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ORIGINAL PAPER

Variations in Biochemical Compounds of Fresh Leaves of *Castanea sativa* in Relation to Elevation and Stand Age

Gamze Savaci[1]

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Abstract

This study investigated the effects of elevation and stand age on the photosynthetic pigments and antioxidant enzymes activities of *Castanea sativa* leaves. Leaves of *C. sativa* were collected at 470 m above sea level in stands ranging in age from 15- to 85-year-old and were compared for 13- to 65-year-old with leaves collected at 810 m. Chlorophyll pigments as chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids, antioxidant enzymes activities (ascorbate peroxidase, superoxide dismutase, catalase), on-enzymatic compounds (total soluble protein levels, proline), flavonoid, and the oxidative level (hydrogen peroxide (H_2O_2), lipid peroxidation (MDA-malondialdehyde)) of fresh leaves were analyzed using a UV–visible spectrophotometer. Significant differences in the biochemical compounds of *Castanea sativa* leaves were found between elevation and stand age. In general, photosynthetic pigments (except for the ratio of chlorophyll *a*:chlorophyll *b*), proline, and soluble protein levels, as well as ascorbate peroxidase and superoxide dismutase, had better results at higher elevations than at lower elevations, whereas flavonoids, malondialdehyde, and hydrogen peroxide concentrations, as well as catalase activity, were lower at higher elevations. Results have shown that total chlorophylls, chlorophyll *b*, proline, at both elevations and catalase enzymes activities at only 810 m were decreased with increasing in stand age whereas chlorophyll *a* : chlorophyll *b* at both elevations and total carotenoids at only 810 m were increased with increasing. However, other chemical compounds (chlorophyll *a*, superoxide dismutase, hydrogen peroxide, lipid peroxidation levels) did not show a significant trend with the stand age. This study has provided valuable insight into the variation in the biochemical compounds of *C. sativa* leaves in relation to the elevation and stand age, and the results of this study can contribute to the determination of ideal site factors for the growth and development of chestnut trees.

Keywords Antioxidants, Ecophysiology · Enzymes · Pigments · Chestnut tree

1 Introduction

Castanea sativa Mill., a multi-purpose forest tree species, dominate forests that cover an average of 0.5% of the forested area of the Black Sea region, Turkey, ranging from 1% at the province level in Kastamonu to 15% district level, e.g., Inebolu and Bozkurt (General Directorate of Forestry 2015). *C. sativa* forests are found from 600 to 1300 m above sea level (a.s.l.), and their timber is widely used for furniture making due to its high-quality wood, with coppiced shoots being used for making hand tools (Anonymous 2013). Therefore, the health and vitality of chestnut stands are of particular interest in the region.

The physiological and photosynthetic characteristics of plants are influenced by several factors (Zheng et al. 2012), such as light intensity, photoperiod, ultraviolet intensity, moisture, temperature, and oxygen availability (Chen et al. 2014). In addition to environmental conditions, photosynthetic metabolism and physiological processes are also affected by endogenous factors, such as plant age. Although the main effects of plant age on photosynthesis are not fully understood, many studies have reported that plant age is an important factor affecting photosynthetic physiology (Munné-Bosch 2007).

The effect of elevation on plant growth is a combination of several factors (Polle et al. 1999; Ahmad et al. 2016). It is known that rainfall, light, and radiation intensity increase with altitude, whereas the pressure of oxygen, temperature, and gravity decrease. Plants experience interacting stresses with elevation, such as dehydration and low temperature (Shepherd and Wynne Griffiths 2006). While light limitation

✉ Gamze Savaci
gsavaci@kastamonu.edu.tr

[1] Department of Forest Engineering, University of Kastamonu, Kastamonu, Turkey

reduces plant growth and reproduction at high altitudes, the growth period of plants is shortened due to low temperatures (Körner 2003). Moreover, trees experience higher climatic stresses at higher elevations and have lower growth rates than at lower elevations (Tranquillini 2012). Plants at high elevations may adopt different physiological strategies for stress compensation, for example, increases in antioxidative protection and carotenoids to reduce the risk of photoinhibition (Polle and Rennenberg 1994).

The photosynthetic capacity of the leaves decreases as the tree age and height increase (Day et al. 2001; England and Attiwill 2006). The reduced growth in mature trees and stands is a commonly observed phenomenon at higher elevations (Gower et al. 1996). The growth of trees, and hence growth rates of forest stands, generally increases initially and then decreases with tree age (Bond et al. 2007; Ambebe and Dang 2009). Osmosis and antioxidant defense systems of the plant change when the morphology, physiology and metabolism of the plant are adversely affected by environmental condition changes (Cui et al. 2016). Tree physiology is rather well-studied in deciduous species. Many researchers have shown that tree physiology changes as tree maturity (De Soyza et al. 1996; Qi et al. 2012). Variations in foliar chemicals with stand age and elevation have also been reported (Greenwood et al. 2008; Zheng et al. 2012; Rajsnerová et al. 2015). However, little information is available on how elevation and stand age affect photosynthetic metabolism, secondary metabolites, oxidative stress, and antioxidant enzymes activities, as well as on physiological characteristics of *C. sativa*.

