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Stomatal density and distribution in different cassava genotypes

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This study aimed to establish the anatomical characteristics, and to determine the density, size and distribution of stomata in the abaxial surface of leaves of different cassava genotypes. The apical, middle and basal portions of fully expanded leaves of genotypes Baianinha, Caapora, Fécula Branca, IAC 12, IAC 13, IAC 14, IAC 15, IAC 576, IAC 90 and IAPAR União were analyzed. The design was completely randomized with a 3 by 3 factorial arrangement and three replications, and the data were analyzed by Anova and Scott-Knott test. The parameters evaluated concerned the number, polar and equatorial diameters of stomata and calculations of density and stomatal functionality. Significant interaction was found among the different genotypes and the distribution and size of stomata in the different parts of the leave, being more frequent in leaf apices. The genotypes Baianinha, Caapora, IAC 576 and IAPAR União characterized the group with the highest stomatal density, while the genotype IAC 90 showed the lowest density and the largest polar diameter, regardless of the location of the stomata. In the analyzed leaves, the lowest polar diameter was detected in the genotypes Baianinha, Caapora, IAC 14 and IAPAR União, while the largest equatorial diameter was found in IAPAR União. In all genotypes, functionality was inversely proportional to stomatal density, with a negative correlation between stomatal density and the polar diameter of stomata.

Key words: *Manihot esculenta* Crantz, Papillae cells, stomata paracytic, scanning electron microscope.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is native to South America which is one of the main carbohydrate source about 500 million peoples around the world, playing an important role in human nutrition (El-Sharkawy, 2007). In addition to its nutritional purpose, the cassava is also a raw material widely used in various industrial segments, providing several byproducts as, chips for domestic use, sale to starch and feed factories or as an intermediate for production of flour (Halsey et al., 2008; Karim and Fasasi, 2009). Brazil is the third largest producer of cassava in the world, behind only Nigeria and Thailand (FAO, 2014). Its production 2013 is approximately 21.178.686 tons of

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roots, covering an area of 2.149.403 ha, with an average yield of 14 t ha^{-1} (IBGE, 2014).

Cassava leaves are simple and composed of palmately lobed leaf blade. The number of lobes in a leaf is variable, but it is usually an odd number ranging from three to nine. The cassava leaves as hypostomatic, with a defined palisade and spongy parenchyma, bundle sheath cells developed with radially distributed chloroplasts. The anatomical aspects of cassava have been little studied, more is necessary, due to the plasticity in leaf anatomy in different genotypes, which would cause differences in the photosynthetic rate, affecting CO_2 diffusion (Nassar et al., 2008). The distribution of stomata on leaves of cassava in the abaxial epidermis and restricted only to the proximity of the largest vascular bundles (Cock et al., 1987).

The reduction in the amount of water lost from the plants occurs by changes in the mechanism, size and distribution of stomata. Stomatal density is an important ecophysiological parameter that also affects gas exchanges and photosynthesis, stomatal density may vary in leaves of plants of the same species (Al Afas et al., 2006). Other possible sources of variation in stomatal density are linked to different genotypes and atmospheric CO_2 (Woodward, 1998), moisture (Serna and Fenoli, 2000), intensity and quality of light (Lake et al., 2001) and solar radiation (Kakani et al., 2003). The different genotypes of the same species can contain variations in the structure of the leaves, which may represent as an excellent tool in the selection process of cassava plants.

The aims of this study were to: establish the anatomical characteristics, as well as determine the density, size and distribution of stomata in the abaxial surface of leaves of different cassava genotypes.

MATERIALS AND METHODS

For the study of stomatal density, were collected of portions apical, middle and basal of plants, portions of fully expanded leaves of genotypes Baianinha, Caapora, Fécula Branca, IAC 12, IAC 13, IAC 14, IAC 15, IAC 576, IAC 90 and IAPAR União, obtained at Pólo Regional do Vale do Paranapanema APTA (São Paulo Agency for Agribusiness Technology), located in Assis, São Paulo (latitude 22° 40'S, longitude 50° 26'W and average altitude of 563 m).

The climate in the region is a subtropical climate with dry winters and hot, humid summers. The leaves were collected on April 30, 2013, from 09:00 to 11:30 when there was no record of rainfall, while the minimum and maximum temperatures recorded were 14 and 28°C, respectively.

The leaf samples were fixed in solution containing 5% formaldehyde, 90% ethyl alcohol 700GL and 5% acetic acid (AA), for 24 h at room temperature. The leaf material was prepared according to the methodology proposed by Johansen (1940), for visualization and characterization of the type of the stomata using scanning electron microscope (SEM).

