

**COMPARATIVE ANATOMY OF LEAVES AND STEMS OF
DAPHNE OLEOIDES SCHREB. (THYMELAEACEAE)**

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Abstract

Variability of morpho-anatomical characteristics of the leaves and stem of the plants from eight distant populations of the species *Daphne oleoides* Schreb. was analyzed in order to establish their differential characteristics possibly affecting their adaptive capability. Statistical data analyses were made for 20 characters. Kruskal-Wallis test for comparing means of characteristics showed statistically significant differences among analyzed samples for all characteristics, except for the stem periderm thickness. Principal component analysis has shown that the most important characters for determining variability among the populations of species *D. oleoides* include characters of leaf shape and size, followed by total thickness of the leaf and thickness of palisade and spongy tissue, as well as the surface area of epidermal cells. It is presumed according to present findings that plants from all which were studied showed xeromorphic structures.

Introduction

The genus *Daphne* L. (Thymelaeaceae) includes 95 species of deciduous, semi-evergreen and evergreen shrubs and trees which are distributed in Asia and Europe, with several representatives in N. Africa, Australia and the Pacific (Brickell and Mathew 1978, Halda 1998, Halda 2001). According to Halda (1998), the genus *Daphne* is divided into 15 subgenera and 10 sections, and primary center of evolution is in SW Asia where the largest number of species is present, and the less distinct centers of evolutionary radiation are in the mountain regions of Eurasia. According to Euro+Med Plantbase (2006), there are 21 species in Mediterranean region and Europe, while in the Flora Europaea (Webb and Ferguson 1968) 17 species are native. In the Balkan peninsula, 12 species are recorded, and they belong to 7 different subgenera (Halda 1998).

Daphne oleoides Schreb. belongs to the subgenus *Keisslera* Halda, and the section *Keisslera* (Webb and Ferguson 1968). Of all the species of the section *Keisslera*, only *D. oleoides* is more widely distributed; its distribution encompasses North Africa, southern Europe (from Spain to the Balkan Peninsula), Asia Minor and Iran, Russian Federation and Transcaucasus region (Meusel *et al.* 1965).

A few reports have discussed the morphological and anatomical characteristics of the genus *Daphne* (Brickell and Mathew 1978, Halda 1998). A general description of the anatomical structure of vegetative organs from Thymelaeaceae, comprising the genus *Daphne*, was given by Metcalfe and Chalk (1950). Recent investigations have been conducted on the anatomical structure of the stems of this genus (Schweingruber *et al.* 2011).

The aim of this study was, on the basis of morphometric research, to describe variability of morpho-anatomical structure of the leaves and stem of the *D. oleoides* and to establish the existing trends in differentiation of its populations which inhabiting mountainous areas in the Balkan Peninsula, in Serbia, Montenegro, Republic of Macedonia and Greece.

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Material and methods

Morpho-anatomical analysis was made on plant samples from eight populations of the species *Daphne oleoides* growing in the Balkan Peninsula (Table 1). The anatomical analysis of leaves and stems was performed on permanent and temporary slides prepared by the standard histological method for light microscopy (Ruzin 1999). The total of 20 quantitative characteristics subjected to statistical analysis: leaf thickness (LT), mesofil's thickness (TM), height of adaxial epidermal cells (HAde), thickness of the palisade tissue (TP), thickness of the spongy tissue (TS), height of abaxial epidermal cells (HAbE), surface area of adaxial epidermal cells (SArAdE), surface area of abaxial epidermal cells (SArAbE), surface area of abaxial stomata (SArAbS), vein density (VD), leaf length (LL), distance between the largest leaf width and the leaf top (DLLWLT), largest width of the leaf (LWL), leaf surface area (LSAr), number of palisade layers (NoPL), number of non-glandular abaxial hairs (NoAbH), stomatal density (NoAbS), stem diameter (SD), stem periderm thickness (SPT), stem cortex thickness (SCT), number of palisade layers (NoPL), number of non-glandular abaxial hairs (NoAbH), stomatal density (NoAbS). The morpho-anatomic measurements were performed on the microscope Leica DM 2500-Leica DFC490-Leica Qwin Standard (Leica Microsystem, Germany). Leaf epidermis, trichomes and stomata were analyzed by a scanning electron microscope (SEM, JEOL 5300). Kruskal-Wallis One-way Analysis of Variance (KW) was performed for each character to evaluate the significant difference among the eight populations and the characters which showed significant variation ($p < 0.05$) only were used to Principal Component Analysis (PCA). PCA analysis was performed in order to determine which characteristics most effectively differentiates among populations. Pearson's correlation analysis was performed to determine the relationship between number of stomata and stomatal area. The results were analyzed in the statistical package SYSTAT 12 (Systat Software Inc. 2007).

