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Variation in chemical compounds of walnut (*Juglans regia L.*) leaves with tree age

Adi ceviz (*Juglans regia* L.) ağacı yapraklarının kimyasal bileşiklerinin ağaç yaşına bağlı değişimi

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Abstract

In this present study, we investigated the chemical compounds in the fresh leaves of ancient walnut trees (*Juglans regia* L.) aged 25, 75, 100, and over 400 year. Under similar environmental conditions, the fresh leaves of walnut trees were collected and analysed for chlorophyll molecules as chlorophyll a, chlorophyll b and carotenoids, enzymatic compounds (ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) activities) and non-enzymatic compounds (proline, total soluble protein, total phenolic compounds), flavonoid and reducing sugars (glucose, sucrose, total soluble sugar). In addition, the oxidative stress level was determined by measuring lipid peroxidation (MDA-malondialdehyde) and hydrogen peroxide (H_2O_2). Significant differences in the chemical composition of the fresh leaves were found between the 4 different tree age classes. In general, the results showed that mean chlorophyll pigments were increased with increasing the age. There was also a general trend that mean glucose and starch concentrations increased with the age, while mean sucrose concentration decreased, but no changes were noted for mean total soluble carbohydrate. On the other hand, mean SOD concentration decreased with increasing the age. Other chemical compounds (mean proline, total soluble protein, malondialdehyde, hydrogen peroxide, APX and CAT), however, did not show clear trends with the age. As a result, these pioneer study have provided valuable insight into the variation in the chemical constituents of walnut tree leaves in relation to the age, and it can be used to better understanding, managing and fighting against pathogens of walnut ecosystems in future studies.

Özet

Bu çalışmada ortalama yaşı 25, 75, 100 ve 400 yaş üzeri Adi ceviz (*Juglans regia* L.) olan ağacı yeş yapraklarının kimyasal bileşikleri araştırılmıştır. Aynı yetişme ortamındaki, adi ceviz ağaçlarında toplanan yeşil yaprak örneklerinin içerdiği fotosentetik pigmentler (klorofil a, klorofil b ve karotenoi askorbat peroksidaz (APX), katalaz (CAT), süperoksit dismutaz (SOD) enzim aktiviteleri, prolin, toplar çözünür protein, toplam fenolik bileşikleri, flavonoid gibi enzimatik olmayan bileşikleri ve indirgenm şekerlerden, glikoz ve sukroz miktarı ile toplam çözünür şeker miktarları belirlenmiştir. Ek olarak, yeş yapraklar oksidatif stres seviyesi ile lipit peroksidasyonu (malondialdehit-MDA) ve hidrojen peroks (H_2O_2) konsantrasyonları için analiz edilmiştir. Bu kimyasal bileşiklerden bazıları, ağaç yaşına bağlı olara istatistiksel farklılıklar göstermiştir. Genel olarak, fotosentetik pigmentler, ağaç yaşı arttıkça art göstermiştir. Glikoz ve nişasta miktarı ağaç yaşı ile artma eğilimi gösterirken, sukroz miktarı azalmı fakat toplam çözünür karbohidrat içeriği ağaçlar arasında önemli bir farklılık göstermemiştir. Bunu yanında, SOD aktivitesi yaş arttıkça azalmıştır. Çalışmada incelenen diğer kimyasal bileşiklerle (prolir toplam çözünür protein, MDA, H_2O_2, APX ve CAT) ağaç yaşı arasında belirgin bir ilişki tespit edilmemişti Sonuç olarak, öncü niteliğindeki bu bulgular, ceviz ağacı yapraklarının yaşa bağlı olarak kimyasa yapılarındaki değişimi hakkında önemli bilgiler sağlamış olup, ceviz ekosistemlerinin daha iyi anlaşılmas yönetimi ve zararlarına karşı uygulanacak mücadele yöntemlerine karar vermede gelecekte çalışmalarda kullanılabilecektir.

