

## Article

# Adaptive Responses of Four Medicinal Plants to High Altitude Oxidative Stresses through the Regulation of Antioxidants and Secondary Metabolites

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**Abstract:** The conservation of medicinal plants, particularly endangered or endemic species, is of the utmost importance, especially in light of inevitable climate change and its consequences. Species inhabiting high altitudes adopt exceptional defense mechanisms in response to abiotic stresses as a survival strategy. The objective of the current study was to investigate the effects of altitudinal variations on secondary metabolite accumulation and antioxidant enzyme capacity in four plants (*Cotoneaster orbicularis*, *Crataegus x sinaica*, *Echinops spinosissimus* subsp. *spinosissimus*, and *Tanacetum sinaicum*) growing naturally on the Sinai Peninsula's high mountains. Plant leaves and soil samples were collected from three altitudes between 1500 and 2250 m a.s.l. to evaluate the adaptive responses of these species in relation to high-altitude oxidative stresses. The results showed that at higher altitudes, the electrical conductivity and the micronutrient contents of the soil decreased, which may be due to the prevalence of silt and clay decreasing at higher altitudes. Chlorophyll a, chlorophyll b, ascorbic acid, and total soluble protein showed similar results in relation to higher altitudes for all species. On the other hand, proline, total soluble sugars, carotenoids, phenols, tannins, and flavonoids increased in response to high altitudes. The activity levels of catalase and ascorbic acid peroxidase showed a significant increase aligned with higher altitudes, while a significant decrease in activity levels was obtained for polyphenol oxidase. In conclusion, the present findings showed that *Cotoneaster orbicularis* exhibited the maximum response for coping with high-altitude stresses, followed by the remaining three species regarding the level of biochemical and physiological responses. The present work will help formulate conservation plans for important medicinal species.

**Keywords:** antioxidant capacity; bioactive compounds; endangered medicinal species; high altitude environment; oxidative stresses; protectorate

## 1. Introduction

One of the main issues regarding the impact of climate change is the destruction of particular natural habitats, specifically in fragile environments, as these changes control the growth dynamics, distribution, and population structure of plants [1–3]. A species' survival in its ecosystem depends on its ability for habitat adaptation, and most plant species have evolved a diversified array of biochemical responses to deal with different environmental challenges [4,5]. To maintain a homeostatic level of photosynthetic efficiency, plants either adapt their physiological and morphological characters or adjust their photosynthetic capacity in response to the environment [6,7].

It is anticipated that the continuous consequences of climate change will increase the frequency of droughts, nutrient deficiency, and temperature increase, and the combined effects of these stresses on species' survival will be more harmful than their individual effects [8].

Although the Sinai Peninsula only comprises 6.1% of Egypt's total land area, it is home to about 1262 different plant species. Approximately 62 endemic plant species exist in Egypt, with the Sinai Mountains acting as home to half of them. According to Boulos [9], Saint Katherine Protectorate (SKP) is one of the Middle East's most floristically diverse locations, with 17 endemic taxa (28% of Egyptian endemic taxa), comprising a sizable fraction of the region's endemism [10–12]. Six microhabitats, including wadis, gorges, caves, basins, slopes, and terraces, can be differentiated in this area due to the variety of SKP's landforms and geologic features. The first two microhabitats include dykes that trap water, resulting in substantial plant cover [13].

Due to the altitude of the SKP mountains, plant growth, diversity, and distribution are limited by harsh and complicated environmental circumstances [14]. High illumination, strong winds, low levels of CO<sub>2</sub> and O<sub>2</sub>, gravely thin soils with low water and nutrient contents, and low temperatures are only a few of these harsh environmental conditions [15,16]. Even though these stressors could retard plant growth, many plants have endured and progressed, developing a variety of adaptive defense mechanisms in response to these abiotic stresses [17,18].

In addition to being a valuable assortment of natural products, plant secondary metabolites (SMs) have also been linked to the activation and augmentation of plant defense mechanisms against biotic and abiotic environmental stresses [19,20]. Likewise, environmental variables such as soil microorganisms, water availability, soil pH, nutrients, temperature (high/low), high altitude, drought, and light conditions strongly affect the quality and quantity of SMs production among plant populations [21–23]. These SMs actually assist plants in maintaining physiological and morphological functions. For instance, they increase flavonoids, tannins, and phenols as antioxidants; accumulate soluble protein, soluble sugars, and proline to fend off drought via the regulation of the water-holding potential; and upregulate the xanthophyll cycle pigment pool by increasing carotenoids relative to chlorophylls, lessening the harmful effect of high light intensity [24–26].

On the other hand, reactive oxygen species (ROS) production, including singlet oxygen, hydroxyl radicals, and superoxide, increases as a result of high-altitude environmental stresses (light intensity, low temperature, and drought), amplifying the possibility of oxidative damage [27–29]. The combination of these stresses can seriously harm plants by limiting photosynthesis, closing stomata, and inhibiting various physiological processes, including antioxidant activities [30–32].

Cells achieve ROS homeostasis when the ratio of ROS synthesis to ROS scavenging is balanced. However, under typical circumstances, ROS production is minimal and typically balanced by antioxidant molecules [33]. Plants can scavenge ROS via two antioxidant systems: 1. the non-enzymatic antioxidant system, comprised of compounds of basic antioxidant characteristics, such as glutathione, flavonoids, phenolic compounds (water soluble), carotenoids, and  $\alpha$ -tocopherols (lipid-soluble); and 2. the enzymatic antioxidant system, which comprises catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and ascorbate peroxidase (APX) [34,35]. Additionally, malondialdehyde (MDA) is considered an important indicator of abiotic stress (especially drought), and it is the end product of cell membrane lipid peroxidation. Frequently, MDA will increase with decreasing soil water content [36–38].

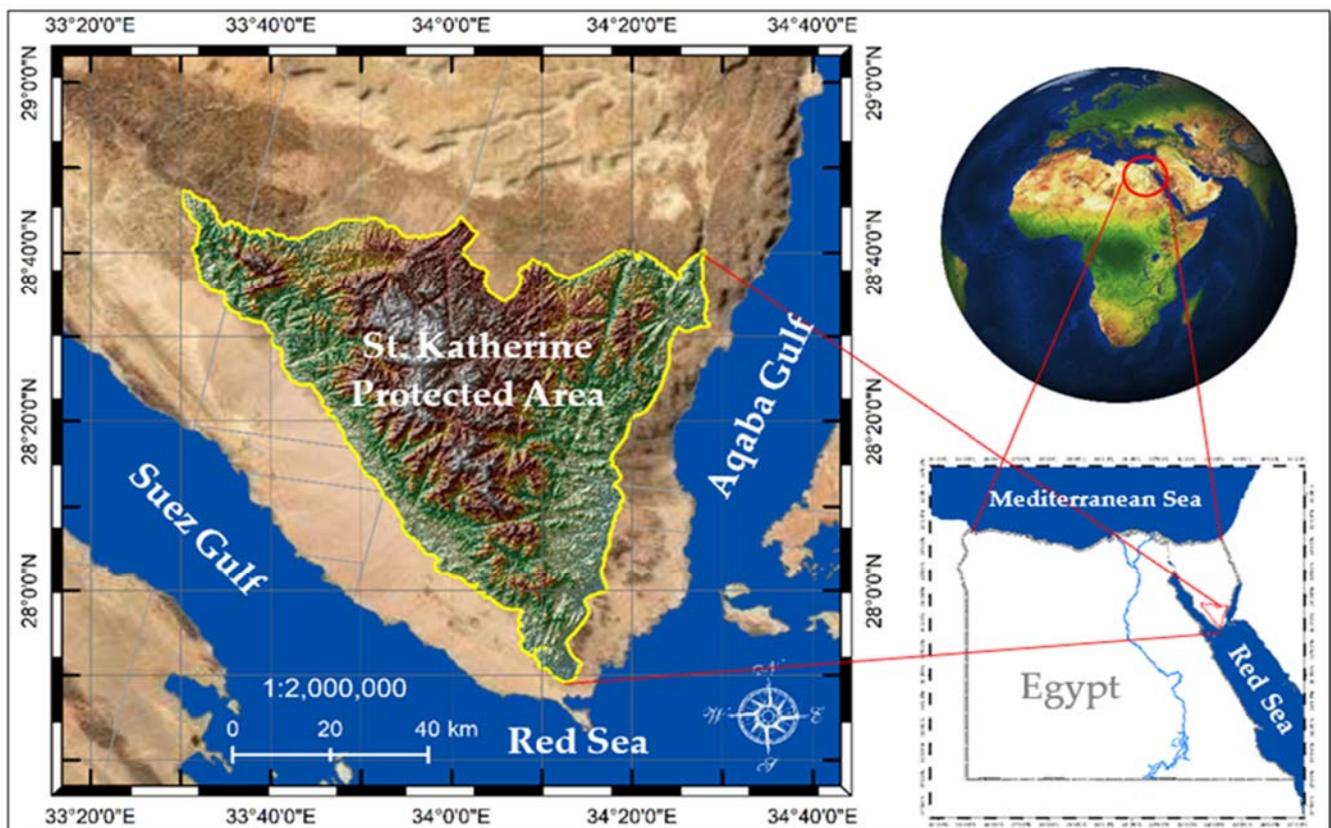
Four plant species of high value (medicinal, solid fuel, and edible) inhabiting the SKP mountains were investigated in this study (namely, *Cotoneaster orbicularis* Schltdl., *Crataegus x sinaica* Boiss., *Echinops spinosissimus* subsp. *spinosissimus* Turra., and *Tanacetum sinaicum* Del.). Despite the medical and ecological importance of these species, little is known about their ecophysiology, particularly at high altitudes in the Sinai Peninsula. Consequently, understanding the different adaptive mechanisms of these species is crucial

for predicting their responses to climate change in the future and assisting researchers in planning to conserve and sustain these species. In this study, the SMs, antioxidant capacity, and physiology of the four plants were analyzed in relation to altitudinal variation. Additionally, the eco-physiological response of these plants to exposure to different stresses at higher altitudes is investigated.

