

Study the morpho-physiological leaves behavior of grafted and ungrafted olive trees (*Olea europaea*) under saline stress

Gharabi Dhia¹, Hassani Abdelkrim^{1*}, Hella Benchaben², Labdaoui Djamel³

¹Laboratory of Agrobiotechnology and Nutrition in dry areas, Ibn Khaldoun University, Tiaret, Algeria

²Laboratory of Geomatics and Sustainable Development, Ibn Khaldoun University, Tiaret, Algeria

³Laboratory of plant protection, Abdelhamid Ibn Badis University, Mostaganem, Algeria

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*Corresponding author email:
karim_hassani2002@yahoo.fr

Abstract

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 took place in a greenhouse with a well-controlled condition. The experimental plan is in a complete randomization of two factors (salinity and varieties); plants without NaCl application were used 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. 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.

Keywords: Olive-tree, Local varieties, Salt stress, RWC, Wax rate

Introduction

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). When it is disposed on a bench, it contributes to reducing the soil erosion and the loss of the fertile soil. (Beaufoy, 2001; 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 (Munns et al., 2006) and insufficient and irregular rainfall (Mezni et al., 2002). This salinization stems also from an inadequately controlled irrigation

(Bennaceur et al., 2001). Salinity and drought additionally have been shown to affect plant growth and protein accumulation (Zhu, 2001; Zhang et al., 2002). It affects, also microorganisms mainly by decreasing osmotic potential, which reduces their activity and alters the composition of the microbial community (Chowdhury et al., 2011). 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, 2013) than the EC of a soil extract. The accumulation of salts in the root zone has adverse effects on plant growth (Belkhdja and 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 and Sunkar, 2005). Therefore, these changes require reflection on the strategies to be undertaken 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. This promotes normal metabolism of plants (Martinez et al., 2007). 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.

Therefore, this experiment was conducted to examine the influence of NaCl or the 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 Arbiquina).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

Material and Methods

Experimental device and plants

The experiment was conducted in a greenhouse in the Ibn Khaldoun University of Tيارت, 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 plantlets used in the experiment were 18-month-old and were obtained by cutting rooted under nebulization' (crop nursery). The initial growing substrate was replaced and the roots of 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, and soil and an organic matter at proportions of four volumes from sand / one volume from soil and one volume from manure or an organic matter.

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 for the production of table olives (Sigoise and Manzanilla) and two for olive oil production (Chemlal and Arbiquina). The cuttings of the local varieties (Sigoise and Chemlal) come from Algiers and the introduced plants (Manzanilla and Arbiquina) come from Spain.

The salt stress induction of the four varieties was performed by adding NaCl to the nutrient solution. Each seedling received a standardized and balanced nutrient solution such as Hoagland and Arnon (1938). In fact the treated plants were watered twice a week with 300 ml (the nutrient solution + 100mM NaCl) and the control plants received only the nutrient solution. And after six (06) weeks of salt stress the following measures were taken:

The relative water content (RWC)

It's determined by method of Barrs and Weatherley, (1962) according by the formula of Clark 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 during 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:

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

The leaf area

The leaf area is directly measured using an electronic planimeter (cm²) using an LICOR-3000A (7) with a resolution of 1 mm².

The stomata density

The stomata density, according to the method of Dohman et al. (1991), used 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 in order 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 wax rate

The excised sheet at its base is introduced into a washed and weighed test tube (P1) to which chloroform is added in order 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: Wax Rate in mg.cm⁻² = P2-P1 / SF.

Statistical analysis

The obtained data were processed by Statistica Software Version 8.0, by analyzing the variance and the correlation matrix. The obtained values were statistically averaged (four repetitions) with a confidence interval calculated at the threshold of 5%.

Results

The relative water content

The above statistical results are seen more significant (Table.1), at the bit error rate threshold of 5%, of the salinity effect and the various interactions on the relative water content (RWC). The relative water content (RWC) of the leaves provides information on the relative turgor of the tissues and is one of the criteria for assessing stress tolerance.

Our statistical results are significant for the varietal effect on the relative water content and not significant for the effect of the plant type. Indeed, it shows that after six weeks of treatment with 100 mM.l⁻¹ of NaCl, the majority 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 01) 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 cm² and then it drops to 27.19 cm² for the leaves of the plants treated with 100 mM.l⁻¹ NaCl, (Fig. 1b).

