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Original scientific paper

BETWEEN-CLONE, BETWEEN-LEAF AND WITHIN-LEAF VARIATION IN LEAF EPIDERMIS TRAITS IN *Iris pumila* CLONES

Danijela MILJKOVIĆ [1*], Stevan AVRAMOV [1], Vukica VUJIĆ [2], Luka RUBINJONI [2], Nataša KLISARIĆ BARISIĆ [1], Uroš ŽIVKOVIĆ [1], Aleksej TARASJEV [1]

[1]Institute for Biological Research "S. Stanković",University of Belgrade, Belgrade, Serbia
[2] Faculty of Biology, University of Belgrade, Belgrade, Serbia

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The goal of this study was to analyze variation and covariation in epidermal characteristics (epidermal cell density -ECD, stomata density - SD, and stomata index - SI) on *Iris pumila* clones on between-clone, between-leaf and within-leaf levels. ECD (similar to the pattern previously observed for SD) increased from the base to the top of leaf, while SI remained constant. Results of profile analyses indicated that clones, individual plants whitin clones (ramets), and three successive leaves on the same plant were not significantly different for examined characteristics, but genetic variation for position effect was detected (significant Zone x clone interaction). Results of the contrast analysis confirmed differences between the base and middle leaf positions for ECD (similar to those for SD) as well as between clone variation for those differences. Observed differences between leaf zones and correlations between analyzed traits were mostly consistent with the expansion hypothesis of stomata differentiation.

Key words: epidermal traits, stomatal density, epidermal cell density, stomata index, *Iris pumila*.

INTRODUCTION

Leaf epidermis traits such as epidermal cell density (ECD), stomata density (SD) and their distribution over the leaf surface, are essential for plants and their relationship with key environmental factors (XU and ZHOU, 2008). Leaf epidermis is interspersed with stomata, as a vital gate between plant and surrounding environmental conditions (such as light, water status,

Corresponding author: Miljković Danijela, Department of Evolutionary Biology,Institute for Biological Research, "S. Stanković"University of Belgrade,Despota Stefana 142,11000 Belgrade, Serbia,phone: +381 11 207 83 76, Fax: +381 11 761 433,Mobtel.:+381 64 24 17 228,e-mail: danijela.miljkovic@ibiss.bg.ac.rs

and CO_2 levels). Stomata are the most important control system of CO_2 influx and water vapor efflux, and as such are the principal leaf epidermal structure for the survival of land plants (NADEAU and SACK, 2002). Investigation of the pattern of stomata distribution, from a whole plant perspective, is very useful because both the epidermal cell number and stomata number present a response to the plant/environment interaction (GALMÉS *et al*. 2007; AVRAMOV *et al.* 2007; XU and ZHOU; 2008. LORANGER and SHIPLEY, 2010; TARASJEV *et al*. 2012).

The pattern of stomata density (of the aerial organs including leaves, stems, flowers, fruits and seeds) is organ and species specific, and depends on the phase of organ's development (young organs have fewer total stomata than mature organs). The frequency and positioning of stomata is also affected by environmental factors (VATÉN and BERGMANN, 2011). Stomata patterns within and between leaves influence plant productivity and water use efficiency (WEYERS and LAWSON 1997). As the plant grows, the epidermis expands by cell division and cell elongation. Stoma pattering presents a result of interaction between plant and the environment, that modified and regulated the stomata density during leaf development (GEISLER and SACK, 2002; POMPELLI *et al*. 2010). Specific epidermal cells differentiate into stomata guard cells, and their correct distribution on leaf surface is important for leaf physiological function in environmental conditions (BROWNLEE, 2000). SD often decreases as neighboring epidermal cells expand during maturation (VATÉN and BERGMANN, 2011).