## 2. Materials and Methods

### 2.1. Study Area

The current study was carried out in the mountainous region of Saint Katherine protectorate (SKP) in the south part of Sinai Peninsula (Figure 1). SKP was declared as a protected area in 1996 by the (EEAA) [39]. It is located between  $28^{\circ}30'$  and  $28^{\circ}35'$  N and  $33^{\circ}55'$  and  $34^{\circ}30'$  E, covering an area of roughly 4350 km<sup>2</sup>. The region is distinguished by the presence of Egypt's highest and steepest mountains, which reach elevations of up to 2624 m above sea level (a.s.l.) [40,41]. As a result of its distinctive geological, climatic, and morphological characteristics, this dry mountainous ecosystem is home to numerous rare, endemic, and medicinal plant species [10,40].

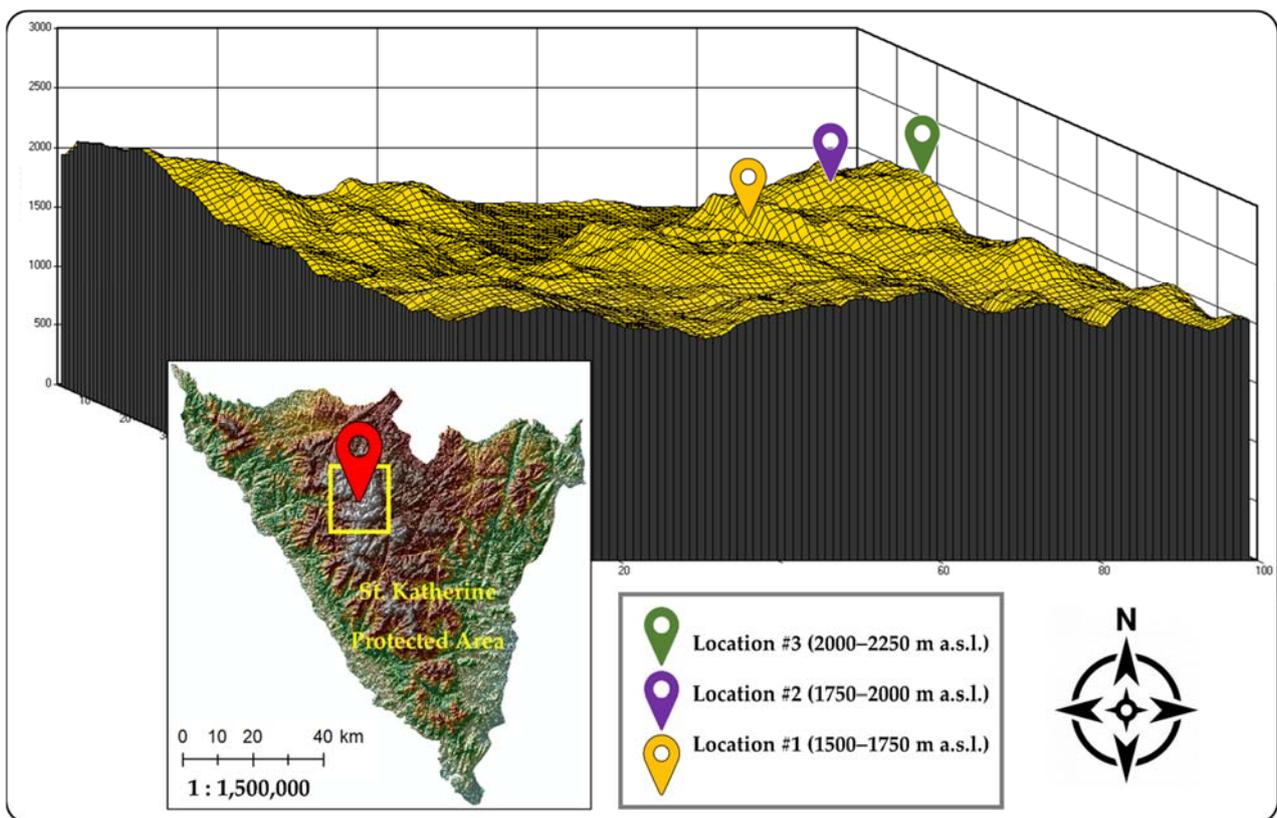


**Figure 1.** Map of the study area for Saint Katherine protectorate, Sinai Peninsula, Egypt (Base map: acquired using Landsat 8-2022-P/R: 178/39).

The plant materials were collected from El-Gebel Al-Ahmer (red mountain) at SKP in April/May 2022. The study area lies in a Saharan-Mediterranean hyper-arid zone, with extremely cold winters and hot, dry summers. Due to its great altitudes, SKP is the coldest place in Sinai [42,43]. The average temperature ranges from 5.4 °C to 25.2 °C, with the mean minimum temperature occurring in February (7.8 °C) and the mean highest temperature occurring in August (36 °C) [44].

## 2.2. Target Species

In Table 1, specifics concerning the target species are given, including their scientific names, their families, habitat, distribution, status, uses, distribution, altitude, and field photos. Four medicinal plant species (namely, *Cotoneaster orbicularis*, *Crataegus x sinaica*, *Echinops spinosissimus* subsp. *spinosissimus*, and *Tanacetum sinaicum*) were collected from three different altitudes (1500–1750, 1750–2000, and 2000–2250 m a.s.l.) of the SKP mountains (Figure 2). Samples were taken from the shoot systems (4 species  $\times$  3 different individuals for each  $\times$  3 altitudes) of the four plant species under investigation, with a total of 36 plant samples, which were stored frozen at ( $-20\text{ }^{\circ}\text{C}$ ). Next, the samples were either extracted to determine the photosynthetic pigments, total antioxidant capacity, and enzyme, proline, ascorbic acid, and malondialdehyde levels, or air-dried to determine the quantity of tannins, carbohydrates, flavonoids, and phenolic compounds.



**Figure 2.** Surface profile showing the three locations of data collection at different altitudes in Saint Katherine protectorate.

Collected taxa were identified according to the work of Boulos, Täckholm, and Zohary [9,45,46]. Taxa names were clarified against Kew's Plants of the World Online [47], which was also used as a reference for family and genera classification.

**Table 1.** Family, scientific name, habitat, status, uses, distribution, and field photos of the four target species.

No.	Family	Plant Species	Habitat, Status, Uses, and Distribution	Field Photo
1	Rosaceae	<i>Cotoneaster orbicularis</i> Schldtl.	<ul style="list-style-type: none"> <li>- Distribution: It is restricted to the rugged South Sinai and Eastern Arabia.</li> <li>- Status: Endangered</li> <li>- Habitat: It flourish in crevices and in soft dykes.</li> <li>- Uses: fuel, folk medicine, and animal grazing.</li> <li>- Refs. [48–50].</li> </ul>	
2		<i>Crataegus x sinaica</i> Boiss.	<ul style="list-style-type: none"> <li>- Distribution: It is confined to the South Sinai mountains.</li> <li>- Status: Endangered</li> <li>- Habitat: It grows in the SKP's elevated high wadis.</li> <li>- Uses: fuel, folk medicine, and food.</li> <li>- Refs. [51–53].</li> </ul>	
3	Asteraceae	<i>Echinops spinosissimus</i> subsp. <i>spinosissimus</i> Turra.	<ul style="list-style-type: none"> <li>- Distribution: It grows well across the Sahara, notably near Sinai and the Red Sea.</li> <li>- Status: Common</li> <li>- Habitat: It grows on coastal calcareous dunes, wadi beds, and on gravelly to rocky surfaces.</li> <li>- Uses: fuel, folk medicine, and animal grazing.</li> <li>- Refs. [48,54,55].</li> </ul>	
4		<i>Tanacetum sinaicum</i> Del.	<ul style="list-style-type: none"> <li>- Distribution: It is confined to the South Sinai mountains.</li> <li>- Status: Endemic to Egypt</li> <li>- Habitat: It grows on the rocky slopes of SKP.</li> <li>- Uses: folk medicine.</li> <li>- Refs. [47,56,57]</li> </ul>	

