Morphological, Physiological, and Biochemical Responses of Some Olive Tree Cultivars to Low Temperature Stress

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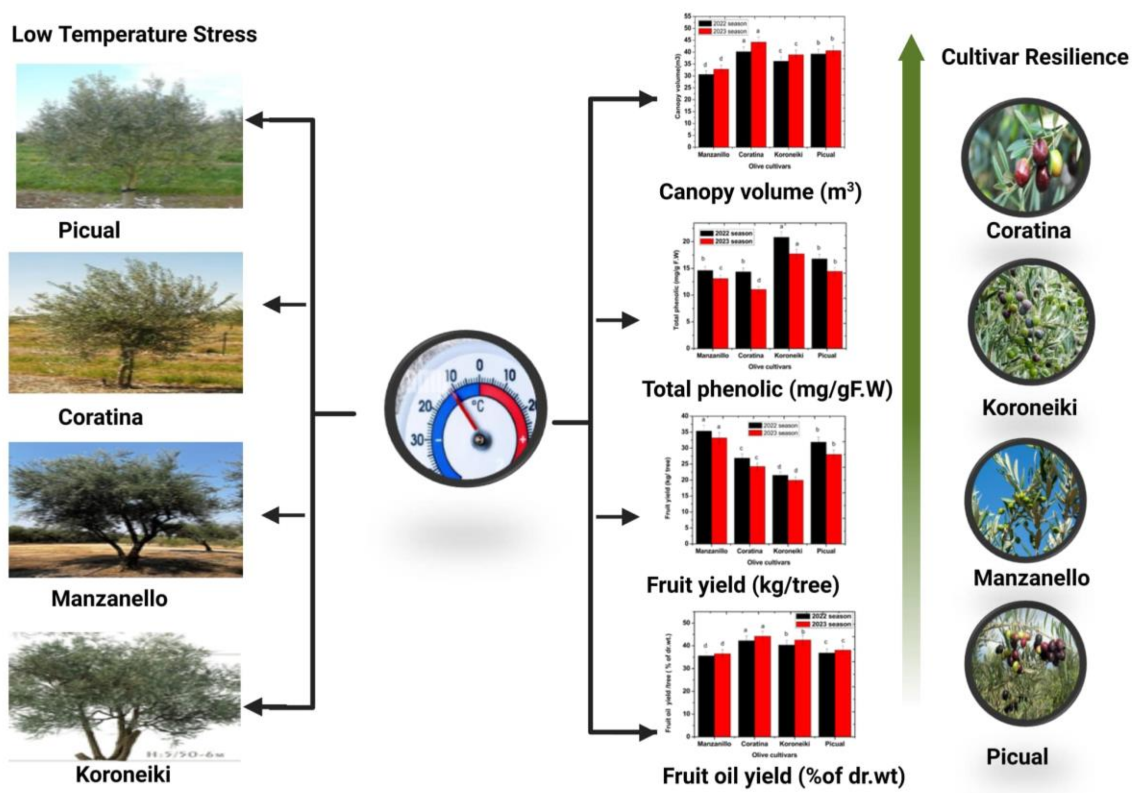
GRAPHICAL ABSTRACT



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This study evaluated the impact of night frost incidents on the biochemical, physiological, and reproductive functions of the olive varieties Manzanillo, Coratina, Koroneiki, and Picual. Certain cultivars were more suited to moderate cold night stress than others, based on the changes in the performance of the stressed plants, including vegetative growth, tree yield, fruit physical characteristics, and fruit chemical characteristics. Compared to other tested cultivars, the biochemical responses of the plants in terms of photosynthetic pigments, relative water content (RWC), total phenolic compounds, total flavonoid, and antioxidant enzyme accumulation demonstrated that some cultivars could withstand the applied stress. The conclusion that some cultivars responded differently to cold stress than others was supported by the plant phenology. This research could be a game-changer for farmers. By understanding how olive trees adapt to cold snaps, a common stressor in open fields, they can make informed decisions about breeding and choosing the best cultivars, ultimately leading to more resilient crops. The results showed that all tested olive tree cultivars differ significantly regarding cold stress conditions. Coratina and Koroneiki were the most resistant tested cultivars in terms of biochemical, physiological, and reproductive functions, followed in ascending order by Manzanello and Picual.

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INTRODUCTION

Olive trees and bushes, scientifically referred to as *Olea europaea* L., thrive in subtropical and temperate regions with temperatures ranging from 30 to 45 °C in each hemisphere (Petruccelli *et al.* 2022). They are a sizeable economic motive force in the Mediterranean Basin, with olive oil extracted from their fruit being a primary contributor, alongside desk olives being a famous food item (Boskou 2015). Olive trees are affected by wintry weather frost, which leads to reduced productivity (Fraga *et al.* 2020). To address this issue, researchers have explored numerous techniques, along with selecting naturally frost-resistant olive types and comparing cultivars' overall performance at some point of real frost occasions. Another approach includes checking the resilience of various olive genotypes under managed freezing conditions in laboratories.

Low temperatures have a widespread effect on the distribution of woody flora and can lead to reduced crop yields because of freezing damage (Medda *et al.* 2022). Optimal plant increase takes place within a selected temperature range, with growth being hindered outside of this range. Factors influencing plant survival in low temperatures consist of cultivars, organ kinds, extent of temperature reduction, and length of exposure to low temperatures (Wahid *et al.* 2007). The freezing tolerance of leaves and stems is closely linked to bloodless acclimation, initiated through shorter day lengths, and decreasing temperatures in autumn, ensuing in expanded freezing tolerance in deciduous woody flora. This process entails adjustments in gene expression, consisting of stress protein encoding, alterations in photosynthesis, antioxidant compounds, and lipid and protein composition. Cold acclimation also includes the accumulation of cytoprotective compounds such as soluble carbohydrates, proline, and proteins, which form ice crystals, facilitate osmotic adjustment, preserve cell turgor, and decorate plant resilience to dehydration stresses (Ershadi *et al.* 2016). Mete *et al*. (2023) evaluated frost tolerance in olive cultivars with the aid of measuring ion leakage in leaves exposed to exclusive temperatures. The tolerance of cultivars varies between seasons. Butko, Memeli, and GemLik confirmed higher tolerance, and at the same time, Edincik and Sinop No. 6 were less tolerant. While adapting to colder temperatures improved olive trees' tolerance, this effect wasn't permanent. The Istarska bjelica variety stood out for its high levels of beneficial plant compounds, particularly oleuropein, which remained consistently elevated. Interestingly, lower air temperature seemed to boost oleuropein levels and antioxidant activity in other varieties. In Egypt, olive growers cultivate various types for different purposes. Toffahi and Egazy are popular for table olives, while Picual and Manzanillo serve as table olives and for oil extraction. Finally, Koroneiki and ChemLali are specifically chosen for their high-quality olive oil production. In this respect, Koroneiki had higher oil content than the Picual olive cultivar (Samra *et al.* 2009). Research by López-Bernal *et al.* (2015) indicates that low temperatures, rather than the length of daylight, are the key environmental cue for olive trees to halt vegetative growth and enter a state of cold acclimation.

