



# Article Cross-Priming Approach Induced Beneficial Metabolic Adjustments and Repair Processes during Subsequent Drought in Olive

Mariem Ben Abdallah<sup>1</sup>, Kawther Methenni<sup>1</sup>, Wael Taamalli<sup>1,2</sup>, Kamel Hessini<sup>3,\*</sup> and Nabil Ben Youssef<sup>1</sup>

- <sup>1</sup> Laboratory of Olive Biotechnology, Centre of Biotechnology of Borj-Cedria, University of Tunis El Manar, P.B. 901, Hammam-Lif 2050, Tunisia
- <sup>2</sup> Department of Biology, Higher Institute of Biotechnology of Beja, University of Jendouba, B.P. 382, Beja 9000, Tunisia
- <sup>3</sup> Department of Biology, College of Sciences, Taif University, P.B. 11099, Taif 21944, Saudi Arabia
- Correspondence: k.youssef@tu.edu.sa

Abstract: Cross-tolerance to abiotic stresses is a typical phenomenon in plants which occurs when exposure to one form of stress confers tolerance to a variety of stresses. Our study aims at investigating whether salinity priming could induce, after a recovery period (2 months), drought tolerance in olive cv. Chétoui. Here, our results revealed that this method of cross-adaptation had further enhanced the olive's subsequent response to drought. In fact, relative to the non-pretreated plants, the saltpretreated ones displayed an enhancement in terms of shoot biomass accumulation, photosynthetic performance, water-use efficiency, and hydration status. Furthermore, the attenuation of oxidative stress and the maintenance of structural lipid contents, as well as their fatty acid composition in salt-pretreated plants, also supported the beneficial effect of this method. From our results, it seems that salt priming substantially modulated the physiological and biochemical responses of olive plants to subsequent drought. Accordingly, metabolite adjustments (soluble sugars and proline), the enzymatic antioxidant system (superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (GP) activities) as well as the nonenzymatic one (phenols), and the increase in leaf density together with the raise of structural lipids content, to a lesser extent, seemed to perform a major role in the development of this improved tolerance to drought. The ameliorative response found in salt-primed olive plants, when subsequently exposed to drought, indicates an efficient cross-tolerance reaction. This could be particularly important in the Mediterranean area, where olive orchards are mainly cultivated under dry-land farming management.

Keywords: cross-tolerance; drought; memory; olive plants; salinity

# 1. Introduction

Due to elevated global climate change, drought is the greatest environmental challenge facing the Earth today. In agriculture, this climatic/environmental stress hampered crop growth, development, and crop productivity [1,2], especially in Mediterranean basin, an area where olive trees were mainly cultivated under rain-fed conditions [3]. In such a region, the cultivation of this tree knows several problems related to various environmental stressors, characterized by long drought periods and soil salinization. This problem will be further aggravated in the long term with the risks of continuous climate change [4,5]. Drought affected olive plants by impairing the  $CO_2$  availability and consequently altered their photosynthetic metabolism. Moreover, it could damage the cell membrane function and alter the redox equilibrium, expressed as oxidative stress, leading to the inhibition of plant growth [6]. In spite of the capacity of olive to overcome water shortage, it is well-documented that dry conditions greatly influence its productive performance. Indeed, under such climatic conditions, extensive research showed that olive growth, flowering, fruit size, yield, and



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**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). notably oil quality could be altered [5,7–11]. Because of the socioeconomic importance of olive in Tunisia and its enhanced cultivation even on drought-affected regions, there is an emergency need for further improvement in its drought tolerance.

Presently, priming, the stimulation of plant defense through prior exposure to an eliciting factor (chemical agents, stress), could develop the so-called "plant stress memory" and improve the plant capacity to survive from later stress events [2,12]. Among priming strategies, cross-priming, also referred to as cross-tolerance, cross-adaptation, cross-resistance, or cross- protection [13], is the process which occurs when exposure to one form of stress confers tolerance to a range of stresses [14–16]. It is well established that abiotic stresses exhibited, generally, many similarities in their effects and plant responses, especially signaling pathways [17]. The overlap in this latest response, and the induction of common defense responses, allows plants to establish efficient acclimation mechanisms for cross-tolerance, such as morphological adjustments, the overproduction of specific transcription factors, the activation of multiple stress-signaling pathways, protective metabolites, and epigenetic modifications [2,18–20]. This acquired tolerance is crucial for the growth and development of plants, since, under natural field conditions, they are frequently exposed to many stresses instantaneously or successively [21]. Such a process has been observed and reported across various herbaceous species. For instance, heat pretreatment increases plant tolerance not only to heat but also to cold [22,23], salinity [22], and drought [24] by reducing leaf water loss, decreasing the oxidative injuries of the photosynthetic apparatus, and increased growth. Saini et al. [25] showed that drought priming allowed chickpea seedlings to maintain membrane stability during cold exposure through the improvement of mitochondrial functioning. In the same context, Li et al. [26] reported that drought priming enhanced subsequent heat tolerance in wheat plants by maintaining water homeostasis, photosynthetic ability, and biomass production. Furthermore, in tomato, Yang et al. [27] showed that salt priming improved temperature tolerance through photosynthetic accommodation. These studies reported that the useful effects of cross-priming are mainly attributed to a plethora of processes, imperatively mediated by osmoregulation, photosynthesis regulation through stomatal control and transpiration, ion homoeostasis, and especially the mitigation of oxidative stress.

