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LEAF ANATOMICAL VARIATION IN *CEPHALARIA LAEVIGATA* (DIPSACACEAE) UNDER DIFFERENT ECOLOGICAL CONDITIONS

KSENIJA JAKOVLJEVIĆ*, NEVENA KUZMANOVIĆ, SNEŽANA VUKOJIČIĆ and D. LAKUŠIĆ

Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade, 11000 Belgrade, Serbia

Abstract - The results of a multivariate morphometric study of leaf anatomical characters in different, geographically distant populations of the taxon *Cephalaria laevigata* from Serbia and Romania are presented with the aim to reveal the trends of population differentiation. Analyses were performed on a cross-section of 105 leaves collected from 10 populations. In order to establish the overall morphological variation and relationships between individuals from all populations, principal component analyses and canonical discriminant analysis were performed. Clustering analyses were carried out to explore whether the observed anatomical differences are a result of adaptive responses. Regression analysis was performed to identify the level of correlation between leaf anatomical characters and basic orographic, geological and bioclimatic habitat characteristics. Unexpectedly, in most of the characters there was discrepancy between leaf anatomy and climatic conditions, and the characters did not show significant regularities in the variability of their dimension neither in the vertical profile nor in relation to geological substrate.

Key words: Cephalaria laevigata, leaf anatomy, statistical analysis

INTRODUCTION

It is well known that climatic factors and geological substrate have a very strong influence on morpho-anatomical differentiation in plants. It is not rare that these environmental factors have a crucial impact on the development of special adaptive structures and the occurrence of a well-defined anatomical differentiation (Rôças and Scarano, 2001; Kuzmanović et al., 2009; Luković et al., 2009; Zorić et al., 2009; Cheng et al., 2010; Lakušić et al., 2010). Numerous anatomical studies have shown that climatic conditions and geological substrate have a strong impact on the anatomical differentiation of populations. In this sense, variations in leaf anatomical characteristics influenced by climatic condition have been widely documented (Chabot and Chabot, 1977; Abrams and Kubriske, 1990; Fahn, 1990; Fahn and Cutler, 1992; Bondada et al., 1996; Ferris et al., 1996; Bosabalidis and Kofidis, 2002; Bacelar et al., 2003; Anyia and Herzogi, 2004; Kulkarni et al., 2010). The significance of the serpentine geological substrate to the morpho-anatomical differentiation of plant populations, plant speciation, and, finally, the total florogenesis in wider geographical regions, was recognized a long time ago and also studied in detail by several authors (Kruckenberg, 1951, 1954, 1967, 1984; Walker, 1954; Whittaker et al., 1954; Tadros, 1957; Ritter-Studnička, 1968; Proctor and Woodell, 1975; Karataglis et al., 1982; Tatić and Veljović, 1990; Stevanović et al., 2003; Kuzmanović et al. 2012).

The genus *Cephalaria* Schrader (Dipsacaceae) comprises ca. 30 species, distributed mainly in the circum-Mediterranean region with an extension in the Middle East and Africa, from Ethiopia to South

Africa. A total of 14 species are distributed in Europe (Ferguson, 1976), mostly in S, SW and SE parts, extending northwards to the Czech Republic and Slovakia, westwards to Portugal and Spain, eastwards to Russia and southwards to Turkey.

Cephalaria laevigata (Waldst. & Kit.) Schrader belongs to the section Leucocephalae. This section is a member of *C.* subgen. *Denticarpus*, which presumably includes species whose achenes have eight apical teeth (Szabó, 1940). This Carpatho-Balkan endemic species is distributed in C and SW Romania, Bulgaria and Serbia (Ferguson, 1976). It inhabits mostly dry rocky places (class Festuco-Brometea Br.-Bl. et R. Tx. 1943), but it also grows in subalpine high mountain pastures and scree vegetation (classes Elyno-Seslerietea Br.-Bl. 1948 and Thlaspietea rotundifolii Br.-Bl. 1948). It inhabits wide altitudinal amplitudes, from lowlands to the montane vegetation belt, mostly on carbonates and more rarely on serpentines.

Bearing in mind that *C. laevigata* inhabits different regions, the aim of this study was to determine on the basis of leaf anatomy, the trends of population differentiation as well as to identify the level of correlation between variations in leaf traits and geological and bioclimatic habitat characteristics.

MATERIALS AND METHODS

Study area and plant sampling

Ten populations of *C. laevigata* (105 individuals) from an almost complete species range were sampled for the analysis of leaf anatomical traits. The samples were taken from Serbia (8 populations, 82 individuals) and Romania (2 populations, 23 individuals).