The observation of the density and the distribution of stomata were performed using the North replica method (1956), where an impression of the leaf surface was obtained with cellulose acetate film adhesive tape over an area of approximately 1.0 cm^2. Sections of the acetate film formed were removed and mounted on slides for microscope assessment. An optical microscope eyepiece lens 15x and objective lens 40x was used, which provided 600x magnification and a field of view of 0.39 mm^2. The images used in the analysis were captured by a capture system (Carl Zeiss, Germany) composed of an AxioPhot I microscope equipped with AxioCam ICc3 camera and Bel View software. Image processing and analysis was performed with public domain software Image J 1.43a, version 64, with the measurement of five fields replicated for each analyzed leaf. For the assessments, polar diameter of stomata (DP) and equatorial diameter of stomata (DQ), slides were prepared with safranin, and traced with camera (Castro et al., 2009).

The stomatal density (DE – number of stomata per unit of area) and stomatal functionality (FUN – regarded as the polar/equatorial diameter ratio of the stomata) were calculated according to Castro *et al.* (2009). For the purposes of statistical analysis, stomatal density on the abaxial surface and the frequency of stomata in the apical, middle and basal portions of leaves were studied. The design was completely randomized with a 10×3 factorial arrangement (genotypes of cassava, with three sampled regions and three replications) and three replications) ad each leaf area was observed in five microscopic fields.

The data were subjected to analysis of variance. Pearson correlation tests were performed ($p \leq 0.05$) between the parameters measured and the averages were grouped according to the criteria of Scott-Knott ($p \leq 0.05$). As the data were normally distributed (Shapiro-Wilk) no transformation was used before analysis of variance. Sisvar software was used for statistical analysis (Ferreira, 2004).

RESULTS AND DISCUSSION

The significant effect was observed for the interaction between genotype x leaf position and the parameters stomatal density (DE), polar diameter (DP) and equatorial diameter (DQ) ($p \leq 0.01$), except for functionality (FUN) ($p \geq 0.01$). This significant interaction shows that the cassava genotypes responded differently to stomatal distribution and size in the different foliar areas (Table 1).

The stomata were classified as paracytic for all the genotypes, being distributed in a waveform on the abaxial surface of the leaves. With the use of SEM, cells forming papillae with a more or less conical dome was observed on the abaxial surface of leaves in all genotypes/cultivars (Figure 1A). These papillae are distributed throughout the epidermis of this surface. However, beside the primary and secondary ribs, they have a round shape, with smaller domes (Figure 1B). In front view, these cells form a crown around the stomata (Figure 1C).

Similar features were found in the epidemis of the adaxial surface of *Manihot caerulescens* (Mendonça, 1983). In *Manihot glaziovii*, authors reported the occurrence of rectangular cells with the external periclinal walls often pointed, similar to the papillae observed in the present study (Mendonça, 1992). The papillae present in the epidermis favor the absorption of light by plants growing under the canopy of tropical forests (Bone et al., 1985; Mantovani and Vieira, 1997).

Given that *M. esculenta* is a plant grown in full sun, the presence of these papillae on the abaxial surface seems to have the primary function of protecting the stomata, spreading like a crown around them. However, more in-

Table 1. Summary of the analysis of variance with the mean squares and coefficients of variation regarding stomatal density (DE), polar diameter of stomata (DP), equatorial diameter of stomata (DQ) and stomatal functionality (FUN).

Cause of variation	DF	DE	DP	DQ	FUN
Genotypes (G)	9	1272.78**	225.36**	8.83**	0.05**
Position (P)	2	173.20**	10.55**	1.78^{ns}	0.02^{ns}
G * P	18	136.57**	58.03**	2.00**	0.04^{ns}
Waste	60	33.37	9.03	0.67	0.02
C.V.(%)		6.92	0.86	3.17	3.21