Even though this process has been noticed, as mentioned above, for many types of stresses, the cross-tolerance ability of salt priming to induce subsequent drought tolerance has only been recently investigated by few studies. Accordingly, Pushpavalli et al. [28] reported that the pretreatment of chickpea by salinity enhanced its tolerance when exposed to terminal drought, resulting in higher grain yield compared to nonprimed plants. In addition, Feng et al. [29] revealed that salt priming regulates growth and photosynthesis in drought-stressed cotton via the regulation of the activation of antioxidant system. Moreover, the results of Yang et al. [30] displayed that salt priming improved the subsequent tolerance to drought in tomato plants, mainly via the mitigation of damage in the photosynthetic apparatus; this study showed that salt priming increased the rate of photosynthetic assimilation and photochemical efficiency in tomato plants compared with non-pretreated plants by inducing nonphotochemical quenching, maintaining cyclic electron flow, and alleviating the damage to the thylakoid membrane. Indeed, this type of cross-tolerance between drought and salinity would be of particular interest, since salinity constitutes a major common abiotic stress under natural environments due to frequent irrigation [31]. In parallel, plants always suffer from the negative effects of drought, particularly in Mediterranean arid regions [32]. Furthermore, these two stresses are regarded as the main factors affecting olive crop performance and productivity. Nonetheless, there is no report regarding the ameliorative efficiency of drought tolerance through salinity priming in olive plants.

Therefore, the objective of the present study was to assess whether and how salt priming regulated plant growth and physiological and biochemical responses under subsequent drought stress, and to unravel the underlying mechanisms for this process in young olive plants cv. *Chétoui*, a drought-sensitive variety. This innovative approach would aim at olive plants raised from priming treatment at nursery scale to perform better in terms of drought stress tolerance once planted in the field.

#### 2. Materials and Methods

# 2.1. Greenhouse Conditions and Cross-Priming Application

Greenhouse conditions for all the experiment cultures were maintained with a temperature regime of 25/17 °C day/night, an air relative humidity of 70–75%, and 16 h photoperiod. Young olive seedlings cv. *Chétoui* were (i) firstly primed with salt stress (200 mM NaCl) for 21 days (d), (ii) recovered for another 60 d, and (iii) finally exposed to drought stress (withholding water) for 30 d.

In detail, olive plants (seven-month-old) were cultivated in 10 L pots filled with inert sand and watered every 2 d with 200 mL of Hoagland nutrient solution for 3 weeks, and subsequently divided, as indicated in Figure 1, into three lots: C— control plants, received every 2 d with 200 mL of Hoagland solution for 111 d; NPPs—nonprimed plants, well-watered for 81 d with 200 mL of Hoagland nutrient solution before being water-stressed for 30 d; SPPs—salt-primed plants, primed by salt exposure to 200 mM NaCl (irrigation every two d with 200 mL of Hoagland solution containing 200 mM NaCl) for 21 d of salt exposure, recovered for 60 d, and subsequently exposed to drought for 30 d.



**Figure 1.** Diagram of the experiment design. C: control plants, well-watered during 111 d experiment; NPPs: nonprimed plants, well-watered for 81 d and then stressed by withholding water for 30 d; SPPs: salt-primed plants, primed by exposure to salinity (200 mM NaCl) for 21 d, recovered for 60 d, and then exposed to water depletion for 30 d as with the NPPs.

A complete randomized design was used in the experiment, and plants from each treatment were chosen by a random sample and independently from plants in other treatments. In total, 300 plants were used, 100 per treatment (C, NPPs, and SPPs). Each studied variable was measured/determined with 6 replicates (6 plants) per treatment.

#### 2.2. Physiological Analysis

# 2.2.1. Growth and Relative Water Content Determination

Plants were collected in the morning and separated into leaves and roots. Dry weights were noted after total desiccation in an oven at 60 °C for approximately two weeks. The relative water content (RWC) of leaves was determined as described in Ben Abdallah et al. [6].

