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Density and size of stomata in the leaves of different hybrids (*Vitis sp.)* and *Vitis vinifera* varieties

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Summary

A number of studies have highlighted differences in the density of stomata between *Vitis* species, but few have examined differences between varieties of *V. vinifera*. The density and size of the stomata in the lower epidermis of leaves belonging to 12 grapevine varieties, a direct producer hybrid (DPH) involving a *V. vinifera* and a non-*vinifera* parent, and the non-*vinifera* rootstocks 'SO4' and '110-Richter', were therefore examined. Transparent nail polish peel prints of the area between the main and right lateral veins were produced for 10 leaves per variety. These prints were then examined under a light microscope and the number of stomata in a unit area of 0.196 mm^2 counted. Image analysis software was then used to measure the length and width of all those counted. Rootstock 'SO4', 'Chasselas Dorée', 'Albariño' and 'Cabernet Sauvignon' had the highest stomatal densities (all > 34 stomata per unit area), while 'Castañal', 'Torrontés' and 'Caiño Blanco' and 'Jacquez' (DPH), had the smallest (all < 26.50 stomata per unit area). 'Treixadura' and 'Caiño Blanco' had significantly longer and wider stomata than all the other varieties examined; the DPH 'Jacquez' had among the shortest and narrowest. No relationship was seen, however, between mean varietal leaf size and the stomatal density or stomatal size; nor was any seen between the variables examined and the condition of belonging to *V. vinifera* or not.

K e y w o r d s : *Vitis vinifera*; HPD; rootstocks; number of stomata; nail polish prints; stoma size.

Introduction

Stomata are small pores through which plants exchange gases and must regulate water loss (Bernard 1978, Düring 1980, Ren *et al.* 2003). Unfortunately, they also provide a route via which fungi and other pathogens can enter the plant. The leaves of grapevines (*Vitis vinifera* L.) have stomata only on the lower epidermis, where they are arranged in no apparent order. All stomata are have two kidney-shaped guard cells that surround the pore or ostiole (Bernard 1978, Düring 1980, Alonso-Villaverde *et al.* 2011, Boso *et al.* 2010), although some authors report differences in stomatal morphology between varieties within different *Vitis* species (Swanepoel and Villiers 1987, Codreanu 2006), as well as in their opening and closing mechanisms, and their involvement in physiological processes (Liu *et al.* 1978, Teixeira *et al.* 2009, Rogiers *et al.* 2011). A relationship has also been reported between the density and size of stomata and susceptibility to downy mildew (Lu *et al.* 2010). Studying grapevine stomata can, however, be difficult since the results obtained may differ depending on a number of factors. Indeed, a single plant of the 'Carignan' variety may show differences in leaves examined at budding, flowering, budset, veraison, and fruit ripening (Bernard 1978), and Düring (1980) has reported the same for other *Vitis* species, e.g. 'Silvaner' and 'Müller-Thurgau'. Results may also vary depending on the position of a leaf on a shoot (Bernard 1978, Martínez and Grenan 1999) or the type of shoot examined, e.g. a fruiting shoot (*i.e.* derived from the previous year's wood), a water shoot (which derives from the trunk), or axillary shoot (derived from a leaf axilla on a green shoot) (Palliotti *et al.* 2000). Thus, plants of the same variety may return very different results if they have not been raised under exactly the same climatic conditions and following the same training and pruning practices, etc. (Kara and Özeker 1999, Gómez del Campo *et al.* 2004, Ben Salem-Fnayou *et al.* 2005). The type of rootstock used may also influence the results (Fregoni *et al.* 1978, Kara and Özeker 1999), as can the vegetative state of the plant. Rigorous sampling is therefore key to success in studies on grapevine stomata.

The aim of the present work was to compare the density and size of the stomata of different *V. vinifera* varieties, and other non-*vinifera* members of *Vitis*, including a direct producer hybrid (DPH).

Material and Methods

P l a n t m a t e r i a l a n d p l o t c h a r a c t e r i s t i c s : The studied plant material included 12 varieties of *V. vinifera*, some of international winemaking importance ('Albariño', 'Alicante Henri Bouschet', 'Cabernet Sauvignon' and 'Chasselas Dorée'), and others little known outside their traditional Spanish growing regions but beginning to awaken interest ('Albarin Blanco', 'Caiño Blanco',

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'Caiño Tinto', 'Castañal', 'Godello', 'Mencia', 'Torrontés' and 'Treixadura'). Three varieties of non-*vinifera* were also examined: the rootstocks '110-Richter' (a hybrid of *V. berlandieri* Resseguier nº2 and Rupestris Martín) and 'SO4' (a hybrid of *V. berlandieri* and *V. riparia* [Oppenheim, selection of Teleki nº4]) (Galet 1995), and the DHP known as Jacquez (a teinturier of uncertain origin) (Santiago *et al.* 2008). Except for the two rootstocks, all were grafted onto '110-Richter' in April 1994 (10 replicas per variety) and grown on trellises at the Misión Biológica de Galicia (CSIC) in northwestern Spain (42° 25´ N, 8° 38´ W; altitude 20 m). All were subject to Sylvoz pruning.

