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Water Relations and Mesophyll Anatomy in Almond Leaves

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Abstract. **Instantaneous water use efficiency (WUE$_i$) is a measure made at the leaf scale, which can be used as a criterion for estimating WUE in breeding programs. To study the WUE$_i$ in different almond (*Prunus dulcis*) genotypes, we measured stomatal conductance (g_S), assimilation rate (A), transpiration, internal concentration of CO_2 (C_i), and leaf hydraulic conductance normalized to leaf area in five mixed crosses of almond trees. For all measured parameters we observed the most significant differences between 'Johnston' × 'Lauranne' and 'Nonpareil' × 'Lauranne'. Nevertheless, 'Carmel' × 'Tarraco' showed the highest WUE$_i$ among the five crosses. The significant correlations among g_S, A, and C_i indicated that A was probably limited by both stomatal and non-stomatal parameters that might be affected by genotype variations. In another experiment, we selected three cultivars of a new set of almond cultivars (Nonpareil, Carmel, and Masbovera) in four replicates for measuring g_S at field capacity. Meanwhile, using a cryo-scanning electron microscopic (SEM) method, we prepared some images from the internal structures of leaves collected from the same cultivars of almond trees. Results showed that 'Masbovera' leaves, in which post-venous hydraulic distance (D$_m$) was higher compared with 'Carmel' and 'Nonpareil', represented significantly lower values of g_S rather than the two other cultivars. Comparing mesophyll anatomy and g_S between these cultivars demonstrated that D$_m$ and the density of mesophyll cells might indirectly affect g_S in almond leaves. In conclusion, our study demonstrated that water relations, WUE$_i$, and leaf anatomy in almond trees differed among genotypes.**

Improving WUE in perennial crops like fruit trees may decrease water use without reducing yield. This can be particularly important for water-limited areas in which crop productivity is dependent on water availability (Bassett et al., 2011; Naor et al., 2008). To this aim, it is necessary to identify the physiological processes involved in improving WUE in crops (Boyer, 1982; Raiabi et al., 2009).

Plant scientists use instantaneous WUE as a direct measure of leaf level WUE at a moment in time (Comstock and Ehleringer, 1992; Ripullone et al., 2004). For measuring WUE$_i$, the instantaneous assimilation rate is compared with the instantaneous transpiration (E) through the stomata. A and E can be influenced by two factors: first is g_S and the other is concentration differences for CO_2 between outside and inside of stomata ($C_a - C_i$). Therefore, A and WUE$_i$ are mostly affected by the function of stomata (Condon et al., 2002; Lambers et al., 2008).

Because stomatal behavior follows the optimality theory for gas exchange regulations, it is also possible that A affects the variations of g_S. According to this theory, assimilating the maximum levels of carbon per unit of water transpired is considered the optimal control of gas exchange (Cowan and Farquhar, 1977).

In addition to the stomatal limitation, internal or non-stomatal limitations may also affect A. In this respect, Marchi et al. (2008) reported that non-stomatal limitations and photosynthetic enzymes may be more important rather than stomatal limitations in restricting A (Marchi et al., 2008). Non-stomatal limitations can be related to biochemical factors; e.g., photosynthetic enzyme activities (Faver et al., 1996) or chlorophyll content (Guerfel et al., 2009), and diffusive limitations, including mesophyll conductance (g$_m$) (Ethier and Livingston, 2004; Grassi and Magnani, 2005). Under non-stress conditions, non-stomatal limitations are dependent more on diffusional rather than biochemical factors. Biochemical limitations on the other hand can be important only under severe water deficit conditions or during leaf development and senescence (Grassi and Magnani, 2005). Previous findings indicate that the anatomical differences in the distances between sub-stomatal pathways to carboxylation sites might be the reasons for the variations in A (Evans and Von Caemmerer, 1996). In this respect, Brodribb et al. (2007) reported the considerable effects of leaf anatomical parameters on both g$_m$ and A, confirming the close link between water and CO_2 pathways in the mesophyll (Brodribb et al., 2007).