Large differences in stomata characteristics were found among species and cultivars (FERRIS *et al.* 2002; LORANGER and SHIPLEY, 2010), as well as for other factors such as light intensity (TUCIĆ *et al*. 1999; CAO and BOOTH, 2001; AVRAMOV *et al.* 2007), nutrient availability (CROXDALE, 2000), or interpopulation variability (BATOS *et al.* 2010).

Monocots and dicots take different paths of stomata development. Numerous studies were published in recent years, with intent to define development proces of stomata, their ontogeny and structure in monocots (ABUBAKAR *et al.* 2011 and references therein). In monocotyledon leaves with polarized growth, stomata are arranged in rows along the main leaf axis and they are characterized by a temporal and spatial gradient. The linear proces of growth (from the "division and expansion zone" in the base, to the middle and top of leaf as the "elongation zone") is characterized for monocot leaves (VAN VOLKENBURGH *et al*. 1998; PEMAC and AVRAMOV, 2001). The dissipated distribution of stomata is characterized for dicot leaf type (CROXDALE, 2000; GEISLER and SACK 2002).

Stomata patterning has been studied in monocotyledons (CHIN *et al.* 1995; CROXDALE, 2000) and, in most cases, the formation of stomata on any mature leaf reflects the contribution of cell divisions and expansion to leaf growth (KOUWENBERG *et al*. 2004). The parallel rows of stomata and epidermal cells that are located between the veins are typical for the *Iris pumila* monocotyledon type leaf.

The ratio between stomata and epidermal cell density, stomata index (SI) is a sensitive parameter that quantifies the effects of epidermal cell expansion due to contrasting light conditions, leaf age, and other environmental conditions (POOLE and KURSCHNER, 1999). Possible source of variation of stomata pattern between individual plants and individual leaves is the different time and mode of leaf growth of monocotyledonous and dicotyledonous leaf types (CROXDALE, 2000). The complexity of this problem indicates that a large number of stratified samples is required to obtain a truly representative value (WEYERS and LAWSON, 1997).

The coordination of two tightly controlled and dynamic processes: cell proliferation and subsequent cell expansion during leaf growth ultimately determines leaf size and shape (SKIRYCZ

et al 2011). The factors that are responsible for stomata differentiation are not completely investigated, but observed patterns can be result of differences in differentiation itself, result of irregular epidermal cell expansion, or result of both (the differentiation, expansion and mixed hypotheses (BEERLING and CHALONER ,1993).

In this work we studied variability and covariation of epidermal traits on *Iris pumila*, monocot clonal plant that was previously extensively used in genetic, ecological and evolutionary studies (TARASJEV *et al.* 2012 and references therein). The goal of this preliminary study was to:

- Examine is there significant between-leaf variation (between three successive leaves) , and within-leaf variation (between the three sampling zones -base, middle and top of the leaf) for analyzed epidermal characteristics.

- Examine is there significant variation between clones in analyzed epidermal characteristics, in order to check for genetic variability of analyzed traits

- Estimate significance and sign of the correlations between stomata density, epidermal cell density and stomata index, in order to test the hypothesis of stomata differences development over the leaf surface of *Iris pumila*.

MATERIALS AND METHODS

Iris pumila is a rhizomatous perennial herb and is very abundant in the dune system at Deliblato Sands, a situated about 50-km northeast of Belgrade (44^o 48' N, 20^o 58' E). Three successive last fully expanded leaves were examined, from three ramets of the same clone. The first leaf is the youngest and the third is the oldest (the period of new leaf appearance is about five to seven days). Stomata frequency could be biased due to the counting of fields overlapping vein areas (which had a low number or zero stoma) (LARKIN *et al.* 1997). Total leaf stomata census is inpractical to count, which is why an optimal sampling strategy must be established. The number of readings per area can be estimated using objective statistical criteria. In this experiment, samples were taken from three zones on every leaf: the base (first cm of the second quarter of leaf); the middle (first cm of the third quarter of leaf), and the top (first cm of fourth quarter of leaf). To obtain the impression of leaf surface replicas of harvested leaf material were made, using clear nail polish applied to the leaf surface. The clear nail polish was painted across the selected leaf part, with a 0.5cm-wide band.

