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The Effect of Low Temperature on Physiological, Biochemical and Flowering Functions of Olive Tree in Relation to Genotype

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Abstract: Olive tree growth and reproduction are severely affected by temperature extremes, compromising fruit yield. In that aspect, the olive varieties "Koroneiki" and "Mastoidis" were employed in a mild cold stress experiment, imitating night frost incidents to assess their biochemical, physiological and reproductive functions in relation to genotype. The physiological performance of the stressed plants was not significantly altered, suggesting that both cultivars were well adapted to mild cold night stress. The biochemical response of the plants, regarding antioxidant enzymes, H_2O_2 and TBARS accumulation, confirmed that both cultivars could cope with the stress applied. The mRNA levels of the *PPO* gene, which participates in hydroxytyrosol biosynthesis and plant defense, were elevated after 24-h stress at 0 °C, in both cultivars with "Mastoidis" plants exhibiting higher levels for a longer period. Three more genes involved in hydroxytyrosol biosynthesis upregulated their expression levels as a response to cold stress. The numerous plant phenology aspects measured reinforced the conclusion that both cultivars responded to the stress applied. The results of the present study may contribute to better understanding olive tree adaptive responses to low temperature events, an abiotic stress condition that is often present in an open plantation, thus assisting farmers on breeding and cultivar selection.

Keywords: antioxidant enzymes; climate change; frost damage; photosynthesis; plant phenotyping

1. Introduction

The olive tree is a crop of high economic significance, and a cornerstone of a healthy diet due to the high nutritional value of its products, namely table olives and olive oil. Olive cultivation and spread are geographically limited by temperature, an important climatic factor [1]. Tree growth, yield and olive oil quality are favored in areas with moderately cold winters as well as warm and dry weather during fruit maturation, thus olive cultivation is located mainly in the Mediterranean-type climate regions. Olive trees are not so resistant to frost and different olive cultivars show diverse reactions to cold stress [2].

As a xerophytic plant, the exposure to drought and low temperatures is a major limiting factor for growing olives. Olive trees cannot tolerate temperatures less than −7 °C, but demand certain exposure

to low temperature, at the range of 5–15 °C, during winter to overcome dormancy and be able to develop flowers in the subsequent spring [3]. Olive flower induction was shown to be regulated by the expression of the *FLOWERING LOCUS T* gene [4]. Low temperature incidents during flowering may be responsible for an increase in shotberry incidence, which are abnormally grown small fruit commonly resulting from parthenocarpy [5].

In addition, leaf injury induced by frost incidents during spring and autumn retards overall tree growth and has a serious impact on plant yields and development [6]. Frost damage is mainly related to the physical disruption of cell structures by ice, and subsequent dehydration [7], due to the increased water potential of the cellular interior compared to the space between cells where ice crystals are formed [8]. In general, olive trees may face dramatic consequences by frost incidents, especially in the first years of plantation, and the most common symptoms on the aerial parts of the plant consist of shoot tip necrosis, leaf fall as well as bark wounding, while below −12 °C, olive trees may be completely damaged and die.

Photosynthesis is a physiological process that is highly susceptible to abiotic stress factors [9]. In olive trees though, previous studies reported that the photosynthetic apparatus is adapted to various types of abiotic stresses, like excess UV radiation and drought [10–12]. Moreover, abiotic stress enhances the built up of reactive oxygen species (ROS), such as superoxide anion radical (O^{2-}), hydroxyl radical (OH^{-}) and hydrogen peroxide (H_2O_2) in plant cells, and a series of cascade reactions for ROS neutralization, including enzymatic (detoxifying enzymes such as superoxide dismutase-SOD, ascorbate peroxidase-APX and guaiacol peroxidase-GPX) and non-enzymatic (antioxidants of low molecular mass, such as ascorbate, glutathione and carotenoids) mechanisms, are being triggered [13,14]. In fact, SOD and GPX activities were increased in stress-tolerant plants [15–17]. In addition, when exposed to cold stress, olive plants increased the activity of two enzymes implicated in response to abiotic stress: phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO). The response of PPO, in particular, was suggested to contribute to the antioxidant protection of plant tissues [18]. The elevated expression of the *PPO* gene in plants as a response to pathogens attack has been demonstrated [19–21], while more recent studies linked increased PPO activity with abiotic stress, such as chilling [22–24]. The *PPO* gene in olive is involved in the production of a strong antioxidant, hydroxytyrosol (HT), along with the genes *TYROSINE DECARBOXYLASE* (*TDC*), *COPPER-AMINE OXIDASE* (*CuAO*) and *ALCOHOL DEHYDROGENASE* (*ALDH*) [25–28].

Climatic conditions, in general, play a decisive role in tree phenology and adaptation [29]. Temperature during spring is considered to have great effect on olive flower development [30], pollen germination [31], fruit set and development [5] as well as the number of flowers per panicle [32]. In addition, temperature levels as low as 0 °C may cause drastic damages on flower buds and flowers [33]. Flowering, pollination, and fruit set were shown to be negatively affected by prolonged periods of low spring temperatures [34]. Low temperature during flowering may also retard pollen germination [31] thus jeopardizing fruit set.

The main aim of the present investigation was to assess olive physiological, biochemical and reproductive responses under the imposition of mild freezing stress (0 ± 2 °C), imitating night frost incidents. We used a multidimensional approach studying visual changes (leaf damage, shoot growth, number of inflorescences), physiological performance (gas exchange characteristics), biochemical response (antioxidant enzymes) and molecular mechanisms (expression levels of HT biosynthetic genes) towards the comprehension of olive capacity to cope with unfavorable environmental conditions. The olive cultivars “Koroneiki” and “Mastoidis” were selected due to their significance in current and new olive plantations.

2. Materials and Methods

2.1. Plant Material and Treatments

Two-year-old plants of *Olea europaea* L. "Koroneiki" (50 plants) and "Mastoidis" (50 plants), were grown in an outdoor plant nursery in the premises of Institute for Olive Tree, Subtropical Crops and Viticulture in Chania, Greece. Olive plants, propagated clonally by rooting cuttings, were grown in pots (12 L in size) filled with a mixture (3:1) Loamy-Sandy soil/perlite and were frequently irrigated to avoid drought stress, as described previously [35]. One application of fertilizer, 500 mL of 10 g L^{-1} of 20:20:20 (NPK), was added to each pot in early March. Climatic data of average monthly minimum and maximum temperatures during the trials are presented in Supplementary Figure S1.

Olive plants were exposed to cold stress (0 ± 2 °C) in a room with controlled conditions in a simulation of night frost incidents (16 h in the cold chamber at 16:00–08:00 in the dark, followed by 8 h outdoors at ambient temperature and sunlight at 08:00–16:00) in March and replicated in April of the same year under similar environmental conditions to confirm our results. The treatment was applied for 4 consecutive days and measurements were done every day (1, 2, 3, 4th). Due to excessive data collection without statistical differences, we present herein data of the main time points. This way, we simulated a frost incident during 2 consecutive nights and a frost incident during 4 consecutive nights. Daily mean air temperature during the experiment was minimum 12.2 °C and maximum 17.8 °C in March and minimum 14.7 °C and maximum 20.1 °C in April. During that period, the plants were under active vegetative growth and flower bud development.

