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Determination of the Total Phosphorus (P) Content and Anatomical Study on Stomata of Plant Species in Urban and Mountainous Environments (Part 2)

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Abstract. The different habitat conditions affect physiology, morphology and anatomy of the plants and provide the necessary information for comparing their ecological plasticity. The aim of the present study was to analyse the degree of changes in the stomatal apparatus and the total phosphorus content in the leaves of species from different habitats, in order to determine the possibility of using them in biological control. The method of comparative anatomy was applied for the analysis of the stomata of four plant species: *Juglans regia* L., *Amorpha fruticosa* L., *Laburnum anagyroides* Medic., *Syringa vulgaris* L. The experimental variants included two locations – the urban area (Plovdiv City) and the mountainous area (Beklemeto Area, Stara Planina Mts.). UV/VIS DR 6705 spectrophotometer (JENWAY) was used for determining the phosphorus content in the samples, the wave length for *P* being 410 nm (BDS ISO 11263:2002). The highest ecological plasticity according to the stomatal characteristics was reported for *A. fruticosa* The mean values for number, width and length of stomata in that species in urban area were 245.6; 17.3 μm; 18.9 μm and in moutainous – 121.2; 19.1 μm; 24.3 μm, respectively. The maximal values of total phosphorus were reported for that same species (1868.32 mg/kg in urban and 2361.2 mg/kg in mountainous area**)**, as well a deviation from the tendency of a decrease of the phosphorus content in mountainous environment. In *J. regia*, in contrast to the other three species, an increase of the number of stomata was observed in the mountainous area. That tendency, in combination with the comparatively small number and the highest size of stomata in both studied regions, determines the species as the least xeromorphic. The correlation dependence between the total phosphorus content and the stomatal characteristics showed that the increase of the phosphorus content in leaves corresponds to the xeromorphic characteristics.

Key words: phosphorus, stomata, epidermis, plant plasticity.

Introduction

The use of plants as bioindicators of the dynamic environmental changes was an object of a number of research investigations (DINEVA, 2004; ZADEH *et al.*, 2013). The analysis of same species from different habitats and the identification of the parameters that are most greatly affected by the environmental conditions, provide the necessary information for comparison of the resistance and ecological plasticity of plants (NIKULA *et al.*, 2010; RAI, 2013). Elevation gradients and the degree of air pollution provide a setting for powerful "natural experiments", in which ecological and evolutionary responses of biota to changing environments can be tested (WANG *et al.*, 2014). Air particulates affect the overall growth and development of plants according to their physical and chemical nature (GUPTA & GHOUSE, 1987). A number of studies show a change in leaf anatomy and morphology as a result of the concentration of air pollutants (GUPTA & MISHRA, 1994; SINGH & STHAPAK, 1999; FAROOQ *et al.*, 2000; PAL *et al.*, 2000; SHRIVASTAVA & JOSHI, 2002).

In the studies carried out by FALLA *et al.* (2000) and HONOUR *et al.* (2009), was mentioned that plant species showed the properties of environment in which they grow, by changes in their anatomy and physiology and those changes in leaf characteristics could be used as rational and precise evaluation of habitat properties.

The different habitat conditions change the physiological processes occurring in the plants, one of them being the total phosphorus content (CHIWA *et al.*, 2002; TESSIER & RAYNAL 2003; VENDERINK *et al.*, 2002). What is more, data about the content of phosphorus compounds have been controversial. ZHANG *et al.*, 2014; WARREN, 2011; KANT *et al.*, 2011 established that *P* deficiencies cause negative effects on plants. ILKUN (1978) pointed out that under the influence of sublethal rates of phytotoxic gases on plant leaves, the total phosphorus content does not change or it slightly decreases. The incidence of insignificant symptoms of damages usually increases the phosphorus content. An increase of the phosphorus content in plant leaves in warmer climate was reported by KÖRNER (1989), while REICH & OLEKSYN (2004), established a decrease of the phosphorus content in tree species in habitats with higher temperatures. RUSSEL *et al.* (1998) note that the total *P* content vary both spatially and temporally, due to, variations in land use and other characteristics. VAN DE WEG *et al.* (2009) underline the poor variability of phosphorus depending on altitude, while GARTLAN *et al.* (1986) establish a strong correlation between elevation of altitude and phosphorus content in soil and plant organs.

