.60 [3.30]a	14.60 [6.20]b	16.40 [3.40]b	16.50 [1.62]b		
90	12.98 (2.74)	17.96 (4.48)	20.08 (4.87)	20.19 (4.90)	K= 24.41	P<0.05
	12.50 [3.50]a	19.40 [8.10]b	22.20 [4.40]b	21.95 [1.97]b		
125	17.38 (3.44)	23.80 (6.95)	26.88 (6.91)	27.34 (6.88)	K= 22.44	P<0.05
	17.40 [5.30]a	26.10 [12.30]b	29.60 [6.90]b	30.11 [2.70]b		
190	24.15 (8.10)	33.82 (10.22)	37.46 (11.01)	39.68 (10.12)	K= 22.52	P<0.05
	23.80 [7.40]a	36.80 [17.30]b	41.50 [12.20]b	43.70 [4.57]b		
285	32.34 (6.88)	47.51 (12.89)	51.66 (15.93)	56.27 (13.69)	K= 26.99	P<0.05
	32.70 [10.60]a	51.50 [21.10]b	56.30 [19.50]bc	61.25 [7.45]c		
420	42.06 (9.35)	62.98 (18.80)	67.61 (23.20)	75.66 (50.18)	K= 24.54	P<0.05
	41.80 [15.70]a	67.70 [26.60]b	72.00 [33.90]b	82.00 [12.57]b		
625	51.23 (11.99)	83.26 (23.17)	87.17 (30.33)	100.62 (28.80)	K= 28.21	P<0.05
	49.40 [16.80]a	83.20 [37.60]b	90.60 [46.40]b	109.35 [21.82]b		
820	57.40 (11.92)	96.15 (27.91)	101.02 (37.35)	118.88 (34.24)	K= 29.38	P<0.05
	57.50 [14.50]a	96.10 [37.20]b	105.00 [56.10]b	125.35 [32.40]b		
1150	64.75 (16.78)	111.55 (34.01)	116.18 (44.01)	144.26 (41.77)	K= 32.84	P<0.05
	61.80 [19.80]a	112.10 [46.80]b	114.00 [64.70]bc	151.15 [47.60]c		
1500	77.29 (18.41)	122.56 (42.30)	127.35 (51.15)	164.86 (55.45)	K= 27.84	P<0.05
	76.90 [18.20]a	126.00 [40.30]b	127.90 [68.00]b	169.15 [70.75]b		

WG – winter-green leaves; F1, F2, and F3 – leaves from the first, second, and third growth flush (a spurt of growth) developed during the growing season; A, mean values \pm SD; B, median values [interquartile range]; C, K values, Kruskal-Wallis test was applied to examine differences among medians; df = degree of freedom. Medians in a row without a common superscript letter differ, as analyzed by the Steel-Dwass-Critchlow-Fligner procedure after the Kruskal-Wallis test (p<0.05); n, sample size (number of leaves).

2021 growing season also showed marked acclimation to changing light conditions. Acclimation at the flush level was also observed in *Q. robur* seedlings [21,38,39]. This enabled higher photosynthetic efficiency, especially under high light conditions in the third growth flush. *Q. pubescens* leaves during the second growing season demonstrated a rapid response to variations in light conditions. These acclimation patterns, coupled with the species' documented drought tolerance [40], could position *Q. pubescens* as one of the winners in a changing climate [41].

The underlying cause of winter-green leaves in species that typically undergo leaf abscission is not fully understood. There are several potential explanations for this phenomenon. In plants, certain phenotypes might display modified senescence as a result of mutations [3]. However, we can largely dismiss this hypothesis, as our seedlings came from a progeny of 20 half-sib lines, with most of them exhibiting this trait. Delayed leaf senescence may be triggered by drought [42], but this possibility can also be discounted, as the *Q. pubescens* seedlings did not experience a lack of precipitation during summer months and were also regularly watered. Another potential explanation may lie in the slowed senescence of *Q. pubescens* leaves that receive higher PPFD [4]. This was observed on adult trees [4], but it may also pertain to juvenile plants. The climate data supports these results in our research. Compared to the 1991–2020 average, total insolation in the growing seasons of 2020, 2021, and 2022 increased by 12.1, 14.8, and 16.1%, respectively. We hypothesized that the high insolation might have prompted a genetic response, activating specific ancestral genes; this could explain why the *Q. pubescens* seedlings resembled their evergreen ancestors [43], indicating adaptation to a warming climate. This, however, may refer only to some specimens of *Q. pubescens*. The hypothesis can only be partly accepted, as the retention of winter-green leaves was not observed in all seedlings, despite experiencing the same light conditions within the micro area. The species is known for exhibiting atavistic traits such as summer flowering with untypical lignified flower branchlets, as reported by Bobinac et al. [25] from the same population in Košutnjak, Belgrade on a specimen determined as *Q. virgiliana* Ten. Winter-green leaves were observed during natural regeneration in the Belgrade area, similar to what was noted on our

seedlings in 2021, 2022, and 2023. The role of the population should be considered as well [12].

