Age-related Changes of Some Chemical Components in the Leaves of Sweet Chestnut (*Castanea sativa* Mill.)

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The aim of this study was to investigate the developmental physiology of sweet chestnut trees (*Castanea sativa* Mill.) of different age groups (≥ 25, ≥ 50, ≥ 100, ≥ 200, and ≥ 400) in the Catalzeytin district of Kastamonu, Turkey. For this purpose, photosynthetic pigments, proline, total soluble protein, glucose, sucrose, total soluble carbohydrate and starch values, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentration, and also ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) activities were measured in leaf samples. Hence, significant differences were found in the amounts and concentrations of all types of chlorophyll, carotenoid, proline, total soluble protein, glucose, sucrose, total soluble carbohydrate, starches, MDA, H_2O_2, APX, CAT, and SOD among the trees of different age groups. While the chlorophyll a value was low in young trees, the chlorophyll b value was low in older trees. It was determined that the MDA content was high in old trees, whereas it was low in young trees. While the amounts of glucose, sucrose, total soluble carbohydrate, and starch were highest in ≥ 400-year-old trees, they were lowest in the young trees. Both APX and CAT activities were high in young trees, whereas SOD activity was lowest in ≥ 400-year-old trees.

Keywords: Chestnut trees; Chemical components; Aging; Vitality

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INTRODUCTION

The sweet chestnut tree (*Castanea sativa* Mill.), a valuable tree found in the broadleaved forests of Turkey, has a high potential for multi-purpose use. In addition, for centuries, it has provided numerous benefits to people and ecosystems with its fruit being rich in carbohydrates, proteins, fats, vitamins, and minerals, its antimicrobial honey, its branches, leaves and bark with antioxidant properties used in dyeing, and its quality timber used for furniture and woodworking.

Although there are 10 to 12 species of chestnut genus around the world, sweet chestnut is the only naturally grown species in Turkey (Kayacik 1981; Davis 2006). There are some rumours that chestnut culture began in Anatolia and was taken to Greece in the 5th century BC, and then from there to Italy. Genetic studies showed that Italian chestnut varieties and Western Anatolian varieties were related to each other and revealed the reliability of historical records. It is thought that the sweet chestnut originates from the coast of the Black Sea, especially around the Kastamonu province, and it is also believed

that the genus name *Castanea* is derived from the name of this province (Ozcagiran *et al.* 2005; Bucak 2006).

The existing chestnut forests in Turkey are under great pressure due to diseases such as branch cancer (*Cryphonectria parasitica* Murr. Bar.) and root rot (*Phytophthora cambivora* (Petri) Buisman). In the fight against such diseases, emphasis is placed more on internal and external quarantine measures and control with cultivation practices (Celiker and Onogur 2009; Akilli *et al.* 2011). In addition, in many European countries, biological control studies are conducted with the help of hypovirulent *C. parasitica* strains (Heiniger and Rigling 1994). Studies on topics such as isolate collection, identification of isolate phenotypes, affinity groups and the mating types, and transfer of hypovirulence, were conducted by several researchers in the Aegean, Marmara, and Black Sea regions of Turkey, where chestnut cultivation is performed (Gurer *et al.* 2001; Açikgöz *et al.* 2007; Akilli *et al.* 2009)

In addition to the biotic and abiotic factors affecting metabolic and physiological events, such as photosynthesis, carbon, respiration, nitrogen metabolism, oxidative stress, and antioxidant defence, researching processes, such as water and mineral intake from the soil by roots and distribution to plant organs, can provide important data for successful forestry and chestnut cultivation (Ertan *et al.* 2007; Atkinson and Urwin 2012). In addition, since the amount of photo-assimilation in the leaf is an indicator of organic compounds to be transported to other organs, as leaves, stem, root and fruit, the photosynthetic capacity of the leaf is very important. In this context, a larger leaf means more yield and more photo-assimilation or photosynthetic capacity (Wünsche and Lakso 2000; Baïram *et al.* 2019). The total photosynthetic capacity of a species varies according to the photosynthetic rate, that is, the rate at which carbon dioxide is assimilated into organic material and also the total leaf number and leaf area (Liu *et al.* 2004; Orians *et al.* 2005). Famiani *et al.* (2000) studied the effect of leaf to fruit ratios on fruit growth in chestnut. According to their result, the development and growth of fruit vary significantly, depending on the leaf characteristic and light condition. Prediction of a tree's biological life, and thus the future of a forest, and determination of its economic life depend on the identification of its cause-effect relationships. In fact, many researchers state that presenting the ecological characteristics of a forest and the growth and development relations of the species constituting this forest is an important step in forest planning (Eler 2006; Bettinger *et al.* 2009).