Studies of NJOGU *et al.* (2014); CHIERA *et al.* (2002) showed that the different content of total *P* in the leaves has an impact mainly on the change in the number and size of stomata and less on the basic epidermal cells and mesophylls. The different studies show a relatively similar correlation between the two indicators. SEKIYA & YANO (2008) established that the higher *P* content leads to greater SD in cowpea plants. SUN *et al.* (2014) did not find an effect of the increased content of *P* on the stomatal number and size in potato leaves. Obviously significant correlation between the phosphorus deficient leaf and the decrease of the number of stomata was established by SARKER *at al.* (2010) in *Zea mays* L. FERNANDES & GUZMAN (2014) found out that increasing plant *P* status resulted in higher stomatal frequencies in wheat.

Morphology and the number of stomata are most often subject to analysis for determining the resistance of a given plant species (DIMITROVA, 2000; GOSTIN & IVANESCU, 2007). SHIELDS (1950), FAHN (1964), NINOVA & DUSHKOVA (1981) and RÔÇAS *et al.* (1997), establish a correlation between the resistance (xeromorphity) of airborne and industrial pollutants and the plasticity of epidermal syndromes. According to the authors the protective capabilities of the epidermis largely correspond to the degree of xeromorphity of the stomatal apparatus. The xeromorphic-oriented plasticity of epidermal syndromes is associated with the reduction of the size of the stomata and an increase in their number. Moreover, the more significant the degree of change of these indicators in plants subject to different environmental stresses (air pollution, drought, etc.), the higher the ecological plasticity of those species (NINOVA & DUSHKOVA, 1981; ROTONDI *et al.*, 2003; GANGRONG *et al.*, 2006; RÔÇAS *et al.*, 1997). The studies of the effects of altitude on the number and size of stomata show contradictory trends. KÖRNER *et al.* (1986) found that at a higher altitude the stomatal density increased, while their size decreased, while GOU *et al.* (2005) established a decrease of stomatal number at higher elevation.

The aim of the present investigation was to follow out the changes occurring in the stomatal apparatus and in the total phosphorus content in leaves, as well as the correlation between the two factors in four plant species: common walnut (*Juglands regia* L.), golden rain (*Laburnum anagyroides* Medic.), lilac (*Syringa vulgaris* L.) and false indigo bush (*Amorpha fruticosa* L.), growing in urban and mountainous environments, as a precondition for using those species in biological control.

Materials and Methods

Samples collection. The method of comparative anatomy was applied for the analysis of the epidermis (METCALFE & CHALK, 1950; ANELI, 1975). The analysis was made on matured leaves of the four species - *Juglans regia* L., *Amorpha fruticosa* L., *Laburnum anagyroides* Medic. and *Syringa vulgaris* L. The reason for choosing those four species was that they inhabit both mountainous and urban areas in Bulgaria. Five plants of each species were labelled from the two locations and an average sample of 50 leaves from each plant was collected, fixed in 70% ethanol. Following the methodology of DRING (1971), the semi-permanent microscopic preparations from the lower and upper epidermis were made from the middle part of the leaf lamella. The following characteristics were studied: stomatal width (SW), stomatal length (SL) and number of stomata (SD) per mm^2 of abaxial epidermis (and of adaxial epidermis for *S. vulgaris* species) stomatal index (SI) – SD/ΣSD+NEC per 1 mm^2. A microscope with a camera was used for taking pictures with 2304X zoom.

Characteristics of the regions. Different areas were chosen in the two locations: Plovdiv City (the urban area) and Beklemeto Area, Stara Planina Mts. (the mountainous area). Both habitats differ significantly with regard to the values of the average monthly temperatures and precipitation. Average monthly temperatures in Plovdiv City for a 30-year period (12.1ºC) are almost two times higher than those of Beklemeto Area (7.9ºC), while the amount of precipitation in Beklemeto Area (1018.4) exceeds approximately two times that in Plovdiv City (514). The specific weather conditions in Plovdiv City – temperature inversions (in about 85% of the days), a large percentage of windless days (about 40% of the days in the year with a wind speed below 1,5 m/s) and fogs leading to retention and accumulation of pollutants – have a significant impact on the environment. The measured annual average values of FDP_{10} (particles less than 10 microns), range from 42 $\mu g/m^3$ to 47,62 $\mu g/m^3$, the adopted average annual rate for human health protection for FDP_{10} being 40 $\mu g/m^3$. The measured average annual value for $FDP_{2.5}$ (particles less than 2.5 microns), is 29,8 $\mu g/m^3$, at an average annual rate for human health protection of 26 $\mu g/m^3$.