Some authors classify *Q. pubescens* (determined as *Q. virgiliana* Ten.) in the Mediterranean as a semi-evergreen oak species [15]. Our results confirm that *Q. pubescens* can be a winter-green species in continental areas as well, as suggested by Holland et al. [14]. Our results also correspond with those indicating that oaks are anticipated to maintain longer canopy duration in future warmer climates characterized by fewer chilling temperatures [44]. Herein we only covered the photosynthetic capacity of *Q. pubescens* leaves. To deepen our understanding of the role of winter-green leaves in the acclimation of *Q. pubescens*, future research should explore this leaf pattern from the perspectives of chlorophyll degradation and phenological studies. The observed retention of green leaves may be a trait worth including in genetic selection, as suggested by Nelson and Isebrands [6]. Considering the significant phenological variability of *Q. pubescens*, winter-green leaves, as a potentially valuable trait in forestry, should be investigated in future research on a broader scale in the region and encompass various growing conditions that might influence this particular trait.

CONCLUSIONS

In the initial stages of development, *Q. pubescens* can alter its typical deciduous leaf habit, potentially extending leaf longevity throughout the entire winter. This adaptation may be attributed to environmental factors, such as heightened insolation and population genetics. In one-year-old seedlings, winter-green leaves retained chlorophyll and sustained approximately 50–60% of the photosynthetic activity observed in subsequent growth flushes during the 2021 growing season. In conclusion, winter-green leaves demonstrate resilience against sub-zero temperatures, maintaining photosynthetic activity until the start of the next growing season. This highlights the species' remarkable plasticity in both phenological and photosynthetic activity patterns.

Funding: This research was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Contracts 451-03-66/2024-03/200053 and 451-03-65/2024-03/200169, and by the Secretariat for Environmental Protection of the City of Belgrade, Project: *Identification and monitoring of the*

gene pool of rare, vulnerable, and endangered plant species in NM Kosutnjak Forest.

Acknowledgments: The authors would like to thank Dr. Srđan Bojović for assistance in statistical processing, analysis, and the presentation of data.

Author contributions: Conceptualization, MB, NŠ and SV-J; methodology, NŠ, SV-J and MŠ-N; validation, MB, NŠ, MŠ-N, IKJ, and SV-J; investigation, MB, NŠ, I-KJ, SV-J; resources, MŠ-N, SV-J; data curation, NŠ; writing – original draft preparation, MB, NŠ, SV-J; writing – review and editing, MB, NŠ, M Š-N, IKJ, SV-J; visualization, NŠ, SV-J; supervision, MB, SV-J; funding acquisition, MB. All authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: The authors declare no conflicts of interest.

