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STUDY REGARDING STOMATAL DENSITY IN *MAGNOLIA* SP.

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ABSTRACT
The purpose of this study is to reveal the structural aspects of the leaf as occurring in the genus Magnolia. The leaves are bifacial and hypostomatic. Secretory oil cells are a constant presence. We have revealed significant dissimilarities in stomatal density and size as occurring in three ornamental species: Magnolia kobus, Magnolia x soulangeana "Soulange-Bodin" (M. denudata x M. liliiflora) and Magnolia x "Susan" (M. kobus var. stellata "Rosea" x M. liliiflora "Nigra"). The highest stomatal density was recorded in the diploid species Magnolia kobus. The stomata are significantly elongated in Magnolia x soulangeana "Soul.-Bod." and wide in Magnolia kobus.
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KEY WORDS: *Magnolia kobus, Magnolia x soulangeana, Magnolia x "Susan", stomatal density, stomatal size, leaf anatomy.*

INTRODUCTION

A histo-anatomical analysis of the vegetative organs allows for the identification of anatomical particularities and ecological adaptations. Structural variations in plants regularly influenced by environmental factors are particularly evident in the morphology and anatomy of leaves (Ianovici et al., 2010b; Ianovici et al., 2011a; Ianovici et al., 2011b). The differentiation and development of stomata are known to be determined by genetic factors and are considered a key event in the evolution of terrestrial plants (Hetherington & Woodward, 2003).

Stomata act as the most important channel that facilitates gas exchange in vascular plants. The parameters of stomata reflect two significant physiological processes: photosynthetic CO_2 assimilation and water transpiration (Ianovici, 2010). Changes in the structure of the epidermis constitute eco-physiological processes (Ianovici, 2011a; Ianovici, 2011b; Ianovici, 2011c). Studies reveal an inversely proportional relationship between stomatal density and the concentration of CO_2 in the atmosphere, which is considered an adaptive response intended to maximise water usage efficiency (Woodward, 1987). Stomatal density and size demonstrated marked phenotypic plasticity, presenting large scale variations depending on water availability (Fraser et al., 2009), temperature (Luomala et al., 2005), exposure (Woodward et al., 2002), leaf position (Woodruff et al., 2008), pollution (Riikonen et al., 2010), light and UV-B radiation (Gitz et al., 2004), O_2 (Ramonell et al., 2001), soil phosphorus levels (Sekiya & Yano, 2008), phytohormones (Davies & Mansfield, 1987), atmospheric humidity (Schulze et al., 1987). While an increase in stomatal size tends to reduce stomatal conductance, an increase in density will have the opposite effect on the latter (Franks et al., 2009). Due to the relationship between stomata and the volume of water lost, stomatal density is a significant eco-physiological trait, particularly in environments with limited water resources (Xu & Zhou, 2008). The number of stomata per unit of surface area has been listed among 10 morphological, anatomical, physiological, and biochemical characteristics (bio-indicators of pollution) (Ninova et al., 1983; Ianovici et al, 2009).

The present paper proposes to investigate the differences in stomatal density and size in three species of *Magnolia*.

MATERIALS AND METHODS

The biological material has been collected from the Simeria Dendrological Park in the spring of 2013. After washing, a share of the plants was fixed in 70% ethyl alcohol, while the others were utilised to perform freehand transverse sections and produce fresh sample preparations. We used the Geneva reactive for staining. The stripping off method was used to detach the epidermis.Ten sample preparations were extracted from both surfaces of the leaf. Part of the epidermal layers were discoloured in a solution of 5% sodium hypochlorite for 30-60 minutes. Stomatal density (SD) corresponds to the number of stomata per mm^2 (Ianovici, 2009). In order to determine SD, we used the micrometric coefficient for each objective lens-ocular pair in the Optika B500 microscope. Photographs were taken with a Canon PowerShot A630. Statistical processing was conducted in Microsoft Office Excel 2007.

