

## COMPARATIVE LEAF MICROMORPHOANATOMY OF *Vitis vinifera* SSP. *vinifera* (Vitaceae) RED CULTIVARS

### MICROMORFOANATOMIA FOLIAR DE CULTIVARES TINTAS DE *Vitis vinifera* SSP. *vinifera* (Vitaceae)

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#### SUMMARY

Aiming to characterize and discriminate between four red grapevine cultivars – ‘Aragonez’ (AR), ‘Cabernet Sauvignon’ (CS), ‘Syrah’ (SY) and ‘Touriga Nacional’ (TN) – grown under Mediterranean field conditions, we studied their leaf micromorphoanatomic characteristics under light (LM) and scanning electron microscopy (SEM). The studied characteristics included those of the epidermis, stomata and hair distribution, and the mesophyll structure. The individual primary leaf area revealed significant differences between cultivars, with the highest value presented by AR and the lowest by CS, while SY and TN gave intermediate values. CS presented a significantly higher leaf specific dry weight value than the other three cultivars, which returned similar values. Under SEM magnification three types of stomata were identified in all the studied genotypes: sunken, at the same level, and raised above the other epidermal cells. Each cultivar displayed different percentages of these types of stomata: the highest raised-above values were observed in AR; TN had the highest same-level values and the lowest sunken ones; CS revealed the highest values for sunken stomata; while SY returned average values for all the types of stomata. Stomatal density was higher in AR and SY and lower in CS and TN. The hairs on the lower surface presented a similar woolly aspect in all the studied cultivars, but the mesophyll structure was quite different: CS presented the highest and AR the lowest values for total thickness of the lamina, thickness of palisade and spongy parenchyma, and length and thickness of upper and lower epidermal cells; the values for these leaf features in TN and SY fell between those for CS and AR. The data suggest that differences in leaf micromorphoanatomy can be used to distinguish between grapevine cultivars. Further studies are needed to confirm whether there is any association between some of these leaf traits – e.g. stomata type and mesophyll structure – and the physiological behaviour observed under field conditions.

#### RESUMO

Este estudo teve por objetivo caracterizar e comparar as características micromorfoanatômicas foliares de quatro cultivares tintas de videira – ‘Aragonez’ (AR), ‘Cabernet Sauvignon’ (CS), ‘Syrah’ (SY) e ‘Touriga Nacional’ (TN) – cultivadas em condições de campo com clima mediterrânico, com recurso à microscopia ótica (LM) e eletrónica de varrimento (SEM). Observaram-se os seguintes caracteres foliares: epiderme superior e inferior, estomas, indumento e estrutura do mesófilo. A área foliar da folha principal apresentou diferenças significativas entre cultivares, apresentando AR o maior valor e CS o menor e SY e TN valores intermédios. CS apresentou uma área foliar específica significativamente superior ao das outras cultivares. Por recurso à microscopia eletrónica, identificaram-se três tipos de estomas nas quatro cultivares – enterrados, ao mesmo nível e elevados em relação às células epidérmicas. A proporção de cada tipo de estoma variou significativamente entre cultivares: AR apresentou uma maior percentagem de estomas elevados; TN os maiores valores de estomas ao mesmo nível e os menores dos estomas enterrados; CS os maiores valores de estomas enterrados; enquanto SY apresentou percentagens intermédias dos três tipos de estomas. A densidade estomática foi maior em AR e SY e menor em CS e TN. O indumento da página inferior foi semelhante nas quatro cultivares. A estrutura do mesófilo apresentou diferenças significativas entre as quatro cultivares. Os maiores e menores valores da espessura total do limbo, do parênquima clorofilino em paliçada e lacunoso e do comprimento e espessura das células da epiderme superior e inferior foram apresentados pelo CS e AR, respetivamente, enquanto que a TN e a SY apresentaram valores intermédios destes caracteres. Os resultados mostram diferenças significativas na micromorfoanatomia foliar dos genótipos estudados sublinhando a necessidade de mais estudos para averiguar possíveis interações entre caracteres anatómicos foliares – tipo de estomas e estrutura do mesófilo, por exemplo – e respostas fisiológicas observadas em condições de campo.

**Key words:** grapevine, leaf, epidermis, stomata, mesophyll.

**Palavras-chave:** videira, folha, epiderme, estomas, mesófilo.

#### INTRODUCTION

It is well known that plants have developed functional and structural features that allow them to survive in unfavourable environments. This diversity of structure is as great as taxa biodiversity. Grapevine (*Vitis vinifera* L. ssp. *vinifera*) leaves are a good example, as they exhibit several adaptive mechanisms – a reduction in leaf size, or an increase in mesophyll

compactness (Esau, 1977; Dickison, 2000), for instance. Under heat and water stress conditions plants decrease the size of their epidermal cells, increase epidermal cell density and thickness, and stomata became more numerous and smaller (Gokbayrak *et al.*, 2008; Salem-Fnayou *et al.*, 2010). Grapevine cultivars have also been reported to adapt to water deficit, mainly by modifying their morphological

and anatomical characteristics (Gómez-del-Campo *et al.*, 2003, 2004; Koundouras *et al.*, 2008; Costa *et al.*, 2012). Descriptive botanical works on the ampelography (Galet, 2000), morphology and anatomy (Pratt, 1974; Esau, 1977; Metcalfe and Chalk, 1979) of grapevines were published in the early twentieth century. More recently, grapevine cultivars have been characterised by ampelographic descriptors based on morphology and morphometry (Mota, 1986; Dettweiler, 1991; Coelho *et al.*, 2004), and in some cases, on microsatellite markers (Santiago *et al.*, 2005; Lopes *et al.*, 2006; Veloso *et al.*, 2010). Grapevine cultivar susceptibility and resistance to downy mildew associated with differences in leaf morphology at the macro- and microscopic levels were studied by Boso *et al.* (2010, 2011). Few attempts have been made to employ leaf micromorphology traits to explain heat and water stress tolerance (Gómez-del-Campo *et al.*, 2003; Koundouras *et al.*, 2008; Costa *et al.*, 2012). Recently, Sadras *et al.* (2012) reported that stomata density of the ‘Shiraz’ cultivar was unaffected by temperature, but stomata length and width increased with heat. They concluded that longer and wider stomata contributed to the enhanced plasticity of stomatal conductance under higher temperatures.

