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Determination of the Total Nitrogen (N) Content and Anatomical Study on the Leaves Epidermal Cells of Plant Species in Urban and Mountainous Environments (Part 1)

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Abstract. The different environment causes change in the plants morphology and anatomy and as well as their physiology. The most informative about the degree of resistance and plasticity of the plant species was epidermis. With respect to physiological processes, the stress environment accelerating the disintegration of nitrogen-containing compounds. The comparative anatomical analysis of the major epidermal cells and the total nitrogen content in the leaves of four plant species (*Juglans regia* L., *Amorpha fruticosa* L., *Laburnum anagyroides* Medic., *Syringa vulgaris* L.) growing in urban and mountainous environments is accomplished. N-content and the number of the basic epidermal cells revealed positive correlation. The highest ecological plasticity, from the four studied species, was reported for *A. fruticosa*, in which the number and size of major epidermal cells in urban environmental conditions is biggest (2090 for upper and 2635.1 for lower epidermis). For that same species was reported maximal contents and the minimal variation in total N [mg/kg] values. On the same indexes, as the least plastic concerning, is determinate *J. regia*. The medium level of plasticity sowed *L. anagyroides* and *S. vulgaris*.

Key words: nitrogen, leaf anatomy, epidermis, plant steadiness, plant plasticity.

Introduction

The plant organs performing photosynthesis and gas-exchange are most subject to the effect of the environment and their response is most obvious (Pilarski, 1999; Rai, 2013; Nikula *et al.*, 2010; Zadeh *et al.*, 2013; Dyulgerski & Kirkova, 2013).

The epidermis as the outer layer of the assimilating organs, having a protective and regulatory function, provides information about the degree of resistance and plasticity of the plant species (Aneli, 1975). The smaller size of the major epidermal cells and their increased number are among the basic indicators about the resistance of the plant organism to the unfavorable environmental conditions (NINOVA & DUSHKOVA, 1977; KOEV *et al.*, 2001; GOSTIN & IVANESCU, 2007; VELICHKOVA *et al.*, 2012).

On the other hand, according to ILKUN (1978), analyzing the so-called "hidden disorders" expressed in reduced plant vitality, is of great importance. One of the most important characteristic in that case, is the result about the changes in the nitrogen, carbohydrate and phosphorus exchange (TESSIER & RAYNAL, 2003; GUSEWELL & BOLLENS, 2003). According to DUSHKOVA & NINOVA (1977a; b), the polluted environment causes deviations in the metabolism of the nitrogen-containing substances, slowing down the process of synthesis and accelerating the disintegration of proteins and the other nitrogen-containing compounds.

The aim of present study was to make a comparative anatomical analysis of the major epidermal cells and to establish the total nitrogen content in the leaves of four plant species (*Juglands regia* L., *Amorpha fruticosa* L., *Laburnum anagyroides* Medic. and *Syringa vulgaris* L.) growing in urban and mountainous environments and to estimate their ecological plasticity.

Materials and Methods

Samples. The method of comparative anatomy was used for the analysis (METCALFE & CHALK, 1950). Mature leaves from the periphery of lower whorl of tree crown of the four species – *Juglands regia* L., *Amorpha fruticosa* L., *Laburnum anagyroides* Medic. and *Syringa. vulgaris* L. were studied. The experimental variants included two locations – urban environment (Plovdiv City) and mountainous environment (Beklemeto Area in the Stara Planina Mts.). The two chosen areas have a significant difference in regards to climate (Table 1). They are different and in regards to air pollution. Plovdiv region is characterized by heavy motor transport traffic, the household sector and the industrial activities, which haves a negative effect on air quality and contributes to higher levels of FDP_{10} and $FDP_{2.5}$ (particles less than 10 and 2.5 microns). According to the regional reports of the Ministry of Environment and Waters of the Republic of Bulgaria (RIOEW-Plovdiv, 2018) on the environmental conditions in 2017 the upper limits of the average daily rates, adopted as permissible for protection of human health, was exceeded several times in the calendar year.

Five plants of each species from the two locations were chosen and an average sample of 50 leaves collected from each plant was fixed in 70% ethanol. Semi-permanent microscope slides were prepared from the lower and the upper epidermis of the middle part of the leaf blade. The following characteristics were studied: shape and number of the basic adaxial and abaxial epidermal cells in 1 mm^2.