INTRODUCTION

Growth and development take place in some stages such as latent period (seeds), pregenerative stages (seedling, juvenile, immature and virginile), generative phases (young, mature and old) and also post-generative period (senile) which are different from other taking place at different times and with different events within itself and with discrete stages in tree species (Evstigneev and Korotkov 2016). For example, it has been reported that photosynthesis metabolism is reduced by changing of leaf structures and biochemistry and also diurnal respiration rate increase rather than photosynthesis (Augspurger and Bartlett 2003). Increasing height, volume and diameter of

a tree with age leads to increase photo-assimilates, energy and nutrient requirements according to proportional of above and underground organs (Koch et al. 2004). Dieback may start in the crown allowing sinks in order to balance with photosynthetic resources due to destructions (Foyer 1988, Del Tredici 1998). Moreover, cell and tissue deaths induce senescence, oxidative stress and lipid peroxidation reactions (Thomas 2013). Determination of the cause-and-effect relation related to growth is a significant step in estimating the lifetime of a tree and understanding the factors affecting it. Length, diameter, basal area, volume and weight of a tree have been subject for many studies. However, there are limited number of studies on age-related changes of chemical components which involving in the formation of organic mass in tree species. Metabolic and chemical reactions are also changed with tree age like phenotypes. Therefore, investigation of the chemical composition of tree leaves in relation to tree age is of great significance.

Walnut trees grow well in areas with a temperate climate. It is reported that Turkey is one of the genetic origins of Juglans regia L. and un-grafted wild walnut trees and all of them show considerable variation in respect of vegetative growth and fruit characters (Akça et al. 2012, Ercisli et al. 2012). Turkey, with 212.140 tons of walnuts is the fourth largest walnut producing country in the world (FAO 2013). Due to its economic importance, many researches have been performed in Turkey especially dealing with the phenological, physical and chemical characteristics of walnuts types and cultivars grown in different areas of Turkey (Muradoglu and Balta 2010, Polat et al. 2015). However, there is no study available having investigated the factors (especially stand characteristics, such as stand or tree age) affecting the leaf chemical constituents of walnut (*Juglans regia* L.) in Turkey. In this study, we therefore aimed at investigating the change in the chemical composition of walnut leaves from different tree ages. Fresh leaves were sampled the walnut trees aged 25, 75, 100, and over 400 year and analsed for macro- and micro-elements, chlorophyll molecules, primary metabolites as soluble protein, proline, sucrose, glucose, and total soluble carbohydrates, secondary metabolites as total phenolic, flavonoids, oxidative stress markers as malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and also activities of antioxidant compounds as ascorbate peroxidase, catalase, and superoxide dismutase.

MATERIAL AND METHODS

Study site description and sampling

This study was carried out in Kastamonu, Northwest Turkey (41°29'12"N, 33°53'07"E) (Fig. 1). Mean altitude was 775 m a.s.l. The aspect was north. The study area is under terrestrial climatic conditions, i.e. winters are long, cold and snowy, whereas summers are short and warm. The seasonal and daily temperatures show high extreme values and rainfall is generally low (Duran 2017). The long term weather data (1950-2015) from Kastamonu Meteorology Station, at 800 m. showed that rainfall was annually 474 mm and the average temperature was 9.8 °C. The average monthly temperatures ranged from 20.2 ^{o}C in July to -0.8 ^{o}C in January. The average wind was 1.2 m/s, whereas average relative humidity was 75.9%. The average duration of sunshine was 5.8 hours. The highest rainfall per day was recorded 104.7 kg/m^2 in 1953. The highest snow depth was measured at 53.25 cm in 1954. According to the geological map, the study area emerged in the Paleozoic-Triassic era and was made of submarine volcanic rocks with sedimentary rocks.

In the study area, three study plots (20 m x 20 cm) were chosen and the diameters of all walnut trees in the plots were measured. It was seen that the diameters of the walnut trees were grouped into three categories as < 100 cm, >100, 150 cm – 200 cm and over 500 cm. Therefore, for each diameter category, mean tree age was measured using three trees in each plot. The four tree age categories were noted as less than 25 year-old, between 75 and 100 year-old and over 400 year-old. Mean age, diameter, and height of the trees are shown in Table 1. Mean diameter at the breast height (DBH) was measured using a diameter tape. Mean tree age was determined using increment borer by counting annual rings at DBH. Tree heights were measured with a Blume-Leiss clinometer (Mackensen et al. 2001).