Micromorphological grapevine leaf traits, such as stomata (Denisov, 1970; Sievers, 1971; Hegedüs, 1974; Pratt, 1974; Düring, 1980; Boso *et al.*, 2011), trichomes (Hegedüs, 1974; Pratt, 1974), mesophyll (Pratt, 1974; Patakas *et al.*, 2003) and leaf surfaces (Salem-Fnayou *et al.*, 2010; Boso *et al.*, 2011), have been studied. Differences have been observed between *V. vinifera* cultivars in leaf morphoanatomical features, although few works have used them to classify the studied cultivars (Swanepoel and Villers, 1987; Boso *et al.*, 2011). The latter researchers concluded that the most important micromorphological characteristics for distinguishing between genotypes are stomatal frequency and trichome distribution.

According to the review by Pratt (1974), grapevine mainly has stomata on its lower epidermis, which may be raised above, at the same level and sunken, relatively to the surface of the epidermis. Swanepoel and Villers (1987) also described all three types of stomata in the genus *Vitis*. Boso *et al.* (2011) reported that the stomata of the cv. ‘Blanco Legítimo’ stood out in that it displayed a type of doming of the epidermis – a feature that was not seen in any other of the genotypes they studied.

According to Schanderl (1968) and Pratt (1974), in grapevine the trichomes appear especially on the lower surface of the leaf and are uni- or pluricellular, dead and woolly, or living and woolly, round or flat, or twisted if long. Boso *et al.* (2011) observed reclining and erect hairs under the scanning microscope at different magnifications. The reclining hairs appeared as filaments of different sizes, while the erect hairs took the shape of small spikes. Patakas *et al.* (2003)

and Boso *et al.* (2011) observed that some genotypes presented differences in palisade and spongy thickness and in exposed upper epidermal cell area.

The knowledge of the structure of epidermis and mesophyll cells is meaningful in terms of environmental species adaptation. The Mediterranean climate experiences periods of high light and drought that greatly influence the plant growth and development, yield and the quality attributes of the grapes. Some differences among grapevine cultivars have been observed with regard to their ability to adapt and produce under drought conditions. For example, under the same Mediterranean field growth conditions the red grapevine cultivars ‘Aragonez’, ‘Cabernet Sauvignon’, ‘Syrah’ and ‘Touriga Nacional’ diverge in their responses to heat and drought in summer conditions (mid-ripening – late August), with ‘Cabernet Sauvignon’ and ‘Touriga Nacional’ being more tolerant than ‘Aragonez’ (Costa *et al.*, 2012).

In the present study leaf micromorphoanatomic-biometric characteristics of the *V. vinifera* ssp. *vinifera* red cultivars ‘Aragonez’, ‘Cabernet Sauvignon’, ‘Syrah’ and ‘Touriga Nacional’ were analysed at the light and scanning electron microscope level, with the goal of distinguishing between the leaf anatomy of the genotypes.

## MATERIAL AND METHODS

### Plant material and growth conditions

Five-year-old field-grown grapevines of the red cultivars ‘Aragonez’ (AR), ‘Cabernet Sauvignon’ (CS), ‘Syrah’ (SY) and ‘Touriga Nacional’ (TN) grafted on SO4 rootstocks were studied at a non-irrigated vineyard located near Torres Vedras, 60 km north of Lisbon, within the Lisbon Winegrowing Region (39° 01’ N, 9° 06’ E). The soil is a sandy clay loam with 1.45% organic matter and pH 5.9. The vineyard has a planting density of 4,000 vines per hectare, spaced 1.0 m within and 2.5 m between north-south oriented rows. Vines were trained on a vertical shoot positioning with two pairs of movable wires, and spur-pruned on a bilateral Royat Cordon system. All vines were uniformly pruned to 14-16 nodes per vine. Shoots were trimmed twice, between bloom and veraison, at a height of about 1.5 m.

The vineyard is a small varietal collection, where the four studied cultivars are planted side by side, with three adjacent rows of 60 plants per cultivar under the same canopy and cultivation management. For data collection, four blocks of 10 vines per cultivar were established along the central row.

### Morphoanatomical analysis

In 2007, six full-expanded leaves per block from each cultivar were taken from the 8<sup>th</sup> node of the shoot, at veraison (end of July). Five small leaf blade sec-

tions were cut from the central leaf part, between the main and lateral veins. All measurements and counts related with morphoanatomical traits were done on random fields under light (LM) and scanning electron microscopy (SEM), always at comparable leaf situations and magnifications.

### Light microscopy (LM)

Fresh material and dried material were placed in sodium hypochlorite until bleached white, and then washed in distilled water. These LM observations focused on the upper and lower epidermal characteristics, such as epidermal cell shape.

At the same time, pieces of fresh leaves of each cultivar were fixed with 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0 (Hayat, 1981). Samples were then washed and tissue dehydration was carried out in an ascendant alcohol series and processed using the paraffin micro-technique (Ruzin, 1999). The blocks were sectioned (10-12 µm) in a Minot microtome and examined with a Nikon Labophot 2 photomicroscope. Images were obtained with a Nikon FX-35W camera equipped with a semi-automatic Nikon PFX adapter (Nikon®). These LM observations focused on the transverse sections and addressed total lamina thickness, thickness of palisade and spongy tissues, upper cuticle thickness, length of upper and lower epidermal cells, and thickness of upper and lower epidermal cells.

