Comparative Studies on Leaf Micromorphology of the Abaxial Surface of *Quercus robur* L. subsp. *robur* and *Quercus robur* L. subsp. *pedunculiflora* (K. KOCH) MENITSKY

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Abstract – This study examined micromorphological traits on the abaxial surface of leaves using a Scanning Electron Microscope (SEM) to compare pedunculate oak and greyish oak taxa close-up. We selected a pedunculate oak population in Hungary and greyish oak population in Romania. The study randomly selected trees over 100 years old from these populations to investigate the differences between the two taxa based on leaf micromorphological characteristics. We focused mainly on indumentum because observing trichomes can be used in practice. Variation was found in trichome types, trichome-ray lengths, and stomata extent and shape. Stellate and fasciculate trichomes were absent on pedunculate oak leaves and densely developed on greyish oak leaves. The average length of the simple-uniseriate trichomes of pedunculate oak was 49.45 µm and 61.96 µm in greyish oak. On average, stomata surfaces on pedunculate oak leaves were 513.09 µm^2 and 440.28 µm^2 on greyish oak leaves. The study found no variation in epicuticular wax layer type. Despite the small sample size, in comparing the two populations, we found that the two taxa were distinguishable based on trichome types, and we believe that forestry practice could utilise this trait.

pedunculated oak / greyish oak / trichome / stomata / epicuticular wax /SEM

Kivonat – Összehasonlító mikromorfológiai vizsgálatok a *Quercus robur* L. subsp. *robur* és *Quercus robur* L. subsp. *pedunculiflora* (K. KOCH) MENITSKY levélfonákán. Pásztázó-elektronmikroszkópos felvételeken mikromorfológiai méréseket végeztünk a kocsányos tölgy és a szürke tölgy taxonok összehasonlítása céljából. A vizsgálatokhoz egy magyarországi kocsányos tölgy populációt és egy romániai szürke tölgy populációt választottunk ki. Ezekből a populációkból 100 év feletti fákat véletlenszerűen választottunk, hogy megvizsgáljuk a két taxon között a különbségeket a levelek mikromorfológiai karakterisztikája alapján. Legfőképpen a szőrözöttségre fordítottunk figyelmet, mert a szőrözöttség megfigyelése a gyakorlatban is alkalmazható. E két taxonon eltérő szőrtípusokat figyeltünk meg, méréseink alapján különbséget találtunk a szőrkarok hosszában és a sztómák méreteiben. A csillagszőrök és a nyalábszőrök a kocsányos tölgy levelek abaxiális oldaláról hiányoztak, ellenben a szürke tölgy levelek fonákán megfigyeltük őket. A kocsányos tölgy levélfonákán a fedőszőrök átlagos hossza 49,45 µm volt, a szürke tölgy esetében átlagban a 61,96 µm-t értek el. A kocsányos tölgyön a sztómák felülete átlagosan 513,09 µm^2 érte el, míg a szürke tölgyön ez az érték 440,28 µm^2 volt. A viaszréteg típusát viszont azonosnak találtuk. A két populáció összehasonlítása során

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megállapítottuk, hogy a kis mintaszám ellenére a két taxon a trichómák típusa alapján megkülönböztethető egymástól, és véleményünk szerint ez a bélyeg az erdészeti gyakorlatban is alkalmazható.