The plant material was collected in the period 2011-2012. Voucher specimens are deposited in BEOU (Index Herbariorum, http://sweetgum.nybg.org/ih/). Voucher numbers and collecting details are given in Table 1.

Leaf cross-section

Anatomical analyses of the leaves were done on permanent slides, prepared by the standard method for light microscopy. Frozen sections of leaves (105 samples) were cut on a Leica CM1859 cryostat (up to 45 μm thick). The sections were cleared in Parazone and thoroughly washed before staining in safranin (1% w/v in 50% ethanol) and alcian blue (1% w/v, aqueous). Permanent slides were mounted with Canada balsam after dehydration.

Morphometric analyses

Measurements were performed on the cross-sections of 105 leaves (each obtained from different individuals) collected in the field. A total of 20 characters were measured: 1. S – total leaf surface; 2. W – width of leaf; 3. TL – largest thickness of the leaf; 4. HCAd – height of cuticle of adaxial side; 5. HCAb – height of cuticle of abaxial side; 6. HEAd – height of epidermis of adaxial side; 7. HEAb – height of epidermis of abaxial side; 8. HPAd – height of palisade tissue of adaxial side; 9. HPAb – height of palisade tissue of abaxial side; 10. HS – height of sponge tissue; 11. HPar – height of parenchyma; 12. NVB – total number of vascular bundles; 13. NVBmaj – number of major vascular bundles; 14. NVBmin – number of minor vascular bundles; 15. HCVB – height of the central vascular bundle; 16. WCVB – width of the central vascular bundle; 17. HLVB – height of the largest lateral vascular bundle; 18. WLVB – width of the largest lateral vascular bundle; 19. WCr – width of the central rib; 20. HScCr – height of sclerenchyma at the central rib.

All measurements were made with Digimizer Image Analysis software (MedCalc Software, Belgium).

For each of the quantitative characters, descriptive statistics (mean, standard deviation, minimum, maximum and standard error, coefficient of variation) were calculated. The significance of differences between the studied populations was established by analysis of variances (ANOVA). For checking ANOVA results, Tukey's HSD post-hoc test was applied.

Principal component analysis (PCA) based on the complete data set (105 individuals × 20 characters) was performed to show the overall morphological variation and relationship between individuals from all populations. The hypothesis of anatomical separation of analyzed populations was tested by canonical discriminant analysis (CDA). Overall differences between the compared groups are presented by Mahalanobis distances, which are used for clustering on the basis of the UPGMA method. All statistical analyses were performed using the package Statistica 5.1 for Windows (StatSoft, 1997).

Cluster analysis of bioclimatic data

In order to evaluate the bioclimatic differentiation between the habitats of the 10 investigated populations, cluster analysis (UPGMA) was performed. Each location was characterized by 19 bioclimatic parameters, extracted from the WorldClim set of global climate layers (Hijmans et al., 2012).

Regression analysis

Regression analysis (linear regression) was performed to estimate the correlation between the variation of anatomical characters of *C. laevigata* and basic orographic, geological and bioclimatic habitat characteristics, as well as the geographic position of each population. The geographic positions were recorded using a hand-held Global Positioning System (GPS Garmin eTrex Vista® C). Orographic characteristics, which included altitude, slope and aspect, were calculated using DIVA-GIS 7.5 software. Bonferroni correction of the significance level was applied to the results of linear regression.

RESULTS

Basic anatomical characteristics

Regarding the basic leaf anatomical characteristics, there is a single layered epidermis on the upper and lower surface of the leaf (Fig. 1a). Palisade parenchyma cells are two-layered on the adaxial and 1-2 layered on the abaxial side. There are 2-3 layered spongy parenchymal cells. Stomata generally present on both sides of the leaf. Vascular bundles of the veins not accompanied by sclerenchyma; either embedded in the mesophyll or vertically transcurrent by thin-walled tissue. Crystals, when present, generally clustered (Fig. 1b). Trichomes are mostly glandular, each having a short stalk and a head composed of a few cells.

