

## Foliar behavior of olive trees (*Olea europaea* L.) grafted and cut under the effect of salt stress

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Olive (*Olea europaea* L.) is an important perennial crop in many agricultural regions of the Mediterranean countries but it is planted, often in salty soils. In this context, the objective of this work is to determine the effect of salinity on the morpho-physiological behavior of young olive plants in cuttings and grafted plants, including two local varieties (Chemlal and Sigoise) and two introduced varieties (Manzanilla and Arbiquina). The experiment was conducted in a laboratory and greenhouse with a well-controlled condition. The experiment was arranged in a completely randomized design of two factors (salinity and varieties) with four replications and two treatments. Not treated plants (without NaCl application) were utilized as control. The plant material (young 2-year-old olive seedlings) was selected and brought from a crop nursery. The experimentation started by the irrigation of the control plant with a nutrient solution. About the salinity treatment, the tree seedlings received a nutrient solution fortified by the addition of 100mM of NaCl. Four repetitions are being done according to the field capacity. The study covered the variation of the relative water content in leaves as well as the leaf surface, stomata density, stomatal size and the wax rate on the upper foliar epidermis. According to the achieved results, the RWC leaf area and stomatal density of the treated plants decreased compared to the control. On the other hand, the wax level increases in the case of salt stress compared to the control, both for the grafted plants and for the plants not grafted.

**Key words:** salt stress; olive-tree; RWC; stomatal density; wax rate; varieties

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### Introduction

The olive tree constitutes an economic and food source for the autochthonous inhabitants. The development of olive growing in Algeria was the subject of an extensive program to plant one million hectares of land in olive groves. Thus, new decisions are taken to improve the management of olive growing by extending it to land where intensification of production is possible. Olive (*Olea europaea* L.) is an important perennial crop in many agricultural regions of the Mediterranean countries. By adaptation to arid conditions, it has a positive impact on the environment and the economy of the region (Besnard et al., 2002). It contributes to reducing the soil erosion and the loss of the fertile soil (Álvarez et al., 2007). Otherwise, the recorded salinization of the arid and semi-arid ecosystems is the result of a high evaporation of water from the ground and insufficient and irregular rainfall (Mezni et al., 2002; Munns et al., 2006). This salinization stems also from an inadequately controlled irrigation (Bennaceur et al., 2001). Salinity and drought additionally have been shown to affect plant growth, protein accumulation (Zhu, 2001). Therefore, osmotic potential provides a better measure of the effect of salt on plant growth (Ben-Gal et al., 2009) and microbial activity (Chowdhury et al., 2011; Setia and Marschner, 2012) than the EC of a soil extract. The accumulation of salts in the root zone has adverse effects on plant growth (Belkhodja et Bidai, 2004). It is due to the low osmotic potential of the soil solution resulting in decreased availability of water to plants, but also due to the ion imbalance and ion toxicity (Pages et al., 2000; Lindsay et al., 2004; Bartels et Sunkar, 2005). Therefore, it must to understand the mechanisms developed by plants to adapt to changing environmental conditions to maintain their growth and productivity (Szabolcs, 1994; Trinchant et al., 2004). Indeed, according to the salinity in the media, glycophytes are exposed to changes in their morpho-physiological (Bennaceur et al., 2001) and

biochemical behavior (Grennan, 2006). So, the plants must modify their growth and development to suit the prevailing environmental conditions.

Indeed, given the salt constraint, activity and morphology of the leaves play a role in plant resistance to stress. According to Garg et al. (2002), Moinuddin et al. (2005) and Hassani et al. (2008), the plant expressed itself by maintaining turgor by reducing transpiration. Therefore, this experiment was conducted to examine the influence of NaCl or the effect of salinity on morpho-physiology of the leaf for four varieties of olive trees derived from cuttings or grafted including two local varieties (Chemlal and Sigoise) and two introduced varieties (Manzanilla and Arbequina). The analysis focused on changes in the relative water content of leaves, area, density of stomata, rate of wax and the internal structure. The induction of salt stress is achieved by adding a NaCl to the nutrient solution

## Materials and methods

### The Experimental Device

The plantlets used in the experiment were 18-month-old obtained by cutting rooted under nebulization (crop nursery). The olive plants were placed into a vinyl polychloride (VPC) cylinders (sixty centimeters long and twenty centimeters diameter) filled with a mixture of soil made by sand, soil<sub>6</sub> and an organic matter at proportions of four volumes from sand / one volume from soil / one volume from manure or an organic matter. The experiment was conducted in a greenhouse in the Ibn Khaldoun University of Tiaret (Algeria) with a daytime temperature of 18°C and nocturnal of 10°C, the relative humidity of the air was 70% and the photoperiod of 10-12 h.

