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Comparative impacts of water stress on the leaf anatomy of a drought-resistant and a drought-sensitive olive cultivar

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SUMMARY

The effects of drought on several major morphological and anatomical features of leaves were investigated in an attempt to explain the origin of the difference in drought resistance between two olive (*Olea europaea* L.) cultivars, ('Chemlali' and 'Meski') previously demonstrated to be drought-resistant and drought-sensitive, respectively. Under water deficit conditions, 'Chemlali' maintained higher rates of photosynthetic assimilation and lower rates of transpiration compared to 'Meski'. In the present study, we found cultivar-dependent differences in leaf morpho-anatomical adaptations to drought stress. When subjected to water stress, the leaves of 'Chemlali' increased the thickness of their upper palisade and spongy parenchyma by 17% and 22%, respectively, compared with only 9% and 13% in the case of 'Meski'. A thicker palisade parenchyma could contain larger numbers of CO_2-fixation sites, while a thicker spongy parenchyma could result in easier diffusion of CO_2 to these sites. Furthermore, stomatal density (SD) in 'Chemlali' leaves increased by 25% (*vs.* 7% for 'Meski' leaves) during drought treatment, which could also enhance the external supply of CO_2. Other morpho-structural traits implicated in the control of water loss were enhanced more in 'Chemlali' than in 'Meski' leaves. Under conditions of lower water availability, leaf size decreased by 24% in 'Chemlali' (*vs.* 15% in 'Meski'), trichome density (TD) increased by 25% (while remaining unchanged in 'Meski'), and the thickness of the upper and lower epidermis increased by 32% and 25%, respectively (while remaining unchanged in 'Meski'). The above morpho-anatomical adaptations should improve the water-use efficiency of the tree. These differential changes in leaf morphology and anatomy can explain, at least in part, the difference in drought resistance between the two cultivars. In particular, the upper palisade parenchyma, the spongy parenchyma, SD, and TD could be considered key structural features of leaves that govern the ability of a tree to withstand water stress. They could therefore be used as criteria to select olive cultivars that are more resistant to drought.

Recent and predicted changes in climate may disrupt the temporal and spatial distribution of rainfall. This will probably increase water shortages in many parts of the World. Drought, in conjunction with high temperatures and solar radiation, poses the most important environmental constraint on plant survival and crop productivity (Boyer, 1982). With increasing aridity and a growing global population, modern agriculture is faced with a major challenge to meet increasing food demand with reducing water resources. This appears to be even more difficult when we know that modern agriculture is essentially based on intensive production systems which require large quantities of irrigation water. Improving water-use efficiency (WUE) can help to meet the higher demand for water with a lower supply. Several approaches can be adopted to achieve improved WUE, including cultivar selection, which is important during the installation of a new plantation.

A better understanding of the effects of drought on plants is vital to improve crop management practices, to guide breeding efforts in agriculture, and to predict the fate of natural vegetation under periods of climate change (Chaves *et al.*, 2003). When faced with drought, plants adopt one of two strategies: drought avoidance, or drought tolerance. The avoidance of dehydration is associated with a variety of adaptive traits to minimise water loss and maximise water uptake. Both can be brought about by a combination of morphological, anatomical, and physiological adaptations. In contrast, drought tolerance refers to the ability of a plant to maintain its normal functions during periods of low tissue water potential. It involves specific metabolic and biochemical reactions at the sub-cellular level. Olive trees are able to delay the onset of water stress (i.e., adopt drought avoidance) both by controlling transpiration and by increasing water uptake through having a large and deep root volume. Furthermore, olive trees are able to tolerate dehydration and thus sustain a large internal water deficit, yet maintain sufficient metabolic activity for survival (Connor, 2005; Ennajeh *et al.*, 2008; 2009). For this reason, olive trees display a wide range of anatomical, physiological, biochemical, and biophysical adaptations.

Most Mediterranean regions, where olive trees are commonly grown, are prone to periods of drought of varying intensity from year-to-year. Indeed, the yields of mature olive orchards are often affected by water deficit

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(Moriana and Orgaz, 2003). Under such conditions, choosing drought-resistant cultivars is of paramount importance for the commercial success of a plantation. The slow-growing nature of olive trees, and the long duration of the juvenile phase, make field trials time-consuming and costly. Therefore, it is useful to take advantage of those morphological and physiological traits relevant to drought resistance in order to facilitate the breeding and selection process. Olive is renowned for its drought tolerance (Spiegel, 1955), however intra-specific differences in its responses to water deficit exist (Bacelar *et al*., 2006; Bosabalidis and Kofidis, 2002; Ennajeh *et al*., 2009).