Several studies indicate that g$_m$ and g_S are highly correlated with each other (Flexas et al., 2012; Perez-Martin et al., 2009), probably because water and CO_2 transfer through a shared pathway in some parts in leaves. Both water vapor and CO_2 cross the aerial sub-stomatal cavity through the stomata (Flexas et al., 2012). Although the directions of water movement and CO_2 diffusion are mostly opposite to each other, they share diffusion pathways in some parts in the post-venous area of the mesophyll (Evans et al., 2009; Terashima et al., 2011). In this respect, Sack and Frole (2006) observed that maximum A is highly dependent on the capacity of the leaf hydraulic system to supply water for mesophyll photosynthetic cells (Sack and Frole, 2006). Therefore, it can be concluded that hydraulic conductance (k_{leaf}) is highly correlated with photosynthetic capacity and thereby indirectly affects g_S by limiting the A in mesophyll cells (Brodribb, 2009; Brodribb et al., 2007). According to previous reports, there is more resistance to water movement in living mesophyll cells compared with highly conductive vessels (Passioura, 1988). Sack et al. (2003) and Sack and Frole (2006) reported that extravascular resistance in

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the leaves of dicotyledons constitutes ≈30% of the hydraulic resistance for the whole plant (Sack et al., 2003; Sack and Holbrook, 2006); therefore, vascular delivery of water is more effective in comparison with water flowing through the mesophyll cells. Based on this concept, it can be concluded that hydraulic distance length is correlated with the photosynthetic capacity of the mesophyll tissues (Brodribb et al., 2007). That is why the spatial arrangement of minor veins in leaves is an important non-stomatal limiting factor for photosynthesis (Brodribb et al., 2007; Ocheltree et al., 2012).

In comparison with other nut crops grown in Mediterranean climates, almond trees are relatively more drought-resistant. For this study we used five almond breeding lines. These genotypes mostly included the progenies of 'Nonpareil' and 'Carmel', which are the most common cultivated almond trees in Australia. We also included 'Masbovera' in our experiment, which is a relatively water use-efficient cultivar compared with other almond cultivars (Gispert et al., 2009). The main aim of this study was to test the assumption that under non-stress conditions, genotypic variation affects water relations and WUE_i in almond plants. We further assessed the hypothesis that genotypic variation in almond may lead to anatomical differences in mesophyll, which may influence g_S.