In Turkey there have not been many studies on the contribution of photosynthetic metabolism to the growth and development events of forest trees, on protein, proline, soluble sugars, damage to cellular membranes and their effects on chemical content, and on the age-related changes of organic compounds such as antioxidant enzymes. Focusing on these issues may provide important data for chestnut cultivation and successful chestnut forest management. For this reason, this study aims to reveal the age-related changes of the chemical components of chestnut tree leaves as photosynthetic pigments, proline, total soluble protein, glucose, sucrose, total soluble carbohydrate and starch values, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentration, and also ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) activities. These chemicals have been chosen because they are necessary for the growth and development of trees and they also improve their adaptation to the change of environmental conditions.

EXPERIMENTAL

Materials

Research area and samples

This study was conducted in the Caglar village, which is 6 km away from the district centre, located in the Catalzeytin district, and 68 km away from Kastamonu province (41° 54' 13. 9716" N and 34° 11' 3. 1776" E). For the purpose of the study, samplings were prepared from chestnut trees of different ages (≥ 25, ≥ 50, ≥ 100, ≥ 200, and ≥ 400 years). Fresh leaves under the canopy trees from each age group in the sampling area were collected from each direction of the trees in the second half of July. In the leaf samples taken, the photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoid), proline, total soluble protein, total amino acid, glucose, sucrose, total soluble sugar content, malondialdehyde and hydrogen peroxide concentrations, and ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) enzyme activities were determined. Their relationships with tree ages were investigated.

Methods

Chemical analysis

In leaf samples taken from trees of different ages, photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid), proline, total soluble protein, total amino acid, glucose, sucrose, total soluble sugars, the amounts of total phenolic compounds and flavonoids, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations, and APX, CAT, and SOD enzyme activities were studied by taking the relationship between the tree ages into consideration. To determine the chlorophyll content, 0.5 g of fresh leaf tissue was thoroughly crushed in liquid nitrogen and was homogenized by adding 5 mL of acetone solution at 80% at 4 °C. The homogenate was centrifuged at 3000 rpm for 10 min, and the spectrophotometric reading of the supernatant taken was performed in triplicate with the values of 450 nm, 645 nm, and 663 nm.

In determining the total chlorophyll content, the Arnon equation (Arnon 1949) was used, whilst the carotenoid content was determined based on Jaspars formula (Witham *et al.* 1971). Proline content of leaf tissues was determined spectrophotometrically following the ninhydrin method described by Bates *et al.* (1973). 500 mg of leaf tissue were extracted with 3% of sulphosalicylic acid. Samples were centrifuged at 10.000 ×g for 15 min, and added on the supernatants 2 mm glacial acetic acid and ninhydrin reagent 83% (w/v) ninhydrin in 60% (v/v) 6 M phosphoric acid) in order. All samples were boiled at 90 °C for 1 h. After ice cooling, 3 mL of cooled toluene was added on the homogenates, and then the absorbance of the upper (toluene) phase was recorded at 520 nm against a zerotime blank. Proline concentrations were estimated by proline standards (0 to 100 μg mL^{-1}). The amount of MDA was measured according to the methods used by Lutts *et al.* (1996). 500 mg sample was homogenized in 5 mL 0.1% (w/v) trichloroacetic acid (TCA) using a chilled mortar and pestle. The extract was centrifuged at 15,000 g for 15 min. To 1 mL aliquot of supernatant, 2 mL 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA was added. The mixture was boiled at 95°C for 60 min. The samples transferred to an ice bath. Then the absorbance of the supernatant was noted at 532 and 600 nm. MDA content was given as μmol g^{-1} of MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1} as μmol. The proline content in leaf samples was determined according to the methods used by Bates *et al.* (1973), whereas the protein content was determined according to the

methods used by Bradford (1976), MDA according to Lutts *et al.* (1996), and H_2O_2 extraction by Velikova *et al.* (2000).