Equipment. The analysis of N in manure samples was done with Kjeldahl digestion unit (VELP Scientifica UDK 142).

Total nitrogen determination. The chemical analyses were conducted with analytical reagent-grade Merck and Fluka chemicals (0.1 N HCl; 20% H_3BO_3; H_2SO_4 (d=1.84); 50% NaOH; mixed indicator – 0.2% alcohol solution of methylene blue and 0.25% alcohol solution of methyl red; Cu catalyst tablets)

All glassware in contact with samples was soaked in HNO_3 solutions (1:1), left for at least 24 hours, and washed repeatedly with double distilled water.

The samples for N-content determination were prepared according to standard methods. They were dried at 65°C in a ventilated oven and kept in dark polyethylene bottles.

Total nitrogen content of samples was done according to the classic Kjeldahl method, which has undergone modifications with regard to temperature and time of digestion, acid concentrations and oxidation catalysts. The principle of the method consists in digestion of organic matter and reduction of nitrates with phenol-sulfuric or salicyl-sulfuric acid in the presence of catalysts, converting the nitrogen in samples to ammonium sulfate. The latter was digested by making the solution alkaline with addition of concentrated NaOH, and the liberated ammonia was distilled, by reaction with boric acid of known concentration, and the excess was titrated with 0.2 N HCl. The analytical method has been modified and proposed from the manufacturer VELP Scientifics. The amount of samples, according to their N content varied from 0.25 g to 1 g.

Analysis procedure: the respective amount of sample is weighed, and to it, 7 g K_2SO_4; 5 mg Se; 7 ml k.H_2SO_4 and 5 ml H_2O_2 (hydrogen peroxide) are added. The thus prepared samples are placed in the digestion unit, previously set for 20-min digestion at 420˚C. After the digestion, the sample is distilled and alkalised with 35% NaOH. The distillate is collected into a flask with 4% boric acid; the ammonia excess is titrated with 2N HCl, containing Toshiro's indicator. The used amounts of hydrochloric acid are used for calculation of total nitrogen content.

Statistics. SPSS (Statistical package for social sciences) for Windows and the method of descriptive statistics were used for statistical data processing. The variation coefficient (VC %) and the mean error (Sx%) were determined.

Results and Discussion

Shape of the epidermal cells

The degree of undulation of the anticlinal walls of the major epidermal cells was one of the diagnostic characteristics in studying of different plants responses to the environmental changes in the locations. The greater undulation of the anticlinal walls is an indicator of a higher susceptibility, i.e. a lower ecological plasticity (Ninova & Dushkova, 1981), and what is more, the response of the upper and the lower leaf epidermis is different.

The highest degree of undulation of the anticlinal cell walls of the upper epidermis was observed in walnut. According to the classification scale of Aneli (1975) they refer to the curvilinear type with their zigzag folded cell walls (Fig. 1a; Fig. 2A). In the other three species the basic epidermal cells of the upper epidermis were broadly curved with folded cell walls (Fig. 1c, e, g; Fig. 2C, E, G). Significant differences in the shape of the adaxial epidermis cells of the plants from the separate locations were reported for false indigo bush, the cells of the plants from Beklemeto Area have a higher degree of folding (Fig. 2C).

The shape of the major epidermal cells of the lower epidermis was polygonal in both mountainous and urban conditions and their anticlinal walls were strongly folded compared to the cells of the upper epidermis (Fig. 1b, d, f, h; Fig. 2B, D, F, H). The degree of folding of the abaxial epidermal cells was significantly higher in walnut and especially in false indigo bush under mountainous environmental conditions (Fig. 2B, D).