Gas-exchange parameters were determined using a portable open-system infrared gas analyzer LCi instrument (Analytical Development Company Ltd., Hoddesdon, UK). Characteristics measurements of net CO<sub>2</sub> assimilation (A), transpiration rate (E), stomatal conductance (gs), and intercellular CO<sub>2</sub> assimilation (Ci) were made from 10.00 to 12.00 h on a fully expanded 3rd leaf (from top) of each plant (6 plants per treatment). The following conditions were used for the measurement:  $398 \pm 1 \mu mol mol^{-1}$  CO<sub>2</sub> concentration;  $30 \pm 0.3 \degree$ C leaf temperature; 1012 mBar atmospheric pressure. Water-use efficiency (WUE) was measured as the ratio of CO<sub>2</sub> assimilation to stomatal conductance (A/gs).

Fluorescence measurement (maximum quantum efficiency of PSII (Fv/Fm) and the effective quantum yield of PSII photochemistry (Y)) and photosynthetic pigments (total chlorophylls (Chl a and b) and carotenoids (Car)) were monitored during the experiment as detailed in Ben Abdallah et al. [6].

# 2.2.3. Determination of Leaf Density

Leaf density was calculated as reported in Guerfel et al. [33];  $D = (DM/FM) \times 1000$ , with DM = leaf dry mass and FM = leaf fresh mass

#### 2.3. Biochemical Analysis

#### 2.3.1. Proline and Sugar Content

The quantitative estimation of proline and sugar contents were carried out according to Bates et al. [34] and Yemm and Willis [35], respectively, as reported in Ben Abdallah et al. [6,36].

# 2.3.2. Estimation of Lipid Peroxidation and Electrolyte Leakage

Malondialdehyde (MDA) was measured in olive leaves to estimate the level of lipid peroxidation, using the method of Heath and Packer [37].

The electrolyte leakage (EL) was quantified according to the method described in Ben Abdallah et al. [6,36].

#### 2.3.3. Protein Extraction and Antioxidant Enzyme Activities

For protein extraction, olive leaves were firstly lyophilized, powdered, and then homogenized in 100 mM potassium phosphate buffer (pH 7.5). The homogenate was centrifuged at  $14,000 \times g$  for 30 min and then the supernatant was used for enzyme estimation.

Using the bovine serum albumin (BSA) as standard, the total protein content in foliar olive samples was calculated by the Bradford reaction [38]. SOD, CAT, and GP activities were assayed as described in Ben Abdallah et al. [36].

#### 2.3.4. Estimation of Total Phenol Amount and Its Antioxidant Activity

For the determination of total phenolic concentration, we used the Folin–Ciocalteu according to the method of Skerget et al. [39], slightly modified. The antioxidant activity of these phenolic extracts was quantified using the DPPH assay method as previously described in Ben Abdallah et al. [36].

# 2.3.5. Lipid Extraction and Fatty Acid Determination

Total lipids were extracted using the method of Folch et al. [40], modified by Bligh and Dyer [41]. Fatty acids from total lipids were methylated according to the method of Metcalfe et al. [42]. The separation and quantification of the methyl esters of fatty acids were determined using a gas chromatography (GC) as detailed in Nouairi et al. [43].

#### 2.4. Statistical Analysis

Statistical analysis was determined using the R software v3.6 (R Core Team, 2013). The data set was checked for normality and homogeneity of variance using the Shapiro–Wilk test and Levene's test, respectively. One-way variance analysis (ANOVA) was performed to evaluate statistical differences among treatments; the HSD Tukey test was used (p < 0.05)

when significant differences were detected. Finally, all studied parameters in C, NPP, and SPP were subjected to principal component analysis (PCA) to explore relationships among variables and treatments.

# 3. Results

### 3.1. Changes in Seedlings Biomass and Relative Water Content

As shown in Figure 2a, under drought stress for 30 days, the growth of the NPPs was markedly reduced by -42% compared to the control. Interestingly, salt priming significantly improved plant growth when grown under drought conditions. Likewise, the total dry weight of the roots was found significantly unchanged between primed and nonprimed plants.



**Figure 2.** Effect of cross-tolerance on shoot biomass (**a**) and root biomass (**b**) of olive plants subjected to subsequent drought stress. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.

Under drought conditions, the NPPs showed a significantly reduced RWC, to values below 40%. In contrast, the SPPs showed a better RWC content (76%) than that of the nonprimed ones (Figure 3).

#### 3.2. Changes in Osmoticums

Our results revealed that drought stress had no significant change on proline content; however, it increased that of total sugar (Figure 4a,b). On the other hand, the SPPs showed higher sugar and proline contents than the NPPs (Figure 4a,b).

#### 3.3. Changes in Photosynthesis Parameters

Water stress reduced net photosynthesis, stomatal conductance (gs), and transpiration rate (E) in the NPPs relative to the control (Figure 5a–c). Interestingly, the SPPs exhibited improved photosynthetic performance when compared to the NPPs, as evidenced by higher leaf A, with a level similar to that of the control. In the SPPs, E and gs diminished slightly compared to the control, but still higher than the NPPs (Figure 5a–c). Moreover, in contrast

to the NPPs, which displayed an increase in Ci, our results showed a maintenance of Ci in the SPPs when compared to the control plants (Figure 6a).