The dry film of polish was pulled from the leaf by a piece of adhesive tape and mounted on a microscope slide. With this replica technique stomata and epidermal cells were visible and easy for counting (Figure 1). Stomata density (SD) and epidermal cell density (ECD) were sampled in 20 randomly chosen microscope fields at each of three zones on individual leaf. Microscope measurements of these preparations were made in a band across the leaf in the middle of a segment, using the Olympus "Vanox" microscope (magnification of 6.7 x 10). Number of stomata and epidermal cells on a given zone of the leaf were counted by frames of dimension 700□m x 436□m (area of 0.327mm^2) (software UTHSCSA Image Tool 3.00). Methodology of cell count was previously given in PEMAC and AVRAMOV (2001).

Stomata index (SI) was calculated according to the formula of SALISBURY 1927 (after CHEN *et al.* 2001; ABUBAKAR *et al.* 2011)

$$SI= (SD/ECD+SD) x100$$

where SD (stomata density) is the number of stomata per unit leaf area and ECD (epidermal cell density) is the number of epidermal cells per unit leaf area.

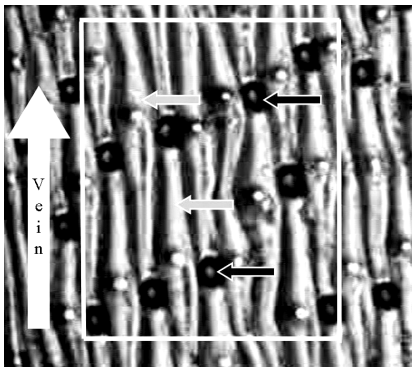

Figure 1. Images of the abaxial surface of *Iris pumila* leaf showing epidermal cells (grey arrows) and stomata (dark grey arrows) and vein (white arrow). White square presents the sampling area for the counting of stomata.

Scheffe's test was used to assess differences between the analyzed epidermal traits for different zones on leaf. Traits from different zones could be treated as a same trait that was repeated thought the time of each leaf development. According to this assumption we analyzed data using multivariate repeated-measures analysis (REPEATED option in SAS procedure). The samples are referred to as the subjects and repeated observations (zones: base, middle, and top) on each leaf as the "within-subject" factor, by repeated terminology (VON ENDE, 1993), while the individual clone was designated as the "between-subject" factor. The pattern of response of the within-subject factor zones (base, middle, top) was analyzed using the profile analysis, which transforms the within-subject repeated observations to a set of differences (contrasts of first and second order), and then makes the univariate analysis on the contrasts.

Linear correlation coefficients between leaf characteristics were calculated for every zone on each of the three successive leaves (PROC CORR). The correlations between SD, ECD and SI may be used to test the hypotheses on the mechanisms of contributing to spatial variation in epidermal characteristics. All statistical analyses were performed using SAS 9.1 *(*SAS, Cary, NC).

RESULTS

Statistically significant differences in epidermal traits between three successive leaves in the three zones along the leaf have not been obtained (Table 1), and therefore the leaf mean values for mean stomata density, epidermal cell density and stomata index are presented (Figure 2). The stomata density (SD) had a range from 61.23 (#/mm^2) for the base to the 94.57 (#/mm^2) to the top. ECD had a same pattern of gradients for all three successive leaves: 145.45 (#/mm^2) for the base to the 216.40 (#/mm^2) to the top. Stomata index (SI) was not significantly different between leaves and, while showed significant differences between positions according to profile analysis (Table 1), no difference was found by Scheffe's test (Figure 2).

Table 1. Results of profile analysis (MANOVA) epidermal cell density (ECD) and stomatal index (SI) observed across three succesive leaves and three adjacent positions on each of Iris pumila leaves.