An image is obtained via scanning electron microscopy (SEM) by detecting and measuring the electronic flows dispersed or issued (secondary electrons) from the surface of the sample preparation under investigation (Stokes, 2008), organ fragments or even the specimen being studied. Stomatal size has been studied via the Fei Quanta 250 Scanning Electron Microscope.

RESULTS AND DISCUSSIONS

1. Histo-anatomical aspects

In the case of the three species of Magnolia under observation, the leaf is dorsiventral, presenting with pinnate reticulate nervation. The tertiary and quaternary nervure network is far denser in *Magnolia kobus* as compared to the studied hybrids. A transverse section through the lamina will reveal the upper and lower epidermis, the mesophyll and nervures (Ianovici, 2010).

The epidermis is single-layered, at mesophyll level presenting with isodiametric cells in *Magnolia kobus*, while the two hybrids reveal cells of varying sizes and alternately positioned. The stomata are present in the lower epidermis, their type being amaryllidaceae paracytic. The cuticle on both surfaces is thin and reveals no xerophytic adaptations. However, in the case of *Magnolia kobus*, a thicker cuticle covers the epidermal cells. Surface ornamentations are absent.

The structure of the mesophyll is bifacial, with typical palisade tissue, organised in 2-3 layers, the cells aligning perpendicularly on the epidermal cells. Lacunar tissue is present towards the lower epidermis, revealing small intercellular spaces and being highly dense in *Magnolia kobus*, while wider spaces are observed in the two hybrids. Oil cells are frequently found in the mesophyll.

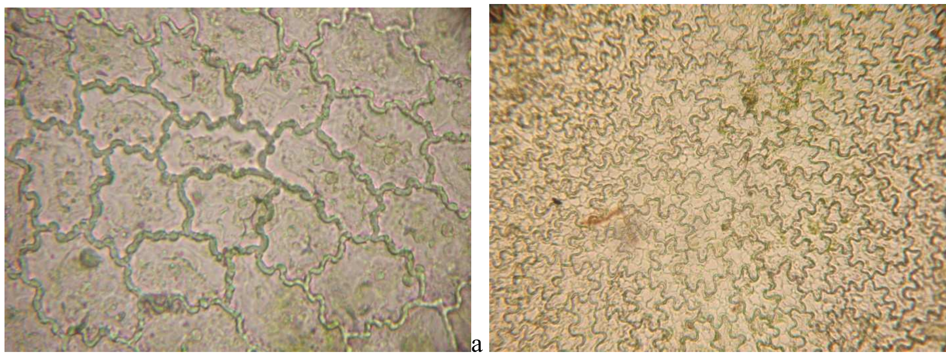
The conducting tissue forms vascular bundles of varying size, which constitute a part of nervures. There are fewer vascular bundles present in *Magnolia x soulangeana*. The vascular bundles are collateral, the xylem lying adaxially and phloem being positioned abaxially, presenting, towards the exterior, with a continuous layer of sclerenchyma, through which it adheres to the upper epidermis. The central nervure area is occupied by parenchyma cells of large sizes, while the periphery is occupied by collenchyma. The central cylinder of the nervure is surrounded by parenchyma structured in 3-4 layers. Calcium oxalate crystals are noticeable, these arising in a larger number in *Magnolia x "Susan"*.

The nervure contour is irregular in *Magnolia x soulangeana*, highly irregular in *Magnolia kobus* and rounded in *Magnolia x "Susan"*.

At nervure level we encounter bicellular flagellated hairs. The number of hairs/nervure falls between 2-4 and they are very long, while in the case of the hybrids the number of hairs increases.