Measurement results

The surface of the leaves varied from 844 619.5 to 3 219 938 μm^2. The width of the leaf varied from 1 658.9 to 8 661.2 μm, and the central rib from 344.9 to 600.5 μm. The greatest thickness of the leaf fluctuated from 278.3 to 500.2 μm. The mesophyll is differentiated into spongy and palisade tissues. Thickness of the spongy tissue varied from 34.6 to 147.8 μm, while the palisade tissue thickness varied from 62.3 to 164 μm on the adaxial side and from 24.3 to 132 μm on the abaxial side. Epidermal cells on the upper side of the leaf were approximately equal in size to cells on the bottom. Their thickness varied from 8.8 to 34.1 μm on the adaxial side and from 8 to 30.8 μm on the abaxial side. The thickness of the cuticle layer was uniform on the adaxial and abaxial sides, and varied from 0.8 to 1.9 μm for the adaxial and from 0.7 to 1.7 μm for the abaxial side of the leaf (Table 2).

The total number of vascular bundles varied greatly, from 6 to 31. The number of major vascular bundles varied from 1 to 7. Minor vascular bundles were without or with hardly noticeable big tracheas. The number of minor vascular bundles varied from 4 to 27. The height of the central vascular bundle varied from 150.8 to 382.4 μm. The width of the central vascular bundle varied from 128.9 to 354.4 μm. The height of the largest lateral vascular bundle varied from 91.3 to 225.4 μm and the width of the largest lateral vascular bundle varied from 62.2 to 233.9 μm.

Morphometric analyses

The analysis of variation of particular anatomical

Table 1. Populations, number of specimens and habitat characteristics of *Cephalaria laevigata* used in this study

	Locality	Coordinate	Altitude (m)	Habitat	Substratum	Voucher	Individuals
1	Romania, Carrasova, Caras valley	44.9481 N, 22.145157 E	480	rocky grounds	limestone	BEOU, 36208	13
2	Romania, Baile Herculane, Prolaz	44.866317 N, 22.420641 E	516	screes	limestone	BEOU, 36207	10
3	Serbia, Đerdap Gorge, Golubac	44.661601 N, 21.684936 E	112	rocky grounds	limestone	BEOU, 36205	11
4	Serbia, Đerdap Gorge, Kazan	44.610457 N, 22.27941 E	317	rocky grounds	limestone	BEOU, 36206	8
5	Serbia, Kučevo	44..485663 N, 21.640195 E	287	road side	limestone	BEOU, 33996	10
6	Serbia, Vukan, Mali Vukan	44.28887 N, 21.53691 E	720	rocky grounds	limestone	BEOU, 34166	10
7	Serbia, Gornjak Gorge	44.27349 N, 21.53456 E	170	screes	limestone	BEOU, 34164	11
8	Serbia, Mt Suva planina, Sokolov kamen	43.20251 N, 22.13236 E	1447	rocky grounds	limestone	BEOU, 34172	11
9	Serbia, Mt Vlaška planina, peak	42.986783 N, 22.588017 E	1440	rocky grounds	limestone	BEOU, 36360	11
10	Serbia, Ibar valley, Maglič	43.61555 N, 20.55078 E	263	rocky grounds	serpentine	BEOU, 33968	10

characteristics in ten populations of *Cephalaria laevigata* showed that the characters exhibit a moderate degree of variability (13-47%). Within the group of highly variable characters, whose coefficients of variation (CV%) were higher than 35%, were the number of minor vascular bundles (47%) and total number of vascular bundles (39%). In contrast, the lowest coefficients of variation (13-15%) were found for characters related to the greatest thickness of the leaf, height of cuticle, parenchyma and width of the central rib (Table 2).

Analyses of variance showed that all the characters significantly contributed to the overall variability of the analyzed populations. However, a *post-hoc* test showed that most of the characters did not show any significant contribution to differentiation or contributed only to a minimum extent. The characters with the most significant contribution were the number of minor vascular bundles and the total number of vascular bundles (Table 2). For most characters, the populations from Gornjak Gorge and Mt. Vlaška Planina contributed the most to differentiation.

Principal component analysis (PCA), based on 20 measured characters from 10 populations, showed that the structural variability of the studied populations was quite complex, since the first three axes comprised only 55.52% of the total variability. Most of the variation was explained by the first axis (27.20%), 18.91% by the second and only 9.41% by the third. The first five principal components accounted for 70.30% of the total variance of all traits, indicating a low degree of correlation among the