### Scanning electron microscopy (SEM)

Plant material was fixed as above, critical-point dried in a Critical Point Polaron BioRad E3500, and coated with gold in a Jeol JFC-1200. Observations were carried out at 15 kV on a Jeol JSM-5220 LV scanning electron microscope equipped with a direct image acquisition system. Measurements and counts were obtained by computer-assisted image analysis.

SEM observations focused on the upper and lower epidermis surface details – type of indumentum, epicuticular waxes, stomata density (stoma mm<sup>-2</sup>), type of stomata and percentage of total of each type, and stomata cell length and width. The type of stomata followed the nomenclature given by Pratt (1974), who considered three types: rose above, at the same level, and sunken to the level of the other epidermal cells.

### Vegetative growth

Individual primary leaf area was estimated using the methodologies proposed by Lopes and Pinto (2000). Leaf dry weight and specific dry weight were assessed at veraison in a random sample of 12 leaves (8<sup>th</sup> node).

### Data analysis

Anatomical data were analyzed by one-way ANOVA in accordance with GLM procedures from the SAS® program package (SAS Institute, Cary, NC, USA), and statistical differences between means were assessed by LSD test ( $\alpha < 0.05$ ). Stomata type (% of total) was analysed in terms of non-transformed and transformed (arcsine of the square root) values. As arcsine transformation did not alter the interpretation of the data, thus non-transformed mean figures for stomata type are presented.

## RESULTS

### Micromorphoanatomical traits

The leaf morphoanatomic-biometric characteristics of the studied *V. vinifera* cultivars are summarised in Tables I and II. The individual primary leaf area of the 8<sup>th</sup> leaf reveals significant differences between cultivars, with the highest value presented by AR and the lowest by CS, while SY and TN gave intermediate values. CS presented a significantly higher leaf specific dry weight value than the other three cultivars, which obtained similar values (Table I).

TABLE I

Means and standard error (in parentheses) of several leaf morphoanatomical features of four red *V. vinifera* ssp. *vinifera* varieties, at veraison. Média e erro padrão da média (entre parêntesis) de vários caracteres morfoanatómicos foliares das quatro cultivares tintas de *V. vinifera* ssp. *vinifera*, ao pintor.

Leaf character	'Aragonez'	'Cabernet Sauvignon'	'Syrah'	'Touriga Nacional'	sig.
<b><sup>a</sup> Macromorphological parameters</b>					
Individual primary leaf area (cm <sup>2</sup> )	262.9 (5.10) a	146.8 (3.71) c	200.8 (5.89) b	186.5 (2.97) b	***
Dry weight (g)	1.5 (0.08) a	1.3 (0.06) b	1.4 (0.09) b	1.2 (0.05) b	***
Specific dry weight (DW/LA; mg.cm <sup>-2</sup> )	5.2 (0.27) b	7.1 (0.40) a	6.2 (0.23) b	6.6 (0.41) b	***
<b><sup>b</sup> Micromorphoanatomical characters</b>					
Total thickness of lamina (µm)	165.8 (1.22) d	201.4 (1.39) a	191.7 (1.63) c	196.7 (1.45) b	***
Thickness of palisade tissue (µm)	48.4 (0.70) c	60.8 (0.71) b	60.1 (0.87) b	64.4 (0.67) a	***
Thickness of spongy tissue (µm)	80.4 (1.14) c	97.0 (0.74) b	109.6 (1.25) a	99.7 (0.95) b	***
Thickness of upper cuticle (µm)	2.66 (0.04) a	2.63 (0.03) a	2.60 (0.01) a	2.60 (0.01) a	ns
Length of upper epidermal cells (µm)	24.3 (1.26) c	34.0 (1.09) a	27.0 (1.04) b	29.6 (0.94) b	***
Thickness of upper epidermal cells (µm)	13.2 (0.40) b	17.5 (0.54) a	13.9 (0.56) b	17.7 (0.85) a	**
Transversal area of upper epidermal cells (µm <sup>2</sup> )	319.5 (19.36) c	595.8 (29.00) a	461.9 (27.13) b	530.9 (37.5) a	***
Length of lower epidermal cells (µm)	22.0 (0.98) a	23.2 (0.68) a	22.2 (1.48) a	25.1 (0.90) a	ns
Thickness of lower epidermal cells (µm)	13.7 (0.65) c	17.2 (0.34) a	13.2 (0.40) c	14.9 (0.40) b	***
Transversal area of lower epidermal cells (µm <sup>2</sup> )	304.2 (22.21) c	398.3 (15.66) a	326.3 (19.33) b	331.7 (16.13) b	***

<sup>a</sup> Means of 12 replicates. <sup>b</sup> Means of 30 replicates.

In each row different letter suffixes show statistically significant differences at  $\alpha < 0.05$  by LSD test. sig – significance; ns – not significant;

\*\* - significant at  $\alpha < 0.01$  and \*\*\* - significant at  $\alpha < 0.001$ .



**TABLE II**

Means and standard error (in parentheses) of stomatal density, raised above, at the same level and sunken stomata types, stomata length and width of four red *V. vinifera* ssp. *vinifera* varieties, at veraison.