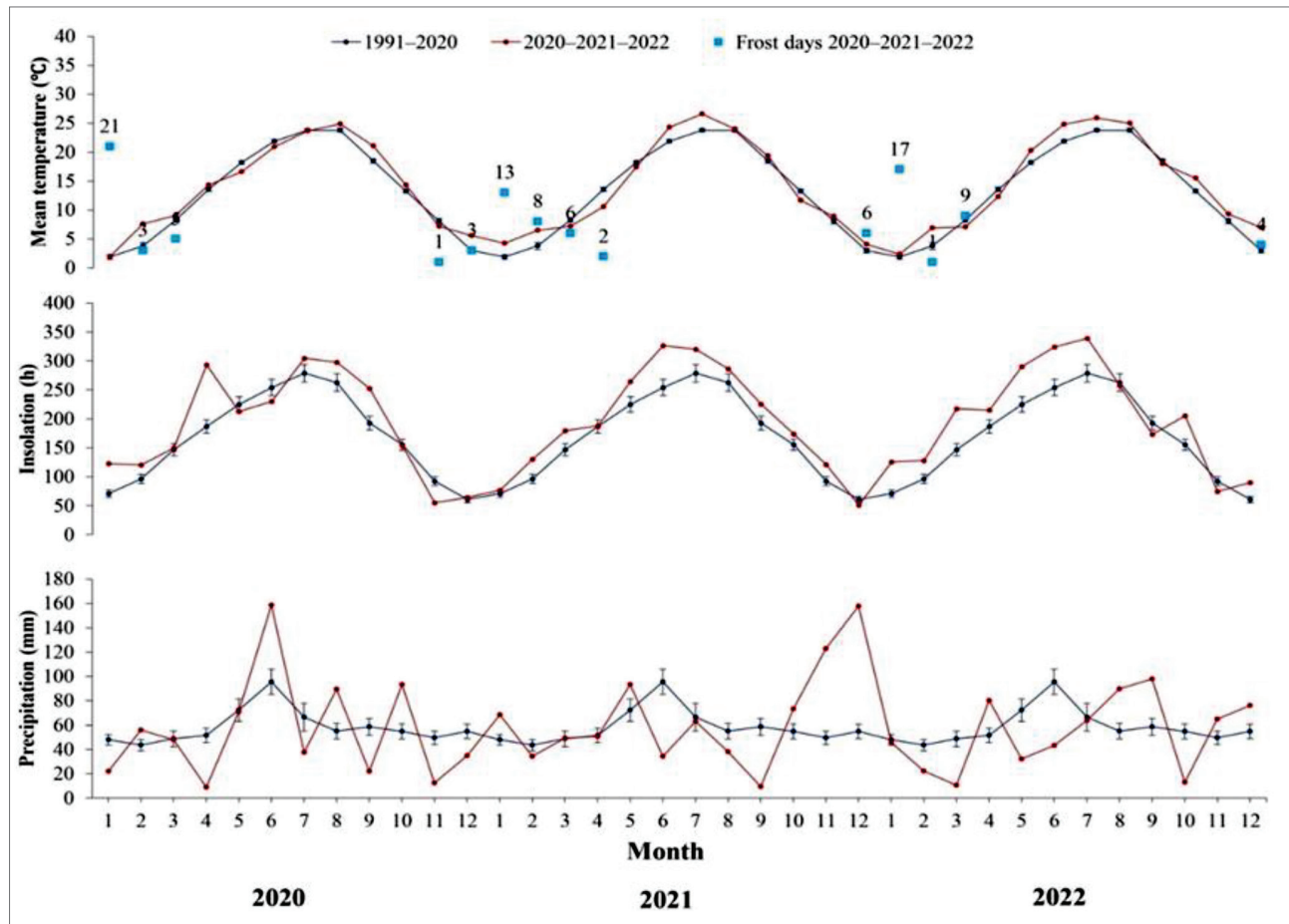
Data availability: Data underlying the reported findings have been provided as a raw dataset and is available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Bobinac%20et%20al_Raw%20Dataset.xlsx