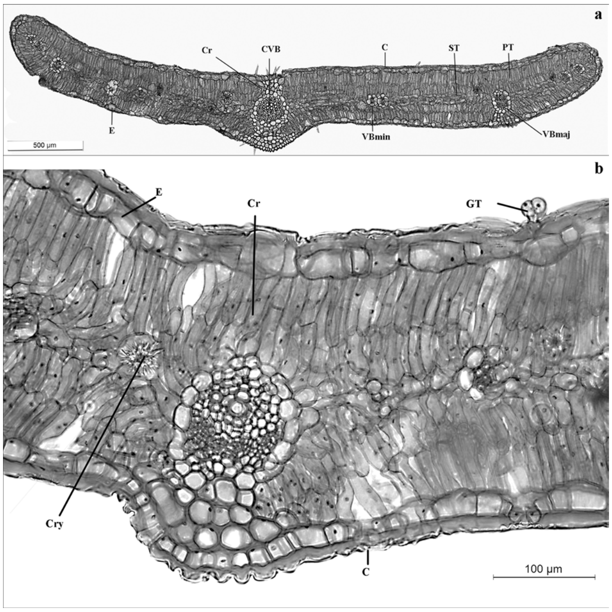

Fig. 1. Leaf cross-section of *Cephalaria laevigata*: a) in total; b) cross-section detail (VBmin – minor vascular bundle, VBmaj – major vascular bundle, PT – palisade tissue, ST – sponge tissue, Cr – central rib, CVB – central vascular bundle, E – epidermis, C – cuticle, GT – glandular trichome, Cry – crystal)

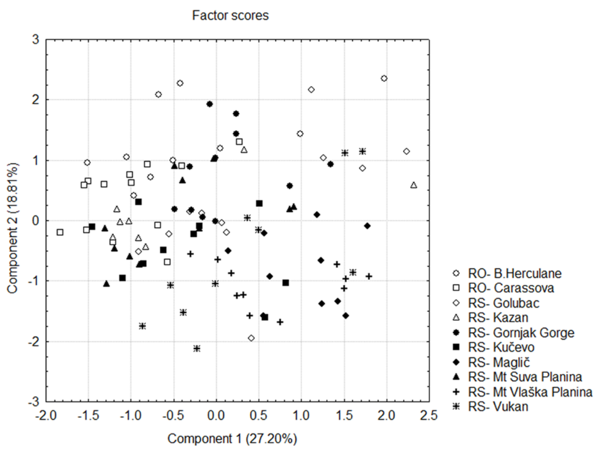

Fig. 2. Principal component analysis (PCA) based on 20 leaf anatomical characters of 10 populations of *Cephalaria laevigata* in investigated area

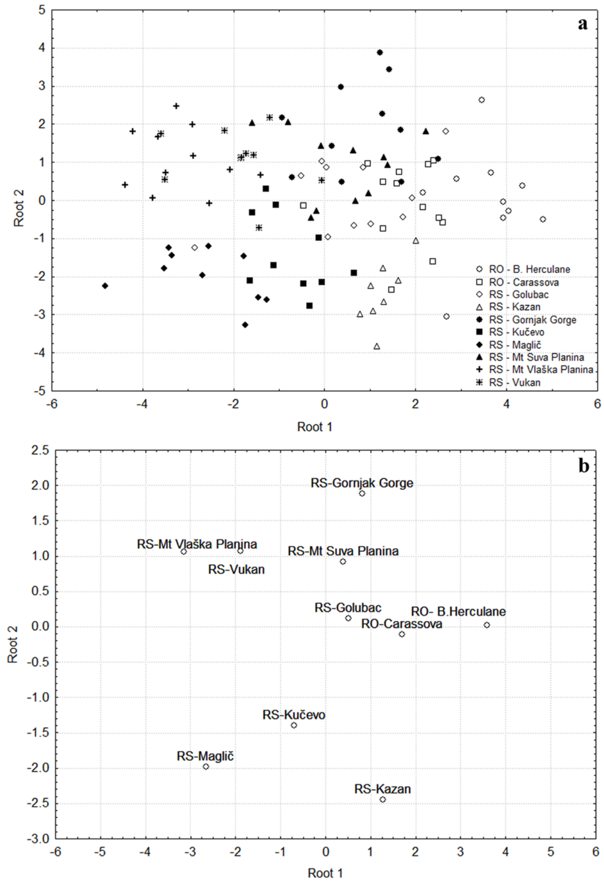

Fig. 3. Discriminant analysis based on: a) 20 leaf anatomical characters of 10 populations of *Cephalaria laevigata* in investigated area; b) multivariate mean values (centroids) of 10 populations

analyzed characteristics. Several characters were responsible for the differentiation along the first axis: total leaf surface, width of leaf, height and width of the largest vascular bundle and width of the leaf central rib. The second axis, responsible for 18.91% of the total variance, was influenced by greatest thickness of the leaf, height of palisade tissue of the adaxial side of the leaf blade and height of parenchyma. The results of principal component analysis showed that there is no clear differentiation between the populations. Namely, some indication of separation could be observed, regarding the populations from Maglič and Mt. Vlaška Planina, but not enough for differentiation in the real meaning of the expression (Fig. 2, Table 2).