*Média e erro padrão da média (entre parêntesis) da densidade estomática, tipos de estomas, comprimento e largura dos estomas nas quatro cultivares tintas de V. vinifera ssp. vinifera, ao pintor.*

Leaf character		'Aragonez'	'Cabernet Sauvignon'	'Syrah'	'Touriga Nacional'	sig.	
<sup>a</sup> Stomatal density (n°.mm <sup>-2</sup> )		285.7 (22.13) a	217.1 (17.14) b	280.0 (16.61) a	206.7 (12.29) b	***	
	<sup>b</sup> Stomata type % of total	Raised above	68.7 (2.65) a	41.8 (3.72) b	39.1 (1.83) b	36.7 (1.85) b	***
		Same level	19.8 (1.36) c	23.4 (3.72) c	37.0 (1.28) b	54.2 (1.85) a	***
		Sunken	11.5 (1.05) c	34.8 (3.03) a	23.9 (2.55) b	9.1 (1.28) c	***
Raised above (µm)	Length	28.3 (0.56) a	28.3 (2.06) a	29.7 (1.10) a	29.8 (1.02) a	ns	
	Width	21.1 (0.42) a	19.0 (1.44) b	16.7 (0.52) c	18.5 (0.46) b	**	
Same level (µm)	Length	24.5 (0.95) a	24.8 (0.96) a	22.3 (0.51) b	24.1 (0.66) a	**	
	Width	14.1 (0.56) b	17.9 (0.85) a	13.9 (0.58) b	17.5 (0.24) a	**	
Sunken (µm)	Length	20.0 (0.88) a	22.5 (0.74) a	18.1 (0.58) b	11.1 (0.60) b	**	
	Width	11.3 (0.88) b	15.4 (0.69) a	22.1 (0.83) a	16.7(0.83) a	**	

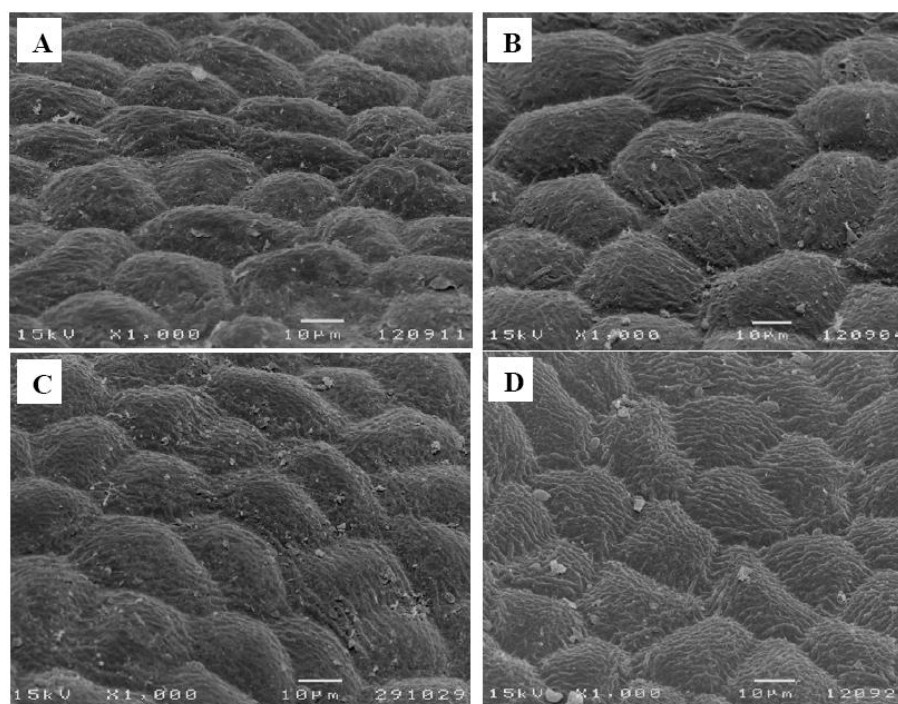
Means of 12 scanning electron micrographs: a x500 and b x750.

In each row different letter suffixes show statistically significant differences at  $\alpha < 0.05$  by LSD test.. sig – significance; ns – not significant;

\*\* - significant at  $\alpha < 0.01$  and \*\*\* - significant at  $\alpha < 0.001$ .

The epidermal cells of the upper leaf surface were uniform, rectangular or slightly polygonal in shape, with straight walls and some striate in the cuticle surface (Fig. 1). In terms of the length of the upper

epidermal cells CS showed the significantly highest values and AR the lowest, while both SY and TN gave intermediate values (Table I).