2.2. Gas Exchange Measurements

Three photosynthetic parameters, net photosynthetic rate (Pn), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci), were assessed by gas exchange measurements. The measurements were made between 10:30 pm and 12:30 a.m. Four leaves (one leaf from each of four plants) from each treatment, variety and date were used to measure Pn, Gs and Ci using a portable gas exchange system (LI-6400, Li-Cor Biosciences, Lincoln, NE, USA). Specifically, we used the third and fourth fully expanded leaves starting from the top of the shoot at a persistent 400 μmol mol^{-1} [CO_2] in all the dates. For each date, all measurements were realised at fixed Photosynthetically Active Radiation (PAR) values, temperature and relative humidity (RH) levels, according to the daily weather conditions.

2.3. Determination of Antioxidant Enzymes Activity

A different set of four leaf samples (third and fourth leaves starting from the end of the shoot) per cultivar and treatment were harvested at the end of each treatment and directly frozen at −80 °C for the analyses of SOD and GPX activities. The medium for extraction contained 0.1 M potassium phosphate buffer (pH 7.6), with 1 mM ethylenediaminetetraacetic acid disodium salt, 0.5 mM ascorbate as well as 1% PVPP (polyvinylpolypyrrolidone). Tissues (0.2 g) were homogenised with 1.5 mL extraction buffer and subsequently we centrifuged the homogenate at 13,000× *g* for 30 min. Then, we used the supernatant to determine the enzyme activity. Crude enzyme extract absorbance was calculated by a Hitachi U-1100 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Total SOD (EC 1.15.1.1) and GPX (EC 1.11.1.7) activities were determined using the methodology described by [10].

2.4. Determination of H_2O_2 and Thiobarbituric Acid Reactive Substances (TBARS)

Olive leaves (0.5 g) were homogenised using 5 mL trichloroacetic acid (TCA) 0.1% (*w*/*v*) inside an ice bath. Then we centrifuged the homogenate at 10,000× *g* for 30 min and we employed the supernatant to determine hydrogen peroxide as well as lipid peroxidation. H_2O_2 content was determined in a spectrophotometer following the reaction with potassium iodide (KI), according to the methodology of [10]. Lipid peroxidation was calculated by the quantity of malondialdehyde (MDA). TBARS was quantified using the methodology presented in [10] based on the absorbance of the supernatant at 532 nm, following the subtraction of non-specific absorption at 600 nm and 440 nm.

2.5. Phenological Measurements

Four shoots per treatment and variety were tagged and several phenotypic characters were measured every 10 days in spring: shoot length, leaf number, leaves with symptoms (browning, tip necrosis, abnormal growth), phenological stage and the total number of inflorescences. All phenological measurements and observations were implemented according to [36].

2.6. Pollen Germination

To determine pollen germination, we used closed flowers to ensure pollen originality. Pollen was subsequently cultured at 25 °C for 24 h with 16 h light/8 h dark, in an incubation chamber (Kottermann 2770, D3162; Hanigsen, Germany). Subsequently, pollen germination as well as pollen tube length were determined. A solid growing medium was used with 15% (*w*/*v*) sucrose, 0.8% (*w*/*v*) agar, 60 ppm tetracycline hydrochloride and 100 ppm boric acid according to [37]. Measurements of germination were done on four petri dish fields with at least 200 pollen grains from each case. Measurements of tube length were realized for 80 pollen tubes for each case. The pollen germination trial was repeated once to double replications.

2.7. RNA Isolation and Gene Expression Analysis

In gene expression study, another group of olive plants of the same varieties, age and growth conditions, were exposed to cold stress (0 ± 2 °C) in a growth chamber for 24 h (9 a.m.–9 a.m.) during the subsequent winter (December and replicated in January). From December to January plants were grown outdoors at ambient temperature and sunlight before transferring to the cold room. Different plants were used in December and in January trial to avoid cumulative responses. The time points for the gene expression study were 0.5, 1, 2, 3, 6 and 24 h after treatment initiation. Leaf samples (third and fourth leaves starting from the top of the shoot) per cultivar and treatment were collected (n = 4) in each time point and were directly frozen at −80 °C. Subsequently, we isolated total RNA by NucleoSpin® RNA Plant kit (Macherey-Nagel, Duren, Germany). The expressions of specific mRNAs were determined by reverse transcription (RT) and then by quantitative PCR (qPCR) employing gene-specific primers according to [28] (Table 1). Complementary DNA synthesis was realized with 0.5–1 μg DNAse-treated RNA with the kit Tetro Reverse Transcriptase (Bioline, London, UK) or PrimeScript Reverse Transcriptase (Takara, Clontech, CA, USA) with three separate biological replicates. The total reaction quantity was 40 μL and then it was diluted five times in nuclease-free water. Quantitative PCR analysis was done in reactions with 1× buffer including KAPA™ SYBR® Green FAST qPCR Kit Master Mix (KapaBiosystems, Cape Town, South Africa). In every 10 μL reaction, we used 2 μL of the RT product. Then we did absolute quantification for qPCR products using *OeTIP41* as a reference gene. Gene expression was presented as reference gene-normalized levels of the target genes. StepOne Software v2.1 was employed to analyze gene expression (Applied Biosystems, Foster City, CA, USA).

2.8. Statistical Analysis

The software SPSS (SPSS Inc., Chicago, IL, USA) was used for data analysis employing one-way analysis of variance (ANOVA). The least significant difference (LSD) test at $p = 0.05$ was employed to detect statistically significant differences among treatments. For gene expression, an additional paired t-test was employed for each time point to detect differences among olive cultivars. Fifty plants of each cultivar were used in total. In each experiment, 4 plants of each cultivar were used for each treatment.

Table 1. Primer nucleotides sequences used in quantitative PCR (qPCR).

Primer Code	Gene Name	Gene Accession Number	Oligonucleotide Sequence (5′–3′)	Strand
KVI106	*OeCuAO*	KP968841	CCTTACCTCCAGCTGATCCAT	Forward
KVI107			GATCATTGGGATCTCCATAGG	Reverse
KVI108	*OeTDC*	KP968842	GGCTACTGCATTCGGGTTAACA	Forward
KVI109			GTGTGCATTGAAACTGAATGAATC	Reverse
KVI110	*OePPO*	KP968843	CTATGAAAGAATATTGGGCAAACTG	Forward
KVI111			ACGCTGCGAATCATTTCACTATAT	Reverse
KVI140	*OeALDH*	KP968844	TGGTACATGGTCTGAATATGTCG	Forward
KVI141			CCTCCAGGCAGATCCAA	Reverse
KVI164	*OeTIP41*	XM_023020543	CAACGGTGTCTCTCTTTTGACAGT	Forward
KVI165			TCATAAGCACTCCATCCACTCTCA	Reverse

3. Results

3.1. Photosynthesis Parameters

Net photosynthetic rate (Pn) measured in both cultivars highlighted their difference, as in control plants, without cold treatment, "Mastoidis" (Figure 1a, blue line) displayed lower Pn than "Koroneiki" (Figure 1b, blue line). In general, cold stress treatment did not affect the photosynthetic rate of the plants. In cv. "Mastoidis", the 4-day treated plants followed the same trend with the control plants while the 2-day stressed plants exhibited an increased value during the third day of the treatment. In "Koroneiki", the decrease in the photosynthetic rate of stressed plants was not statistically important. Stomatal conductance (Gs), also measured as a photosynthetic parameter, followed the same trend in both cultivars as Pn (Figure 1c,d). "Mastoidis" control plants (Figure 1c) had lower Gs than "Koroneiki" control plants (Figure 1d). During cold stress, "Mastoidis" 4-day stressed plants responded as the control plants, while the 2-day stressed plants had a non-significant increased Gs value the third day, as also observed for Pn. "Koroneiki" 2-day stressed plants exhibited decreased Gs in the last two days, same as Pn measurements, but this reduction was not statistically significant.

