

Image analysis of the leaf vascular network: physiological considerations

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Abstract

The study of leaf vascular systems is important in order to understand the fluid dynamics of water movement in leaves. Recent studies have shown how these systems can be involved in the performance of photosynthesis, which is linked to the density of the vascular network per unit of leaf area. The aim of the present study was to highlight the correlation between a leaf vein density (V_D) and net photosynthetic rate (P_N), which was undertaken using a digital camera, a stereoscopic microscope, and a light source. The proposed hypothesis was tested, for the first time, on the leaves of two cultivars of *Vitis vinifera* (L.). A significant difference was found between the V_D of mature leaves of the two cultivars. V_D was also significantly correlated with the maximum leaf P_N . These findings support the hypothesis that the vascular system of grape leaves can be correlated with leaf photosynthesis performance.

Additional key words: gas exchange; leaf; net photosynthetic rate; vascular network; vein density.

Introduction

One physiological aspect, which is shared among living organisms, is a high level of branching and structural diversity of the vascular system (Parsons-Wingerter *et al.* 2011, Sack *et al.* 2012). Over the eons of plant evolution, the mass of water transported by leaves (per unit of pressure gradient) has increased in terms of performance. The structure of the xylem, which is able to increase hydraulic efficiency and maintain water column continuity, is crucial for the survival of vegetal species. However, the factors which have driven this development are still being studied. The vascular system of a leaf has two principal functions: to transport nutritive substances and to provide mechanical support. Water, solutes, and hormones pass through the xylem, while carbohydrates (which are produced within the leaves) are conducted *via* the phloem (Roth-Nebelsick *et al.* 2001). Leaf vein architecture plays a crucial role in defining the maximum limit of photosynthetic efficiency, in addition to being

essential as mechanical support (Brodrribb *et al.* 2007, Sack 2013). Recent studies have underlined how vein density (V_D) per unit area and mesophyll thickness determine the efficiency of the hydraulic system (Brodrribb *et al.* 2007). According to Brodrribb *et al.* (2007), V_D (defined by various authors as the total length of veins per unit area) is correlated to photosynthetic rate. In turn, V_D can be correlated with the distance that the water must travel through the mesophyll. The differences in V_D between species may be functional for different water-use efficiency (WUE) behaviors, a fact that can be of great help in selecting plant material for cultivations, especially in light of current climate change. The aim of the present study is to highlight the correlation between leaf V_D and net photosynthetic rate (P_N). This is the first known study of this type to be carried out on leaves from the cultivar *Vitis vinifera* (L.).

Materials and methods

The research involved two ten-year-old cultivars of *Vitis vinifera* (L.): Sangiovese (clone SS-F9-A5-48) and

Montepulciano (clone AP-MP-3). Eight vines per cultivar were trained upwards on a single spur cordon and grafted

Received 31 August 2015, accepted 29 April 2016, published as online-first 4 May 2016.

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Abbreviations: BSEs – bundle sheath extensions; C_i – intercellular CO_2 concentration; E – transpiration rate; g_s – stomatal conductance; LA – leaf area; LED – light emitting diode; P_N – net photosynthetic rate; ppm – parts per million; RH – relative humidity; V_D – leaf vein density; WUE – water-use efficiency.

Acknowledgments: We are grateful to the anonymous reviewers for their precious advices, as well as Lori Hetherington and Maria Sole Valleccoccia for the kind collaboration. All authors contributed equally to the manuscript.

onto SO4 (*V. berlandieri* \times *V. riparia*) rootstock. The vines were placed in an open field and exposed to the same agricultural and environmental conditions [27.10°C, 54.1% of relative humidity (RH)] inside the experimental vineyard in Arezzo (43°28'N, 11°49'E), Italy. All measurements were carried out on fully expanded leaves (usually the sixth to eighth on the shoot axes). All determinations were made on eight leaves, one from each of eight different vines randomly chosen along the row. Leaf gas exchanges were performed throughout the natural photoperiod (16-h daylight, 8-h darkness) by a *CIRAS-I* portable photosynthesis system (*PP Systems*, Hitchin, UK) with the leaves attached to the plant. Each measurement was carried out between 10:00 and 11:00 h. The reference CO₂ concentration and RH were set at 400 ppm and 70%, respectively, during measurements. The leaf temperature measured in the chamber was 27°C. Forty-day-old mature leaf samples were selected according to the maximum net photosynthetic rate (P_N) (Kriedemann *et al.* 1970). The eight leaves per cultivar, used for leaf gas-exchange determinations, were sampled from the main shoot. To keep the leaves fresh for image analysis, each sample was temporarily placed in a refrigerated chamber at 4°C for 24 h before image acquisition. Images were acquired using an *EOS 700D* camera (Canon, Japan) with EF 100 mm f/2.8 Macro USM objective (Canon, Japan), and a *Dinolite* digital microscope (AM-313T5, *AnMo Electronics Corporation*, Hsinchu, Taiwan) placed over the LED illumination panel (Fig. 1). Leaves were stretched to allow maximum focus rate during image acquisition. After leaf shape was taken by the camera, the vein image was