The growing demand for olive oil has brought about the growth of olive plantations beyond the traditional Mediterranean region into better-range areas (Gómez *et al.* 2014). This shift has had positive and negative consequences. In chillier areas, dropping winter and early spring temperatures can result in freezing or frost damage to the plants because of ice crystal formation and fast temperature fluctuations. On the other hand, cool autumns can improve the excellence of olive oil through more desirable enzyme activities within the lipoxygenase pathway, leading to advanced flowering within the following spring (Ozturk *et al.* 2019). While olive timber displays mild resistance to freezing temperatures, subzero situations can cause extensive harm. Studies imply that maximum olive cultivars enjoy leaf drop and twig desiccation at temperatures below -7 °C, with temperatures below -12 °C resulting in extensive reductions in productiveness and tree lifespan (León *et al.* 2016). The variability in freezing tolerance among olive cultivars underscores the importance of understanding the biochemical and physiological mechanisms underlying cold tolerance (Lodolini *et al.* 2022). This knowledge can be a resource in figuring out precise genes or new cultivars with greater bloodless resistance, using improvements in genetic research. Low temperatures control these physiological and biochemical changes by causing major changes in gene expression (Yurtsever and Vural Korkut 2019). More recently, the transcriptome alterations brought on by low temperatures in olives were monitored using RNA-Seq analysis (Guerra *et al.* 2015). Both RNA-seq experiments showed upregulation of genes encoding enzymes involved to modifications in the lipid composition of membranes, as well as stress-related genes such as cold-regulating genes and dehydrin, and downregulation of genes involved in photosynthesis, and activation of carbohydrate metabolism **(**Petruccelli *et al*. 2022). Several biochemical and physiological factors play an important function in choosing freezing-tolerant olive sorts, which include stomatal density, stomatal length, phenolic compounds, ion leakage, soluble sugars, general soluble proteins, and antioxidant enzymes. However, counting on a single parameter is inadequate for pinpointing precise cultivars due to the quantitative nature of cold tolerance. The main aim of the present investigation was to assess olive physiological, biochemical and reproductive responses under the imposition of low- temperature stress, imitating night frost incidents. A multidimensional approach was used to study visual changes shoot length (cm), leaf area index, and canopy volume (m^3). Tests also included the initial fruit set, final fruit set, fruit weight, and fruit yield of all cultivars considered, physiological performance, chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids. Other tests included relative water content and proline, fruit oil yield/ tree, 7otal soluble solids (%) (TSS) %, total acidity %, and T.S.S acid ratio %. Also measured were the total phenolics and total flavonoids, and biochemical response (antioxidant enzymes) towards the comprehension of olive capacity to cope with unfavorable environmental conditions. Manzanillo, Coratina, Koroneiki, and Picual olive cultivars were chosen because of their importance in new and existing olive plantations.

EXPERIMENTAL

Experimental Site

This study was conducted for two years and examined four olive cultivars (Manzanillo, Coratina, Koroneiki, and Picual trees) during the 2022 and 2023 seasons at a private orchard, Al-Salehia district in El-Sharkia Governorate (30°39′14″N, 31°52′ 28″E, and 30.653862°N 31.874371°E). Weather Data of Egypt- Al-Salehia, El-Sharkia -Daily-2022-2023 is represented in Table 1. The trees were planted using an artesian water drip irrigation system at 4 m × 6 m (1250 trees/ha) in sandy soil. Every 50 cm on the hose, there were two JR hoses for every tree. A 2 L per hour discharging point was present. The Manzanillo, Coratina, Koroneiki, and Picual trees were planted in 2010 Owen rooted. The care practices for the orchard involved consistent watering and nutrient application, ensuring the trees were free of pests and any signs of nutrient deficiencies. The impact of low temperature on the aboveground portions of all trees was evaluated during May 2022 and 2023. Mature leaves were collected from the study trees' final autumn cycle (four shoots in each of the four origin directions around the tree canopy, namely North, East, South, and West) per tree in May of each season to perform various measurements on them.

Field and Laboratory Determinations

Vegetative growth

The number of damaged leaves per shoot was determined by counting the total number of leaves per shoot (four shoots in each of the four origin directions around the tree canopy, namely North, East, South, and West) and recording this information in early May of each year. To measure growth metrics such as shoot length (in cm), a random sample of nine shoots/trees from each of the two seasons was selected and labeled on each tree. Also, we estimated the height of trees at the end of growth in September in each season. In additionally, a sample of nine mature leaves per tree was abscised in May, and the leaf area (cm^2) was calculated using Eq. 1 (Ahmed and Morsy 1999),

$$\text{Leaf area} = 0.53\ (L \times W)\ \text{-}1.66 = \ldots\ldots cm^2 \tag{1}$$

where L is maximum leaf length and W is width, respectively.
Equation (2) was used to estimate the volume of the olive tree canopy, using the field measurements (Sola-Guirado *et al.* 2017),

$$\text{Ground Volume} = 1/6\pi D_1 \times D_2 \times (H_t - H_s) \tag{2}$$

where D_1 and D_2 are the diameters of the tree crown measured in two different directions, H_t is the height of the tree, and H_s is the average distance of the lowest point of the tree crown from the ground, measured in the same positions as the diameter.

Table 1. Daily Weather Data of Egypt- Al-Salehia, El-Sharkia (2022-2023)

Month	T_{2M}	T_{MIN}	T_{MAX}	T_{DEW}	RH_{2M}	RAIN	WIND	SRAD
	2022							
Jan.	11.01	6.23	17.57	4.59	68.17	0.97	2.53	12.14
Feb.	12.89	7.04	20.45	6.10	67.87	0.35	2.34	15.48
March	13.75	7.36	21.78	4.90	61.32	0.43	2.75	19.49
April	22.22	13.60	32.30	6.28	46.89	0.03	2.91	22.89
May	25.07	16.78	34.25	9.03	45.01	0.12	3.13	25.93
June	29.54	21.13	38.59	13.47	44.44	0.02	2.87	28.59
July	30.35	21.92	39.44	13.72	43.78	0.02	2.60	28.63
Aug.	30.65	23.39	39.35	16.56	48.85	0.06	2.72	26.26
Sept.	28.59	21.59	37.17	16.15	52.84	0.03	2.67	22.69
Oct.	24.49	18.68	31.73	14.04	57.29	0.13	2.44	17.94
Nov.	19.64	14.45	26.39	10.59	60.61	0.02	2.17	13.90
Dec.	16.89	11.90	24.01	9.23	65.64	0.22	1.99	11.57
	2023							
Jan.	14.03	8.93	21.42	7.72	69.45	1.31	2.10	12.98
Feb.	12.56	7.00	19.95	6.13	69.08	0.48	2.27	15.02
March	18.07	11.37	26.76	6.50	53.46	0.24	2.60	20.13
April	21.70	13.76	30.99	7.17	47.11	0.21	2.73	24.15
May	25.20	17.32	34.20	10.22	46.68	0.03	3.14	26.29
June	29.36	21.27	38.47	12.92	42.80	0.06	3.10	27.34
July	31.90	23.06	41.62	14.35	43.22	0.00	2.77	28.92
Aug.	31.08	23.44	40.54	16.16	47.58	0.00	2.68	26.44
Sept.	23.06	16.07	31.54	10.72	53.80	0.25	2.58	21.57
Oct.	25.72	19.94	32.99	15.77	59.75	0.09	2.30	17.61
Nov.	21.01	15.91	27.99	12.89	64.74	0.59	2.16	13.43
Dec.	17.34	12.35	24.10	10.31	67.20	0.15	2.35	10.91

T_{2M}: Average Temperature (°C), T_{MIN}: Minimum Temperature (°C), T_{MAX}: Maximum Temperature (°C), T_{DEW}: Dew/Frost Point (°C), RH_{2M}: Average Relative Humidity (%), RAIN: Precipitation (mm), WIND: Average Wind Speed (m/s) and SRAD: Total Solar Radiation ($MJ/m^2/day$)

Photosynthetic pigments

From fresh leaves of four different olive cultivars, chlorophyll A, B, total chlorophyll, and total carotenoids were extracted. This was accomplished by homogenizing fresh leaves (0.2 g) with 10 mL of acetone (80% v/v) and then centrifuging the resulting mixture at 12,000 × g for 10 min. Three mL of the liquid extract was used for spectrophotometric measurements (Wellburn 1994; Brito *et al.* 2011).

Determination relative water content (RWC)

The Yamasaki and Dillenburg (1999) method was used to calculate the relative water content (R.W.C.) of leaves,

$$\text{RWC (\%)} = [(\text{FM-DM}) (\text{TM} - \text{DM})] \times 100. \quad (3)$$

In each repetition, two leaves were randomly selected from the middle sections of the plants. Initially, the fresh masses (FM) of the leaves were determined after they were detached from the stems. To determine the saturation mass (TM), the samples were sealed in distilled water for a full day at 22 °C to ensure they reached their maximum saturation mass. Following this, the samples were weighed. After, leaves were kept in an electrical oven with an air temperature of 80 °C for 48 h, during which time their dry mass (DM) was determined. Every measurement was completed.