**Figure 3.** Changes in relative water content (RWC) of primed and nonprimed olive leaves subjected to subsequent drought. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.



**Figure 4.** Effect of cross-tolerance on soluble sugar (**a**) and proline (**b**) concentrations of olive leaves subjected to subsequent drought stress. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.



**Figure 5.** Changes in CO<sub>2</sub>-assimilation rate, A (**a**), stomatal conductance, gs (**b**), transpiration rate, E (**c**), of primed and nonprimed olive leaves subjected to subsequent drought. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.



**Figure 6.** Changes in water-use efficiency (WUE) (**a**) and intercellular CO<sub>2</sub> assimilation (Ci) (**b**) of primed and nonprimed olive leaves subjected to subsequent drought. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.

# 3.4. Changes in Leaf Density, Intercellular CO<sub>2</sub> Assimilation, Water-Use Efficiency, *Photosynthetic Pigments, and PSII Photochemistry*

Our results showed that, relative to unstressed plants, drought stress increased leaf density in both the NPPs and the SPPs. Nonetheless, leaves from the latest exhibited a higher density than the leaves of the NPPs (Table 1).

Additionally, Figure 6b indicates that drought increased Ci in the NPPs; however, the SPPs showed a maintenance of this parameter when compared to the control plants (Figure 6b).

**Table 1.** Effect of cross-tolerance on maximum efficiency of PSII photochemistry, Fv/Fm, and efficiency of PSII photochemistry, Y, and density of olive leaves subjected to subsequent drought stress. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.

Treatment	Fv/Fm	Y	D
С	$0.79\pm0.01~^{\rm b}$	$0.76\pm0.03^{\text{ b}}$	$435\pm60~^{a}$
SPP	$0.75\pm0.02~^{\rm b}$	$0.65\pm0.02^{\text{ b}}$	$853\pm40~^{ m c}$
NPP	$0.13\pm0.02$ a	$0.20\pm0.03$ a	$769\pm50~^{\rm b}$

Likewise, Figure 6a reveals a large increase of the WUE in the SPPs when compared to the NPPs.

Moreover, Figure 7a,b shows that the content of photosynthetic pigments (Chl and Car) both in the NPPs and SPPs showed no significant differences as compared to the control.



**Figure 7.** Changes in total chlorophyll content (**a**) and carotenoid content (**b**) of primed and nonprimed olive leaves subjected to subsequent drought. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.

Results also revealed that water deficit negatively affected the photochemistry of the PSII of the NPPs, as verified by the concomitant decrease in the Fv/Fm ratio and Y in comparison with the control plants (Table 1). However, the SPPs displayed attenuation in stress effects, as their Fv/Fm ratio and Y became close to those of the nondrought conditions (Table 1).

#### 3.5. Effect on Lipid Metabolism

Our results showed that drought generated a significant reduction in the lipid concentration (-71%) in the NPPs relative to the control (Figure 8a). However, a marked increase was found in the SPPs when compared to the NPPs and to the control (Figure 8a).



**Figure 8.** Changes in total lipid content (**a**), UFAs/SFAs × (unsaturated-to-saturated fatty acid ratio) (**b**) and in DBI × (double-bond index = R (unsaturated fatty acid × number of double bonds) (**c**) of olive leaves subjected to subsequent drought stress. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means ± standard error (n = 6), where 'n' is the number of replicates for each group.

In Table 2, we show the change in the fatty acid composition induced by water stress. Our results revealed that drought increased saturated fatty acid levels associated with a decrease of the major unsaturated ones: C18:1 and C18:3 in the NPPs. The SPPs displayed a modification in fatty acids composition, which is marked by a decrease in the majority of fatty acid percentages, except that of C18:3.

**Table 2.** Fatty acid composition (%) of leaf lipids of olive plants cv. 'Chetoui' subjected to subsequent drought stress. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.