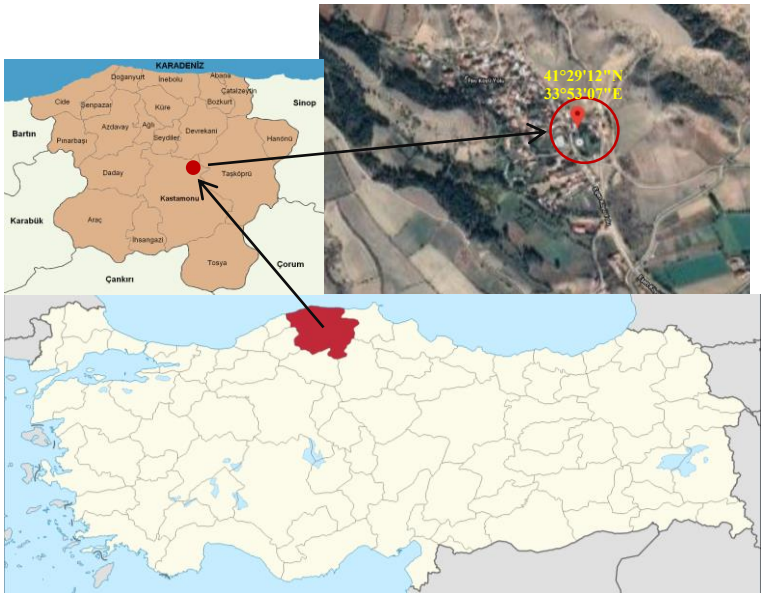

Figure 1. Location of the study area

Table 1. Mean age, diameter at the breast height (DBH), height of walnut (Juglans regia L.) trees in the study site

Age (year)	DBH (cm)	Height (m)	Number tree
>400	>500	20	1
>100	150-200	15-20	2
≥75	>100	15-20	4
≥25	<100	>15	4

Depending on the number of trees in the classes, at least twenty leaves were collected from the lower parts of the trees with different directions and placed into the paper bags. Then, the leaf samples were mixed and brought to the laboratory fort he chemical analyses.

Analysis of the fresh leaves

For photosynthetic pigment analyses, 0.5 g leaf samples were crushed with 10 ml of 80% acetone in a porcelain mortar and centrifuged at 5000 rpm for 5 minutes (Witham et al. 1971). The clear supernatant was used for reading absorbance at 663, 645 and 450 nm. Chlorophyll (Chl) content was determined following the method of Arnon (1949). The Carotenoid amount was estimated by Jaspars Formula. Proline level was measured by the methods of Bates et al (1973). 500 mg samples crushed and homogenized in 3% aqueous sulfosalicylic acid and estimated by using acidic ninhydrin reagent. The absorbance of homogenate was read at 520 nm. The amount of proline was estimated using the calibration curve and described as µmol/g fresh weight.

Lipid peroxidation was calculated as MDA in the leaf tissues following the Lutts et al. (1996) method. 500 mg samples were extracted in 5ml 0.1% (w/v) TCA solution and centrifuged at 12000 g for 15 min. 1ml of the supernatant was put into 1 ml 5% (w/v) TBA in 20% TCA. After heating the extract at 95°C for 30 min, the reaction was stopped by cooling mixtures in an ice bath. The cooled extract was centrifuged for 10 min at 12000 g and the absorbance of the supernatant was taken at 532 and 600 nm. The amount of MDA was estimated by its molar extinction coefficient of 155 mM-1 cm-1 as nmol MDA. Hydrogen peroxide concentration of leaf was measured following the method of (Velikova et al. 2000). The total phenolic amount was conducted by the spectrophotometric Folin-Ciocalteu method (Singleton et al. 1999). Total flavonoid estimation was performed spectrophotometrically (Kumaran and Karunakaran 2006). The amount of soluble sugars were determined using the method of Pearson et al. (1976).

Antioxidant analysis of the leaf samples was carried out by using 500 mg of fresh leaf samples, and all extractions

and enzyme preparations were done in an ice bath. The samples were ground in nitrogen liquid. The powdered samples were extracted with 5 mL of 100 mM sodium phosphate buffer (pH 7.4 with 0.1 mM of EDTA (ethylene diamine tetraacetic acid).Then, this extract was centrifuged at 10000g for 20 min at 4ºC. Final mixtures used for measurement of soluble protein content, APX, CAT and SOD, activities. The activity of SOD was determined by measuring its ability to inhibit the photochemical reduction of NBT (nitroblue tetrazolium), adopting the method of Cakmak (1994). Catalase activity (CAT) was estimated according to the method of Bergmeyer and Grabl (1983) considering the destroying of H_2O_2, measuring the decrease of the absorbance at 240 nm. Ascorbate peroxidase (APX) activity was evaluated by following the procedure described by noting the decline in absorbance at 290 nm due to a reduction in the amount of ascorbic acid by Nakano and Asada (1981). APX and CAT were expressed per mg protein, and one unit represented 1 µmol of a substrate undergoing reaction per mg protein per min. The amount of soluble protein leaf tissues was determined following the Bradford method (1976) using bovine serum albumin as the standard. The leaf samples were also analyzed for macro (Ca, Mg, P, K, and S) and micro (Na, Mn, Fe, Si, Al, and Zn) nutrient concentrations using SPECTRO brand XEPOS model XRF instrument at Central Research Laboratory at Kastamonu University.