kocsányos tölgy / szürke tölgy / trichóma / sztóma / viaszréteg /SEM

1 INTRODUCTION

The German botanist Karl Koch determined the greyish oak as a species in 1849, but currently, it is classified as a subspecies of pedunculate oak (*Quercus robur* L.) and listed under the description of Yuri Menitsky (*Q. robur* L. subsp. *pedunculiflora* (K. Koch) Menitsky) (Bartha 2021). Genetic studies also confirmed the relationship of greyish oak (ssp. *pedunculiflora*) to pedunculate oak (subsp. *robur*), and it was shown that the ecological speciation of subsp. *pedunculiflora* has not been completed yet (Curtu et al. 2011). This xeromorphic dendrotaxon spreads from the Balkan Peninsula through Asia Minor and Crimea to the Caucasus and the northwestern part of Iran. Interestingly, the two subspecies show horizontal vicariance in Turkey, along the Anatolian axis (Uslu et al. 2011) and are well separated ecologically (Yilmaz – Yilmaz 2016). Carella (2018) reported new occurrences in southern Italy from the Bari region. The greyish oak is probably not native to Hungary, but hybrids or intermediate forms may occur in the peripherals of the Hungarian Lowlands (Mátyás 1967, Gencsi – Vancsura 1992). The taxon is listed as 'introduced' in the Royal Botanic Garden (Kew Garden) database in London (https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:60459295-2). In climate change context, KEFAG Kiskunsági Erdészeti és Faipari Ltd. has established experimental plots in Hungary to compare the growth capacities of native pedunculate oak seedlings versus greyish oak seedlings from Romania. The results have thus far revealed that the greyish oak seedlings show better growth than the pedunculate oak seedlings (Hegedüs 2021). In comparison, both oak taxa might be distinguishable by generative (the length of female inflorescence, the length of peduncle/the ratio of the length of lamina, the pattern of cupule) and vegetative (the width of lamina, the segmentation of lamina) morphological characters (Mátyás 1967, Gencsi – Vancsura 1992). Describing and characterising the morphological traits of generative organs is often challenging due to the irregular crop of oaks, and many authors (Halácsy 1904, Hayek 1927, Koch 1849, Enescu 2017) emphasised the differences in the abaxial surface of leaves ("subtus glauca, puberula"), which is considered the most important morphological trait in taxonomic differentiation.

Trichomes perform several functions, including reducing evaporation and protecting against environmental influences. Ecological conditions determine their development (Hülskamp, 2019), so it is possible to infer environmental factors from indumentum. Hardin (1976) described 10 types of trichomes in oak leaf hairs. Bussotti – Grossoni (1997) provided a classification of indumentum for Central European and Mediterranean oaks but did not study greyish oak. In their research on oak species in Iran, Panahi et al. (2012) investigated the leaf abaxial surface of subsp. *pedunculiflora* and compared them with six other oak taxa in Iran; however, *Quercus robur* was not in their examinations; only greyish oak samples were. Uzunova – Palamarev (1992) provided an overview of the epidermis of five *Quercus* taxa, including greyish oak, using herbarium leaf samples.

Stomata characteristics are also often used as morphological traits in taxonomic studies. Stomata location and density offer insights into transpiration mechanisms and ecological associations (Yocum, 1935). A comparison of stomatal density of *Quercus* petraea and *Q. robur* populations in northern Turkey found higher density in *Q. robur* (mean value: 517 stomata/mm^2). Yücedağ et al. (2019) examined the micromorphological traits of *Quercus robur* and *Quercus petraea* in natural populations in northern Turkey. They found variation in stomatal density that correlated with various genetic and environmental factors. In general, oak taxa have an anomocytic stomata type, and cells surrounding stomata are undifferentiated from the regular epidermal cells (Bačić 1981, Panahi et al. 2012). Bačić (1981) studied the stomata structure of various oak taxa and distinguished *Q. robur, Q. conferta* and *Q. cerris* samples due to the specific structure and shape of stomata cells in each. Nikolić et al. (2003) analysed stomatal and indumental characteristics of pedunculate oak (subsp. *robur*) provenances and found variations in 17 genotypes.

The epicuticular waxes on leaves are composed of lipids (Gülz – Boor, 1992), and the wax layer has specific functions in light and heat reflection and in the regulation of evaporation (Tschan – Denk, 2012). Gülz – Boor (1992) observed that the wax layer structure in *Q. robur* changed with age, and the number of wax crystalloids increased during May and concentrated around the stomata. Barthlott et al. (1998) classified epicuticular waxes in the genus *Quercus* and established a determination key based on the wax layer. By combining the above three characteristics, significant differences were reported in various oak taxa from the Iberian Peninsula (del Rio et al. 2014), Italy (Gellini et al. 1992), the Balkan Peninsula (Uzunova – Palamarev 1992) and Iran (Panahi et al. 2012). A smooth wax coating was observed on *Q. pedunculiflora* leaves, and a crystalline wax coating in other taxa (Panahi et al. 2012).