### 2.3. Soil Analysis

Twelve soil samples were taken from each studied altitude at a depth of 0–30 cm (one soil sample for each plant individual at each altitude). The samples were then air-dried to determine their physical and chemical characteristics. Mechanical analysis (texture) was determined according to the technique of [58] using the hydrometer method to separate sand, silt, and clay. Using the potentiometric method [59], pH value, total dissolved salts (TDS), and electric conductivity (E.C.) were determined in the soil–water extracts (1:5). Using the method designed by Johnson and Ulrich (versene titration method) [60], magnesium and calcium were measured volumetrically. According to the method of Shapiro and Brannock [61], potassium and sodium were determined by flame photometry. Bicarbonates and chlorides were determined by the titration method according to the work

of [62,63]. Sulphates and calcium carbonates were determined according to the method of Bardsley and Lancaster [63]. Organic carbon (O.C.) and organic matter (O.M.) were determined using the titration method described by Piper [64].

#### 2.4. Phytochemical Assay

All the chemicals used in this investigation were of high purity, purchased from Sigma Aldrich Chemical Co., Germany, and all organic solvents were of AR grade.

##### 2.4.1. Extraction and Estimation of Photosynthetic Pigments

The photosynthetic pigments in terms of Chl a, Chl b and carotenoids were extracted in 80% acetone. Measurements were performed using a spectrophotometer (Spectronic 601, Milton Roy Company, Ivyland, PA, USA). Pigment contents were calculated according to the method of Metzner et al. [65].

##### 2.4.2. Extraction and Estimation of Total Soluble Sugars

Following the method of Homme, et al. [66], sugars were extracted. The method of Loewus [67] was used to measure the total soluble sugar contents by reacting 0.1 mL of the ethanolic extract with 3 mL of newly made anthrone reagent. Utilizing the glucose standard curve, the soluble sugar concentration was quantified and represented as  $\mu\text{g}$  glucose equivalent/g dry weight.

##### 2.4.3. Extraction and Estimation of Total Phenolic, Flavonoid, and Tannin Content

According to Malik and Singh's method [68], the total phenolic molecules were extracted, and their content was measured (concentrations were estimated based on the pyrogallol standard curve as gallic acid equivalents/g). According to the Harborne method [69], after being extracted, the total flavonoids were calorimetrically determined by reacting with  $\text{AlCl}_3$  (the concentration of total flavonoids, given as  $\mu\text{g/g}$  dry weight, was determined using the quercetin standard curve). Finally, following the method outlined by Ejikeme et al. [70], the tannins were extracted and then quantified.

#### 2.5. Biochemical Assay

##### 2.5.1. Extraction and Estimation of Total Soluble Proteins and Proline Content

Total proteins were extracted by grinding 0.5 g of fresh leaf tissue in 1 mL of phosphate buffer (0.1 M, pH 7.0), before being preserved in ice. Then, the concentration of soluble proteins was assessed according to the method of Bradford [71]. The amount of free proline expressed as  $\mu\text{g/g}$  fresh weight was calculated according to the method Bates et al. [72].

##### 2.5.2. Extraction and Estimation of Malondialdehyde

According to the method of Heath and Packer [73], the quantity of lipid peroxidation was assessed by counting the MDA amount generated by the thiobarbituric acid reaction (results were expressed as  $\mu\text{mol g}^{-1}$  fresh weight).

#### 2.6. Enzymes Extraction and Assays

The enzymes were extracted following the method of Mukherjee and Choudhuri, [74]. A total of 250 mg of fresh tissue were crushed coarsely using a pestle in a cooled mortar after being frozen in liquid nitrogen. Then, the ground powder was added to 10 mL of 100 mM phosphate buffer ( $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ , pH 6.8), and centrifuged at  $20,000\times g$  for twenty minutes. Using the same buffer, the supernatant was diluted to a specified volume and used as an enzyme extract to determine the activity of various enzymes.

The super oxide dismutase (SOD) activity level was estimated following the method of Dhindsa et al. [75]. The SOD activity was measured as unit/mg protein. The CAT activity level was determined following the method of Hermans et al. [76]. The Catalase (CAT) activity was measured as mM of  $\text{H}_2\text{O}_2/\text{g FW}/\text{min}$ . The peroxidase (POD) activity was calculated according to the Kar and Mishra method [77], with slight modifications. Quinone/g

f.w./min was used to express POD activity. Finally, ascorbate peroxidase (APX) activity was assessed according to the method of Koricheva et al., with slight modifications [78]. The APX activity was expressed as mM of ascorbate oxidized/g f.w./min.

### 2.7. Determination of Ascorbic Acid (AsA) and Total Antioxidant Capacity (TAC)

According to the method of Kampfenkel et al. [79], ascorbic acid extracted from fresh tissue weighing (0.1 g) was homogenized in one milliliter of trichloroacetic acid solution (6% (w/v)); the homogenate was then centrifuged at  $12,000\times g$  and  $4\text{ }^{\circ}\text{C}$  for ten minutes. Ascorbic acid estimation was performed using the supernatant. TAC was measured according to the method of Prieto et al. [80] using phosphomolybdenum.

### 2.8. Statistical Analysis

Data of four species (*Co. orbicularis*, *Cr. sinaica*, *Ec. spinosissimus*, and *Ta. sinaicum*) at three different altitudes (1500–1750, 1750–2000, and 2000–2250 m a.s.l) was analyzed to obtain the physiological and ecological parameters using Excel 365, Minitab 20, IBM SPSS 26, and PC-ORD 5. Three plant and soil samples were collected from each species at each altitude, with a total of 36 soil and plant samples (3 samples  $\times$  4 species  $\times$  3 altitudes). Data was cleansed before running any statistical analyses. Missing data and mistyping errors were checked. Descriptive statistics, including mean and standard error of mean, were calculated for the physiological parameters (CAT, SOD, APX, POX, total phenols, flavonoids, tannins, TAC, AsA, soluble sugar, total sol. protein, proline, MDA, chl. a, chl. b, and carotenoids) and soil parameters (pH, E.C., TDS,  $\text{Cl}^{-}$  (meq/L),  $\text{CaCO}_3^{-}$  (%),  $\text{SO}_4^{-}$  (Mg/L),  $\text{HCO}_3^{-}$  (%),  $\text{Ca}^{++}$  (meq/L),  $\text{Mg}^{++}$  (meq/L),  $\text{Na}^{+}$  (mlq/L),  $\text{K}^{+}$  (mlq/L), O.C. (%), O.M. (%), sand %, Clay%, and silt%). Inferential statistics were used to compare the results of different groups. All variables parametric assumptions were tested, and the Box Cox transformation for non-normal dependent variables was applied using the optimal  $\lambda$  method, whenever needed.

Two-way ANOVA was used to evaluate the physiological and soil parameters for each altitudinal variation and different species, using the general fit linear model in Minitab 20. Bar charts of all variables, showing the mean and SE of each variable in relation to different factors, were produced using Excel 365. The results showed a good fit for different models, while normal residual probability plots showed a linear attitude for all analyses after data transformation. The  $p$  value was considered significant at  $\alpha < 0.05$ . Post hoc analyses of the interactions among all groups were conducted using the Tukey test for pairwise comparisons. The results of the post hoc analyses are represented with letters, where groups that share the same letters are non-significantly different, while different letters express significant differences among different groups.

Multivariate analyses of physiological and soil parameters were performed using PC-ORD 5. Two-way cluster analysis (TWCA) and principal component analysis (PCA) were conducted for all parameters regarding different species from different altitudes. Two-way cluster analysis was achieved using Euclidean distance as a distance measurement and Ward's method as a linkage method. PCA was plotted using PC1 and PC2, and correlations of all parameters and species from different altitudes were obtained. The TWCA dendrogram and the PCA joint plot were graphed using PC-ORD5.