REFERENCES

- Kikuzawa K, Lechowicz MJ. Ecology of leaf longevity. 1st ed. Tokyo: Springer; 2011. 147 p. <https://doi.org/10.1007/978-4-431-53918-6>
- Pallardy SG. Physiology of woody plants. 3rd ed. Academic Press; 2008. 454 p. <https://doi.org/10.1016/B978-012088765-1.50004-X>
- Lim PO, Kim HJ, Nam HG. Leaf senescence. Annu Rev Plant Biol. 2007;58:115-36. <https://doi.org/10.1146/annurev.arplant.57.032905.105316>
- Abadía A, Gil E, Morales F, Montañés L, Montserrat G, Abadía J. Marcescence and senescence in a submediterranean oak (*Quercus subpyrenaica* E.H. del Villar): photosynthetic characteristics and nutrient composition. Plant Cell Environ. 1996;19(6):685-94. <https://doi.org/10.1111/j.1365-3040.1996.tb00403.x>
- Nelson ND, Dickmann DI, Gottschalk KW. Autumnal photosynthesis in short-rotation intensively cultured *Populus* clones. Photosynthetica. 1982;16(3):321-33. <https://doi.org/10.1007/s10530-015-0887-2>
- Nelson ND, Isebrands JG. Late-season photosynthesis and photosynthate distribution in an intensively-cultured *Populus nigra* x *laurifolia* clone. Photosynthetica. 1983;17(4):537-49.
- Ewing PM, Lauko D, Anderson M. Elevated, but highly variable, acetylene reduction in soils associated with the invasive shrub *Rhamnus cathartica* in a Midwestern oak forest. Biol Invasions. 2015;17:2229-34. <https://doi.org/10.1007/s10071-015-0887-2>
- Bobinac M, Andrašev S, Šušić N, Bauer-Živković A, Jorgić Đ. Growth and structure of Italian alder (*Alnus cordata*/Loisel./Duby) linear plantation at age 11 and 16 years at Fruška gora (Serbia). Šumarski list. 2020;144(9-10):455-62. <https://doi.org/10.31298/sl.144.9-10.2>
- Karban R. Deciduous leaf drop reduces insect herbivory. Oecologia. 2007;153(1):81-8. <https://doi.org/10.1007/s00442-007-0709-z>
- Pan VS, Pepi A, Goidell J, Karban R. Retention of green leaves not brown leaves increases spring cynipid diversity on large valley oaks. Arthropod Plant Interact. 2021;15:353-62. <https://doi.org/10.1007/s11829-021-09815-7>
- Modrušan Z, Wrisher M. Seasonal changes in chloroplasts of blackberry leaves. Acta Bot Croat. 1987;46(1):23-31. <https://hrcak.srce.hr/159297>
- Morin X, Roy J, Sonié L, Chuine I. Changes in leaf phenology of three European oak species in response to experimental climate change. New Phytol. 2010;186(4):900-10. <https://doi.org/10.1111/j.1469-8137.2010.03252.x>
- Pasta S, De Rigo D, Caudullo G. *Quercus pubescens* in Europe: distribution, habitat, usage and threats. In: San-Miguel-Ayanz J, de Rigo D, Caudullo G, Houston Durrant T, Mauri A, editors. European atlas of forest tree species. Publications Office of the European Union; 2016. p. e019e5c+.
- Holland V, Koller S, Brüggemann W. Insight into the photosynthetic apparatus in evergreen and deciduous European oaks during autumn senescence using OJIP fluorescence transient analysis. Plant Biol. 2014;16(4):801-8. <https://doi.org/10.1111/plb.12105>
- Lovrić AŽ. Some rare and interesting semi-sempervirent oaks in the Adriatic littoral karst [In Croatian with English abstract]. Šumarski list. 1981;105(3-4):119-32. <https://www.sumari.hr/sumlist/198103.pdf#page=17>
- Wellstein C, Spada F. The status of *Quercus pubescens* Willd. in Europe. In: Box E, Fujiwara K, editors. Warm-Temperate Deciduous Forests around the Northern Hemisphere. Cham: Springer; 2015. p. 153-63. (Geobotany Studies). https://doi.org/10.1007/978-3-319-01261-2_8
- Harmer R. Relation of shoot growth phases in seedling oak to development of the tap root, lateral roots and fine root tips. New Phytol. 1990;16(4):23-7. <https://doi.org/10.1111/j.1469-8137.1990.tb00917.x>
- Bobinac M. Multi-stage height growth of pedunculate oak (*Quercus robur* L.) one-year seedlings and some aspects of economical significance. Šumarstvo. 1994;1-2:47-57.
- Kuster TM, Dobbertin M, Günthardt-Goerg MS, Schaub M, Arend M. A phenological timetable of oak growth under experimental drought and air warming. PLoS One. 2014;9(2):e89724. <https://doi.org/10.1371/journal.pone.0089724>
- Jensen AM, Gardiner ES, Vaughn KC. High-light acclimation in *Quercus robur* L. seedlings upon over-topping a shaded environment. Environ Exp Bot. 2012;78:25-32. <https://doi.org/10.1016/j.envexpbot.2011.12.020>
- Masarovičová E. Leaf shape, stomata density and photosynthetic rate of the common oak leaves. Biologia plantarum. 1991;33(6):495-500. <https://doi.org/10.1007/BF02897727>
- Gaytán Á, Moreira X, Castagneyrol B, Halder IV, Frenne PD, Meeussen C, BGH Timmermans, Ten Hoopen, JJJG, Rasmussen PU, Bos N, Jaatinen R, Pulkkinen P, Söderlund S, Covelo F, Gotthard K, Tack AJM. The co-existence of multiple oak leaf flushes contributes to the large within-tree variation in chemistry, insect attack and pathogen infection. New Phytol. 2022;235(4):1615-28. <https://doi.org/10.1111/nph.18209>

