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METHOD FOR ESTIMATING STOMATAL DENSITY IN GRASS-TYPE LEAVES: AN EXAMPLE USING IRIS PUMILA. **Danijela Pemac and S. Avramov,** *Department of Evolutionary Biology, Institute for Biological Research „Siniša Stankovic"*, 11060 Belgrade, Yugoslavia.

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It has been experimentally demonstrated that stomatal density can vary greatly over the leaf surface. Large differences were found among species and cultivars (Meidner and Mansfield 1968; Jones 1977), as well as within the same plant species encountering different growth conditions (Gay and Hurd 1975, Hasio and Fisher 1975, Tucic *et al.* 1999). Summarizing the results presented in many papers Ticha (1982) has stated that stomatal density differences may exist even among successive leaves on a plant or along a single leaf blade. Therefore, when one attempts to estimate stomatal density, maximum care should be taken when selecting sampling strategy and interpreting experimental results (Poole *et al.* 1996).

expansion zone") and enter the "elongation zone", where the process of cell expansion continues until they leave the elongation zone. As a consequence of such growth pattern, the leaf tip matures before its base. Monocotyledonous leaves cease to grow after reaching a given size. Their final size is determined by number of cells in the division zone, and the mean cell elongation rate. Because of their relatively narrow shape, such leaves are known as "grass-type" leaves (Kubinova 1993).

The aim of this study was to define an unbiased sampling strategy for estimating stomatal traits that should be used in comparative analyses, as well as for obtaining representative data for any other intentions.

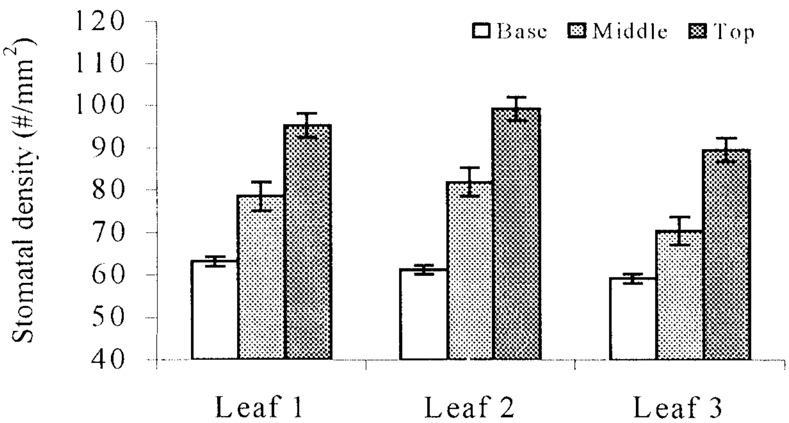

Fig. 1. Mean stomatal density (#/mm^2) ± SE of the three *Iris pumila* successive leaves and three adjacent zones, on each leaf blade. Leaf 1 = the last fully developed leaf; Leaf 2 = the last but one fully developed leaf; Leaf 3 = the last but two fully developed leaf.

In *Iris pumila*, a rhizomatous perennial monocot, the stomatal density is non-randomly distributed over the leaf surface. Number of stomata *per* leaf area appeared to be higher at the leaf tip than near the base. A recent study has shown that the stomata density in *I. pumila* species can be affected by ambient light conditions prevailing in its natural habitats, as well (Tucic *et al.* 1999).

In monocotyledonous plants such as *I. pumila*, leaf growth is a linear process (Van Volkenburgh *et al.* 1998). The meristematically active cells occupy a basal position on the leaf, where the production and elongation of new cells occur. Because of the continuous cell division and expansion in this zone, the produced daughter cells displace away from the leaf base (the "division and

Rhizome segments (three replicas of each) of six *I. pumila* clonal genotypes were single potted in 500 cm^3 pots. Plants were raised for 18 months under constant photosynthetic photon flux density (PPFD over the canopy level was 116.1 μmol m^{-2} s^{-1} and the ratio of red to far-red light amounted 8.2). The ambient temperature in the growth room was 21°C with $8h/16h$ light/dark cycle. For stomatal density analyses the last three fully expanded leaves on each of the three clonal replicas were sampled. To obtain the impression of leaf blades, three zones on the surface of each harvested leaf (base: the first *cm* of the second quarter of the leaf blade; middle: the first *cm* of the third quarter of the leaf blade; top: the first *cm* of the fourth quarter of leaf blade;) were arbitrary chosen and painted with a 0.5 *cm*-wide band of the clear nail polish. The dry polish copy of leaf surface was peeled off by a peace of adhesive tape and mounted on the microscope slide. Stomata counts were done in 20 randomly chosen microscope fields from the middle of each sampling zone on every individual leaf. These microscope fields (0.327 mm^2, under magnification of 6.7 x 10 of an Olympus "Vanox" microscope) were projected on the screen of a personal computer and the number of stomata contained assessed.