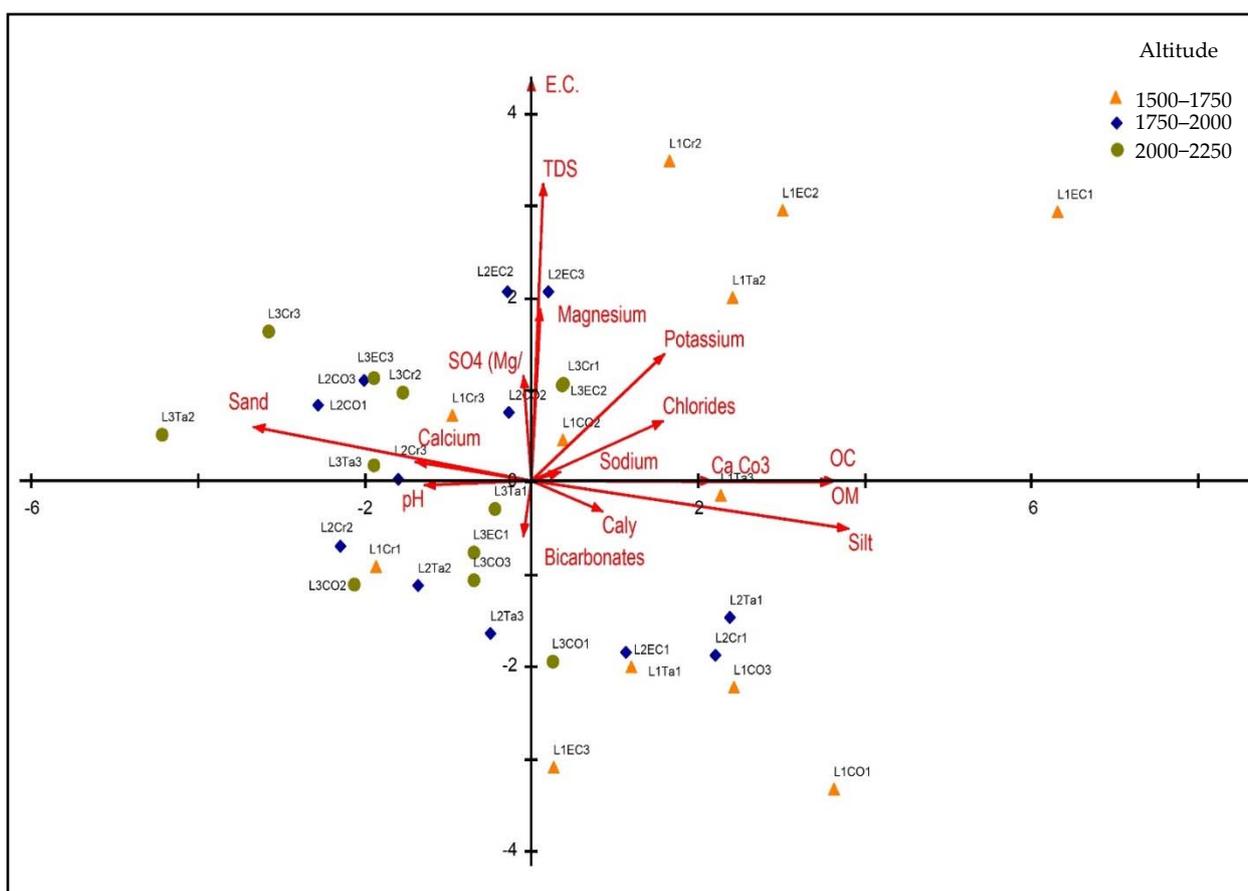
## 3. Results and Discussion

### 3.1. Soil Analysis

In natural environments, such as mountains, the main determinant of plant species distribution and growth are moisture and temperature gradients, which combine the influence of numerous physical factors. The moisture content depends on the land form type, slope degree, nature of the soil surface, soil texture, and soil organic content, whereas the temperature is a net product of the elevation and the slope aspect [81–83]. Moreover, in the present work, there is a marked decrease in the distribution and intensity of species

with increasing altitude. This may be correlated with changes in the physical and chemical characteristics of the soil collected from the three locations at different altitudes.

Performing a principal component analysis (PCA) showed that altitude is a major factor separating the 36 soil samples. PCA output showed that the first altitude (1500–1750) tends to correlate positively with axis 1, while the third altitude (2000–2250) showed a negative correlation with axis 1. The second altitude (1750–2000) showed no correlation with axis 1. On the contrary, axis 2 showed no correlation with any altitude. Generally, pH value, sand, and  $\text{Ca}^{+2}$  contents showed a correlation with the highest altitude (2000–2250), while lower altitudes were correlated with silt, clay, (OC), (OM),  $\text{Cl}^-$ ,  $\text{K}^+$ ,  $\text{Mg}^{+2}$ , and E.C. (Figure 3).



**Figure 3.** PCA joint plot showing target species at different altitudes in relation to soil parameters. Abbreviations: L1 = altitude 1; L2 = altitude 2; L3 = altitude 3; Co. = *Cotineaster orbicularis*; Cr. = *Crataegus sinaica*; Ec. = *Echinops spinossissmus*; Ta. = *Tanacetum sinaicum*.

From the obtained results shown in Figure 3, it is clear that electrical conductivity and some free salts, such as  $\text{K}^+$ ,  $\text{Na}^+$ , OC, OM, and  $\text{Mg}^{+2}$ , are negatively correlated with high altitudes (they tend to accumulate at low altitudes). The EC value, OC, and OM content of the soil exhibits a positive relationship with the fertility and nitrogen content in the soil [81,84]. Moreover, the EC value of soil decreased with an increase in the altitude due to the decrease in water content and free salts [85–87]. This was accompanied by a decrease in the ratio of silt and clay contents (lowest values of 24.06% and 4.03%) and an increase in the ratio of sand as it reached its highest value (71.75%) at the highest altitude (2000–2250 m a.s.l.), as shown in Table 2. Collectively, these conditions cause the plants grown at high altitudes to suffer from stress due to deficiencies in water and nutrients, which may affect their growth and distribution [81,84].

**Table 2.** (a) Mean  $\pm$  SE of soil samples characteristics (pH, E.C, TDS, O.C, O.M, clay, sand, and silt contents) for the studied species at different altitudes. Abbreviations: L1 = altitude 1; L2 = altitude 2; L3 = altitude 3; Co. = *Cotineaster orbicularis*; Cr. = *Crataegus sinaica*; Ec. = *Echinops spinosissimus*; Ta. = *Tanacetum sinaicum*. (b) Mean  $\pm$  SE of the chemical analysis of soil samples (anions and cations content) for the studied species at different altitudes. Abbreviations: L1 = altitude 1; L2 = altitude 2; L3 = altitude 3; Co. = *Cotineaster orbicularis*; Cr. = *Crataegus sinaica*; Ec. = *Echinops spinosissimus*; Ta. = *Tanacetum sinaicum*.