Total carbohydrate quantitation was completed using the "Anthron Method" of Pearson *et al.* (1976). Approximately 1 g of powder sample was homogenized in 80% ethanol. Absorbance of some of the homogenate was recorded at 630 nm using a spectrophotometer, and glucose and total starch quantitation was performed. The remaining filtrate was extracted with 52% perchloric acid, and its absorbance values were taken at 620 nm wavelength by a spectrophotometer, and then they were used for sucrose and total soluble carbohydrate estimation. The damage to cellular membranes and their effects on chemical content, and on the age-related changes of organic compounds, such as antioxidant enzymes, was recorded. Focusing on these issues may provide important data for chestnut cultivation and successful chestnut forest management. For this reason, this study aimed to reveal the age-related changes of the chemical components of chestnut tree leaves. While determining the enzyme activities of fresh leaf samples, 0.5 g of fresh leaf sample was crushed inside liquid nitrogen and then was homogenized with 5 mL of 50 mM (pH 7.6) KH_2PO_4 (pH = 7), which was a buffered solution containing 0.1 mMNa-ethylene diamine tetra acetic acid (EDTA).

The homogenized samples were centrifuged at 15000 g and at 4 °C for 15 min. Enzyme activities were measured in this supernatant. The APX was determined spectrophotometrically with an ultraviolet/visible (UV/VIS) spectrometer (Model: T80; PG Instruments Limited, Lutterworth, United Kingdom) according to the method applied by Nakano and Asada (1981) by measuring the oxidation rate of ascorbate at 290 nm (E = 2.8 mM cm^{-1}), while CAT activity was determined spectrophotometrically according to Bergmeyer (1975), and SOD enzyme activity was determined according to the method applied by Cakmak (1994).

Statistical analysis

An analysis of variance (ANOVA) was performed to determine whether each parameter of the chemical compounds detected on the leaves differed significantly by age. These findings were presented using the SPSS program (version 11, IBM Corporation, Armonk, NY, USA). The difference that existed between each of the processes, according to the ANOVA results, the significant differences among main values of processes was determined by the Tukey's test. The relationship between the measured characters was revealed by correlation analysis.

RESULTS AND DISCUSSIONS

Changes in the amount of photosynthetic pigment

Significant differences in the amount of photosynthetic pigment were found in chestnut trees of different age groups ($p < 0.05$). The chlorophyll a content was low in young chestnut trees, while it was higher in old trees. The chlorophyll a content was highest in the trees older than 200 and 400 years old, and lowest in the trees older than 25 years old. However, while the chlorophyll b content had higher values in young trees, lower values were found in old trees. While a significant variance was determined in terms of total chlorophyll values (0.317 mg to 0.413 mg) depending on the tree age groups, no significant change could be determined in terms of total carotenoids (Table 1).

Table 1. Changes in Photosynthetic Pigment

Age Group	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total Chlorophyll (mg/g)	Chlorophyll a/b (mg/g)	Total Carotenoid (mg/g)
≥ 400	0.159 ± 0.0003c	0.159 ± 0.0004b	0.318 ± 0.001b	0.993 ± 0.004d	11.53 ± 0.04c
≥ 200	0.162 ± 0.0002d	0.139 ± 0.0010a	0.301 ± 0.001a	1.168 ± 0.010e	11.24 ± 0.03a
≥ 100	0.156 ± 0.0010b	0.249 ± 0.0007c	0.405 ± 0.00d	0.627 ± 0.006c	11.31 ± 0.04b
≥ 50	0.149 ± 0.0001a	0.250 ± 0.0003c	0.399 ± 0.001c	0.595 ± 0.001b	11.25 ± 0.02a
≥ 25	0.149 ± 0.0002a	0.264 ± 0.0004d	0.413 ± 0.001e	0.562 ± 0.001a	11.39 ± 0.06b
F	161.13	9820.37	10.488.11	2832.10	10.19
Significance	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*

Note: Each letter represents the homogeneous group formed by multiple test analysis