Source	df	ECD (#/mm^2) F-value	SI (%) F-value
Between subject			
clone	5	1.65	2.79
Within subject			
Leaf	2	1.90	0.68
Leaf x clone	10	0.66	0.55
Zone	2	75.27****	5.87**
Zone x clone	10	2.87*	1.09
Leaf x Zone	4	1.43	0.98
Leaf x Zone x clone	20	1.12	0.69

* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001

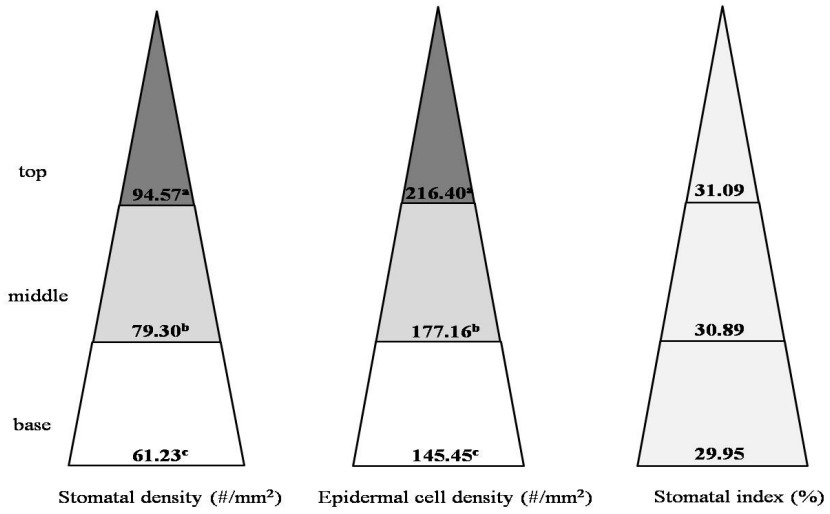

Figure 2. Mean values of SD, ED and SI on leaf surface of *Iris pumila* leaves on three sampling zones base, middle and top.

According the REPEATED profile analysis (Table 1), between-subject source variation was not significantly different among clones for all of the examined traits (SD - PEMAC and AVRAMOV 2001, ECD and SI (Table 1)). For within-subject factor, zones, statistically significant difference was observed for all three traits ($p < 0.05$). Different zones on the same leaf vary among clones, according the observed statistically significant zones and clone interaction for SD (PEMAC and AVRAMOV, 2001) and ECD ($p < 0.05$) (Table 1).

Table 2. *Individual ANOVAs on each of the contrasts of within-subject factor between three adjacent zones on each leaf first order (Base-Middle) and second order (Middle-Top).*

	df	ECD (#/mm^2) F-value	SI (%) F-value
Contrast variable			
First order (Base-Middle)			
Mean	1	199.46****	2.20
clone	5	6.29****	0.73
plant (clone)	12	1.85	0.87
Leaf	2	1.27	0.88
Contrast variable			
Second order (Middle-Top)			
Mean	1	0.04	0.79
clone	5	1.65	0.55
plant (clone)	12	1.01	0.39
Leaf	2	1.53	0.38

* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001

Individual Repeated ANOVA was computed on each of the contrast variables: first order (base-middle) and second order (middle-top) contrast variable (Table 2). Mean effects were statistically significant for SD and ECD, indicating that these two traits display a gradient along the leaf for base-middle contrast. A significant clone effect was observed for the contrast variable of first order (Base-Middle) for SD and ECD. This result suggests that the zone patterns of SD and ECD were clone specific, at the leaf part between base and middle zone (SD – (PEMAC and AVRAMOV, 2001), ECD, and SI (Table 2)).

A significant positive linear relationship was found between SD and ED ($r_{SD\ vs.\ ECD} = 0.65$; $p<0.0001$) and significant negative correlation between ECD and SI ($r_{ECD\ and\ SI} = - 0.60$;

p<0.0001) (Figure 3). In both cases lines produced for different leaves were parallel, e.g relationship between traits was similar in successive leaves.