Results and Discussion

It is shown that *Daphne oleoides* grows in the mountains and river gorges and optimal habitats of this plant are high-mountain natural grasslands (Table 1). Actually, in this type of vegetation *D. oleoides* is so dominant that it forms its own vegetation belt which belongs to the vegetation class *Daphno-Festucetea*. According to EUNIS habitat classification (EUNIS 2016), this class belongs to calcareous alpine and subalpine grassland and it is included in a Resolution 4 habitat type at a higher level (E4.4) of the Bern Convention. This species can also be found in basic and ultra-basic inland cliffs (EUNIS H3.2) (EUNIS 2016), of the vegetation class *Aplenieta trichomanis* as well as in perennial calcareous grassland of vegetation class *Festuco-Brometea* (Table 1). In the Balkan Peninsula, it grows preferably on carbonate, but rarely can be found on ultramafite or silicate, at the altitude from 450 m up to 2800 m (Markova and Cherneva 1979, Aldén 1986).

In all populations the leaves of *D. oleoides* are evergreen, greyish-green, hairy, with a short leaf stalk simple, and obovate oblong or elongated elliptical. The leaves of all the plants studied are 10 to 36 mm long and 3 to 9 mm wide. The most significant variation among the plants in analyzed populations was recorded in the character of leaf surface area (Table 2).

The sparse leaf indumentums always occurred at the abaxial side of the leaves, composed of non-glandular trichomes which are simple, unicellular, basally widened, sharp on the top and they vary in size and distribution within populations (Fig. 1B, D, Table 2). The leaves of plants from all the investigated populations have dorsiventral structure (Fig. 2A-H). Their thickness in all the plants ranged between from 292 and 603 μm (Table 2).

The cuticle is slightly thicker on the leaf abaxial epidermis than that of adaxial epidermis. The single layered epidermis is thicker on adaxial than on abaxial leaf side. The mesophyll is

differentiated into well-developed, palisade parenchyma and spongy parenchyma; the ratio of these two tissues is generally 1 : 1 (Fig. 2A-H, Table 2). The vascular tissue is well developed and represented by a large central and a number of smaller vascular bundles.

Table 1. Ecological characteristics of the habitats of the analyzed populations.

Locality	Vegetation	Substratum	Altitude (m)	Voucher
SERBIA – Mt. Suva Planina (Trem)	Perennial calcareous grassland (<i>Festuco-Brometea</i>)	Limestone	1811	HMN 5509
SERBIA - Mt. Vlaška Planina (Dren)	Perennial calcareous grassland (<i>Festuco-Brometea</i>)	"	1250	HMN 5511
MONTENEGRO - Mt. Durmitor (Canyon of Piva river)	Open vegetation with ferns and mosses in rock and wall crevices (and rarely on screes) (<i>Asplenietea trichomanis</i>)	"	1705	HMN 5512
R. MACEDONIA - Mt. Galičica (Magaro peak)	Oromediterranean calciphilous grasslands and phrygana (<i>Daphno-Seslerietea</i>)	"	2000-2200	HMN 5513
GREECE - Mt. Olympus (Prionia)	Open vegetation with ferns and mosses in rock and wall crevices (and rarely on screes) (<i>Asplenietea trichomanis</i>)	"	1057	BEOU 24726
GREECE - Mt. Smolikas (2 km SW from peak)	Oromediterranean calciphilous grasslands and phrygana (<i>Daphno-Seslerietea</i>)	Ultramafile	1800-2000	BEOU 27791
GREECE - Mt. Parnassus (near the ski center)	Open vegetation with ferns and mosses in rock and wall crevices (and rarely on screes) (<i>Asplenietea trichomanis</i>)	Limestone	1500	BEOU 27702
GREECE - Mt. Chelmos (Aristarchos Telescope)	Oromediterranean calciphilous grasslands and phrygana (<i>Daphno-Seslerietea</i>)	"	2000-2100	BEOU 27675

The leaves of plants from all the investigated populations have dorsiventral structure (Fig. 2A-H). Their thickness in all the plants ranged between 292 and 603 μm (Table 2). The cuticle is slightly thicker on the leaf abaxial epidermis than that of adaxial epidermis. The single layered epidermis is thicker on adaxial than that of abaxial leaf side. The mesophyll is differentiated into well-developed, palisade parenchyma and spongy parenchyma; the ratio of these two tissues is generally 1 : 1 (Fig. 2A-H, Table 2). The vascular tissue is well developed and represented by a large central and a number of smaller vascular bundles.