There are almost no study on the effect of elevation and stand age on the leaf biochemical compounds of *C. sativa* in TurkeyHowever, several studies have been performed in Turkey especially dealing with its ethnobotanical uses, morphological, phenological, biochemical, mechanical, chemical characteristics and disease of *C. sativa* (Ertan and Kılınç 2005; Ay and Şahin 2011; Kendir et al. 2016; Turna et al. 2017; Tuttu et al. 2021).

2 Materials and Methods

2.1 Description of the Study Area and Sampling

This study was carried out in Inebolu and Bozkurt districts, 94 km from Kastamonu city, north-western Turkey (Figure S1). The climate is characteristic of the Black Sea, where winters are warm in the coastal areas and cold and snowy in the interior. Summer is warm and rainy on the coasts and less rainy in the interior (Partal and Yavuz 2020). Well-drained and sandy clayey loam soils overlay volcanic sedimentary rocks and schist (Akbaş et al. 2002). The soils are orthic acrisols (IUSS WGWRB) and are very acidic with a base saturation < 50% (Atalay 2006).

Inebolu meteorology station (64 m a.s.l.) is 20.1 km from the site at 470 m elevation and 32 km from the site at 810 m elevations. Mean monthly temperature and precipitation values have been re-interpolated (Çepel 1995; Kılınç et al. 2006), and the mean monthly rainfall was 146.07 mm, the monthly temperature was 13 °C, and the mean solar radiation was 3.96 kWh/m^2 at lower elevations. The mean monthly rainfall was 161.37 mm; the monthly temperature was 11.3 °C and 4.06 kWh/m^2 at higher elevations (State Meteorological Service 2016).

Castanea sativa forests are native to the study area. For each stand age category, three plots (20 m × 20 m) were chosen at 470 m a.s.l. (3 age categories × 3 replicate plots = 9 plots) and at 810 m (2 age categories × 3 replicate plots = 6 plots). Trees were grouped by diameter into three categories: 1, 10–15; 2, 25–30; and 3, 90–99 cm. Therefore, for each diameter category, the mean tree age was measured using three trees in each plot. The three age categories were noted as 15 years old, 30 years old, and 85 years old. At 810 m, the trees were grouped into two diameter categories: 13 to 15 and 65 to 70 cm, corresponding to 13 and 65 years old of stand age. The mean age, diameter, height and canopy closure of the tree are shown in Table 1. The diameter at breast height (DBH) and tree height measurements were made on a total of 45 chestnut trees with three from each stand age class. Diameter at breast height of *C. sativa* trees was measured using a diameter tape. The mean tree age of *C. sativa* was determined using increment borer by counting annual ring at diameter at breast height. Tree heights were measured with a Blume-Leiss clinometer (Mackensen et al. 2001). The stand canopy closure (%) was measured with a CanopyApp digital covering meter. Depending on the number of trees in classes, at least twenty leaves were collected from the lower parts of the trees with different directions in August 2016 from different stand ages. Then, samples of *C. sativa* leaves were brought to the laboratory for chemical analyses.

2.2 Chestnut Leaf Analysis: Bioactive Composition and Antioxidant Enzymes Activities

2.2.1 Photosynthetic Pigment Contents

Chlorophyll and carotenoids concentrations in fresh leaves were analyzed using a UV–visible spectrophotometer (PG Instruments Ltd., Alma Park, Wibtoft, Leicestershire, England). The certified qualification kit reference material (NIST srm 2034) was used to assess the wavelength

Table 1 Description of study areas, meteorological data and stand characteristics of *Castanea sativa* Mill. leaf collection locations in 2016 (Devlet Meteoroloji İşleri 2016)

Geographic data		Low elevation		High elevation	
Elevation (m)		470		810	
Latitude (North)		41° 55′ 9.76″		41° 55′ 9.10″	
Longitude (East)		33° 52′ 8.59″		33° 57′ 4.84″	
Stand age (year)		15-, 30-, 85-		13-, 65-	
Mean annual temperature (°C) (Min–max)		9.6–23		7.9–21.3	
Total annual rainfall (mm)		1040.3		1223.9	
Wind speed (m sec^{-1}) (Minimum–maximum)		1.3–9.6		2.8–13.3	
Wind direction		Southwest		Northwest	
Solar radiation (kWh m^{-2})		3.91–4.00		4.01–4.10	
Stand age (year old)	15	30	85	13	65
Height (m)	13	16	30	10	24
Diameter (cm)	10–15	25-30	90–99	13-15	65–70
Canopy closure (%)	11–40%	11-40%	41–70%	<10%	41–70%

accuracy of spectrophotometer analyses. The amount of chlorophyll was performed with 10 ml of 80% acetone extract (Whitham et al. 1971). Fresh leaf samples (500 mg) were extracted with 10 ml of 80% acetone extract and centrifuged at 5000 rpm for 10 min. The absorbance value of the supernatant was recorded at $\lambda = 663$, 645, and 450 nm by a spectrophotometer. All analyses were performed in triplicate. Chlorophyll concentrations were calculated using Arnon's (1949) formula, as shown in Eqs. (1) to (3). Carotenoids were spectrophotometrically quantified using the equations of Jaspars formula (Whitham et al. 1971) (Eq. 4):

$$Chla = \left(12.7 \times A_{663} - 2.69 \times A_{645}\right) \times (V/1000 \times FW) \quad (1)$$

$$Chlb = \left(22.9 \times A_{645} - 4.68 \times A_{663}\right) \times (V/1000 \times FW) \quad (2)$$

$$TotalChl = \left(20.2 \times A_{645} + 8.02 \times A_{663}\right) \times (V/1000 \times FW) \quad (3)$$

$$Car = \left(4.07 \times A_{450}\right) - (0.0435 \times Chla + 0.367 \times Chlb) \quad (4)$$

where *Chl a* is chlorophyll *a*, *Chl b* is chlorophyll *b*, *Total Chl* is total chlorophylls, *Car* is carotenoids, A_λ is absorbance reading at wavelength λ (nm), *FW* is the fresh weight of leaf, and *V* is the total volume of filtrate.