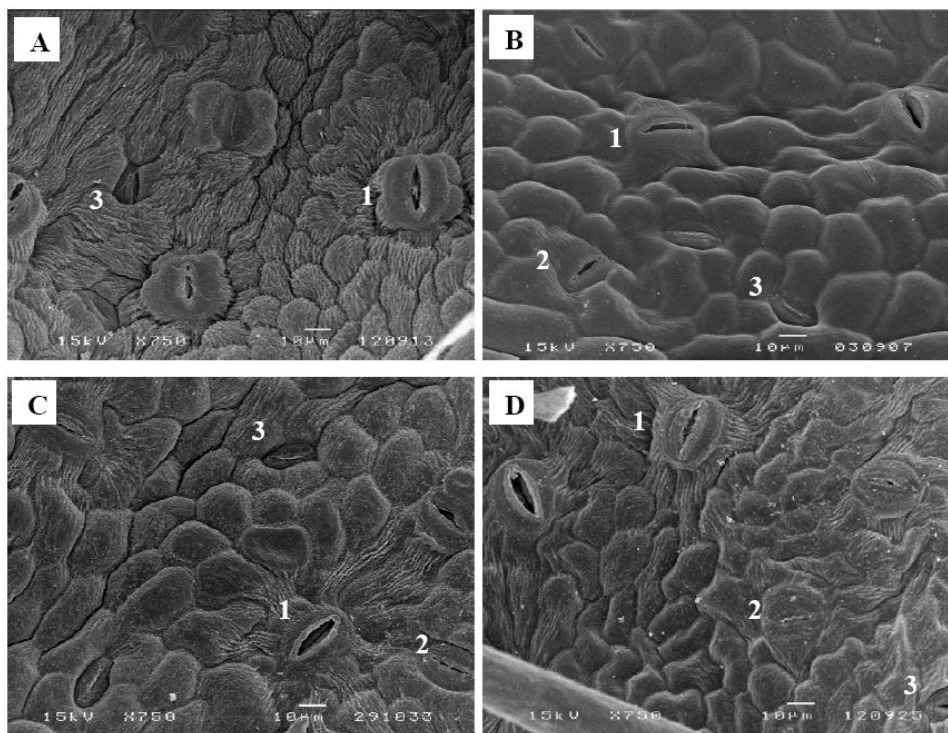


**Figure 1** - Scanning electron micrographs of the upper epidermis surface of mature leaves of *V. vinifera* ssp. *vinifera* with polygonal epidermal cells showing epicuticular striate wax deposits. A - 'Aragonez', B - 'Cabernet Sauvignon', C - 'Syrah' and D - 'Touriga Nacional' (SEM x1000).

*Microfotografias da epiderme superior de folhas adultas de V. vinifera ssp. vinifera obtidas por microscopia eletrônica de varrimento, evidenciando células epidérmicas poligonais com depósitos epicuticulares estriados cerosos. A - 'Aragonez', B - 'Cabernet Sauvignon', C - 'Syrah' and D - 'Touriga Nacional' (SEM x1000).*

In all the cultivars the lower epidermis revealed rectangular or slightly polygonal cells with sinuous cell walls and similar length (Fig. 2, Table I). Cuticular striations were seen in all cultivars, but were scarcer in CS (Fig. 2B).

not always decreasing with deepness level (Table II and Fig. 2). The raised-above type stomata exhibited a similar length in all cultivars, but significant differences in width. SY presented the smallest and AR the greatest width, differing significantly from that



**Figure 2** - Scanning electron micrographs of the lower epidermis surface of mature leaves of *V. vinifera* ssp. *vinifera*. A - 'Aragonez', epicuticular striate wax deposits can be seen; B - 'Cabernet Sauvignon' - Epicuticular striate wax deposits are scarce; C - 'Syrah' and D - 'Touriga Nacional' - Epicuticular striate wax deposits can be seen in both varieties. There are also different types of stomata: (1) raised above; (2) at the same level; (3) sunken (x750).

*Microfotografias da epiderme inferior de folhas adultas de V. vinifera ssp. vinifera obtidas por microscopia eletrônica de varrimento. A - 'Aragonez', observam-se depósitos epicuticulares estriados cerosos, B - 'Cabernet Sauvignon', depósitos epicuticulares estriados cerosos escassos; C - 'Syrah' e D - 'Touriga Nacional' - depósitos epicuticulares estriados cerosos nas duas cultivares. Observam-se também os diferentes tipos de estomas (1) elevados; (2) ao mesmo nível; (3) enterrados (SEM x750).*

Stomata density was determined from SEM images, with a significantly higher value in AR and SY compared to CS and TN (Table II). Three types of stomata coexisted in the lower epidermis of all cultivars (Fig. 2): raised above (the guard cells are above, and each flanked by curved subsidiary cells), same level (the guard cells are flattened with the subsidiary cells) and sunken (the guard cells are buried in the subsidiary cells). The prevalence of one type over the others varied significantly with genotype (Table II): AR showed a significantly higher percentage of raised-above stomata than the other three cultivars, which presented statistically similar values. In terms of the proportion of same-level stomata, TN produced the highest values and AR and CS the lowest, while the SY values were intermediate. CS presented the highest percentage of sunken stomata, TN and AR the lowest, and SY intermediate values.

All types of stomata presented a random distribution and an irregular orientation, with size generally but

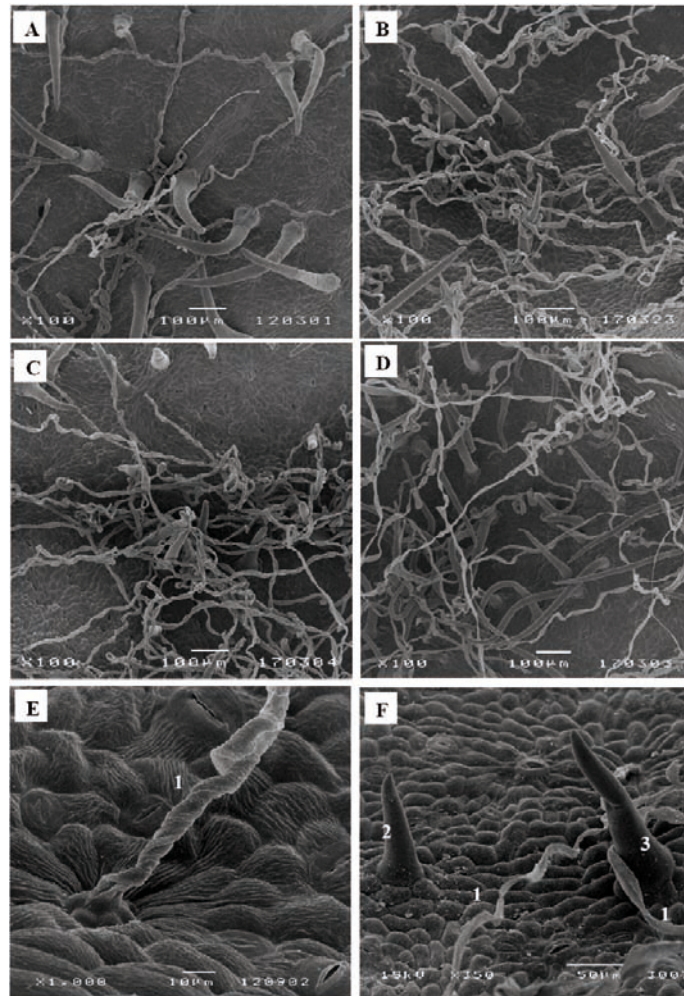
of TN and CS, which returned similar values (Table II). For the same-level type stomata, SY presented a significantly shorter stomata length than the other three cultivars, which presented statistically similar values; however, the width was significantly lower for AR and SY, compared to that of CS and TN. Regarding to the sunken type stomata, SY again presented significantly short stomata length than the other three cultivars, which presented statistically similar values. The width of the sunken type was significantly smaller in AR and SY, compared to that of CS and TN (Table II).