The epidermal cells have almost straight anticlinal walls on both leaf sides in the plants from all populations (Fig. 1A,B). Some epidermal cells contain mucilaginous substances but their number varied as among the plants as well as among populations (Fig. 2A-H). The main constituents of mucus are polysaccharides, which have the ability to bind water and reduce the harmful effects of excessive solar radiation on the palisade tissue (Bredenkamp 1999).

The leaves are always hypostomatic with more or less sunken stomata (Fig. 2A-H), of anomocytic type (Fig. 2B, C, D). The variation of density and size of stomata among the investigated populations in species *D. oleoides* has revealed statistically significant differences.

The negative Pearson correlation coefficient for number of stomata on the surface ($R = -0.502$) indicates that an increase in the number of stomata reduces their surface area in all populations. Higher stomata density and smaller stomata size were also confirmed for other species (Smith *et al.* 1989). These data suggest that the environmental factors are closely associated with the distribution, density and size of the stomata (Schluter *et al.* 2003).

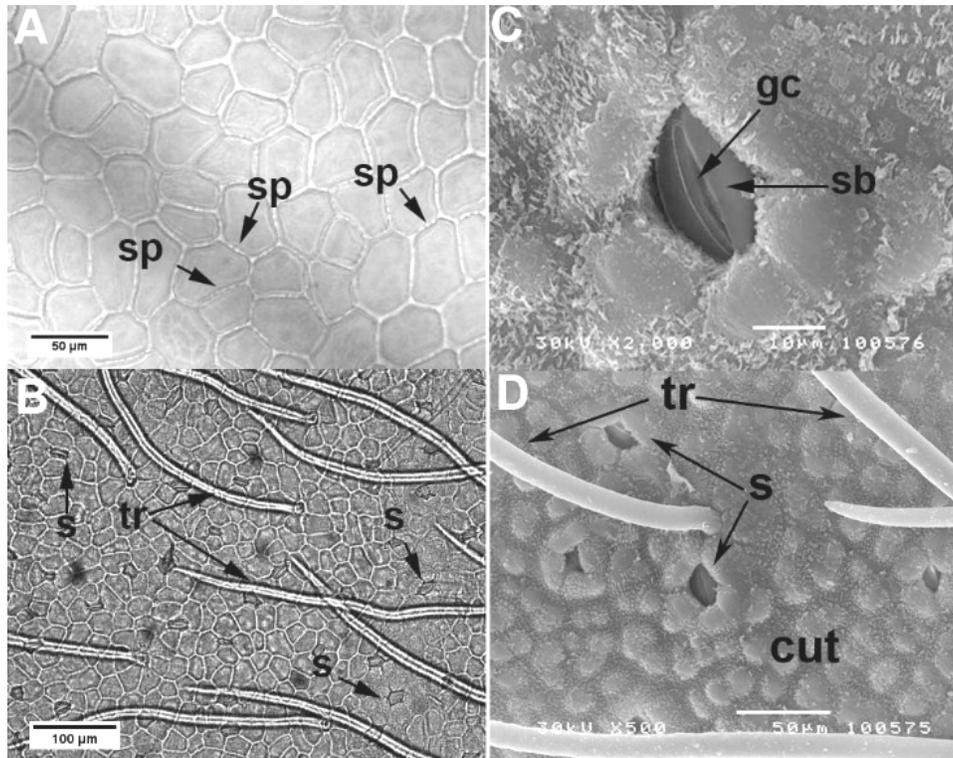


Fig. 1. Light and scanning electron micrographs showing epidermis of *D. oleoides* leaves. (A) Mt. Vlačka Planina; characteristics of adaxial epidermal cells - polygonal cells with straight anticlinal walls and simple pits. (B) Mt. Smolikas; abaxial leaf surface showing trichomes and anomocytic stomatal complexes. (C) Mt. Suva Planina; abaxial surface with stomata (SEM). (D) Mt. Galičica; abaxial surface with stomata and non-glandular trichomes (SEM); sp - simple pit; s - stomata; tr - trichomes; gc - guard cell; sb - subsidiary cell; cut - cuticle. Scale bars: 100 μm (C), 50 μm (A, D) 10 μm (B).