2 MATERIALS AND METHODS

This study investigated lower surface characteristics in subsp. *robur* and subsp. *pedunculiflora* leaves to find distinctive micromorphological traits for both taxa. This study compares three characteristics on the abaxial surface of leaves: 1. indumentum, 2. stomata, and 3. type of epicuticular wax, as these traits can be studied throughout the vegetation period and life cycle. Natural stands in the forest-steppe climatic zone were selected; in total, two populations (subsp. *robur* and subsp. *pedunculiflora*) located 808.5 km apart. The collection site of subsp. *robur* was in the Kunpeszér Forest (47.097934ºN, 19.292653ºE and 47.087969ºN, 19.314133ºE) in Hungary at 94 m altitude. The subsp. *pedunculiflora* samples were collected in the Danube Delta in Romania (45.037231ºN, 29.401113ºE and 45.029099ºN, 29.406569ºE) at 5 m altitude. The pedunculate oak was collected in August 2020; the greyish oak in August 2019. Both sites are located in the forest-steppe climatic zone characterised by continental and arid climatic conditions. Two trees were selected according to macromorphological characteristics to exclude hybrid phenotypes in each population. Age and height (≥ 100 years and ≥ 20 m) were also considered to exclude non-autochtonous material as much as possible.

Shoots were sampled from the outer and lower part of the crown to analyse mature, fully-developed leaves. Five leaves per individual tree were taken and desiccated. A HitachiS3400N Electron Scanning Microscope was used for the micromorphological studies at the Natural Resources Research Centre (NRRC) of the University of Sopron.

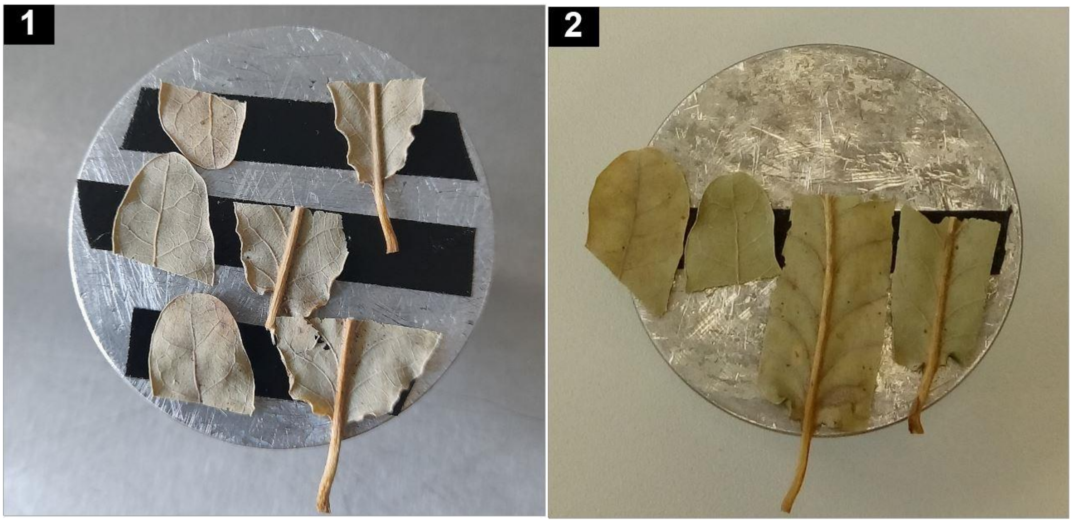


Figure 1-2. The greyish oak (1) and pedunculate oak (2) samples are fixed to an aluminium stub

The present study examined the trichome types, stomata, and wax layer on the abaxial surface of 10 leaves adjacent to the mid-vein and on the mid-petiole and compared these micromorphological features of both taxa (*Figure 1-2*). It did not examine the adaxial sides of the leaves. Trichome densities were observed at 100× magnification, while stomata and the wax layers were observed at 1000× magnification. The length of the trichome rays and the length, width, extent, and density of the stomata were measured using ArchiCAD 24. Trichome density was not determined. We only measured unsevered, whole and visible trichomes. Trichome type classification was based on Hardin (1976) and Bussotti – Grossoni (1997) and the categories of Barthlott et al. (1998) to determine the characteristics of the wax layer. Wax layer parameters were not measured due to technical reasons.