In all cultivars only the lower epidermis surface was pubescent (Fig. 3A, B, C and D). All cultivars presented two types of trichome on the main veins and over different areas of the limbus: i) uni- and pluricellular uniseriate non-glandular hairs, erect or slightly curved (Fig. 3A and 3F); ii) very long hairs, probably unicellular and usually flat with a helicoidal rolling (Fig. 3E), producing a network with a fluffy



effect. Some of these long hairs are dead because they do not retain their protoplasts. All the kinds of hairs presented an irregular distribution, no orientation and a variable density within each cultivar (Fig. 3A, B, C and D). These features and the fluffy long hairs make it impossible to assess trichome density.

in TN, intermediate in CS and SY, and smaller in AR (Table I). The spongy parenchyma cells were much more heterogeneous in shape and were partly separated from one another by irregular intercellular air-spaces, which were larger and more clearly visible on the leaves of SY (Fig. 4C) and AR (Fig. 4D),

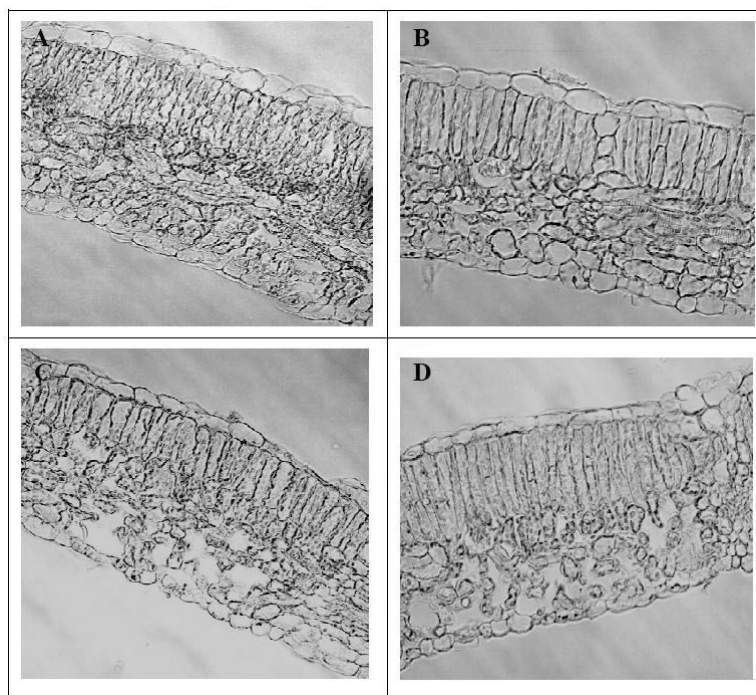


**Figure 3** - Scanning electron micrographs of the lower epidermis surface of mature leaves of *V. vinifera* ssp. *vinifera* showing the indumentum with a woolly effect and a heterogeneous hair distribution. A - 'Aragonez'; B - 'Cabernet Sauvignon'; C - 'Syrah' and D - 'Touriga Nacional' (x100); E - 'Aragonez' with a long, flat and helicoidal hair (1), (x1,000). F - 'Syrah', with a long, flat and helicoidal hair (1), uni- (2) and pluricellular (3) uniseriate non-glandular hairs (x350).

*Microfotografias da epiderme inferior de folhas adultas de V. vinifera ssp. vinifera obtidas por microscopia eletrônica de varrimento evidenciando o indumento com um aspeto lanoso e uma distribuição irregular dos tricomas. A - 'Aragonez'; B - 'Cabernet Sauvignon'; C - 'Syrah' e D - 'Touriga Nacional' (SEM x100). E - 'Aragonez', com um tricoma comprido, achatado e helicoidal (1), (x1,000). F - 'Syrah', com um tricoma comprido, achatado e helicoidal (1) uni- (2) e pluricellular (3) tricoma uniseriado não-glandular (x350).*

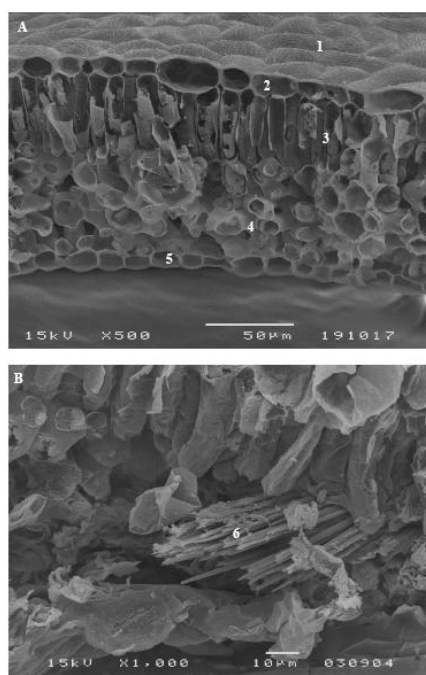
Leaf cross sections revealed dorsiventral leaves with a single layer of protection tissue. AR and SY presented a tendency towards flattened epidermal cells, while CS and TN tended to have almost circular cells in both the upper and the lower epidermis (Table I and Figures 4 and 5A). The upper cuticle thickness was similar in all cultivars. Palisade parenchyma displayed compact cells arranged in 1-2 layers in all cultivars (Fig. 4), but in CS three layers were also observed in some transverse sections. Despite this, the thickness of the palisade parenchyma was greater