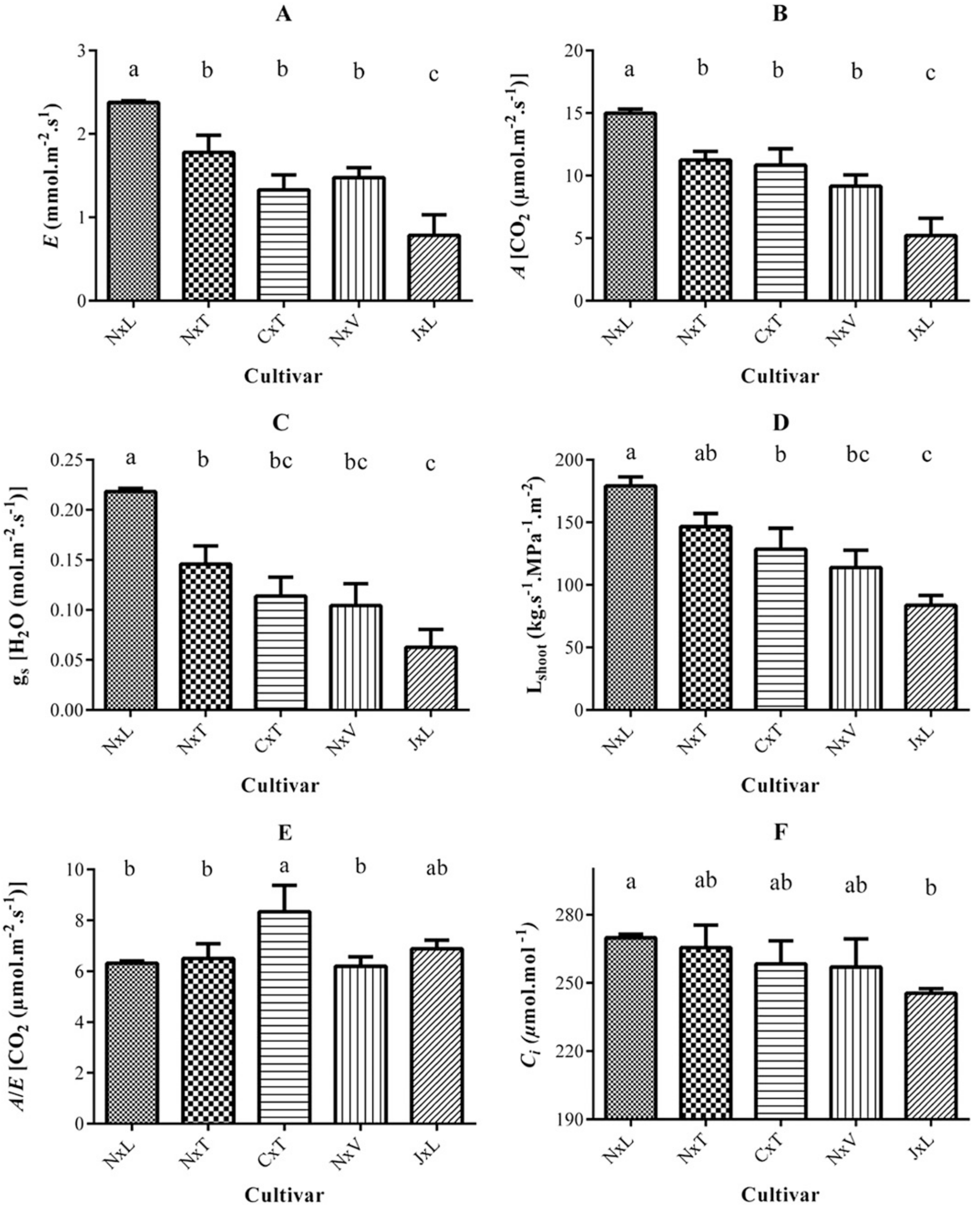

Fig. 1. Transpiration rate [E (**A**)], assimilation rate [A (**B**)], stomatal conductance [g_S (**C**)], leaf hydraulic conductivity normalized to leaf area [L_{shoot} (**D**)], instantaneous water use efficiency WUE_i [A/E (**E**)], and internal concentration of CO_2 [C_i (**F**)] for five mixed crosses of almond: 'Nonpareil' × 'Lauranne' (N×L), 'Nonpareil' × 'Tarraco' (N×T), 'Carmel' × 'Tarraco' (C×T), 'Nonpareil' × 'Vayro' (N×V), and 'Johnston' × 'Lauranne' (J×L). Each column represents the average of four replicates ± SE. Different letters indicate statistical differences (Duncan's test at $P < 0.05$).