Two olive cultivars planted widely in Tunisia were used in the present study. Our choice was based more on their physiological properties than on their economic importance. Indeed, these two olive cultivars differ greatly in their ability to withstand water stress. The cultivar 'Chemlali' is known for its drought tolerance, whereas 'Meski' is drought-sensitive (Ennajeh *et al*., 2006; 2008; 2009).

The leaf is the most adaptable organ in its response to environmental conditions (Marchi *et al*., 2008; Nevo *et al*., 2000). Leaf structures reflect the effects of water stress more clearly than those of stems and/or roots. In this study, morphometric data and the quantitative anatomy of water-stressed leaves of these two olive cultivars are reported. The effects of drought on several major morphological and anatomical leaf characteristics were investigated in order to reveal the origin of the variability in drought resistance between these two olive cultivars.

MATERIALS AND METHODS

Plant material and treatments

Two-year-old self-rooted cuttings of the olive (*Olea europaea* L.) cultivars 'Chemlali' and 'Meski' were transplanted into 10 l pots (one plant per pot) filled with a 4:1 (v/v) mixture of sandy soil (85% coarse-to-fine-grained sand, 15% silt) and vermiculite. Plants ($n = 20$) of each of the two cultivars had comparable leaf areas and stem heights (120 – 150 cm). All pots were covered with a water-impermeable plastic film and aluminium foil to reduce evaporation from the soil surface and to minimise solar heating of the substrate.

The experiment was performed outdoors at the Faculty of Sciences of Gabès (southern Tunisia; 33°50' N, 10°5' E). The plants were watered to field capacity once a week, alternately with tap water or a complete nutrient solution (N 1.8 mM, P 0.35 mM, K 0.64 mM, Ca 1.0 mM, Mg 0.35 mM, S 0.35 mM, Fe 0.03 mM, Zn 0.4 µM, Mn 5 µM, Cu 0.1 µM, and B 0.023 mM). After 1 month of growth under these conditions, and when all plants showed good shoot growth, they were divided into two groups of ten of each cultivar.

Two different watering regimes were applied to each cultivar over the following 3 months (May – July 2007). In Tunisia, this period is hot and dry. For each cultivar, one group of ten plants was used as controls (well-watered; WW) in which plants were watered every 2 – 3 d to maintain their soil water content near to field capacity (Ψ_{soil} = –0.01 MPa). The other group of ten plants was under-watered (UW; i.e., irrigated once every 15 d) to subject it to drought stress during which the Ψ_{soil} was allowed to reach –1.5 MPa. Soil water potential was measured using a thermocouple psychrometer (PST-55-15-SF; Wescor, UT, USA) connected to an HR-33T probe (Wescor) placed at a depth of 20-cm .

Fully-expanded mature leaves formed during each treatment were sampled for measurements at the end of the experiment. Leaf anatomy and sclerophylly were studied to determined to elucidate the long-term effects caused by 3 months of water deficit.

Stomatal and trichome densities

To determine stomatal (SD) and trichome densities (TD) on the abaxial leaf-surface, a thin layer of nail polish was applied to the abaxial epidermis after peltate trichomes had first been removed using adhesive tape. Peltate trichomes were eliminated because they prevent accurate stomatal counts. Once dry, the nail polish layer was carefully peeled-off with adhesive tape, then fixed on a microscope slide and examined under a light microscope (MCX300; Micros, Vienna, Austria) equipped with a camera (CAM2800-XP 3.0; Micros) interfaced to a computer. The numbers of stomata and trichome scars per unit leaf area were then determined. Ten leaves per treatment were used for each cultivar, and three separate counts were carried out on each leaf.

Leaf anatomy study

Small pieces (1 cm^2) were taken from the centre of ten leaves of both WW- and UW-treated olive plants of each cultivar and fixed in FAA solution [50% (v/v) ethanol, 5% (v/v) acetic acid, 10% (v/v) formaldehyde] for ≥ 6 h at 4°C. The samples were then dehydrated in an ascending series of ethanol solutions followed by infiltration with paraffin (CP-EMB44026; Melun, France) in which they were finally embedded. Semi-thin (1 – 2 µm-thick) sections were cut with an ultramicrotome (RM2135; Leica, Nussloch, Germany). All sections were stained with 1% (v/v) toluidine blue O, and observed under a light microscope. Windias software (Version 1.4; Voith, Crailsheim, Germany) was used for morphometrical analysis of the sections.