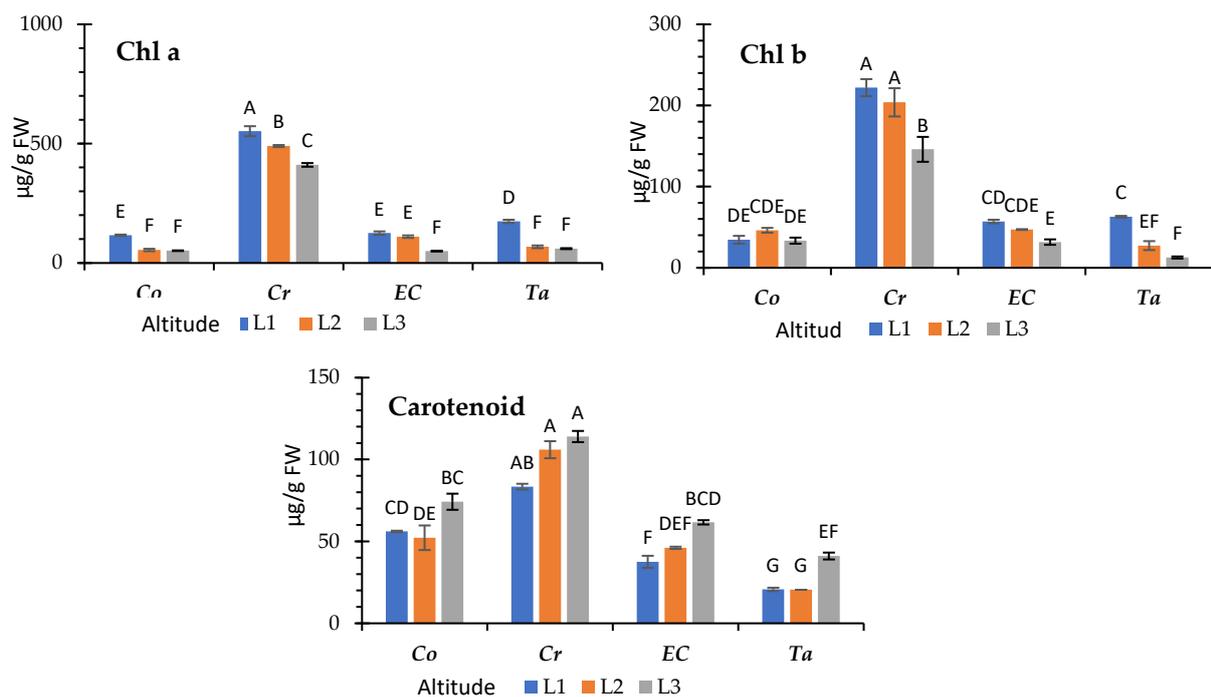
(a)								
Species	pH	E.C. (ds/m)	TDS	O.C. (%)	O.M. (%)	Clay%	Sand%	Silt%
L1 CO.	7.43 $\pm$ 0.07 A	0.94 $\pm$ 0.18 A	408 $\pm$ 113 A	1.23 $\pm$ 0.16 A	2.12 $\pm$ 0.28 A	8.48 $\pm$ 0.28 AB	39.82 $\pm$ 4.89 ABC	51.71 $\pm$ 5.18 AB
L1 Cr.	7.53 $\pm$ 0.03 A	1.03 $\pm$ 0.18 A	657 $\pm$ 115 A	0.83 $\pm$ 0.13 A	1.43 $\pm$ 0.22 A	10.65 $\pm$ 1.58 AB	53.64 $\pm$ 4.4 ABC	35.71 $\pm$ 3.23 ABC
L1 EC.	7.57 $\pm$ 0.09 A	0.89 $\pm$ 0.15 A	569.6 $\pm$ 97.9 A	1.01 $\pm$ 0.19 A	1.75 $\pm$ 0.32 A	16.31 $\pm$ 4.04 AB	27.02 $\pm$ 5.01 C	56.66 $\pm$ 3.41 A
L1 Ta.	7.5 $\pm$ 0 A	0.9 $\pm$ 0.11 A	576.9 $\pm$ 71.4 A	1.21 $\pm$ 0.12 A	2.09 $\pm$ 0.2 A	20.26 $\pm$ 4.98 A	37.66 $\pm$ 4.4 BC	42.08 $\pm$ 0.75 ABC
L2 CO.	7.53 $\pm$ 0.03 A	0.88 $\pm$ 0.05 A	501.5 $\pm$ 50.6 A	0.76 $\pm$ 0.03 A	1.31 $\pm$ 0.05 A	7.35 $\pm$ 1.33 AB	64.17 $\pm$ 5.41 AB	28.48 $\pm$ 6.33 BC
L2 Cr.	7.57 $\pm$ 0.03 A	0.72 $\pm$ 0.04 A	496 $\pm$ 53.1 A	1.05 $\pm$ 0.19 A	1.81 $\pm$ 0.32 A	8.69 $\pm$ 2.98 AB	61.2 $\pm$ 11.7 AB	30.15 $\pm$ 8.76 BC
L2 EC.	7.47 $\pm$ 0.03 A	0.82 $\pm$ 0.14 A	588.4 $\pm$ 90.3 A	0.98 $\pm$ 0.05 A	1.69 $\pm$ 0.09 A	6.93 $\pm$ 0.74 AB	58.69 $\pm$ 6.9 AB	34.38 $\pm$ 6.16 ABC
L2 Ta.	7.5 $\pm$ 0 A	0.71 $\pm$ 0.04 A	484.7 $\pm$ 23.5 A	0.93 $\pm$ 0.15 A	1.61 $\pm$ 0.25 A	5.91 $\pm$ 0.21 AB	62.22 $\pm$ 1.65 AB	31.87 $\pm$ 1.44 ABC
L3 CO.	7.6 $\pm$ 0 A	0.67 $\pm$ 0.04 A	437.5 $\pm$ 24.9 A	0.87 $\pm$ 0.04 A	1.49 $\pm$ 0.08 A	6.37 $\pm$ 3.66 AB	51.25 $\pm$ 6.73 ABC	25.9. $\pm$ 3.28 ABC
L3 Cr.	7.47 $\pm$ 0.09 A	0.68 $\pm$ 0.01 A	540.6 $\pm$ 37 A	0.81 $\pm$ 0.03 A	1.4 $\pm$ 0.06 A	6.58 $\pm$ 3.9 AB	65.22 $\pm$ 7.9 AB	25.2 $\pm$ 4.47 C
L3 EC.	7.53 $\pm$ 0.03 A	0.6.9 $\pm$ 0.1 A	533.8 $\pm$ 67 A	0.85 $\pm$ 0.08 A	1.47 $\pm$ 0.14 A	4.03 $\pm$ 0.76 B	65.78 $\pm$ 2.93 AB	26.19 $\pm$ 2.2 BC
L3 Ta.	7.53 $\pm$ 0.07 A	0.71 $\pm$ 0.07 A	548.1 $\pm$ 43.5 A	0.8 $\pm$ 0.12 A	1.38 $\pm$ 0.2 A	4.19 $\pm$ 1.34 B	71.75 $\pm$ 6.67 A	24.06 $\pm$ 5.4 C
(b)								
Species in Different locations	CL (meq/L)	CaCO <sub>3</sub> (%)	SO <sub>4</sub> (Mg/L)	HCO <sub>3</sub> (%)	Ca (meq/L)	Mg (meq/L)	Na (meq/L)	K (meq/L)
L1 CO.	2.66 $\pm$ 0.1 B	9.33 $\pm$ 1.17 A	7.2 $\pm$ 1.46 BC	0.61 $\pm$ 0 A	4.42 $\pm$ 0.54 A	2.16 $\pm$ 0.52 AB	0.62 $\pm$ 0.09 A	0.98 $\pm$ 0.3 A
L1 Cr.	2.19 $\pm$ 0.19 B	5.5 $\pm$ 1.04 A	8.44 $\pm$ 0.33 ABC	0.61 $\pm$ 0 A	5.04 $\pm$ 0.03 A	1.69 $\pm$ 0.06 AB	0.47 $\pm$ 0.06 A	3.12 $\pm$ 1.79 A
L1 EC.	4.66 $\pm$ 0.91 A	9.5 $\pm$ 5.57 A	11.57 $\pm$ 2.06 AB	0.51 $\pm$ 0.1 A	2.83 $\pm$ 0.51 A	3.3 $\pm$ 0.9 AB	0.6 $\pm$ 0.24 A	2.18 $\pm$ 1.3 A
L1 Ta.	2.28 $\pm$ 0.17 B	5 $\pm$ 1.32 A	9.56 $\pm$ 0.12 ABC	0.51 $\pm$ 0.1 A	4.42 $\pm$ 0.54 A	1.76 $\pm$ 0.04 AB	0.71 $\pm$ 0.1 A	2.18 $\pm$ 0.54 A
L2 CO.	2.66 $\pm$ 0.1 B	4.5 $\pm$ 1.5 A	11.74 $\pm$ 0.32 AB	0.51 $\pm$ 0.1 A	4.95 $\pm$ 0.94 A	2.78 $\pm$ 0.49 AB	0.33 $\pm$ 0.03 A	0.64 $\pm$ 0.08 A
L2 Cr.	2.19 $\pm$ 0.1 B	6.5 $\pm$ 1.26 A	8.77 $\pm$ 1.49 ABC	0.61 $\pm$ 0 A	4.48 $\pm$ 0.57 A	1.59 $\pm$ 0.05 B	0.33 $\pm$ 0.03 A	0.77 $\pm$ 0.13 A
L2 EC.	3.14 $\pm$ 0.17 AB	6.83 $\pm$ 1.09 A	7.32 $\pm$ 1.37 BC	0.61 $\pm$ 0 A	4.52 $\pm$ 0.59 A	2.74 $\pm$ 0.54 AB	0.42 $\pm$ 0.06 A	1.54 $\pm$ 0.29 A
L2 Ta.	2.47 $\pm$ 0.34 B	6.67 $\pm$ 1.42 A	5.03 $\pm$ 0.39 C	0.61 $\pm$ 0 A	3.9 $\pm$ 0.56 A	1.65 $\pm$ 0.02 B	0.53 $\pm$ 0.14 A	0.99 $\pm$ 0.04 A
L3 CO.	2.47 $\pm$ 0.1 B	4.67 $\pm$ 0.88 A	10.15 $\pm$ 1.48 ABC	0.61 $\pm$ 0 A	4.42 $\pm$ 0.54 A	1.71 $\pm$ 0.04 AB	0.48 $\pm$ 0.12 A	0.34 $\pm$ 0.04 A
L3 Cr.	2.47 $\pm$ 0.25 B	2.33 $\pm$ 0.44 A	13.45 $\pm$ 0.43 A	0.61 $\pm$ 0 A	4.48 $\pm$ 0.57 A	3.26 $\pm$ 0.08 A	0.68 $\pm$ 0.2 A	0.53 $\pm$ 0.31 A
L3 EC.	2.85 $\pm$ 0.44 AB	5.83 $\pm$ 1.09 A	9.45 $\pm$ 1.4 ABC	0.61 $\pm$ 0 A	4.43 $\pm$ 1.09 A	1.69 $\pm$ 0.06 AB	1.36 $\pm$ 0.92 A	1.41 $\pm$ 0.45 A
L3 Ta.	1.33 $\pm$ 0.53 B	9.33 $\pm$ 1.17 A	12.31 $\pm$ 0.66 AB	0.61 $\pm$ 0 A	4.39 $\pm$ 1.05 A	1.65 $\pm$ 0.02 B	0.41 $\pm$ 0.08 A	1.24 $\pm$ 0.37 A

Data are means of three replications  $\pm$  SE, and at  $p < 0.05$ ; cells with different letters are differ significantly.