2.2.2 Total Flavonoid and Total Soluble Protein Level

Total flavonoid content was determined using the aluminum chloride method (Luximon-Ramma et al. 2002). Leaves (500 mg) were extracted in 10 ml of 80% acetone using mortar and pestle and filtered through a Buckner's funnel using Whatman No. 1 filter paper. The volume of the filtrate was adjusted to 50 ml with 80% acetone. The reaction mixture contained 1.5 ml of the plant extract and 1.5 ml of 2% methanolic aluminum chloride (2 g aluminum chloride dissolved in 100 ml pure methanol) and was prepared using distilled water. The absorbance of the reaction mixture was measured at 368 nm on a UV–visible spectrophotometer, and the values were expressed as g/100 g FW. The flavonoid contents were calculated with help of a standard curve of rutin (0.3 mg ml^{-1}).

The total soluble protein level of leaves was determined according to Bradford (1976). Three replicates from each chestnut leaf sample were pooled. Briefly, 0.5 g of each leaf sample was homogenized with 5 ml of 50 mM KH_2PO_4 (pH 7.0). A 10 µl suspension was taken from homogenates and centrifuged at 15,000 rpm for 20 min in an Eppendorf tube, 2.5 ml Coomassie Brilliant Blue G-250 dye was added to each well, and the microplate was incubated for 10 min in the dark at room temperature. The chestnut leaf samples were measured spectrophotometrically at 595 nm. Total soluble protein level content was determined using a standard graphic prepared with the Bradford assay kit (Bio-Rad Protein Assay, Bio-Rad Laboratories) with bovine serum albumin (Sigma Chemical, A 7638) standards. The standards were taken 0.01 mg ml^{-1} (100 µl stock BSA and add on 9.900 ml 50 mM Na phosphate tampon), 0.02 mg ml^{-1} (200 µl stock BSA and added on 9.800 ml 50 mM Na phosphate tampon), 0.04 mg ml^{-1} (400 µl stock BSA and added on 9.600 ml 50 mM Na phosphate tampon), 0.08 mg ml^{-1} (800 µl stock BSA and added on 9.200 ml 50 mM Na phosphate tampon), 0.16 mg ml^{-1} (1600 µl stock BSA and added on 8.400 ml 50 mM Na phosphate tampon), and 0.32 mg ml^{-1} (3200 µl stock BSA

and added on 6.800 ml 50 mM Na phosphate tampon) and expressed as mg g^{-1} FW.

2.2.3 Total Proline Content, Lipid Peroxidation, and Hydrogen Peroxide

Total proline content in the leaf tissue was estimated according to Bates et al. (1973). Fresh leaves (0.5 g) were homogenized in 5 ml of 3% sulfosalicylic acid in a pod. The top phase of the filtrate was separated, its precipitates were washed twice with 5 ml of 3% sulfosalicylic acid, and the top phase was added to the previous upper chamber. The alcoholic extract was kept in a refrigerator at 4 °C. Acid ninhydrin (2 ml) and glacial acetic acid (2 ml) were added to a 2 ml sample in a glass tube and covered with foil. The mixture was placed in a boiling water bath at 100 °C for 1 h. The reaction was stopped by placing test tubes in cold water. The samples were mixed with 4 ml toluene. The light absorption of the toluene phase was estimated at 520 nm using a UV–visible spectrophotometer (Terzi et al. 2014). Total proline content was expressed as µmol g^{-1} fresh weight (FW) of leaves.

The level of lipid peroxidation was estimated using the thiobarbituric method by determining the malondialdehyde (MDA) content (Lutts et al. 1996). Fresh leaves (500 mg) were extracted in 5 ml of 1% trichloroacetic acid (TCA). The supernatant (750 µl) was extracted and centrifuged at 10,000 rpm for 15 min in a tube, and thiobarbituric acid (750 µl) was added to the sample. The mixture was placed in a boiling water bath for 1 h at 100 °C. After cooling in an ice bath, the samples were incubated in the dark at room temperature for half an hour. The chestnut leaf sample extracts were measured spectrophotometrically at 532 and 600 nm. The level of lipid peroxidation obtained a curve and equation from the standards prepared at 100 µl MDA stock (1% TCA in 4900 µl TCA), 200 µl MDA stock (1% TCA in 4800 µl TCA), 400 µl MDA stock (1% TCA in 4600 µl TCA), 800 µl MDA stock (1% TCA in 4200 µl TCA), and 1000 µl MDA stock (1% TCA in 4000 µl TCA).