acquired by *Dinolite* microscope at 230 \times magnification. *DinoXcope 1.9.9* (*AnMo Electronics Corporation*) software was used to identify the vein. The microscope was set vertically to the sample and each image was collected with the optimal focus allowed by *DinoXcope* software. Image acquisition was performed by three rectangles of one mm² areas into a virtual grid. The rectangles were drawn to include at least two 2° order of veins. For each leaf, we subsampled three rectangular boxes (according to Sack *et al.* 2012). The boxes represented typical fragments and were located between the midvein and margin. Images were processed using *Fiji 1.50i* software (*ImageJ*, *National Institutes of Health*, USA) which allows the user to measure the physical parameters that characterize the leaf network. Images were binarized and processed using a triangle *Fiji* plug-in (Fig. 2). We tried various algorithms available within *Fiji*. The triangle algorithm (Zack *et al.* 1977) yielded the most robust output. Skeleton image processing consists of the following steps: open image; process; binary; make binary; image; adjust; threshold; triangle algorithm; light background. V_D data were collected manually on the major veins from skeletonized binary images. Image analysis was performed on the same leaves used for the gas exchange measurements. Binary images made possible to highlight the vein profiles and helped carry out clear measurements of the image using the software. The V_D (measured from veins visible from their bundle sheath extensions, considering that this may slightly underestimate the total vein network) were measured using *ImageJ* segmented line tool on the images skeletonized.

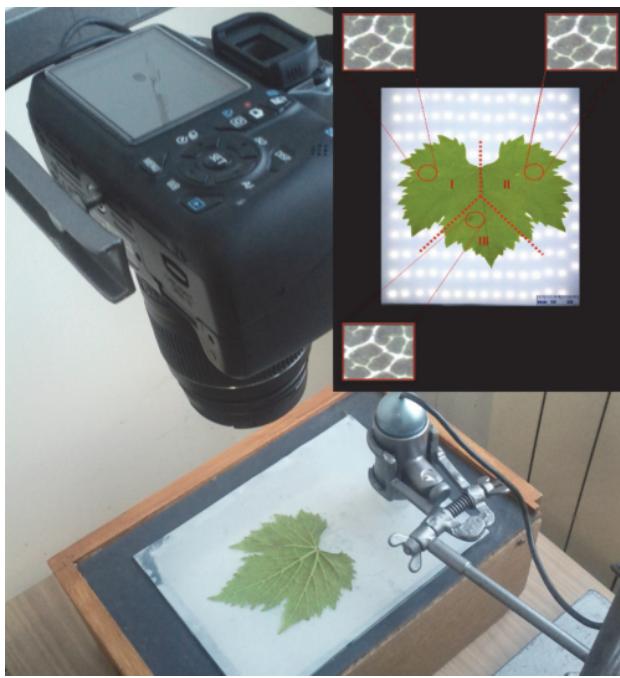


Fig. 1. Image acquisition set-up and crop (1 mm²) image collection method. Leaf LED device (L \times W \times H: 12 \times 7.5 \times 5 cm) was equipped with 180 LEDs (*SMD chip Samsung 5630*; 6,000 K, thickness 2.4 mm). It was created to increase the sharpness of the data achieved and to highlight accurately the profiles of vein texture.