The quantification of intercellular CO_2 (Ci) (Figure 1e,f) revealed no differences between the control plants of the two cultivars. Moreover, the Ci values of the stressed plants were not affected by the night frost treatment, implying that both cultivars were able to tolerate the stress applied.

3.2. Antioxidant Enzymes Activities, H_2O_2 and TBARS

Significant downregulation of GPX activity was observed in the leaves of "Mastoidis" plants at the 4th day of stress compared to the control (Figure 2a) but not at all in "Koroneiki" (Figure 2b). No significant effect on SOD, H_2O_2 and TBARS was observed in either two or four day-low temperature treatment in both olive varieties (Figure 2c–h). The extent of H_2O_2 production was significantly lower in "Koroneiki" trees under the 4-days low temperature treatment, compared to 2-days stressed trees of the same cultivar (Figure 2f) and also decreased, but not significantly, in "Mastoidis" (Figure 2e).

3.3. Phenology

The number of leaves on each plant was counted for two months revealing similar numbers for stressed and control plants (Figure 3a,b). Longer periods of low temperatures, both in M/4D and K/4D samples, seemed to have a positive effect on shoot length, however the differences were not statistically significant. Likewise, the shoot length in all the plants exhibited comparable values, whereas the plants exposed to more days of cold stress had a slight increase in their shoot length, although not statistically important. (Figure 3c,d).

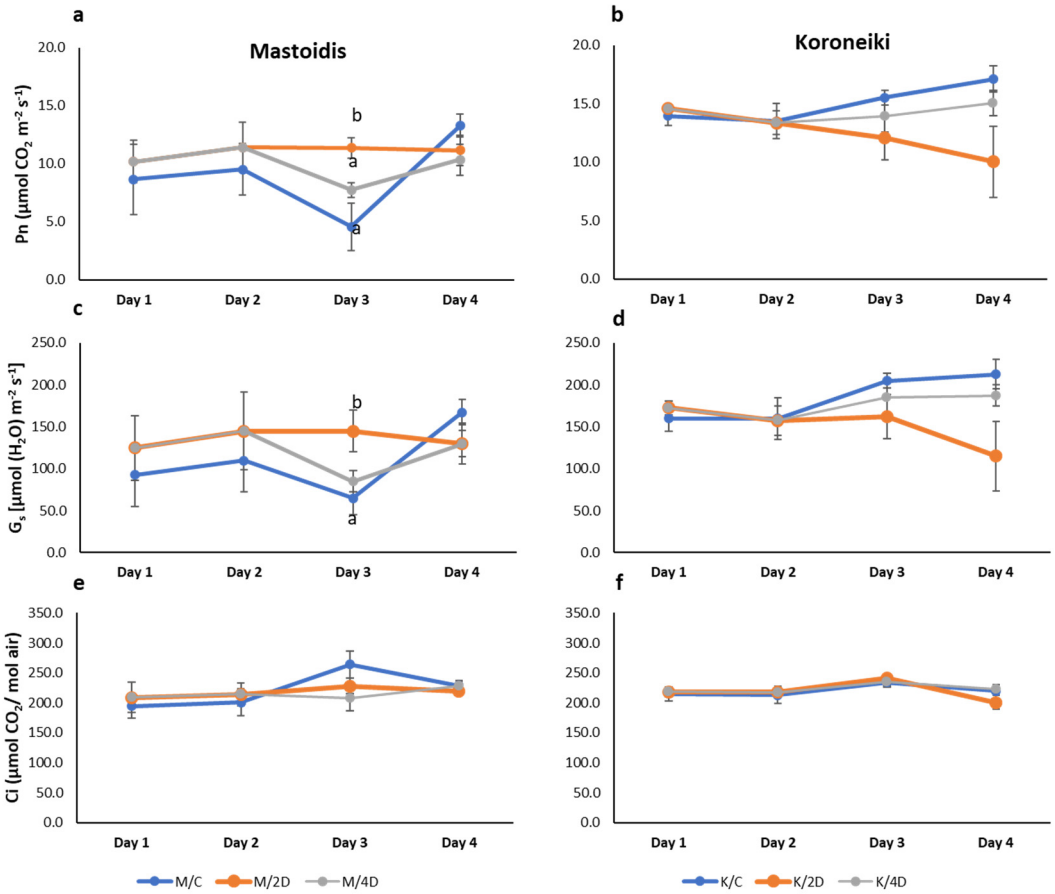

Figure 1. (**a**,**b**) Net photosynthetic rate (Pn); (**c**,**d**) Stomatal conductance (Gs); (**e**,**f**) Intercellular CO_2 (Ci), in cultivars "Mastoidis" and "Koroneiki" in response to 2-days (2D) and 4-days (4D) low temperature stress. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test), sample size n = 4. (K/C) "Koroneiki" control; (M/C) "Mastoidis" control. The error bars represent standard error.

The flowering phenological stage of the plants during the 2-month study period revealed that all plants of "Koroneiki" were premature compared to "Mastoidis" (Figure 3e,f). During the first month of the analysis, "Koroneiki" plants were always in a more advanced stage while at the second month "Mastoidis" plants accelerated flower development and finally reached the same stage at the end of the 2 months.

Other phenological aspects studied, like the number of leaves with symptoms, showed differences between the stressed and the non-stressed olive trees. In general, "Koroneiki" plants had a significantly higher number of leaves with symptoms at the first date of the analysis, but at the end of the study period both cultivars had almost the same number (Figure 4a). At the end of May, the 4-days stressed "Koroneiki" plants had significantly more leaves with symptoms than the 2-days stressed plants, while "Mastoidis" 2-days stressed plants had a significantly higher number of leaves than the control plants in the last date of measurements (Figure 4b).

"Mastoidis" stressed plants had a higher number of inflorescences at the beginning (Figure 5a), compared to "Koroneiki" plants, which kept almost the same number of inflorescences during the two months of study. The total number of inflorescences did not alter significantly between the treated plants in "Koroneiki" (Figure 5b).