23. Löf M. Establishment and growth in seedlings of *Fagus sylvatica* and *Quercus robur*: influence of interference from herbaceous vegetation. *Canad J For Res.* 2000;30(6):855-864. <https://doi.org/10.1139/x99-257>
24. Ponton S, Dupouey JL, Dreyer E. Leaf morphology as species indicator in seedlings of *Quercus robur* L. and *Q. petraea* (Matt.) Liebl.: modulation by irradiance and growth flush. *Ann For Sci.* 2004;61(1):73-80. <https://doi.org/10.1051/forest:2003086>
25. Bobinac M, Tucović A, Isajev V. Summer flowering properties of pedunculate oak and Virgilius's oak. *Glasnik Šumarskog fakulteta.* 2000;83:55-65. <https://scindeks.ceon.rs/article.aspx?artid=0353-45370083055B>
26. Fernández I, Bouffaud ML, Martínez-Medina A, Schädler M, Tarkka MT, Weinhold A, van Dam NM, Herrmann S, Buscot F. Endogenous rhythmic growth and ectomycorrhizal fungi modulate priming of antiherbivore defences in subsequently formed new leaves of oak trees. *J Ecol.* 2024. <https://doi.org/10.1111/1365-2745.14263>
27. Bobinac M, Batos B, Miljković D, Radulović S. Polycyclism and phenological variability in the common oak (*Quercus robur* L.). *Arch Biol Sci.* 2012;64(1):97-105. <https://doi.org/10.2298/ABS1201097B>
28. de Sousa CAF, de Paiva DS, Casari RADCN, de Oliveira NG, Molinari HBC, Kobayashi AK, Magalhães PC, Gomide RL, Souza MT. A procedure for maize genotypes discrimination to drought by chlorophyll fluorescence imaging rapid light curves. *Plant Methods.* 2017;13:61. <https://doi.org/10.1186/s13007-017-0209-z>
29. Vastag E, Coccoza C, Orlović S, Kesić L, Kresoja M, Stojnić S. Half-sib lines of pedunculate oak (*Quercus robur* L.) respond differently to drought through biometrical, anatomical and physiological traits. *Forests.* 2020;11(2):153. <https://doi.org/10.3390/f11020153>
30. Döweler F, Case BS, Buckley HL, Bader MK. High light-induced photoinhibition is not limiting seedling establishment at abrupt treeline ecotones in New Zealand. *Tree Physiol.* 2021;41(11):2034-45. <https://doi.org/10.1093/treephys/tpab061>
31. Schreiber U, Gademann RRPJ, RalphPJ, Larkum AWD. Assessment of photosynthetic performance of *Prochloron* in *Lissoclinum patella* in hospite by chlorophyll fluorescence measurements. *Plant Cell Physiol.* 1997;38(8):945-51. <https://doi.org/10.1093/oxfordjournals.pcp.a029256>
32. White AJ, Critchley C. Rapid light curves: A new fluorescence method to assess the state of the photosynthetic apparatus. *Photosynth Res.* 1999;59:63-72. <https://doi.org/10.1023/A:1006188004189>
33. Ralph PJ, Gademann R. Rapid light curves: A powerful tool to assess photosynthetic activity. *Aquat Bot.* 2005;82(3):222-37. <https://doi.org/10.1016/j.aquabot.2005.02.006>
34. Republic Hydrometeorological Service of Serbia [Interenet]. [cited 2024 Feb 22]. Available from: <https://www.hidmet.gov.rs/>
35. Tomić Z. Community *Orno-Quercetum cerris-virgiliana* Jov. et Vuk. 77. at the southern boundary of Pannonia. *Glasnik Šumarskog fakulteta.* 1991;73:23-32.
36. Genty B, Briantais JM, Baker NR. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim Biophys Acta Gen Subj.* 1989;990:87-92. [https://doi.org/10.1016/S0304-4165\(89\)80016-9](https://doi.org/10.1016/S0304-4165(89)80016-9)
37. Kikuzawa K, Lechowicz MJ. Toward synthesis of relationships among leaf longevity, instantaneous photosynthetic rate, lifetime leaf carbon gain, and the gross primary production of forests. *Am Nat.* 2006;168(3):373-83. <https://doi.org/10.1086/506954>
38. Jensen AM, Löf M, Gardiner ES. Effects of above-and below-ground competition from shrubs on photosynthesis, transpiration and growth in *Quercus robur* L. seedlings. *EnvironExp Bot.* 2011;71(3):367-75. <https://doi.org/10.1016/j.envexpbot.2011.02.005>
39. Šušić N, Milić Komić S, Živanović B, Jelušić A, Marković S, Sedlarević Zorić A, Veljović-Jovanović S. Acclimation of pedunculate oak seedlings to different light conditions in the first months after germination. In: Šerbula S, editor. 30th International Conference Ecological Truth and Environmental Research – EcoTER'23; 2023 Jun 20-23; Stara planina, Srbija. Belgrade: University of Belgrade, Technical Faculty in Bor; 2023. p. 135-40. https://hdl.handle.net/21.15107/rcub_rimsi_1984
40. Laoué J, Havaux M, Ksas B, Tuccio B, Lecareux C, Fernandez C, Ormeño E. Long-term rain exclusion in a Mediterranean forest: response of physiological and physico-chemical traits of *Quercus pubescens* across seasons. *Plant J.* 2023;116(5):1293-308. <https://doi.org/10.1111/tbj.16424>
41. Früchtenicht E, Neumann L, Klein N, Bonal D, Brüggemann W. Response of *Quercus robur* and two potential climate change winners—*Quercus pubescens* and *Quercus ilex*—To two years summer drought in a semi-controlled competition study: I—Tree water status. *Environ Exp Bot.* 2018;152:107-17. <https://doi.org/10.1016/j.envexpbot.2018.01.002>
42. Vander Mijnsbrugge K, Vandepitte J, Moreels S, Mihaila VV, De Ligne L, Notivol E, Van Acker J, Van den Bulcke J. Timing of autumnal leaf senescence in a common shrub species depends on the level of preceding summer drought symptoms. *Environ Exp Bot.* 2023;216:105539. <https://doi.org/10.1016/j.envexpbot.2023.105539>
43. Manos PS, Stanford AM. The historical biogeography of Fagaceae: tracking the tertiary history of temperate and subtropical forests of the Northern Hemisphere. *Int J Plant Sci.* 2001;162(S6):S77-93. <https://doi.org/10.1086/323280>
44. Vitasse Y, Francois C, Delpierre N, Dufrêne E, Kremer A, Chuine I, Delzon S. Assessing the effects of climate change on the phenology of European temperate trees. *Agric For Meteorol.* 2011;151(7):969-80. <https://doi.org/10.1016/j.agrformet.2011.03.003>