Climate data: The mean temperature for the area for the last 50 years was 14.2 °C, and the mean rainfall 1650 mm (with wide annual variation). Mean temperature, maximum temperature, minimum temperature and total rainfall were also recorded for the study period at the Salcedo weather station (nº1485) next to the experimental plot.

Sampling methods and variables measured: Adult leaves were sampled on the same day between budset and veraison in 2008 (July 18) and 2009 (July 16). When the majority of green shoots had produced between 12 and 14 internodes, the 8th leaf from the base of a fertile green shoot (sun exposed) was taken from 10 plants per variety. These leaves were labelled and placed in plastic bags for transport to the laboratory. A transparent nail polish peel print was then made of an approximately 1 × 1 cm area of the underside of each leaf, close to the petiolar sinus between the main vein and the right left lateral vein (D'Ambrogio de Argüeso 1986). For this, a fine layer of nail varnish was applied to the lower surface and allowed to dry. It was then peeled off with the help of a scalpel. All prints were preserved in aluminium foil at room temperature until use. Each print was placed on a microscope slide onto which a drop of distilled water had been placed, and covered with a coverslip. Observations were made using a Nikon Eclipse E200 optical microscope (objective 40×). The number of stomata within a visual field of 0.196 mm^2 (hereafter unit area) was counted at three different points. Representative photographs were taken for each variety (objective 40×) and the stomatal lengths and widths recorded using NIS-Elements Basic Research v.3.1 software.

Statistical analysis: Differences between the mean stomatal density and size for each variety were analysed by ANOVA. An F test was then performed, comparing each fixed factor (varieties) with its error. Significant F values were subjected to comparison of means using Fisher's protected least significant difference (LSD) test. All calculations were performed using SAS System v. 9.1 software.

Results and Discussion

Fig. 1 shows the weather data over the two years in which the study was performed. The mean annual temperature for 2008 was 14.29 °C and the rainfall was 1509.1 mm; for 2009 these figures were 13.90 °C and 1954.1 mm. The year 2009 was therefore wetter and colder. Indeed, in June 2009, the rainfall recorded was 157.8 mm compared to 14.40 mm in 2008, and with the exception of March and May, the temperatures were always cooler than in 2008.

Results collected over both years for "stomatal density", "stomatal length" and "stomatal width" were subjected to ANOVA. Significant differences were detected bewteen the varieties ($p < 0.01$) and between years for stomatal length ($p < 0.05$) and density ($p < 0.01$). The interaction variety × year had a significant ($p < 0.01$) effect on all these

