

Macro- and microscopic leaf characteristics of six grapevine genotypes (*Vitis spp.*) with different susceptibilities to grapevine downy mildew

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Summary

This work reports the leaf morphology of six grapevine genotypes, five belonging to *Vitis vinifera* and one to *Vitis riparia*. Earlier studies on these genotypes showed different levels of susceptibility to grapevine downy mildew (*Plasmopara viticola*). The aim of this work was to detect differences between the leaf morphology of these cultivars at the macro- and microscopic levels, and to characterize morphological traits which could be associated with susceptibility and resistance to downy mildew. An ampelographic description of each genotype was used to develop a scheme illustrating the characteristic leaf morphology. The density and morphology of the trichomes and the stomatal index was assessed by means of microscopical techniques. Distinct macro and microscope differences among the genotypes were seen. No clear relation between ampelographic characteristics and susceptibility to downy mildew was observed. The two cultivars that in earlier studies were found to be the least susceptible to downy mildew were the most similar in terms of their spongy mesophyll. Both showed very little or no wax on the abaxial surface of their leaves.

Key words: *Vitis vinifera* L., leaf morphology, spongy mesophyll cells, susceptibility, grapevine downy mildew.

Introduction

Several studies have described the cultivars of *Vitis vinifera* and *Vitis riparia* (HUGLIN 1986, GALET 2000) and show distinct macroscopic differences between them. Among the cultivars a great variety of leaf shape especially of the form of the leaf sinuses occur (GALET 2000, MARTÍNEZ *et al.* 2005). Morphological observations at the macroscopic level carried out both at the field (SANTIAGO *et al.* 2005) and in the laboratory (unpubl.), have led to detect differences between the grapevine cultivars 'Cabernet Sauvignon', 'Albariño', 'Tempranillo', 'Touriga Nacional' and 'Pinot noir' or in *Vitis riparia*. The variability in leaf morphology regarding to the size and thickness of blade, the entire limbe of the leaves or with deep lateral sinuses,

the degree of opening of petiole and lateral sinuses, the goffering, undulations or blistering of blade, the profile of blade in a cross section (flat, involute, revolute, twisted), could indicate a different leaf development in each variety. These characteristics may reflect variations in the histologic structure and therefore be related to different levels of susceptibility to downy mildew. The overlapped sinus, the goffering of blade, etc., could also create favourable conditions for the growth or spread of this pathogen. Differences can also be seen in terms of the types of the trichomes on the upper and lower leaf surfaces. Some cultivars ('Albariño') have only reclining threadlike trichomes of different length (Martínez *et al.* 2005), while others (*Vitis riparia*) have erect trichomes like small transparent spines (GALET 1956). Some cultivars ('Cabernet Sauvignon') have both types of trichomes (GALET 2000). The density of trichomes varies from leaves with a very dense cover resulting in a light-coloured leaf surface to a nearly bare leaves. Whereas no relationship between ampelographical traits and susceptibility is known, the role of trichomes as a barrier against infections by grapevine downy mildew has been described (KORTEKAMP *et al.* 1999). Different authors have studied the histological characteristics of vine leaves at different stages in the vegetative cycle (BERNARD 1978), the differences between leaves from primary green shoots and green shoots arising from axillary buds (PALLIOTTI *et al.* 2000), and those between leaves of the same cultivar when cultivated in more arid or more humid climates (BEN SALEM-FNAYOU *et al.* 2005). All these studies reported differences in terms of histological parameters.

In the second half of the 20th century many histological studies were performed on grapevines with different goals in mind, although very few focused on comparing the differences between one cultivar and another. A number of observations made during work on the resistance of grapevine cultivars against fungal diseases (GINDRO *et al.* 2003, BOSO *et al.* 2004 b, 2006, 2007, MUSETTI *et al.* 2005, ALLÉGRE *et al.* 2006, ALONSO -VILLAVARDE *et al.* 2008) and on the water needs (DAMI and HUGHES 1995, GOMEZ DEL CAMPO *et al.* 2004, SOAR *et al.* 2006), suggest that the histological characteristics of some cultivars may have a determinant effect in these respects. Studies performed on the same genotypes showed *Vitis riparia* and 'Cabernet Sauvignon' to be the least susceptible to downy mildew (BOSO and KASSEMAYER. 2008).