SUPPLEMENTARY MATERIAL



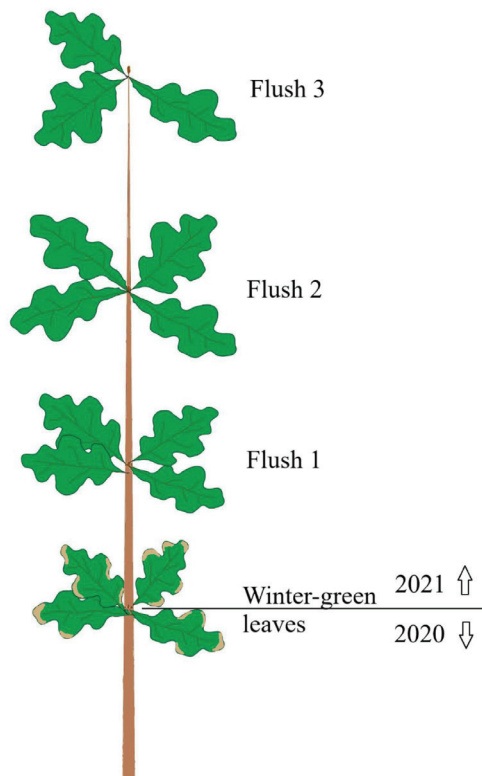
Supplementary Fig. S1. Basic climate parameters in 2020, 2021, and 2022 compared to the 1991-2020 average (\pm standard error of the mean).



Supplementary Fig. S2. One-year-old seedlings of *Quercus pubescens* Willd. in a progeny test in the nursery of the University of Belgrade, Faculty of Forestry, with both abscised and retained green leaves from the 2020 growing season (16/03/2021).



Supplementary Fig. S3. One-year-old *Quercus pubescens* Willd. seedlings from a single half-sib line, with both abscised and retained green leaves from the 2020 growing season (16/03/2021).



Supplementary Fig. S4. Schematic representation of the sampled physiological stages: winter-green leaves developed in 2020 and leaves from the three flushes developed during the growing season 2021.