Leaf morphology and sclerophylly

A further ten mature leaves per treatment were collected from each cultivar. The following parameters were examined: leaf area (LA), measured using an AM300 Area Meter (ADC; BioScientific Ltd., Hoddesdon, UK), fresh mass (FM), turgid mass (TM), and dry mass (DM) per leaf. The olive leaves were weighed immediately after harvest to determine their FM. Turgid mass was determined after the leaves had been immersed in distilled water for 12 h. After the determination of TM, leaves were oven-dried at 80°C for 48 h and their DM was then determined.

Several indices of sclerophylly and leaf water status were calculated. These included: specific leaf area (SLA = LA/DM; in m^2 kg^{-1} DM), density of foliar tissue [D = (DM/FM) × 1,000; in g kg^{-1}], succulence [S = (FM – DM)/LA; in mg H_2O cm^{-2}], and water content at saturation [WCS = (TM – FM)/DM; in g H_2O g^{-1} DM].

Statistical analysis

Data variance was analysed using ANOVA procedure in the SAS statistical software package Version 6.12

TABLE I
Mean values of leaf tissue thicknesses (µm) and the densities of stomata and trichomes (no. mm^{-2}) of two olive cultivars grown under two water availability regimes (WW: well-watered and UW: under-watered)

	Thickness (µm)							Densities (no. mm^{-2})	
Parameter	Total lamina	Upper epidermis	Upper palisade	Spongy parenchyma	Lower palisade	Lower epidermis	Trichome layer	Stomata	Trichomes
Cultivar									
'Chemlali'	476.0 b[†]	20.2	205.6 a	191.4 b	36.5 b	18.1 a	35.0 b	501 b	182 a
'Meski'	496.4 a	23.5	185.0 b	229.7 a	41.0 a	16.4 b	41.2 a	561 a	151 b
Watering regime									
WW	457.0 b	21.7	185.9 b	196.9 b	35.3 b	16.4 b	34.6 b	514	156 b
UW	523.1 a	21.8	209.3 a	225.8 a	43.0 a	18.5 a	43.7 a	548	177 a
Cultivar × watering regime									
'Chemlali'									
WW	439.5 b	18.0 b	192.4 b	196.3 b	33.0	16.5 b	30.6	445 b	160 b
UW	534.9 a	23.8 a	226.9 a	215.7 a	42.2	20.7 a	40.5	558 a	203 a
'Meski'									
WW	481.1 b	26.8	176.9 b	224.9	38.5	16.3	37.9	538 b	152
UW	512.5 a	20.1	193.5 a	234.8	43.7	16.4	47.9	584 a	150
Two-way ANOVA (*P*-values)[‡]									
Cultivar (C)	0.039	0.352	< 0.001	< 0.001	0.003	< 0.001	0.046	< 0.001	< 0.001
Watering regime (W)	< 0.001	0.876	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.065	0.010
C × W	< 0.001	0.020	0.004	< 0.001	0.052	< 0.001	0.976	< 0.001	0.004

[†] Mean values (n = 10) flanked by different lower-case letters within a column indicate statistically significant differences at $P < 0.05$ (LSD test).
[‡] The level of significance (two-way ANOVA; *P*-values) for differences between cultivars (C), watering regimes (W), and C × W are reported.

(SAS Institute, Cary, NC, USA). The experiment was arranged as a complete randomised design with ten single replicate trees per treatment for each cultivar. The appropriate means were separated by the LSD test ($P < 0.05$).

RESULTS

Leaf tissue structure and stomatal and trichome densities

A cross-section of an olive leaf shows that it has an asymmetric, heterogeneous structure (Figure 1). Two unequal palisade parenchyma exist. The first, in contact with the upper epidermis, is composed of three compacted layers of elongated cells; while the second, in contact with the lower epidermis, is composed of one layer of relatively elongated cells. In olive leaves, the stomata are protected by umbrella-shaped peltate trichomes that create a thin layer to limit water loss by transpiration.