The three factors experiment was laid out in Randomized Complete Block Design (RCBD) with four replications (four blocks) were set up to study the different parameters that demonstrate the physiological effects of salt water on the olive plants tested. The experiment is carried out on four varieties, two of which are to produce table olives (Sigoise and Manzanilla) and two for olive oil production (Chemlal and Arbequina). The cuttings of the local varieties (Sigoise and Chemlal) come from Algiers and the introduced plants (Manzanilla and Arbequina) come from Spain.

The induction of the salt stress of the four varieties was achieved by adding NaCl to the nutrient solution. Each plantlet received a standardized and a balanced nutrient solution as Hoagland and Arnon (1938). Treated plants were watered with the nutrient solution and twice a week with 300ml of 100 mM NaCl solution. Control plants received only the nutrient solution. Analyzes and measurements were done after twelve (12) weeks of saline stress

**The relative water content (RWC).** It was determined by method of Barrs and Weatherley, (1962) according by the formula of Clarck and McCaig (1982) and used by Rascio et al. (1988).

After excising the leaf, the initial fresh weight (ifw) was determined. Then, the leaf introduced into a test tube containing of distilled water. The unit is placed at the darkness at 4 °C for 12 hours. The leaves were weighed again (weight in full turgidity, wft). The dry weight (dw) is obtained by drying in the oven during 48 hours at 80 °C. The relative content water of the leaves is estimated by the equation:

$$\text{RWC (\%)} = [ \text{ifw} - \text{dw} / \text{wft} - \text{dw} ] \times 100 \%$$

**The leaf area.** The leaf area is directly measured by electronic planimeter LICOR-3000A (Cheverry, 1995) with a resolution of 1 mm<sup>2</sup>.

**The Wax Rate.** The excised sheet at its base is introduced into a washed and weighed test tube (P1) to which chloroform is added to extract the wax. The test tube with the wax and the chloroform is dried in an oven for 24 h at 45 °C and then weighed (P2). The wax ratio is determined according to the formula:

$$\text{Wax Rate in mg.cm}^{-2} = \text{P2-P1} / \text{SF}$$

**Density and size of the stomata.** The stomata density, according to the method of Dohman *et al.* (1991) adopted by Denden and Lemeur (1999). The stomata density is evaluated on the lower and the upper surface in the central portion of the penultimate leaf that was dusted off and, after removing the epidermal hairs by applying an adhesive tape, thin layer of a clear nail varnish was applied. After two minutes, the varnish layer is removed with the stomata print by using another diaphanous adhesive tape, then spreading it on the microscope slide which has been beforehand washed and dried, and we observed it using an optical microscope to determine the number and the size of the stomata (length and width). The measurements are carried out using a ZEISS type microscope (with microscope and OPTIKA photo tube).

**The structure of the leaf.** Operated on limiting the water loss were assessed by performing an anatomic cut of the leaves and the histological study was carried out by the method of (Martoja et Martoja, 1968). Fragments with a length of two centimeters taken from young leaves were placed in a fixative based on alcohol, and formalin, and acetic acid for twenty-four hours. Then the samples were washed and dehydrated with increasing doses of the ethanol (70° and 100°). We went ahead with an impregnation of the samples: toluene then paraffin-toluene (V/V) during four hours at 60°C before including them in a pure and warm paraffin. The use of the microtome (type LEICA RM 2145) allowed to obtain lices of 12 µm thicknesses. They placed on to a slide in gelatin water then colored, fixed and using a microscope with an ocular micrometer ZEISS type.

**Statistical Analysis.** The obtained data were processed with *Statistica* 12.0, by analyzing the variance and the correlation matrix. The analysis of variance and the means were compared to the Tukey test at 5% probability level.

## Results and discussion

### The relative water content.

**Table 1.** Analysis of the variance of the Relative Water Content (RWC) and the leaf area of stressed and unstressed olive trees.