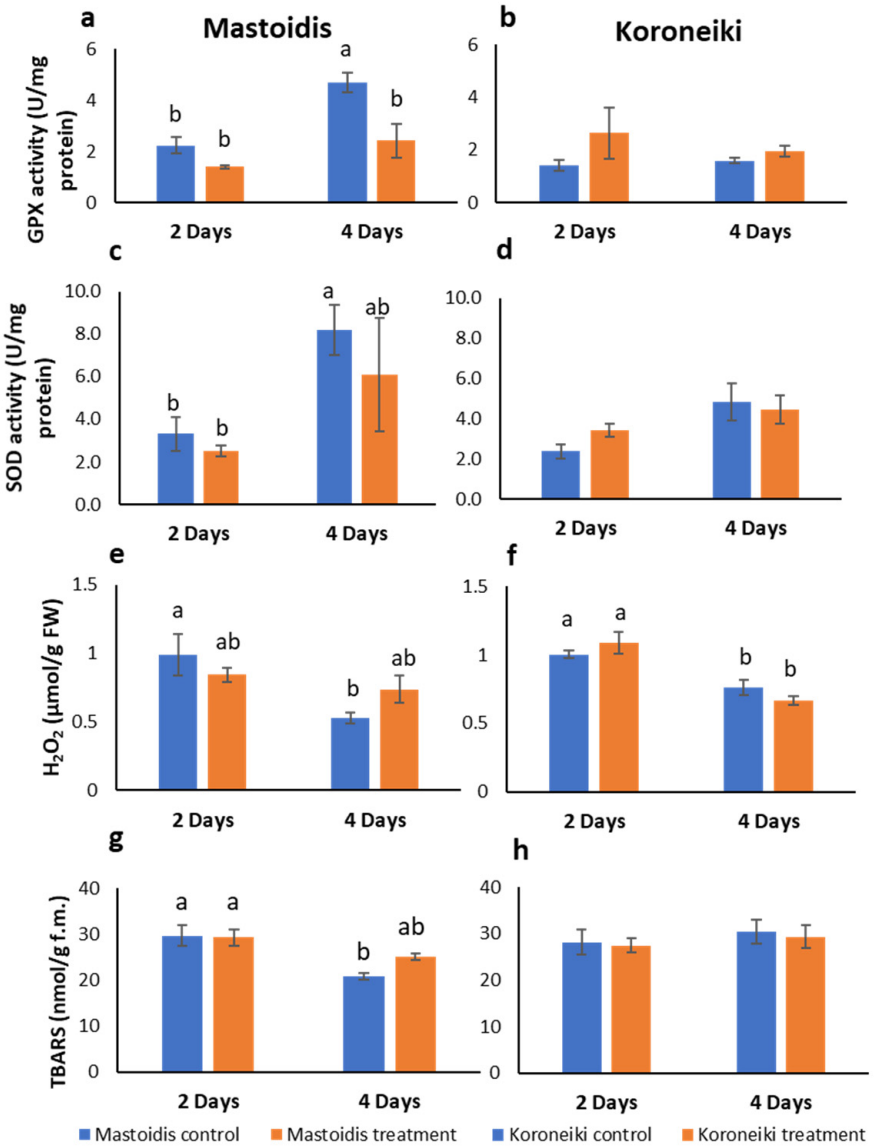

Figure 2. (**a**,**b**) GPX activity; (**c**,**d**) SOD activity; (**e**,**f**) H_2O_2 concentration; (**g**,**h**) TBARS concentration in cultivars "Mastoidis" and "Koroneiki" in response to 2-days and 4-days low temperature stress. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test), sample size n = 4. The error bars represent standard error.

3.4. Pollen Germination

The pollen germination rate was not affected in "Mastoidis" (Figure 6a), while it significantly decreased in the "Koroneiki" 2-day treatment (Figure 6b). Pollen tube length was significantly decreased at 4-day stressed plants in "Mastoidis" (Figure 6c) but not in "Koroneiki" (Figure 6d).

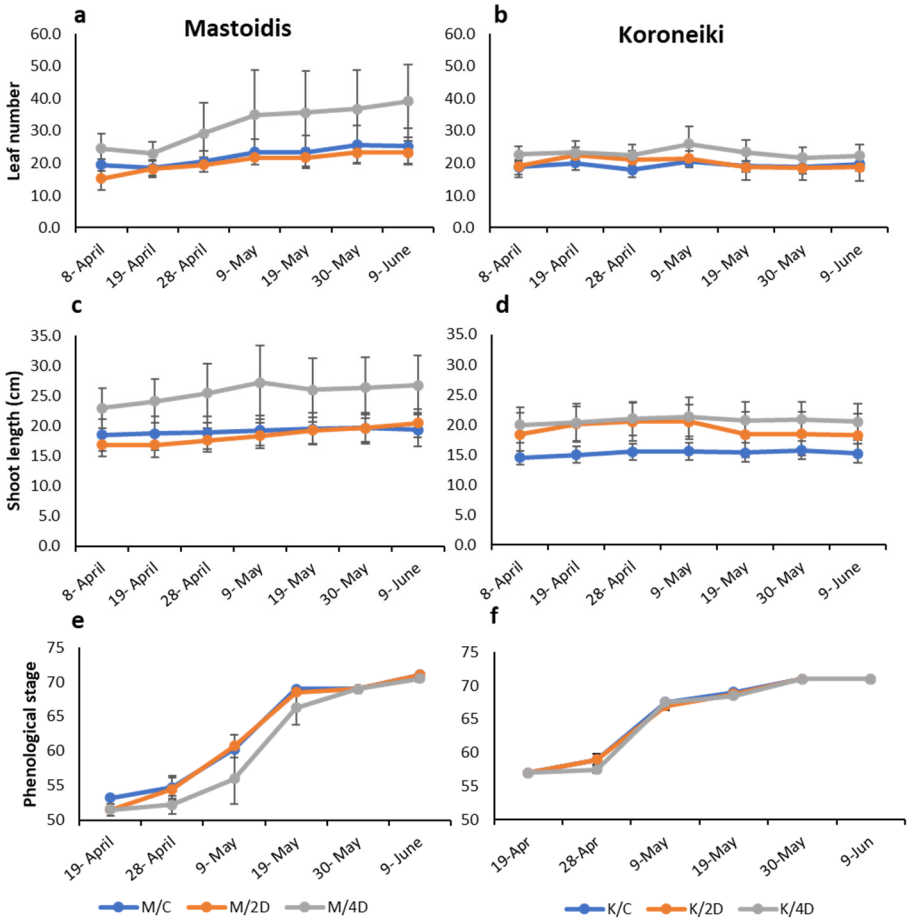

Figure 3. (**a**,**b**) Leaf number; (**c**,**d**) shoot length; (**e**,**f**) flowering phenological stages according to [4] of cultivars "Mastoidis" and "Koroneiki" under the 2-days (2D) and 4-days (4D) cold treatments for 2 post-treatment months. (K/C) "Koroneiki" control; (M/C) "Mastoidis" control. Sample size n = 4. The error bars represent standard error (LSD test). For example, flowering phenological stage 55 corresponds to "flower cluster totally expanded, floral buds start to open'.