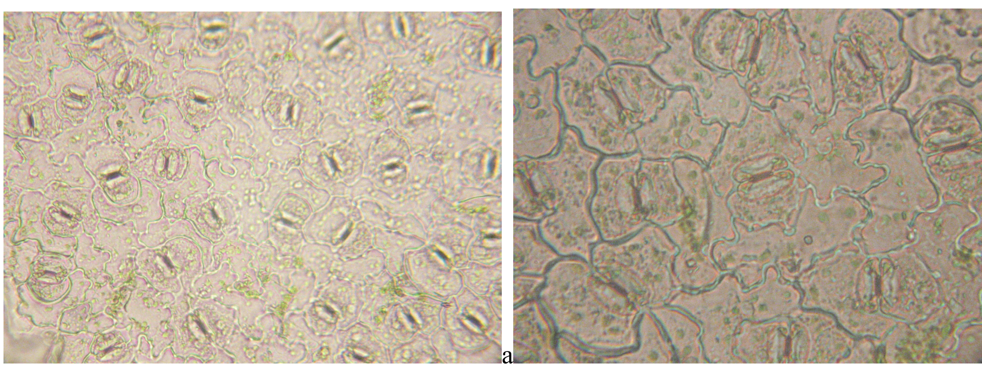
A hypodermic layer has often been observed under the adaxial surface. This aspect was mentioned by Baranova (1972) in regards with Magnoliaceae. As such, in the case of the three species, between the main nervure and the mesophyll, under the upper epidermis, there are 1-2 layers of larger sized, non-chlorophyllous aquiferous cells, which extended as a hypodermis. The hypodermis is not continuous, as it becomes thinner in the presence of secondary nervures.

The structure of the petiole is highly similar to the nervure. It presents with collateral vascular bundles, typically more than ten, organised circularly, outlined by a sclerenchyma sheath. A cross-section of the sheath reveals two instances of outpunching which extend progressively, separated by a channel. The periphery is constituted by assimilatory angular collenchyma and parenchyma, the latter also present between vascular bundles and the central area of the petiole.



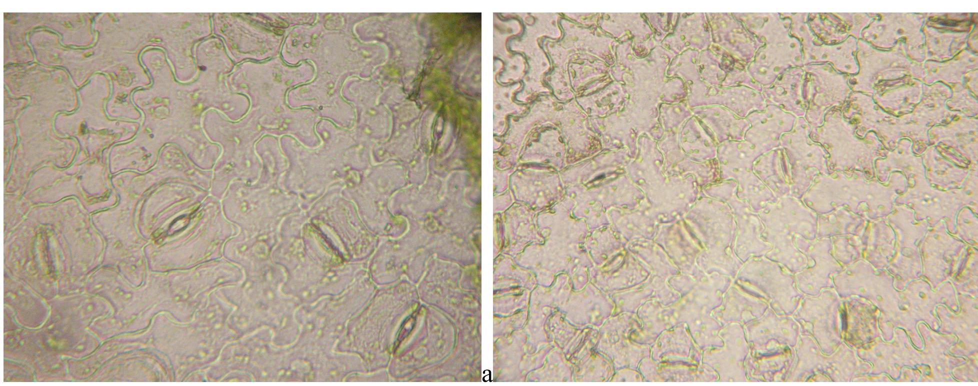
a b

Figure 1. Appearance of the upper epidermis: a – *Magnolia kobus* (400x, zoom4); b-*Magnolia x soulangeana* (100x, zoom4)