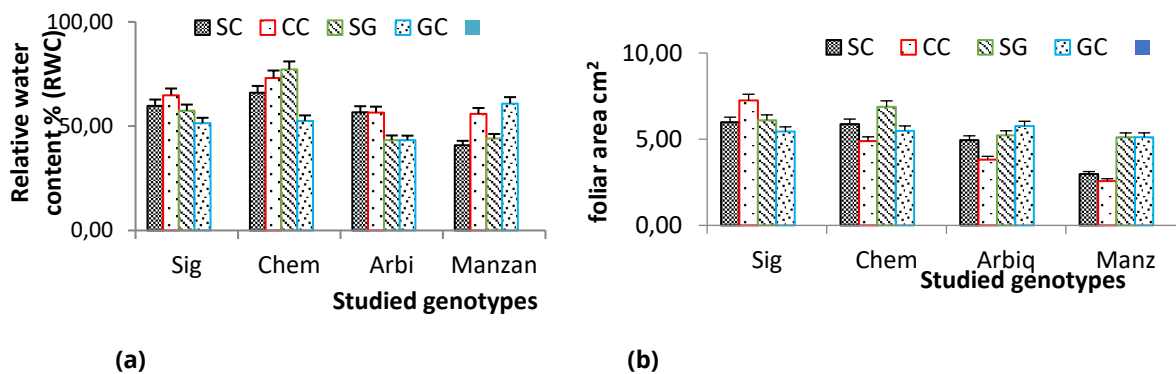
Variable	Variety effect (F1)	Plant effect (F2)	Stress effect (F3)	F1*F2	F2*F3	F1*F3	F1*F2*F3
RWC	<b>0.026</b>	0.221	0.630	0.573	0.855	0.763	0.098
Leaf area	<b>0.007</b>	0.074	0.462	0.132	0.726	0.942	0.588

Our statistical results are significant for the varietal effect on the relative water content and nonsignificant for the effect of the plant type (the bit error rate threshold of 5%). Indeed, it shows that after six weeks of treatment with 100 mM.l<sup>-1</sup> of NaCl, most of the genotypes studied did not show a significant decrease in the relative water content. The reduction of the RWC of all the tested plants fluctuates between 1% and 2% compared to the control (Fig. 1a).

Thus, the varieties of olive trees studied (local and introduced) have retained a high RWC in the presence of salt stress, indicating that the olive tree is of the "stay green" type. The olive tree retains green leaves and photosynthetically active allowing having reasonable yields even in the presence of abiotic stresses. However, a high content can be found in local varieties of olive trees compared to introduced varieties. For the local plants treated with 100 mM NaCl and in absence of salinity, the sheet exhibited a RWC of 70% and 60% respectively for Chemlal and Sigoise, and 50% on average for the introduced varieties.

### The leaf area

The same effect (Table 1) is observed when analyzing the variance of the leaf area. statistical calculations appear to be significant for the varietal effect on the leaf area and not significant for the effect of the plant type under the saline treatment effect and the various interactions at the 5% error threshold. Saline stress affects the leaf area slightly (p = 7.4%). Indeed, the foliar area recorded in the control plants is 49.34 cm<sup>2</sup> and then it drops to 27.19 cm<sup>2</sup> for the leaves of the plants treated with 100 mM.l<sup>-1</sup> NaCl, (Fig. 1b). Except for the effect of the plant type, the impact of salinity is very highly significant on the stomatal density (p=0%), as well as the varietal effect and the various interactions, particularly at leaf level of the plants in 100 mM.l<sup>-1</sup>NaCl, (Table 2).



**Figure 1.** Relative water content (RWC) (a) and the leaf area (cm<sup>2</sup>) (b) as measured from the penultimate leaf of the studied plantlets (SC: Stressed Cut, CC: Cutting Control, SG: Stressed Graft, GC: Grafted Control).

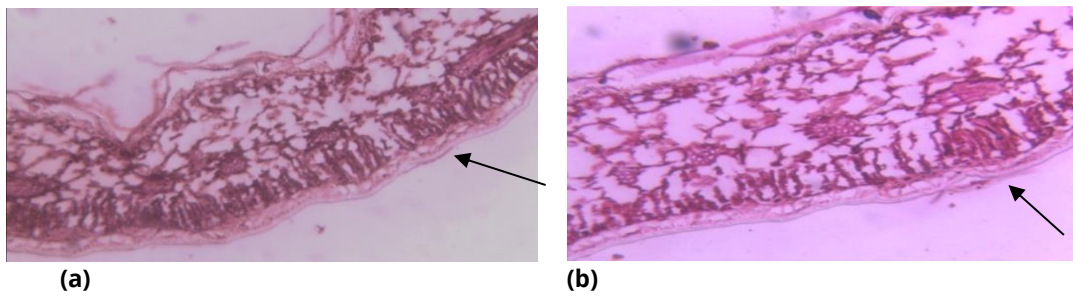
### The wax rate.