Hydrogen peroxide (H_2O_2) level was determined according to Sergiev et al. (1997). Chestnut leaf tissues (500 mg) were homogenized with 5 ml of 0.1% TCA in an ice bath. The homogenate was centrifuged at 12, 000 × *g* for 15 min, and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI (Kumar et al. 2013). The absorbance of the supernatant was read at 355 nm (Velikova et al. 2000). The level of H_2O_2 was determined with the help of the graph obtained from the standards prepared at 2 µl stock (0.1% TCA in 498 µl), 4 µl stock (496 µl), 8 µl stock (492 µl), 12 µl stock (488 µl), 16 µl stock (484 µl), 20 µl stock (480 µl), and 24 µl (476 µl) concentrations.

2.2.4 Antioxidant enzymes Activities

To determine the enzymes activities of *C. sativa* leaves, a 500 mg leaf sample was ground in liquid nitrogen and homogenized with 7 ml of 50 mM KH_2PO_4 (pH 7.0) containing 0.1 mM sodium ethylenediaminetetraacetic acid. The extracted solution was centrifuged at 15.000 rpm and 4 °C for 15 min. Superoxide dismutase (SOD; enzyme commission number [EC] 1.15.1.1) enzyme activity was determined by superoxide radical reduction of nitroblue tetrazolium chloride under light, and the results were described as EU (endotoxin units)/mg protein (Cakmak 2002). Ascorbate peroxidase (APX; EC 1.11.1.11) enzyme activity was determined according to Nakano and Asada (1981). Catalase (CAT; EC 1.11.1.6) enzyme activity was determined according to Wahlefeld and Bergmeyer (1974).

2.3 Statistical Analysis

SPSS statistical software package for IBM Corporation SPSS version 22.0 (Armonk, NY, USA) was used for statistical analyses. All analyses were performed in triplicate, and results are shown as mean ± standard error of the mean. A two-way ANOVA (analyses of variance) was applied for analyzing the effects of stand age and elevation on biochemical compounds of *C. sativa* leaves. Following the results of ANOVAs, the Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$) was used for significance.

3 Results

Mean chlorophyll *a*, chlorophyll *b*, total chlorophylls, chlorophyll *a*:chlorophyll *b*, and carotenoid concentrations of *C. sativa* leaves with different stand ages and elevations are given in Table 2. The photosynthetic pigments (except for chlorophyll *a*) variables changed significantly between stand age and elevations ($p < 0.01$). Sixty-five-year-old trees at higher elevations had the highest chlorophyll *a* (0.180 mg g^{-1} FW) and carotenoids (12.09 mg g^{-1} FW) concentrations, while 85-year-old trees at lower elevations had the lowest chlorophyll *a* (0.130 mg g^{-1} FW) and carotenoids (9.54 mg g^{-1} FW) concentrations (Table 2). Chlorophyll *b* and total chlorophylls contents were the highest for 85-year-old trees (0.036 mg g^{-1} FW and 0.132 mg g^{-1} FW, respectively) at lower elevations, while they were the highest for 13-year-old trees at higher elevations (0.152 mg g^{-1} FW and 0.273 mg g^{-1} FW, respectively). Chlorophyll *a*:chlorophyll *b* ratio was the highest for 85 years old at lower elevation and the lowest for 13 years old at higher elevation (Table 2). In addition, there were significant effects of elevation on the photosynthetic pigment variables ($p < 0.001$). The effects of stand age were significant for chlorophyll *b*, total

Table 2 Comparison of leaf photosynthetic pigment variables among stand age and between elevations, showing results of the two-way analyses of variance

Photosynthetic pigment variables	Elevation zone/stand age class (year)							
	Low elevation, 470 m			**High elevation, 810 m**		**Significance**		
(mg g^{-1})	**15**-	30-	85-	13-	65-	SA	E	SA x E
Chl *a*	$0.161^{c} \pm 0.0036$	$0.144^{b} \pm 0.0008$	$0.134^{a} \pm 0.0001$	$0.168^{d} \pm 0.0014$	$0.180^{e} \pm 0.0001$	n.s	***	***
Chl *b*	$0.111^{d} \pm 0.0005$	$0.057^{b} \pm 0.0001$	$0.036^{a} \pm 0.0001$	$0.152^{e} \pm 0.0005$	$0.105^{c} \pm 0.0004$	**	***	ns
Total Chl	$0.227^{c} \pm 0.0020$	$0.161^{b} \pm 0.0005$	$0.132^{a} \pm 0.0003$	$0.273^{e} \pm 0.0015$	$0.235^{d} \pm 0.0003$	**	***	ns
Chl *a*:Chl *b*	$1.451^{b} \pm 0.039$	$2.529^{d} \pm 0.022$	$3.757^{e} \pm 0.015$	$1.104^{a} \pm 0.005$	$1.710^{c} \pm 0.008$	***	***	*
Total carotenoid	$9.64b^{c} \pm 0.063$	$9.66^{c} \pm 0.014$	$9.54^{a} \pm 0.012$	$9.55^{ab} \pm 0.010$	$12.09^{d} \pm 0.047$	***	***	***

Results are expressed as means ± standard error. Where letters in superscript differ, data are significantly different ($p < 0.05$). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; *ns*, not significant; *Chl a*, chlorophyll *a*; *Chl b*. chlorophyll b; *Total Chl*, total chlorophyll; *Chl a:Chl b*, chlorophyll *a*:chlorophyll *b*; *SA*, stand age; *E*, elevation; *SA x E*, stand age elevation

chlorophylls, chlorophyll *a*:chlorophyll *b*, and carotenoids concentrations ($p < 0.01$). Two-way (stand age × elevation) interactions were not significant for chlorophyll *b* and total chlorophylls ($p > 0.05$). For the remaining photosynthetic pigments, there were significant differences considering stand age × site interactions ($p < 0.05$). There was an increase with elevation at both sites for these variables; however, the magnitude of the increase varied among the stand ages (Table 2).