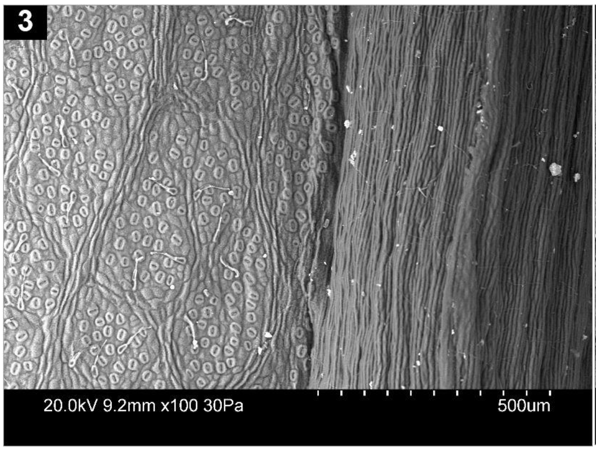
The present study used TIBCO Statistica 14.0.1 for data processing and T-test for data evaluation. The significance interval was $p<0.05$; sample numbers were above 220. *Table 1* lists the abbreviations used and their corresponding units of measurement and explanations.

Table 1. Abbreviations, their units and explanations

Abbreviations	Units	Description
Taxa		
Q.R.R.	-	*Quercus robur* L. *robur*
Q.R.P.	-	*Quercus robur* L. subsp. *pedunculiflora* (K. Koch) Menitsky
Trichomes		
RL	μm	Ray length
Stomata		
SL	μm	Stomata length
SW	μm	Stomata width
SD	no./mm^2	Stomata density
SS	μm^2	Stomata scope
LSP	μm	Length of stomatal pore

3 RESULTS

Glandular, simple-uniseriate trichomes were observed on the abaxial surface of *Quercus robur* L. subsp. *robur* leaves (*Figure 3*). The rays were thin-walled, short, and extended parallel to the epidermis, thus protecting the underlying cells. The stomata formed a disordered spatial structure and were protrusively distinct from the epidermis, with an elliptical shape and thick-walled guard cells. A crystalline, densely laminated wax layer structure covered the stomata and the stomatal epidermis (*Figure 4*).



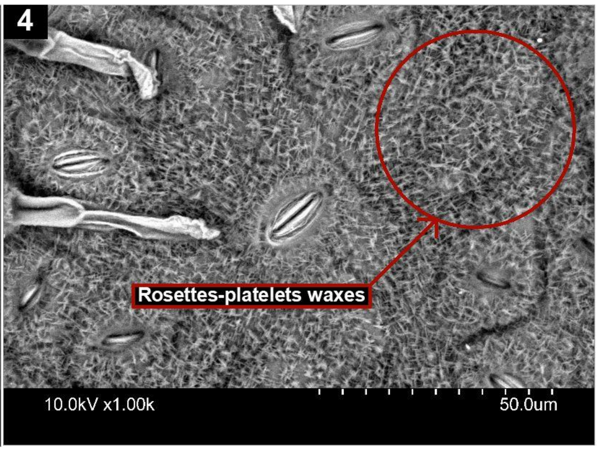
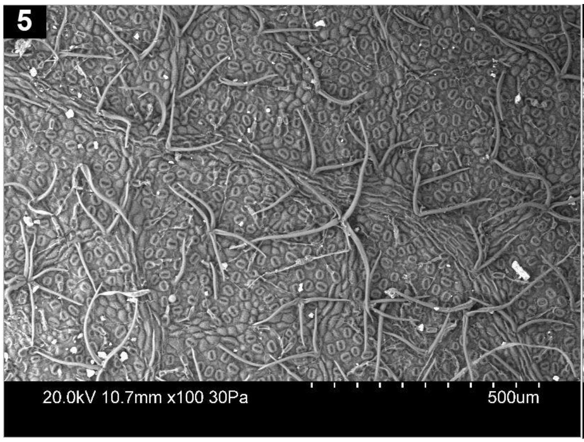