With the exception of 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⁻¹NaCl, (Table 02).

The stomata density

According to the obtained analysis (p=0%), (Table 2) the reaction of the plants followed the same tendency about the expression of this parameter. The Figure 2 shows a significant variation in the number of stomata. In fact, Sigoise cuttings have 40 stomata / us (control) versus 26 (stressed) stomata. The Chemlal presents 45 against 24, the Arbiquina 44 against 24 and the Manzanilla 30 against 23 stomates / us. For the genotypes grafted on oleaster the fall is more important. According to Fig. 05, the values are 49 (control) versus 14 for the Sigoise, 45 against 24 for Chemlal, 62 against 21 for Arbiquina and 48 against 21 stomata/us for the variety Manzanilla.

The size of the stomata

The impact of salinity on the length of stomata, particularly on the leaves of plants treated with 100 mM.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 2).

Indeed, the values of these parameters, shows that the length and width of stomata in the leaves of plants treated with 100 mM·1⁻¹ NaCl react differently. The two local varieties (Sigoise and Chemlal) in cuttings and grafted mark an increase in the length of the stomata with the exception of the grafted sigoise which records an average decrease of 4 µm. For the local varieties, the length of the stomata varies between 0.4 and 4.5 µm. 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 µm, with the exception of the grafted Manzanilla variety which marks an increase of the width of the stomata 0.75 µm relative to the control.

The wax rate

The analysis of wax variance shows a very highly significant influence for all factors tested at p<0.05, in particular 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 04). The histograms displayed in figure (02b) show that all the genotypes studied local and introduced develop a cuticle by the deposition of wax, (Fig.03).

The anatomical structure of the leaf

That 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 and Martoja, (1968).

Table 01. Analysis of the variance of the Relative Water Content (RWC) and the leaf area of olive trees used stressed and unstressed.

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

Table 02. Analysis of the variance of the stomatal density, the length and width of the stomata (µm) 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 stomata	0.000	0.481	0.001	0.000	0.000	0.020	0.037
width stomata	0.000	0.239	0.000	0.007	0.024	0.000	0.000

Table 03: 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**

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

Variable	Variété	Plant	Salinity	RWC	Leaf area	Stomatal Number	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		
stomatal Number	-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

Very more significant***: p < 0.001, more significant **: p < 0.01, Significant *: p < 0.05; no significant: p > 0.05



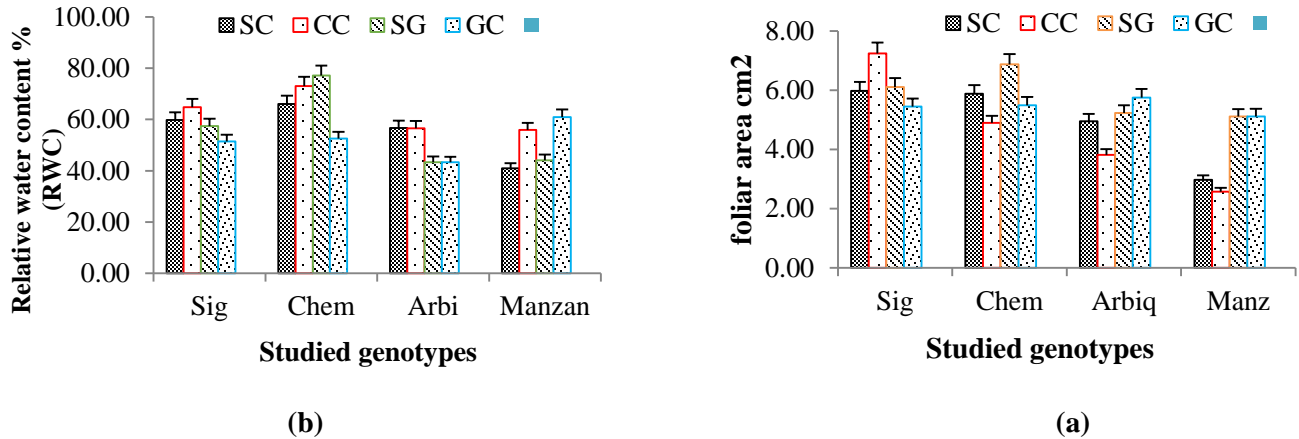


Figure 01. Relative water content (RWC)(a) and the leaf area (mm²) (b) as measured from the penultimate leaf of the studied plantlets (SC: Stressed Cut, CC: Cutting Control, SG: Stressed Graft, GC: Grafted Control)