** - Significant effect by F test at 1% probability; D.F. - degrees of freedom; n.s. - non-significant.

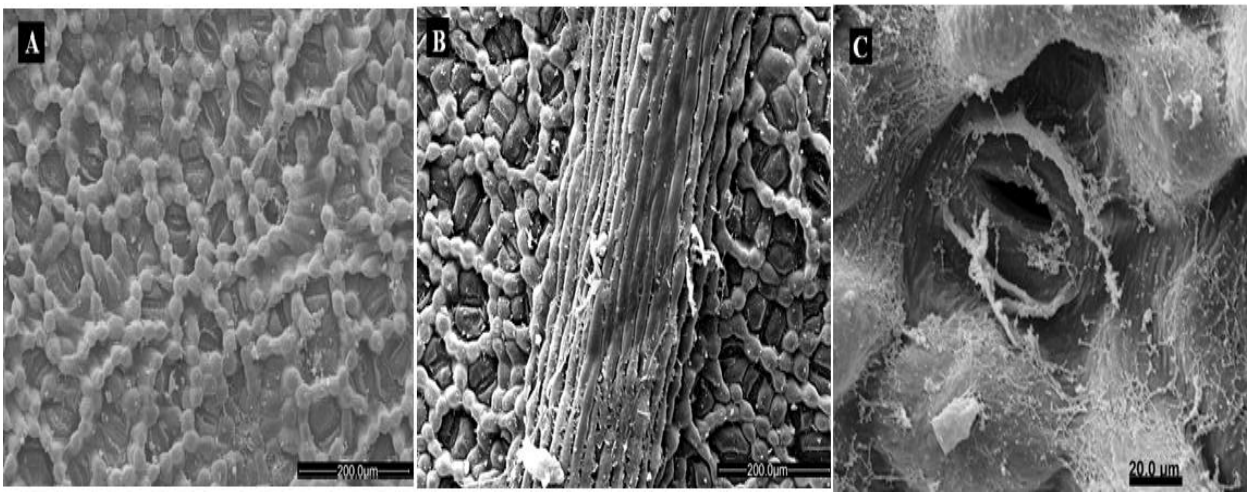

Figure 1. Scanning electron microscopy of stomata on the abaxial surface of the leaf lobe of leaves of cassava cultivar IAC 14. Cells forming papillae in A, papillae adjacent to primary rib, in B, highlight of stoma with papillae arranged in crown shape (C).

depth studies are needed to prove that papillae protect stomata or else that they act as lenses that could converge sun rays to the cells that contain chlorophyll.

The means observed for DE varied in the apical portion of the leaf between 67 to 100 stomata per mm^2, combining cassava genotypes in three groups, according to Scott-Knott (Table 2). In this part of the leaf, the following genotypes deserve mention: Baianinha (100), Caapora (94), IAC 15 (97), IAC 576 (92) and IAPAR União (100). The other genotypes Fécula Branca, IAC 12, IAC 13 and IAC 14 were placed in an intermediate group, with 75, 75, 78, and 79 stomata per mm^{-2}, respectively (Table 2). The smallest DE observed between genotypes in the apical portion was found in IA 90, with 67 stomata per mm^2.

Mean variation of DE in the middle portion of the leaf: 61 to 104 stomata per mm^2 (Table 2) were observed. The largest DE was observed for genotype IAPAR União, while the genotypes Fécula Branca, IAC 12, IAC 14 and IAC 90 formed the group with the smallest amount of stomata per area. The other genotypes are grouped in an intermediate group for DE.

Concerning DE averages in the basal portion, there was an increase of 7.8% over the highest average of stomata in the middle portion. Also, the genotypes in this portion were grouped, with Caapora with the highest DE (112). The average results of lowest DE on the base of the leaf were observed in genotypes IAC 90, IAC 15, IAC 14 and Baianinha (Table 2).

DE changes observed in different parts and on the surface of cassava leaves are consistent with those found in several other plants (Heichel, 1990). Similar results were obtained in leaves of *Zebrina purpusii* Bruckn, which revealed a stomatal density of 62.5 stomata per mm^2, arranged in rows parallel to the main rib at the apex of the leaf (Schooner and Bukovac, 1972). Observations in stomatal density on the adaxial surface of leaves of *Sorghum halepense* L. varied between 25 and 106 stomata per mm^2 and on the abaxial surface stomatal density varied between 76 and 129 stomata per mm^2, both distributed on the apex of the leaf (Mcwhorter et al., 1993).

The effect of water scarcity on leaf anatomy of cassava genotypes, found high plasticity in DE for genotype UFLA

Table 2. Stomatal density (stomata per mm^2) of the apical, middle and basal portions of the abaxial surface of leaf lobes in different cassava genotypes.