### 3.2. Changes in Photosynthetic Pigments

Chlorophyll plays an important role in light harvesting, and its biosynthesis in chloroplasts is adversely affected when exposed to low-temperature stress [88]. As the altitude increased from 1500 to 2250 m a.s.l., a significant gradual reduction in the content of chloro-

phylls a and b was observed in the four species (*Co. orbicularis*, *Cr. sinaica*, *Ec. spinosissimus*, and *Ta. sinaicum*) in this study (Figure 4). The reduction in chlorophyll pigments may be due to the decreased biosynthesis and/or increased photo-oxidation of chlorophyll. This reduction in chlorophyll contents was concomitant with a decrease in the concentration of  $Mg^{+2}$  in the soil at the highest altitude locations (Table 2). Magnesium is an important constituent in the chlorophyll structure and a cofactor of various enzymes in the photosynthetic process [83]. Moreover, the reduction in chlorophyll at high altitudes could indirectly represent the oxidative stress level on plants, which is triggered by the production of ROS. This may inhibit chlorophyll synthesis, as it inhibits the enzymes responsible for chlorophyll biosynthesis or destroys the mesophyll chloroplasts, reducing the number of chloroplasts in the leaves and enhancing their degradation, which leads to a reduction in the photosynthetic rate [89,90]. On the other hand, this reduction was accompanied by a significant increase in the carotenoid content in all species investigated (Figure 4). A more pronounced value was recorded in *Cr. sinaica* at the highest altitude (2000–2250 m a.s.l.). At high altitudes, there are aggressive stressors, such as high light intensity and UV-B radiation [91], which cause the release of electrons from excited chlorophylls and their transfer photosystem I of the photosynthetic process to  $O_2$  to form superoxide radicals which initiate a chain of free radical liberation [92,93]. Carotenoids act as potent antioxidants, as they serve as scavengers of singlet oxygen species, quench the triplet state of chlorophyll molecules [94], and protect the photosynthetic apparatus from damage caused in response to stress conditions [95,96].



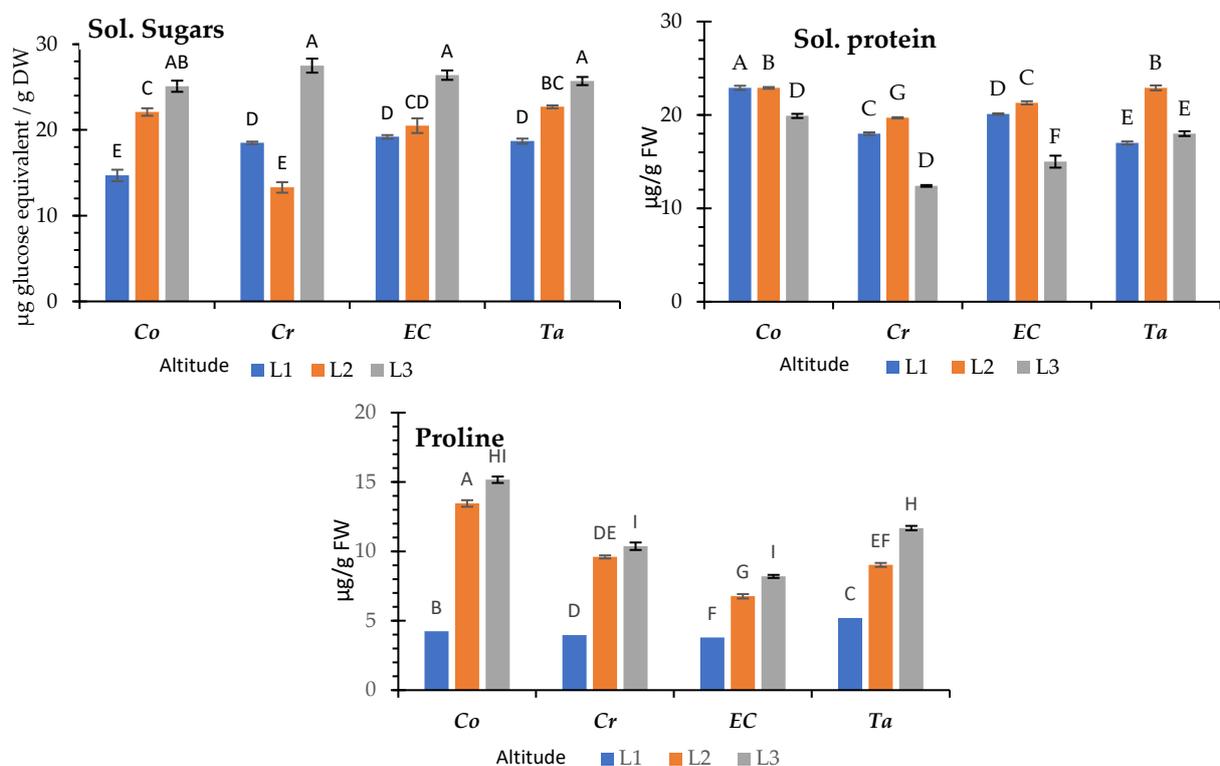
**Figure 4.** Photosynthetic pigment content (chlorophyll a, chlorophyll b, and carotenoids) in the leaves of four plant species from different altitudes. Each value is the mean of three replicates  $\pm$  SE. At  $p \leq 0.05$ , bars with different letters are significantly different. Abbreviations: L1 = altitude 1; L2 = altitude 2; L3 = altitude 3; *Co.* = *Cotineaster orbicularis*; *Cr.* = *Crataegus sinaica*; *Ec.* = *Echinops spinosissimus*; *Ta.* = *Tanacetum sinaicum*.

### 3.3. Variation in Total Soluble Sugar, Total Soluble Proteins, and Proline (Osmolytes)

In natural environments, plants are challenged by adverse abiotic stressors, resulting in various physiological changes that help plants to cope with these stressors. The accumulation of different types of compatible solutes founds in plants is one of the main strategies for coping with various types of environmental stresses [97]. In fact, these solutes help in

the maintenance of the integrity of the cellular membrane, protein stabilization, cellular osmotic balance, and ROS scavengers [98].

The data presented in Figure 5 show that with the increase in altitude, there was a significant increase in the content of total soluble sugar in the four investigated species, reaching the highest level of 27.59  $\mu\text{g}$  glucose equivalent/g DW in *Cr. sinaica*, 26.48  $\mu\text{g}$  glucose equivalent/g DW in *Ec. Spinossissmus*, and 25.75  $\mu\text{g}$  glucose equivalent/g DW in *Ta. Sinaicum*, and the lowest level was recorded in *Co. orbicularis*, with 25.10  $\mu\text{g}$  glucose equivalent/g DW. This increase in total soluble sugars may be due to the acceleration of photosynthetic performance. The photosynthetic process typically becomes accustomed to the environment and maintains homeostasis through various adaptations. [99]. The formation of some SMs helps plants cope with stressors, since they act as scavengers of free radicals and protect plant cells from oxidative damage [100]. Furthermore, the SMs pathway is supported by the derivation of photosynthetic products with a variety of intermediates, increasing the photosynthetic rate through a positive-feedback mechanism, so that the soluble sugars, which are the primary products of photosynthesis, increase. In accordance with these results, Hashim et al. [17] stated that endemic species growing naturally at high altitudes tend to increase their total soluble sugar content.



**Figure 5.** Total soluble sugar, total soluble proteins, and proline contents in the leaves of four plant species from different altitudes. Each value is the mean of three replicates  $\pm$  SE. Bars with different letters are significantly different at  $p < 0.05$ . Abbreviations: L1 = altitude 1; L2 = altitude 2; L3 = altitude 3; Co. = *Cotineaster orbicularis*; Cr. = *Crataegus sinaica*; Ec. = *Echinops spinossissmus*; Ta. = *Tanacetum sinaicum*.