Data for the area of Beklemeto Area show that in 2017 the levels of controlled indicators of air quality (AQ) were below the established standards for human health protection.

Determination of total P content [mg/kg]. The samples were washed with distilled water and dried at 65°C in a fan oven to constant weight. The dried samples were ground, then homogenized and stored in polyethylene bottles until analysis. UV/VIS DR 6705 spectrophotometer (JENWAY) was used for determining the phosphorus content in the samples. An amount of 0.2 g of samples was taken into digestion tubes and 5 ml of HNO_3 (65%), 1 ml of HCl and 3 ml of H_2O_2 (30%) were added. The acid-soluble phosphorus compounds were determined in a decomposition solution using a vanadate molybdate reagent,the wave length for *P* being 410 nm. Duplicated analysis was performed on the samples. Blank digestion was also carried out in the same way.

Statistics. SPSS (Statistical package for social sciences) for Windows. The method of descriptive statistics were used for statistical data processing, determining the variation coefficient (VC%), the mean error (Sx%) and correlation coefficient (R). Differences in the average values of stomatal characteristics and total *P* mg/kg for each species from both habitats were calculated following Fisher's LSD Multiple Comparison Test.

Results and Discussion

Stomatal Characteristics

Stomatal distribution on leaves is a morphological feature of each plant species (ANELI, 1975). According to NINOVA & DUSHKOVA (1981) the presence of stomata on both leaf surfaces is an indicator of lower resistance to air pollution.

Out of the four species included in the present study, only lilac has amphistomatic leaves, the stomata on the upper epidermis being of a transitional type from paracytic and anisocytic to anomocytic. The other three species have hypostomatous leaves with anomocytic type of stoma (Fig. 1). The presence of stomata on the upper epidermis determines *S. vulgaris* as the most mesomorphous, i.e. possessing the least ecological plasticity according to that indicator.

Stomata density (SD) - number of stomata per mm^2

SUN *et al.* (2014) mentioned that the stomatal size and density are controlled by both genetic and environmental factors. The decrease of the size and the increase of the number of stomata is an indicator of xeromorphity (resistance), (DIMITROVA, 2000). WANG *et al.* (2015) pointed out that at the species level, plant species at lower latitudes had higher SD that those distributed in higher latitudes, whereas the observed latitudinal trend of SL was opposite.

Despite the amphistomatous structure of the lilac leaves in urban region, a xeromorphic maximum was reported referring to their number on the abaxial epidermis, i.e. 276.6 in average. Out of the three species with hypostomatic leaves, the largest number of stomata was reported in false indigo bush from urban region, while in mountainous region a xeromorphic minimum of 121.2 was also established for the same species, which determines the highest ecological plasticity of that species referring to that characteristic (Table 1).

In *J. regia*, in contrast to the other three species, a slight increase of the number of stomata was observed in the plants from mountainous area. That tendency, in combination with the comparatively small number of stomata, determines the species as the least plastic (Table 1).

Stomatal index (SI) was higher under urban conditions in all the species except for *J. regia*. Differences were not established in walnut in both habitats. The highest SI under urban conditions was reported for *A. fruticosa* and in the lower epidermis of *S. vulgaris*. The maximum value under mountainous conditions was established in the latter, while the minimum value was recorded in the lower epidermis of *A. fruticosa*. This confirms the high ecological plasticity of the species *A. fruticosa* regarding that indicator (Fig. 2).