In the study, chlorophyll a and chlorophyll a/b contents were high in 400- and 200-year-old chestnut trees of different age groups and low in 25- and 50-year-old chestnut trees of different age groups. The highest amount of carotenoids was found in the 400-year-old tree group (Table 1). Chlorophyll a, b, and chlorophyll a/b data indicated that older trees have a higher capacity to receive light than young trees (Bailey *et al.* 2001; Tanaka *et al.* 2010). Tanaka *et al.* (2001) found that chlorophyll b accumulation increased in poor light conditions and decreased in high light conditions, and thus chlorophyll a/b ratio decreased in shade conditions. Moreover, carotenoid ponds also decrease in poor light conditions (Rosevear *et al.* 2001). The high MDA and H_2O_2 content (Dekker and Boekema 2005) and low proline, protein (Goral *et al.* 2011), carotenoid contents (Green and Durnford 1996), as well as low APX and SOD activity in trees older than 400, 200, and 100 years old may also have affected the chlorophyll b and total chlorophyll contents (Foyer and Noctor 2003).

MDA and H_2O_2 concentration changes with the proline and total soluble protein amount

Significant age-related differences were determined in chestnut trees of different age groups in terms of proline, total soluble protein amount, and MDA and H_2O_2 concentration values ($p < 0.05$). The MDA content was high in the leaves of old chestnut trees, while it was low in the young trees. In addition, H_2O_2 concentration was higher in the tree age groups of ≥ 100 (Table 2).

Low proline content in trees older than 400 years old was associated with high MDA content and low APX and SOD activities (Kreslavski *et al.* 2012). In such trees, the fact that tissue deformations in the trunk and branches (Day *et al.* 2002) reduce the metabolite and assimilate requirement (Cabrera 2004). The hydrolytic resistance (Burg and Ferraris 2008) may have affected the proline content as well. The trees older than 200 years old have H_2O_2-induced proline accumulation (Yang *et al.* 2009). Researchers have stated that ROS's played a role in increasing the hydrolytic resistance in cellulose wall accumulations, vascular tissue differentiation through apoptotic cell deaths, and in transmission of water from soil to leaves (Donald *et al.* 2001). High levels of MDA, glucose, and sucrose contents in such trees confirm this result (Koch *et al.* 2004).

Table 2. Changes in the Amounts of Proline, Total Soluble Protein, MDA, and H_2O_2

Age Group	Proline (µmol/g)	Total Soluble Protein (mg/g)	MDA (µmol/g)	H_2O_2 (nmol/g)
≥ 400	7.11 ± 0.17a	20.96 ± 0.25a	0.413 ± 0.002d	177.14 ± 0.48c
≥ 200	9.90 ± 0.11c	22.60 ± 0.25b	0.484 ± 0.002e	192.98 ± 0.38d
≥ 100	8.88 ± 0.17b	28.73 ± 0.16d	0.400 ± 0.001c	204.07 ± 0.25e
≥ 50	7.39 ± 0.19a	20.50 ± 0.28a	0.341 ± 0.001b	161.02 ± 0.50b
≥ 25	9.57 ± 0.18c	25.31 ± 0.28c	0.210 ± 0.001a	142.55 ± 0.25a
F	72.83	193.63	9056.38	4063.58
Significance	*0.000*	*0.000*	*0.000*	*0.000*

Note: Each letter represents the homogeneous group formed by multiple test analysis.

The low content of total soluble protein in the trees older than 400 years old was due to high levels of H_2O_2, glucose, sucrose, and carbohydrate content (Mishra *et al.* 2009) and low APX and SOD activity (Munné-Bosch and Alegre 2002). The acceleration of senescence due to tissue deformations in the trunk and branches (Mencuccini *et al.* 2007) may have affected the decrease in the amount of protein (Millard and Grelet 2010). In fact, high sucrose and soluble carbohydrate values (Nebauer *et al.* 2011) indicate that senescence is stimulated in tissues (Ally *et al.* 2010).

Tissue differentiation (Gunawardena *et al.* 2004) may have affected MDA content to be highest in trees older than 200 years old. In trees of this age group, protein may degrade and cause proline accumulation (Sharma *et al.* 2011). This explains the result of high proline and glucose values and low protein value (Verbruggen and Hermans 2008). In addition to this, chlorophyll a content being the highest and chlorophyll b content being the lowest indicated that there was photo-oxidative stress in leaves of trees older than 200 years old (Yamasato *et al.* 2005).