3.5. Gene Expression Analysis

The application of a 24-h cold treatment at 0 °C in "Koroneiki" and "Mastoidis" plants verified an increase in *OePPO* expression levels in both cultivars. A rapid 10-fold increase was observed ($p < 0.0001$, LSD test) immediately after stress was applied, at 0.5 h (Figure 7a). Thereafter, expression levels dropped in "Koroneiki" leaves whereas in "Mastoidis" leaves kept increasing, reaching maximum levels at 2-h of cold treatment ($p < 0.0001$, LSD test). The expression analysis of three more genes, with a potential role in HT biosynthesis and may be involved in stress response, after 24-h of cold stress revealed differences between cultivars. The *OeTDC* gene expression was unaffected in "Koroneiki" plants ($p > 0.9$, LSD test), whereas in "Mastoidis" a significant increase in expression was observed at 2-h after stress ($p = 0.0488$, LSD test), at about 3-times higher than control (Figure 7b). The expression of *OeCuAO* was significantly increased in both cultivars, to 4-fold in "Koroneiki" ($p = 0.0063$ after one hour of treatment, LSD test) and 3-fold in "Mastoidis" ($p = 0.0116$ after two hours of treatment, LSD test), dropping thereafter (Figure 7c). The *OeALDH* gene expression increased rapidly in both cultivars, reaching a peak at 0.5-h in "Mastoidis" leaves ($p < 0.0001$, LSD test) and one hour in "Koroneiki" leaves ($p < 0.0001$, LSD test) and remaining at elevated levels during the 24-h stress (Figure 7d).

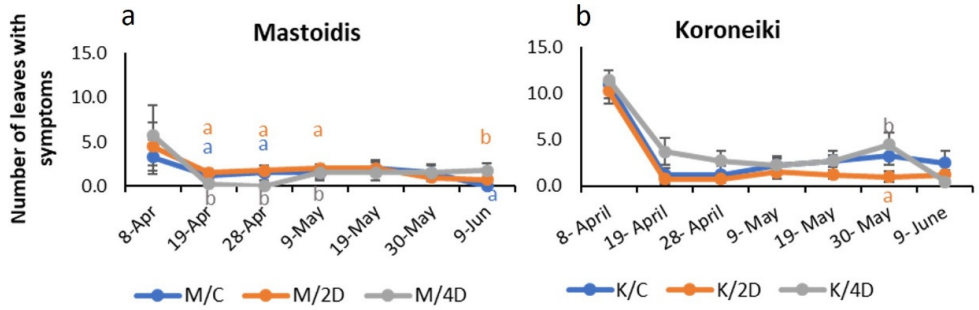

Figure 4. The number of leaves per shoot with symptoms of both cultivars (**a**) "Koroneiki" and (**b**) "Mastoidis" under the 2-days (2D) and 4-days (4D) low temperature treatments for 2 months. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test), sample size n = 4. (K/C) "Koroneiki" control; (M/C) "Mastoidis" control. Error bars depict standard error.

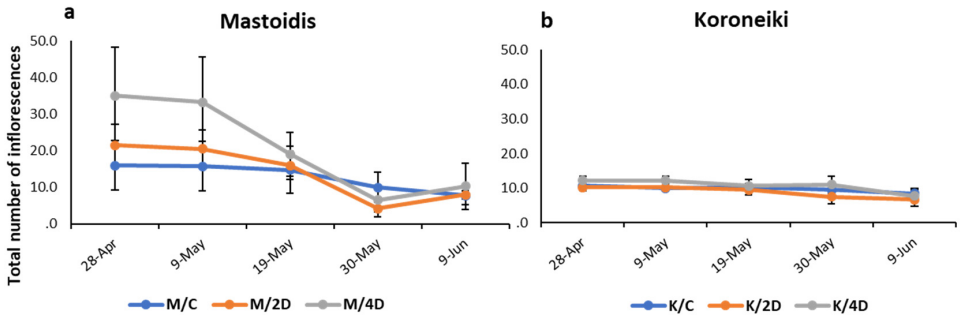

Figure 5. "The total number of inflorescences of (**a**) "Mastoidis" and (**b**) "Koroneiki" under the 2-days (2D) and 4-days (4D) low temperature treatments for one month. (K/C) "Koroneiki" control; (M/C) "Mastoidis" control. Sample size n = 4. Error bars depict standard error (LSD test).

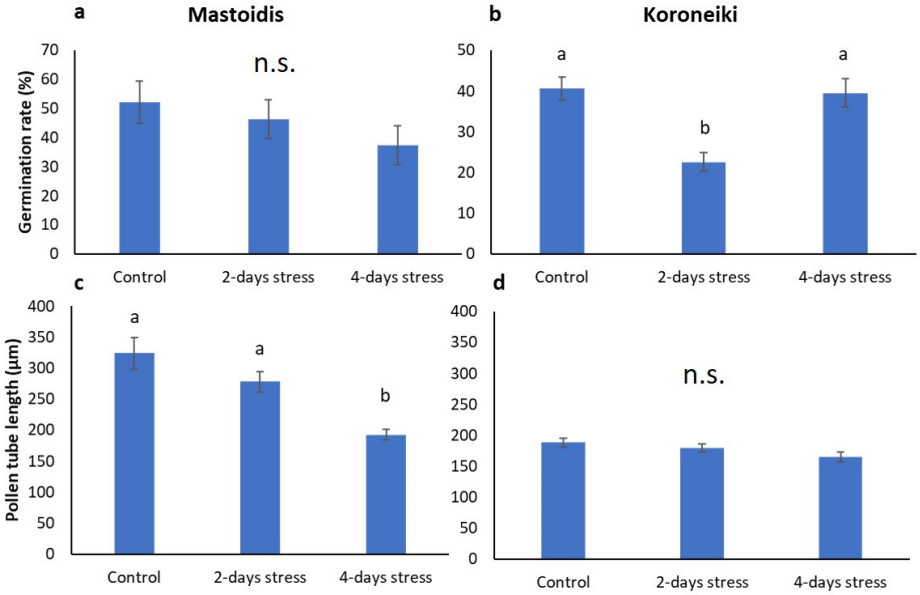

Figure 6. (**a**,**b**) germination rate; (**c**,**d**) pollen tube length of both cultivars "Mastoidis" and "Koroneiki" under the 2-days and 4-days low temperature treatments. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test). Error bars depict standard error. "n.s.' stands for non-significant differences.