Determination proline content

A rapid colorimetric method was used to determine proline content (Bates *et al.* 1973). Ninhydrin (1.25 g) was dissolved in a solution of glacial acetic acid (30 mL) and phosphoric acid (6 M, 20 mL). This mixture was heated for clarity, cooled, and stored at 4 °C. Following homogenization in sulfosalicylic acid (0.5 g explant material in 10 mL, 3% aqueous), the mixture was filtered. The filtrate (2 mL) was then treated with glacial acetic acid (2 mL) and freshly prepared ninhydrin solution (2 mL). The reaction mixture was heated to 100 °C for 1 h, then cooled on ice. Toluene (4 mL) was added with vigorous shaking (15 to 20 s), allowing for phase separation. Proline concentration was determined by measuring the chromophore's absorbance at 520 nm with a spectrophotometer using toluene as a blank. The standard curve was used to calculate proline concentration based on the fresh weight of the sample.

Determination of antioxidant enzymes

To extract peroxidase activity (POX), polyphenol oxidase (PPO), and catalase (CAT) enzyme, fresh leaves (0.5 g) of four different olive cultivars were mashed in a mortar with 5 mL of 0.1 M cold phosphate buffer (pH 7.1), and the mixture was centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was used in an enzyme activity experiment (Esfandiari *et al.* 2007). POX activity was measured using an approach that was based on Amako *et al.*'s study (1994). PPO activity was measured in compliance with Kavrayan and Aydemir (2001). The activity of the CAT enzyme was measured in compliance with Aebi (1984).

Physical characteristics of fruits

The study characterized a detailed flower and fruit phenotyping process for different tree cultivars. Researchers recorded flowering dates, average panicles per tree, and flower counts at full bloom (including perfect and male hermaphrodite flowers) from five representative panicles. Similarly, at harvest, fruits were collected in a bag and then transferred to the chemical analytical laboratories of the horticulture and biochemistry departments at the Faculty of Agriculture in Cairo, Al-Azhar University. Samples of twenty-seven fruits of each tree were replicated three times and devoted to determining the following parameters: fruit number and weight (average from five fruits) were assessed at harvest. This approach aligns with Ferguson and Gratten's study (2005), which counted fruits on designated shoots before harvest.

The yield per tree (kg) was calculated using fruit weight. According to Khattab *et al.* (2021), the four tested olive oil cultivars were harvested on September 15th in both seasons. There was a yield recorded (kg/tree). The percentage was calculated using Eq. 4 (Hamdy *et al.* 2022):

$$\text{Yield increase (\%)} = \frac{\text{Yield}_{\text{treatment}} - \text{Yield}_{\text{control}}}{\text{Yield}_{\text{control}}} \times 100 \quad (4)$$

Chemical characteristics of fruits

A digital refractometer (force-Gouge ModelIGV-O.SA to FGV-100A. Shimpo instruments) was used to measure the total solids (TSS%) in fruit oil. Titration was used to determine total acidity, which was then expressed as citric acid in accordance with AOAC (McKie and McCleary 2016). The values of total soluble solids divided by the values of total acids and oil content percentage were used to compute the total soluble solids/acid ratio (McKie and McCleary 2016).

Total phenolic compounds and total flavonoid

According to Singleton *et al.* (1999), the total phenolic content of four olive tree leaves measured using the Folin–Ciocalteu colorimetric method. The results were expressed as (mg/g) using gallic acid as a standard. According to Chang *et al.* (2002), aluminum chloride ($AlCl_3$) colorimetric method was used to evaluate the total flavonoid in fresh leaves of the four olive cultivars. Rutin was used as a standard and the results were represented as mg/g.

Statistical Analysis

The design of the present study was a complete randomized block design. The analysis of variance as one-way ANOVA was used through Costat software Snedecor and Cochran (1980), and means of different treatments were compared using the Duncan test ($p \leq 0.05$).

RESULTS

Vegetative Growth of Olive Cultivars

The results in Fig. 1 indicate that there were significant differences between all tested olive cultivars regarding the vegetative growth parameters such as shoot length (cm), leaf area index, and canopy volume (m^3) during the two studied seasons (2022 and 2023) under low temperature conditions. In the same time, there were significant differences between the two tested seasons (2022 and 2023) regarding the vegetative growth parameters in all tested olive cultivars, where that data is consistent with an impact of low temperature stress on vegetative growth parameters in all tested olive cultivars in the first season was higher than the impact in the second season under the same condition, as a result of the decrease in temperature in the first season from the second season.

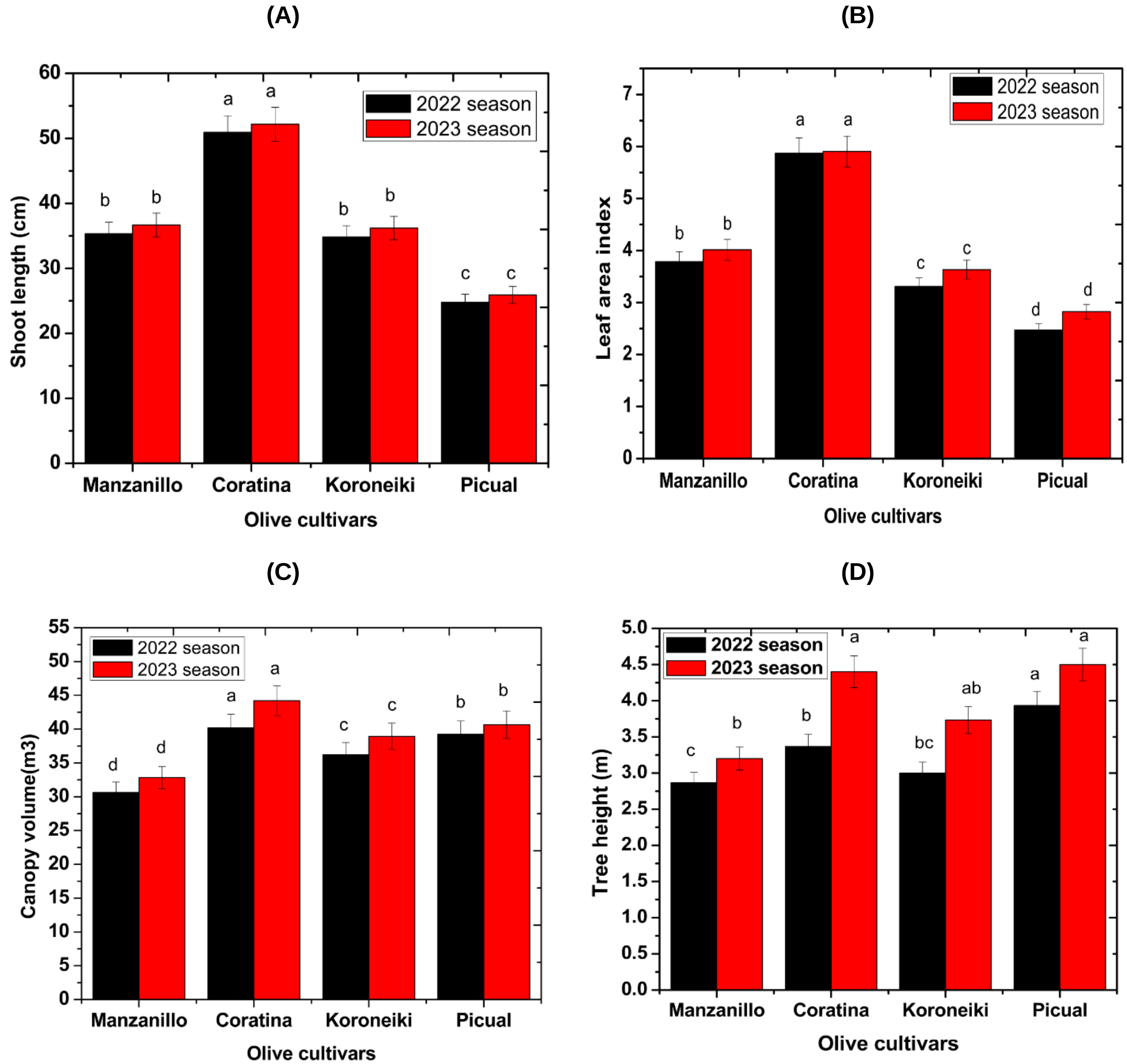

Fig. 1. The shoot length (cm), leaf area index, canopy volume, and tree height of all tested cultivars (Manzanillo, Coratina, Koroneiki, and Picual) under low-temperature conditions for three months. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test) and error bars depict standard error.