C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0
$\begin{array}{c} 0.60 \pm 0.03 \ ^{a} \\ 0.78 \pm 0.04 \ ^{b} \\ 1.74 \pm 0.32 \ ^{a} \end{array}$	$\begin{array}{c} 1.10 \pm 0.12 \; ^{a} \\ 0.87 \pm 0.09 \; ^{b} \\ 2.47 \pm 0.38 \; ^{a} \end{array}$	$\begin{array}{c} 17.70 \pm 1.55 \ ^{a} \\ 20.01 \pm 0.49 \ ^{b} \\ 21.48 \pm 1.23 \ ^{b} \end{array}$	$\begin{array}{c} 0.80 \pm 0.05 \; ^{a} \\ 0.94 \pm 0.09 \; ^{a} \\ 0.93 \pm 0.03 \; ^{a} \end{array}$	$\begin{array}{l} 3.00 \pm 0.04 \; ^{a} \\ 2.88 \pm 0.07 \; ^{b} \\ 4.16 \pm 0.21 \; ^{a} \end{array}$	$\begin{array}{c} 20.49 \pm 0.46 \; ^{a} \\ 19.44 \pm 1.23 \; ^{a} \\ 19.20 \pm 0.91 \; ^{a} \end{array}$	$\begin{array}{c} 10.50 \pm 0.52 \; ^{a} \\ 10.57 \pm 0.27 \; ^{b} \\ 11.51 \pm 0.43 \; ^{b} \end{array}$	$\begin{array}{c} 40.00 \pm 1.41 \ ^{b} \\ 41.73 \pm 1.64 \ ^{a} \\ 35.30 \pm 0.48 \ ^{c} \end{array}$	$\begin{array}{c} 3.20 \pm 0.20 \; ^{a} \\ 2.76 \pm 0.10 \; ^{a} \\ 3.19 \pm 0.32 \; ^{a} \end{array}$

Furthermore, Figure 8b,c shows that drought decreased the ratio the UFAs/SFAs and the DBI index in the nonprimed plants when compared to the control. In contrast, the SPPs showed stability in the DBI index and an increase in the ratio of UFAs/SFAs.

# 3.6. Changes in MDA Contents and Electrolyte Leakage

Drought stress significantly increased the foliar oxidative damage. Indeed, compared to the control plants, drought induced an increase in the level of the MDA content and EC in the NPPs relative to the control (Figure 9a,b). Contrarily, the SPPs exhibited significantly lower EC and MDA values than those of the NPPs (Figure 9a,b).

# 3.7. Changes in Antioxidant Enzymes Activity

In the NPPs, the SOD activity remained unchanged, that of the GP was reduced, and only the CAT activity was increased. Nonetheless, NaCl priming increased greatly CAT, SOD, and GP activities in the SPPs relative to the NPPs and to the control (Table 3).



**Figure 9.** Changes in malondialdehyde, MDA (**a**), and electrolyte leakage, EC, percent (**b**) of olive leaves subjected to subsequent drought stress. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.

**Table 3.** Effect of cross-tolerance on CAT, superoxide dismutase, SOD, and guaiacol peroxidase, GP, activities (U mg<sup>-1</sup> protein) of olive leaves subjected to subsequent drought stress. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.

Treatment	CAT	SOD	GP
С	$2.60\pm0.31~^{a}$	$119.20\pm11.12~^{\rm a}$	$3.17\pm0.50~^{\rm b}$
SPP	$8.08\pm0.33~^{\rm b}$	$187.73\pm9.67^{\text{ b}}$	$5.28\pm0.26$ $^{\rm c}$
NPP	$6.97\pm0.44~^{\rm b}$	$143.50 \pm 12.50 \ ^{\rm a}$	$1.18\pm0.08~^{\rm a}$

#### 3.8. Changes in Phenolic Content and on Antioxidant Activity (IC50)

Drought increased the concentrations of phenolic compounds in the NPPs. However, the SPPs displayed greater accumulation of these compounds compared to the NPPs and to the control (Figure 10a).

The IC50 values of phenolic extracts exhibited a decrease in the SPPs when compared to both the NPPs and the control (Figure 10b), confirming a better antioxidant activity in the primed plants than either.

#### 3.9. PCA Analysis

In order to find the best parameters that can explain the real ameliorative status of salt-primed olive plants under the drought condition, we performed a principal component analysis (PCA) (Figure 11) that correlated all of the investigated variables, including growth parameters (shoot and root biomass accumulation), water status (RWC), gas exchange and PSII photochemistry parameters (A, E, gs, WUE, Ci, Fv/Fm, Y), photosynthetic pigments (total content of Chl and Car), leaf density (D), osmolytes (contents of total sugar and proline), oxidative stress parameters (contents of MDA and EL percent), enzymatic antioxidants (activities of SOD, CAT, GP), nonenzymatic antioxidant (content of phenols and their antioxidant activity), and lipids and their fatty acid composition (total lipids, DBI, UFAs) with the three treatments (C, SPPs, and NPPs).



**Figure 10.** Effect of cross-tolerance on phenol content (**a**) and antioxidant activity (**b**) of olive leaves subjected to subsequent drought stress. Significant differences between the means (at p < 0.05, according to ANOVA) appear with different letters. Values are means  $\pm$  standard error (n = 6), where 'n' is the number of replicates for each group.