Figure 3. Abaxial surface of Quercus robur L. subsp. robur leaf at 100× magnification, with the simple-uniseriate trichomes next to the midrib

Figure 4. The epicuticular wax layer, stomata, and simple-uniseriate trichome at 1000× magnification on the abaxial surface of Quercus robur L. subsp. robur leaves

Simple-uniseriate trichomes similar to *Quercus robur* L. subsp. *robur* leaves were found on the abaxial surface of *Quercus robur* L. subsp. *pedunculiflora* leaves. As *Table 2* summarises, ray length was one of the differences between the two taxa. In addition, the simple-uniseriate trichome type, stellate trichomes and fasciculate trichomes were also observed on leaves of greyish oak. Stellate trichomes were also found on the thinner secondary veins (*Figure 5*), which were parallel to the epidermis. The number of rays varied from one to seven and were straight, short, thick-walled, and cylindrical, branching directly above the epidermis and flattening at the branching point. The fasciculate trichomes were observed only on and nearby the midrib (*Figure 6*). The long, relatively straight rays were disordered, convoluted and not adherent to the epidermis. The number of rays varied from one to four, branching directly above the epidermis. *Table 2* summarises the statistical data on trichome types.



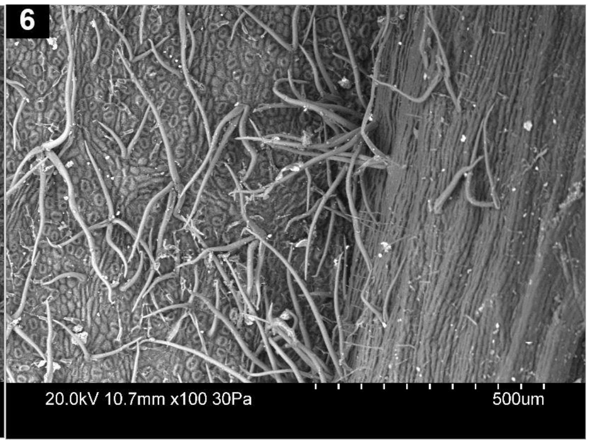


Figure 5. Stellate trichomes observed on the abaxial leaf surface of Quercus robur L. subsp. pedunculiflora (K. Koch) Menitsky
Figure 6. Fasciculate trichomes and stellate trichomes observed on the abaxial surface of the leaf of Quercus robur L. subsp. pedunculiflora (K. Koch) Menitsky, adjacent to the midrib

Table 2. Descriptive Statistics of Indumentum (Interpretation of variables: simple=simple-uniseriate trichome; stellate=stellate trichome; fasciculate=fasciculate trichome)

Variable	Number of measurements	Mean	Median	Minimum	Maximum	Std.Dev.
Q.R.R. RL by simple	236	49.45	48.20	19.00	98.60	15.09
Q.R.P. RL by simple	228	61.96	60.00	25.90	113.90	18.36
Q.R.P. RL by stellate	319	112.38	107.60	47.10	251.00	28.63
Q.R.P. RL by fasciculate	140	258.61	234.50	132.80	485.10	78.50

Stomata shape was elliptical, and the guard cells were ascending as on the *Quercus robur* L. subsp. *robur* leaves observed. In this regard, the extent of stomata on *Quercus robur* L. subsp. *robur* leaves were greater than those of *Quercus robur* L. subsp. *pedunculiflora* (K. Koch) Menitsky. *Table 3* summarises the descriptive statistical parameters of the stomata. The epicuticular wax layer of greyish oak also had a crystalline structure (rosettes-platelets wax, see *Figures 7* and *8*).