Other authors (KROSTANOVA 1989, GABLER *et al.* 2003) indicate the existence of a positive correlation between resistance to cryptogamic diseases and the thickness of the epidermis, hypodermis, cuticle and wax content.

The aim of the present work was to determine whether differences between cultivars at the morphological level of the leaf are accompanied by differences at the histological level, perhaps explaining differences in susceptibility to this disease.

Material and Methods

Plant material: Five grapevine genotypes were studied: 'Albariño' (two clones), 'Tempranillo', 'Pinot Noir', 'Cabernet Sauvignon', 'Touriga Nacional', plus the species *V. riparia* 'Gloire de Montpellier'.

Ampelographic and histological studies were made on leaves from plants grown in the field.

Ampelographic descriptions: Ten leaves were taken from each cultivar, all from node 8 or 9 on fruiting green shoots sprouting from the current year's wood. All leaves were pressed, dried and stored until use. By means of the Analysis v.3.0 imaging software (Soft Imaging System), leaf characteristics were measured according to MARTINEZ and GREAN (1999) and by stereomicroscopy (Nikon SMZ 800), the density of the erect and reclining trichomes and other mature leaf parameters were determined following the method proposed by the OIV (1983) (codes OIV 072, 073, 074, 075, 084, 085, 086, 087).

Histological description; optical microscopy: Ten leaves were selected from each cultivar. Samples of leaf limbus were randomly taken from each and fixed in 4 % paraformaldehyde/2.5 % glutaraldehyde in PBS buffer (Phosphate buffered saline). Once fixed these samples were preserved in 0.05 M cacodylate buffer at 4 °C (KARNOVSKY 1965) before setting in EPON resin. Histological sections (1 µm) were made using a Leica RM 2165 rotary microtome. All sections were stained with methylene blue and photographed using a Zeiss AxioCam camera mounted on the microscope. The upper and lower cuticle-cell wall complexes, the thickness of the upper and lower epidermises, and the thickness of the palisade and spongy mesophyll were measured.

Epifluorescence microscopy; number of stomata: Epifluorescence microscopy was used to determine the number of stomata. Three fresh leaf fragments were taken from each cultivar, cleared in 1 M KOH, autoclaved for 15 min at 121 °C, and washed three times in deionised water. They were then stained with 0.05 % aniline blue in 0.067 M K₂HPO₄ (pH 9) (HOOD and SHEW 1996). Observations were made using a Zeiss AxioPhot microscope equipped with Plan-Neofluar objectives and with epifluorescence capability. Photographs were taken using a Zeiss AxioCam digital camera, and the images analysed using Zeiss AxioVision software. The number of stomata between the veins were counted and the inter-microvein area calculated for each specimen. The stomatal index was determined as: n° stomata / inter-microvein area.

Low temperature electron microscopy: Small pieces of fresh leaves were cut. Samples were mounted on SEM-stubs using a low-temperature mounting medium and then rapidly frozen by plunging them into liquid nitrogen. They were then placed on a Balzer's specimen table under liquid nitrogen. Using a manipulator equipped with an anti-contamination cup, the table was transferred under nitrogen gas flow conditions to a Balzer's SCU 020 cryopreparation unit attached to a Jeol JSM 6300 electron microscope (MÜLLER *et al.* 1991). Photomicrographs were taken of the most important details in each section.

Statistical analysis: ANOVA was performed on the quantitative data recorded for the different sections and on the stomatal index figures. The GLM method of the SAS System v 9.1 software package was used to determine whether any differences between cultivars were significant. Fisher's protected least significant differences test was performed for each fixed factor and its error.

Results and Discussion

Ampelographic description of leaf shape and structure of the trichomes: In Fig. 1 the characteristic leaf morphology is illustrated showing distinct differences in the aperture of the petiole sinus, the presence of upper and lower lateral sinuses and their depth, the number of teeth and their width, and the superimposition of the lobules. Only the two clones of 'Albariño' showed very similar leaf morphology; however the base of the petiole sinus in clone 1 was limited by a vein; this was not the case in clone 2. Regarding to the profile of the blade in a cross section, goffering, undulations or blistering of the blade, all genotypes showed the lowest OIV note (1). Only 'Cabernet Sauvignon' (medium) and 'Pinot Noir' (strong) showed blistering of the upper side of blade. 'Pinot Noir' also showed twisted profile of blade and *Vitis riparia* presented undulated leaves. In view of these results, it is not possible to determine the existence of any correlation between the ampelographic characteristics and the different levels of susceptibility to downy mildew in these genotypes, previously reported by Boso *et al.* (2008).