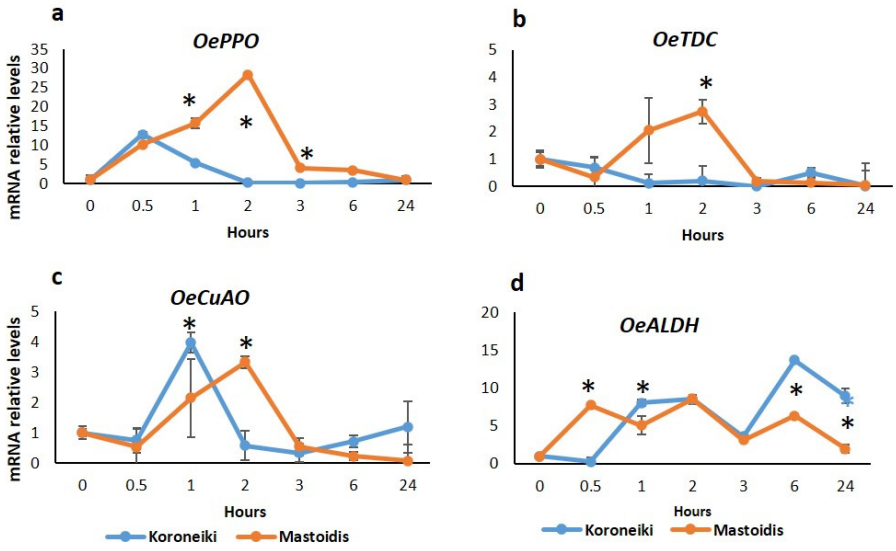

Figure 7. Relative mRNA levels of four potential HT biosynthetic genes (**a**) *OePPO*; (**b**) *OeTDC*; (**c**) *OeCuAO*; (**d**) *OeALDH* for both cultivars "Koroneiki" and "Mastoidis", during a period of 24-h cold stress. Error bars depict standard error. The least significant difference (LSD) test at $p = 0.05$ was employed to detect statistically significant differences among time points for each cultivar (not presented in the graphs for clarity of view). For gene expression, an additional paired t-test was employed for each time point to detect differences among olive cultivars and are marked with asterisks ($p \leq 0.05$).

4. Discussion

A down-regulation of the photosynthetic performance caused by decreased air temperature levels is a widely documented response of cold-prone plants [38]. In the present work, the results of photosynthetic rate, stomatal conductance and intercellular CO_2 in stressed and control plants of "Mastoidis" and "Koroneiki" reveal only minor effects of cold stress. However, we have to mention that "Mastoidis" stressed plants kept higher photosynthetic values than "Koroneiki" stressed plants, suggesting that "Mastoidis" plants were able to cope better with night-frost incidents. The exposure of cold-stressed plants to ambient outdoor temperature for 8 h/day might be enough to maintain a stable photosynthetic activity, explaining the non-significant effects on photosynthesis parameters. Previous studies reported that, when exposed to minimum temperatures, olives responded with a decrease in stomatal conductance [39,40]. Additionally, disorder in water balance was observed even when soil moisture was sufficient, if low temperature incidents occurred [41]. Fast recovery of olive to short-term stress, as reported in our study, has also been shown for high temperature and drought implying great hardiness and adaptive capacity of olive trees to mild stresses [42]. However, under severe stress or longer exposure, permanent damages in leaf physiological functioning were observed [43].

When plants are exposed to an unfavorable environment, enhanced activities of ROS-scavenging are usually observed as a protection mechanism. The defense of plants to stress is linked to the antioxidant capacity, and higher levels of antioxidants play a protective role against stress damage [44]. The variations in the tolerance of olive cultivars to freezing were also associated with other biochemical indicators such as the content of soluble sugars and phospholipids [45] as well as soluble carbohydrate and proline content, DPPH (1, 1-diphenyl–2-picrylhydrazyl) scavenging capacity, and malondialdehyde (MDA) [46]. Antioxidant enzymes have a variable response to stresses. For example, the activity of catalase was down-regulated under frost stress, while the activity of APX was not affected before temperature dropping below −5 °C [47]. In this study, the measurements of antioxidant enzymes activity did not show any statistically significant differences between the 2-days and 4-days stressed plants of either cultivar. Nonetheless, "Mastoidis" stressed plants slightly increased the activity of

both enzymes studied in response to cold stress, while "Koroneiki" stressed plants only marginally increased SOD activity. This could be an indication that "Mastoidis" plants respond better to cold stress, making the cultivar more resistant, and this observation could be verified under severe cold stress. The fact that none of the stressed plants accumulated hydrogen peroxide or TBARS is an indication that no severe oxidation damaged occurred in the plants under the stress applied.

The numerous plant phenology aspects measured reinforced the conclusion that both cultivars were resistant to the mild stress applied. It was also observed, by the flowering phenological stage, that "Koroneiki" is a precocious cultivar compared to "Mastoidis", as it was proposed by the literature [48]. It could be assumed that the phenological characteristics of the plants studied were not impaired by a few days treatment and differences may be revealed under prolonged periods of stress.

Studies in other plant species have shown that cold stress caused a decrease in pollen germination rate and reduced pollen tube length [49,50]. In the olive stressed plants investigated in this study, pollen germination rate and pollen tube length showed some differences, but overall, these changes were not observed in both parameters, and neither were consistent in both treatments, thus as a conclusion the stress applied did not affect these aspects.

Shorter and longer periods of low temperatures are known to affect gene expression profile in plants [51] including olives [24]. For instance, *OePPO* gene expression was found elevated under abiotic stress [22,23]. As an indication of responsiveness to the 24-h cold stress applied, the mRNA levels of the *OePPO* gene were quantified, as this gene is known to increase its expression and subsequently the enzymatic activity of the produced protein, under stress [22–24]. The immediate and significant increase in *OePPO* expression in all the stressed plants suggests that both cultivars sensed the cold stress and altered their transcription levels to act in response. As *OePPO* plays a decisive role in the biosynthesis of the antioxidant hydroxytyrosol, which is part of the olive tree defense system, more genes involved in this pathway were evaluated. It was shown that "Mastoidis" stressed plants had a stronger response regarding the HT biosynthetic genes. This was observed in *OeTDC* as well as in *OePPO* gene. Additionally, "Mastoidis" plants increased faster their gene expression levels under low temperatures, except for *OeCuAO* where "Koroneiki" cultivar reached first a significant increase. The fast increase in the HT biosynthetic gene expression levels as well as the stability of the physiological and phenological parameters assessed in "Mastoidis" stressed plants may be an indication of the adaptation of this cultivar to mild cold stress. "Koroneiki" plants were also able to cope with mild frost incidents, despite the less extended gene expression alteration. "Koroneiki" has been classified as moderately tolerant in a previous experiment based on electrolyte leakage in leaves [46]. "Mastoidis" is also considered relatively tolerant to low temperatures since it has been selected for centuries for growing in mountainous areas of Greece. A fast and temporary enhancement of gene transcription was also previously detected for geranylgeranyl reductase gene (*OeCHLP*) in leaves of various age treated with low temperature (4 °C) [52]. The underlying mechanism is worth studying in the future to elucidate whether adaptation or recovery strategies are activated similarly to increased gene expression in plants exposed to low temperatures and other environmental stresses such as water deficit [42].

The estimation of the physiological parameters assessed during this study, in combination with quantification of the mRNA levels of *OePPO* and other genes involved in antioxidant biosynthesis, could be exploited as suitable indicators for cultivar stress tolerance discrimination, elucidating olive tree response under unfavorable environmental conditions in the era of climate change.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/12/23/10065/s1, Figure S1: Monthly minimum and maximum temperature during the trials.