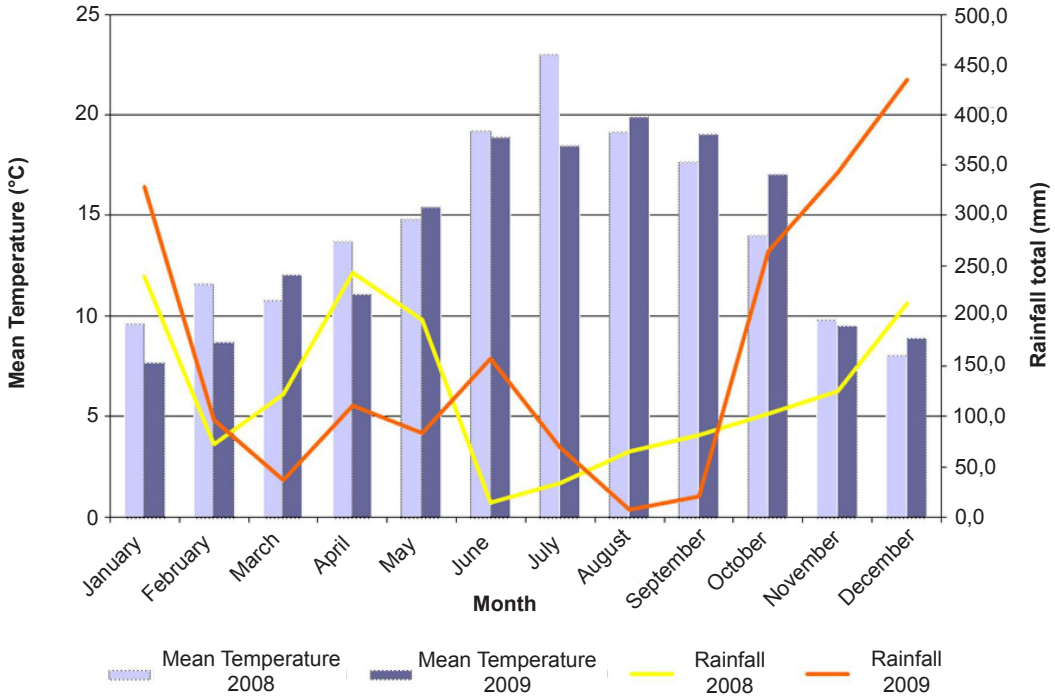

Fig. 1: Temperature (°C) and rainfall (mm), as recorded at the Salcedo nº1485 weather station (near the plot) over the experimental period (2008 and 2009).

variables (Tab. 1). This agrees with reports by other authors (Tichá 1982, Wentworth *et al.* 2006) which indicate weather conditions (rainfall, atmospheric CO_2 concentration, light intensity, air temperature), photoperiod and crop management etc., to affect these variables. Thus, ANOVAs were performed separately for the different variables and year. Again, significant differences ($p < 0.01$) were detected between varieties (Tab. 1). Tab. 2 shows the mean stomatal densities, lengths and widths for each year

In 2009, the varieties had a larger stomatal density than in 2008 (35.63 stomata per unit area compared to 30.33 per unit area). They were also longer in 2009 at a mean 42.1 μm compared to 41.37 μm in 2008, and wider, at a mean 26.9 μm compared to 26.06 μm (Tab. 2). At first, these findings might be the result of the greater rainfall in 2009 during early-mid summer (157.8 and 69 mm in June and July respectively) compared to the same months in 2008 (14.4 and 33.4 mm) when leaf development is greatest). However, Palliotti *et al.* (2001) demonstrated that the increase of individual leaf area in well watered vines produced the decrease of stomatal density compared to low watered vines. The same and other authors (Palliotti *et al.*

Table 1

ANOVAs involving stomatal density, length and width

	Variable		Varieties (V)	Year (Y)	Variety × Year[d]	Error
2008 + 2009	Stomatal length (μm)	g·L^{-1} [b]	14	1	14	2070
		C.M.[c]	686.69***[e]	143.38*	238.09***	38.60
	Stomatal width (μm)	g·L^{-1}	14	1	14	2070
		C.M.	478.00***	31.95ns	306.18***	36.29
	Stomatal density[a]	g·L^{-1}	14	1	14	2070
		C.M.	4283.51***	16448.78***	1211.40***	414.01
2008	Stomatal length (μm)	g·L^{-1}	14			949
		C.M.	454.336***			36.96
	Stomatal width (μm)	g·L^{-1}	14			949
		C.M.	306.77***			33.84
	Stomatal density[a]	g·L^{-1}	14			949
		C.M.	1755.73***			337.50
2009	Stomatal length (μm)	g·L^{-1}	14			1121
		C.M.	470.09***			39.99
	Stomatal width (μm)	g·L^{-1}	14			1121
		C.M.	475.45***			38.37
	Stomatal density[a]	g·L^{-1}	14			1121
		C.M.	3637.61***			478.77

[a]Number of stomata in a unit area of 0.196 mm^2; [b]Degrees of freedom; [c]Mean square; [d]Interaction variety x year; *Year; [e]ns, not significant; *$p < 0.05$; ***$p < 0.001$.

Table 2

Comparison of mean stomatal densities, lengths and widths in each year of the study