The high contents of proline, protein, MDA, and H_2O_2 in the leaves of trees older than 100 years old indicated high metabolic activity in such trees (Cooke and Weih 2005). The average value of pigment and sugar indicates that the tree is going through the maturity stage (Borchert 1976), and that the amount of assimilate and the pool capacity are balanced (Cosgrove 2016). The low amount of proline in the leaf samples of trees older than 50 years old was associated with the fact that the tree was in a shaded area. Plants growing in the shade may have less need for water (Sharma *et al.* 2011). As a matter of fact, researchers have found that when the water content of the cell decreased, proline accumulation of cytoplasm and vacuole increased (Szabados and Savoure 2009).

The H_2O_2 concentration in chestnut trees increased with age. While the highest H_2O_2 content was obtained from trees older than 200 and 100 years old, the lowest H_2O_2 belonged to young trees older than 25 and 50 years old (Table 2). H_2O_2 content may have increased respiratory reactions in trees of the 400-year-old age group. In this tree age group, the high content of glucose, sucrose, and total carbohydrate (Amthor 1994), and low content of soluble protein indicated an increased catabolism (Gibson 2005). Furthermore, low APX activity may have caused an increase in H_2O_2 content (Jajic *et al.* 2015). The fact that the level of H_2O_2 was the highest in trees older than 100 years old was associated with high metabolic reactions in such trees (Fleming *et al.* 1997). In fact, the contents of proline, protein (Johnson 2006), pigment (Hansen *et al.* 2002), and APX and SOD activity (Hernández *et al.* 2001) reinforced the level of developmental events in the trees.

The low H_2O_2 in young trees older than 50 and 25 years old was associated with low MDA content and high enzyme activities (Farmer and Mueller 2013). The H_2O_2 accumulation in trees may have changed depending on the genotype (Liao *et al.* 2012), environmental factors, pathogenic attacks, injuries (Lim and Nam 2007), and silvicultural interventions (Mund 2004).

It was stated that abiotic and biotic stress factors stimulated the oxidative stress in plants and increased MDA and H_2O_2 accumulation in cells and tissues depending on the plant species, and a stressor caused a decrease in vegetal resistance by triggering another stress factor (Atkinson and Urwin 2012). These results indicated that photosynthetic activity is more closely associated with leaf characteristics than the tree age. The results of chestnut trees in different age groups were similar to the results that the authors have performed with different age groups of the leaves of oriental beech (*Fagus orientalis* Lipsky.) (Turfan *et al.* 2019). In addition, many researchers have stated that the photosynthetic activity of the leaf changes depends on the developmental status of the leaf, the leaf's position on the plant, and its capacity to receive light (Lusk and Reich 2000; Niinemets 2007).

In trees older than 100 years old, the monosaccharide, total carbohydrate, and starch values suggested that the photosynthesis-respiratory balance was normal in such trees (Atkin *et al.* 2007). Proline, protein (Gomez and Faurobert 2002), and pigment values (Brown *et al.* 1991) coincided with this result.

Changes in the amount of carbohydrate

Significant differences were determined between tree age groups in terms of glucose, sucrose, total soluble carbohydrate, and starch quantities. While the glucose, sucrose, total soluble carbohydrate, and starch amounts showed the highest values in tree age groups of ≥ 400 years old and above, they showed the lowest values in tree age groups of ≥ 25 and ≥ 50 (Table 3).

In chestnut trees, the glucose and starch amounts were the highest in trees older than 400 years old, and the lowest in trees older than 50 years old. The total amount of soluble carbohydrate was the highest in trees older than 400 years old, and the lowest in trees older than 25 years old (Table 3).