It was clear that Picual and Coratina trees were also superior to those of Manzanello and Koroneiki with respect to tree height under the same conditions. Also, the results in Fig. 1A showed that Coratina trees had the longest shoots, followed by Manzanello, Koroneiki, and then Picual. Similarly, the leaf area index data in Fig.1B follows the same trend. Coratina trees had the largest leaf area, followed by Manzanello. Koroneiki and Picual trees had the smallest leaf area in two tested seasons. The data in Fig. 1C indicated that Coratina trees had the largest canopy volume, followed by Koroneiki and then

Manzanello. On the other hand, Picual trees had the smallest canopy volume among the cultivars studied. According to Fig. 1D, Picual and Coratina trees were higher than both Koroneiki and Manzanello. Olive trees halt their vegetative growth and enter a dormant stage in the autumn when the temperature drops below the ideal range for growth (5 to 10 °C) and the photoperiod shortens.

It can be concluded that in terms of all vegetative growth parameters, including shoot length, leaf area, canopy volume, and tree height, Coratina trees outperformed the other olive cultivars.

Leaf Photosynthetic Pigments

Differences between olive cultivars in the values of plant pigments, whether chlorophyll a, b, and carotenoids under cold conditions are shown in Figs. 2 (A to D).

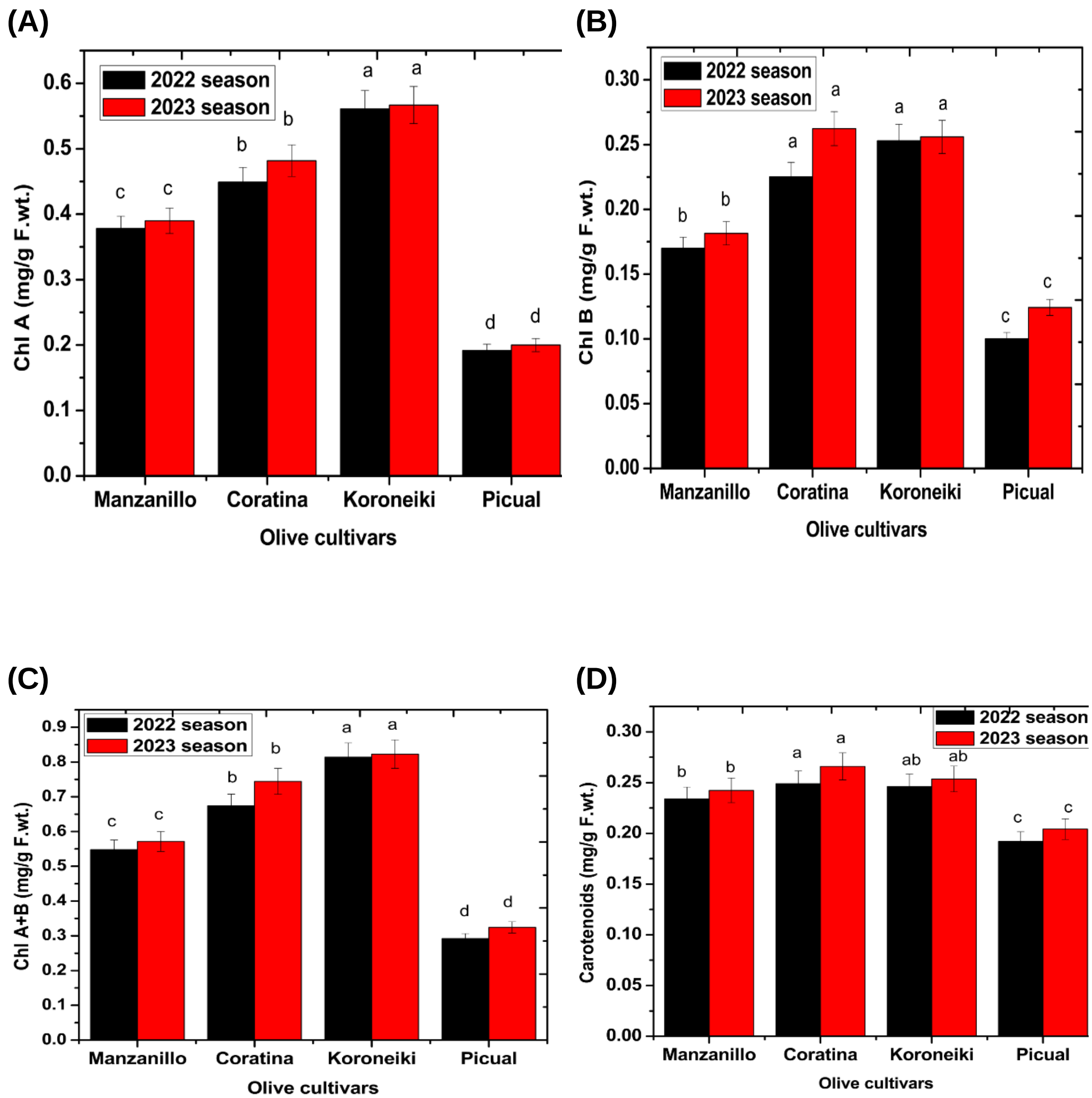

Fig. 2. Chlorophyll a, chlorophyll b, a total of chlorophyll, and carotenoids of all tested cultivars (Manzanillo, Coratina, Koroneiki, and Picual) under low-temperature conditions for three months. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test) and error bars depict standard error.

Koroneiki cv. had the highest values in chlorophyll (a (0.567), b (0.256), and a + b (0.823)), followed by Coratina cv. which shared the highest values in chlorophyll (b (0.262)), and carotenoids (0.266 mg/g F. wt.). On the other hand, Picual cv. had the lowest values of plant pigments; chlorophyll (a (0.192), b (0.100)), and carotenoids (0.192) compared to other cultivars.

Leaf chemical characteristics.

Figure 3 (A and B) show the effect of low-temperature conditions on relative water content and proline accumulation in the leaf of the four tested olive varieties. It was established that the relative water content in the leaves of Manzanello, Coratina, Koroneiki, and Picual was decreased under cold conditions. There were significant differences between all tested olive tree cultivars in relation to cold stress conditions, where Coratina gained the highest relative water content followed in ascending order by Manzanello, Koroneiki, and Picual. On the other hand, growing all tested olive tree varieties under low-temperature conditions caused an increase in the proline content of all tested olive tree cultivars (Fig. 3). There were no significant differences between all tested varieties in relation to the effect of cold stress on leaf proline content where Coratina gained the highest proline content followed in ascending order by Manzanello, Koroneiki, and Picual.

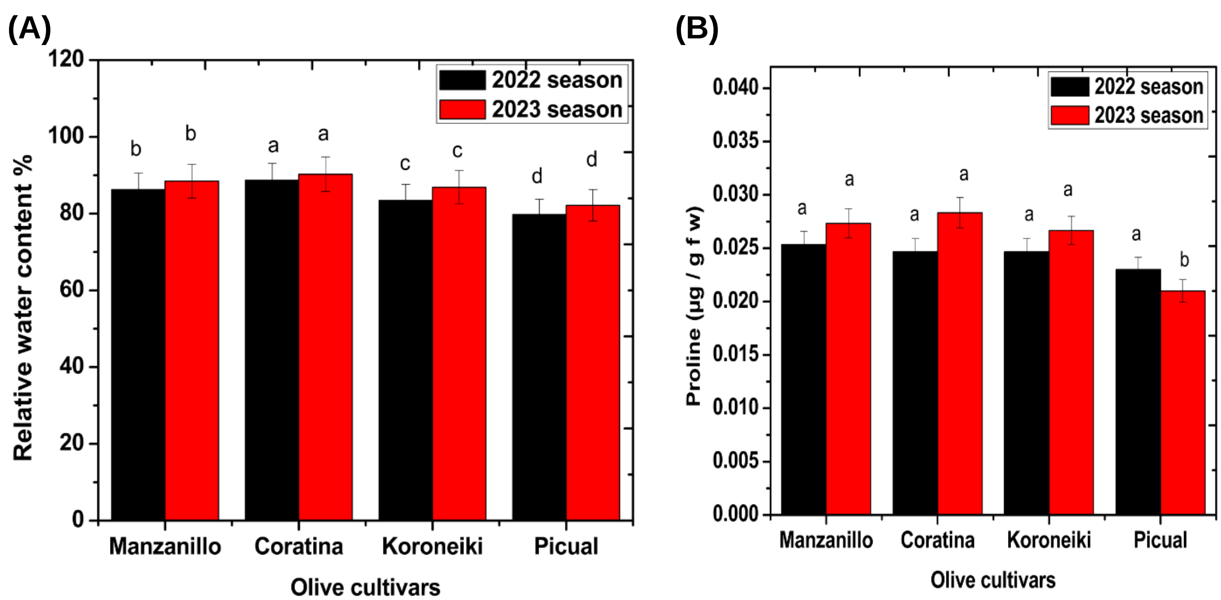

Fig. 3. Relative water content and proline of all tested cultivars (Manzanillo, Coratina, Koroneiki, and Picual) under low-temperature conditions for three months. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test) and error bars depict standard error.