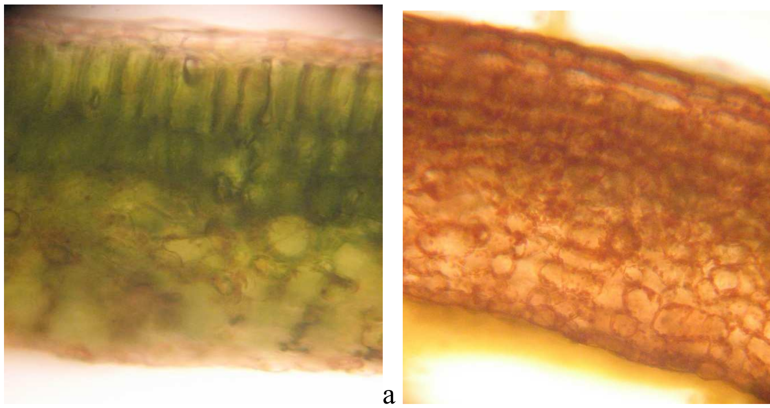
a b

Figure 2. Appearance of the lower epidermis: *Magnolia kobus*: a-200x; b-400x



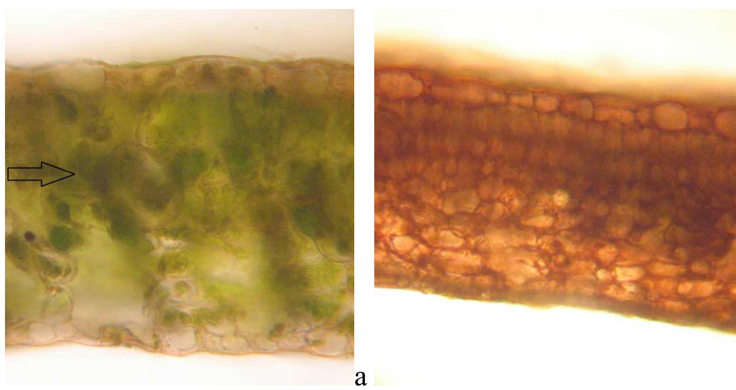
a b

Figure 3. Appearence of the lower epidermis: a-*Magnolia x soulangeana* (400x); b- *Magnolia x "Susan"* (200x)



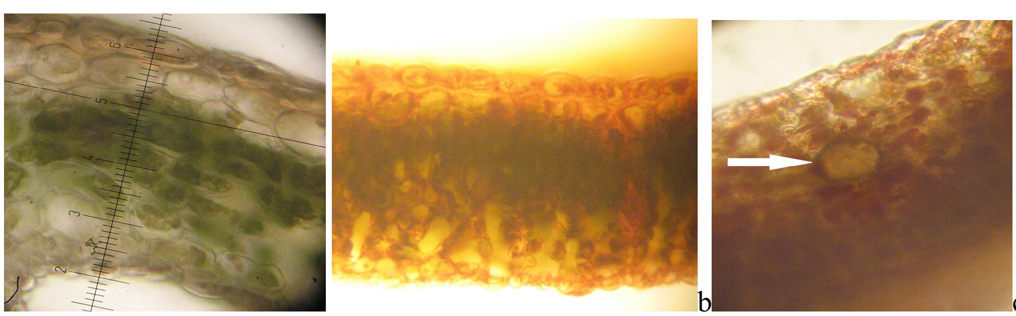
a b

Figure 4. Transverse section of the foliar lamina in *Magnolia x "Susan"* (a-fresh sample preparation; b-preparation preserved in ethyl alcohol, javelized and stained with Geneva reactive)



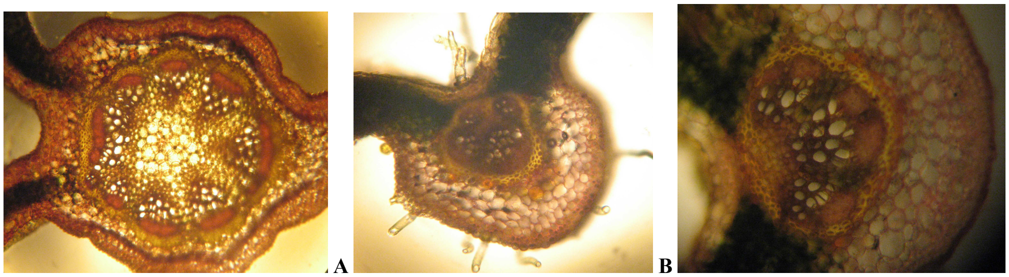
a b

Figure 5. Transverse section of the foliar laminain *Magnolia x soulangeana* (a-fresh sample preparation; b-preparation preserved in ethyl alcohol, javelized and stained with Geneva reactive; the arrow indicates the position of the oil cell)



b c

Figure 6. Transverse section of the foliar lamina in *Magnolia kobus* (a-fresh sample; b,c-preparation preserved in ethyl alcohol, javelized and stained with Geneva reactive; the arrow indicates the position of the oil cell)



A B C

Figure 7. Transverse section of the main nervure: A-*Magnolia kobus*, B-*Magnolia x soulangeana*, C- *Magnolia x "Susan"* (preparations preserved in ethyl alcohol, javelized and stained with Geneva reactive)