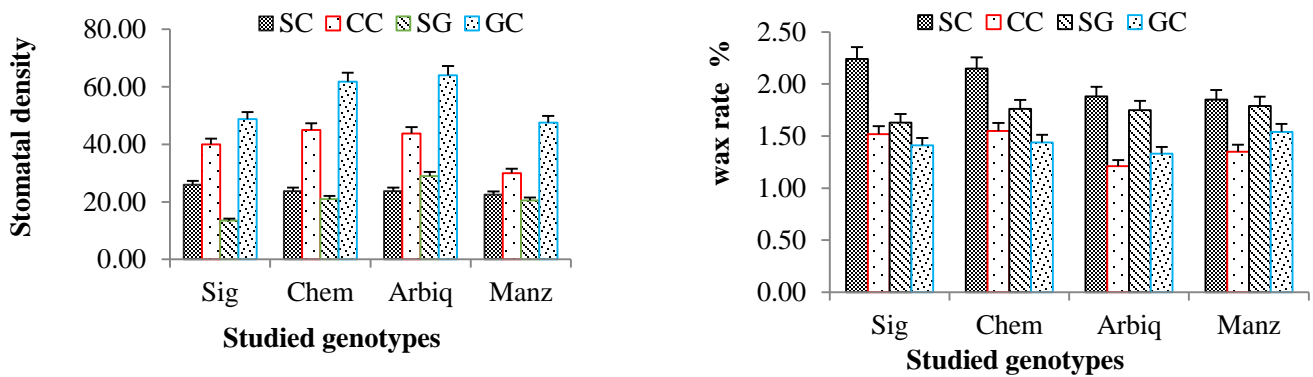


Figure 02. The stomatal density (/mm²) of lower surface of the leaves (a) and the wax rate of the upper surface of the leaves (b)(SC: Stressed Cut, CC: Cutting Control, SG: Stressed Graft, GC: Grafted Control)