The stem anatomical characters the young (one-year-old) stems showed similar anatomical characteristics in all the investigated populations (Table 2). On the cross section, the stems were rounded to oval in shape, without ribs (Fig. 3).

The young stems were covered by unicellular non-glandular trichomes (Fig. 3a). However, the older stem had a denser indumentum than younger ones. The single-layered epidermal cells were thick-walled and covered by a thick cuticle. The stem cortex is well developed. This cortex consisted of three to four layers of collenchyma and about a dozen layers of thin-walled parenchyma cells (Fig. 3c). The vascular tissue was arranged as continuous ring, divided into phloem on the outside and particularly well-developed xylem on the inside of the stem (Fig. 3d). The stem pith consisted of rounded parenchyma cells without intercellular spaces (Fig. 3b). The

Table 2. Results of Kruskal-Wallis one-way analysis of Variance for comparing means of morphometric characteristics in eight populations of *D. oleoides*. Values are the means \pm Sd. Data were analysed by Kruskal-Wallis test (* $p < 0.05$).

Characters	Mt. Durmitor	Mt. Galičica	Mt. Vlaška Planina	Mt. Suva Planina
Leaf				
LT (μm)	442.94 \pm 79.60	413.29 \pm 62.14	450.44 \pm 55.26	377.84 \pm 34.87
TM (μm)	351.17 \pm 69.84	313.25 \pm 53.76	361.59 \pm 52.57	289.14 \pm 30.13
HAdE (μm)	45.51 \pm 4.45	42.37 \pm 4.03	43.22 \pm 5.04	42.34 \pm 4.97
TP (μm)	176.06 \pm 37.77	142.47 \pm 25.08	180.46 \pm 28.61	135.62 \pm 17.41
TS (μm)	177.05 \pm 37.38	170.36 \pm 31.42	181.66 \pm 30.60	152.40 \pm 20.61
HAbE (μm)	28.02 \pm 2.72	26.16 \pm 2.13	23.97 \pm 2.02	26.21 \pm 3.97
SArAdE (μm^2)	2738.32 \pm 453.52	1739.60 \pm 293.37	1086.74 \pm 171.59	1410.13 \pm 565.15
SArAbE (μm^2)	1515.64 \pm 258.06	844.10 \pm 122.12	773.27 \pm 124.53	819.53 \pm 230.14
SArAbS (μm^2)	1848.83 \pm 173.10	1467.43 \pm 146.02	1397.67 \pm 106.09	1297.58 \pm 207.85
VD (mm/mm^2)	10.30 \pm 0.67	11.40 \pm 0.99	10.86 \pm 0.81	12.08 \pm 1.09
LL (mm)	24.24 \pm 5.24	19.85 \pm 3.84	20.23 \pm 3.43	18.99 \pm 1.49
DLLWLT(mm)	7.74 \pm 1.23	7.97 \pm 1.63	7.31 \pm 1.26	2.48 \pm 0.65
LWL (mm)	7.47 \pm 1.09	5.97 \pm 1.05	6.33 \pm 0.96	6.65 \pm 0.63
LSAr (mm^2)	115.34 \pm 36.33	79.84 \pm 27.96	84.03 \pm 24.29	80.83 \pm 11.77
NoPL	3.30 \pm 0.47	3.40 \pm 0.50	3.27 \pm 0.45	3.13 \pm 0.51
NoAbH (no/mm^2)	5.18 \pm 2.36	4.45 \pm 2.01	3.01 \pm 1.63	8.98 \pm 4.26
NoAbS (no/mm^2)	79.61 \pm 12.96	114.41 \pm 17.70	119.69 \pm 14.31	110.28 \pm 22.84
Stem				
SD (mm)	2.04 \pm 0.31	1.79 \pm 1.47	1.94 \pm 0.39	2.06 \pm 0.13
SPT (μm)	29.23 \pm 2.93	27.97 \pm 2.95	28.59 \pm 2.54	29.75 \pm 2.80
SCT (μm)	449.88 \pm 69.61	368.73 \pm 52.18	393.60 \pm 93.27	398.59 \pm 54.33

Table 2 (contd).