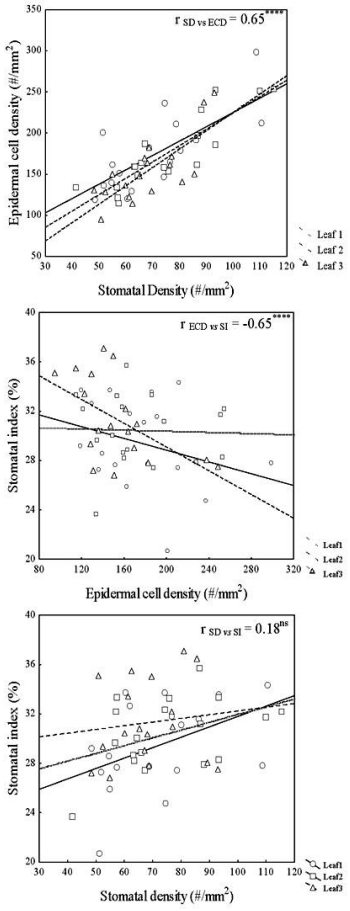

Figure 3. Correlation analysis among epidermal traits for three successive leaves of *Iris pumila.* Scatter plots showing the relation between epidermal cells, stomatal density and stomatal index. The trandlines and values of Pearson's coefficients for all three comparations of epidermal traits were also presented ($^{ns}P > 0.05$, $^{****}P < 0.0001$). Individual values of epidermal traits circles present data for first leaf, squares for second and triangles for third leaf.

DISCUSSION

Fully expanded leaves of plants grown continuously in the same environment are expected to have similar SD irrespective of their positions on the plant (GAY and HURD, 1975).

After stomata initiation in complete, leaf expansion could still be modified by a change in light level. The Stomata density is a trait that can change after exposure to new light conditions, and SD modifications were observed after the light regime change (GAY and HURD, 1975, TUCIĆ *et al.* 2003). Differences between the three successive leaves were tested in this study because first leaf was the youngest (relatively to the second and third leaf), and we did not detect differences in epidermal traits patterns between three successively developed leaves (Table 1). In some species, differences between cultivars, clones and genotypes in stomata parameters were observed (WILLMER and FRICKER 1996; MISHRA, 1997, PEKŞEN *et al*. 2006). In our study there was no difference between clones for analyzed epidermal traits (no significant clone effect in Table 1) but we detected significant Zone x clone interactions for SD and ECD (Table 1. and PEMAC and AVRAMOV, 2001) that was consequence of genetic difference in differentiation between base and the middle part of *I.pumila* leaves (significant Clone effect - Table 2). Therefore, genetic aspects should be taken into account in further analysis of epidermal traits variation in *I. pumila*.

Stomata density is often not found to be constant over the whole leaf surface (GITZ and BAKER, 2009). Leaf elongation in monocots is a linear process and new cells were produced in a basal meristem so leaf size can vary with cell length, cell number, or both (VAN VOLKENBURGH *et al.* 1998). Cells are displaced away from the base, in parallel longitudinal cell files, by the continuous production and elongation of new cells in this zone. After leaving the 'division and expansion zone' these cells go through the 'elongation zone' (SCHNYDER *et al*. 1990). In *Iris pumila* leaves, stomata were denser on top of the leaf (PEMAC and AVRAMOV, 2001) and this study showed the same pattern for epidermal cells, too (Figure 2, Tables 1 and 2).

The factors that are responsible for stomata differentiation are not completely investigated (BEERLING and CHALONER, 1993) but three possible explanations for stomata differences over leaf surfaces are given:

1. Stomata differentiation was uneven over the leaf surface, resulting in different densities of stomata, or epidermal cells, or both (the differentiation hypotheses).

2. The leaf undertakes irregular expansion after the cells had differentiated, resulting in uneven spacing of the stomata (the expansion hypothesis).