**Table 2.** Analysis of the variance of the wax rate of the stressed and unstressed olives genotypes.

Variable	Variety effect (F1)	Plant effect (F2)	Stress effect (F3)	F1*F2	F2*F3	F1*F3	F1*F2*F3
The wax rate	0.012**	0.000***	0.000***	0.000	0.000	0.001	0.004**

\*\*\*p<0.001, \*\*p<0.01, \*p<0.05; insignificant: p>0.05

The analysis of wax variance shows a very highly significant influence for all factors tested at p<0.05, the varietal effect on stomatal density, "plant type" effect, Saline treatment effect and plant genotype-type interactions, genotype-stress, genotype-treatment and genotype-type plant-treatment (Table 2). The histograms displayed in Fig. 3a show that all the genotypes studied (local and introduced) developed a cuticle by the deposition of wax.



**Figure 2.** The foliar cuticle in olive tree "Sigoise" BS: Stressed Cut (a) and Stressed Graft (b) (photo by Gharabi, Hassani, 2015)

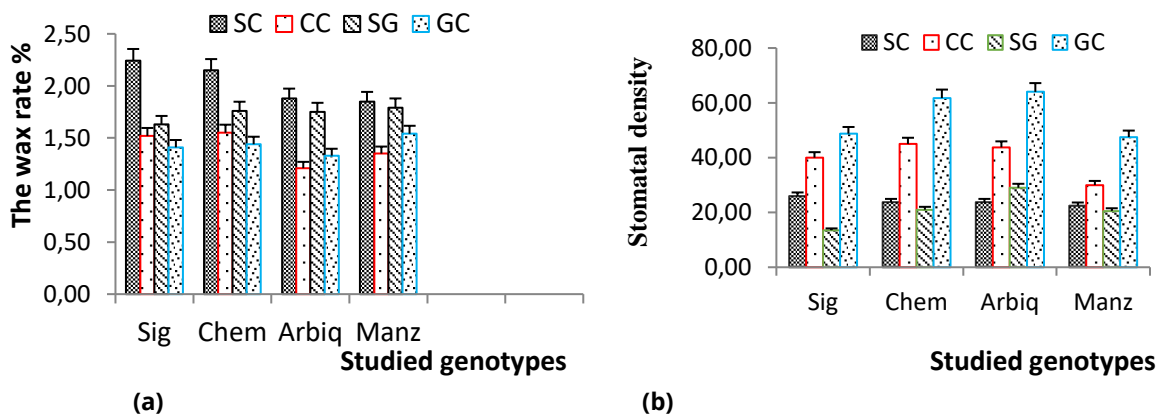
In addition, Hopkins, (2003), have established the relationship between transpiration and stomatal resistance in *Arabidopsis thaliana*, by increasing this resistance during salt stress to minimize water loss and sweating, becomes more important in case of thin cuticle leaves. According to our results, the stressed genotypes have cuticles thicker than those under normal conditions; this can explain the resistance of our plants by keeping their leaves throughout the duration of the stress applied (Fig. 2).

### The stomata density

**Table 3.** Analysis of the variance of the stomatal density ( $\text{cm}^2$ ), the length and width of the stomata ( $\mu\text{m}$ ) of stressed and unstressed olive trees

Variable	Variety effect (F1)	Plant effect (F2)	Stress effect (F3)	F1*F2	F2*F3	F1*F3	F1*F2*F3
Stomatal Density	0.000	0.378	0.000	0.000	0.003	0.041	0.036
length of stomata	0.000	0.481	0.001	0.000	0.000	0.020	0.037
width of stomata	0.000	0.239	0.000	0.007	0.024	0.000	0.000