The assessment of the shape and density of trichomes on the leaves showed distinct differences among the genotypes. *Vitis riparia* had no reclining trichomes on either side of the leaf but showed a medium density of erect trichomes on the main veins of the lower leaf surface. A small number of reclining trichomes was seen between the veins on the upper side. On the lower leaf side of 'Cabernet Sauvignon' a medium density of reclining trichomes between the main veins occurred; on the veins the density was low. The lower side showed a medium density of erect trichomes on and between the veins. The leaves of 'Tempranillo' had a low density of reclining trichomes between and a very low density on the veins. Erect trichomes occurred between the veins with a high and on the veins with a medium density. The lower leaf side of 'Touriga Nacional' showed a medium density of reclining trichomes between and a low den-

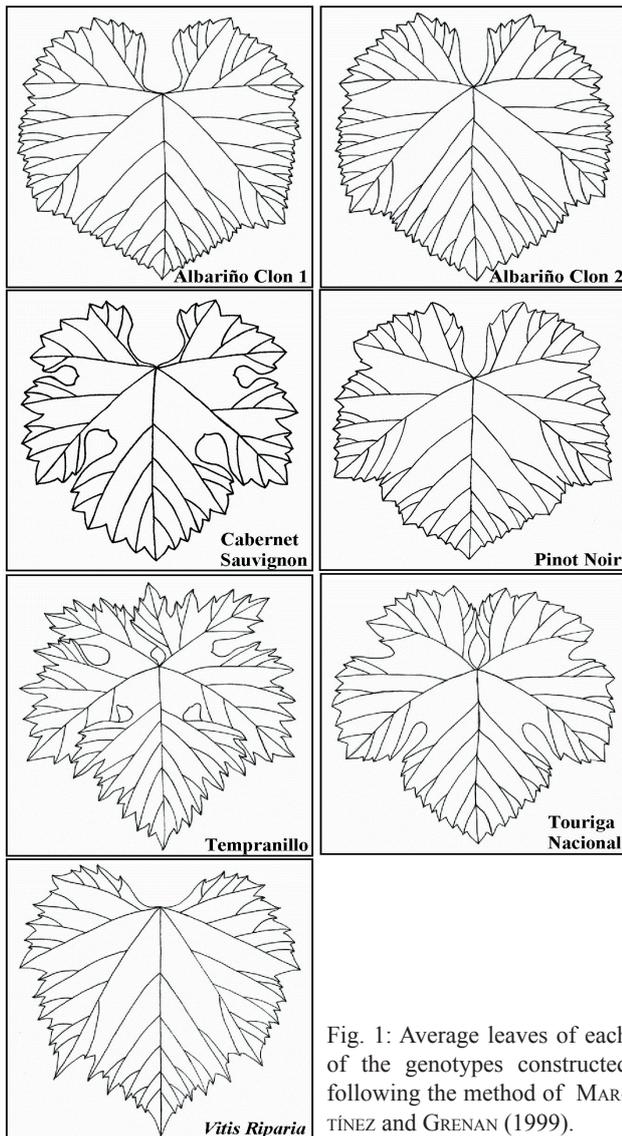


Fig. 1: Average leaves of each of the genotypes constructed following the method of MARTÍNEZ and GRENNAN (1999).