**Figure 11.** PCA biplot showing the clustering of nonprimed (NPP), salt-primed (SPP), and control (C) olive seedlings based on growth, physiological and biochemical traits. Shoot dry biomass production (PS shoots); root dry biomass production (PS roots); relative water content (RWC); net CO<sub>2</sub> assimilation (A), transpiration rate (E), stomatal conductance (gs), and intercellular CO<sub>2</sub> assimilation (Ci); water-use efficiency (WUE); maximum quantum efficiency of PSII (Fv/Fm); effective quantum yield of PSII photochemistry (Y); total chlorophylls (Chl); carotenoids (Car); leaf density (D); total sugar (sugar); proline (proline); lipid peroxidation (MDA); electrolyte leakage (EL); superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (GP); phenols(phenols); antioxidant activity (DPPH. IC50); total lipid content (tot. lipids); unsaturated-to-saturated fatty acid ratio (UFAs); double-bond index (DBI).

Our results showed that the first two components (PCs) explained 85,4% of total variance (55% and 30.4% for PC1 (Dim1) and PC2 (Dim2), respectively (Figure 11)).

The PCA analysis revealed a clear separation between the C, NPP, and SPP olive seedlings grown under the subsequent drought condition. The PCA biplot indicated the involvement of osmoprotectants (proline and sugar), the enzymatic antioxidant system (SOD, CAT, and GP), as well as the nonenzymatic one (phenols and, at less degree, carotenoids), and an increase in leaf density together with, to a lesser extent, the raise of structural lipids content in the acquisition of the subsequent drought tolerance in the SPPs.

Interestingly, the PCA analysis indicated that a high WUE was mostly associated with the salt pretreatment under the subsequent drought stress, implying that this priming method positively regulates the WUE.

#### 4. Discussion

Recently, cross-priming was used in many plant species to induce tolerance to abiotic stresses. However, in trees such as olive, there is scarce information about the effectiveness of this approach. In the present work, we have used salt priming as an elicitor to induce tolerance for subsequent drought stress in olive plants cv. *Chétoui*, a drought-sensitive olive cultivar. Here, physiological and biochemical signatures linked to both damage (effects) and defense were determined under the upcoming drought-stress events.

# 4.1. Improvement of Growth, Water Homeostasis, Photosynthetic Activity, Structural Membrane Lipids Contents, and ROS Elimination Confirming the Beneficial Effects of Salt Priming to Mitigate Subsequent Drought Damages in Olive Seedlings

Under stressful conditions such as drought, whole-olive-plant physiology, and thus growth, can be impaired with the alteration of leaf-water status, the inhibition of photosynthesis ability, including photochemical efficiency, and the oxidative degradation of membrane lipids [6,36,44]. Here, as expected, when exposed to water stress for 30 d, the NPPs exhibited significantly reduced growth, as evidenced with the reduction in aerial biomass accumulation compared to the control, which could be the consequence of the loss of cellular turgor as manifested by the significant reduction of the RWC to values below 40%, leading to the inhibition of expansion and cell division [45,46]. Moreover, this reduction in growth parameters might be due to the inhibition of photosynthesis. Indeed, our findings showed that drought severely affected the photosynthetic ability in the NPPs, probably by the photoinhibition of the photochemical apparatus, since these plants showed a very low level of Y and Fv/Fm. Furthermore, drought was observed to reduce the lipid concentration relative to the control, which could result from alterations in the lipid synthesis under water deficit and/or an increase in lipid peroxidation [47]. In fact, the enhanced MDA accumulation in our study, which reflected a high lipid peroxidation rate, and therefore an overproduction of the ROS, confirmed that drought stress in olive plants was associated to oxidative processes resulting in membrane instability. In addition, the observed increase in the EC percent could also support the high level of membrane injury resulting from the oxidative damage.

In contrast, it is clear from our results that salt priming promoted better response to subsequent drought stress in olive by the improvement of the RWC content (70%) and the shoot biomass production. On the other hand, the root biomass production was found significantly unchanged between the NPPs and SPPs, which is a classical response of olive under drought.

Importantly, despite the total restriction of water during 30 d, our physiological data revealed that the SPPs displayed net photosynthesis rate similar to the control, while in the NPPs, the values were severely reduced. Indeed, the SPPs can maintain a healthy photosynthetic capacity despite the decrease of gs and E compared to the control, but these values are higher than those of the NPPs. Therefore, salt priming seemed to help olive seedlings maintain a functional, although low, level of gas exchange. Thus, under water-limited conditions, the SPPs, which displayed a higher RWC, might be able to maintain greater stomatal conductance, eventually resulting in maintaining better photosynthetic activity

than in the NPPs. In this sense, the SPPs showed the maintenance of Ci when compared to the control plants. Contrarily, the Ci increased in the NPPs, reflecting a predominance of nonstomatal limitations to photosynthesis [48]. It was also interesting in this study that, in the SPPs, even after 30 d of water withholding, the Y and Fv/Fm were not affected by stress and exhibited similar values as the control plants, indicating that the PSII integrity was not affected. In accordance with our results, Feng et al. [29] revealed that salt priming enhanced subsequent drought tolerance in wheat plants by maintaining water homeostasis, photosynthetic ability, and biomass production. Furthermore, Yang et al. [30] showed that salt priming increased the rate of photosynthetic assimilation and photochemical efficiency under drought conditions in tomato plants compared with the non-pretreated ones.