Characters	Mt. Chelmos	Mt. Olympus	Mt. Smolikas	Mt.Parnassus	p value
Leaf					
LT (μm)	391.85 \pm 28.56	374.96 \pm 33.97	394.03 \pm 27.83	376.44 \pm 25.48	0.00*
TM (μm)	311.08 \pm 32.53	300.84 \pm 26.76	306.83 \pm 27.30	298.18 \pm 22.40	0.00*
HAdE (μm)	44.87 \pm 7.49	44.39 \pm 5.55	46.26 \pm 6.11	44.20 \pm 6.30	0.03*
TP (μm)	156.99 \pm 21.93	153.45 \pm 14.85	156.72 \pm 16.41	153.42 \pm 15.56	0.00*
TS (μm)	155.17 \pm 18.82	149.37 \pm 17.64	151.65 \pm 15.87	144.12 \pm 15.13	0.00*
HAbE (μm)	24.08 \pm 1.60	25.95 \pm 2.40	25.33 \pm 2.21	25.66 \pm 2.50	0.00*
SArAdE (μm^2)	1446.08 \pm 298.34	1443.81 \pm 353.08	1916.95 \pm 276.96	1412.36 \pm 201.41	0.00*
SArAbE (μm^2)	787.07 \pm 108.80	1008.04 \pm 183.56	873.65 \pm 88.32	754.77 \pm 87.33	0.00*
SArAbS (μm^2)	1028.49 \pm 126.97	1381.79 \pm 173.42	1487.53 \pm 165.28	1484.53 \pm 138.17	0.00*
VD (mm/mm^2)	10.59 \pm 0.55	10.51 \pm 0.79	11.02 \pm 0.63	9.75 \pm 0.57	0.00*
LL (mm)	19.28 \pm 6.41	18.10 \pm 2.63	13.75 \pm 2.41	16.64 \pm 4.77	0.00*
DLLWLT(mm)	4.59 \pm 1.35	4.93 \pm 1.21	3.71 \pm 1.34	2.94 \pm 0.93	0.00*
LWL (mm)	5.21 \pm 2.37	6.00 \pm 1.35	4.23 \pm 0.55	5.18 \pm 0.64	0.00*
LSAr (mm^2)	75.23 \pm 54.81	75.00 \pm 18.02	46.29 \pm 12.63	60.28 \pm 20.23	0.00*
NoPL	3.17 \pm 0.38	3.10 \pm 0.48	3.13 \pm 0.43	2.93 \pm 0.37	0.01*
NoAbH (no/mm^2)	14.08 \pm 2.93	8.79 \pm 5.10	6.55 \pm 3.37	21.22 \pm 14.18	0.00*
NoAbS (no/mm^2)	142.33 \pm 14.63	116.28 \pm 17.39	140.22 \pm 15.74	111.17 \pm 20.24	0.00*
Stem					
SD (mm)	1.84 \pm 0.14	1.67 \pm 0.16	1.82 \pm 0.24	1.80 \pm 0.19	0.00*
SPT (μm)	30.11 \pm 2.18	28.87 \pm 2.18	28.98 \pm 2.01	29.30 \pm 2.58	0.10
SCT (μm)	409.44 \pm 31.77	361.43 \pm 52.63	400.03 \pm 53.72	351.58 \pm 35.40	0.00*