2. Stomatal density

Modifications in stomatal density, distribution and morphology on a foliar surface can be considered as significant traits in plants (Bettarini et al., 1998). Stomata, regulating the mechanisms of gas exchange in leaves, offer the possibility to study the interactions between plants and their environment. Plants are able to control their stomatal characteristics, which means, in the short term, influencing the opening and closing of the stomata in order to optimise the exchange of CO_2 and water vapors, and on a larger time scale, influencing the creation of new leaves (Robinson et al, 1998; Elagoz et al, 2006).

Stomatal density may vary within the same leaf, the leaves of the same plant, and between individuals of the same species (Al Afas et al., 2006). In amphistomatic leaves, the frequency at which they occur is typically higher in the abaxial epidermis (Volenikova & Ticha, 2001; Ianovici, 2006). Their number may also vary according to environmental factors, such as light, atmospheric humidity, water availability and atmospheric concentration of CO_2. Generally, stomatal density decreases with the increase of CO_2 (Woodward & Kelly, 1995).

Table 1. Stomatal density at lower epidermis level in *Magnolia sp.*

Magnolia kobus (stomata/mm^2)	*Magnolia* x *soulangeana* (stomata/mm^2)	*Magnolia x "Susan"* (stomata/mm^2)
502.79	102.42	270.01
586.59	93.1	242.08
633.14	111.73	251.39
502.79	111.73	223.46
549.34	121.04	260.7
614.52	65.17	288.64
558.65	121.04	279.32
502.79	139.66	260.7
567.97	111.73	279.32
512.1	102.42	297.95
521.41	148.97	260.7
512.1	121.04	242.08
540.03	111.73	232.77
502.79	148.97	242.08
558.65	121.04	297.95

In the case of the species included in this study, the average stomatal density has varied. *Magnolia kobus* presents the highest value – 544.3773 stomata/mm^2. The lowest number of stomata occurred in *Magnolia x soulangeana*, averaging at 115.4527 stomata/mm^2, while the hybrid *Magnolia x "Susan"* presents an intermediary average value of 261.9433 stomata/mm^2.

Regarding the maximum and minimum stomatal density (table 1), we can state the following: the maximum value is evident in *Magnolia kobus* (633.14 stomata/mm^2), while the minimum value is encountered in *Magnolia x soulangeana* "Soulange-Bodin", where we notice 65.17 stomata/mm^2.

Table 2. Analysis of stomatal density at *Magnolia sp*.

	Magnolia kobus	*Magnolia* x *soulangiana*	*Magnolia x "Susan"*
Min	502.79	65.17	223.46
Max	633.14	148.97	297.95
Sum	8165.66	1731.79	3929.15
Mean	544.377	115.453	261.943
Std. error	10.9365	5.51224	6.01837
Variance	1794.1	455.772	543.311
Stand. dev	42.3569	21.3488	23.309
Median	540.03	111.73	260.7
Skewness	0.836815	-0.446751	0.109956
Kurtosis	-0.20963	1.29446	-0.994574
Geom. mean	542.889	113.404	260.976
Levene's test for homogeneity of variance, based on means/p = 0.009179			

The stomatal complexes are situated at epidermis level (Schneider, 2007). The stomata are restricted to the abaxial surface of leaves (hypostomatic). Annexed cells and lateral neighbouring cells are distinguishable. The stomata correspond to the paracytic type (Metcalfe & Chalk, 1957), respectively the brachyparacytic type (Dilcher,1974). Krausel and Weyland (1959) incorrectly interpreted stomata as being of the anomocytic type. The subsidiary cells are narrow. These do not entirely surround annexed cells. On some leaves, the frequency of stomata increases from base to tip. According to Rao (1939), stomatal frequency in Magnoliaceae is uniform on the lamina of leaves. Generally, the increase of stomatal size is inversely proportional to the decrease of stomatal density (Schneider, 2007). The leaves of the hybrids reveal a stomatal density of less than 300, which indicates the fact that these are leaves developed in the shadow. Leaves developed in low intensity light conditions have lower stomatal densities than leaves developed in sunny areas (Givnish, 1988). Higher stomatal densities are found in young leaves, which may maximise photosynthetic gas exchange and water conductance before the introduction of senescence (Menghiu et al., 2012). An increase in stomatal density along with a decrease in stomatal size leads to an optimal adjustment, in general, of the regulation of gas exchange and, in particular, of the admission of pollutants through the stomata (Alves et al., 2008).