Statistical analysis: Measurements taken on different samples were considered together in order to obtain an average trend valid for the whole leaf. Statistical analyses were performed using *Prism v. 5.0 (GraphPad software, San Diego, California, USA)*. In particular, we tested for

Results

Image analysis technique has long been used in many fields, such as medicine, engineering, and architecture in order to obtain quantitative and qualitative information about the object under study. Image processing (Fig. 2) made it possible to acquire different kinds of V_D profiles

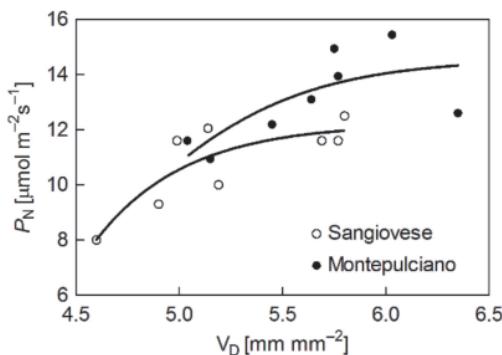
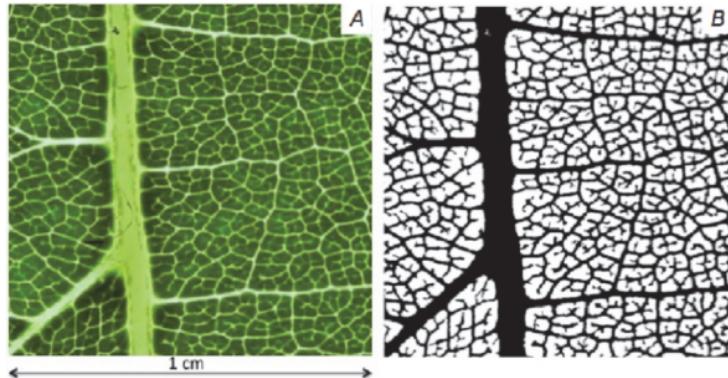


Fig. 3. Relationship between net photosynthetic rate (P_N) and leaf vein density (V_D) in Montepulciano and Sangiovese cultivars. Each point contains an average of three crops (24 measurements per cultivar) and one *CIRAS* measurement.

t-test ($p < 0.05, n = 8$) showed that the Montepulciano cultivar was characterized by a significantly higher mean density/area of veins per unit compared to Sangiovese (5.70 ± 0.40 vs. $4.98 \pm 0.51 \text{ mm mm}^{-2} \pm \text{SD}$). Furthermore, maximum P_N correlated with V_D [mm mm^{-2}] for both cultivars ($r^2 = 0.55$ and 0.72 respectively, $n = 8$) (Fig. 3), confirming its positive link with density. The gas-exchange parameters in Sangiovese and Montepulciano (Table 1) highlighted a significant increase of the P_N in the cultivar with higher V_D . In the correlation graph (Fig. 3), each point contains an average of three crops (24 measurements per cultivar) and one *CIRAS* measurement. The relationship was not linear: after the first V_D point,

differences in correlation between net photosynthesis and V_D in order to verify the best fit. The best relationship was determined using a nonlinear regression (log agonist vs. response; *GraphPad Prism 5.0*). Instead, a linear correlation between the V_D and leaf area (LA) was the best fit.

on the sample leaves. A clear image of the skeleton was needed to accurately determine data by hand measurements. Crops (1 mm^2) taken from the leaves by *Dino-lite* microscope were used to measure V_D .

Fig. 2. Example of skeleton image processing acquired through the following *ImageJ* steps: open image; process; binary; make binary; image; adjust; threshold; triangle algorithm; light background. Vein data were collected manually from skeletonized binary images (A – original image; B – skeletonized image).

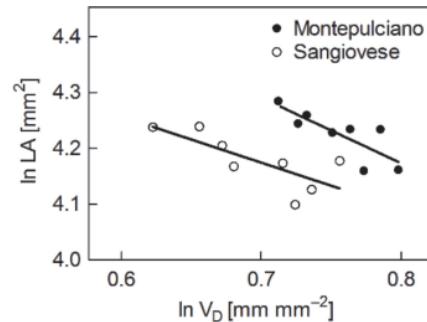


Fig. 4. Significant linear correlation between leaf area (LA) and leaf vein density (V_D) in Montepulciano ($p < 0.05, n = 8$) and Sangiovese cultivars ($p < 0.05, n = 8$). The vein density per unit, as expected, decreases as leaf size grows up.

the curve become flat and the P_N did not increase further. Compared to the Sangiovese, Montepulciano showed statistically significantly higher values of mean \pm SD: P_N [13.10 ± 1.71 vs. $10.82 \pm 1.56 \text{ μmol m}^{-2} \text{ s}^{-1}$], stomatal conductance (g_s) [191.79 ± 50.85 vs. $125.56 \pm 27.07 \text{ mmol (H}_2\text{O) m}^{-2} \text{ s}^{-1}$] and transpiration (E) [4.09 ± 0.78 vs. $3.15 \pm 0.61 \text{ mmol (H}_2\text{O) m}^{-2} \text{ s}^{-1}$] (Table 1). The photosynthetic active radiation was not different between the two groups [PAR of $1,394.31 \pm 266.65$ vs. $1,387.14 \pm 190.82 \text{ μmol(photon) m}^{-2} \text{ s}^{-1}$]. In addition, we found a significant correlation between leaf size and V_D ($r^2 = 0.65$ in Montepulciano, $r^2 = 0.57$ in Sangiovese), where the V_D per unit, as expected, decreased as the leaf size increased (Fig. 4).