Genotypes	Area of the leaf		
	Apical	Middle	Basal
Baianinha	100^{aA1}	93^{bA}	78^{cB}
Caapora	94^{aA}	84^{bC}	112^{aA}
Fécula Branca	75^{bA}	61^{cB}	71^{dA}
IAC 12	75^{bA}	68^{cA}	73^{dA}
IAC 13	78^{bA}	83^{bA}	84^{cB}
IAC 14	79^{bA}	74^{cA}	79^{cA}
IAC 15	97^{aA}	85^{bB}	84^{cA}
IAC 576	92^{aA}	89^{bA}	92^{bA}
IAC 90	67^{cA}	69^{cA}	64^{dA}
IAPAR União	100^{aA}	104^{aA}	99^{bA}

[1]Means followed by the same lower case in the column and uppercase in the line belong to the same group, according to Scott-Knott (1974), at 5% probability.

E, under dry conditions, and the level of plasticity was different for the different genotypes studied (Cerqueira, 1992). Thus, a higher DE may allow stomatal opening in a shorter period of time, with proper capture of CO_2 and reducing the period of time that these stomata are open, and, therefore, reducing transpiration, allowing better adaptation of cassava genotype to conditions of low availability of water (Mcwhorter et al., 1993; Cerqueira, 1992).

For some authors, there is a direct response of stomatal density to increased shading (Khan et al., 2003). This finding in their analysis of the growth of *Spondias purpurea* L. (*Anacardiaceae*) in response to different sunlight levels, reporting that stomatal density decreased with decrease in light levels (Barrios and Hernández, 2003). Another factor that can influence the number of stomata in the leaves is their architecture, and in studies with *Xylopia brasiliensis* Sprengel, smaller plants with more branches showed lower stomatal density values. Also, a low positive correlation was observed between stomatal density and average CO_2 assimilation in the plants of this study. These results are directly related with the cassava crop, since many genotypes usually have trichotomous stems, with three branches (Justo et al., 2005). The cassava is a compact plant where competition for light is intense, which can lead to reduced availability of solar radiation to the leaves located in the lower third of the plant. The size and location of stomata also affect CO_2 absorption (Boeger and Wisniewski, 2003). The genotypes showed different characteristics regarding the DP parameter ($p \leq 0.05$), demonstrating that cassava leaves have plasticity for this parameter. In this study we found an inverse relationship between DE and DP (Tables 2 and 3), which is consistent with the findings obtained by other authors (Hetherington and Woodward, 1993; Pearce et al., 2006).

The genotype IAC 90 showed the highest DP values, regardless of the position of the leaf, and genotype IAC 14 showed the lowest value in the apical portion. The lowest DP values in the middle portion of the leaf were obtained for genotypes Baianinha (43.13 µm), IAC 576 (43.26 µm) and IAPAR União (42.93 µm). However, for the basal portion, the lowest DP value of stomata was found for genotye Caapora (42.16 µm) (Table 3). The reduction in the size of the stomata is an event recognized as important in the regulation of gas exchanges, since leaves with smaller stomata have greater water use efficiency because of the smaller size of stomatal pores, resulting in reduced water loss through transpiration.

Thus higher stomatal density associated to reduction in stomatal size (Tables 2 and 3) may ensure an adequate supply of the CO_2 needed for photosynthesis, without excessive water loss to the detriment of stomata with smaller pores. No significant difference ($p \geq 0.01$) was observed for variable DQ in the different areas of the leaf lobe (Table 1). The evaluation of each analyzed area showed the following: in the apical area, genotypes IAC 12, IAC 13 and IAPAR União showed the highest DQ values, unlike the other genotypes that showed the lowest equatorial diameter values in µm (Table 4). In the middle portion of the leaf, the highest DQ values were obtained for the abovementioned genotypes and also for genotypes Fécula Branca, IAC 15 and IAC 90, and the other genotypes had the lowest DQ values. Unlike the apical and middle portions, the basal portion of the leaves of genotypes showed the most significant differences in DQ values among the groups (Table 4).

Reduction in stomatal opening has greater effect on water than CO_2 diffusion, thus, maintaining the uptake of CO_2 needed for photosynthesis with lower water loss through transpiration (Beaulieu et al., 2008; Hodgson et al., 2010; Bergmann and Sack, 2007).

Changes in stomatal behavior, in what concerns

Table 3. Polar diameter (µm) of the apical, middle and basal portions of the abaxial surface of leaf lobes in different cassava genotypes.