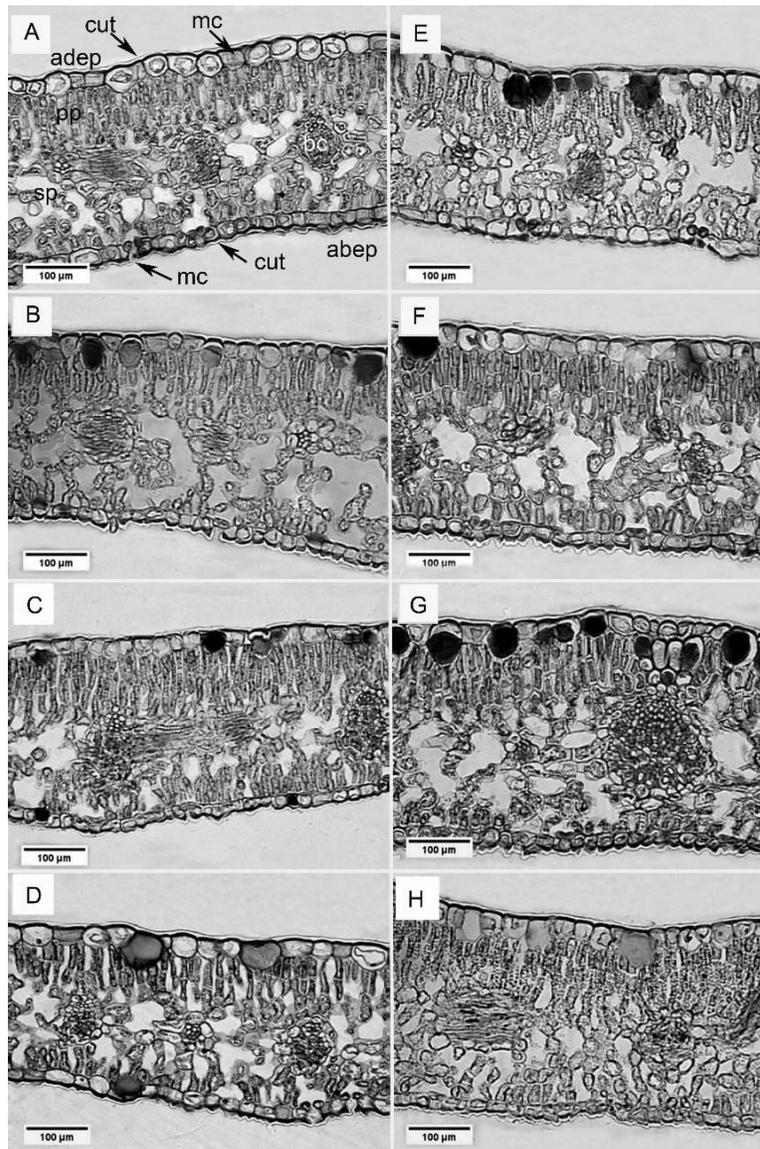


Fig. 2. Cross section of the leaves of plants. (A) Mt. Vlaška Planina, (B) Mt. Parnassus, (C) Mt. Chelmos, (D) Mt. Suva Planina, (E) Mt. Olimp, (F) Mt. Smolikas, (G) Mt. Durmitor, (H) Mt. Galičica; cut - cuticle, adept - adaxial epidermis, abep - abaxial epidermis, mc - mucilaginous cells, pp - palisade parenchyma, bs - vascular bundles, sp - spongy parenchyma, s - stomata. Scale bar: 100 µm.

ratio of the cortex and the whole stem diameter ranged from 0.388 to 0.445, even in a one-year-old stem, and this fitted well with Fahn's data for xeromorphic stems ranging from 0.271 to 0.833 (Fahn and Cutler 1992, Yiotis *et al.* 2006, Lakušić *et al.* 2010). The wide stem cortex around the narrow central cylinder may provide good protection for the vascular tissue in a time of low temperature and high temperature with drought (Jušković *et al.* 2010, Lakušić *et al.* 2010).

Kruskal-Wallis one-way analysis of variance for comparing means of characteristics showed statistically significant differences among analyzed samples for all characteristics except for the stem periderm thickness (Table 2).

Principal component analysis (PCA) results showed that the three PCA axes account for 50, 97% of the total variability, indicating that the structural variability of the studied populations is very complex. The first principal component (PC1) accounted for 25.82%, the second principal component (PC2) 14.69% and the third principal (PC3) component of explains 10.50% of the total variation. The most significant loadings on PC1, PC2 and PC3 were leaf thickness, mesophyll and spongy tissue thickness, surface area of abaxial and adaxial epidermal cells, surface area of abaxial stomata, leaf surface area, leaf length, and the distance between the largest leaf width and the leaf top. All variables had positive and very high contributions to PC1, which can be interpreted as a size variation (Table 3).

Table 3. Principal component analysis (PCA) of measured parameters of the analyzed populations of *D. oleoides*. The first three principal components accounted for 50.97% of the variance.