Leaf anatomical characteristics varied both by cultivar and by watering regime (Table I). Indeed, we observed differences for most of the anatomical parameters studied (e.g., leaf tissue thickness, SD, and TD). The upper palisade and lower epidermis of 'Chemlali' were 11% thicker than those in 'Meski' leaves. Similarly, 'Chemlali' had a higher density of trichomes compared to 'Meski'. In contrast, 'Meski's spongy parenchyma and lower epidermis were 20% and 12% thicker, respectively, than those of 'Chemlali'. 'Meski' leaves also had 12% more stomata per unit leaf area. Furthermore, the low watering (UW) regime increased the thickness of all leaf tissues with the exception of the upper epidermis, and increased TD.

This study shows that morpho-anatomical changes in olive leaves under water deficit conditions are cultivar-dependent. In fact, when well-watered, 'Chemlali' had a thinner leaf lamina than that of 'Meski'. Water deficit increased the total thickness of the leaf lamina in both cultivars, but more so for 'Chemlali'. In fact, all leaf tissues in 'Chemlali' (except the lower palisade and trichome layers) became considerably thicker under water stress. In 'Meski', only some tissues became thicker. The thickness of the upper palisade and spongy parenchyma layers increased by 17% and 22%, respectively, for 'Chemlali', but by only 9% and 13% for 'Meski'.

The thickness of the upper and lower epidermis increased under drought conditions in 'Chemlali' leaves, but not in 'Meski'. The thickness of the trichome layer was increased by water deficit. Stomatal and trichome distributions depended on cultivar and watering regime.

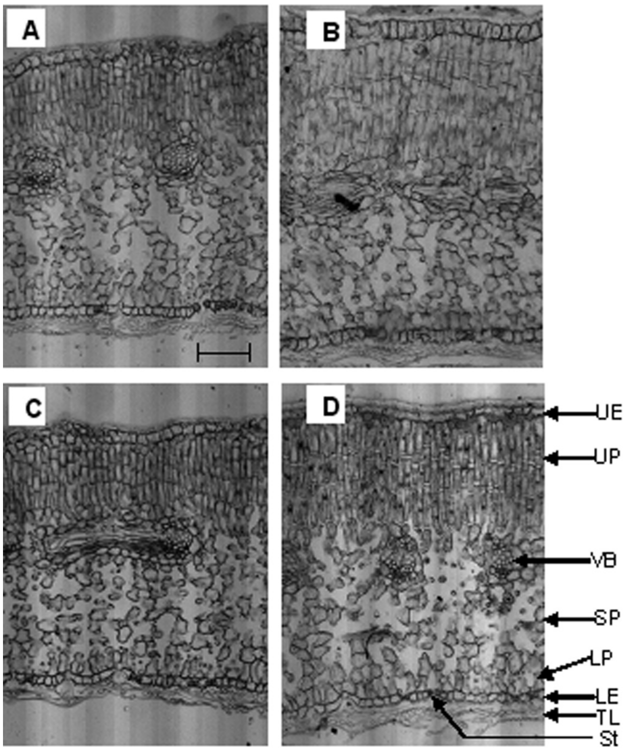

FIG. 1
Cross-sections of the leaves of olive cultivars 'Chemlali' (Panel A: well-watered, Panel B: under-watered) and 'Meski' (Panel C: well-watered, Panel D: under-watered). LE = lower epidermis, LP = lower palisade, SP = spongy parenchyma, St = stomata, TL = trichome layer, UE = upper epidermis, UP = upper palisade, VB = Vascular bundle. Scale bar = 100 µm.

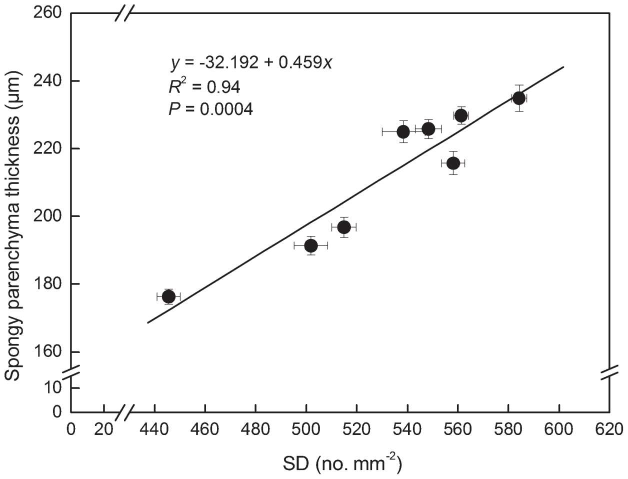

FIG. 2
Relationship between stomatal density (SD) and thickness of the spongy parenchyma in olive trees (*Olea europaea*). The regression equation, squared correlation coefficient (R^2) and significance level (P) are also reported.