Table 3. Descriptive Statistics of Stomata

Variable	Number of measurements	Mean	Median	Minimum	Maximum	Std.Dev.
Q.R.R. LSP	262	12.30	12.38	5.20	18.90	2.08
Q.R.R. SL	222	25.68	25.60	18.90	34.20	2.46
Q.R.R. SW	226	20.30	20.30	15.80	26.40	2.27
Q.R.R. SS	222	513.09	507.00	295.20	779.60	88.45
Q.R.P. LSP	218	11.11	10.90	5.90	19.40	2.33
Q.R.P. SL	218	24.21	24.27	16.90	32.70	2.89
Q.R.P. SW	219	18.44	18.30	12.70	25.80	2.18
Q.R.P. SS	221	440.28	437.70	264.20	740.50	82.14

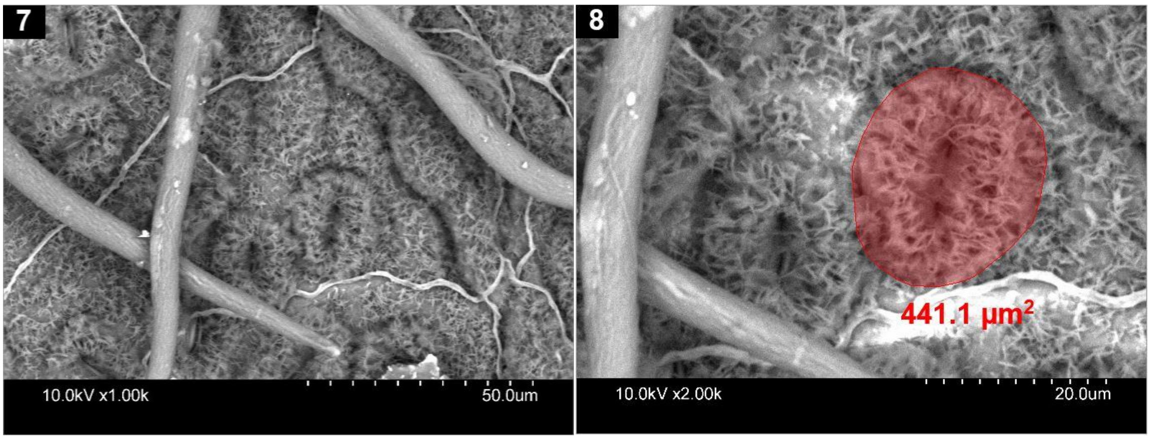


Figure 7. Greyish oak stomata with crystalline wax layer at 1000× magnification.
Figure 8. Area measurement of the stomata on the abaxial surface of a greyish oak leaf at 2000× magnification ArchiCAD 24

In comparison, significant differences were found in trichome types, measured for both taxa, respectively. On average, pedunculate oak had shorter rays (49.45 µm), while greyish oak had longer rays (61.96 µm) (*Figure 9*) on their trichomes. In addition, stellate and fasciculate trichomes were absent on pedunculate oak leaves but densely developed on greyish oak leaves. The average length of stellate and fasciculate trichomes were 112.38 µm and 258.61 µm, respectively.

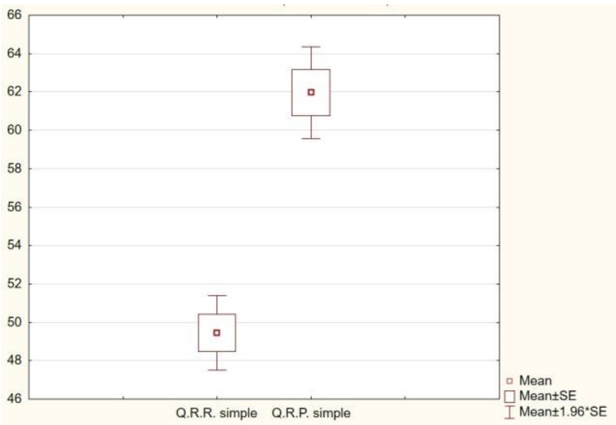


Figure 9. Comparison of the length of simple-uniseriate trichomes

Significant differences in the dimensional parameters of stomata appeared, specifically in length, width at their widest point, surface area, and length of stomatal pore. The stomata were dimensionally more extensive on pedunculate oak leaves than on greyish oak leaves. *Figures 10–13* show the comparison of stomatal parameters. Based on the measurements, the average stomatal density on the abaxial surface of pedunculate oak was 301.19 stomata/mm^2, and the stomatal density on the abaxial leaf surface of greyish oak was 313.75 stomata/mm^2. Nevertheless, the stomatal density of greyish oak was probably even higher than measured since trichomes intensively covered the surface, reducing stomatal density in many cases. (See *Figures 5–8.*, with trichomes of greyish oak).