Statistical analysis

Analysis of variance (ANOVA) has been carried out for analyzing the differences in the chemical composition of walnut leaves between 4 tree age classes using the SPSS program (Version 11 for Windows). Following the results of ANOVAs, Tukey's honestly significant difference (HSD) test (α= 0.05) was used for significance. The relations between leaf nutrients and chemical compounds were examined by leaf samples with the Pearson correlation coefficients.

RESULTS

Nutrient concentration of walnut leaves according to the age classes

Mean macro- and micro-nutrient concentrations in the fresh leaves are given in Table 2 and Table 3, respectively. Mean P, Ca and Mg concentrations were highest for the 75-year-old trees (2772 ppm, 22060 ppm, and 7138 ppm, respectively), while mean S concentration was highest for the 25-year-old trees (3271 ppm). However, the 25-year-old trees had the lowest K and Mg concentrations (30260 ppm and 5435 ppm, respectively). Over 400-year-old trees had the lowest mean Ca and S concentrations (17130 ppm and 2807 ppm, respectively). Mean P concentration for the 100-year-old trees (2361 ppm) was the lowest (Table 2).

Table 2. Macronutrient concentrations (ppm) in the leaves from the different age classes.

Age (year)	P	K	Ca	Mg	S
>400	2556c±4	39620d±40	17130a±20	5696b±36	2807a±3
>100	2361a±4	32875b±30	22010c±30	5626b±37	3105c±4
≥75	2772d±5	33520c±40	22060c±30	7138c±41	3066b±4
≥25	2530b±4	30260a±30	18800b±20	5435a±36	3271d±4
F	85302.000	47061075.000	34255.330	1843804.750	132719.200
Sig.	<0,001	<0,001	<0,001	<0,001	<0,001

Mean Fe, Mn, and Zn concentrations were lowest for the 25-year-old trees (76 ppm, 22.2 ppm, and 8.2 ppm, respectively), while mean Si concentration was lowest for the over 400-year old trees (1092 ppm). However, the 75-year-old trees had the highest Mn and Zn concentrations (112.8 ppm and 28.7 ppm, respectively), while the 100-year-old trees had the highest Fe concentration (251 ppm). Mean Si concentration was highest for the 25-year-old trees (2271 ppm) (Table 3).

Table 3. Micronutrient concentrations (ppm) in the leaves from the four different age classes.

Age (year)	Fe	Mn	Si	Zn
>400	113b±0.9	59.6b±0.5	1092a±4	28.1c±0.3
>100	251d±14	91.9c±0.6	2057c±6	24.6b±0.3
≥75	137c±1.0	112.8d±06	1472b±5	28.7c±03
≥25	76a±0.5	22.2a±0.6	2271d±6	8.2a±0.3
F	17082.750	3344733.544	873022.000	208048.184
Sig.	<0,001	<0,001	<0,001	<0,001

Photosynthetic pigments

Variation in the chlorophyll a, and b, total chlorophyll, carotenoids, total phenolic compounds (TFC), flavonoids levels in the leaves with the different age classes are given in Table 4. The photosynthetic pigments changed significantly between the age classes ($p < 0.001$). Over the 400-year-old trees had the highest chlorophyll a, total chlorophyll, carotenoids, and total phenolic compounds, while mean flavonoids concentration and the ratio of chlorophyll a/b were highest for the 100-year-old walnut trees (Table 4). Only, mean amount of chlorophyll-b was highest for the 75-year-old walnut trees.

Table 4. Mean concentrations (mg/g) of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (TCh) and total carotenoid (TCa), total phenolic compounds (TFC), flavonoids (Fla.) and the ratio of chlorophyll a/b in walnut leaves of the four different age classes.