Discussion

The study of leaf vascular system and their evolution is the main point to understand the fluid dynamics involved into the vascular tissues. The present research is a first contribution to the investigation of leaf veins and their correlation with photosynthesis performance in *V. vinifera* L. Comparing the veins of the two cultivars analysed, Montepulciano had a higher density area resulting as a

thicker grid than that of Sangiovese. A different texture of the V_D in Sangiovese and Montepulciano cultivars is a novel information about these widely grapevine genotypes cultivated in Italy. This diversity in V_D might be functional to a different behavior for the water-use efficiency (WUE). Furthermore, a major V_D plays an important role in water conduction (Roth-Nebelisck *et al.* 2001, Sack and

Table 1. Gas-exchange parameters in Sangiovese and Montepulciano cultivars (*t*-test, $n = 8$, \pm SD, * – significant for $p < 0.05$, ** – significant for $p < 0.01$, ns – not significant).

Parameter	Sangiovese	Montepulciano	
P_N [$\mu\text{mol m}^{-2} \text{s}^{-1}$]	10.82 ± 1.56	13.10 ± 1.71	*
g_s [$\text{mmol}(\text{H}_2\text{O}) \text{m}^{-2} \text{s}^{-1}$]	125.56 ± 27.07	191.79 ± 50.85	**
E [$\text{mmol}(\text{H}_2\text{O}) \text{m}^{-2} \text{s}^{-1}$]	3.15 ± 0.61	4.09 ± 0.78	*
PAR [$\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$]	$1,394.31 \pm 266.65$	$1,387.14 \pm 190.82$	ns

Holbrook 2006, Zwieniecki *et al.* 2007) and in biomechanical support of the leaf (Roth-Nebelisck *et al.* 2001). Furthermore, we found a significantly positive correlation between V_D and maximum P_N (Fig. 3) in mature leaves of *V. vinifera*. This finding supports the hypothesis that the vascular system is directly linked to the P_N performance. The relationship is not linear, however, this is probably due to a maximum rate of a saturation point, beyond which the P_N decreases. We are aware that photosynthesis can be influenced, beyond the leaf age, by different types of environmental factors, such as temperature, CO_2 , light, drought stress, cultivar genotype (Crafts-Brandner *et al.* 2000), but we also believe that V_D can play a fundamental role for P_N performance. A correlation between the ratio of photosynthesis per area and V_D has been shown for different species in recent studies (Brodribb *et al.* 2007, Sack *et al.* 2013). In this study, the major vein texture density in the leaves declined with the increasing leaf size (Fig. 4). This result is in accordance with the findings of Sack *et al.* (2012) who noted that the major veins showed a negative scaling in V_D with a leaf size. Moreover, the correlation between the leaf size and V_D can be explained as an increase of areole area among vein perimeter, which do not necessarily mean a

higher physiological performance per unit (Fiorin *et al.* 2016). Sack and collaborators also highlighted that the density of minor veins was independent of a leaf size. Furthermore, Nikolopoulos *et al.* (2002) showed that the existence of veins appears to be an adaptation to save water and to protect mesophyll from water stress. Future studies could be directed toward the construction of predictive models able to integrate knowledge gained through the ‘reading of the leaves’: for example, to highlight the potential risk of water stress and the resulting demands for irrigation requirements in vineyards. Other investigations may be oriented toward understanding the correlation between stomata density per unit of area and V_D and how they are linked to transpiration (Brodribb 2009). Use of image analysis in order to characterize V_D with the aim of recognizing variety could be also conceived. Ultimately, the imaging setup used in this study (LED illumination panel, a digital camera and microscope) could also be used for measurements on similar species. In conclusion, the Montepulciano cultivar was characterized by a significantly higher mean density/area of veins per unit compared to Sangiovese. The maximum P_N correlated with V_D in both cultivars. There was a significant increase in P_N rate in the cultivar with the higher V_D .

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