Size of stomata

The tendency of an increase of the number of stomata and a decrease of their size, established in regions with pronounced air pollution (NINOVA & DUSHKOVA, 1981; DIMITROVA, 2000), showed contradictory res-

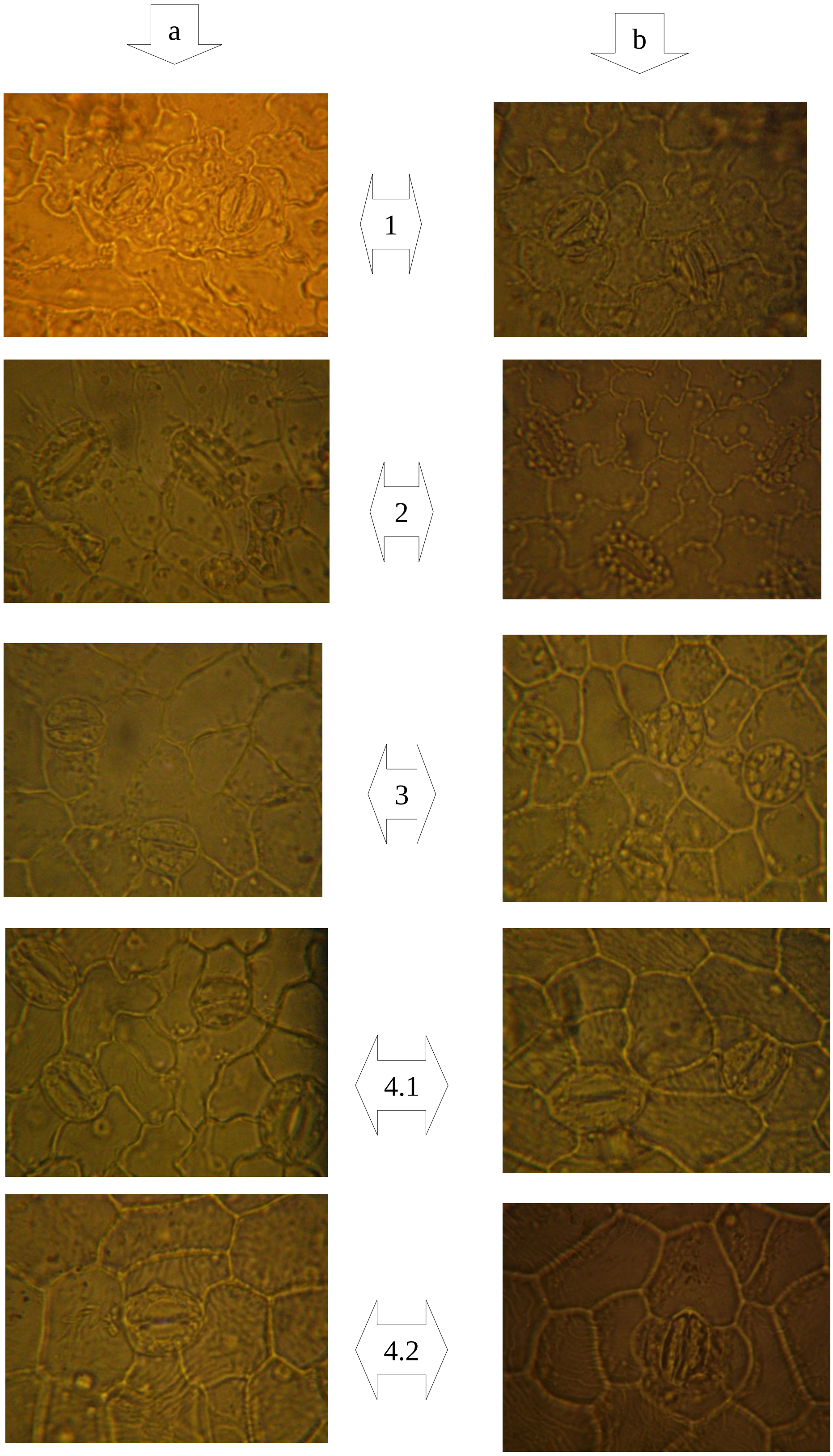

Fig. 1. A general appearance of the stomata of the studied species (2304X):
1. *A. fruticosa* L.; 2. *J. regia* L.; 3. *L.anagyroides* Medic. 4. *S. vulgaris* L. – 4.1. lower epidermis; 4.2. upper epidermis a) urban environments; b) mountainous environments.