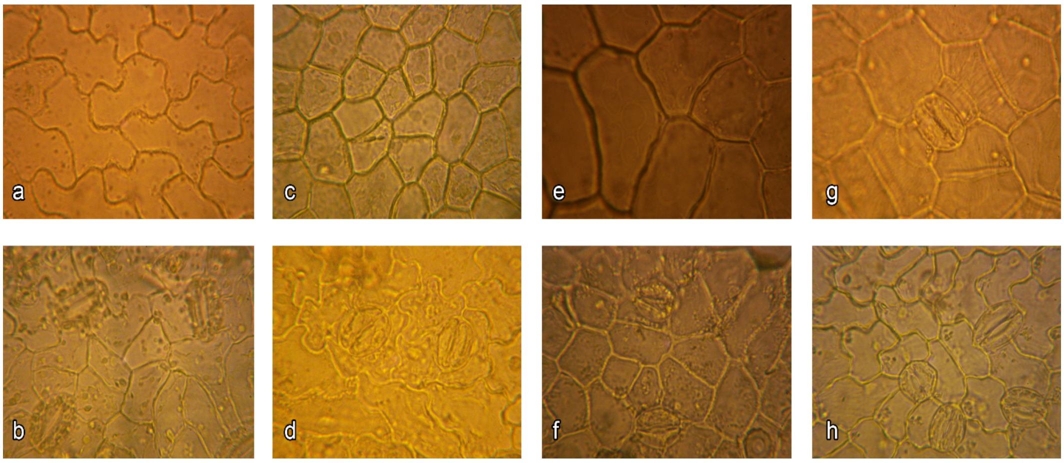

Fig. 1 Structure of the adaxial (a, c, e, g) and abaxial (b, d, f, h) epidermis of the species in Plovdiv City: a,b - *Juglans regia* L.; c,d - *Amorpha fruticosa* L.; e,f - *Laburnum anagyroides* Medic.; g,h - *Syringa vulgaris* L.

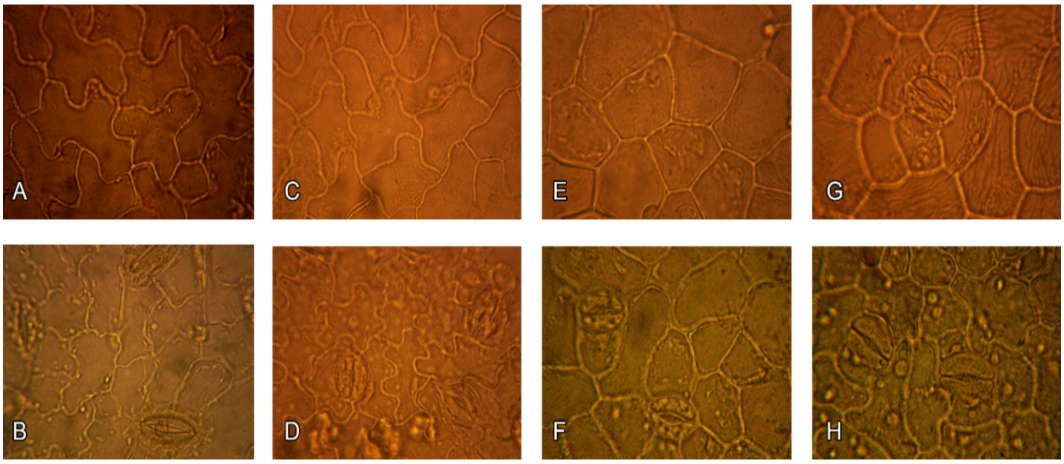

Fig. 2. Structure of the adaxial (A, C, E, G) and abaxial (B, D, F, H) epidermis of the species in Bekleme area A,B - *Juglans regia* L.; C,D - *Amorpha fruticosa* L.; E,F - *Laburnum anagyroides* Medic.; G,H - *Syringa vulgaris* L.

Table 1. Climatic data for the Plovdiv City and Beklemeto Area for 1980 – 2015.

Plovdiv City (urban environment) Altitude 100 to 190 m a.s.l.	Mean annual temperature, °C	12.1
	Mean annual rainfall, mm	514
Beklemeto Area (mountainous environment) Altitude 1500 m a.s.l.	Mean annual temperature, °C	7.9
	Mean annual rainfall, mm	1018.4

Number of the epidermal cells in 1 mm²

Changes in the number of the epidermal cells in 1 mm², and in their size, respectively, as a result of the environmental factors, are an indicator of their adaptability according to a number of authors (VASILEVSKAYA, 1965; NINOVA & DUSHKOVA, 1977; VELICHKOVA *et al.*, 2011). The small cell size is an indicator of xeromorphity, i.e. of plant plasticity (MIROSLAVOV, 1974; DIMITROVA, 2000).

S. vulgaris had the largest in size and the smallest in number upper epidermal cells in both studied locations (776 in average in mountain area, 781.9 in average in urban, respectively). Those values showed the lowest degree of changes in the number of cells depending on the environmental conditions, i.e. according to that characteristic the species demonstrated the lowest ecological plasticity (Table 2).