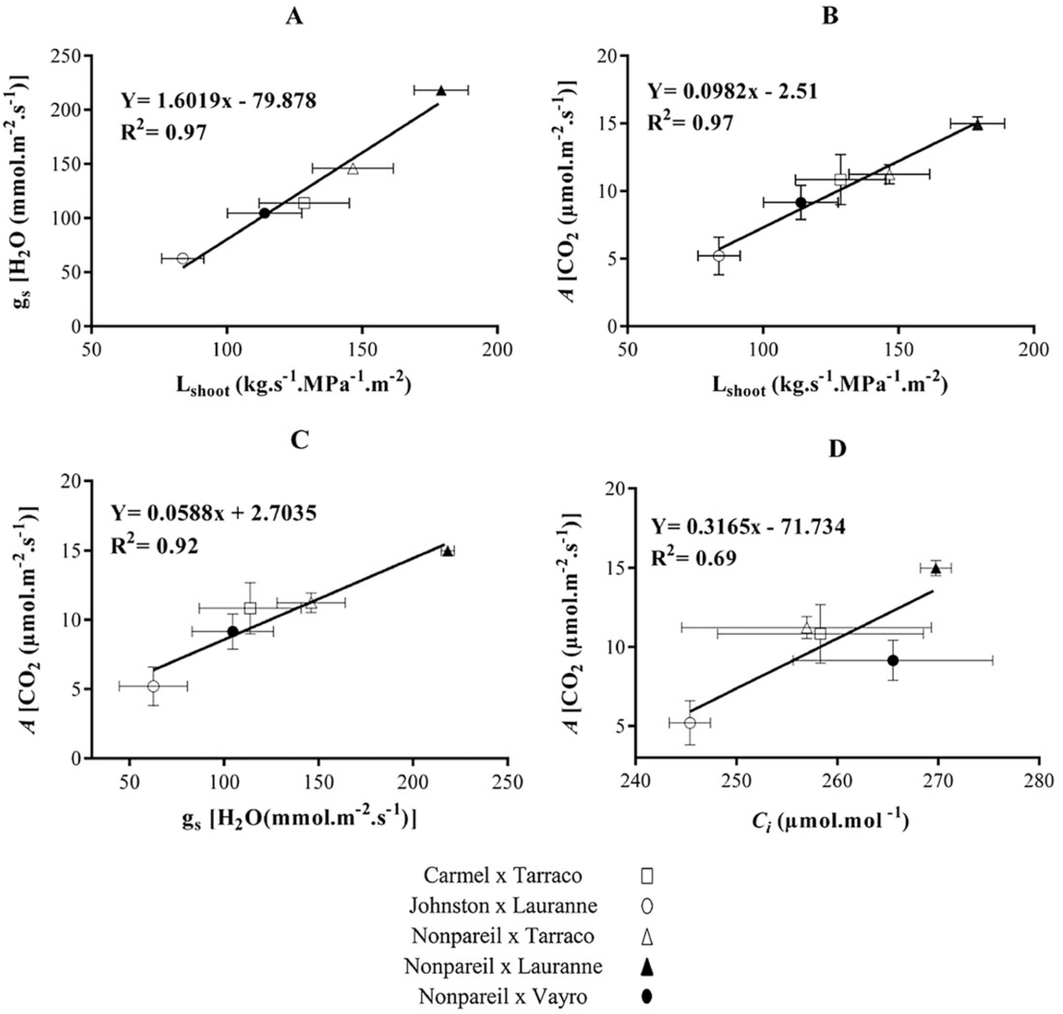

Fig. 2. The relationships between hydraulic conductance normalised to leaf area (L_{shoot}) with stomatal conductance [g_S (**A**)] and carbon assimilation [A (**B**)]. The relationships between carbon dioxide assimilation rate A with g_S (**C**) and internal concentration of CO_2 [C_i (**D**)]. Measurements are taken in leaves of five mixed crosses of almond: 'Nonpareil' × 'Lauranne', 'Nonpareil' × 'Tarraco', 'Carmel' × 'Tarraco', 'Nonpareil' × 'Vayro', and 'Johnston' × 'Lauranne'. Error bars represent the average of four replicates ± SE for each point.

Materials and Methods

EXPT. 1. In this experiment, we selected five mixed crosses of almond comprised of 'Carmel' × 'Tarraco' (C×T), 'Johnston' × 'Lauranne' (J×L), 'Nonpareil' × 'Tarraco' (N×T), 'Nonpareil' × 'Lauranne' (N×L), and 'Nonpareil' × 'Vayro' (N×V) with four replicates. These crosses were selected based on the parental kernel quality characteristics assessed in the breeding program. Pots were arranged randomly in each block on four separate benches as replicates. Trees were 3 years old at the time of the experiment. Each tree was grown in a 30-cm pot containing coco peat mix (two peat:one sand) plus slow-release fertilizer. Trees were own-rooted because they were progeny from the breeding program. Pots were maintained in a greenhouse set at 26 °C with a 12-h day/night light regime.

Every week one replicate, comprised of all five mixed crosses, was moved to the growth chamber. The reason for moving the plants to the growth chamber was that the levels of light, humidity, and temperature were under constant control in the chamber, whereas in the glasshouse, because of forecast variations, these elements may not be constant from day to day. The temperature in the growth chamber was 22 °C and the light regime was set at 12-h light/dark. For limiting the evaporation rates and, therefore, reducing the possible effects of water deficiency on plants, the temperature of the chamber was set on 22 °C, which was 4 °C less than that of the glasshouse. The light sources in the chamber were metal-halide lamps. The light intensity on the upper surface was 300 ± 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After 1 week, A, E, g_S, and C_i of leaves were measured using a portable photosynthesis system (Model LI-6400; LI-COR, Lincoln, NE). In this respect, WUE_i was calculated as A/E (Condon et al., 2002). For each plant, the first three fully expanded leaves were measured. It is important to note that the leaf chamber was equipped with an extra light source for measuring light-saturated photosynthesis. The light saturation point was obtained by measuring the light response curve for each genotype. In this regard, the light saturation point was set at 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The external CO_2 concentration was set at 400 $\mu mol \cdot mol^{-1}$, temperature was 22 °C, and air flow rate was 350 $mmol \cdot s^{-1}$. The relative humidity was kept nearly constant throughout the experiment (50% to 55%).