Under WW conditions, 'Meski' had a higher SD but a lower TD than 'Chemlali'. Under water deficit (UW) conditions, the SD increased by 25% in 'Chemlali', but only by 7% in 'Meski'. Similarly, the TD increased by 25% in 'Chemlali', but not in 'Meski'. Furthermore, there was a strong positive correlation (r^2 = 0.94; P < 0.01) between SD and the thickness of the spongy parenchyma (Figure 2).

Leaf morphology and sclerophylly

Morpho-structural parameters were also examined in WW and UW trees of both olive cultivars (Table II). Intra-specific differences were revealed for most parameters. 'Chemlali' trees had lower LA and D values, and higher SLA and WCS values compared to 'Meski'.

Water availability also had an effect on olive leaf morphology. Water stress decreased LA, SLA, D, and WCS, but increased S. These morpho-structural changes were cultivar-dependent. Under lower water availability (UW), the average LA decreased by 24% for 'Chemlali', but by only 15% for 'Meski' compared to the WW regime. Leaf succulence (S) remained unchanged for both cultivars. Water content at saturation (WCS) was not affected by water availability in 'Chemlali', but decreased sharply in 'Meski'.

DISCUSSION

Olive trees exhibit an intra-specific variability in their ability to withstand water stress (Bacelar *et al.*, 2007; Chartzoulakis *et al.*, 1999; Sofo *et al.*, 2007). Previously, we reported that 'Chemlali' was more drought-resistant than 'Meski' (Ennajeh *et al.*, 2008; 2009) and was able to maintain higher photosynthetic assimilation rates (A) under drought conditions. This variability can be attributed to differences in the efficacy of the defense mechanisms used by these cultivars. Similar adaptations were reported in other olive cultivars such as 'Koroneiki' (Chartzoulakis *et al.*, 1999), 'Cobrançosa', and 'Madural' (Bacelar *et al.*, 2006) which have a high A, despite low stomatal conductance values caused by drought. Under drought conditions, olive trees develop a wide range of morphological, anatomical, and physiological adaptations in their leaves, the main organs of internal water loss.

Photosynthetic assimilation rates are regulated by the intrinsic photosynthetic capacity of the mesophyll and by the conductance of CO_2 from ambient air to the sites of carboxylation in the chloroplasts (Syvertsen *et al.*, 1995). However, olive leaves have a uniform stomatal distribution (i.e., they are homobaric) and the resistance to gas circulation should depend mainly on the packing of the mesophyll cells (Marchi *et al.*, 2008). In our study, water stress resulted in significant but different changes in leaf tissue anatomy, and in stomatal and trichome densities in the two cultivars. Total lamina thickness increased in both cultivars, but more so for the drought-resistant cultivar, 'Chemlali'. Similarly, drought caused an increase in the thickness of the upper palisade parenchyma which was more important in 'Chemlali'. This should increase the number of CO_2 assimilation sites per unit leaf area, helping to maintain high A values despite the low stomatal conductance values caused by drought. Furthermore, the present work suggests that the

TABLE II
Leaf area (LA), specific leaf area (SLA), density of leaf tissue (D), succulence (S), and water content at saturation (WCS) of two olive cultivars grown under two water availability regimes (WW: well-watered and UW: under-watered)

Parameter	LA (mm^2)	SLA (m^2 kg^{-1} DM)	D (g kg^{-1})	S (mg H_2O cm^{-2})	WCS (g H_2O g^{-1} DM)
Cultivar					
'Chemlali'	471.1 b[†]	3.50 a	511.3 b	27.35	0.13 a
'Meski'	690.3 a	3.34 b	532.8 a	26.71	0.07 b
Watering regime					
WW	641.6 a	3.55 a	539.0 a	24.11 b	0.13 a
UW	519.9 b	3.28 b	503.6 b	29.95 a	0.09 b
Cultivar × watering regime					
'Chemlali'					
WW	537.2 a	3.59	535.0 a	24.23 b	0.14
UW	405.0 b	3.41	485.5 b	30.45 a	0.12
'Meski'					
WW	746.0 a	3.51	543.0 a	23.99	0.10 a
UW	634.7 b	3.17	521.7 b	29.44	0.06 b
Two-way ANOVA (P-values)[‡]					
Cultivar (C)	< 0.001	0.021	< 0.001	0.739	< 0.001
Watering regime (W)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
C × W	0.028	0.238	< 0.001	0.448	0.010