The minimum-maximum values in *Tables 2–3* determine the overlap between the extent but the 95% confidence intervals are well separated, and no outliers were obtained (*Figures 9–13*).

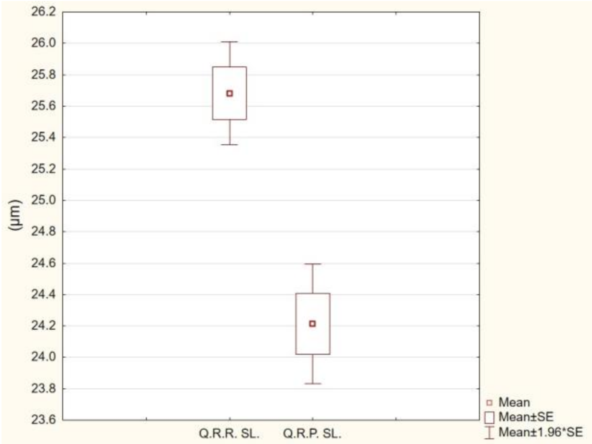


Figure 10. Comparison of stomata lengths.

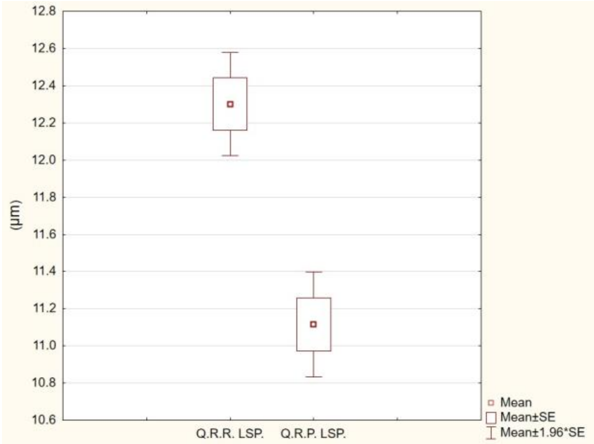


Figure 11. Comparison of stomata pore lengths

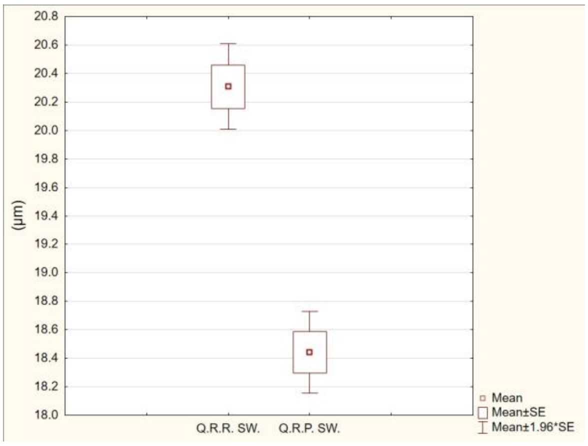


Figure 12. Comparison of stomata widths.

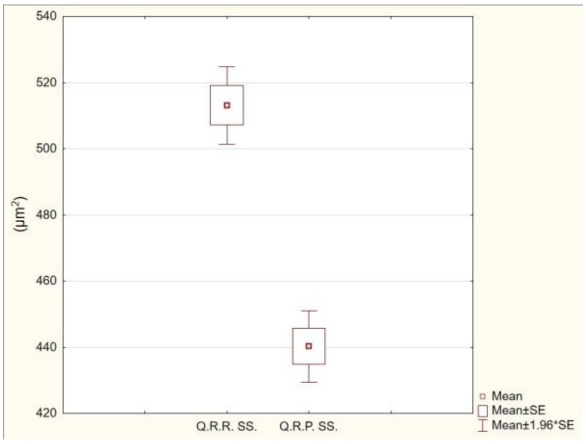


Figure 13. Comparison of stomata areas

4 DISCUSSION

The differences observed on trichome types and stomata were suitable to distinguish *Quercus robur* L. subsp. *robur* and *Quercus robur* L. subsp. *pedunculiflora* (K. Koch) Menitsky, whereas the wax layer type was unsuitable to distinguish the two taxa.