Total flavonoid contents and total soluble protein levels of *C. sativa* leaves samples are presented in Fig. 1a and b. At lower elevations, 30-year-old trees had the highest total flavonoid content (17.34 mg g^{-1}), and 85-year-old trees had the lowest (7.62 mg g^{-1}), while 85-year-old trees had the highest soluble protein level (66.52 mg g^{-1}), and 15-year-old trees had the lowest (34.72 mg g^{-1}) levels (Table 2). There were significant effects of elevation and two-way (stand age × elevation) interactions on total flavonoid content and soluble protein levels ($p < 0.001$). The effects of stand age were only significant for total flavonoid content ($p < 0.001$). Total soluble protein levels showed higher concentrations at higher elevations than at lower elevations, whereas flavonoid concentrations at higher elevations were much lower than at lower elevations (Fig. 1). In this study, the total flavonoid content of *C. sativa* leaves at lower elevations was more abundant, and significant differences ($p < 0.05$) were observed between the values, with lower values from higher elevations and mature chestnut stands. However, the values at lower elevations were higher. The soluble protein levels showed a significant difference ($p < 0.05$) and were determined from higher elevations (Fig. 1a).

The total proline content for *C. sativa* leaves for the two elevations studied was significantly different ($p < 0.05$) at higher elevations, with greater concentrations than at lower elevations (Fig. 2a). Eighty-five-year-old trees at lower elevations had the lowest total proline content (20.91 µmol g^{-1} FW), while 13-year-old trees had the

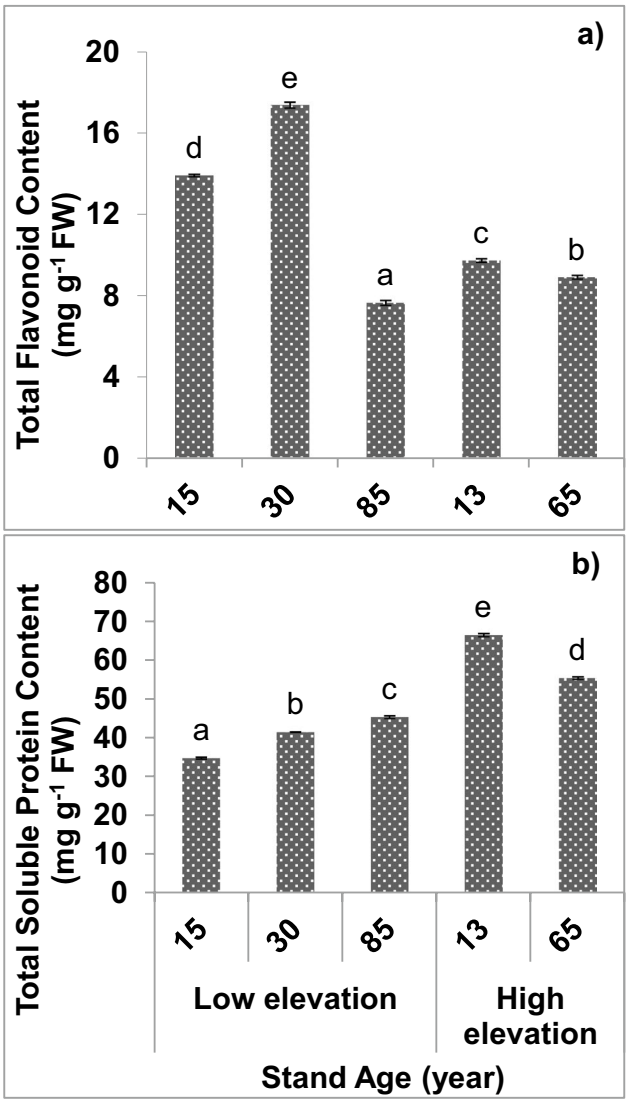

Fig. 1 *C. sativa* leaf treatment results. (**a**) Total flavonoid content. (**b**) Total soluble protein for different stand ages at lower and at higher elevations. Values indicate mean ± SE; means followed by different letters are significantly different according to Tukey's test ($p < 0.05$)