Table 3. Changes in the Amount of Glucose, Sucrose, Total Soluble Carbohydrate, and Starch

Age Groups	Glucose (mg/g)	Sucrose (mg/g)	Total Soluble Carbohydrate (mg/g)	Starch (mg/g)
≥ 400	329.49 ± 0.36e	119.14 ± 0.25d	385.55 ± 0.29e	204.32 ± 0.22e
≥ 200	299.58 ± 0.35c	114.40 ± 0.15b	361.32 ± 0.21b	185.77 ± 0.22c
≥ 100	308.83 ± 0.37d	108.32 ± 0.16a	364.36 ± 0.16c	191.51 ± 0.23c
≥ 50	189.35 ± 0.40a	115.40 ± 0.16c	368.97 ± 0.25d	117.42 ± 0.25a
≥ 25	279.72 ± 0.24b	107.90 ± 0.05a	355.32 ± 0.06a	173.46 ± 0.15b
F	24959.513	861.994	3068.809	24959.514
Significance	*0.000*	*0.000*	*0.000*	*0.000*

Note: Each letter represents the homogeneous group formed by multiple test analysis

The glucose and starch values showed similarities in the trees. Researchers reported that starch accumulation was high in trees with high viability (Renaud and Mauffette 1991). In addition, the chlorophyll a and chlorophyll a/b ratio influenced the glucose and starch amounts, as they were the highest in this tree group.

It was concluded that the amount of glucose of leaves being the lowest in the group of trees older than 50 years old may have been caused by the low amounts of chlorophyll a, chlorophyll a/b (Horie *et al.* 2009), total soluble protein, and MDA, and by the trees being located in the shade (Eggink *et al.* 2001). The low amount of starch and the high amount of sucrose and total soluble sugars supported this result. The sugars, starch value, and pigment values in trees older than 25 years old indicated that the tree was in the shade area (Sato *et al.* 2009) and that metabolic functions were in equilibrium (Walter *et al.* 2005).

Changes in antioxidant enzyme activities

Antioxidant enzyme activities showed significant age-related changes in chestnut trees ($p < 0.05$). Both APX and CAT activities were high in young trees and low in old trees. The SOD activity was lowest with the protein value of 56.79 EU/mg in ≥ 400-year-old trees (Table 4).

The low protein content in the trees older than 50 years old was associated with low assimilation requirements, depending on the tree's size and volume (Majewska-Sawka and Nothnagel 2000). In addition, low proline, protein, and glucose contents, in spite of enzyme activities, indicate that tissue differentiation and tree activities are not high (Liu *et al.* 2013). The proline, MDA, H_2O_2, sucrose, and glucose contents, and CAT and SOD activity in trees older than 25 years old affected the amount of protein. The balance of such compounds indicates that metabolic activity is normal in such trees (Rocha 2013).

The low chlorophyll a, proline, protein, and glucose contents in the chestnut trees older than 50 years old indicated the photo-oxidative stress effect on the tree (Foyer *et al.* 2012). However, high CAT and SOD activity suggests that photooxidative damage is prevented in trees (Das and Roychoudhury 2014). Senescence (Bhattacharjee 2005) and stress factors (Jajic *et al.* 2015) prevent the growth and development by increasing the MDA amount in tissues. The MDA content being the lowest in chestnut trees older than 25 years old is associated with proline content (Gomez and Faurobert 2002) as well as CAT and SOD activity (Foyer *et al.* 2017).

Table 4. Changes in the Activities of APX, CAT, and SOD

Age Groups	APX (EU/mg Protein)	CAT (EU/mg Protein)	SOD (EU/mg Protein)
≥ 400	0.264 ± 0.002b	0.257 ± 0.002c	56.79 ± 0.08a
≥ 200	0.252 ± 0.001a	0.206 ± 0.002b	71.46 ± 0.18b
≥ 100	0.269 ± 0.004b	0.148 ± 0.001a	99.32 ± 0.24d
≥ 50	0.276 ± 0.002c	0.266 ± 0.001d	106.59 ± 0.13e
≥ 25	0.311 ± 0.001d	0.275 ± 0.003e	91.76 ± 0.16c
F	124.044	899.957	15650.082
Significance	*0.000*	*0.000*	*0.000*

Note: Each letter represents the homogeneous group formed by multiple test analysis.

The lipid peroxidation and ROS accumulation in living cells are in equilibrium under normal conditions. However, abiotic and biotic stress factors, senescence, and silvicultural interventions may increase the severity of oxidative stress by disturbing such balance (Zimmermann and Zentggraf 2005). In this case, the continuation of growth and development is sustained in resistant species and types by either stimulating the synthesis of non-enzymatic antioxidants (Adams, III *et al.* 2013), such as phenolic compounds, and enzymatic (Arnholdt-Schmitt *et al.* 2006) carotenoids, such as APX, CAT, GPX, POD, and SOD, or by preventing or repairing the start of oxidative damage (Ally *et al.* 2010) (Table 4).