Tree Age	Chl.l a mg/g	Chl. b mg/g	Chl. a/b mg/g	Total Chl. mg/g	Total Carotenoids mg/g	Total Phenolic mg/g	Flavonoids mg/g
>400	0.172c±0.001	0.208c±0.001	0.828c±0.003	0.380d±0.001	10.30d±0.003	185.73d±0.15	91.94±0.12
>100	0.162b±0.00	0.158a±0.001	1.031d±0.005	0.320a±0.001	10.13c±0.021	177.87c±0.18	93.51d±1.01
≥75	0.156a±0.00	0.220d±0.001	0.709a±0.002	0.376c±0.001	9.94b±0.023	165.94b±0.12	91.34b±0.22
≥25	0.161b±0.001	0.198b±0.001	0.814b±0.004	0.359b±0.001	9.83a±0.019	143.30a±0.08	89.62a±0.10
F	340.09	3865.78	1674.14	3014.46	131.18	2142.18	121.59
Sig.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Proline, total soluble protein, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2)

Quantity variation of all mentioned chemicals in the leaf samples are presented in Table 5. The amount of proline, total soluble protein and H_2O_2 were highest in the leaves for the 75-year-old trees (87.7 µmol, 23.5 mg/g, and 93.9 µmol respectively), while the content of MDA was highest for the over 400-year-old trees (8.82 µmol). The 25-year-old trees had the lowest proline (7.4 µmol) and H_2O_2 (67.0 µmol) concentrations, and the 100-year-old trees had the smallest amount of total soluble protein (15.5 mg/g) and MDA (3.42 µmol) (Table 5).

Table 5. Variation in proline, total soluble protein, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations with the four age classes.

Age (year)	Proline (µmol/g)	Protein (mg/g)	MDA (µmol/g)	H_2O_2 (µmol/g)
>400	76.3b±0.25	19.3c±0.28	8.82d±0.20	88.8c±0.26
>100	81.9c±0.03	15.5a±0.28	3.42a±0.24	83.1b±0.33
≥75	87.7d±0.25	23.5d±0.23	4.37b±0.27	93.9d±0.19
≥25	75.4a±0.27	17.2b±0.05	5.67c±0.15	67.0a±0.22
F	1098	225	118	2088
Sig.	<0.001	<0.001	<0.001	<0.001

Soluble sugars and total carbohydrates

The amount of glucose, sucrose and total soluble carbohydrates in the leaf samples are shown in Table 6. According to the results, mean glucose and starch concentrations increased, while mean sucrose concentration decreased compared to others, but no variation in mean total soluble carbohydrate (Table 6).

Over 400-year-old trees had the maximum glucose concentration (85.2 mg/g) and starch (101.1 mg/g), while the 25-year-old tree had the maximum sucrose concentration (197.8 mg/g).

Table 6. Variation in glucose, sucrose and total soluble carbohydrate concentrations with the four age classes

Age (year)	Glucose mg/g	Sucrose mg/g	Total Carbohydrate mg/g	Starch mg/g
>400	85.2d±0.16	184.4a±0.01	23.3c±0.01	101.1d±0.12
>100	75.1c±0.03	187.8c±0.02	23.1b±0.01	93.6c±0.05
≥75	60.5a±0.01	184.3b±0.03	22.5a±0.01	80.8a±0.01
≥25	63.2b±0.03	197.8d±0.08	23.3c±0.01	84.5b±0.03
F	19068.05	24068.21	3111.04	21356.83
Sig.	<0.002	<0.003	<0.001	<0.002

Enzyme Activity

Variation in APX, CAT and SOD enzyme activities in the leaves with the tree age are presented in Table 7. Their activities changed significantly between the age classes ($p<0.001$). In general, the over 400-year-old trees showed maximum APX and CAT activities (0.148 and 0.449 EU, respectively), whereas the 75-year-old trees had the smallest enzyme activities (0.114 and 0.293 EU, respectively). The older walnut trees (over 400- and 100-year-old trees) showed lower mean SOD concentration than the younger walnut trees (75- and 25-year-old trees) (Table 7).

Table 7. Variation in ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) activities with the four age classes.