[†] Mean values (n = 10) flanked by different lower-case letters within a column indicate statistically significant differences at P < 0.05 (LSD test).
[‡] The level of significance (two-way ANOVA; P-values) for differences between cultivars (C), watering regimes (W), and C × W are reported.

increase in the thickness of the spongy parenchyma could improve the diffusion of CO_2 through the inter-cellular spaces from the sub-stomatal cavity to the outer surface of the mesophyll cells. Drought increased the thickness of the spongy parenchyma by 22% in 'Chemlali', but by only 4% in 'Meski'. 'Madural', a drought-resistant olive cultivar, showed similar increases under conditions of low water availability (Bacelar *et al.*, 2006).

There was a strong positive correlation between SD and the thickness of spongy parenchyma in 'Chemlali' under drought conditions. A high SD can improve the external supply of CO_2 and a higher fraction of inter-cellular space (attributed to a thicker spongy parenchyma) could improve internal CO_2 diffusion in 'Chemlali' leaves. The relative importance that bio-mechanical and bio-chemical leaf traits could have on photosynthetic capacity would depend on a complex interaction of internal leaf architecture and physiological differences (Marchi *et al.*, 2008). The higher photosynthetic assimilation rate in 'Chemlali' under water stress conditions (Ennajeh *et al.*, 2008) can be attributed to: (i) an increase in the palisade parenchyma fraction, which can increase the number of CO_2 fixation sites; (ii) the improvement of internal CO_2 diffusion due to abundant intercellular spaces attributed to the increase in the thickness of the spongy parenchyma; and (iii) an improvement in the external supply of CO_2 due to the increase in SD on the lower epidermis.

It is generally accepted that a higher SD allows better control of transpiration (Bolhar, 1987; Bosabalidis and Kofidis, 2002). Water loss through transpiration in 'Chemlali' should be further minimised by the high density of trichomes around the stomata and the thicker upper and lower epidermis providing more protection against desiccation for the inner leaf tissues (Bacelar *et al.*, 2004; Baldini *et al.*, 1997).

Water deficit decreased the individual LA in both cultivars, but more so in 'Chemlali'. With its smaller leaves, 'Chemlali' appeared better adapted to drought. Generally, those leaves that develop under drought conditions have lower SLA values than those that develop under optimum watering conditions (Bacelar *et al.*, 2006). A lower SLA is usually a consequence of an increase in the density or thickness of foliar tissue and normally occurs when the costs of the assimilatory apparatus are increased (Centritto, 2002), such as during long periods of drought. In our study, SLA values remained unchanged by 3 months of water deficit in both cultivars. Contrary to a previous report by Bacelar *et al.* (2006), we found that water stress reduced leaf tissue density in both cultivars, but especially in 'Chemlali', possibly because of the significant fraction of the leaf volume occupied by intercellular spaces.

In 'Chemlali', water deficit did not significantly affect WCS. This indicates that the leaves of water-stressed plants needed only a small amount of water to reach water saturation and have a greater capacity to withstand arid environments (Abd-El-Rahman, 1966). In contrast, leaves of 'Meski' showed an acute decrease in WCS (40%) under water deficit conditions.

Drought-induced morpho-anatomical changes in 'Chemlali' leaves appeared to improve *A* and to reduce water loss. These adaptations should increase the WUE of this cultivar, helping to maintain vegetative growth and productivity under drought conditions.

In conclusion, leaf morpho-anatomical adaptations to water deficit could explain the difference in drought resistance between the two olive cultivars. In particular, the thicknesses of the palisade parenchyma and spongy parenchyma, and stomatal and trichome densities could be considered key structural adaptations responsible, in part, for the observed intra-specific variability in the response of olive trees to drought. These leaf morpho-anatomical features could therefore be used as selection criteria in screening tests for drought-resistant olive cultivars. However, more studies with more cultivars are needed to confirm these results. Such studies should also encompass the root system and the xylem, in order to obtain a more complete picture of the drought resistance strategies of this species.

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