Genotypes	Stomatal length (μm)		Stomatal width (μm)		Stomatal density	
	2008	2009	2008	2009	2008	2009
Albarín Blanco	40.00 def	38.71 fg	21.74 d	26.22 efg	26.50 efgh	41.50 abc
Albariño	41.60 bcd	44.42bc	27.88 bc	29.04 b	34.00 abcd	43.50 a
Alicante Henri Bouschet	41.94 bcd	41.73 de	26.04 c	24.45 gh	37.50 ab	34.50 cd
Cabernet Sauvignon	38.89 f	41.97 de	26.26 c	28.76 bc	36.50 abc	41.00 abc
Caiño Blanco	42.51 bc	44.80 abc	30.27 a	31.59 a	22.00 gh	30.50 de
Caiño Tinto	41.56 bcd	41.20 de	26.80 c	28.24 bcde	30.00 cdef	34.50 cd
Castañal	43.68 b	37.89 g	29.23 ab	22.32 i	25.50 fgh	25.00 e
Chasselas Dorée	41.70 bcd	41.40 de	23.36 d	26.50 defg	31.00 bcdef	44.50 a
Godello	39.13 ef	38.78 fg	26.78 c	25.38 fg	33.00 bcde	32.00 de
Jacquez	35.92 g	37.89 g	22.84 d	22.32 i	26.50 efgh	25.00 e
Mencia	41.80 bcd	44.21 bc	26.94 c	27.39 bcdef	28.00 defg	43.50 a
SO4	41.78 bcd	41.77 de	27.19 bc	28.14 bcde	40.00 a	47.00 a
Torrontés	38.78 f	46.88 a	21.59 d	26.80 cdef	20.50 h	25.50 e
Treixadura	48.79 a*	45.99 a	27.11 c	22.77 hi	34.00 abcd	30.50 de
110-Ritcher	42.45 bc	43.04 cd	26.74 c	28.38 bcd	30.00 cdef	36.00 bcd
LSD (0.05)	2.21	2.12	2.11	2.08	6.68	7.36

* Values with the same letter are significantly different ($p < 0.05$).

2000, Gómez del Campo *et al.* 2007) add that rainfall is not the only limiting factor; light intensity and high temperatures (the maximum reached in July 2008 was 32.5 °C) also appear to be involved. Data on the individual leaf area of the studied vines are being recorded at present in order to verify this behaviour.

The *V. vinifera* varieties 'Treixadura' and 'Caiño Blanco' always had the longest stomata, while the DHP 'Jacquez' always had among the shortest (Fig. 2, Tab. 2). Those of 'Jacquez' were also the narrowest, while the varieties 'Castañal', 'Caiño Blanco', 'Albariño' and the rootstock 'SO4' had the widest. 'Castañal' actually showed different behaviours in the different years. In 2008 it returned the highest values for both variables, while in 2009 it returned the lowest of all. Torrontés showed the opposite behaviour: in 2008 it returned the lowest values for both variables, and in 2009 it returned among the highest.

Tab. 2 shows the rootstock 'SO4' and the *V. vinifera* varieties 'Chasselas Dorée', 'Albariño' and 'Cabernet Sauvignon' had the largest stomatal densities (all > 34 stomata per unit area), while 'Castañal', 'Torrontés' and 'Caiño Blanco' plus the DHP 'Jacquez' had the smallest (all < 26.5 per unit area). Swanepoel and Villiers (1987), who studied the stomatal density and stomatal index (number of stomata/number of epidermal cell) of different hybrids (including 'Jacquez'), *V. Vinifera* cultivars ('Pinot noir', 'Muscat d'Alexandrie') and American roostocks ('99R', '143B Mgt', etc.), reported that American spp. always had highest values. In the present work, SO4 returned the highest stomatal density, but 'Jacquez' fell within the varieties with the smallest values.

No direct relationship was seen between number of stomata and any leaf characteristic (such as size or the depth of the lateral sinuses). For example, 'Castañal' and 'Jacquez' had among the lowest stomatal densities but have very large leaves (Santiago *et al.* 2008, Gago *et al.* 2009; www.vitis.mbg.csic.es/vitis/), while Caiño Blanco had a low stomatal density yet has small leaves (Santiago *et al.* 2007).

No clear relationship was seen between the stomatal density and either stomatal length or width. For example, 'Jacquez' had a low stomatal density and its stomata were small, while 'Caiño Blanco' had a low stomatal density but its stomata were large, and 'Castañal' had both large and small stomata on the same leaf in the same year. Neither was any clear relationship seen between stomatal density and known susceptibility to downy mildew (Boso *et al.* 2010, Boso and Kassemeyer 2008); this agrees with that reported by other authors (Mendgen 1996, Allègre *et al.* 2007, Jürges *et al.* 2009). Work performed by our group has shown that 'Cabernet Sauvignon' and the rootstocks '110-Richter' and 'SO4' are more resistant to this disease than others (Boso and Kassemeyer 2008), yet they had high stomatal densities, while 'Caiño Blanco' and 'Treixadura', which had low stomatal densities, have been shown rather susceptible (Boso *et al.* 2010). Both these results are the opposite of what one might expect. Similarly, Paolocci *et al.* (2014) reported the downy mildew-resistant variety Solaris to have a high stomatal density.