sity on the veins. The density of erect trichomes between the veins was medium, on the veins the density was very low density. No reclining and erect trichomes between and a low to very low density on the veins was observed on the leaf lower leaf side of 'Pinot Noir', the two clones of 'Albariño' showed on the lower leaf side a medium to high density of reclining trichomes between and on the veins, and no erect trichomes. These results are similar to those reported by other authors (FÖEX, 1891, VIALA and VERMOREL 1901-1910, GALET, 1956, CATALOGUE DES VARIÉTÉS ET CLONES DE VIGNE 1995, SANTIAGO *et al.* 2005, MARTÍNEZ *et al.* 2006). A dense layer of trichomes in the intercostals field of the lower leaf surface may act as a constitutive barrier against infections by grapevine downy mildew (STAUDT and KASSEMAYER 1995, KORTEKAMP and ZYPRIAN 1999, KORTEKAMP *et al.* 1999) when they prevent a proper leaf wetness which is necessary for zoospore release and targeting of the stomata (KIEFFER *et al.* 2002). However, the present data show no relationship between trichome density and shape and the level of susceptibility. The less susceptible cultivar 'Cabernet Sauvignon' (BOSO and KASSEMAYER 2008) has a medium density of both types of trichomes

on the intercostals field of the lower leaf side comparable with 'Touriga Nacional' and 'Tempranillo' belonging to the group with a significant higher susceptibility according to BOSO and KASSEMAYER (2008). 'Albariño', also a member of the group with a high level of susceptibility, has no erected but a medium to high density of reclining trichomes on the abaxial leaf side (LOUREIRO *et al.* 1998, MARTÍNEZ *et al.* 1994). 'Müller-Thurgau', show high susceptibility in previous works (KORTEKAMP *et al.* 1999, BOSO *et al.* 2006), and low trichome density (BOIDRON *et al.* 1995, AMBROSI *et al.* 1998), or 'Caíño Blanco', also susceptible (unpubl.), with high density of reclining trichomes (SANTIAGO *et al.* 2005), could be two examples of no correlation between hair density and resistance to downy mildew.

In conclusion, a high number of hairs does not automatically lead to resistance, and plants may also be resistant without hairs, but in some cases the hairs seem to be the only suitable lifesaver and they might be therefore part of a resistance mechanism (KORTEKAMP and ZYPRIAN 1999). However, it is important to keep in mind that a major number of cultivars or species should be investigated to conclude if hairs play an important role or not.

Structure of epidermis and mesophyll: All the genotypes studied showed an unstratified adaxial epidermis with cells that, in transverse section, were elongated with thin walls. In the most cultivars the cuticle was very thin; however the clones of 'Albariño' showed a significantly thicker cuticle-cell wall complex and a thicker epidermis (Fig. 2, Table). The epidermis of both sides of 'Tempranillo', 'Touriga Nacional' and 'Pinot Noir' was thinner than that of 'Cabernet Sauvignon' and 'Albariño' (Table). According to GALET (2000), the epidermis of *Vitis spp.* is usually composed of rectangular cells, the dimensions of which vary depending on the species. According to MANZONI (1954), in *V. vinifera* the epidermal cells are smaller and less uniform than in *V. riparia*. In transverse section the abaxial epidermal cells appeared small and no differences occurred between the different genotypes. The cuticle on the abaxial leaf side was thinner than on the adaxial side.

The thickness of the palisade mesophyll cells was very similar in most of the genotypes, except in the clones of 'Albariño'. In these clones the palisade-cells were significantly thicker (Table), more elongated, and showed abundant chloroplasts (Fig. 2). The palisade mesophyll was, only one cell layer thick in all cultivars, and in all the cells were elongated and arranged in a compact manner. In some cases ('Albariño' clone 1, *V. riparia* and 'Touriga Nacional'), a transverse cell wall was seen towards the lower third of these cells; this was also reported by BERNARD (1978) and GALET (2000). The palisade mesophyll cells of *V. riparia* could not be differentiated from those of the other cultivars by their thickness or any other feature (Fig. 2). Most of the cultivars showed a narrow spongy mesophyll with 4-5 layers of polygonal cells separated by an intercellular space; the exceptions were 'Cabernet Sauvignon' and *V. riparia* which had smaller, more loosely packed cells with an extended intercellular space. These results agree with those of GALET (2000) and TEIXEIRA *et al.*

Table

Comparison of average leaves in terms of leaf variables (thicknesses, μm)