compared to those of CS (Fig. 4A) and TN (Fig. 4B and 5A). The thickness of spongy tissue was greater in SY, intermediate in CS and TN, and smaller in AR (Table I). Total thickness of the lamina showed significant differences between all cultivars (Table I), with CS being the cultivar with the thickest leaf lamina, followed by TN, SY and AR. These anatomic features are in accordance with the specific dry weight values, as CS has the smallest and thickest leaf and AR the largest and thinnest one, while SY and TN presented intermediate values (Table I).



**Figure 4** - Mature leaf cross sections (c.s.) of the four red *V. vinifera* ssp. *vinifera* varieties (MO x 200). Two distinct genotype groups are seen, considering the epidermal cells structure and the spongy parenchyma arrangement, 'Cabernet Sauvignon' & 'Touriga Nacional' and 'Syrah' & 'Aragonez'. A - 'Cabernet Sauvignon' leaf c.s. with enlarged epidermal cells, 2-3 layers of palisade parenchyma and spongy parenchyma with almost no intercellular air-spaces; B - 'Touriga Nacional' leaf c.s. also with enlarged epidermal cells, 1-2 layers of palisade parenchyma and spongy parenchyma with almost no intercellular air-spaces; C - 'Syrah' leaf c.s. with few enlarged epidermal cells, 1-2 layers of palisade parenchyma and spongy parenchyma with many, large intercellular air-spaces; D - 'Aragonez' leaf c.s. with few enlarged epidermal cells, 1-2 layers of palisade parenchyma and spongy parenchyma with many, large intercellular air-spaces.

*Corte transversal (c.t.) do mesófilo de folhas adultas das quatro cultivares tintas de V. vinifera ssp. vinifera (MO x200). Dois grupos distintos de genótipos são evidenciados ao considerar a estrutura das células da epiderme e o arranjo dos parênquimas clorofilino lacunoso, 'Cabernet Sauvignon' & 'Touriga Nacional' e 'Syrah' & 'Aragonez'. A - 'Cabernet Sauvignon' c.t. da folha evidenciando células epidérmicas grandes, 2-3 camadas de parênquima clorofilino em paliçada e parênquima clorofilino lacunoso quase sem espaços intercelulares; B - 'Touriga Nacional' c.t. da folha evidenciando também células epidérmicas grandes, 1-2 camadas de parênquima clorofilino em paliçada e parênquima clorofilino lacunoso quase sem espaços intercelulares; C - 'Syrah' c.t. da folha evidenciando poucas células epidérmicas grandes, 1-2 camadas de parênquima clorofilino em paliçada e e parênquima clorofilino lacunoso com muitos e grandes espaços intercelulares; D - 'Aragonez' c.t. da folha evidenciando poucas células epidérmicas grandes, 1-2 camadas de parênquima clorofilino em paliçada e e parênquima clorofilino lacunoso com muitos e grandes espaços intercelulares.*



**Figure 5** – Mature leaf cross section of *V. vinifera* ssp. *vinifera*: A - 'Touriga Nacional' (SEM x 500): (1) surface view of upper epidermal cells with epicuticular striate wax deposits; (2) upper epidermal cells (3) palisade parenchyma; (4) spongy parenchyma; (5) lower epidermal cells; B - 'Cabernet Sauvignon' (SEM x 1000): (6) raphides.

*Corte transversal do mesófilo de folhas adultas de V. vinifera ssp. vinifera: A - 'Touriga Nacional' (SEM x500): (1) superfície das células epidérmicas da página superior com depósitos cerosos estriados epicuticulares; (2) células epidérmicas da página superior (3) parênquima clorofilino em paliçada; (4) parênquima clorofilino lacunoso; (5) células epidérmicas da página inferior; B - 'Cabernet Sauvignon' (SEM x 1000): (6) ráfides.*

During the observation of cross sections, enlarged idioblasts were visible, widely distributed in the parenchyma tissues, with two different types of calcium oxalate crystals inside: raphides (Fig. 5B) and druses.

## DISCUSSION

All four genotypes displayed upper and lower unistratified epidermal cells with thin walls and a thin cuticle. They had almost the same rectangular or slightly polygonal cell shape – features similar to those reported by Galet (2000) and Boso *et al.* (2010).

In CS, values for transverse epidermal cell surface area and epidermal thickness were higher than those obtained by Boso *et al.* (2010, 2011) in Spain, while SY epidermal thickness values were lower than the ones reported by Patakas *et al.* (2003) in Greece. Under our conditions, TN presented transverse epidermal cell surface values similar to those for SY, but AR presented the lowest ones. These differences seem to indicate that ecological conditions may influence grapevine anatomical features. Some authors have pointed out that larger cellular lumen might be involved in water storage, which may have some physiological significance (Esau, 1977; Dickison, 2000). Salem-Fnayou *et al.* (2010) observed that under heat stress the grapevine cultivars ‘Razegui’ and ‘Muscat Italia’ exhibited elongated convex epidermal cells with a less sinuous shape than those of control leaves that were not submitted to heat stress.