Age (year)	APX EU/mg Protein	CAT EU/mg Protein	SOD EU/mg Protein
>400	0.148d±0.003	0.449d±0.002	53.72b±0.14
>100	0.124b±0.003	0.327c±0.003	46.46a±0.29
>70	0.114a±0.003	0.293a±0.002	68.13d±0.47
≥25	0.137c±0.003	0.310b±0.004	61.87c±0.26
F	30.15	641.16	925.68
Sig.	<0.001	<0.001	<0.001

DISCUSSION

Variation in photosynthetic pigments with the tree age

The results in this study have pointed out significant variation in the chemical compounds of walnut leaves with the age. Although not statiscally significant, there was a relationship between the photosynthetic pigment concentration, total phenolics, and flavonoids (Annex 1). On the otherhand, there was a good relationship between pigments, the other chemical commpounds (soluble and non-soluble carbohydrate, proline, protein, MDA, H_2O_2, APX, CAT, SOD) and minerals (Annex 1). In this study, mean chlorophyll a, total chlorophyll, carotenoid, and total phenolic compounds were maximum for the over 400-year-old walnut trees, while mean flavonoids concentration and the ratio of chlorophyll a/b were highest for the 100-year-old walnut trees, and mean chlorophyll-b was maximum for the 75-year-old walnut trees. On the other side, in general, mean chlorophyll-a and total chlorophyll enhanced with increasing total phenolic, while mean chlorophyll-b and total chlorophyll decreased with increasing mineral elements, especially Fe concentration. Chlorophyll pigments as photoreceptors are involved in photosynthesis and play important roles in the productions of carbonaceous compounds. They are considered to be indicative of transitions of growth phases with the level of tolerance to environmental conditions such as soil properties, climatic factors and disease (Bertamini et al. 2001, Green 2003). Similarly, a number of authors (Cakmak and Engels 1999, Martinez-Finley et al. 2013) have stated that chlorophyll pigments may be reduced with higher mineral concentrations and

heavy metals. Because excess heavy metals and minerals may inhibit chlorophyll biosynthesis enzymes due to oxidative stress and senescence leaf tissue. Mg, Fe and Mn are shown to be important elements for chlorophyll biosynthesis, electron transport, and enzyme activation, splitting of water in photosystem II and chlorophyll a molecule transfer in orderly (Kusunoki 2011).Therefore, mineral elements can have a significant influence on pigment content and photosynthetic metabolism. On the other side, Augspurger and Bartlett (2003), Louis et al. (2009), have showed that chlorophyll is highly unstable in contrast to other biological pigments such as carotenoids, tannins, and melanins, and total chlorophyll content may significantly vary with the life-cycle, environmental conditions and phase transition like juvenility, maturation and aged (senility).

Phenolic compounds and fluorine are secondary metabolites which are active in the physiological processes such as increasing cellular resistance against UV, high light intensity and pathogen attacks, the formation of unique aromas of plants (Amaral et al. 2004). The phenolic and chlorophyll-a concentrations can decrease under low light conditions but chlorophyll-b may increase (Louis et al. 2009).

Lower amounts of phenolic compounds, flavonoids and also chlorophyll-a for the younger walnut trees may be associated with the adaptation for sink/pool organization (Krapp et al. 1993). Diameters values and sugars level confirms this result. However phenolic levels may change due to changes in allelopathic properties and richer in volatile compounds of walnut trees (Willis 2000). Khanna-Chopra (2012) determined that older leaves were harder than the younger leaves because phenolic and volatile compounds can result in increasing rigidity in leaf structure.

Variation in reducing sugar as glucose, sucrose and total soluble carbohydrates

Mean sucrose concentration was highest for the youngest tree, but no variation in mean total soluble carbohydrate was seen among the tree ages (Table 6) (Schaffer et al. 1986, Paul and Driscoll 1997). Carbonaceous compounds such as glucose, fructose, and sucrose produced by photosynthesis are essential molecules in joining the structure and in the control of growth and development as source of repair, regulation or energy (Talon et al. 2002). But high concentrations may induce senescence and lead to aging and death of tissues and organs (Foyer, 1988, Paul and Driscoll 1997). The highest mean glucose and starch concentrations for the over 400-year-old trees could be attributed to higher the chlorophyll-a, total chlorophyll and K concentrations. In our study, the results showed that the chlorophyll-a, total chlorophyll, and K concentrations were higer in the older aged tree.

Some authors showed that photosynthetic electron transfer amount, ATP, NADPH+H deposition increased with increasing chlorophyll-a (Scholes and Fleming 2005). It has been stated that higher K concentration increases light tolerance of tree (Cakmak 1994) and activates the RubisCO enzyme and therefore stimulates glucose and starch accumulation in plants (Schafer et al. 1992). The reason why the amount of starch was highest for the over-400-year-old trees may also be due to an adaptation for the balanced production of photoassimilates with reduced assimilate pool (Del Tredici 1998, Genet et al. 2010). It can be concluded that the lowest levels of chlorophyll-a and chlorophyll a/b, K, Mg, Fe, Mn and Zn in the walnut leaves from the 25-year-old trees could have influenced the amount of photoassimilates by reducing the electron transfer in pigment systems (Scholes and Fleming 2005).