Genotype	Upper cuticle-cell wall complex	Lower cuticle-cell wall complex	Upper epidermis	Lower epidermis	Palisade mesophyll	Spongy mesophyll	Stomatal index
Alb Clon1	3.49 a	2.62 b	16.85 a	15.89 a	81.73 b	141.46 a	1.39d
Alb Clon2	3.90 b	2.86 a	14.71 b	13.89 b	93.10 a	105.98 b	2.22a
Tempranillo	1.89 cd	0.78 f	10.02 de	8.60 c	20.24cd	42.46 d	1.35d
Pinot noir	1.93 c	1.37 c	11.56 cd	8.55 c	18.87 d	45.30 cd	1.55bc
Cabernet Sauvignon	1.63 ef	1.08 de	12.24 c	9.94 c	23.94 c	46.47 cd	1.28d
Touriga Nacional	1.39 f	1.29 cd	9.08 e	8.99 c	23.51 c	48.89 c	1.72b
<i>Vitis riparia</i>	1.66 de	1.06 e	12.00 c	8.79 c	22.31 cd	44.08 cd	1.30d
LSD (0.05)	0.25	0.23	1.72	1.46	4.60	6.28	0.32

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

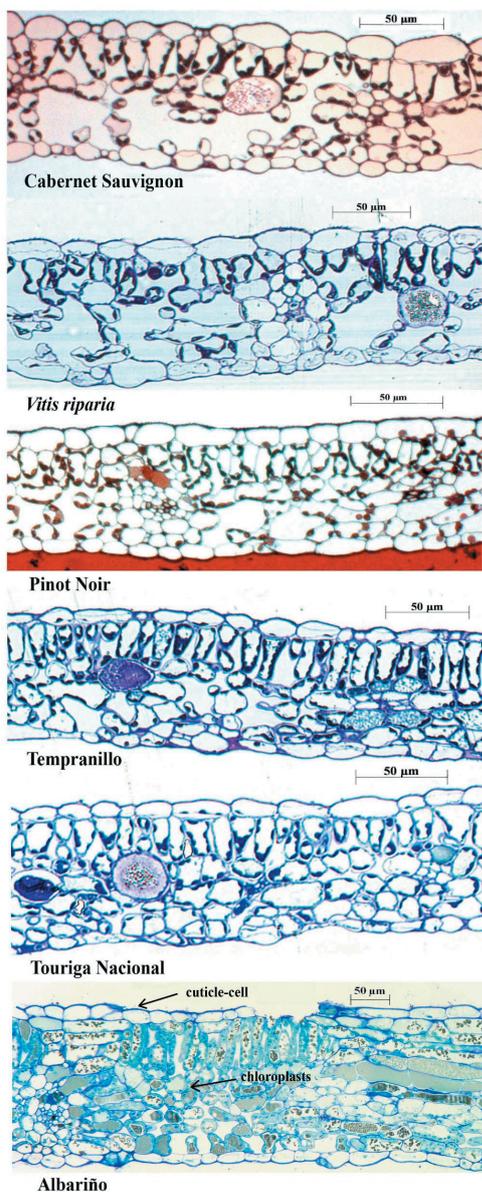


Fig. 2: Leaf sections of the genotypes studied (50 μm , 200x). 'Albariño': Very compact palisade mesophyll cells showing with many chloroplasts.

(2009). The leaf diameter of 'Albariño' clones was significantly higher, whereas among the other genotypes no significant differences occurred. For necrotrophic grapevine pathogens such as *Botrytis cinerea* a positive correlation between the structure of the epidermis including the cuticle and the susceptibility for the pathogen has been suggested (KROSTANOVA *et al.* 1989, MLIKOTA *et al.* 2003). However, the causal agent of grapevine downy mildew, *Plasmopara viticola*, is not able to penetrate the cuticle and epidermis of its host plant, rather the biotrophic pathogen takes advantage of the stomata to invade its host plant via these natural openings (ROYLE and THOMAS 1973, RIEMANN *et al.* 2002, KIEFER *et al.* 2002). Therefore the thickness of the cuticle and epidermis is insignificant for the penetration efficacy of the pathogen, but probably the structure of the outer surface of the host plant, especially of the cuticle may play a pivotal role for the adhesion of sporangia and the attachment of zoospores around the stomata. The analysis of the mesophyll cells revealed that *V. riparia* and 'Cabernet Sauvignon' had smaller and more compact cells than the other genotypes. The pathogen colonizes the intercellular space of the mesophyll, and consequently it is highly probable that a compact mesophyll may impede the growth of the mycelium in the host tissue considerably. In fact, both cultivars with a narrow intercellular space, *V. riparia* and 'Cabernet Sauvignon', have a high resistance and lower susceptibility respectively against *P. viticola* (BOSO and KASSEMAYER 2008). These findings confirm the suggestion of RIBEREAU-GAYÓN and PEYNAUD (1982) that the mesophyll structure may influence the spread of pathogens in grapevine. Recently UNGER *et al.* (2007) described the bundle sheet cells as a structural barrier within the host tissue impeding an extended spread of *P. viticola* within leaf tissue.