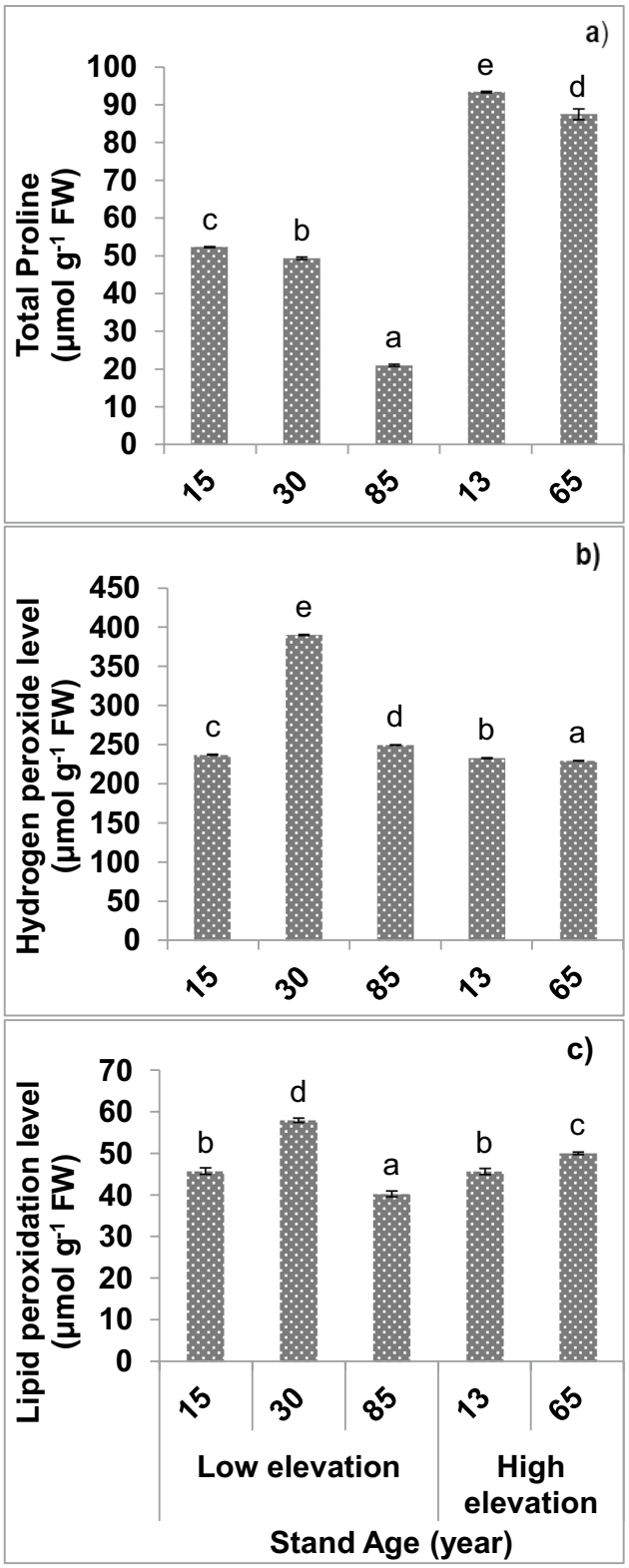

Fig. 2 *C. sativa* leaf treatment results. (**a**) Total proline. (**b**) Hydrogen peroxide. (**c**) Lipid peroxidation for different stand ages at lower and at higher elevations. Values indicate mean ± SE; means followed by different letters are significantly different according to Tukey's test ($p < 0.05$)

highest (93.24 µmol g^{-1} FW) at higher elevations. Also, total proline contents showed clear trends and have decreased with increasing trends in stand age at both elevations, but no significant differences ($p > 0.05$) were observed between stand ages (Fig. 2a). Variation in the hydrogen peroxide and malondialdehyde levels of *C. sativa* leaves with different stand age classes and elevations are given in Fig. 2b and c. There were no significant effects ($p > 0.05$) of elevation, stand age, and two-way (stand age × elevation) interactions on the malondialdehyde and hydrogen peroxide levels. At both elevations, malondialdehyde and hydrogen peroxide variables showed first decreasing and then increasing trends according to stand ages (Fig. 2b and c).

The mean antioxidant enzyme activities (ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT)) of the *C. sativa* leaf samples are given in Table 3. Thirty-year-old trees had the lowest ascorbate peroxidase and superoxide dismutase activities (1.21 EU mg^{-1} protein and 64.95 EU mg^{-1} protein, respectively) at lower elevations, while 65-year-old trees had the highest ascorbate peroxidase activity (2.52 EU mg^{-1} protein) and 13-year-old trees had the highest superoxide dismutase activity (120.03 EU mg^{-1} protein). The highest catalase activity was obtained for 15-year-old trees (0.58 EU mg^{-1} protein), and the lowest catalase activity was in 65-year-old trees (0.43 EU mg^{-1} protein). There were significant effects of elevation ($p < 0.001$), stand age ($p < 0.001$), and stand age × site interactions ($p < 0.01$) on the ascorbate peroxidase activity. The effects of stand age and two-way (stand age × elevation) interactions on catalase enzymes activities were significant ($p < 0.05$), but the effects of elevation were not significant ($p > 0.05$). Superoxide dismutase activity was only significant with elevation ($p < 0.05$). For these variables, there were first decreasing and then increasing trends with elevation and stand age at both sites (Table 3).