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References

1. Langgut, D.; Cheddadi, R.; Carrión, J.S.; Cavanagh, M.; Colombaroli, D.; Eastwood, W.J.; Greenberg, R.; Litt, T.; Mercuri, A.M.; Miebach, A.; et al. The origin and spread of olive cultivation in the Mediterranean Basin: The fossil pollen evidence. *Holocene* **2019**, *29*, 902–922. [CrossRef]
2. Bartolozzi, F.; Fontanazza, G. Assessment of frost tolerance in olive (Olea europaea L.). *Sci. Hortic. (Amsterdam)* **1999**, *81*, 309–319. [CrossRef]
3. Campoy, J.A.; Ruiz, D.; Egea, J. Dormancy in temperate fruit trees in a global warming context: A review. *Sci. Hortic. (Amsterdam)* **2011**, *130*, 357–372. [CrossRef]
4. Haberman, A.; Bakhshian, O.; Cerezo-Medina, S.; Paltiel, J.; Adler, C.; Ben-Ari, G.; Mercado, J.A.; Pliego-Alfaro, F.; Lavee, S.; Samach, A. A possible role for flowering locus T-encoding genes in interpreting environmental and internal cues affecting olive (Olea europaea L.) flower induction. *Plant Cell Environ.* **2017**, *40*, 1263–1280. [CrossRef]
5. Koubouris, G.C.; Metzidakis, I.T.; Vasilakakis, M.D. Influence of cross pollination on the development of parthenocarpic -shotberries- olive (Olea europaea L.) fruits. *Exp. Agric.* **2010**, *46*, 67–76. [CrossRef]
6. Woodward, F.I. *Climate and Plant Distribution (Cambridge Studies in Ecology)*; Cambridge University Press: Cambridge, UK, 1987; ISBN 0521282144.
7. Pearce, R. Plant Freezing and Damage. *Ann. Bot.* **2001**, *87*, 417–424. [CrossRef]
8. Larcher, W. Temperature stress and survival ability of mediterranean sclerophyllous plants. *Plant Biosyst.* **2000**, *134*, 279–295. [CrossRef]
9. Gururani, M.A.; Venkatesh, J.; Tran, L.S.P. Regulation of photosynthesis during abiotic stress-induced photoinhibition. *Mol. Plant* **2015**, *8*, 1304–1320. [CrossRef]
10. Koubouris, G.C.; Kavroulakis, N.; Metzidakis, I.T.; Vasilakakis, M.D.; Sofo, A. Ultraviolet-B radiation or heat cause changes in photosynthesis, antioxidant enzyme activities and pollen performance in olive tree. *Photosynthetica* **2015**, *53*, 279–287. [CrossRef]
11. Liakoura, V.; Stavrianakou, S.; Liakopoulos, G.; Karabourniotis, G.; Manetas, Y. Effects of UV-B Radiation on Olea Europaea: Comparisons Between a Greenhouse and a Field Experiment. *Tree Physiol.* **1999**, *19*, 905–908. [CrossRef]
12. Sofo, A.; Manfreda, S.; Fiorentino, M.; Dichio, B.; Xiloyannis, C. The olive tree: A paradigm for drought tolerance in Mediterranean climates. *Hydrol. Earth Syst. Sci.* **2008**, *12*, 293–301. [CrossRef]
13. Mittler, R.; Vanderauwera, S.; Gollery, M.; Van Breusegem, F. Reactive oxygen gene network of plants. *Trends Plant Sci.* **2004**, *9*, 490–498. [CrossRef]
14. Bacelar, E.A.; Santos, D.L.; Moutinho-Pereira, J.M.; Lopes, J.I.; Gonçalves, B.C.; Ferreira, T.C.; Correia, C.M. Physiological behaviour, oxidative damage and antioxidative protection of olive trees grown under different irrigation regimes. *Plant Soil* **2007**, *292*, 1–12. [CrossRef]
15. Bowler, C.; Montagu, M.V.; Inze, D. Superoxide Dismutase and Stress Tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1992**, *43*, 83–116. [CrossRef]
16. León, A.M.; Palma, J.M.; Corpas, F.J.; Gómez, M.; Romero-Puertas, M.C.; Chatterjee, D.; Mateos, R.M.; Del Río, L.A.; Sandalio, L.M. Antioxidative enzymes in cultivars of pepper plants with different sensitivity to cadmium. *Plant Physiol. Biochem.* **2002**, *40*, 813–820. [CrossRef]
17. Masoumi, H.; Masoumi, M.; Darvish, F.; Daneshian, J.; Nourmohammadi, G.; Habibi, D. Change in several antioxidant enzymes activity and seed yield by water deficit stress in soybean (Glycine max L.) cultivars. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2010**, *38*, 86–94. [CrossRef]
18. Ortega-García, F.; Peragón, J. The response of phenylalanine ammonia-lyase, polyphenol oxidase and phenols to cold stress in the olive tree (Olea europaea L. cv. Picual). *J. Sci. Food Agric.* **2009**, *89*, 1565–1573. [CrossRef]
19. Mayer, A.M. Polyphenol oxidases in plants and fungi: Going places? A review. *Phytochemistry* **2006**, *67*, 2318–2331. [CrossRef]
20. Thipyapong, P.; Hunt, M.D.; Steffens, J.C. Antisense downregulation of polyphenol oxidase results in enhanced disease susceptibility. *Planta* **2004**, *220*, 105–117. [CrossRef]