Similar to the principal component analysis, the results of canonical discriminant analysis (CDA), were very complex and only some indications of separation could be observed (Fig. 3a). Populations

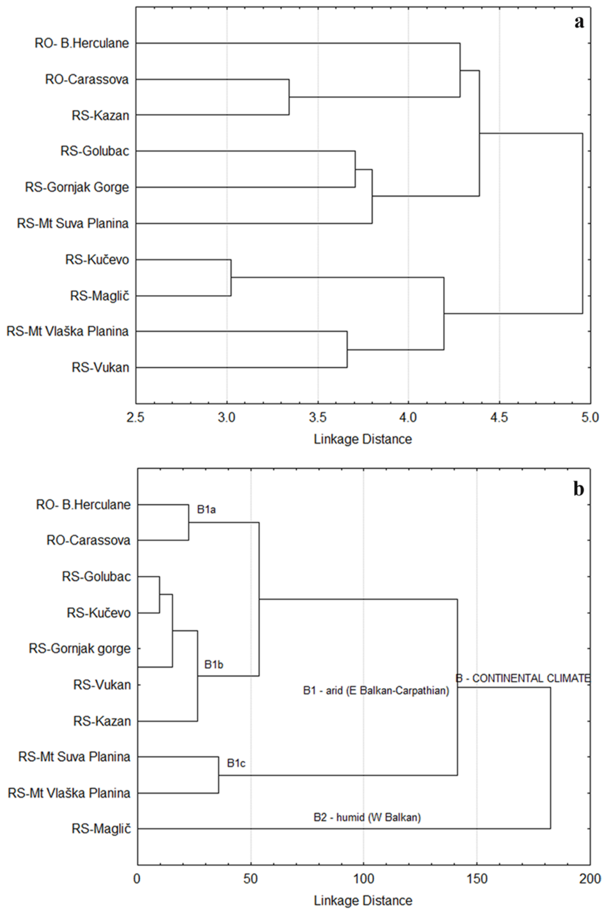

Fig. 4. Cluster analysis of 10 populations of *Cephalaria laevigata* based on: a) 20 leaf anatomical characters; b) habitat climatic characteristics

from Romania (Baile Herculane and Carassova) and NE Serbia (Kazan) formed one group along the first axis, while populations from Maglič, Vukan and Mt. Vlaška Planina were located at the negative part of the first axis forming the second group. There was an overlap between these two groups, including populations from Golubac, Gornjak Gorge, Kučevo and Mt. Suva Planina. Furthermore, along the second discriminant axis there was a trend of separation of the population from Kazan compared to the other populations on the positive part of the first axes, especially those from Golubac, Gornjak Gorge and Mt. Suva Planina. On the other side of the axis, its negative part, the populations from Maglič and Kučevo were separated from those from Vukan and Mt. Vlaška Planina. The plot of population centroids in the space of the first two vectors provided a much clearer view of variability, with larger distances among the populations (Fig. 3b).

The same pattern was shown by UPGMA cluster analysis, in which two completely separated clusters were formed, both consisting of two subgroups (Fig. 4a). The first cluster consisted of populations from Baile Herculane, Carassova and Kazan that formed one subgroup, and populations from Golubac, Gornjak Gorge and Mt. Suva Planina that formed the second subgroup. Two equal subgroups within the second cluster were formed by the populations from Kučevo and Maglič, included into the first, and populations from Vukan and Mt. Vlaška Planina included into the second subgroup.

Cluster analysis of bioclimatic data

The results of the cluster analysis based on bioclimatic data (Fig. 4b) showed that two main "types" (clusters) could be differentiated: habitats of the arid continental climate of the eastern Balkans and the lowland and hilly region of the South Carpathians (type B1), and habitats with a humid continental climate of the western Balkans (type B2). Within the B1 type, strong differentiation was apparent on 3 subtypes: B1a - Carpathian (B. Herculane and Carassova), B1b – lowland east Serbian (Golubac, Kučevo, Gornjak Gorge, Vukan and Kazan) and B1c – mountainous east Serbian (Mt. Suva Planina and Mt. Vlaška Planina).