According to Wilkinson *et al.* (1995), the stomatal index varies little between varieties, while the stomatal density differs more strongly. However, controversy surrounds whether the stomatal density is genetically predetermined or whether it can vary according to conditions such as light intensity, humidity, air temperature or soil temperature, etc. Palliotti *et al.* (2000) argue the former case, while others (Lake *et al.* 2001, Casson and Gray 2008, Rogiers *et al.* 2011) argue the latter. Some authors (Gómez del-Campo *et al.* 2003, Rogiers *et al.* 2011), suggest that while the latter may be true there are genetic differences as well. According to Meisel *et al.* (2011), plants have a "specialised vision system", that allows them to perceive light signals via specialised photoreceptors. These initiate biochemical events that regulate the activity of the genome, thus allow-

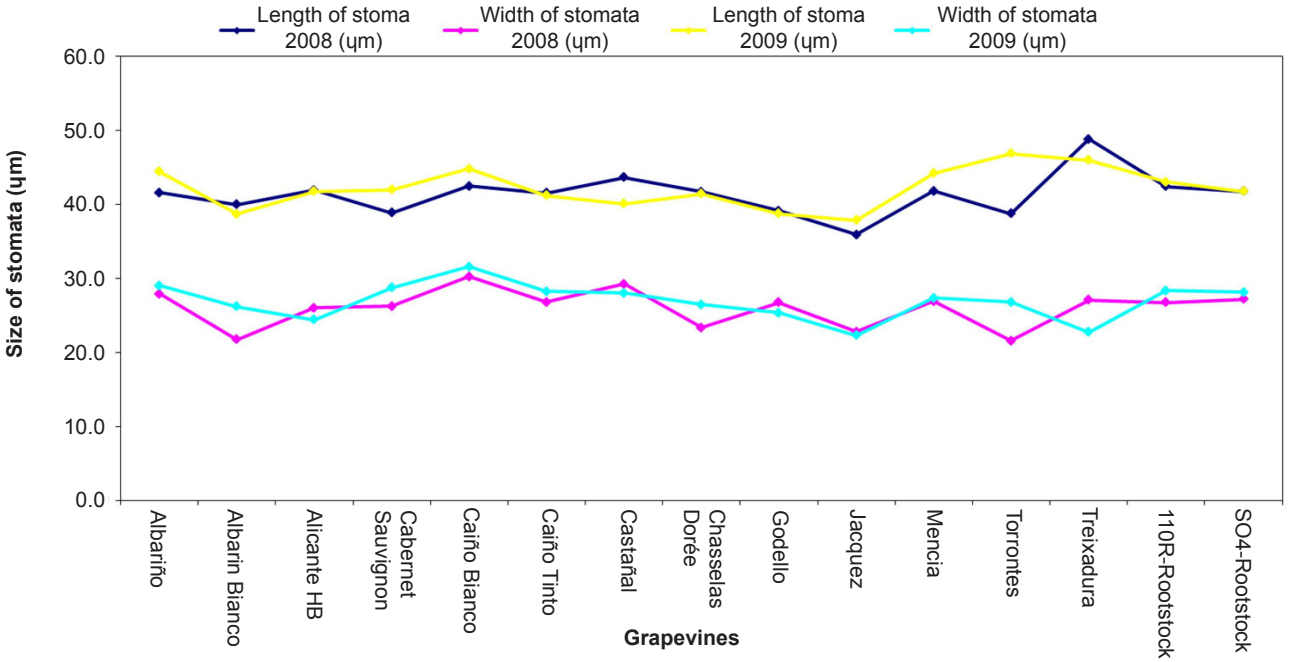

Fig. 2: Size of stomata (length and width) in 2008 and 2009.

ing responses to environmental stimuli to be made. Sugano *et al.* (2010) recently reported that the mesophyll cells of immature Arabidopsis leaves express an intercellular signalling factor (stomagen) that interacts with epidermal cell factors to influence stomatal density. It may be, therefore, that these photosynthetic cells, and not the epidermal cells, ultimately regulate this variable.

The differences detected in the present work cannot be due to having taken the examined leaves at different stages of development or from different places on the shoot; all were taken at the same time from node 8, which, according to various authors (Martinez and Grenan 1999) is that at which grapevine leaves are structurally and morphologically most stable.

Conclusion

In conclusion, no relationship was seen between leaf size and stomatal size or density, nor between stomatal density and size. Neither did belonging to *V. vinifera* or not appear to influence any of the variables examined. Finally, no clear relationship was seen between stomatal density or size and known susceptibility to downy mildew.

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