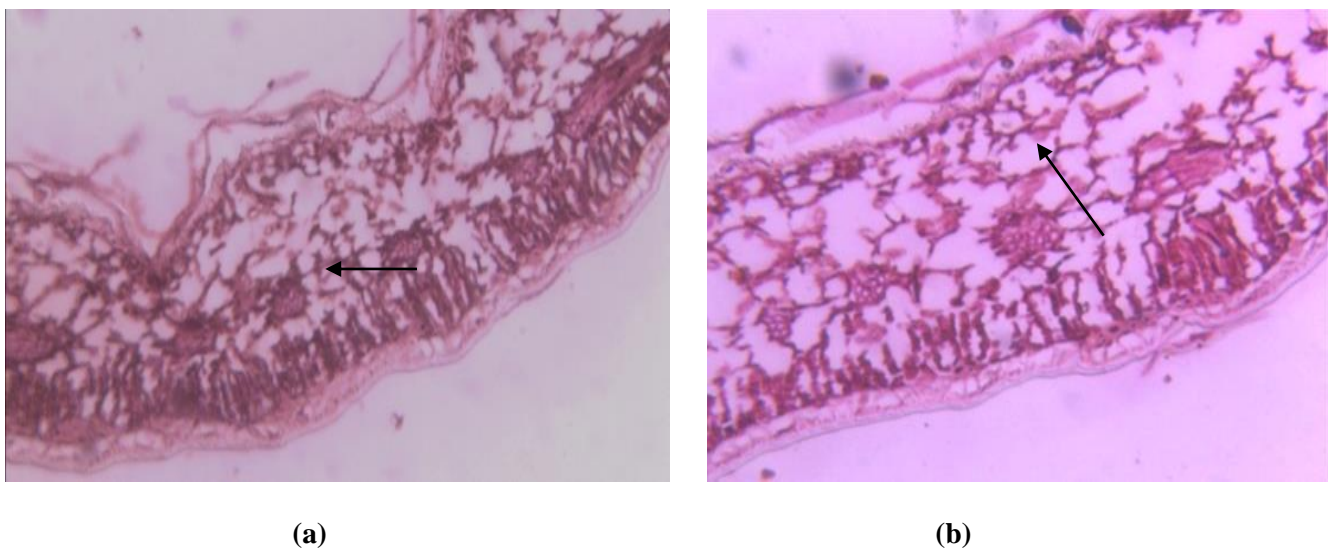


Figure 03. The foliar cuticle in olive tree "Sigoise" BS: Stressed Cut (a) and Stressed Graft (b) (photo gharabi and hassani, 2015)

Discussion

Our study emphasizes the irrigation of olive trees of local origin and introduced or local (Spanish) with brackish water which is about 6g/l of soluble salts (NaCl at a 100 mM.l-1), For 06 weeks. It enabled us to determine certain characteristics of the morpho-physiological behavior of this plant species in a situation of salt stress. The water state of the plant, expressed by the relative water content, was sensitive to the applied treatments ($r = - 0.200 *$). 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 to 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 stressed varieties varies with salinity. However, the reduction is small compared to that of the control (between 1 and 2%). Local varieties retained a higher RWC than the introduced varieties. It is also noted that saline treatment did not cause a significant reduction in water content. The analysis of the relative water content allows describing in a global way 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 noted by (Wang and Nil, 2000) and that decreased vegetative growth, expressed as leaf area reduction or leaf area, is generally the first response of glycophytes exposed to saline stress (Munns et al., 2006). As regards our results, it's observed that the decrease in the leaf area of all the genotypes studied shows only a slight drop which is between 1 and 2% compared to the control. This is confirmed by the results of the analysis of the correlation matrix between variety and leaf area ($p = - 0.421*$)(Table 4). For cuttings and grafted plants, it was found that the local ecotypes grafted and stressed showed an increase in the leaf area compared to their control and those from cuttings and stressed showed a decrease in leaf area by compared to their control (less than 1.1 cm² compared to the control). Thus, decreasing leaf area is 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 (Bidinger and Witcombe, 1989; Acevedo, 1991; Hassani et al., 2008).

The stomatal density of leaves of all stressed genotypes decreased compared to that of no salt (Fig. 04). Moreover, this decrease is greater in the ecotypes obtained from herbaceous cutting (it is 65% for Sigoise, 52.78% for Chemlal, 54.29% 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 during 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 CO₂ in the air as well as in the chamber under stomatal conditions (Teare and Kanemasu, 1972; 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, the results obtained 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 ($r = 0,825***$) 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 in order to reduce water losses and increase the RWC. Indeed, 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 CO₂ and decrease the loss of water. In fact, a large number of stomata can cause small stomata and rapid closure (Heller et al., 2004).

In addition (Hopkins, 2003), have established the relationship between transpiration and stomatal resistance in *Arabidopsis thaliana*, by increasing this resistance during salt stress, in order 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.

Conclusion

The application of brackish water resulted in a lower, but relatively high, TRE compared to other plants in the same situation. This characteristic can be attributed to the osmotic adjustment of the stressed plant. This control of hydration reveals a good ability to adjust the osmotic potential of the olive tree in general and the varieties studied. For this purpose, the olive varieties studied have retained a high RWC in the presence of salt stress, which indicates that this plant is of the "stay green" type, which keeps the green leaves and photo synthetically active, even in the presence of stress abiotic.

In arid climates and ion stress conditions accompanied by osmotic stress, the plant must maintain a dynamic equilibrium between opening and closing stomata. This activity allows it to increase carbon fixation and better transpiration, thus avoiding the heating of the plant. In the Mediterranean regions, physiological drought (due to excessive salinity) is often chronic, leading to 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, thus reducing leaf transpiration. This can explain the resistance of the olive tree in general and in particular of the genotypes studied allowing him to keep his leaves during all the stress applied. We can conclude that thanks to its leaves, the olive tree can survive in an arid environment. The results obtained in this study show that the olive tree is a salinity-resistant plant characteristic of arid and semi-arid zones. Olive trees are therefore a promising alternative to improve the productivity of marginal lands

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