Variation in proline, total soluble protein, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2)

Total soluble protein and proline are nitrogen compounds that have important functions in metabolic reactions involving growth and development (Dickson et al. 2000). It is reported that their amounts increase during the changing of environmental conditions and stimulate resistance by preventing lipid peroxidation and oxidative stress (Szabados and Savoure 2009). Cellular membranes and the walls are the areas that are most exposed to the changes in the ontogenic and morphogenic processes in trees. It is also stated that MDA content may increase during those phases (Spiteller 2001, Thomas 2013). A number of authors reported that lower amounts of

proline and protein concentrations in the older trees were associated with higher MDA, H_2O_2, and starch, and tissue deformation due to tree age (Sofo et al. 2004). Schaffer et al. (1986) and Talon et al. (2002) and explained that excessive starch accumulation in tree leaves and H_2O_2 damaged the chloroplast membrane. In addition, H_2O_2 is synthesized in photosynthetic active chloroplasts near transmission bundles in leaf and transported from there to tissues and organs and triggers deterioration of tissues and organs (Ros Barceló 1998, Groover and Jones, 1999). Turfan et al. (2016) explained that tree species, age and soil characteristics of chemical components, photosynthetic pigments were very prominent in the amount of proline and protein. Many authors found out that phosphorus stimulated the synthesis of nitrogenous compounds and soluble compounds such as proline and protein to protect the enzyme and membrane structure (Cakmak and Engels 1999).

In this present study, however, it was noted that there were no clear trend in mean proline, total soluble protein, malondialdehyde, and hydrogen peroxide concentrations in the walnut leaf samples with increasing or decreasing with the tree age. The amount of proline, total soluble protein and H_2O_2 increased in the leaves for the 75-year-old trees, while mean MDA concentration was highest for the over 400-year-old trees. The 25-year-old trees had the lowest proline concentrations, and the 100-year-old trees had the minimum total soluble protein. In the cases where the MDA content is low, the amount of proline is high and when the MDA is high, the APX, GPOX, and CAT activities are high, and these compounds are more effective in eliminating MDA damage (Turfan et al. 2016).

Variation in antioxidant enzyme activity

Enzymes such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD, GPOX) are also nitrogen compounds are compounds that work in many processes. In our study, there was no clear trend in mean APX and CAT concentrations in the walnut leaf samples with increasing or decreasing with tree age. However, there was a clear trend that mean SOD concentration decreased with increasing the tree age. The lowest concentration of SOD concentration in older trees could be attributed to the lower proline, protein and sucrose concentrations (Koch 2004, Szabados and Savoure, 2009). Additionally, the iron concentration may have shown toxic effects in the enzyme activity (Weinstein and Robbins 1955). It was reported that lower concentrations of MDA and H_2O_2 and moderate activities of SOD, APX and CAT may have been an adaptation for anabolic and catabolic reactions in order to be proportional with the age, volume and size of younger trees (Foyer and Shigeoka 2011, Thomas 2013). Higher concentrations of pigment, proline, protein, glucose, K, Fe, Mn and Zn in older walnut trees support these results (Schafer et al. 1992, Cakmak 1994, Martinez-Finley et al. 2013). Excessive mineral intake may suppress growth and development by influencing metabolic reactions such as cell growth, photosynthesis, and breathing (Weinstein and Robbins 1955, Marschner, 1995).

CONCLUSION

The results in this present study have indicated that the chemical constituents produced by plants for essential functions in the leaves of walnut trees under similar environmental conditions in Northwest Turkey can significantly vary with the age. However, significant variations are clearly noted between the over 400 and 25-year old tree ages. In general, K, Mg, Fe, Mn, Zn, chl. a, chl. b, total chlorophyll, total carotenoid, total phenolic compounds, flavonoids, proline, protein, MDA, H_2O_2, glucose, starch, APX and CAT concentrations in the leaves of the 400-year old trees increased compared to the leaves of the 25-year old trees, whereas Ca, S, Si, sucrose concentrations and SOD activity enhanced significantly with the 25-year old trees. All those chemical compounds in plant leaf are essential for the synthesis of structural components of the cell, tissue, and organs and other metabolic reactions and plant defense against the attack by herbivores and disease-causing microorganisms as well as plant litter decomposition. However, a more detailed analyses of the chemical substances and their functions within and among plants and with time are needed and will be useful to complement studies on growth-defense relationships.