3. Stomatal size

Knowing stomatal size, we can determine an inversely proportional relation to stomatal density. Measuring stoma cell dimensions is easily achieved via scanning electron microscopy; as such, we will present concrete data regarding stomata size in *Magnolia.*

Magnolia kobus reveals the widest stomata, with a maximum value of 25088.01 nm and a minimum of 19294.51 nm, as well as the lowest length value (15692.69 nm). Elongated stomata are found in *Magnolia x soulangeana*, with a maximum value of 33672.21 nm and a minimum of 25303.71 nm. *Magnolia x "Susan"* has an intermediate stomata length and the lowest width values (13398.00 nm).

Table 3. Measurements regarding length and width of stomata in *Magnolia sp.*

N°.	Width of stomata			Length of stomata		
	Magnolia kobus	***Magnolia x soulangeana***	***Magnolia x "Susan"***	***Magnolia kobus***	***Magnolia x soulangeana***	***Magnolia x "Susan"***
	Width nm	Width nm	Width nm	Lengh nm	Lengh nm	Lengh nm
1	19798.46	18076.57	17551.70	24128.70	31606.47	25855.17
2	19781.30	20071.08	17910.88	21374.19	25303.71	26552.81
3	18912.23	21053.96	17785.33	23774.16	30109.45	22535.62
4	18512.88	19798.46	16894.30	20298.26	28438.60	20889.76
5	18132.85	21626.96	15488.45	21907.84	30244.52	25241.19
6	17840.14	19312.11	18462.12	22759.54	30982.36	24327.52
7	17840.14	20826.87	14093.00	19582.83	28581.57	26988.96
8	18659.09	20071.08	15549.25	19294.51	32937.90	24039.79
9	16991.74	18254.21	18230.69	23443.25	31520.38	23698.85
10	18067.17	20356.75	15662.61	20981.23	32186.84	21086.44
11	18659.09	16941.69	13398.00	20851.32	33505.35	22676.49
12	19539.42	20498.09	15435.06	21760.05	29683.39	23975.92
13	17210.23	20431.70	15242.83	21681.86	30778.88	22171.57
14	19408.60	20826.87	15358.46	23290.61	28700.16	24288.71
15	19824.18	18244.90	16027.34	25088.01	27834.99	22006.05
16	21500.94	18595.27	16775.20	21190.65	31086.34	26181.55
17	19312.11	21775.65	15857.19	20390.09	32059.96	26109.37
18	20473.22	19373.57	16326.26	22154.52	28706.08	27375.13
19	16119.78	19815.61	18891.52	24029.95	28847.72	25487.63
20	17571.56	18291.39	18230.69	22632.32	28005.31	27409.57
21	20231.22	19952.27	15250.57	20989.33	26948.36	21855.47
22	21053.96	19789.88	15457.96	23959.17	28270.89	24592.72
23	15692.69	21013.59	19451.35	23959.17	33672.21	24812.34
24	18831.23	19660.73	17917.47	22737.14	28005.31	24973.43
25	18595.27	18291.39	21325.59	24149.81	28158.88	25001.75
Average stomata size	**18742.38**	**19718.02**	**16742.95**	**22256.34**	**29847.03**	**24405.35**