In addition, it seemed that priming had a positive effect on leaf membrane integrity. In fact, drought was observed to raise the total lipid content of the SPPs when compared to that of the NPPs and the control, indicating that priming increases the foliar lipid synthesis. This finding could therefore reflect a stability of the functional properties of cellular biomembranes under water stress. In support of this notion, our results revealed that the MDA content and the EC percent in the SPPs were similar to the control (and lower than those of the NPPs), suggesting that no oxidative damage occurred. Similar results was obtained in wheat seedlings [29].

The above results were consistent with that of our PCA (Figure 11), which revealed that the SPPs attained the same extent of tolerance against stress when compared to the control plants.

From these results, it is clear that the first exposure of the olive plants to salinity made them more effective in responding to the subsequent stress (Figure 12). This is because there are many similarities between salinity and drought stresses. In fact, it is well-known that these two stresses activate common signaling pathways and defense responses [17], which allows olive plants to establish efficient acclimation mechanisms for cross-tolerance. Evidently, all these beneficial effects of cross-priming observed in the olive plants under the subsequent drought event are the complex result of many physiological, metabolic, and genetic mechanisms, and we considered that changes in all of which could be implicated in this observed drought acclimation. The possible physiological and metabolic modulations linked to this process are further discussed below (Figures 11 and 12).



**Figure 12.** A model scheme for cross-tolerance responses (physiological and biochemical) responses in salt-primed plants relative to nonprimed ones.

# 4.2. Physiological and Metabolic Adjustments Induced by Cross-Priming under Subsequent Drought in Young Olive Seedlings

4.2.1. Cross-Priming Induced Accumulation of Osmoticums and Morphofunctional Mechanisms in Olive Leaves

Indeed, the maintenance of the RWC at 76% under limited water conditions would help the SPPs to have minimal cell turgidity, indicating a good ability of osmotic adjustment, a famous physiological mechanism adopted by the olive tree, through the accumulation of osmoticums, notably proline and sugar, to mitigate drought effects [49]. These small compounds could have double roles, either in osmoregulation or in osmoprotection, as lowmolecular-weight chaperons and ROS scavengers [50,51]. In our study, the SPPs exhibited a significant increase in both total sugar and proline amounts relative to the NPPs. This greater increase on osmoticums would favor the better maintenance of water homeostasis in the SPPs, leading to drought acclimation towards activating other morphofunctional processes of adaptation.

One of the best-studied morphofunctional mechanisms acting as first lines of defense against immediate dehydration is stomata functionality [52,53]. We have already reported that the SPPs are able to maintain a healthy photosynthetic capacity despite the decrease of gs and E. Similar results were found by Ennajeh et al. [54] in olive plants; this study showed that under drought conditions, *Chemlali*, a drought-tolerant olive cultivar, maintained high levels of photosynthetic assimilation despite a decrease in E and gs. These authors have explained this finding by morphoanatomical changes. In the same way, it has been reported that modifications in leaf anatomical features impair the components of CO<sub>2</sub> conductance diffusion from the substomatal cavities to the sites of carboxylation, ensuring photosynthetic activity even at a low level of stomatal conductance [55,56]. Recently, Shinga et al. [57] reported that both the improved plant water status and reduced ABA signaling improved the stomatal opening and thus significantly increased leaf photosynthesis under the salinity stress. Moreover, it has been reported that variations in leaf thickness and/or leaf density that contribute to drought tolerance could be the reasons for the decrease in the leaf size and differences in specific leaf area [33,58]. Our results showed that leaves from the SPPs exhibited an increase in their density compared to those of the NPPs. This characteristic would confer a more mechanic stability to leaves of the SPPs [59]. Previous studies suggested that the ameliorative action of this priming method was due to the fact that salt-stress signal cascades might induce downstream overlapping transduction pathways that improve the photosynthetic acclimation of seedlings under low-temperature stress, which is consistent with the mechanisms of cross-tolerance [27,60,61].

Interestingly, our results indicated a significant increase of the WUE in the SPPs relative to the NPPs. Further, the PCA analysis indicated that a high WUE was mostly associated with salt pretreatment under the subsequent drought stress, implying that this priming method positively regulates the WUE to help maintain the growth and survival of olive seedlings under drought conditions. In agreement with our results, Shinga et al. [57] found that drought priming clearly enhanced the WUE level of wheat plants under subsequent salinity stress. Concerning photosynthetic pigments, their contents remain unchanged as compared to the control both in the SPPs and the NPPs.