The smallest size and the largest number of cells were reported in the adaxial epidermis in false indigo bush from Plovdiv region (2090 in average). That determined the species as the most xeromorphic by that characteristic and the reported minimal difference in the mean values in the plants from both locations determined it as the species with the highest plasticity.

Concerning the other two species, walnut exhibited a more obvious response and the number of the epidermal cells in the upper epidermis increased significantly in Plovdiv region (799.4 for mountain area vs. 1173.7 for urban area, respectively).

Referring to the abaxial epidermis, the highest value was reported in *A. fruticosa* for Plovdiv region (2635.1), followed by *S. vulgaris* for mountain area (1639.8).

The maximal number of cells in the abaxial and adaxial epidermis of false indigo bush and the biggest difference caused by the growing location determined the species as the most adaptable concerning that characteristic, despite the fact that the minimal value was reported for that same species growing in mountain area (1480.1 in average).

Data showed an obvious tendency to an increase in the number of cells in both the lower and upper epidermis under urban conditions. Exceptions were reported for the lower epidermis of common lilac and walnut, the average value for the mountain region being 1639.8 and 1735 and for urban region – 1582.5 and 1504.7, respectively. The low degree of variation and the deviation from the general tendency determined both species as the least plastic concerning that characteristic.

Nitrogen (N) content [mg/kg]

N-content and the number of the basic epidermal cells revealed positive correlation. The determined correlation coefficient for cells of the upper epidermis was significantly higher as compared to that for the lower layer, + 0.48 and + 0.13, respectively.

The leaves of false indigo bush from both habitats are characterized with the highest content of N (Table 3). The minimum registered values were for walnut and lilac in Plovdiv region: 22346.87 mg/kg and 22756.41 mg/kg, respectively. Similar results were obtained by DUSHKOVA & NINOVA (1977a). They established lowest protein contents in highly sensitive species, growing in regions with highest air pollution. Reduction of N content in plants from polluted areas were observed by CHIWA *et al.* (2003).

According to the study of REICH & OLEKSYN (2004), N-content in plants from regions with warm climate and low rainfalls was decreased. Similar results regarding increased N-content in plants from high mountain regions were obtained by VAN HEERWAARDEN *et al.* (2003).

The variations of average N-contents with respect to the four studied species in both studied regions were the highest for *J. regia.* For urban areas the observed mean value was 22346.87 mg/kg, while for mountain areas it increased to 32856.83 mg/kg. The latter determines the studied species as the most sensitive according to this index, which corresponds to its high sensitivity towards epidermal parameters (Fig.3).

Table 2. The values of the measure epidermal indexes at the four studied species.

Number of the epidermal cells in 1 mm^2		**Urban environments min(x±Sx)max**	**Sx%**	**VC%**
J. regia L.	Upper epidermis	946.9(1173.7±14.7)1298.2	1.2	6.8
	Lower epidermis	1596.5(1735.1±19.2)1982.5	1.1	6
L.anagyroides	Upper epidermis	982.5(1227.5±19.6)1350.9	1.5	8.7
	Lower epidermis	1754.4(2161.9±44)2578.9	2	11.1
S. vulgaris	Upper epidermis	666.7(781.9±16.4)947.4	2	11.5
	Lower epidermis	1315.8(1582.5±32.1)2105.3	2	11.1
A. fruticosa	Upper epidermis	1719.3(2090±32.8)2421.1	1.5	8.6
	Lower epidermis	1929.8(2635.1±118.1)3122.8	4.4	14.1
		Mountainous environments min(x±Sx)max		
J. regia	Upper epidermis	701.8(799.4±12.4)982.5	1.5	8.5
	Lower epidermis	1385.9(1504.7±21.62)1912.3	1.4	7.9
L.anagyroides	Upper epidermis	877.2(1019.3±9.7)1122.8	0.9	5.2
	Lower epidermis	1456.1(1609.9±18.3)1842.1	1.1	6.2
S. vulgaris	Upper epidermis	596.5(776±16.3)929.8	2	11.4
	Lower epidermis	1491.2(1639.8±18.6)1947.4	1.1	6.2
A. fruticosa	Upper epidermis	1175.4(1424.6±23.3)1701.8	1.6	8.9
	Lower epidermis	1263.2(1480.1±26.7)1719.3	1.8	8.4

Table 3. Total N content [mg/kg] in the leaves at the four studied species.