As can be inferred from Table 3, these two clusters differed mainly in bioclimatic characteristic related to precipitation. Generally, the continental climate of the western Balkans (type B2) was characterized by slightly colder and more humid conditions than the arid continental climate of the eastern Balkans and the lowland and hilly region of the Carpathians (type B1). The greatest differences in precipitation were observed in the driest and the wettest quarters, as well as in the annual precipitation values.

Table 2. Basic statistic parameters of analyzed populations of *Cephalaria laevigata* in investigated area; Values are expressed in μm (Legend: Valid N – Number of measured cases, Mean – Mean value, Min. – Minimum value, Max. – Maximum value, Std. Dev – Standard Deviation, Std. Error – Standard Error, CV% – coefficient of variation, F - distance between individual distributions, p - probability of error, PC1, PC2 and PC3 - factor loadings of principal component analysis).

	Valid N	Mean	Minimum	Maximum	Std.Dev.	CV (%)	F	p	PCA1	PCA2	PCA3
*Total leaf surface	105	1676973	844619.5	3219938.0	491516.6	29.30975	2.88495	0.004672	0.851037	0.232182	-0.084824
*Width of leaf	105	5062	1658.9	8661.2	1367.1	27.00824	3.07979	0.002768	0.781887	-0.130118	-0.234651
*Largest thickness of the leaf	105	374	278.3	500.2	49.3	13.21094	3.40597	0.001150	0.320701	0.790165	0.283581
*Height of cuticle of adaxial side	105	1	0.8	1.9	0.2	14.65941	3.40239	0.001161	-0.113008	0.394705	-0.252677
*Height of cuticle of abaxial side	105	1	0.7	1.7	0.2	15.93853	7.75560	0.000000	-0.129859	0.403862	-0.145448
*Height of epiderm of adaxial side	105	22	8.8	34.1	4.0	18.17896	3.55752	0.000764	-0.341850	0.291231	-0.282817
*Height of epiderm of abaxial side	105	21	8.0	30.8	4.8	22.78849	8.82343	0.000000	-0.350706	0.331662	-0.456428
*Height of palisade tissue of adaxial side	105	118	62.3	164.0	22.4	19.01946	4.13647	0.000161	0.153112	0.788876	0.360892
*Height of palisade tissue of abaxial side	105	68	24.3	132.0	23.2	34.23301	3.87033	0.000329	0.313299	0.343172	0.569682
*Height of sponge tissue	105	79	34.6	147.8	21.6	27.29577	8.99722	0.000000	0.126787	0.554931	-0.264823
*Height of parenchyma	105	272	189.2	386.9	41.9	15.39032	3.30860	0.001495	0.276542	0.850797	0.343106
*Number of vascular bundles	105	15	6	31.0	6.0	39.02854	19.45582	0.000000	0.612912	-0.565310	0.268712
*Number of major vascular bundles	105	3	1	7.0	1.0	30.10959	3.94378	0.000270	0.426949	-0.259444	0.109626
*Number of minor vascular bundles	105	12	4	27.0	5.6	47.16486	16.73321	0.000000	0.573091	-0.552675	0.265163
*Height of the central vascular bundle	105	207	150.8	382.4	36.3	17.55129	4.76331	0.000031	0.656658	0.225314	-0.228320
*Width of the central vascular bundle CVB	105	216	128.9	354.4	45.3	21.01840	5.83274	0.000002	0.617733	-0.068940	-0.458847
*Height of the largest lateral vascular bundle	105	138	91.3	225.4	25.7	18.53779	5.87386	0.000002	0.738421	-0.046623	0.050971
*Width of the largest lateral vascular bundle	105	110	62.2	233.9	24.5	22.27808	6.72814	0.000000	0.744905	-0.066282	0.032154
*Width of the central rib	105	448	344.9	600.5	63.8	14.25194	3.98634	0.000241	0.757194	0.173997	-0.348743
*Height of sclerenchyma at the central rib	105	120	54.5	178.1	29.1	24.35179	8.21838	0.000000	0.382125	0.131868	-0.424312
*ANOVA effects significant at p < 0.05											

Table 3. Bioclimatic characteristics of the studied localities (Climate types B1a, B1b, B1c and B2 correspond to the groupings obtained from the cluster analysis of bioclimatic parameters (see Figure 4b)).