21. Thipyapong, P.; Hunt, M.D.; Steffens, J.C. Systemic wound induction of potato (Solanum tuberosum) polyphenol oxidase. *Phytochemistry* **1995**, *40*, 673–676. [CrossRef]
22. Boeckx, T.; Winters, A.L.; Webb, K.J.; Kingston-Smith, A.H. Polyphenol oxidase in leaves: Is there any significance to the chloroplastic localization? *J. Exp. Bot.* **2015**, *66*, 3571–3579. [CrossRef]
23. Raimbault, A.K.; Marie-Alphonsine, P.A.; Horry, J.P.; Francois-Haugrin, M.; Romuald, K.; Soler, A. Polyphenol oxidase and peroxidase expression in four pineapple varieties (Ananas comosus L.) after a chilling injury. *J. Agric. Food Chem.* **2011**, *59*, 342–348. [CrossRef]
24. Hashempour, A.; Ghasemnezhad, M.; Ghazvini, F.R.; Sohani, M.M. Olive (Olea europaea L.) freezing tolerance related to antioxidant enzymes activity during cold acclimation and non acclimation. *Acta Physiol. Plant.* **2014**, *36*, 3231–3241. [CrossRef]
25. Satoh, Y.; Tajima, K.; Munekata, M.; Keasling, J.D.; Lee, T.S. Engineering of L-tyrosine oxidation in Escherichia coli and microbial production of hydroxytyrosol. *Metab. Eng.* **2012**, *14*, 603–610. [CrossRef]
26. Saimaru, H.; Orihara, Y. Biosynthesis of acteoside in cultured cells of Olea europaea. *J. Nat. Med.* **2010**, *64*, 139–145. [CrossRef]
27. Trantas, E.; Navakoudis, E.; Pavlidis, T.; Nikou, T.; Halabalaki, M.; Skaltsounis, L.; Ververidis, F. Dual pathway for metabolic engineering of Escherichia coli to produce the highly valuable hydroxytyrosol. *PLoS ONE* **2019**, *14*, e0212243. [CrossRef]
28. Mougiou, N.; Trikka, F.; Trantas, E.; Ververidis, F.; Makris, A.; Argiriou, A.; Vlachonasios, K.E. Expression of hydroxytyrosol and oleuropein biosynthetic genes are correlated with metabolite accumulation during fruit development in olive, Olea europaea, cv. Koroneiki. *Plant Physiol. Biochem.* **2018**, *128*, 41–49. [CrossRef]
29. Geßler, A.; Keitel, C.; Kreuzwieser, J.; Matyssek, R.; Seiler, W.; Rennenberg, H. Potential risks for European beech (Fagus sylvatica L.) in a changing climate. *Trees Struct. Funct.* **2007**, *21*, 1–11. [CrossRef]
30. Koubouris, G.; Limperaki, I.; Darioti, M.; Sergentani, C. Effects of various winter chilling regimes on flowering quality indicators of Greek olive cultivars. *Biol. Plant.* **2019**, *63*, 504–510. [CrossRef]
31. Koubouris, G.C.; Metzidakis, I.T.; Vasilakakis, M.D. Impact of temperature on olive (Olea europaea L.) pollen performance in relation to relative humidity and genotype. *Environ. Exp. Bot.* **2009**, *67*, 209–214. [CrossRef]
32. Malik, N.S.A.; Bradford, J.M. Inhibition of flowering in "Arbequina" olives from chilling at lower temperatures. *J. Food Agric. Environ.* **2009**, *7*, 429–431.
33. Connor, D.J.; Fereres, E. The Physiology of Adaptation and Yield Expression in Olive. In *Horticultural Reviews*; John Wiley & Sons, Inc.: Oxford, UK, 2010; Volume 31, pp. 155–229.
34. Martin, G.C.; Sibbett, S.G. Botany of the olive. In *Olive Production Manual*; Sibbett, S.G., Ferguson, L., Coviello, J.L., Lindstrand, M., Eds.; University of California, Agriculture and Natural Resources: Oakland, CA, USA, 2005; pp. 15–19.
35. Sofo, A.; Dichio, B.; Xiloyannis, C.; Masia, A. Antioxidant defences in olive trees during drought stress: Changes in activity of some antioxidant enzymes. *Funct. Plant Biol.* **2005**, *32*, 45–53. [CrossRef]
36. Sanz-Cortés, F.; Martinez-Calvo, J.; Badenes, M.L.; Bleiholder, H.; Hack, H.; Llacer, G.; Meier, U. Phenological growth stages of olive trees (Olea europaea). *Ann. Appl. Biol.* **2002**, *140*, 151–157. [CrossRef]
37. Koubouris, G.C.; Tzortzakis, N.; Kourgialas, N.N.; Darioti, M.; Metzidakis, I. Growth, photosynthesis and pollen performance in saline water treated olive plants under high temperature. *Int. J. Plant Biol.* **2015**, *6*, 28–32. [CrossRef]
38. Allen, D.J.; Ort, D.R. Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trend Plant Sci.* **2001**, *6*, 36–42. [CrossRef]
39. Pavel, E.W.; Fereres, E. Low soil temperatures induce water deficits in olive (Olea europaea) trees. *Physiol. Plant.* **1998**, *104*, 525–532. [CrossRef]
40. Pérez-López, D.; Gijón, M.C.; Mariño, J.; Moriana, A. Water relation response to soil chilling of six olive (*Olea europaea* L.) cultivars with different frost resistance. *Spanish J. Agric. Res.* **2010**, *8*, 780. [CrossRef]
41. López-Bernal, Á.; García-Tejera, O.; Testi, L.; Orgaz, F.; Villalobos, F.J. Low winter temperatures induce a disturbance of water relations in field olive trees. *Trees* **2015**, *29*, 1247–1257. [CrossRef]
42. Araújo, M.; De Oliveira, J.M.P.F.; Santos, C.V.; Moutinho-Pereira, J.M.; Correia, C.; Dias, M.C. Responses of olive plants exposed to different irrigation treatments in combination with heat shock: Physiological and molecular mechanisms during exposure and recovery. *Planta* **2019**, *249*, 1583–1598. [CrossRef]

43. Trabelsi, L.; Gargouri, K.; Hassena, B.A.; Mbadra, C.; Ghrab, M.; Ncube, B.; Amoo, S.O.; Gargouri, R. Impact of drought and salinity on olive water status and physiological performance in an arid climate. *Agric. Water Manag.* **2019**, *213*, 749–759. [CrossRef]
44. Monk, L.S.; Fagerstedt, K.V.; Crawford, R.M.M. Oxygen toxicity and superoxide dismutase as an antioxidant in physiological stress. *Physiol. Plant.* **1989**, *76*, 456–459. [CrossRef]
45. Gulen, H.; Cansev, A.; Eris, A. Cold hardiness of olive (*Olea europaea* L.) cultivars in cold-acclimated and non-acclimated stages: Seasonal alteration of soluble sugars and phospholipids. *J. Agric. Sci.* **2009**, *147*, 459–467. [CrossRef]
46. Saadati, S.; Baninasab, B.; Mobli, M.; Gholami, M. Measurements of freezing tolerance and their relationship with some biochemical and physiological parameters in seven olive cultivars. *Acta Physiol. Plant.* **2019**, *41*, 51. [CrossRef]
47. Cansev, A.; Gulen, H.; Eris, A. The activities of catalase and ascorbate peroxidase in olive (*Olea europaea* L. cv. Gemlik) under low temperature stress. *Hortic. Environ. Biotechnol.* **2011**, *52*, 113–120. [CrossRef]
48. Koubouris, G.C.; Metzidakis, I.T.; Vasilakakis, M.D. Phenological, morphological and functional indicators of genetic variability and their implication in the sexual reproductive system of *Olea europaea* L. (Oleaceae). *Sci. Hortic.* **2010**, *123*, 547–550. [CrossRef]
49. Chakrabarti, B.; Singh, S.D.; Nagarajan, S.; Aggarwal, P.K. Impact of temperature on phenology and pollen sterility of wheat varieties. *Aust. J. Crop Sci.* **2011**, *5*, 1039–1043.
50. Kumar, S.; Nayyar, H.; Bhanwara, R.; Upadhyaya, H. Chilling stress effects on reproductive biology of chickpea. *J. SAT Agric. Res.* **2010**, *8*, 1–14.
51. Thomashow, M.F. PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms. *Annu. Rev. Plant Biol.* **1999**, *50*, 571–599. [CrossRef]
52. Bruno, L.; Chiappetta, A.; Muzzalupo, I.; Gagliardi, C.; Iaria, D.; Bruno, A.; Greco, M.; Giannino, D.; Perri, E.; Bitonti, M.B. Role of geranylgeranyl reductase gene in organ development and stress response in olive (*Olea europaea*) plants. *Funct. Plant Biol.* **2009**, *36*, 370–381. [CrossRef]

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