4 Discussion

4.1 Changes in Photosynthetic Pigments with Elevation and Stand Age

Chlorophylls and carotenoids are the main components of photosynthetic metabolism and play an active role in capturing sunlight and transforming it into chemical energy (Shevela and Bjorn 2018). Also, they are involved in the absorption and conversion of light and directly affect photosynthetic capacity (Ashraf and Harris 2013). The results in this study have indicated significant differences in the photosynthetic pigments of *C. sativa* leaves with elevation and stand age. However, there was no relationship of chlorophyll *a* with stand age (Table 2). Also, the results showed that chlorophyll *b*, total chlorophylls, and carotenoids were

Table 3 Comparison of leaf activities of antioxidant enzymes among stand age and between elevations, showing results of the two-way analyses of variance

Antioxidant activity (EU mg^{-1} protein)	Elevation zone/stand age							
	Low elevation, 470 m			High elevation, 810 m		Significance		
	15-	30-	85-	13-	65-	SA	E	SA x E
APX	$1.38^{b} \pm 0.01$	$1.21^{a} \pm 0.03$	$1.90^{c} \pm 0.01$	$2.14^{d} \pm 0.03$	$2.52^{e} \pm 0.04$	***	***	**
CAT	$0.58^{e} \pm 0.004$	$0.44^{b} \pm 0.005$	$0.52^{c} \pm 0.004$	$0.56^{d} \pm 0.004$	$0.43^{a} \pm 0.005$	*	ns	*
SOD	$96.76^{c} \pm 0.22$	$64.95^{a} \pm 0.57$	$92.14^{b} \pm 0.31$	$120.03^{e} \pm 0.37$	$105.24^{d} \pm 0.35$	ns	*	ns

Results are expressed as means ± standard error. Means followed by different lowercase letters in the same row are significantly different according to Tukey's test ($p < 0.05$). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; *ns*, not significant; *APX*, ascorbate peroxidase; *CAT*, catalase; *SOD*, superoxide dismutase; *SA*, stand age; *E*, elevation; *SA* × *E*, stand age × elevation

lower in the older aged tree at lower elevations. On the contrary, Zhou et al. (2003) noted that, at higher elevations, the amounts of both chlorophyll *a* and chlorophyll *b* decreased, but the chlorophyll *a*:chlorophyll *b* ratio increased. Huang et al. (2015) stated that, as the elevation increased, chlorophyll content tended to decline, and the chlorophyll *a*:chlorophyll *b* ratio tended to increase. The reduced growth in mature stands is a commonly observed phenomenon at higher elevations (Gower et al. 1996). The growth of trees and hence growth rates of forest stands generally increase initially and then decrease with tree age. The results from high elevations showed differences between 65-year-old and 13-year-old stands in some of the measured variables. Similarly, Turfan et al. (2016) reported the highest chlorophyll *a*, total chlorophylls, chlorophyll *b*, and carotenoids in 90-year-old Uludağ fir stands. In contrast, Jan et al. (2011) found that the total chlorophyll contents decreased with increasing elevation, and the statistical analysis for chlorophyll content was insignificant. Other research also showed a decrease in the photosynthetic capacities of older trees (England and Attiwill 2006; Seo et al. 2007). Although the underlying effects of plant age on photosynthesis are not fully understood, many studies have reported that plant age is an important factor affecting photosynthetic physiology (Munné-Bosch 2007).

Carotenoids were higher in mature chestnut trees growing at higher elevations than at lower elevations, indicating that environmental factors at higher elevations can promote carotenoid biosynthesis (Cui et al. 2018). Light is needed for carotenoid biosynthesis, and an increase in light intensity at elevation favors carotenoid synthesis (Simkin et al. 2003). Therefore, the increase in carotenoids content with increasing elevation can be linked to the protective function of the pigments in the distribution of excess energy and the discharge of free radicals (Havaux and Niyogi 1999). It is reported that carotenoids increased at high elevation, and similar results were also found (Ahmad et al. 2016; Cui et al. 2018). The significant decline in chlorophyll *a* and chlorophyll *b* and an increase in carotenoids at higher elevations may be associated with a decrease in photosynthesis and an increase in photo-inhibition (Demmig-Adams and Adams lii 1992). Similarly, Prasad et al. (2005) reported that carotenoids are a scavenger of single oxygen molecules during exposure to high-intensity light and protect chlorophylls from photo-oxidative damage. For this reason, increased carotenoids at higher elevations due to ultraviolet radiation could be a protective mechanism for chlorophylls (Cui et al. 2018).

4.2 Changes in Total Flavonoid Content and Soluble Protein Levels with Elevation and Stand Age

Changes in total flavonoid compounds can be significant owing to the susceptibility of plants to disease (Caldwell and Flint 1994). Plants induce the production of defensive substances, such as flavonoids, as well as enzymes to reduce pathogen injuries (Han et al. 2011). In this study, the total flavonoid content of *C. sativa* leaves at lower elevations was more abundant. Significant differences were observed between the values, with stand age and elevations ($p < 0.001$). Similarly, Liu et al. (2017) found that the total flavonoid content in the *Pueraria lobata* (Willd.) sample at 100 m altitude was significantly higher than at altitudes of 1400 and 1700 m, and it was determined that different tree species at different altitudes have higher total flavonoid content than at lower altitudes. Total flavonoid variables showed first decreasing and then increasing trends according to stand age at both elevations (Fig. 1a). Sommavilla et al. (2012) stated that the sub-Mediterranean species *Celtis australis* L. has higher phenolic acid and flavonoid contents in the earlier leaf development stages.