Antioxidant enzymes activities

The enzymatic activities of POX, PPO, and CAT in leaves of four olive cultivars were measured under frost during the 2022 and 2023 seasons (Fig. 4 A to C). The highest POX activity was recorded in Coratina *cv.* (25.14 U/mg F.wt.), followed by Manzanillo (17.41 U/mg F. wt.), and Koroneiki (17.25 U/mg F. wt.), whereas the lowest POX activity was assayed in *cv.* Picual (8.14 U/ mg F. wt.) (Fig. 3A). The highest PPO activity was found in Coratina *cv.* (56.86 U/mg F. wt.), followed by Manzanillo (49.02 U/mg F. wt.), whereas the lowest PPO activity was observed in *cv.* Koroneiki (33.41 U/ mg F. wt.) (Fig.

4B). The highest and the lowest CAT activities were detected in Manzanillo (8.19 U/mg F. wt.) and Koroneiki (3.77 U/ mg F. wt.) cultivars, respectively (Fig. 4C).

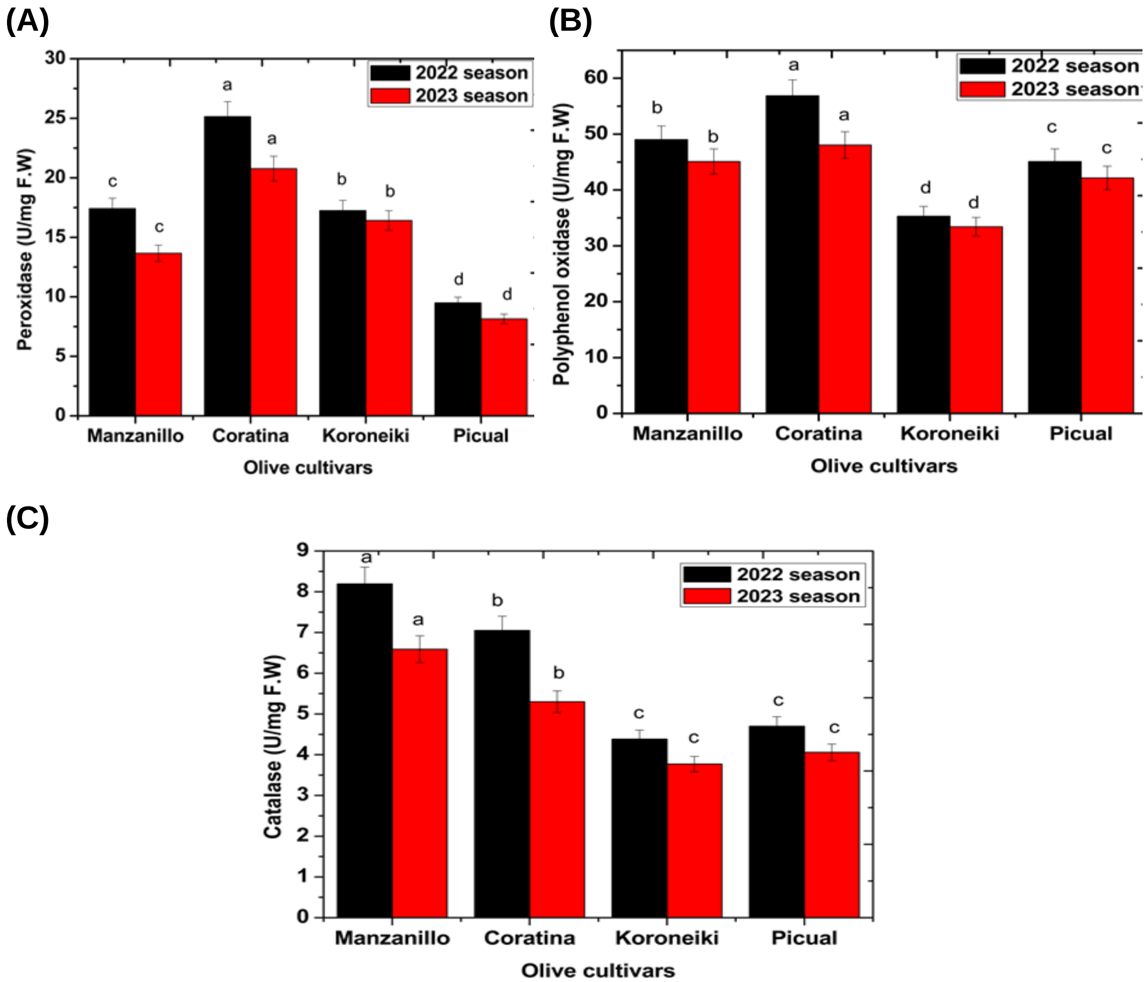

Fig. 4. Peroxidase (POX), polyphenol oxidase (PPO), and catalase (CAT) of all tested cultivars (Manzanillo, Coratina, Koroneiki, and Picual) under low-temperature conditions for three months. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test) and error bars depict standard error.

Fruit physical characteristics at harvest

Figure 5 shows significant differences between fruit physical characteristics among the four studied olive cultivars under frost conditions. Coratina trees were superior to those of Manzanello, Koroneiki, and Picual varieties in terms of initial and final fruit set percentage during the two studied seasons (2022 and 2023). On the other hand, the growth of olive trees under frost conditions through 2022 and 2023 led to a reduction in the fruit weight and yield of the four tested olive tree cultivars, where the Manzanello olive cultivar was superior to the other tested cultivars, followed by the descending order of Picual, Coratina, and Koroneiki.