	Characters	PCA 1	PCA 2	PCA 3
Leaf anatomy	LT	0.73	-0.64	0.04
	TM	0.71	-0.69	0.01
	HAdE	-0.02	0.03	-0.41
	TP	0.59	-0.64	-0.06
	TS	0.68	-0.58	0.06
	HAbE	0.29	0.28	-0.43
	NoPL	0.19	0.02	0.04
Leaf surface characters	SArAdE	0.52	0.33	-0.55
	SArAbE	0.61	0.37	-0.47
	SArAbS	0.59	0.24	-0.49
	NoAbH	-0.40	0.02	-0.16
	NoAbS	-0.49	-0.35	0.29
	VD	-0.02	0.05	0.38
Leaf shape characters	LL	0.62	0.47	0.45
	DLLWLT	0.69	0.11	0.28
	LWL	0.61	0.47	0.45
	LSAr	0.62	0.50	0.47
	SD	0.29	0.03	0.02
Stem	SPT	-0.15	0.27	0.11
	SCT	0.32	0.00	-0.01
Variance explained by components		5.16	2.93	2.10
Percent of total variance		25.82	14.69	10.50

One part of variability of analyzed characters may be explained by adaptive response to different ranges and ecological factors, while the other part is caused by genetic and evolutionary factors. On the other hand, structural similarity of analyzed populations may be considered an

indicator of some kind of morpho-anatomical conservatism of this species, also recorded in other species from Balkan Peninsula (Jušković *et al.* 2010, Lakušić *et al.* 2010, Jakovljević *et al.* 2013). The stable form of leaf and stem structure is characteristic of plants that grow in stressful environments, as an adaptation that allows them to avoid production of structures too “expensive” to be sustained (Valladares *et al.* 2000).

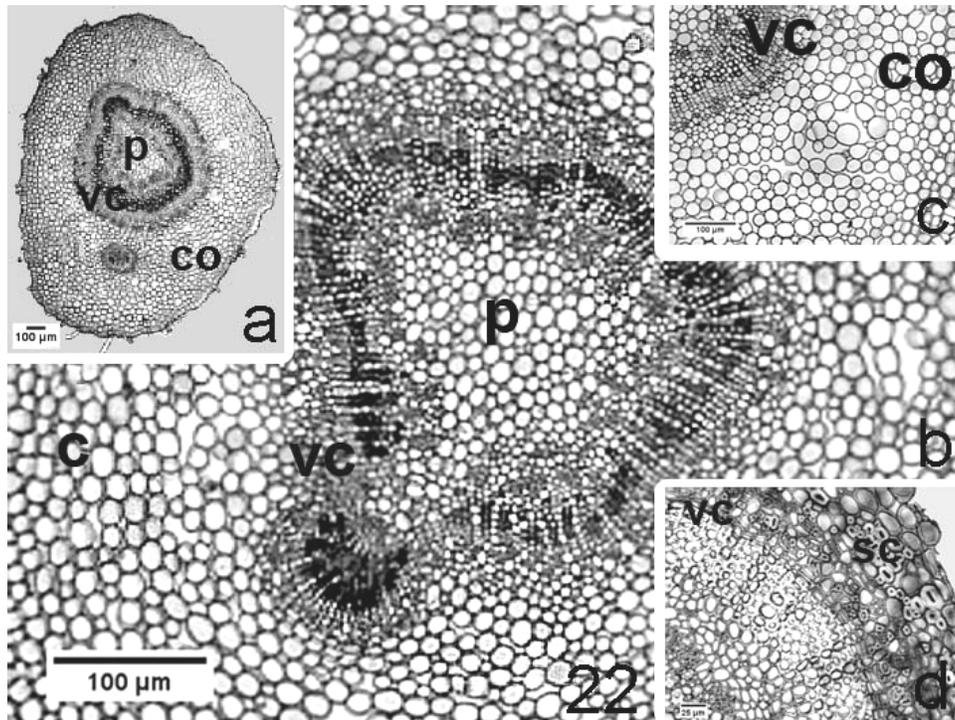


Fig. 3. Cross section of the one-year-old stem of *D. oleoides*. (a) The cross section stem, (b) the stem pith, (c) the stem cortex, (d) the vascular tissue. co - stem cortex, sc - sclerenchyma cells, vc - vascular cylinder, p - pith. Scale bars: 100 µm (a, b, c), 25 µm (d).

Numerous characteristics of leaves and stems of plants from eight distant populations indicated that species *D. oleoides* belongs to the xeromesophytic type of adaptation. More pronounced variability in some of the analyzed characters in plants from a certain population indicates both the variability of habitat and the phenotype plasticity and adaptive ability of species *D. oleoides*. Therefore, in order to ensure more efficient conservation of a species it is also necessary to protect their habitats. Anatomical studies of this genus are very limited and the results of our studies may contribute to a better knowledge the genus *Daphne*.

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