Morphology of the stomata and the stomatal index: Transverse sections of the leaf limbus showed stomata only on the abaxial epidermis, and as reported by BERNARD (1978) and DUERING (1980), none of the genotypes showed stomata on the adaxial surface. In all the genotypes the stomata showed the characteristic morphology with two kidney shaped guard cells surrounding

the pore and a substomatal cavity underneath the stoma. The assessment of the frequency of stomata on the abaxial surface revealed distinct differences between the genotypes. The results of ANOVA showed significant different stomatal indices between the genotypes (Table). The lowest stomatal index was observed on 'Cabernet Sauvignon', but 'Albariño' clone 1, 'Tempranillo', and the species *V. riparia* had also a low index significantly different from the other genotypes. The highest stomatal index, significantly different from all of the genotypes, was showed by clone 2 of 'Albariño'. 'Touriga Nacional' and 'Pinot Noir' had a medium stomatal index. According to the findings on the stomatal targeting of the zoospores of *P. viticola* it can be speculated that a high density of stomata on the host surface is responsible for an increased susceptibility against the pathogen. However, as a consequence of the statistical analysis of the data it can be noticed that a low stomatal index is not related to a low susceptibility and vice versa even though the clone of 'Albariño' showed the highest stomatal index and is also very susceptible against *P. viticola*. However, the clone 1 of 'Albariño' and 'Tempranillo', also belonging to the group with a distinct susceptibility (BOSO *et al.* 2006; BOSO and KASSEMAYER 2008), have a low stomatal index compared with the low susceptible 'Cabernet Sauvignon' and the resistant species *V. riparia*. Probably the high stomatal index of 'Albariño' clone 2 may be responsible for the high agronomic performances in the field (BOSO *et al.* 2004 a) because a high stomata density is associated with an increased photosynthetic capacity (DÜRING and HARST 1996, ROSS-KARSTENS *et al.* 1998).

Although a high number of stomata on a given surface is not correlated with an increased level of susceptibility, there are some other resistance mechanisms implicated: the hypersensitivity reaction (HR) causing programmed cell death (PCD) around the infection site, observed in resistant genotypes like *Vitis riparia* (BARLASS *et al.* 1987, BROWN *et al.* 1999 a, BOSO and KASSEMAYER 2008); the resistance induced by fungal attack with synthesis of phytoalexins (LANGKAGE and PRYCE 1977); the lignification processes induced by peroxidases (DAI *et al.* 1995, KORTEKAMP and ZYPRIAN 2003) or the formation of callose in stomata, (GINDRO *et al.* 2003), etc. Furthermore, the specific structure of a given stoma, like the presence of an additional cuticular rim at the junction between substomatal cavity and stomatal aperture, may also act as a physical barrier towards downy mildew zoospores (JÜRGES *et al.* 2009).

The interaction "variety x leaf sample" was not significant. KOUWENBERG *et al.* (2004) and BEN SALEM-FNAYOU *et al.* (2005) reported that the stomatal index in grapevine and other plants varies depending on the developmental stage of the leaf development and whether the leaves examined come from plants grown in the field or in the glasshouse (DÜRING 1980). WILKINSON (1992) indicated a variation of the stomatal index of the genotypes among a species, and PALLIOTTI *et al.* (2000) demonstrated a genetically controlled stomatal index.