3. Spatial variation in both leaf expansion and stomata differentiation contributed to the uneven spacing of stomata (the mixed differentiation and expansion hypothesis).

To test this hypothesis, correlation between SD, ECD, SI was examined (Figure 3). Our results (statistically positive correlation between SD and ECD, and negative correlation between ECD and SI, as well as similar patterns of differentiation between zones both for SD and ECD) indicate the differences of stomata density mainly confirm predictions from expansion hypothesis (different expansion of epidermal cells, with larger cells reducing density – BOCCALANDRO, 2009). The cells at the top of the leaf were smaller then cells at the base, which is the reason why stomata and epidermal cell density differs between zones, and changes of stomata density depend on epidermal cell size. The positive correlation between SD and ECD has been previously reported in other papers (SALISBURY, 1927; POMPELLI *et al*. 2010). and for example, detected in the earlier studies in *Alnus glutinosa*, a deciduous broad-leaved tree (POOLE *et al*. 2000) and of various vine species (TAY and FURUKAWA, 2008). Other studies also found that stomata index remains relatively constant (SALISBURY, 1927; TICHA, 1982, ROYER 2001). However, significant zone effect for SI in this study (Table 1), while not detected in pairwise

comparisons by Scheffe's test (Figure 2) indicate that there is possibility that differentiation also has some role in observed patterns.

Stomata end epidermal frequency is correlated while Because of the observed within–leaf variation in epidermal traits, for future examination of epidermal traits in *Iris pumila* (and especially for their analysis as means of plant adaptation to different environmental conditions) we suggest that it would better to take measurements on two different positions of the *Iris pumila* leaf (middle and top).

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VARIRANJE EPIDERMALNIH OSOBINA LISTA *Iris pumila* KLONOVA (IZMEĐU KLONOVA, IZMEĐU LISTOVA I U OKVIRU LISTA)

Danijela MILJKOVIĆ [1 *], Stevan AVRAMOV [1], Vukica VUJIĆ [2], Luka RUBINJONI [2], Nataša KLISARIĆ BARISIĆ [1], Uroš ŽIVKOVIĆ [1], Aleksej TARASJEV[1]

[1]*Institut* za biološka istraživanja "S. Stanković",Univerzitet u Beogradu, Beograd, Srbija,
[2] Biološki fakultet, Univerzitet u Beogradu, Beograd, Srbija

Izvod

Cilj ovog istraživanja je analiza variranja i kovariranja epidermalnih osobina lista (gustina epidermalnih ćelija ECD, gustina stoma SD i stomatalni indeks SI) između i unutar lista kao i između klonova *Iris pumila*. Gustina epidermalnih ćelija (slično obrascu gustina stoma, ranije publikovano) raste od baze ka vrhu lista, dok vrednost stomatalnog indeksa ostaje konstantna. Rezultati Profile analize (MANOVA) ukazuju da se klonovi, individualne biljke svakog klona (rameta), kao i tri sukcesivna lista na svakoj od biljaka ne razlikuju za ispitivane karakteristike epidermisa lista (epidermalna gustina lista i stomatalni indeks). Međutim, variranje unutar lista na različitim pozicijama lista (baza, sredina i vrh lista), uslovljeno razlikama genotipa (klonovima) je potvrđeno statistički značajnom interakcijom zona (pozicija na listu) x klon ($P < 0.05$). Razlike između baze i sredine lista za gustinu epidermalnih ćelija ($F = 199.46$, $P < 0.0001$), kao i značajna razlika između klonova između pomenutih pozicija je prema rezultatima analize kontrasta statistički značajna ($F = 6.29$, $P < 0.001$). Dobijene razlike između pozicija na listu i korelacija između analiziranih epidermalnih osobina lista *I.pumila* klonova su potvrda hipoteze širenja epidermalnih ćelija prilikom diferencijacije stoma unutar lista.

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