Table 1. Stomatal indexes of the four studied species.

Stomata characteristic			Urban environments min(x ± Sx) max	Sx%	VC%
J. regia L.	Number of stomata		70.2(125.1 ± 5.7)210.5	4.5	24.9
	Width µm.		16.5(20.6 ± 0.4)24.5	1.9	10.9
	Lenght µm.		23.5(28.4 ± 0.7)39.4	2.6	13.5
L.anagyroides Medic.	Number of stomata		105.3(180.7 ± 8.2)280.7	4.5	24.9
	Width µm.		15.8(19.4 ± 0.3)22.1	1.4	7.9
	Lenght µm.		17.8(22.7 ± 0.4)28.6	1.8	9.8
S. vulgaris L.	Upper epidermis	Number of stomata	0(19.9 ± 2.5)52.6	12.4	68.4
		Width µm.	19.7(23 ± 0.3)27.12	1.5	8.2
		Lenght µm.	24.2(28.9 ± 0.4)32.1	1.2	6.8
	Lower epidermis	Number of stomata	140.4(276.6 ± 10.2)280.7	3.7	20.3
		Width µm.	16.3(20.6 ± 0.3)23.5	1.4	7.8
		Lenght µm.	22(27.2 ± 0.6)32.6	2.2	12.2
A. fruticosa L	Number of stomata		140.4(245.6 ± 20.1)350.9	8.1	25.8
	Width µm.		13.4(17.3 ± 0.3)20.4	1.7	9.8
	Lenght µm.		18.9(21.9 ± 0.3)26.1	1.3	7.8
			Mountainous environments min(x ± Sx) max		
J. regia L.	Number of stomata		87.7(126.9 ± 4.9)175.4	3.9	21.3
	Width µm.		16.8(21 ± 0.4)25.9	2	11
	Lenght µm.		24.9(30.6 ± 0.6)37.2	2	11.3
L.anagyroides Medic.	Number of stomata		52.6(138 ± 7.4)210.5	5.3	29.2
	Width µm.		16.3(18.6 ± 0.2)21.1	1.3	7.2
	Lenght µm.		19.7(23.1 ± 0.4)26.9	1.6	8.8
S. vulgaris L.	Upper epidermis	Number of stomata	0(8.8 ± 2.1)35.1	24.7	135.9
		Width µm.	18(21.5 ± 0.4)26.6	1.9	10.6
		Lenght µm.	23.3(28.4 ± 0.4)32.4	1.2	6.9
	Lower epidermis	Number of stomata	87.7(161.4 ± 6.9)263.2	4.3	23.6
		Width µm.	16.8(19.6 ± 0.3)24.2	1.6	9.4
		Lenght µm.	20.4(26.9 ± 0.4)30	1.4	7.9
A. fruticosa L	Number of stomata		87.7(121.2 ± 4.3)175.4	3.5	16.6
	Width µm.		16.6(19.1 ± 0.3)22.6	1.4	8.1
	Lenght µm.		20.1(24.3 ± 0.6)32.4	2.2	12.5