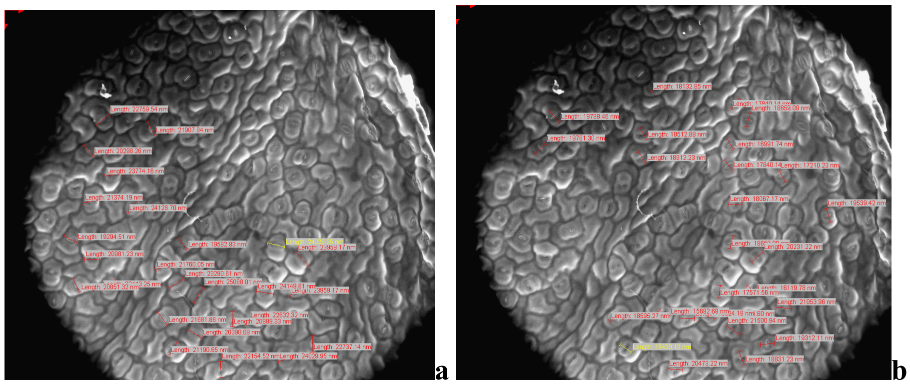


Fig. 8-SEM images with measurements of stomata cells in *Magnolia kobus*: a- length, b-width (a,b-500x)

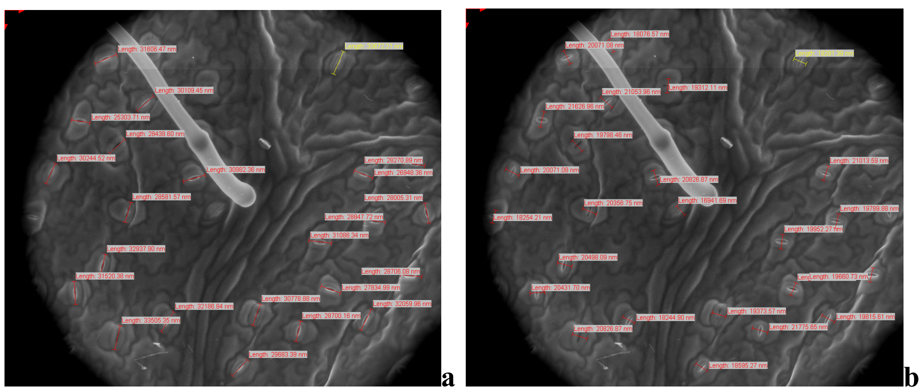


Fig. 9- SEM images with measurements of stomata cells in *Magnolia x soulangeana "Soul.-Bod."*: a- length, b- width (a,b-500x)

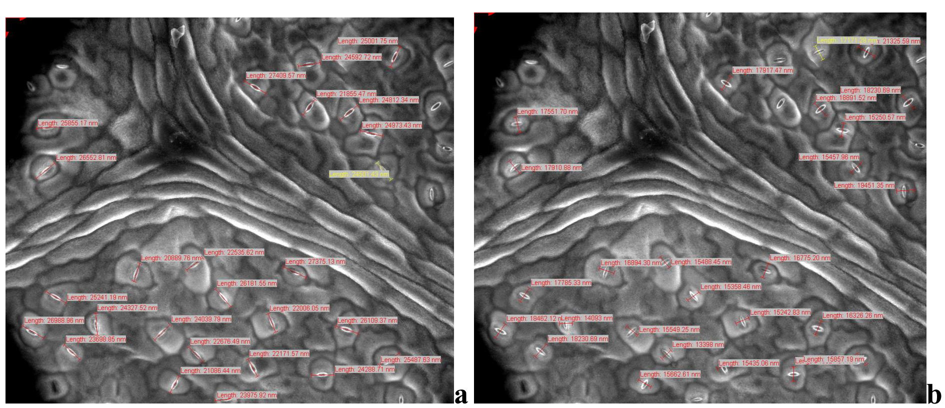


Fig. 10- SEM images with measurements of stomata cells in *Magnolia x "Susan"*: a- length, b- width (a,b-500x)

CONCLUSION

According to the study performed, stomata are present in very large numbers in *Magnolia kobus*, while the two hybrids reveal a lesser number of stomata. Regarding stomatal size, we can state that *Magnolia x soulangeana* presents longer stomata, while *Magnolia kobus* reveals wider stomata.

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