Genotypes	Area of the leaf		
	Apical	Middle	Basal
Baianinha	43.30^{dB1}	43.13^{fB}	45.73^{cA}
Caapora	43.34^{dB}	44.80^{eA}	42.16^{fC}
Fécula Branca	43.50^{dB}	46.80^{bA}	43.26^{eB}
IAC 12	47.80^{bA}	47.86^{aA}	47.60^{aA}
IAC 13	45.70^{bB}	46.23^{cA}	46.63^{bA}
IAC 14	45.07^{cA}	45.65^{dA}	45.13^{cA}
IAC 15	43.30^{dC}	45.30^{dA}	44.30^{dB}
IAC 576	43.16^{dB}	43.26^{fB}	45.73^{cA}
IAC 90	47.66^{aA}	47.96^{aA}	47.90^{aA}
IAPAR União	43.13^{dA}	42.93^{fA}	43.16^{eA}

[1]Means followed by the same lower case in the column and uppercase in the line belong to the same group, according to Scott-Knott (1974), at 5% probability.

stomatal size, vary greatly among plants depending on the environment where they are grown (Melo et al., 2004), on the genetic constitution of the species, and occur frequently in plants under different levels of stress (Camargo and Marenco, 2011).

Changes in stomatal shape directly affect their functionality, the elliptical shape being more typical of functional stomata, while the spherical shape is more frequently associated to stomata with poor functional responses, and both shapes are related to higher or lower DQ values, respectively (Yukawa, 1992). These results are consistent with differences in DQ values of stomata of the genus Dendrobium, for different individuals and between individuals of the same species. The equatorial diameter can be advantageous as a taxonomic marker of species. Changes in the DQ values of stomata of barley were observed in studies conducted in flooded regions by. However, other authors did not find changes in the amount and size of stomata of vegetables in flooded regions, indicating that stomatal changes are not very clear for all species, even when exposed to the same type of environmental change (Yordanova et al., 2005; Striker et al., 2005).

The behavior of the genotypes regarding stomatal functionality (FUN) revealed that Caapora, IAC 12, IAC 14 and IAC 90 showed the highest average values for this characteristic (Figure 2). No significant difference ($p \geq 0.05$) was found between the other cultivars regarding this characteristic, except for genotype IAPAR União that showed the lowest FUN. Compared to the other cultivars, except genotype IAPAR União that showed the lowest FUN (Figure 2).

Obtained different results where the cassava genotypes studied showed the highest DE and FUN values (Ribeiro et al., 2012). The importance of FUN results is the decrease in. The decrease in transpiration was observed in plants with better stomatal functionality (Souza et al., 2010; Boeger and Wisniewski, 2003; Castro et al., 2009).

The correlation coefficients showed a negative and significant association ($p \leq 0.05$) between DE and DP for all the genotypes studied. However, there was no correlation (positive or negative) for the other parameters (Table 5). The opposite is also true, that is, the lower this correlation, the less ellipsoidal is the shape of the stomata, which are also less functional, although this study did not establish a correlation of DE and DP with FUN (Table 5).

The relationship between DP and DE provides a good indication of the shape of the stomata, since the higher this correlation, the more ellipsoidal is the shape of stomata, and they are also more functional (Beaulieu et al., 2008).

The study on stomatal features in cassava demonstrates that the leaves, which are responsible for the synthesis of carbohydrates and root tuberization, present important features related to stomatal distribution and size, depending on the genotype, as well as their own and specific anatomy, e.g. the presence of papillae on the abaxial surface of the epidermis, which may confer protection to stomata and ensure they remain open for a longer period of time even under dry conditions.

Conclusions

The results obtained in this study allow us to conclude that there are differences in stomatal density, size and distribution depending on their position in the leaves of the different cassava genotypes, and that stomatal density is higher in the apical portions of leaf lobes. Also, functionality is inversely proportional to density and there was a negative correlation between stomatal density and the polar diameter of stomata.

Table 4. Equatorial diameter (µm) of the apical, middle and basal portions of the abaxial surface of leaf lobes in different cassava genotypes.

Genotypes	Area of the leaf		
	Apical	Middle	Basal
Baianinha	25.95[b1]	25.04[b]	25.27[c]
Caapora	24.94[b]	24.87[b]	23.26[d]
Fécula Branca	25.91[b]	27.01[a]	23.91[d]
IAC 12	26.56[a]	25.99[a]	26.44[b]
IAC 13	27.55[a]	26.40[a]	26.58[b]
IAC 14	24.77[b]	24.73[b]	24.41[d]
IAC 15	25.43[b]	26.26[a]	25.20[c]
IAC 576	25.85[b]	25.51[b]	26.97[b]
IAC 90	25.68[b]	27.57[a]	25.72[c]
IAPAR União	27.59[a]	27.09[a]	28.37[a]

[1]Means followed by the same lower case in the column and uppercase in the line belong to the same group, according to Scott-Knott (1974), at 5% probability.