Bioclimatic parameters	Investigated area			Climatic type			
	Min	Avg	Max	Continental arid (B1a) Avg	Continental arid (B1b) Avg	Continental arid (B1c) Avg	Continetal humid (B2) Avg
BIO1	7.67	9.27	9.94	9.02	9.65	8.81	8.79
BIO2	8.91	9.32	10.18	8.95	9.24	9.94	9.28
BIO3	30.30	31.41	33.50	30.34	31.02	33.34	31.66
BIO4	716.11	762.87	788.64	778.70	773.92	732.71	736.31
BIO5	23.10	25.12	26.20	24.75	25.66	24.65	24.10
BIO6	-3.90	-4.56	-6.10	-4.75	-4.12	-5.15	-5.20
BIO7	29.20	29.68	30.40	29.50	29.78	29.80	29.30
BIO8	14.38	16.61	17.38	16.58	17.13	15.73	15.78
BIO9	0.22	2.36	13.27	0.47	1.30	7.63	0.88
BIO10	16.18	18.30	19.00	18.22	18.80	17.50	17.52
BIO11	-1.37	-0.43	0.43	-0.87	-0.20	-0.47	-0.62
BIO12	621.00	711.40	857.00	739.50	706.60	622.50	857.00
BIO13	72.00	92.00	108.00	106.00	93.40	72.00	97.00
BIO14	40.00	44.40	54.00	44.50	43.80	41.00	54.00
BIO15	18.08	25.54	32.53	31.95	26.99	19.25	18.08
BIO16	193.00	244.30	277.00	272.50	248.20	194.00	269.00
BIO17	131.00	137.60	173.00	134.00	134.60	131.00	173.00
BIO18	166.00	220.90	259.00	254.00	225.40	167.00	240.00
BIO19	142.00	149.20	184.00	145.50	146.60	142.00	184.00

Regression analysis (linear regression)

The results of regression analysis (Table 4) indicated that 9 of 23 bioclimatic parameters displayed a statistically significant correlation with most of the analyzed anatomical characters. With regard to all the analyzed anatomical features, the most significant correlation was found between the mean temperature of the wettest (BIO8) and driest quarters (BIO9) and precipitation seasonality (BIO15). However, the anatomical variability was not significantly correlated with the largest number of bioclimatic factors. As could be inferred from regression analysis, leaf surface and width, greatest thickness of the leaf, the height of the cuticle, epidermis and parenchyma of the adaxial and abaxial sides (S, W, TL, HCAd, HCAb, HEAd, HEAb, HPAd and HPAb) displayed the least significant correlation with the analyzed environmental factors. In contrast, the greatest dependency in regard to environmental factors was shown by characters related to vascular bundles: total number of vascular bundles, number of minor vascular bundles (NVB and NVBmin), height and width of the central and the largest lateral vascular bundles (HCVB, WCVB, HLVB and WLVB) and width of the central rib (WCr).

DISCUSSION

Despite the significant differences in climatic conditions and type of geological substrate in which the analyzed populations are recorded, the variability

Table 4. Summary statistics of regression (R^2) for independent geological, orographical and bioclimatic variables (ALT - Altitude, BIO3 – Isothermality (2/7) (* 100), BIO4 – Temperature seasonality (STD * 100), BIO7 – Temperature Annual Range (BIO5-BIO6), BIO8 – Mean temperature of the wettest quarter, BIO9 – Mean temperature during the driest quarter, BIO10 – Mean temperature of warmest quarter, BIO15 – Precipitation seasonality (coefficient of variation), BIO17 – Precipitation of driest quarter, BIO19 – Precipitation during the coldest quarter, * – P<0.05; ** – P<0.01, *** – P<0.001 after Bonferroni correction)