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Annex 1. Correlation analysis of leaf nutrients and chemical compound properties.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
2	-,534																		
3	,529	-,999***																	
4	-,410	,978***	-,976***																
5	-,003	-,829*	,827*	-,872***															
6	-,405	-,514	,512	-,581	,900***														
7	-,169	-,719*	,718*	-,768*	,980***	,965***													
8	,818**	-,899***	,901***	-,840**	,505	,088	,345												
9	,841**	-,112	,114	-,019	-,455	-,795**	-,609*	,534											
10	,940***	-,563	,566	-,476	,010	-,418	-,167	,867**	,883***										
11	,676*	-,979***	,979***	-,939***	,699*	,329	,563	,970***	,312	,720*									
12	-,841**	,109	-,111	,015	,459	,797**	,612*	-,531	-1,000***	-,882***	-,309								
13	-,841**	,876***	-,877***	,812**	-,460	-,037	-,296	-,999***	-,578	-,892***	-,956***	,575							
14	,323	,591	-,589	,651*	-,935***	-,996***	-,985***	-,180	,735*	,333	-,414	-,738*	,129						
15	-,897***	,259	-,261	,164	,319	,696*	,484	-,654*	-,989***	-,943***	-,450	,988***	,692*	-,627*					
16	,822**	-,103	,105	-,018	-,458	-,797**	-,613*	,525	,996***	,876***	,303	-,996***	-,569	,738*	-,984***				
17	,926***	-,414	,410	-,334	-,117	-,526	-,300	,742**	,898***	,935***	,575	-,897***	-,772**	,449	-,934***	,880***			
18	-,540	,999***	-1,000***	,976***	-,820**	-,500	-,708*	-,907***	-,128	-,577	-,982***	,125	,884***	,578	,274	-,120	-,423		
19	-,741**	,952***	-,953***	,904***	-,622*	-,229	-,475	-,990***	-,409	-,788**	-,995***	,406	,981***	,317	,540	-,400	-,651*	,957***	
20	-,934***	,405	-,407	,312	,169	,576	,343	-,763**	-,954***	-,983***	-,583	,953***	,796**	-,498	,988***	-,948***	-,950***	,420	,664*
21	-,620*	-,267	,265	-,351	,754**	,964***	,861**	-,180	-,927***	-,645*	,066	,929***	,231	-,935***	,862**	-,927***	-,717*	-,252	,038
22	-,942***	,644*	-,646*	,560	-,112	,325	,067	-,913***	-,832**	-,994***	-,787**	,830**	,933***	-,236	,905***	-,825**	-,919***	,657*	,846**
23	,897***	-,253	,255	-,158	-,324	-,700*	-,490	,649*	,990***	,941***	,444	-,989***	-,688*	,631*	-1,000***	,985***	,935***	-,268	-,535
24	-,083	-,778**	,777**	-,818**	,99[1]***	,939***	,996***	,427	-,536	-,079	,634*	,539	-,379	-,966***	,405	-,542	-,218	-,768**	-,551
25	-,939***	,664*	-,666*	,581	-,138	,300	,041	-,924***	-,817**	-,991***	-,802**	,815**	,942***	-,211	,894***	-,809**	-,913***	,676*	,860**
26	,925***	-,725*	,726*	-,646*	,221	-,218	,044	,953***	,766**	,977***	,850**	-,763**	-,967***	,127	-,853**	,757**	,888***	-,736*	-,900***
27	-,937***	,672*	-,674*	,590	-,149	,289	,029	-,928***	-,811**	-,990***	-,809**	,809**	,946***	-,200	,889***	-,803**	-,910***	,685*	,866**

*: p<0.05; **: p<0.01, ***: p<0.001

1-Chla; 2-Chl b; 3-Chla:Chl b; 4-Total Chl; 5-Total Carotenoit; 6-Total phenolic; 7-Flavonoid; 8-Glucose; 9-Sucrose; 10-Total soluble carbohydrate; 11-Starch; 12-Pr
18-SOD; 19-P; 20-K; 21-Ca; 22-Mg; 23-S, 24-Fe, 25-Mn; 26-Si; 27-Zn