Significant differences in trichome density of the abaxial leaf surface were observed. Similar to the results reported by Gülz – Boor (1992), Bussotti – Grossoni (1997) and Nikolić et al. (2003), only simple-uniseriate trichomes were found on *Quercus robur* L. subsp. *robur* leaves. Simple-uniseriate, stellate trichomes were observed on the leaf abaxial surface of greyish oak. Additionally, fasciculate trichomes were found along the midrib of the abaxial surface. Those micromorphological characters were suitable to distinguish greyish oak from pedunculate oak. Our observations on trichome types and density support the results of Enescu (2017), who reported the indumentum of leaves as an appropriate trait for species identification, as observed by several authors in the past (Koch 1849, Schwarz 1937, Tutin et al. 1964). Analysing greyish oak and pedunculate oak by Kissling's method, Ecaterina-Nicoleta Apostol found that the descriptive data on trichomes differentiated between these two taxa in Romania (Apostol 2019).

Measurements made by Nikolić et al. (2003) on *Quercus robur* L. subsp. *robur* leaves resulted in values (SL = 25.98 µm; SW = 19.05 µm) similar to our results (SL = 25.68 µm; SW = 20.30 µm). However, the stomatal density of *Quercus robur* L. subsp. *robur* leaves was higher in their study (SD = 654.4 no./mm^2) than in ours (SD = 301.19 no./mm^2).

A more concentrated wax layer around the stomata characterises the crystalline wax layer type (Barthlott et al. 1998). Prasad – Gülz (1990) also support our observations on *Quercus robur*; however, they analysed the chemical composition of the wax and the percentage of the components that determined the wax layer structure. The wax layer in *Q. robur* comprises aldehydes, alcohols, alkanes, fatty acids, esters, and triterpenes, which explains the crystalline structure (Prasad – Gülz 1990). No variation was found for wax layer type: a crystalline structure was observed on leaves of both pedunculate oak and greyish oak trees. This was contrasted with the results of Panahi et al. (2012), where smooth wax layers were reported on *Quercus robur* ssp. *pedunculiflora* leaves sampled in populations in Iran. Gülz – Boor (1992) observed that the wax layer could melt in mid-summer under high temperatures due to its plasticity and recrystallise towards the end of the vegetation period.

Nevertheless, the uncertainty of the wax layer type as a micromorphological characteristic – smooth or crystalline – might depend on sampling time.

The micromorphological differences supported our hypothesis that greyish oak was well-adapted to arid and continental ecological conditions, and its adaptation to the specific ecological requirements also manifested in micromorphological traits, such as trichome types and stomata extent. The micromorphological traits observed in greyish oak suggest that it is more drought-tolerant, which is particularly important for the forestry sector in a climate change context. In forestry practice, the presence of stellate and fasciculate trichomes on the abaxial surface of the leaves of *Quercus robur* L. subsp. *pedunculiflora* (K. Koch) Menitsky provides an appropriate morphological trait to distinguish greyish oak from pedunculate oak, as phenotypes of *Quercus robur* tolerant versus less tolerant to drought.

5 CONCLUSIONS

This study compared greyish oak with pedunculate oak based on leaf morphological characteristics using scanning electron microscopy. We found that scanning electron microscopy can better reveal micromorphological differences. However, we conclude that a random sample of a greyish oak leaf and a pedunculate oak is enough to distinguish between the presence of stellate and fasciculate trichomes and the absence of stellate and fasciculate trichomes in the case of pedunculate oak. Among the morphological traits, the indumentum was found to be the most useful because it can distinguish greyish oak from pedunculate oak and can be used in forestry practice. We also found a difference in stoma sizes, but this is not relevant in practice. We analysed wax layer types and concluded that these cannot be used to separate these two taxa. Our study included only two populations, and our results suggest that it would be useful to include more populations in the future, including hybrids from South-East Europe and the Balkan region. In addition to the leaf morphological studies, we will also aim to observe the micromorphology of the generative organs to separate greyish oak and pedunculate oak.

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