In this study, the soluble protein levels of chestnut leaves at higher elevations were more abundant. Significant differences were observed between the values ($p < 0.05$), for lower elevations and 15- and 30-year-old chestnut stands. However, higher values at higher elevations were obtained for 13-year-old chestnut stands (Fig. 1b). The increase in soluble proteins with elevation can have raised the functional

protein level and ensured all the chemical reactions that take place inside cells, including anabolism and catabolism (Cui et al. 2018). Activation of protein synthesis in plants, combined with antioxidant activities, plays a protective role in the stressful conditions of high mountains. The synthesis of specific proteins is an important mechanism that plays a role in increasing cold tolerance. Low temperature may cause the synthesis of stress proteins (Hughes and Dunn 1996; Unal et al. 2013). Similarly, Bano et al. (2009) reported that all herbaceous species showed an increase in protein level with an increase in elevation from 3000 to 3500 m. Turfan et al. (2018) noted that the soluble protein content was lower in young *P. nigra* Arnold. trees. They also stated that 100-year-old trees have the highest proline content which was probably related to higher irradiance. However, Turfan et al. (2016) also reported in fir trees that soluble protein levels were higher in 38-year-old trees than in 100-year-old trees.

4.3 Changes in Total Proline Content, Lipid Peroxidation, and Hydrogen Peroxide with Elevation and Stand Age

Total proline accumulation at higher elevations is a well-known protection mechanism to avoid the detrimental effects of photodestruction by minimizing membrane damage (Unal et al. 2013). These results showed a tendency of proline contents to increase along elevation, but non-significant differences were observed between stand ages ($p > 0.05$). Similarly, Cui et al. (2018) also noted that proline content increased significantly along elevation. However, Turfan et al. (2018) found that, in Anatolian black pine trees, the proline content was lower in younger trees than in older trees. The higher concentration of proline in Anatolian chestnut leaves at 810 m compared to the leaves at 470 m provides an important protective property to the plant, highlighting the importance of proline accumulation in plant adaptation. Some authors have reported that a lower proline amount in older trees was associated with higher lipid peroxidation, hydrogen peroxide, and tissue deformation due to tree age (Sofo et al. 2004; Turfan et al. 2020). Öncel et al. (2004) reported that high proline content has a very important role in the stress resistance of alpine plants. The increase in the proline content of plants at high elevations may be a part of the tolerance mechanism against climate characteristics (minimum temperature and high wind) and other high elevation-related stresses. Koç et al. (2010) noted that altitudinal stress leads to increased proline synthesis and decreased chlorophyll pigment levels. Also, proline increased the chlorophyll content and net photosynthetic rate of some stressed plants (Yan et al. 2011). Induced protection mechanisms may be insufficient to prevent damages to the photosynthetic and accessory pigments against stress factors arising at high elevations (Macar and Kalefetoğlu 2018).

Hydrogen peroxide levels are synthesized in photosynthetically active chloroplasts near vascular bundles in the leaf and transported from there to tissues and organs. In this case, it triggers the deterioration of tissues and organs (Groover and Jones 1999; Turfan et al. 2020). However, it was noted that there was no clear trend in lipid peroxidation and hydrogen peroxide levels of *C. sativa* leaves with stand age and elevations in this present study.

4.4 Changes in Antioxidant Enzymes Activities with Elevation and Stand Age

Enzymes such as APX, SOD, and CAT are also nitrogen compounds that work in many processes (Turfan et al. 2020). A significant increase in SOD enzyme activity with increasing elevation may be required to cope with oxidative stress by maintaining $O_2^{-\bullet}$ and H_2O_2 at low levels (Cui et al. 2018). In this study, there was a clear trend in ascorbate peroxidase and catalase enzymes activities of *C. sativa* leaves with increasing of elevation. However, there was no clear trend in catalase enzymes activities. In addition, the ascorbate peroxidase and catalase activities of *C. sativa* have increased initially and then decreased with stand age (Table 3). However, Turfan et al. (2018) reported that SOD, CAT, and APX enzymes activities were higher in 500-year-old black pine trees than 25- and 50-year-old black pine trees. They also reported that high enzyme activity might be associated with catabolic reactions for tissue deformation and aging.

5 Conclusions

The study has shown some supporting evidence for the effects of elevation and stand age on the biochemical compounds of *C. sativa* leaves. In general, chlorophyll *b*, total chlorophylls, chlorophyll *a*:chlorophyll *b*, total carotenoids, total flavonoid content, soluble protein levels, proline content, and ascorbate peroxidase enzymes activities in the *C. sativa* leaves varied between elevation and stand age. Chlorophyll *a* and chlorophyll *b* concentrations in *C. sativa* leaves of the 85-year-old trees decreased compared to other stand ages. A decrease in chlorophyll *a* and chlorophyll *b* concentrations in older chestnut trees can help plants decrease light absorption and prevent photodamage in chloroplasts. APX and SOD enzymes activities were higher at higher elevations than at lower elevations. The change of APX and SOD enzymes activities can reflect the higher reactive oxygen species scavenging capacity at higher elevations. However, the results showed that ecological site factors may have affected the biochemical compounds used by chestnut trees to adapt and develop in each environment. Also, the adaptive capacity of chestnut trees could be attributed to

the combined effects of many site factors. Therefore, the same tree species may use different compounds to adapt to the specific site factors of each area. Further studies are also necessary to understand how these variations in biochemical compounds in fresh chestnut leaves affect the growth and development of Anatolian chestnut.

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Declarations

Conflict of Interest The author declares no competing interests.

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