Low temperature electron microscopy: The erect trichomes observed, which had the appearance of small white spines (Fig. 3 A, B), were seen on the main veins of the leaf (and frequently on the second-

ary and tertiary veins as well), and on different areas of the limb. Around the base of these trichomes, especially those on the main veins, cells were arranged in a rosette (BERNARD 1978, OIV 1983, PAVAN and PETERLUNGER 1999) (Fig. 4 A). Since several authors have attempted to classify the type of plant trichomes (GALET 1956, UPHOF 1962, JOHNSON 1975), here we report two different types within the reclining hairs as the first time in *Vitis*. The reclining trichomes observed had the appearance of long filaments. Some of these filaments were cylindrical, somewhat shorter in length and rolled into a spiral, while others were longer, flattened, and formed a network over the lower limb surface (Fig. 3 C, D). 'Cabernet Sauvignon' had both types of reclining hair while the longer, flattened type was more common in the other cultivars. The literature contains no references on the existence of two types of reclining hair on grapevine leaves. On the adaxial surface, cuticular striations (cuticular waxes) were seen overlying the epidermal cells in all the genotypes studied, and at similar densities. Similar striations were also seen on the abaxial surface, especially around the stomata. Disperse accumulations of wax deposits were also seen (Fig. 4), except on the abaxial surfaces of 'Cabernet Sauvignon' and *V. riparia*. Epicuticular wax protects against water loss by evaporation and against the leaching of inorganic and organic constituents from the tissues (RIEDERER and MARKSTÄDTER 1996). The waxes of higher plants also provide a mechanical obstacle that prevents the entry of phytopathogenic bacteria and fungi (MENDGEN 1996). In addition, the cuticle plays a significant role in host recognition in certain fungi (PODILA *et al.* 1993, DAI *et al.* 1995, MENDGEN 1996). Wax can also be incorporated in the cuticle and in absence of inhibitory compounds, these incorporations may reduce leaf wettability and thus infection risk caused by fungal spores depending on liquid water. However, the present results show the two most resistant genotypes ('Cabernet Sauvignon' and *V. riparia*) (BOSO and KASSEMAYER 2008) to be those with the least wax. This might be explained by the facts that, in these earlier works, the leaves examined were infected by fungi, unlike in the present work; the majority of these papers describe the presence of wax as a response to infection. In conclusion, no clear relation between ampelographic characteristics and susceptibility to downy mildew could be determined. However, some of the macro- and microscopic differences among the leaves of the studied genotypes may be related to resistance to downy mildew. The two cultivars that in earlier studies were found to be the least susceptible to downy mildew were the most similar in terms of their spongy mesophyll. Both showed very little or no wax on the abaxial surface of their leaves. Regarding to the trichomes, although they seem the only suitable lifesaver and might be therefore part of a resistance mechanism, more studies are needed to conclude if hairs play an important role or not.

Acknowledgements

This research was supported by a fellowship from the Fundación Martín Escudero and Ministerio de Educación y Ciencia

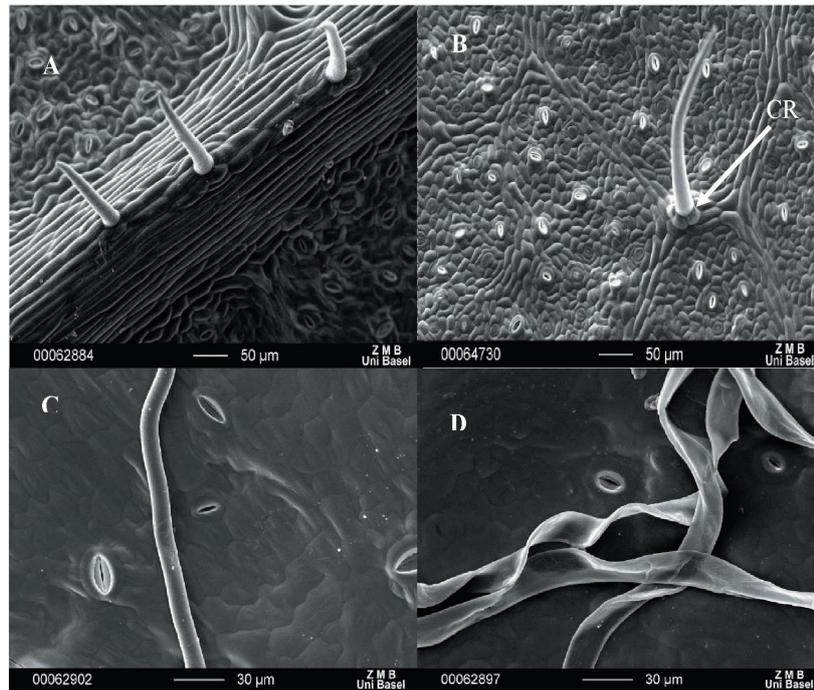


Fig. 3: Erect (A and B) and reclining trichomes (C and D) under low temperature electron microscopy. CR: cells arranged in a rosette.