	ALT	BIO3	BIO4	BIO8	BIO9	BIO10	BIO15	BIO17	BIO19
Total leaf surface	0.010	0.012	0.031	0.014	0.006	0.012	0.029	0.018	0.020
Width of leaf	0.030	0.043	0.078	0.034	0.034	0.030	0.082	0.028	0.030
Largest thickness of the leaf	0.027	0.039	0.038	0.026	0.076	0.023	0.030	0.004	0.004
Height of cuticle of adaxial side	0.086	0.045	0.094	0.079	0.056	0.078	0.075	0.032	0.027
Height of cuticle of abaxial side	0.014	0.028	0.022	0.004	0.002	0.003	0.066	0.033	0.029
Height of epiderm of adaxial side	0.014	0.034	0.046	0.010	0.009	0.008	0.090	0.046	0.046
Height of epiderm of abaxial side	0.002	0.140**	0.078	0.000	0.010	0.001	0.223***	0.030	0.031
Height of palisade tissue of adaxial side	0.050	0.045	0.076	0.058	0.107*	0.052	0.053	0.001	0.001
Height of palisade tissue of abaxial side	0.042	0.080	0.082	0.029	0.006	0.028	0.074	0.020	0.016
Height of sponge tissue	0.188***	0.285***	0.271***	0.143**	0.180***	0.132**	0.247***	0.005	0.003
Height of parenchyma	0.081	0.046	0.082	0.083	0.119	0.078	0.051	0.001	0.001
Number of vascular bundles	0.162***	0.178***	0.364***	0.177***	0.143**	0.161***	0.375***	0.183***	0.183***
Number of major vascular bundles	0.017	0.010	0.055	0.024	0.012	0.021	0.073	0.098	0.105*
Number of minor vascular bundles	0.163***	0.184***	0.357***	0.174***	0.146**	0.160**	0.360***	0.157**	0.156**
Height of the central vascular bundle	0.038	0.016	0.003	0.045	0.009	0.050	0.001	0.027	0.024
Width of the central vascular bundle CVB	0.138**	0.007	0.075	0.145**	0.100	0.144**	0.028	0.036	0.033
Height of the largest lateral vascular bundle	0.009	0.002	0.025	0.018	0.000	0.016	0.036	0.169***	0.178***
Width of the largest lateral vascular bundle	0.041	0.000	0.061	0.053	0.006	0.051	0.067	0.224***	0.233***
Width of the central rib	0.138**	0.036	0.113*	0.169***	0.182***	0.167***	0.027	0.000	0.000
Height of sclerenchyma at the central rib	0.097	0.096	0.097	0.109*	0.173***	0.104*	0.017	0.063	0.063

described by multivariate statistical analysis showed that at the anatomical level clear differences could not be observed between the populations of the species *C. laevigata*. There were some indications of slight grouping of the populations, but not enough for excessive levels of separation. Therefore, it cannot be easily determined which are the factors that affect the grouping and separation.

Many studies of leaf anatomy, performed on those species whose populations inhabit ecologically and geographically very distant and heterogeneous habitats, showed that populations are anatomically clearly differentiated as well. For example, in most cases habitat characteristics (climate, geological substrate, the type of the soil, the type of community) had a crucial influence on the appearance of well-defined anatomical groups, and differences between the groups were interpreted as adaptations to environmental conditions (Fahn, 1964; Todorović and Stevanović, 1994; Bosabalidis and Kofidis, 2002; Lakušić et al., 2006, 2010; Kuzmanović et al., 2012). Much less common were cases where determined differentiation based on anatomical variability was not significantly correlated with ecological and geographical factors, but seem to be related to genetic differentiation within the populations of the analyzed taxon (Mereda et al., 2011; Rakić et al., 2012; Jakovljević et al., 2013). Variations in the anatomy of *C. laevigata* leaves do not fit into any of these two mentioned patterns. Namely, given that the climatic and geological conditions are very heterogeneous, it was expected that the analysis would show the existence of differentiated groups whose differences could be explained as adaptations to environmental conditions. Contrary to expectations, the analyses have not resulted in clear anatomical differentiation, but only in slight variability, so this case represents a third possible pattern of leaf anatomy variation in heterogeneous environments.

Although our results indicate that anatomical variability is not significantly correlated to the type of geological substratum or geographical position, and that a clear geographical or ecological pattern does not exist, some anatomical characters vary considerably according to varying environmental conditions. Thus, characters that varied greatly were the total number of vascular bundles and number of minor vascular bundles. The largest number was observed in serpentine populations from Maglič, and the lowest in populations from the northernmost part of the *C. laevigata* range (NE Serbia and W Romania). These results are consistent with the results of cluster analysis of bioclimatic data. In addition, regression analysis showed that number of total and minor vascular bundles is highly correlated to precipitation, and as shown in Table 3, Maglič was characterized by the most humid conditions of all the studied localities. However, in most of the characters there was discrepancy between the leaf anatomy and the climatic conditions in the studied area. The characters did not show significant regularities in variability of their dimension neither in the vertical profile nor in relation to geological substrate.

Finally, in order to reveal the basis of the described leaf anatomical plasticity, it is necessary to carry out a comparative morphological analysis of the reproductive organs and a complete molecular and phylogenetic study. This work is in progress.

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