According to the obtained analysis (Table 2) the reaction of the plants followed the same tendency about the expression of this parameter. The Figure 3b shows a significant variation in the number of stomata. In fact, Sigoise cuttings have 40 stomata (control) versus 26 (stressed) stomata. The Chemlal presents 45 against 24, the Arbiquina 44 against 24, and the Manzanilla – 30 against 23 stomates/ $\text{cm}^2$ . For the genotypes grafted on oleaster the fall is more important. According to Fig. 5, the values were 49 (control) versus 14 for the Sigoise, 45 against 24 for Chemlal, 62 against 21 for Arbiquina, and 48 against 21 stomata for the variety Manzanilla



**Figure 3.** The wax rate of the upper surface of the leaves(a) and stomatal density ( $/\text{mm}^2$ ) of lower surface (b). (SC: Stressed Cut, CC: Cutting Control, SG: Stressed Graft, GC: Grafted Control)

Otherwise, the impact of salinity on the length of stomata, particularly on the leaves of plants treated with  $100 \text{ mM} \cdot \text{l}^{-1}$  NaCl except for the plant type, is highly significant ( $p = 0\%$ ). It is the same for the varietal effect and the different interactions. The width of the stomata had the same fate (Table 3). Indeed, the values of these parameters, shows that the length and width of stomata in the leaves of plants treated with  $100 \text{ mM} \cdot \text{l}^{-1}$  NaCl react differently.

The two local varieties (Sigoise and Chemlal) in cuttings and grafted mark an increase in the length of the stomata except for the grafted Sigoise which records an average decrease of  $4 \mu\text{m}$ . For the local varieties, the length of the stomata varies between  $0.4$  and  $4.5 \mu\text{m}$ . On the other hand, the width of the stomata of all the genotypes in their two forms (cuttings and grafted) shows a remarkable drop which oscillates between  $1.33$  and  $6.51 \mu\text{m}$ , apart from the grafted Manzanilla variety which marks an increase of the width of the stomata  $0.75 \mu\text{m}$  relative to the control.

**Table 4.** Correlation effect between salinity and the leaf physio-morphological parameters

Variable	Variété	plant	Salinity	RWC	Leaf area	Number stomata	Wax rate
Variety	1.000						
Type de plant	0.000	1.000					
Salinity	0.000	0.000	1.000				
RWC	-0.249*	0.147	0.058	1.000			
Leaf area	-0.421*	0.211	0.086	0.268*	1.000		
Number stomata	-0.022	0.043	0.825***	-0.088	-0.011	1.000	
Wax rate	-0.178	0.269*	0.383*	0.033	0.169	-0.221	1.000

\*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ ; insignificant:  $p > 0.05$

## Discussion

Our study focuses on the irrigation of native and introduced (Spanish) olive trees with salt water at the rate of 6g / l of soluble salts, i.e. approximately 100 mM.l<sup>-1</sup> of NaCl for 6 weeks. It enabled us to determine certain characteristics of the morpho-physiological behavior of this species under the salt stress. The water state of the plant, expressed by the relative water content, was sensitive to the applied treatments ( $r = -0.2$ ). Indeed, salt stress causes a decrease in the values of the relative water content. This parameter, is one of the criteria for evaluating abiotic stress tolerance because it indicates the state of turgescence of the plant tissues and its ability to maintain a level of hydration of the tissues. The last could guarantee the continuity of its metabolism and the water state of a plant can be expressed by its RWC (Morant-Manceau et al, 2004; Mehani et al., 2012).

The results obtained during our study demonstrate that the RWC of all the stressed varieties changed with the salinity. However, the reduction was quite small compared to the control (between 1 and 2%). Local varieties retained a higher RWC than the introduced ones. We also noted that saline treatment did not cause a significant reduction in water content. The analysis of the relative water content is a good indicator of the water status of the plant.

On the other hand, salinity is a complex phenomenon that often leads to osmotic stress due to reduced amounts of water available at the root level, due to the reduced ability of plants to absorb water. The immediate response to salt stress is expressed by a reduction in leaf area as was noted by (Wang and Nil, 2000) and that decreased vegetative growth, expressed as leaf area reduction. Leaf area is generally the first response of glycophytes exposed to saline stress (Munns et al., 2006).