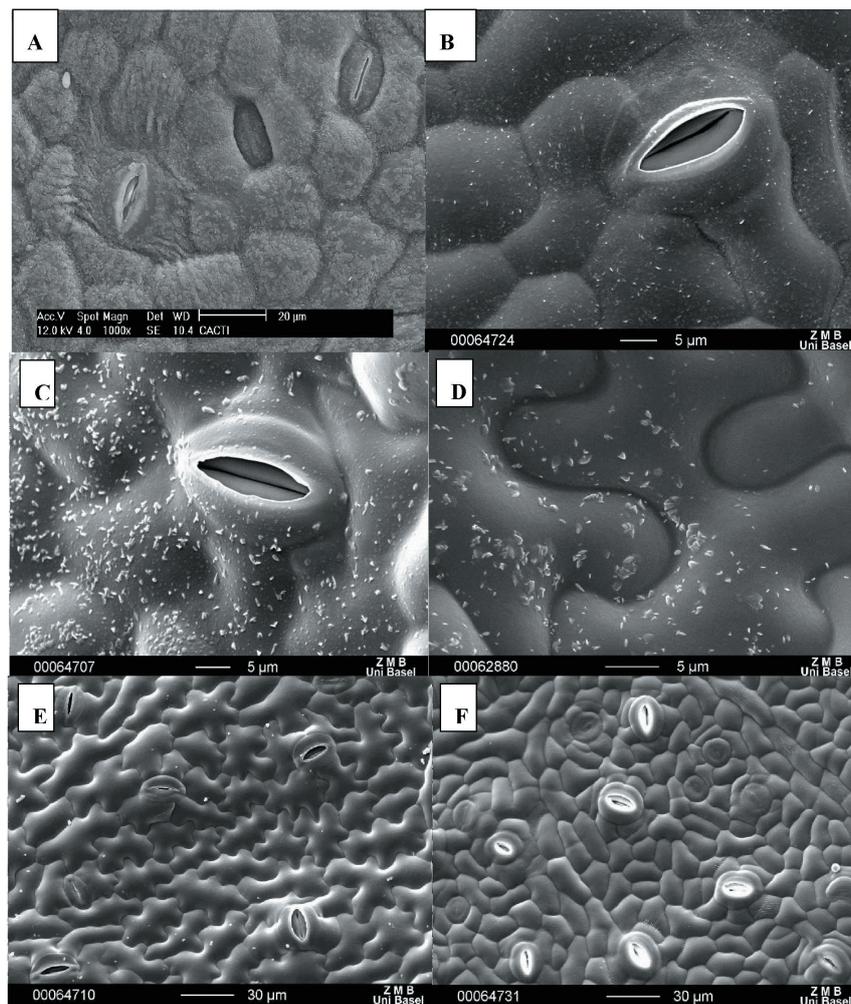


Fig. 4: Wax on the abaxial surface of all the genotypes studied. A: 'Albariño' (1000x); B: 'Touriga Nacional' (2000x); C: 'Pinot Noir' (2000x); D: 'Tempranillo' (3000x); E: 'Cabernet Sauvignon' (500x); F: *Vitis riparia* (500x).

(Spain). The authors thank Dr. EIRAS-DIAS (Estação Vitivinícola Nacional Dois Portos, Portugal) and Dr. MARTÍNEZ DE TODA (Universidad de La Rioja) for supplying the portuguese and Spanish cultivars. We thank Dr. PETERS and Dr. SCHWEIKERT (Staatliches Weinbauinstitut Freiburg) for his help with the German translation. The electronic microscope observations were realized in SEM-Laboratory University of Basel (Switzerland) and CACTI-University of Vigo (Spain).

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Received May 5, 2009