Martin and Juniper (1970) stated that the study of cuticles provides important information about plant habitat and its response to abiotic stresses. In our study, cuticular striations were seen on both upper and lower surfaces, especially around the stomata. Wax deposits were very few or scarce – e.g. in CS – with Boso *et al.* (2011) reporting a similar observation.

Average cuticular thickness for all the cultivars was 2.6  $\mu\text{m}$ , a value that is higher than the one Salem-Fnayou *et al.* (2010) reported for the table grape cultivars ‘Muscat Italia’ and ‘Razegui’. These differences might be determined by the genotype and the growth conditions – namely drought and heat stress (Wahid *et al.*, 2007). Indeed, under heat stress conditions Salem-Fnayou *et al.* (2010) reported a significant decrease in cuticle thickness in their cultivars, followed by an increase in epidermal cell wall thickness.

Stomata classification varies from one author to another, making comparison between studies difficult. In a review published by Pratt (1974), three types of stomata – raised above, same level, and sunken – were identified in the genus *Vitis*. This classification is not easy, inasmuch as it was only with magnifications of more than 750x that it was possible to identify these three types of stomata. Indeed, their small size and position relative to the other epidermal cells meant that sunken stomata were not clearly distinguished with lower magnifications, with implications for stomatal densities, for example. In our study, with

LM microscopy it was impossible to apply the Pratt classification and distinguish between the three types of stomata.

All the genotypes exhibited the three types of stomata, but the proportion of each type varied with genotype. Swanepoel and Villers (1987) also observed that stomata on the same leaf could be raised, same level and sunken; however, they did not present any data on the relative proportion of each type. Working at electron microscope level on the variability in leaves with 11 *Vitis vinifera* and three non-vinifera genotypes, Boso *et al.* (2011) stated that the stomata of the ‘Blanco Legítimo’ cultivar stood out because it displayed a type of doming of the epidermis that corresponds to the raised-above type. However, they did not see this type of stomata on the other studied genotypes.

In our study, stomata density varied significantly with genotype, with values of the same order of magnitude as those presented for several other *Vitis* genotypes by Swanepoel and Villers (1987). Having said this, for the same grapevine cultivars we found higher stomatal densities than the ones reported by other authors (Boso *et al.*, 2010, 2011; Costa *et al.*, 2012). One explanation for the observed differences may be the method we used for stomata counting and differentiation with a 750x scanning electron micrograph. When we compared the counts obtained by LM with the SEM results, we realized that neither the sunken, nor the raised-above stomata were visualized by LM. This methodological limitation could be the main reason why the literature reports stomatal densities below our data. Salem-Fnayou *et al.* (2010) observed no significant differences in stomata densities between heat-stressed and control grapevines, and concluded that grapevine plants maintained fairly constant stomata density in order to avoid excess transpiration due to heat stress, and that stomata density is affected more by drought than by heat stress.

In addition, within each type of stomata significant differences between the four cultivars were found in stomata length and width. Boso *et al.* (2011) reported the same results, and concluded that there was no clear relationship between leaf blade area and stomata size.

Our study showed that when the stomatal index was determined with LM, it presented no significant differences between the genotypes. These results can be explained by the low tissue amplification (the deepest stomata were probably just not seen) and also by the inherent difficulty of distinguishing the epidermal cells from one another, thus affecting the ability to count them. Wilkinson (1979) and Stace (1989) stated that it is better to record the most frequent size or the length-to-width ratio of the stomata, rather than the stomatal index.

Regarding the indumentum found in the lower epidermis, our results pointed to the presence of uni- and pluricellular uniseriate non-glandular hairs, which



were erect or slightly curved, and very long hairs, which were usually flat with helicoidal rolling. The pattern of hair distribution is either absent or essentially the same in all the cultivars. Working on six grapevine genotypes, including CS, Boso *et al.* (2010) were the first to mention the same types of trichome in *Vitis* leaves. The influence of the indumentum on the development of plant organs and its latter's role in several aspects of physiological and ecological adaptation are well known (Theobald *et al.*, 1979).

Mesophyll organization and thickness presented significant differences between cultivars. CS and TN displayed compact cells in both palisade and spongy parenchyma, while SY and AR presented an opposite pattern, inasmuch as their spongy parenchyma exhibited air spaces that were both numerous and large. The same differences were also observed between other cultivars by Salem-Fnayou *et al.* (2010) and Boso *et al.* (2010). It is known that this mesophyll arrangement may be related with downy mildew resistance (Gindro *et al.*, 2003; Boso *et al.*, 2010) and leaf gas exchanges (Evans *et al.*, 1994; Syvertsen *et al.*, 1995; Patakas *et al.*, 2003).

In our study we noticed the coexistence of raphids and druses in all the samples, at veraison. Calcium oxalate crystals are a major bio-mineralization product that is typically developed within specialized cells called idioblasts, but their biological function and plant use are still not properly understood (Prychid and Rudall,

1999; Jáuregui-Zúñiga *et al.*, 2003; Nakata, 2003).

## CONCLUSIONS

Our data showed that many leaf micromorphoanatomical features of the grapevine cultivars 'Aragonez', 'Cabernet Sauvignon', 'Syrah' and 'Touriga Nacional' differ significantly. The leaf traits that contributed most to distinguishing between grapevine cultivars were: stomata density, type of stomata (%), size of stomata cells, and mesophyll characteristics.

Discrepancies in stomata type and density recorded in the literature for the genus *Vitis* indicate that in future works, methodological standardization – e.g. of sample preparation and microscope magnification – is recommended in order to clarify this important anatomical feature.

Further studies are needed to confirm whether there is any association between some of these leaf traits – stomata type and mesophyll structure, for example – and the physiological behaviour observed under field conditions.

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