The measured leaves were separated from plants to measure the leaf-specific hydraulic conductance using a hydraulic conductance flow meter (HCFM; Dynamax, Houston, TX)

(Vandeleur, 2008). k_{leaf} normalized to leaf area [L_{shoot} (kilograms per second per megapascal per square centimeter)] was obtained by dividing the measured conductance by total leaf area. To this aim, leaf area was measured with a portable leaf area meter (AM300; ADC BioScientific, Hoddesdon, U.K.).

Expt. 2. In another experiment, g_S at field capacity was measured in four replicates in three cultivars of a new set of almond cultivars (Nonpareil, Carmel, and Masbovera). Nemaguard seedlings were used as rootstocks for these trees. Plants were 2 years old and were grown in the same soil conditions as the previous experiment. The temperature of the glasshouse was set at 26 °C with a 12-h day/night light cycle. Measurements were recorded between ≈1000 and 1200 hr. Every second day we watered the pots adequately and during 5 d recorded the g_S of leaves daily using a leaf porometer (AP4; Dynamax). The obtained data were statistically analyzed in SAS/STAT (Version 9.1; SAS Institute, Cary, NC).

In addition, some images were prepared from the internal structures of fully expanded leaves collected from the same cultivars (Nonpareil, Carmel, and Masbovera). To this aim, we used a cryo-SEM method at Adelaide Microscopy (Adelaide, Australia). Cryo-SEM is an imaging technique for those samples that contain moisture in their tissues. In fact, in this method tissues can be imaged without removing their water. First, small pieces of leaves (≈1 mm in length) were cut and placed in aluminium planchettes (Müller and Moor, 1984). Before loading the samples in the cryo-SEM, they were physically fixed by a rapid freezing process in liquid nitrogen. After that samples were clamped between a sample holder and finally were cleaved with a cold knife for scanning their internal anatomy (Bastacky et al., 1995; Walther, 2003). Cross-sections were made from the middle parts of the leaves. Rotating the sample holder in the cryo-chamber allowed imaging the samples from different angles; hence, we were able to image clearly three veins for each section. Therefore, data obtained in this section (cryo-SEM imaging) were deduced from three veins of one sample for each cultivar.

Because measuring the accurate distance of water movement through the mesophyll is still controversial (Ye et al., 2008), instead of estimating the exact water pathway through the post-venous area, we calculated an index for this distance, which includes the distance between the end of the veins and evaporation sites. To this aim, D_m was calculated by measuring the horizontal length (x) between the vascular bundle and nearest stomata and the vertical distance (y) from the vascular tissue to the leaf surface (Ocheltree et al., 2012):

$$\mathrm{Dm} = \sqrt{x^2 + y^2} \qquad [1]$$

Results and Discussion

Expt. 1. For all measured parameters, differences were most significant between J×L and N×L. Both A and E values in N×L and J×L were significantly different from N×T, N×V, and C×T. Our results for C_i data showed that only N×L and J×L were significantly different (Fig. 1F), whereas for A, E, and g_S values, N×L was significantly higher than the other four crosses. L_{shoot} values of J×L were significantly lower than C×T, N×T, and N×L.

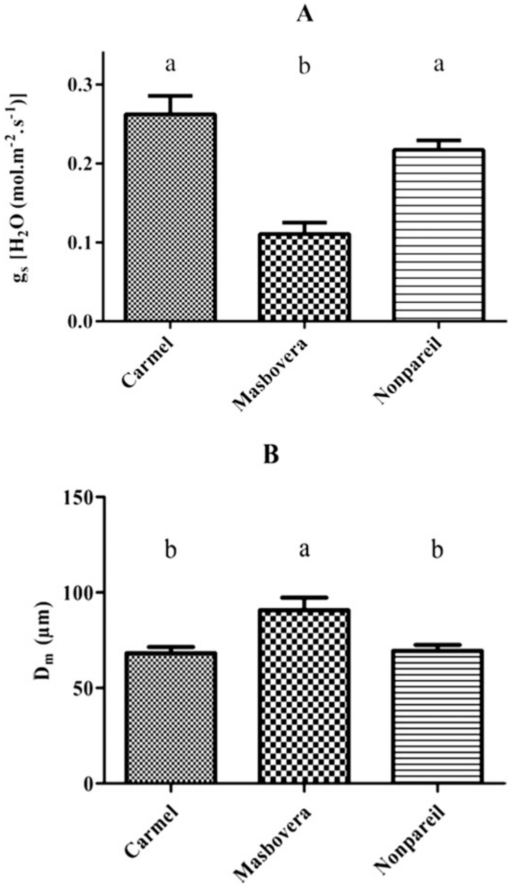

Fig. 3. The variation of stomatal conductance [g_S (**A**)] and post-venous distance [D_m (**B**)] for 'Carmel', 'Masbovera', and 'Nonpareil' almond. Measurements are taken in almond leaves. The means ± se (n = 3 and 8) are shown for D_m and g_S, respectively. Error bars with the same letter are not significantly different (Duncan's test at $P < 0.05$).