Species - environment	N [mg/kg]		
	min	mean	max
J. regia - urban	22451.55	22346.87	22734.86
J. regia - mountainous	32968.67	32856.83	32645.82
L. anagyroides - urban	26538.57	26107.36	26726.24
L. anagyroides - mountainous	29848.21	29368.65	29942.62
S. vulgaris - urban	22801.23	22756.41	23156.74
S. vulgaris - mountainous	26480.98	26211.83	26754.57
A. fruticosa - urban	33655.93	33285.74	36785.53
A. fruticosa - mountainous	34371.89	34295.67	35475.21

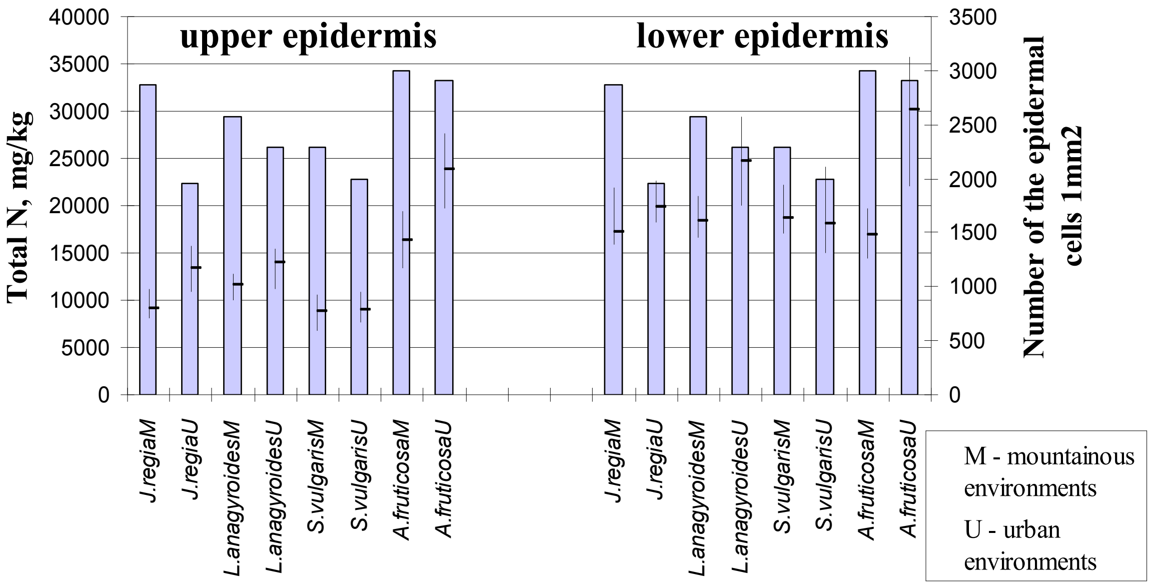

Fig. 3. The variation in the number of the epidermal cells in $1mm^2$, and the total N content, [mg/kg].

The detected minimal variation in N-content for *A. fruticosa* (33285.74 mg/kg Plovdiv and 4295.67 mg/kg mountain aria) corresponds to the dearly registered value of resistance of the species towards epidermal syndromes. Regarding to other two species, higher N-content was determined in the leaves of golden rain. However, the reduction in N-content was more intense for lilac (26211.83 mg / kg mountain area and 22756.41 mg / kg urban area).

Conclusions

In the present study, the highest ecological plasticity according to the results about the major epidermal cells was reported for *A. fruticosa.* Common walnut and lilac proved to be the least xeromorphic referring to both the shape of the major epidermal cells, on the one hand, and their number and size on the other.

The response of *L. anagyroides* was of interest in the present study, however there is not any information in literature. Concerning the epidermal cells and the chemical characteristics of nitrogen, the species showed a medium level of plasticity.

An increased N content in the plants growing in mountain conditions was observed in all the studied variants.

The obvious susceptibility of walnut was expressed in a decreased N content and in the greatest degree of variation of the characteristic in mountain and in urban environmental conditions. The high plasticity of false indigo bush correlated with the reported maximal nitrogen (N) values and the minimal difference established between both environmental locations.

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