We observed that the decrease in the leaf area of all the genotypes studied shown only a slight drop which was between 1 and 2% compared to the control. This was confirmed by the results of the analysis of the correlation matrix between variety and leaf area ( $p = -0.421$ , see Table 4). For cuttings and grafted plants, it was found that grafted and stressed local ecotypes showed an increase in leaf area compared to the control, and cuttings and stress decreased leaf area compared to control (less 1.1 cm<sup>2</sup> at the control). Thus, decreasing of leaf area was considered as a form of adaptation to salt stress, by reducing water losses through perspiration, but it may also cause a decrease in yields due to the reduction of photosynthesis (Acevedo, 1991; Hassani et al., 2008).

The stomatal density of leaves of all stressed genotypes decreased compared to that of no salt (Fig. 4). Moreover, this decrease was greater in the ecotypes obtained from herbaceous cutting (it was 65% for Sigoise, 52.78% for Chemlal, 54.29% for Arbiquina, and 75% for Manzanilla) than those grafted (69% for Sigoise, 34.01% for Chemlal, 45.31% for Arbiquina, and 43.2% for Manzanilla). According to Guyot (1998), stomatal transpiration accounts for 90% of total sweating for 24 hours.

The opening and closing of the stomata are controlled by the turgidity of their guard cells, which depend on soil and air moisture, sheet temperature, incident radiation, wind and concentration CO<sub>2</sub> in the air as well as in the chamber under stomatal conditions (Hassani et al., 2014). However, whenever the plant reduces its transpiration by closing the stomata, it causes the reduction of the production of dry matter following the reduction of the chlorophyll assimilation (Zhang and Shi, 2013).

Moreover, obtained results show a strong positive relationship between salt stress and stomatal density in the lower leaf epidermis. Indeed, the high correlation obtained between salinity and stomatal density ( $r = 0.825$ ,  $p < 0.001$ ) shows that the presence of NaCl leads to an increase in the number of stomata, which is not logical when we know that they are small stomata to reduce water losses and increase the RWC.

According to Erchidi et al. (2000), plants that live in dry environments have many stomata with small sizes and that the presence of small and large stomata allows a much more effective regulation of sweating than that of large and small stomata and the increase in the number of stomata per unit area could be one of the factors of resistance to water deficit if accompanied by a good physiological activity (Slama et al., 2005).

The increase in stomatal density can increase the net assimilation of CO<sub>2</sub> and decrease the loss of water. In fact, many small stomates are fast closing (Heller et al., 2004).

In addition, Hopkins (2003) established the relationship between transpiration and stomatal resistance in *Arabidopsis thaliana* by increasing this resistance during salt stress, to minimize water loss and sweating, becomes more important in the case of thin cuticle leaves.

According to our results, the stressed genotypes have cuticles thicker than those under normal conditions that can explain the resistance of our plants by keeping their leaves throughout the duration of the stress applied.

## Conclusions

The morpho-physiological parameters of the leaf, retained in this study, would be closely involved in the regulation of the water state of the leaf tissues. Salt resistance is a polygenic character that can be controlled at different levels of organization, from the cell to the entire plant. However, the diversity of salt effects on plants offers a wide range of physiological and biochemical criteria that can be the basis for rapid testing for large-scale selection. Application of salt water resulted in a reduction in RWC (relative water content), but relatively higher compared to other plants subjected to the same salt stress. This characteristic can be attributed to the osmotic fit of the stressed plant. This control of the hydration reveals a good ability to adjust the osmotic potential in the olive tree in general and especially in the varieties studied. For this purpose, it can be deduced that the varieties of olive trees studied have retained a high RWC in the presence of saline stress, indicating that this plant is of the "stay green" type, which keeps synthetically active green and photo leaves, have reasonable yields even in the presence of abiotic stress. Under arid climates and in conditions of ionic stress accompanied by osmotic stress, the plant must maintain a dynamic balance between the opening and closing of the stomata. This activity allows it to increase the fixation of the carbon and a better transpiration thus avoiding the overheating of the plant. In Mediterranean regions, physiological drought (due to excess salinity), is often chronic, resulting in a decrease in photosynthesis. Our results showed that all the varieties studied under the effect of salinity, developed a thick cuticle compared to those under normal conditions, that decreasing leaf transpiration. This develops the resistance of the olive tree in general and especially the studied genotypes that keep their leaves during the applied stress. The results obtained in this study show that the olive tree is a salinity-resistant plant that is a characteristic of arid and semi-arid areas. Olive trees, then, is a promising alternative to improve the productivity of marginalized lands.

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