The significant decrease in A values from N×L to J×L in the first experiment (Fig. 1B) was coupled with notable reductions in g_S and C_i (Fig. 1C and F). According to previous studies, C_i variations indicate that A is probably affected by stomatal limitations (Flexas and Medrano, 2002; Garland et al., 2012). Therefore, the lower values of C_i in J×L compared with N×L (Fig. 1F) imply that in J×L stomatal closure is presumably the limiting factor for A (Figs. 1B and 2D). However, according to previous studies, the large scale of variation in C_i may be the result of the variations in photosynthetic capacity between different genotypes (Blum, 2004; Farquhar and Sharkey, 1982).

Regarding WUE_i, C×T trees showed significantly higher WUE_i compared with N×V, N×T, and N×L (Fig. 1E). Although, A, E, g_S, L_{shoot}, and C_i were not significantly different among C×T, N×V, and N×T, the WUE_i of C×T was significantly higher than N×V and N×T. In this respect, N×L and J×L represented the most significant differences for both A and E (Fig. 1A–B), but their WUE_i was not significantly different (Fig. 1E). On the other hand, the highest values of WUE_i were observed in C×T, in which A and E values were not the highest and lowest values, respectively, compared with other crosses. Such results demonstrate that WUE_i in a plant with high A and E may show the same values as another plant with relatively lower A and E (Condon et al., 2002).

We observed a highly significant ($P < 0.01$) correlation between L_{shoot} and g_S and also between L_{shoot} and A (Fig. 2A–B). Although A was highly ($P < 0.01$) correlated with g_S and C_i,