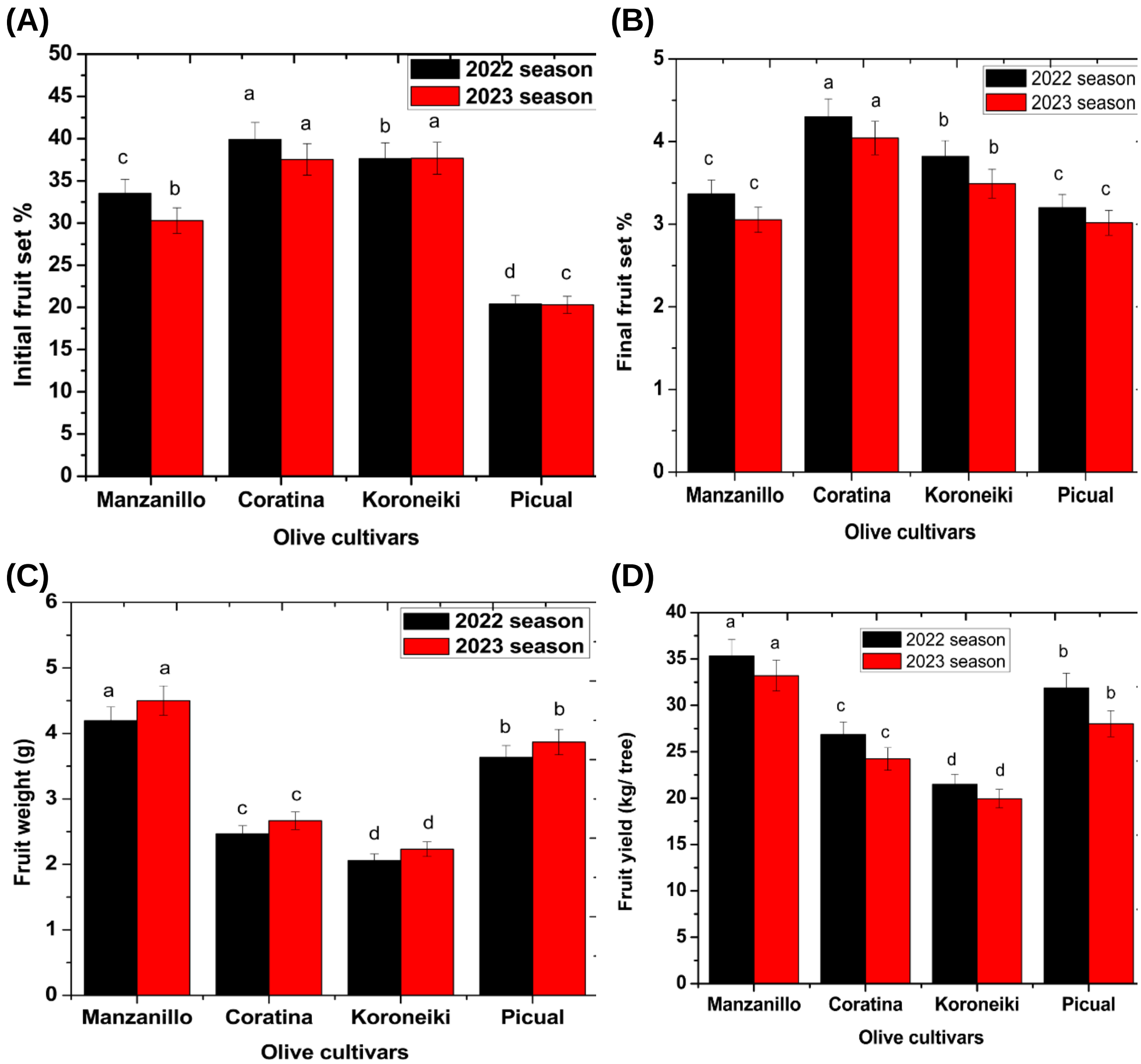

Fig. 5. Initial fruit set, final fruit set, fruit weight, and fruit yield of all tested cultivars (Manzanillo, Coratina, Koroneiki, and Picual) under low-temperature conditions for three months. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test) and error bars depict standard error.

Fruit chemical characteristics at harvest

Figure 6 shows the effect of cold stress on fruit chemical characteristics such as fruit oil yield/tree, T.S.S %, total acidity %, and T.S.S acid ratio % of the four tested olive cultivars as follows, Manzanello, Coratina, Koroneiki, and Picual. There were significant differences between all tested olive tree cultivars regarding the effect of low temperature on fruit chemical characteristics of all tested cultivars where the Coratina olive tree cultivar possessed the highest values of fruit chemical characteristics such as fruit oil yield/tree, T.S.S %, Total acidity %, and T.S.S acid ratio %, followed in ascending order by those of Manzanello, Koroneiki, and Picual varieties. Picual olive tree cultivars gained the highest value of total acidity followed in ascending order by Koroneiki, Manzanello, and Coratina.

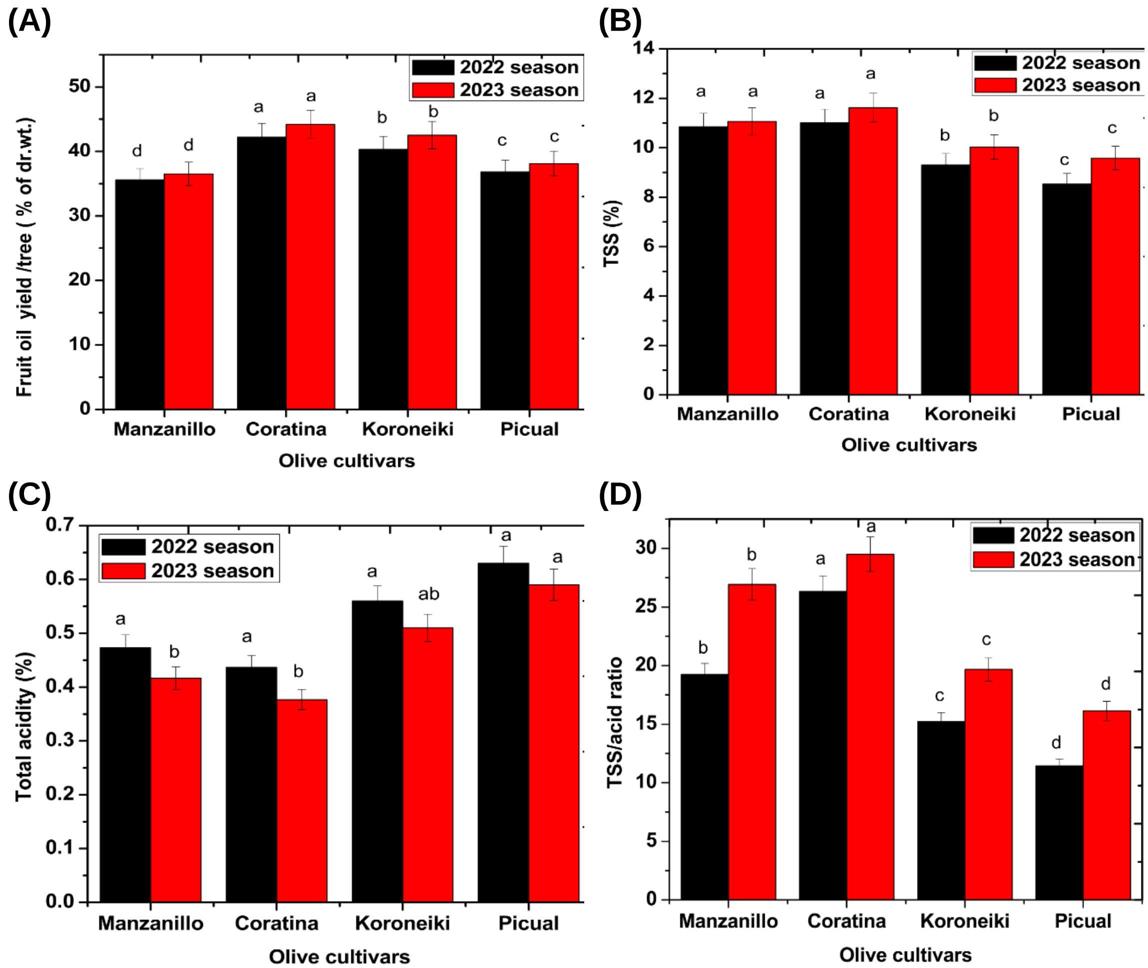

Fig. 6. Fruit oil yield/ tree, T.S.S %, Total acidity %, and T.S.S acid ratio % of all tested cultivars (Manzanillo, Coratina, Koroneiki, and Picual) under low-temperature conditions for three months. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test) and error bars depict standard error.

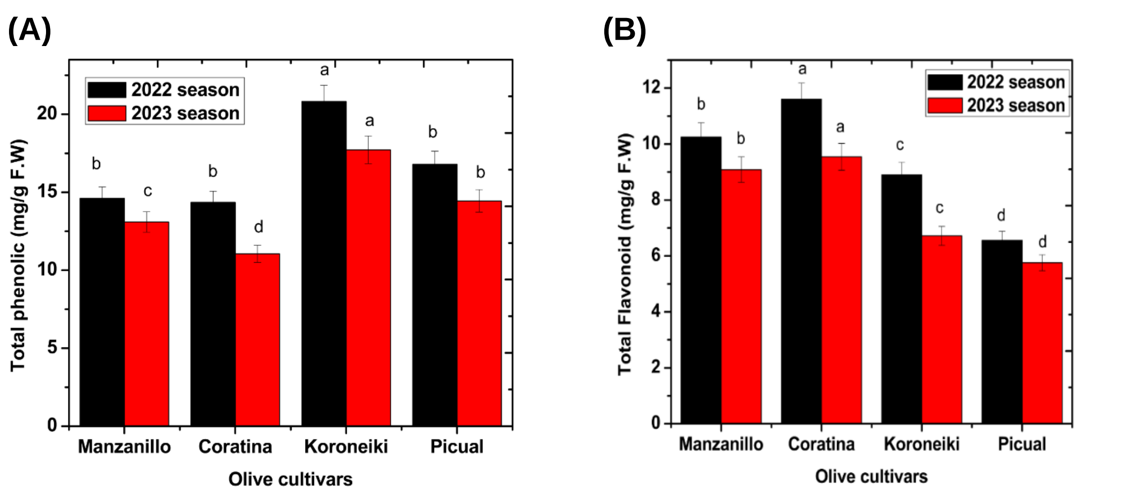

Fig. 7. Total phenolic and total flavonoid of all tested cultivars (Manzanillo, Coratina, Koroneiki, and Picual) under low-temperature conditions for three months. Different letters depict statistically significant changes ($p \leq 0.05$, LSD test) and error bars depict standard error.