4.2.2. Cross-Priming Allowed to Olive Plants to Maintain Their Structural Lipid Contents as Well as Their Fatty Acid Composition to Protect the Photosystem Functionality

It is well-known that the polyunsaturated fatty acid deficiency made the PSII extremely sensitive to photoinhibition in Kanervo et al. [62]. In fact, polyunsaturated fatty acids were known to be involved in the photosystem's integrity and functionality [63] and for the establishment of the appropriate balance of the bilayer and nonbilayer lipids within photosynthetic membranes [64,65]. In the current study, the total lipid content of the SPPs increased when compared to that of the NPPs and the control, indicating that priming increases the foliar lipid synthesis. Despite the modification in fatty acids composition, which is marked by a decrease in the majority of fatty acid percentages, except that of C18:3, the SPPs showed, compared to the control, a slight diminution in the UFAs/SFAs ratio. Nonetheless, this latest remains higher than that of the NPPs, although the DBI index remained constant. Such increase in the fatty acids content, as well as their unsaturation level, could therefore contribute to the protection and the stability of the functional organization of photosynthetic membranes under water stress. Indeed, many works had associated the development of acquired cross-tolerance with a change in the fatty acid and lipid metabolism. From these works, it seems that increasing the unsaturation rate of fatty acids is positively correlated with abiotic stress tolerance, as it maintains membrane stability and enhances subsequent stress tolerance [66,67]. For example, the research on Kentucky bluegrass revealed that, under heat stress conditions, drought-primed plants exhibited, compared to the control, higher total fatty acids [66], with a greater increase in the content of linolenic acid (C18:3). Furthermore, the rise in C18:3 and the unsaturation degree has been reported to have a major role in acquiring drought, heat, and cold tolerance [65,67].

#### 4.2.3. Cross-Priming Induces Antioxidant Defense to Survive Drought Stress

Here, unlike the NPPs, the maintenance of redox homoeostasis in the SPPs reflected the efficacy of salt priming to protect them against drought-induced oxidative injuries. To prevent stress-induced cell destruction, plants usually require the enzymatic antioxidant defense. Importantly, NaCl priming increased greatly the activities of the SOD, CAT, and GP in the SPPs relative to the NPPs, indicating an enhanced superoxide radical and  $H_2O_2$  scavenging ability in the SPPs. It seems that the regulatory effect of salt priming greatly depends on the ROS scavenging ability by enhancing the activities of enzymatic antioxidant. Accordingly, Feng et al. [29] noticed that the activation of the enzymatic antioxidant system through salt priming plays a key role in the acquisition of drought tolerance in cotton seedlings.

Further, during drought stress, plants often rely on phenolic compounds due to their role in detoxifying free radicals [10]. Our results revealed a higher augmentation in the phenol contents of the SPPs when compared with the NPPs. Importantly, our results indicated a better antioxidant activity in the SPPs than either, reflecting an effective phenolic–antioxidant defense system in these plants. Additionally, the great accumulation of proline and sugar observed in the SPPs could also participate to the protection against oxidative damage [50,51]. This better antioxidant system in the SPPs might be directly linked to improved resistance and hence reflects that salt priming enhanced tolerance defense responses, and thus physiological performance and growth, in olive seedlings. In the same line of ideas, Hossain et al. [68] hypothesized that the retention of the imprint of heat priming protects the plants from subsequent salt- and drought-induced oxidative injuries by amplifying the antioxidative enzyme defense systems.

Based on the PCA investigations, it is clear that metabolite adjustments (soluble sugars and proline), the enzymatic antioxidant system (SOD, CAT, and GP activities), as well as the nonenzymatic one (phenols and, at less degree, carotenoids), and the increase in leaf density as well as, to a lesser extent, the raise of structural lipids content are the principal strategies employed by the SPP plants to prevent stress damages under subsequent drought.

#### 5. Conclusions

The results of this study support the key role of cross-priming in improving drought tolerance in olive plants. Consistently, our findings revealed that salt priming substantially modulated the physiological and biochemical responses of olive plants to subsequent drought (Figure 12). Accordingly, the possible strategies that seemed to perform a major role in the development of this improved tolerance to drought in olive seedlings involved the accumulation of osmoticums (total sugar and proline), improved the ROS scavenging system, and the increase in leaf density together with, to a lesser extent, the raise of structural lipids content. Importantly, these extensive modulations suggests that crosspriming can potentially confer immunity to the olive plant organism against drought through the storage of salt-stress stimuli. Interestingly, for the first time in the olive tree, cross-priming could be considered as an efficient approach not only to improve plant responses to drought stress but also to convert this stressful spell to beneficial ones in terms of the WUE (Figure 11), a highly researched parameter in agronomy to improve crop yield.

Therefore, cross-priming might be a promising alternative for the amelioration of olive stress tolerance; however, these results need to be further validated under field conditions.

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