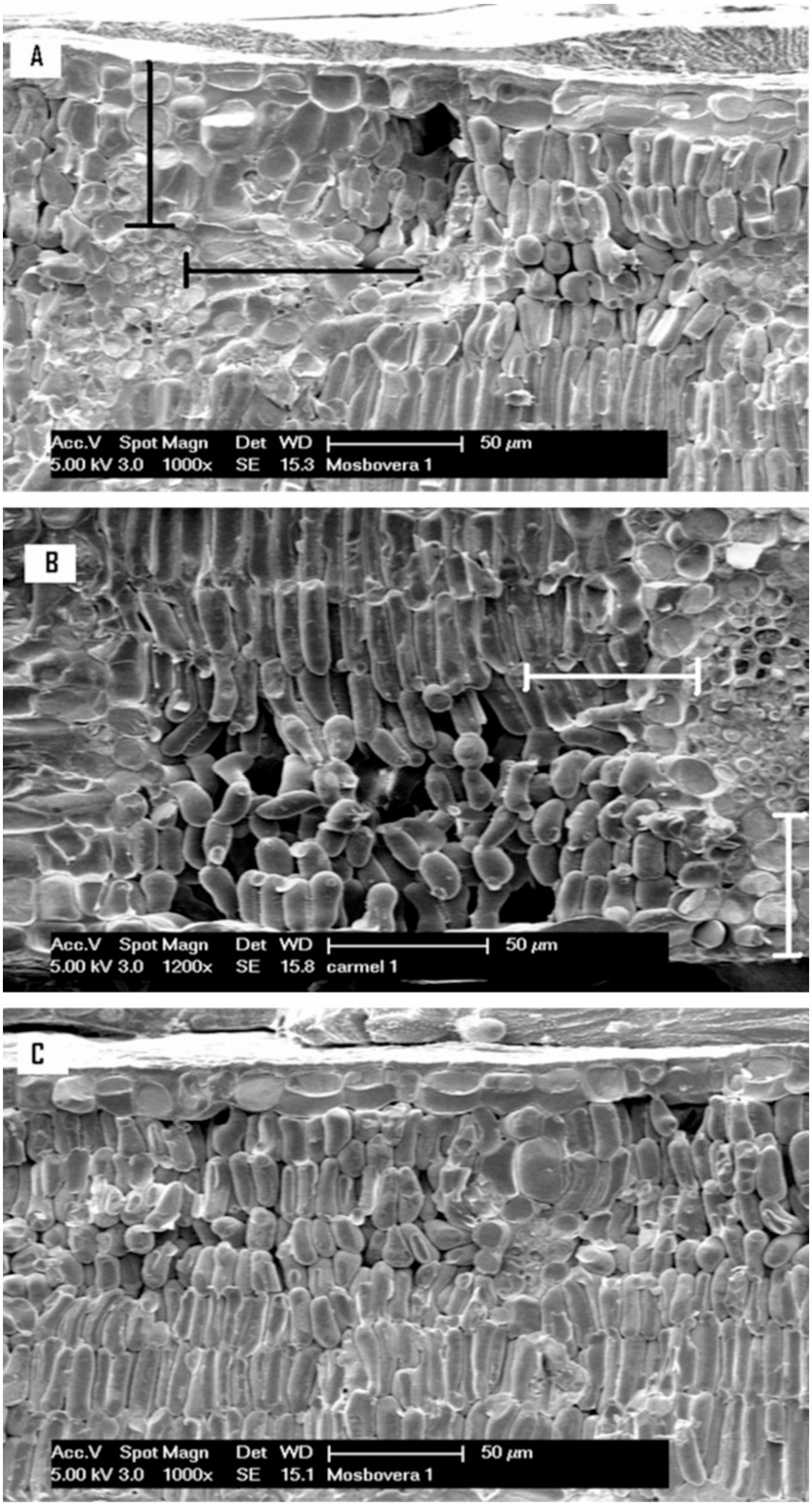

Fig. 4. Scanning electron microscope images of almond leaves. Horizontal (x) and vertical (y) distances of vascular bundles from stomata in ‘Masbovera’ (**A**) and ‘Carmel’ (**B**). The compact arrangement of mesophyll tissue in ‘Masbovera’ (**C**).

there was a higher correlation between A and g_S compared with A and C_i (Fig. 2C–D). The close correlation between A and g_S (Fig. 2C) can indicate that stomatal closure might be affected by the photosynthetic capacity of the mesophyll cells (Wong et al., 1979). Based on the theory of stomatal optimality, stomata tend to maintain the C_i at a constant level (Cowan and Farquhar, 1977; Manzoni et al., 2011; Wong et al., 1979). Bearing in mind that the high levels of A lead to a reduction in the partial pressure of C_i, it can be concluded that the higher g_S for N×L (Fig. 1C) might be the result of its higher A in comparison with other almond crosses (Figs. 1B and 2C) (Wilson et al., 2000). In such conditions, stomata need to open to let in more CO_2 to compensate for the reduction in C_i (Yu and Wang, 1998). It might be that the high correlation between g_S and A (Fig. 2C) decreases the range of variation of C_i. Therefore, there is less dramatic differences in C_i and WUE_i in comparison with substantial differences in g_S, E, and A (Fig. 1A–C and E–F) (Cernusak et al., 2011, 2013); thus, A shows a higher correlation with g_S rather than C_i (Fig. 2C–D). In another words, reducing g_S usually comes together with a decline in A; therefore, WUE_i variations are not dramatic. However, there are variations in C_i among different genotypes (Fig. 1F) (Cernusak et al., 2013).