Total phenolic and total flavonoids

Figure 7 (A and B) illustrates significant differences noted in leaf total phenolic and total flavonoid contents among olive cultivars. Koroneiki *cv.* was revealed to have the highest values of phenolics (20.82 mg/g F. wt.), whereas the lowest content was found in Coratina *cv.* (11.05 mg/g F. wt.) (Fig. 7A). According to Fig. 7B, the maximum and minimum contents of total flavonoids were detected in Coratina and Picual (11.60 and 5.75 mg/g FW), respectively.

DISCUSSION

Low temperatures are one of the main environmental conditions that restrict the dispersion and productivity of plants (Macek *et al*. 2012). Frost-related leaf damage that occurs in the spring and autumn slows down a tree's overall growth and has a big effect on plant development and yields (Zouari *et al.* 2016). The main cause of frost damage is ice breaking down cell structures physically and causing dehydration (Yadu *et al.* 2017). This is because the interior of a cell has a higher water potential than the area where ice crystals form (Ashraf and Foolad 2007). Olive trees are susceptible to severe damage from frost, especially in their early planting years. Shoot tip necrosis, leaf fall, and bark wounding are typical symptoms. Olive trees may completely die in temperatures below -12 °C. Plants that are susceptible to cold temperatures have been shown to respond by down regulating their photosynthetic efficiency in response to lowered air temperatures (Allen and Ort 2001; Larcher 2000). In the present work, the results of morphological, biochemical, and enzyme activity in leaves or fruits of all stressed tested olive cultivars Manzanillo, Coratina, Koroneiki, and Picual reveal only minor effects of cold stress. However, it should be mentioned that Coratina and Koroneiki stressed plants exhibited higher morphological, biochemical parameters values than Manzanillo and Picual stressed plants, suggesting that Coratina and Koroneiki trees were able to cope better with night-frost incidents. The results showed that in terms of all vegetative growth parameters, including shoot length, leaf area, canopy volume, and tree height, Coratina trees outperformed other olive cultivars (Fig.1). The phenological stages of the plant and the season in which low temperatures occur determine the temperature thresholds that cause frost damage to olive trees (early frost, winter frost, and late frost). The younger parts of the tree (leaves, one-year shoots) are usually injured at minimum temperatures of 0 °C/−3 °C (slight damage), showing a reduction in growth and production; almost all of the plant's organs are damaged at minimum temperatures of −6 °C/−7 °C (moderate damage); and the entire plant may be compromised at temperatures below −12 °C/−18 °C (severe damage) (Rahemi *et al*. 2016). Low temperatures (<12 °C) can be harmful to all stages of plant development in plants that are susceptible to chilling, such as subtropical and tropical plants. This can result in phenotypic symptoms such as reduced plant growth, shorter leaf area, reduction in root length, reduced leaf expansion, loss of leaves, chlorosis, and necrosis, as well as damage to reproductive organs such as decreased pollen production and viability, reduced pollen tube growth, and increased flower abortion (Lukatkin *et al*. 2012). Strong vegetative growth may be linked to good fruit yields, suggesting that tree growth may reflect the ability to adapt to new environments (Rallo *et al*. 2018). The interplay between cultivar genotype and the particular environment had an impact on growth of olive trees as height and trunk diameter growth (Kassout *et al*. 2023). Since all of the olive trees in this study were grown at the same location with the same soil, climate, and cultivation practices, genotype was the primary factor influencing the variations in height and diameter growth among cultivars. As a result, olive trees can thrive and endure. Different climates, soil types, and farming practices may be the cause of regional variations in growth (Arenas-Castro *et al*. 2020). According to the present study, olive trees recover quickly from short-term stressors like drought and high temperatures, suggesting that they are very resilient and adaptive to mild stressors (Araújo *et al*. 2019). However, long-term exposure or extreme stress caused irreversible harm to the physiological functioning of the leaves (Trabelsi *et al*. 2019). In the present results, Coratina and Koroneiki trees were able to cope better with night-frost incidents. Thus, the present results are in line with those of other researchers. For example, Saadati *et al*. (2019) found that "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.

The primary pigments in leaf tissue that absorb light energy are chlorophyll and carotenoids. Also, plant photosynthesis strength is represented by the levels of chlorophyll (Zhao *et al*. 2020). The process of photosynthesis is sensitive to low temperatures. Exposure to (frost) low temperatures leads to the disruption of all the main components of the process, such as inhibiting electron transfer in photophosphorylation and reducing the efficiency of photosystem II (PSII). PSII is one of the most vulnerable components of photosynthesis (Banerjee and Roychoudhury 2019). Low temperatures cause a decrease in the rate of net photosynthesis, transpiration, stomatal conduction, and damage to photosystem II in olive cultivars (Saadati *et al*. 2019). In this study, photosynthetic pigments in Koroneiki and Coratina cultivars were considered the least affected under frost conditions (Fig 2.). The physiological process of photosynthesis can be significantly impacted by abiotic stressors (Gururani *et al*. 2015). On the other hand, previous studies on olive trees have shown that their photosynthetic system is tolerant of a variety of abiotic stresses, such as drought and high UV radiation (Koubouris *et al*. 2010). These findings concur with those of Mohajeri *et al.* (2022), who examined how cold stress affected the photosynthetic pigments in the leaves of two different cultivars of olive seedlings. According to a report, when two tested olive seedling cultivars' leaves were cooled from 10 to -10 ° C, their levels of chlorophyll a, b, total chlorophyll, and carotenoids were lower than those of the control group.

Low temperatures are known to cause cell dehydration, and RWC is an easy method to gauge this phenomenon (Ghosh *et al*. 2016). The capacity of plants to preserve cellular water by raising the relative moisture content is one of the most crucial elements in ensuring their survival under cold stress (Mohajeri *et al*. 2022). As shown in Fig. 3A, the relative water content in the leaves of Manzanello, Coratina, Koroneiki, and Picual was decreased under cold conditions. There are significant differences between all tested olive tree cultivars in relation to cold stress conditions where Coratina gained the highest relative water content. The leaves' water content decreased due to cold adaptation, which also increased the concentration of solutes in the cells, increasing the cell's capacity. The ability of the leaf to retain more water under stress is, in theory, indicated by an increase in the relative moisture content of the leaf. This is accomplished by osmotic regulation or the root's capacity to absorb water (Mishra *et al.* 2023). In a similar way, Li *et al.* (2018) on tea plant (*Camellia sinensis*) reported that the RWC under freezing temperature decreased significantly, even in relation to the duration of the treatment (from 3 h to 12 h). Because of an imbalance between the amount of water absorbed by roots and transpiration, low temperatures can also cause dehydration symptoms in shoots and leaves (Centeno *et al*. 2018).

As the main osmotic regulator in plant cells, proline is important in stabilizing the cell structure and inhibiting the production of reactive oxygen species. The most significant basic physiological marker for abiotic stress reflection is proline (Chen *et al.* 2018). In a similar vein, transgenic plants that overexpressed the proline or glycine betaine biosynthesis genes also displayed decreased levels of lipid peroxidation in the presence of abiotic stress (Khan *et al.* 2015). The molecules share the ability to accumulate at high levels without disrupting intracellular biochemistry. These compatible solutes primarily include proline and glycine betaine. Complementary solute accumulation in plant cells is positively correlated with increased stress tolerance. This is because (i) the plant cell's potential water decreases, increasing water absorption from the rhizosphere; (ii) free radical scavenging; (iii) activation of enzymatic and non-enzymatic antioxidant systems; and (iv) protection of the photosynthetic apparatus from damage (Dawood *et al.* 2014; Zouari *et al.* 2016; Yadu *et al.* 2017; Youssef *et al.* 2018). Ashraf and Foolad (2007) state that proline and glycine betaine can function as antioxidants directly or indirectly by activating antioxidant responses and thereby reduce the damage caused by oxidative stress. Zhou *et al.* (2022) reported that in maize, there is a close correlation between proline accumulation and improved cold tolerance. Many reports show a dramatic relationship between proline accumulation and tolerance in plants under freezing stress (Sarikhani *et al.* 2014; Ershadi *et al.* 2016).