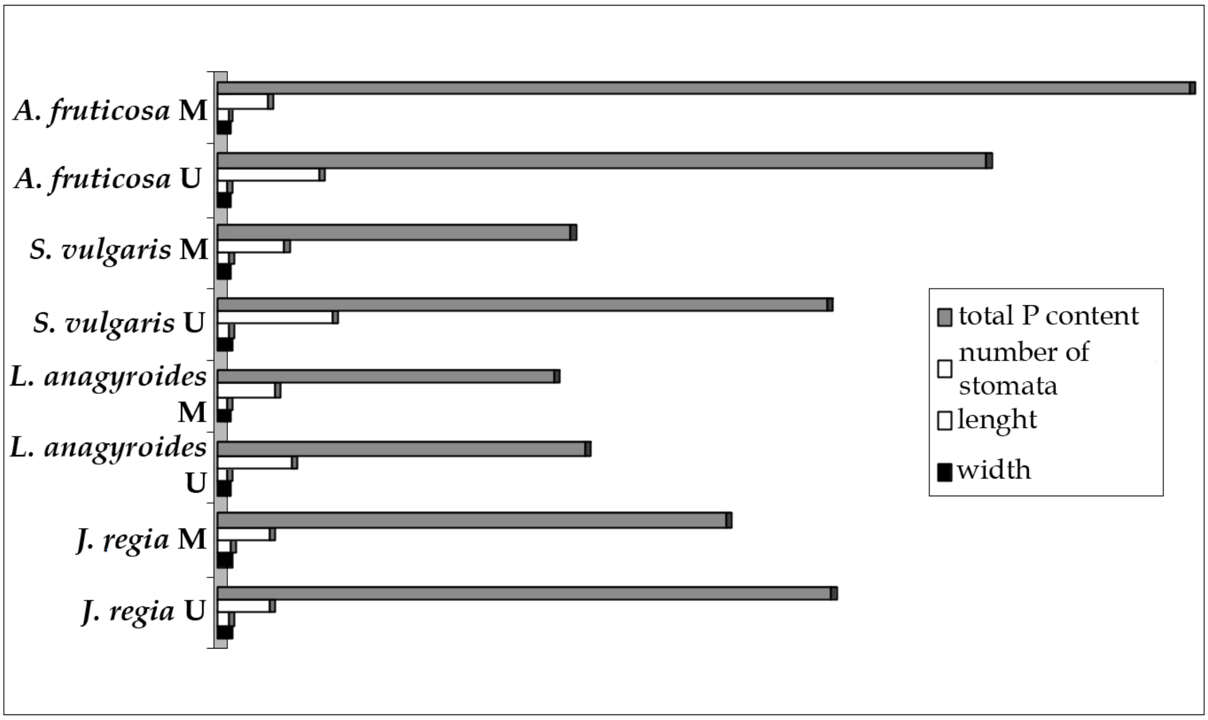

Fig. 2. The average value of the stomata indexes and the total *P* content mg/kg in the leaf of the four studied species.

ults in the present study. In golden rain species from urban region stomata had a bigger width, while in lilac from urban region, despite the established largest number of stomata, they were bigger in size, although the difference was small (Table 1).

The smallest length and width of stomata was established in false indigo bush from urban region, i.e. 17.3 µm. and 21.9 µm., respectively. In common walnut, which is determined as relatively resistant according to the scale of ILKUN (1978), the values reported about the size of stomata in both studied regions were the highest and that shows the species is of low plasticity.

Total phosphorus content, [mg/kg]

The tendency to an increased content of total phosphorus in the leaves in urban environment, mentioned by ILKUN (1978), KÖRNER (1989), CORDELL et al. (1998), was observed in all the studied species with an exception of *A. fruticosa* (Fig. 2). The most likely cause of this is the established strong xeromorphity and ecological plasticity of the species.

In false indigo bush, in accordance with data obtained by REICH & OLEKSYN (2004), the highest content of total phosphorus was reported along with a deviation from the tendency reported in the rest three species. In that species, higher phosphorus values were established in the leaves of plants from mountainous region – 2361.2 mg/kg, while in the urban region the reported value was 1868.32 mg/kg.

The lowest values of total phosphorus content in mg/kg, as well as the smallest difference between the two studied locations was reported in golden rain species (891.53 mg/kg in urban and 818.9 mg/kg in mountainous area). Almost similar values of total phosphorus content were established for *J. regia* and *S. vulgaris* in the urban region: 1492.54 mg/kg and 1482.48 mg/kg, respectively. A greater decrease of the phosphorus content in the mountainous environment was reported in lilac – 858.45

mg/kg, while in common walnut it was 1233.3 mg/kg (Fig. 2).

Statistical correlations between the studied parameters

Strong positive correlation between stomatal width (SW) and stomatal length (SL) was observed in both habitats (Table 2). Such a dependence was also found between SI and SD. SW and SL exhibit an average negative correlation with SI in urban conditions and slightly positive in mountainous. The correlation between SD and the size of the stomata (S and SL) is negative. Such a strong negative correlation between the number and size of stomata in tree and shrub species was reported by WANG *et al.* (2014).