According to previous reports (Brodribb et al., 2007; Sack and Holbrook, 2006), the higher A in N×L is presumably the result of its higher L_{shoot} in comparison with other crosses (Figs. 1D and 2B). In fact, the higher values of L_{shoot} in N×L trees indicate that the capacity of the leaf vascular system to supply water for photosynthetic mesophyll cells is probably higher than C×T, N×V, and J×L (Figs. 1D and 2B) (Sack and Holbrook, 2006). The high correlation between L_{shoot} and A, which indicates the close relationship between water transport capacity and carbon gain, is shown by several studies (Brodribb et al., 2007; Johnson et al., 2009; Zhang and Cao, 2009). Such a high correlation between L_{shoot} and A is mediated through the control of stomatal movements that consequently regulate C_i (Zhang and Cao, 2009). Low L_{shoot} works as a hydraulic signal inducing the stomatal closure (Fig. 2A) (Vadez et al., 2014). Nevertheless, it also might be that L_{shoot} indirectly affected g_S by limiting A. The close correlation among L_{shoot}, A, and g_S is observed in various species of woody plants. The mechanism behind such strong correlation among L_{shoot}, A, and g_S is based on the theory of stomatal optimality. The concept of this theory is optimization of carbon uptake and water loss (Brodribb, 2009). Such variations in L_{shoot} are probably the result of the anatomical differences between various genotypes (Sack and Frole, 2006; Schreiber et al., 2011). The high coefficient between L_{shoot} and A and g_S indicates that C_i is not of major importance in explaining the other observed differences. However, the details of the mechanisms involved in this high coordination between g_S and A are still not clearly understood (Cernusak et al., 2013). In addition, among different leaves with different developmental stages, nitrogen content and photosynthetic enzymes might have a role more important than that played by stomatal regulation in differentiating A (Marchi et al., 2008). Nevertheless, in this study all the measured leaves were collected at the same developmental stage (fully expanded).

This study demonstrated that WUE_i and water relations in almond trees can change depending on genotype. The lowest values of WUE_i were observed in 'Nonpareil' progenies (N×L, N×T, and N×V) (Fig. 1E). The highest and the lowest values of L_{shoot}, A, E, g_S, and C_i were observed between N×L and J×L, which both are progenies of 'Lauranne' (Fig. 1A–F); hence, comparing water relation parameters between 'Nonpareil' and 'Johnston' might demonstrate even more differences.

Expt. 2. In this experiment, 'Masbovera' leaves, in which D_m values were higher compared with 'Carmel' and 'Nonpareil' (Figs. 3B and 4A–B), represented significantly lower values of g_S rather than the two other cultivars (Fig. 3A). It is probably because of the higher D_m that increases the extravascular resistance in 'Masbovera' leaves. Thus, the higher hydraulic resistance in the mesophyll tissues of 'Masbovera' leaves might lead to a lower A that presumably is the reason for the lower g_S in this cultivar (Brodribb et al., 2007; Sack and Holbrook, 2006). In contrast with 'Masbovera', both D_m and g_S values were not significantly different between the cultivars Nonpareil and Carmel. Several studies in this regard indicated that the spatial arrangement of veins in leaves, which determines the D_m, is highly correlated with k_{leaf}, g_S, and A (Brodribb et al., 2007; Ocheltree et al., 2012; Sack and Frole, 2006).

SEM images revealed that palisade mesophyll layers in 'Masbovera' leaves were more compacted in comparison with the other cultivars (Fig. 4B–C). Such compact arrangement of mesophyll cells might also be the reason for the lower g_S in 'Masbovera' compared with 'Carmel' and 'Nonpareil'. It has been previously reported that a compact mesophyll tissue leads to a lower A (Pavlovic et al., 2007; Tomás et al., 2013). Another study on peach (*Prunus persica*) and olive (*Olea europaea*) revealed that genotypic variations might lead to morphological differences and therefore may affect A (Marchi et al., 2008). Subsequently, g_S might be limited in response to the reduction of A (Flexas et al., 2007). Related studies on peach (Marchi et al., 2008), tobacco [*Nicotiana tabacum* (Evans and Loreto, 2000)], and bean [*Phaseolus vulgaris* (Singsaas et al., 2003)] demonstrated high correlations between g_S and A (Flexas et al., 2007, 2012). Thereby, compact mesophyll tissue limits the amounts of water loss during the hot and dry summers of Mediterranean climates. Related studies on olive trees showed that compact palisade mesophyll layers increase the mechanical strength of parenchyma tissue and protect the leaves against extra water loss (Bacelar et al., 2004; Marchi et al., 2008).

Although the results of this experiment are obtained from three veins for each cultivar, conducting a similar experiment with more replicates may help to confirm the existing results. However, there are some reports that the thickness of palisade mesophyll in leaves can also be increased by age (Kositsup et al., 2010; Xie and Luo, 2003). For minimizing errors between young and old leaves, the first fully expanded leaves were collected for this experiment. Bearing in mind that mesophyll anatomical differences may affect g_S (Evans and Loreto, 2000; Flexas et al., 2012), the lower g_S in 'Masbovera' compared with 'Nonpareil' and 'Carmel' could be linked to the compact arrangement of mesophyll cells and lower D_m in 'Masbovera' compared with the two other cultivars.

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