Low temperatures cause oxidative stress due to excessive production of reactive oxygen species (ROS): hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical (OH^-) (Nievola *et al.* 2017). According to Jamshidi Goharrizi *et al.* (2021), H_2O_2 accumulated as cold stress increased. Similar to this, *Fagopyrum tataricum and Arabidopsis thaliana* have shown a considerable increase in H_2O_2 and O_2^- accumulation in response to increased cold stress (Yao *et al.* 2018). The ability of several antioxidant enzymes to function simultaneously is essential for shielding plant cells from ROS toxicity (Das and Roychoudhury 2014). Plant species that are tolerant to freezing-induced oxidative stress tend to exhibit greater resilience against such stressors. This resilience can also be reinforced by enhancement of antioxidant enzyme activities (Luo *et al.* 2007; Cansev *et al.* 2011). Catalase and POX convert free radicals such as hydrogen peroxide (H_2O_2) into water and oxygen (Møller *et al.* 2007). In the current study, Coratina cv. had the highest activity of POX and PPO enzymes under low temperatures, and this may be related to frost tolerance (Fig. 4A to B). Higher values of antioxidant enzymes such as APX, PPO, and CAT were reported in olive cultivars under low temperatures in other studies (Cansev *et al.* 2011; Ortega-García and Peragón 2009). Hashempour *et al.* (2014) reported that there is a substantial correlation between olive trees' ability to frost tolerance and their APX, CAT, and POX activities. According to Ortega-García and Peragon (2009), the activities of PPO and phenylalanine ammonia-lyase (PAL) are crucial in plants' defense against freezing stress because they can prevent major oxidative damage brought on by freezing.

In this study, Coratina cv were superior in terms of initial and final fruit set percentage, while Manzanello olive cultivar was superior in the fruit weight and yield under frost conditions during the two studied seasons (2022 and 2023) (Fig. 5). A temperature drop (from −0.4 to −3 °C) can cause drupe wilting and dehydration, surfaces with brownish blisters and spots, fully frozen drupes, internal browning around the pit, skin browning and mild flesh discoloration, and a decrease in oil accumulation (Bheemanahalli *et al*. 2019). Research conducted on different plant species has demonstrated that cold stress results in a decrease in pollen tube length and germination rate (Geßler *et al*. 2006). In general, the climate has a significant impact on the phenology and adaptation of trees (Zhu *et al*. 2013). Springtime temperatures have a significant impact on the development of olive flowers, pollen germination, fruit set and development, and panicle number of flowers (Ben-Ari *et al*. 2021; Koubouris *et al*. 2010). Furthermore, flowers and flower buds may sustain severe damage at temperatures as low as 0 °C (Kudo and Ida 2013). Long-term low temperatures have been shown to have a negative impact on fruit set, flowering, and pollination (Francini and Sebastiani 2019). Pollen germination may be hampered by low temperatures during flowering, which could compromise fruit set (Koubouris *et al*. 2010).

In the present study it was shown that Coratina olive tree cultivar possessed the highest values of fruit chemical characteristics such as fruit oil yield/tree, T.S.S %, Total acidity %, and T.S.S acid ratio %, followed in ascending order by those of Manzanello, Koroneiki, and Picual varieties (Fig. 6). Olive yield and quality are known to be significantly impacted by ecological factors and cultivation conditions (Calvo-Polanco *et al*. 2016). The differences in oil content in olive fruits are also related to fruit size, which is influenced by exogenous and endogenous factors. Frost, or low air temperatures of 0 ~C or below, can harm olive fruits by causing the water in their pulp to freeze, lowering enzymatic and biochemical reactions, and suppressing microbial activity (Bubola *et al.* 2020). According to Mafrica *et al.* (2021) high altitude-typical low temperatures and heavy rainfalls postpone fruit development and ripening while improving the quality of olive oil. On the other hand, differences in the tolerance of olive cultivars to cold stress were associated by chemical indicators such as the amount of proline, soluble sugars, as well as the content of soluble carbohydrates and phospholipids (Gulen *et al*. 2009). Abiotic stresses affect different cellular processes such as growth, photosynthesis, carbohydrate and lipid metabolism, osmotic homeostasis, protein synthesis and gene expression. Soluble sugars may either act directly as negative signals or as modulators of plant sensitivity and thus, they can also play important roles in cell responses to stress-induced remote signals (Rosa *et al.* 2004). Soluble sugars actively protect plant cells from damage caused by cold stress. In addition, accumulation of TSS, and thus enhanced the cold tolerance of Chinese olive fruit (Lin *et al*. 2016). According to Martínez *et al*. (2004), the buildup of compatible solutes in plants is believed to help stressed cells either by serving as cytoplasmic osmolytes to aid in water uptake and retention or by shielding macromolecules (such as proteins, membranes and carbohydrates) and their structural integrity from stress-induced damage.

Titratable acidity (TA) was used as an indicator to evaluate the quality of fresh produces under abiotic stress (Lin *et al*. 2020). As the primary components of oil production, oil content and fruit production are the most crucial economic factors for characterizing olive cultivars (Gómez-del-Campo 2013). Mediterranean-produced olive oil has generally been identified by its acidity, fatty acid composition, peroxide levels, and polyphenol content (Fernández-Espinosa 2016). The present results indicated that the difference in total acidity values between different olive cultivars is due to a response to stress resulting from low temperature. Additionally, plants use fatty acids as part of their defense mechanism against biotic and abiotic stressors (Okazaki and Saito 2014). Biomembranes are the first to suffer damage when plants are stressed by low temperatures.

Plant cold resistance is directly correlated with membrane fluidity and stability. In general, unsaturated fatty acids are thought to be advantageous for preserving membrane integrity (Upchurch 2008). Consequently, in low-temperature, increasing the amount of unsaturated fatty acids in membrane lipids can improve cold resistance (wang *et al*. 2019).

At low temperatures, the accumulation of secondary metabolites such as phenolic compounds and anthocyanins occur (Francini and Sebastiani 2019). Cold stress leads to increased cellular damage caused by increased production of ROS. Therefore, the development of the antioxidant defense system, which includes antioxidants such as phenolic and flavonoids, may be partly related to cold stress resistance. The most abundant secondary metabolites in plants, phenolic compounds, have a potent ability to donate electrons and hydrogen atoms, which allows them to neutralize ROS and stop lipid peroxidation (Huang *et al.* 2019). In this study, the high amounts of phenolic and flavonoids in the Koroneiki and Coratina cultivars, respectively, may have a direct relationship to the resistance of these cultivars to frost. The values of total phenolic and total flavonoids can be used as biomarkers to distinguish olive varieties under frost stress. These results agree with Ortega-García and Peragón's study (2009). They found changes in total phenolic and proline content in different olive cultivars under cold stress.

CONCLUSIONS

In summary, the findings demonstrated that the tested olive cultivars responded differently to the growing region's environmental conditions. The analysis of the many different parameters considered in this article revealed that low temperatures below the limits of adaptation have a significant impact on morphology, physiology, biochemical, and productivity of olive trees. Based on the findings of this study on olive cultivar behavior, the length of the event, the genotypes, the stage at which the plant is growing, and the interactions with the environment can determine differences in how different cultivars respond to the cold acclimation process, thereby influencing their intrinsic tolerance to cold stress. There were significant variations in olive tree cultivar responses to cold stress. Coratina and Koroneiki emerged as the most resilient cultivars, demonstrating superior performance in biochemical, physiological, and reproductive functions compared to Manzanillo and Picual. These findings empower farmers to make informed decisions regarding olive cultivar selection and breeding programs, ultimately promoting improved adaptation to low-temperature events frequently encountered in open plantations.

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Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization, H.F.A and A.E.H.; methodology H.F.A;IAE;A.N.A;H.K.;A.F. and E.M.H software, A.E.H. and A.N.A; validation, H.F.A and A.F; formal analysis, H.F.A and A.E.H.; H.K.; E.M.H and A.F.A. investigation, H.F.A.; ANA , E.M.H and I.A.F resources, A.N.A.; data curation, I.A.E.; writing—H.F.A.; writing—review and editing, M.H.F. and E.M.H visualization, A.E.H.; supervision, A.E.H. and M.H.F.; project administration, A.M.S.; funding acquisition, A.M.S. All authors have read and agreed to the published version of the manuscript.

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