Table. 1. Results of profile analysis (MANOVA) for stomatal density observed across three successive leaves and three adjacent zones on each leaf in the six clones of Iris pumila.

	Stomatal density (#/mm^2)			
Between-subject				
Source	MS	DF	F	P>F
Clone	0.26	5	1.32	0.3192
Error	0.20	12		
Within-subject				
Source	F	NumDF	DenDF	P>F
Leaf	1.67	2	11.00	0.2335
Leaf x Clone	2.36	10	20.00	0.0486
Zone	144.66	2	11.00	0.0001
Zone x Clone	3.33	10	20.00	0.0106
Leaf x Zone	1.71	4	9.00	0.2295
Leaf x Zone x Clone	0.70	20	30.00	0.7967

Since in this experiment the measurements of stomatal density were repeated three times on each leaf (in three zones), the data were analyzed by using a multivariate repeated-measures analysis (REPEATED option in SAS ANOVA procedure; SAS Institute 1989). Using repeated-measures terminology, the three successive leaves and the three observations on each of these leaves are referred to as the within-subject factor or the repeated factor, while the individual clone were designated as the between-subject factor (von Ende 1993). All raw data were log transformed prior to analyses.

There was a general tendency for the average stomatal density in the three successive leaves (L1, L2 and L3) of *I. pumila* to increase from the base to the top along a leaf blade (Fig. 1). The multivariate comparison of the stomata density in three successive leaves of six clonal genotypes revealed a statistically significant Leaf x Clone interaction (Table 1), indicating that the successive leaves of different clones exhibited the contrasting patterns of stomatal frequency on their surfaces. A statistically significant Zone x Clone interaction (Table 1), indicates that stomata density in different zones of the same leaf varied among the clones. Since the Zone main effect was also statistically significant (Table 1), this implies that the mean stomatal density increased from the base to the top of the leaf. Because we were interested in identifying particular leaf and a particular zone in which stomatal density differed among clonal genotypes, a PROFILE analyses was performed. Individual ANOVAs (F-test) computed on each of the contrast variable concerning leaf insertion level (*i.e.*, L1 - L2 and L2-L3) failed to find any statistically significant effect (Table 2A), implying that when averaged over successive leaves all clones had similar stomatal densities. Conversely, the main effect of Zone was statistically significant when averaged over clones in both contrast variables (Table 2B). This indicates that, in general, stomatal density displayed a gradient along the leaf blade. A statistically significant clone effect revealed for the contrast variable "middle-top" (Table 2B), suggests that the zonal pattern of stomatal density was clone-specific at the leaf segment.

Table 2. Individual ANOVAs on each of the contracts of within-subjectt factor: A. contracrs between three successive fully expanded leaves Iris pumula; B. contracts between three adjacent zones on each leaf

	Stomatal density (#/mm^2)			
A. Source	DF	MS	F	P>F
Contrasts variable L1-L2				
Mean	1	0.64	1.82	0.2018
Clone	5	0.95	2.71	0.0732
Error	12	0.35		
Contrasts variable L2-L3				
Mean	1	0.98	3.55	0.0838
Clone	5	0.58	2.12	0.1334
Error	12	0.28		
B. Source	DF	MS	F	P>F
Contrasts variable: Base-Middle				
Mean	1	8.11	88.49	0.0001
Clone	5	0.21	2.37	0.1028
Error	12	0.09		
Contrasts variable: Middle -Top				
Mean	1	6.81	118.38	0.0001
Clone	5	0.20	3.47	0.0360
Error	12	0.06		

In conclusion, our results strongly suggest that the best sampling strategy for comparative analyses of stomatal density between genetically different individuals of *I. pumila* would be to take two impressions of the leaf surface from the middle and top zone of the last but one fully developed leaf and count stomata number in 20 randomly chosen microscope fields in the middle of each zone.

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