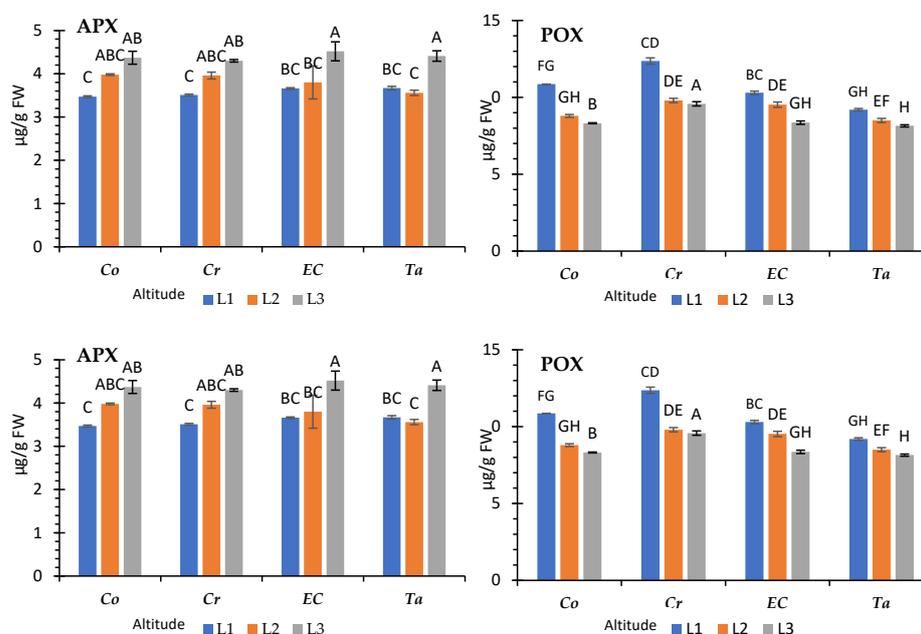
In the current study, the increase in altitude was accompanied by a noteworthy accumulation in proline content in the four species (Figure 5). Proline accumulation was found to be higher in *Co. orbicularis*, which reached 13.46  $\mu\text{g/g}$  Fwt. In addition, the most important roles of proline included increasing the photochemical activities of PS II in thylakoid membranes, adjusting the acidity of cytosol, stabilizing the  $\text{NAD}^+/\text{NADH}$  ratio, and diminishing the lipid peroxidation products, which increased stress tolerance in the investigated species [101]. Proline is a multifunctional amino acid acting as a signaling

molecule. It regulates the osmotic pressure inside the cell, restricts the denaturation of proteins, maintains membrane consistency, stabilizes enzymes, protects the cell against stress, limits ROS-induced damage, and maintains nutrient balance through water transport [102,103]. In response to abiotic stress, the accumulation of proline in plants was observed, which serves as a metabolic signal and acts as an osmo-protectant [104].

Protein synthesis or degradation is one of the mechanisms affected by environmental stresses in plants. Additionally, soluble protein content is considered one of the main signs of the physiological and biochemical status of plants grown under stress conditions [105]. In this study, a remarkable increase in total soluble protein contents was observed in the four species studied (Figure 5) at altitude 2 (1750–2000). However, at altitude 3, the total soluble protein content generally decreased with increased stress conditions. The decline in protein content in response to abiotic stress is suggested to be due to diminishing protein synthesis and a switch to an accumulation of amino acids, especially proline, which is in accordance with our results.

### 3.4. Enzymatic Antioxidant System

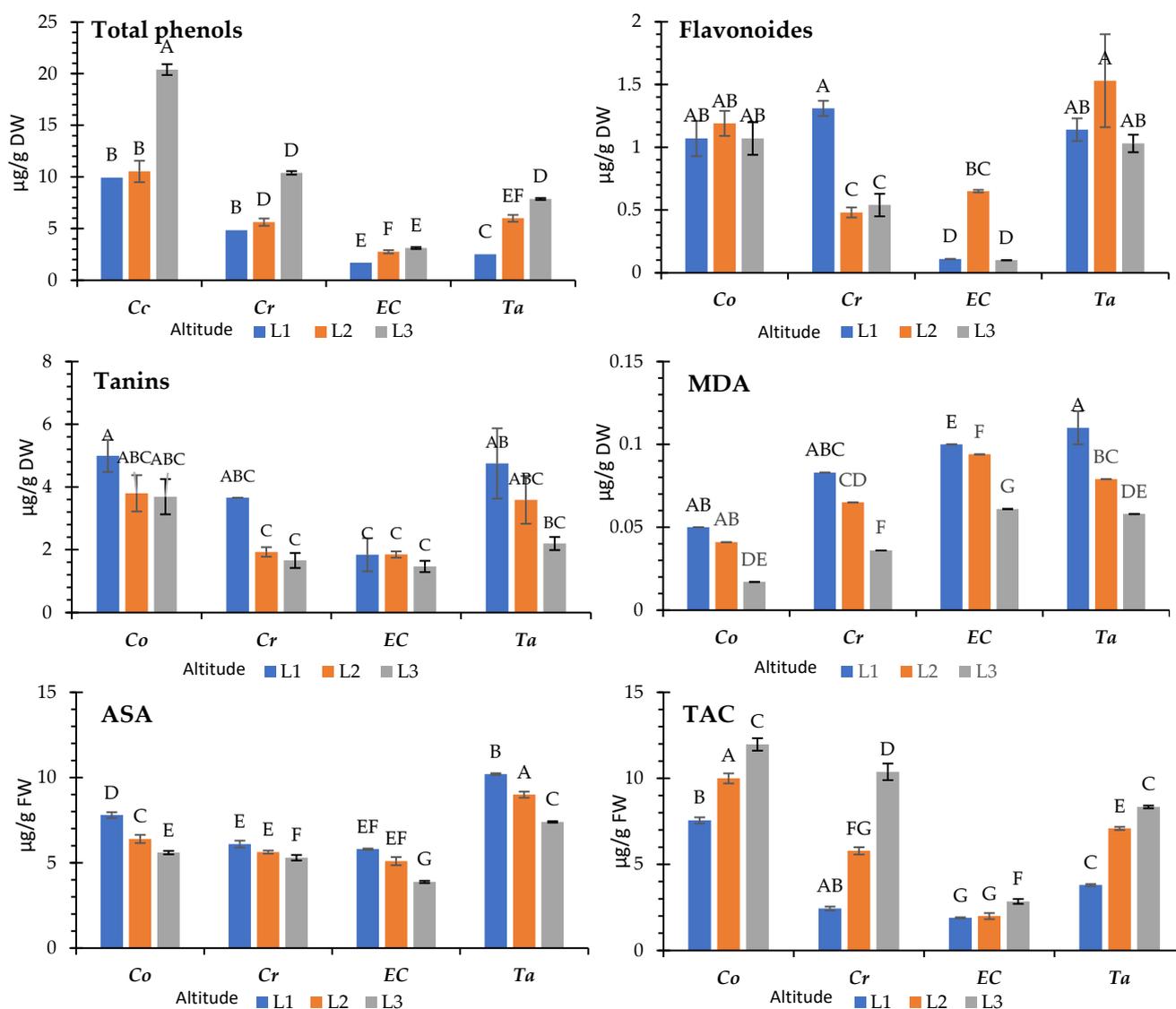
As a consequence of the oxidative stress prevailing at the high altitudes, plants should evolve a strong antioxidant defense system to control the production of free radicals, regulate cellular homeostasis, and alleviate oxidants [106]. Therefore, many plants possess both enzymatic and non-enzymatic antioxidants that can attenuate the increased production of ROS [106]. Enzymatic antioxidants studied in the present work included SOD, POX, CAT, and ascorbate APX. Superoxide dismutase is regarded as the first line of defense in the contradiction of ROS by converting superoxide radicals to oxygen and hydrogen peroxide [107]. The present results reveal that plants that are grown at high altitudes generally show lower activity of SOD, thus indicating that a diminished capacity to detoxify the superoxide radical (Figure 6). However, evidence regarding CAT activity under different abiotic stresses shows different results. CAT is one of the antioxidant enzymes that catalyze  $H_2O_2$  to oxygen and water molecules [108].



**Figure 6.** The activities of some antioxidant enzymes, including catalase (CAT), super oxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase (POX) in the leaves of the target species from different altitudes. Each value is the mean of three replicates  $\pm$  SE. At  $p \leq 0.05$ , bars with different letters are significantly different. Abbreviations: L1 = altitude 1; L2 = altitude 2; L3 = altitude 3; Co. = *Cotinaeaster orbicularis*; Cr. = *Crataegus sinica*; Ec. = *Echinops spinosissmus*; Ta. = *Tanacetum sinicum*.

In our study, the CAT enzyme’s activity was mostly upregulated in the four investigated species grown at differential altitudes, from low to high (Figure 6). Moreover, a higher CAT activity under oxidative stress incites the capability of these plants to scavenge and inhibit ROS overaccumulation. The most pronounced CAT activity was observed in *Co. orbicularis*, which reached 0.843 enzyme activity/g Fwt/min.