Table 5 shows the correlation analysis results of 16 chemical variables measured within the study.

Table 5. Correlations Analysis Between the Measured Characters

Characters / P.C.	Chlorophyll a	Chlorophyll b	Chlorophyll a/b	Total Chlorophyll	Carotenoid	Proline	Protein	MDA	H_2O_2	Glucose	Sucrose	Starch	APX	CAT	SOD
Chlorophyll a	1	-.87**	.89**	-.84**	.067	.21	-.02	.88**	.78**	.68**	.36	.27	.684**	-.82**	-.54*
Chlorophyll b		1	-.99**	.99**	-.171	-.004	.46	-.79**	-.45	-.51	-.69**	-.459	-.505	.75**	.08
Chlorophyll a/b			1	-.99**	.106	.112	-.41	.79**	.47	.51	.61*	.35	.51	-.74**	-.13
Tot. chlorophyll				1	-.180	.019	.507	-.77**	-.405	-.476	-.712**	-.461	-.476	.730**	.032
Carotenoid					1	-.40	-.10	-.15	-.18	.52*	.27	.56*	.52*	.15	.31
Proline						1	.53*	-.06	.11	.25	-.66**	-.84**	.25	.16	-.35
Protein							1	-.19	.33	.37	-.87**	-.52*	.371	.263	-.682**
MDA								1	.849**	.351	.523*	.404	.351	-.98**	-.56*
H_2O_2									1	.48	.07	.19	.48	-.81**	-.9**
Glucose										1	-.02	.22	1.**	-.26	-.41
Sucrose											1	.82**	-.02	-.59*	.36
Starch												1	.22	-.47	.16
APX													1	-.26	-.41
CAT														1	.50
SOD															1

According to Table 5, strong positive relationships were determined between chlorophyll a and chlorophyll a/b (r = 0.892); chlorophyll b and total chlorophyll (r = 0.998); carotenoid and starch (r = 0.563); MDA and chlorophyll a (r = 0.884); H_2O_2 and MDA (r = 0.849); glucose and chlorophyll a (r = 0.824); sucrose and starch (r = 0.824; chlorophyll a); APX and chlorophyll a (r = 0.684); and CAT and chlorophyll b (r = 0.784). In addition, strong negative relationships were found between the variables of proline and starch (r = -0.842); protein, sucrose (r = -0.869), and SOD (r = -0.682); MDA and CAT (r = -0.982); and H_2O_2 and SOD (r = -0.9).

CONCLUSIONS

1. Significant differences were found in the amounts and concentrations of all types of chlorophyll, carotenoid, proline, total soluble protein, glucose, sucrose, total soluble carbohydrate, starches, MDA, H_2O_2, APX, CAT, and SOD among the trees of different age groups.
2. In the study, chlorophyll a and chlorophyll a/b contents were high in 400- and 200-year-old and low in 25- and 50-year-old chestnut trees of different age groups. While the chlorophyll a value was low in young trees, the chlorophyll b value was low in older trees. In addition, the highest amount of carotenoids was found in the 400-year-old tree group.
3. The H_2O_2 concentration in chestnut trees increased with age. While the highest H_2O_2 content was obtained from trees older than 200 and 100 years old, the lowest H_2O_2 belonged to young trees older than 25 and 50 years old.
4. In chestnut trees, the glucose and starch amounts were the highest in trees older than 400 years old, and the lowest in trees older than 50 years old. The total amount of soluble carbohydrate was the highest in trees older than 400 years old, and the lowest in trees older than 25 years old.
5. The MDA, glucose, sucrose, total soluble carbohydrate, and starch contents were high in old trees, whereas they were low in young trees.
6. The results indicated that photosynthetic activity was more closely associated with leaf characteristics than the tree age.

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AUTHOR CONTRIBUTIONS

NT, and SA conceived and designed the research; NT, SMO and ENYC carried out the field works; NT and ENYC performed laboratory analysis; SA and HBO supervised the research and NT, SA, HBO, SMO, and ENYC wrote the manuscript.

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