Regarding the total *P* content in mg/kg and stomatal characteristics, the reported correlations are mainly negative in mountainous conditions and slightly positive for SL and SD in urban conditions. As a whole, the established tendency shows that with the increase of the total *P* content in mg/kg, the sizes and SD decrease in mountainous conditions, while in urban conditions they rather increase. A similar tendency was established by NJOGU *et al.* (2014) in *Camellia sinensis* – the number of stomata is in inverse correlation with the content of *P* at a low altitude, while at a higher altitude the tendency is reverse.

The reported correlation of total *P* mg/kg with stomata characteristics defines the increased content of total *P* mg/kg in the leaves, as an indicator corresponding to xeromorphity. Such a relation between the highly polluted urban environment and the high *P* content in the leaves, resulting in an increase of the size and a reduction of the number of stomata, was published by SANTOS *et al.* (2015).

Comparing the four studied plant species, the highest level of variation of the average values of the reported indicators was established in *A. fruticosa* (Table 3). Regarding the studied indicators, the highest level of variation in both habitats was reported for the total *P* mg/kg (Table 3).

According to WANG *et al.* (2006), the *P* content in the leaves depends to a greater extent on the plant species than on the altitude. The greatest difference in the average total *P* content in mg/kg in the two habitats was established for *A. fruticosa*. Significant difference in the average content of total *P* mg/kg was reported between *A. fruticosa* and *L. anagyroides*, as well as between A. *fruticosa* and *S. vularis* (Table 3).

Table 2. The correlation coefficient (R) between total phosphorus content (*P*, mg/kg) and stomatal indexes.

		SWμm	SL μm.	SD mm²	P, mg/kg
urban	SLμm.	0.88**	1		
	SD mm²	-0.30*	-0.28	1	
	P, mg/kg	-0.42*	0.053	0.36	1
	SI	-0.44*	-0.37	0.99**	0.47
mountainous	SLμm.	0.99**	1		
	SD mm²	-0.13	-0.002	1	
	P, mg/kg	-0.06	-0.16	-0.71*	1
	SI	0.32	0.44*	0.896**	-0.74*

**Values with significant difference at 0.01 * Values with significant difference at 0.05

Table 3. Fisher's LSD Multiple Comparison Test of variation of the difference in the average values of the stomata indicators and total *P* content mg/kg among the species from both habitats. Within each column, means sharing the same letter are not significantly different at the 5% level.

Species/Indexes	SWμm	SLμm	SD mm^2	P, mg/kg	Total
J.regia L.	20.8^{a}	29.5^{a}	126^{a}	1362.92^{ab}	384.80^{b}
L.anagyroides Medic.	19^{ab}	22.9^{b}	159.3^{a}	855.21^{b}	264.11^{b}
S. vulgaris L.	20.1^{ab}	27.05^{a}	183.4^{a}	1170.46^{b}	359.15^{b}
A.fruticosa L.	18.2^{b}	23.1^{b}	219^{a}	2114.76^{a}	584.86^{a}
Total	19.52^{b}	25.63^{b}	171.93^{b}	1375.84^{a}	
LSD5%	2.21	3.24		824.11	160.7

Stomatal width showed significant difference between *J. regia* and *A. fruticosa*.

Regarding SL, similar values were reported for *J. regia* and *L. anagyroides*, as well as for *L. anagyroides* and *A. fruticosa*. The difference between the two groups was significant.

Significant difference between the average values of SD in both habitats was not detected.

Conclusions

The established highest values in number and lowest in size of stomata in urban area for *A. fruticosa*, determine the species as the most xeromorphic. The most pronounced variation of those two indicators in the same species in both habitats also determines it as the most ecologically plastic.

The xeromorphic minimum was shown by *Juglands regia*. In both studied regions, comparatively small number of stomata and the largest stomatal size were established for that species.

Leaf amphistomaticity in *Syringa vulgaris* determined it as the most mesomorphic species, however, the xeromorphic maximum was reported for the species in the urban environment referring to the number of stomata on the abaxial epidermis.

Laburnum anagyroides species, for which available data in literature is quite poor, showed a medium degree of resistance according to stomatal characteristics. The minimum total phosphorus values were reported for the species.

The correlation dependence between the total phosphorus content and the stomatal characteristics showed that the increase of the phosphorus content in leaves corresponds to the xeromorphic characteristics.

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