In this study, a noticeable increase in the activity of the APX enzyme (Figure 6) was correlated with a decrease in ascorbic acid content (Figure 7). The increase in APX activity was accompanied by a general reduction in POX activity. APX utilizes ascorbate as a specific electron donor to scavenge H<sub>2</sub>O<sub>2</sub> in water, while POX scavenges H<sub>2</sub>O<sub>2</sub> in the extra-cellular space [92]. The changes in SOD, CAT, APX, and POX activities and ROS accumulation in the investigated four species were not equal due to oxidative stress, indicating that these plants use various strategies in response to stresses. Consequently, the influence of abiotic stresses on antioxidant enzyme activity depends on the type of plant and the degree of stress experienced.



**Figure 7.** Total phenols, flavonoids, tannins, total antioxidant capacity (TAC), ascorbic acid (ASA), and malondialdehyde (MDA) contents in the leaves of the target species from different altitudes. Each value is the mean of three replicates  $\pm$  SE. At  $p \leq 0.05$ , bars with different letters are significantly different. Abbreviations: L1 = altitude 1; L2 = altitude 2; L3 = altitude 3; Co. = *Cotineaster orbicularis*; Cr. = *Crataegus sinaica*; Ec. = *Echinops spinosissimus*; Ta. = *Tanacetum sinaicum*.

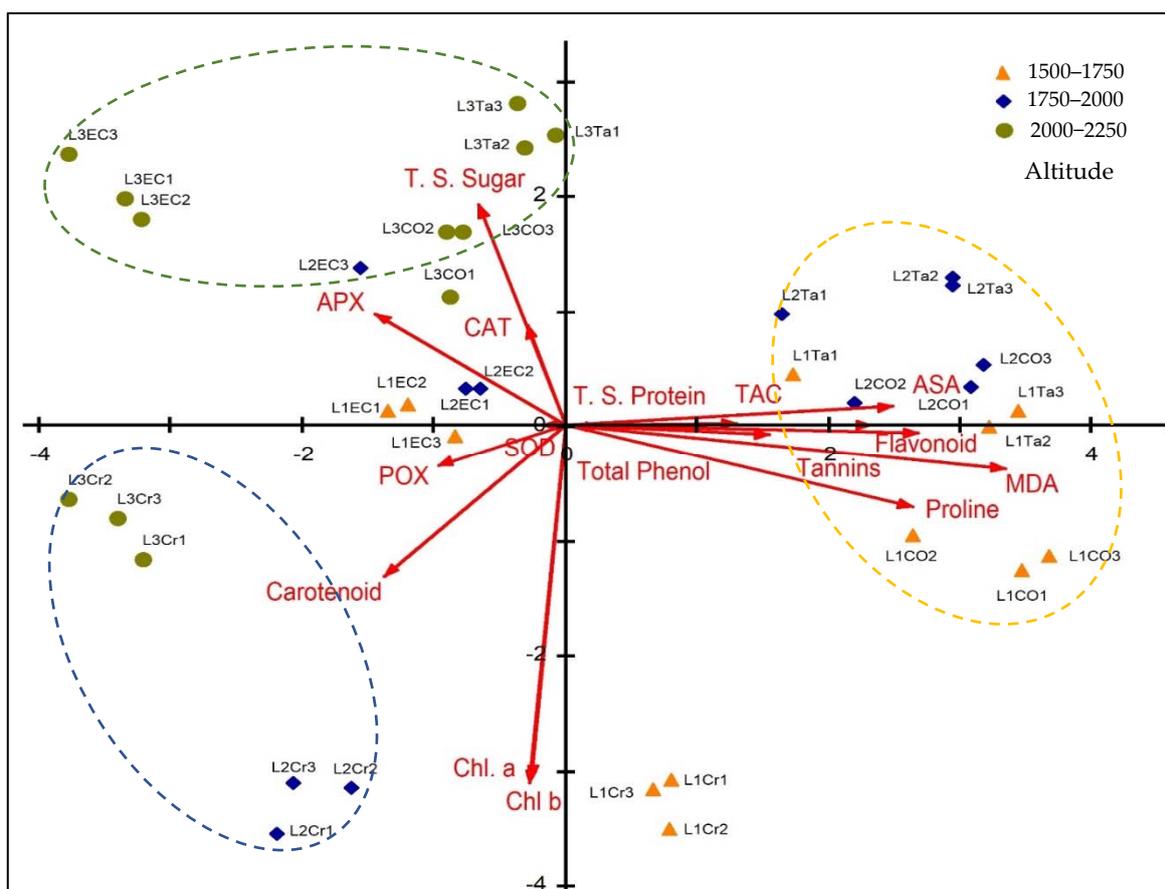
### 3.5. Non-Enzymatic Antioxidant Systems

Regarding the abiotic stresses at high altitudes, there is a notable qualitative and quantitative increase in many SMs, including phenolic compounds, flavonoids, tannins, carotenoids, steroids, alkaloids, and terpenoids [8]. In the current study, the total phenols in the four target species increased significantly with increasing altitude (Figure 7), while flavonoids did not show a clear trend, and tannins decreased with increasing altitude. The increase in the total phenols with the increase in altitude (Figure 7) was concomitant with a general reduction in the activity level of the POX enzyme (Figure 6). Phenolic compounds are considered a highly important class of antioxidants, and they are mostly associated with antioxidant activity in plants, as they inhibit lipid peroxidation by scavenging free radicals. Thus, phenolic constituents promote abiotic oxidative stress adaption [109,110]. Moreover, tannins can bind to membranes, forming a tannin–phospholipid complex that may help maintain membrane morphology and permeability [111].

Malondialdehyde (MDA) is the main cytotoxic product of lipid peroxidation, and its abundance signifies the degree of lipid oxidation by oxidants such as free radicals [112]. The present results showed that the level of MDA decreased parallel to a rise in altitude, which indicated that low temperatures reduced the levels of MDA in the four investigated species (Figure 7). The results showed that higher total phenols content was recorded in the two plants of the family Rosaceae, which reached 20.04 mg/g Fwt in *Co. orbicularis* in accordance with more pronounced levels of antioxidant capacity (11.97 mg/g Fwt), based on FRAP assays at the highest altitude in the investigated area (Figure 7). This was followed by *Cr. Sinaica*, which recorded 10.38 mg/g Fwt, while in this study, plants belonging to the family Asteraceae recorded lower values of phenolic compounds and antioxidant capacity, with the lowest value being observed in *Ec. spinosissimus* (Figure 7). At this point, we can say that the plants of the family Rosaceae were more adapted to grow at high altitudes than plants belonging to the family Asteraceae, especially *Co. orbicularis*, which recorded the highest values of CAT and SOD enzyme activity levels; the highest content of total phenols, flavonoids, tannins, and total antioxidant capacity; and the lowest value of MDA, and this was reflected in the higher frequency of *Co. orbicularis* species than other four plants at the highest altitude.

### 3.6. Principal Component Analysis (PCA)

Applying principal component analysis (PCA) to the present data showed that there are three groups of physiological parameters in relation to the distribution of the four investigated species at three different altitudes. First, photosynthetic pigments (Chl. a, Chl. b, and carotenoids) were positively correlated with *Cr. sinaica* at all altitudes, where the highest altitude (L3) showed a lower correlation. The second group, represented by total soluble sugars, CAT, and APX, was correlated with *Co. orbicularis* and *Ta. sinaicum* at the highest altitude (L3), and with *Ec. spinosissimus* at all altitudes. The third group of physiological parameters was ASA, flavonoids, proline, tannins, and MDA, which correlated positively with *Co. orbicularis* and *Ta. sinaicum* at the second (L2) and third (L3) altitudes (Figure 8).



**Figure 8.** PCA joint plot showing target species at different altitudes in relation to physiological parameters. Abbreviations: L1 = altitude 1; L2 = altitude 2; L3 = altitude 3; Co. = *Cotineaster orbicularis*; Cr. = *Crataegus sinaica*; Ec. = *Echinops spinosissimus*; Ta. = *Tanacetum sinaicum*.

#### 4. Conclusions

Plants may respond to various abiotic stresses by escalating SMs or antioxidant capacity. The present investigation concluded that many significant variations were noticed in the antioxidant activity and chemical composition of the four targeted species collected at three different altitudes. Our results demonstrated that *Co. orbicularis* relies on accumulating SMs and increasing the activity of its antioxidant enzymes to adjust to high altitudes, while as a survival strategy for high altitudes, the other investigated species typically rely on antioxidant enzyme activity or the accumulation of some SMs, either in combination or alternatively. This shows that *Co. orbicularis* is more adaptable than the other species to the oxidative stresses caused by the altitudinal effect. It is highly recommended to further investigate *Co. orbicularis*'s high adaptation capabilities to different environmental factors. Moreover, our findings are highly relevant for the future cultivation of high-grade medicinal herbs, given that many medicinal plants are used due to their bioactive ingredients. However, most medicinal plants have more than one class of bioactive secondary metabolites. Thus, investigating how these other groups of molecules change at various altitudes will also be of interest.

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