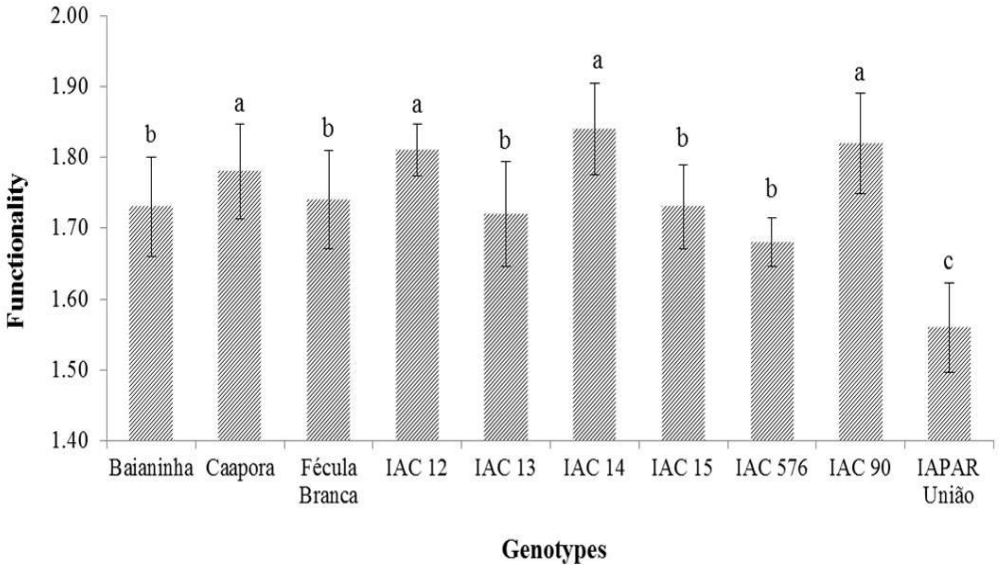

Figure 2. Functionality of stomata of the abaxial surface of leaf lobes in the different cassava cultivars. The letters indicate differences between the cultivars. Mean ± S.E.M = Mean values ± Standard error of means of six experiments.

Table 5. Correlation coefficients between the variables assessed in the leaf lobes of different cassava genotypes.

Genotypes	Variables	DP	DQ	FUN
Baianinha	DE		0.187[ns]	-0.324[ns]
	DP	-0.854**	-0.122[ns]	0.393[ns]
	DQ			-0.201[ns]
Caapora	DE		-0.546[ns]	0.082[ns]
	DP	-0.857**	0.580[ns]	-0.028[ns]
	DQ			-0.089[ns]
Fécula Branca	DE	-0.752**	-0.356[ns]	-0.230[ns]

Table 5. Contd.

	DP		0.325^{ns}	-0.066^{ns}
	DQ			-0.434^{ns}
	DE		0.274^{ns}	-0,350^{ns}
IAC 12	DP	-0.964**	0.262^{ns}	-0.017^{ns}
	DQ			-0.260^{ns}
	DE		-0.103^{ns}	0.289^{ns}
IAC 13	DP	-0.765**	-0.245^{ns}	0.466^{ns}
	DQ			-0.371^{ns}
	DE		-0.466^{ns}	0.375^{ns}
IAC 14	DP	-0.887**	0.174^{ns}	0.183^{ns}
	DQ			0.135^{ns}
	DE		-0.303^{ns}	-0.014^{ns}
IAC 15	DP	-0.711**	0.315^{ns}	0.280^{ns}
	DQ			-0.321^{ns}
	DE		0.083^{ns}	0.167^{ns}
IAC 576	DP	-0.810**	0.213^{ns}	0.077^{ns}
	DQ			-0.517^{ns}
	DE		0.553^{ns}	-0.615^{ns}
IAC 90	DP	-0.865**	0.170^{ns}	0.025^{ns}
	DQ			-0.180^{ns}
	DE		0.068^{ns}	0.022^{ns}
IAPAR União	DP	-0.736**	0.326^{ns}	0.026^{ns}
	DQ			-0.236^{ns}

**Significant by test F, ($P \leq 0.05$); n.s – non-significant.

Conflict of Interest

The authors have not declared any conflict of interest.

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