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

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, Erçisli 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 analysed 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 (H₂O₂) 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 °C in July to -0.8 °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/m² 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).
Variation in chemical compounds of walnut (Juglans regia L.) leaves with tree age

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 for the 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

126 | N.Turfan, G. Savacı, T. Sarıyıldız / AÇÜ Orman Fak Derg 21(1):124-134 (2020)
Variation in chemical compounds of walnut (Juglans regia L.) leaves with tree age

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 were used for the 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 H₂O₂, 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       | 2361c±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 | 47061.075.000 | 34255.330 | 124380.750 | 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).
Variation in chemical compounds of walnut (Juglans regia L.) leaves with tree age

### 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| 1472d±5 | 28.7c±0.3|
| ≥25        | 76a±0.5 | 22.2a±0.6| 2271d±6 | 8.2a±0.3 |
| F          | 17082.750| 33473.544| 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 (year) | Chl. 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          | 93.51±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          | 91.34±0.22         |
| ≥25             | 0.161b±0.00   | 0.198b±0.001  | 0.814b±0.004    | 0.359b±0.001      | 9.83a±0.019             | 143.30a±0.08          | 89.62±0.10         |
| F               | 340.9         | 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 (H₂O₂)

Quantity variation of all mentioned chemicals in the leaf samples are presented in Table 5. The amount of proline, total soluble protein and H₂O₂ 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 H₂O₂ (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 (H₂O₂) concentrations with the four age classes.

| Tree Age (year) | Proline (µmol/g) | Protein (mg/g) | MDA (µmol/g) | H₂O₂ (µmol/g) |
|-----------------|------------------|----------------|--------------|---------------|
| >400            | 76.3±0.25        | 19.3±0.28      | 8.82±0.20    | 88.8±0.26     |
| >100            | 81.9±0.03        | 15.5±0.28      | 3.42±0.24    | 83.1±0.33     |
| ≥75             | 87.7±0.25        | 23.5±0.13      | 4.37±0.27    | 93.9±0.19     |
| ≥25             | 75.4±0.27        | 17.2±0.05      | 5.67±0.15    | 67.0±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       | 85.2±0.16    | 184.4±0.01   | 23.3±0.01                | 101.1±0.12  |
| >100       | 75.1±0.03    | 187.8±0.02   | 23.1±0.01                | 93.6±0.05   |
| ≥75        | 60.5±0.01    | 184.3±0.03   | 22.5±0.01                | 80.8±0.01   |
| ≥25        | 63.2±0.03    | 197.8±0.08   | 23.3±0.01                | 84.5±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       | 0.148±0.003        | 0.449±0.002        | 53.7±0.14         |
| >100       | 0.124±0.003        | 0.327±0.003        | 46.46±0.29        |
| ≥70        | 0.114±0.003        | 0.293±0.002        | 68.13±0.47        |
| ≥25        | 0.137±0.003        | 0.310±0.004        | 61.87±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 statically significant, there was a relationship between the photosynthetic pigment concentration, total phenolics, and flavonoids (Annex 1). On the other hand, there was a good relationship between pigments, the other chemical compounds (soluble and non-soluble carbohydrate, proline, protein, MDA, H$_2$O$_2$, 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 higher 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 (H₂O₂)**

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, H₂O₂, 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 H₂O₂ damaged the chloroplast membrane. In addition, H₂O₂ 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 H₂O₂ 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 H₂O₂ 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, sucrose, 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, H₂O₂, 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.
REFERENCES

Akçay O, Ozgen M, Erturk U, Ercisli S (2012) The effects of AVG and GA3 treatments on pistillate (female) flower abortion in 'Sebin' walnut cultivar. Acta Scientiarum Polonorum Hortorum Cultus 11(4):179-185

Amaral JS, Seabra RM, Andrade PB, Valen too PA, Pereira JÁ, Ferreres F (2004) Phenolic profile in the quality control of walnut (Juglans regia L.) leaves. Food Chemistry, 88: 373-379

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in Beta vulgaris. Plant Physiology, 24: 1-15

Augspurger CK, Bartlett EA (2003) Differences in leaf phenology between juvenile and adult trees in a temperate deciduous forest. Tree Physiology, 23: 517-525

Bates L, Waldern RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant and Soil, 39: 205-207

Bergmeyer J, Grabl M (1983) Methoden der Enzymatischen Analyse (Methods of enzymatic analysis) Akademie Verlag (Academy publishing house), 1: 190-302

Bertamini M, Nedunchezian N, Broghi B (2001) Effect of iron deficiency induced changes on photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase, and photosystem activities in field grown grapevine (Vitis vinifera L. cv. Pinot Noir) leaves. Photosynthetica, 39(1): 59-65

Bradford MM (1976) A rapid sensitive method for the quantitation of micro program quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254

Cakmak I (1994) Activity of ascorbate-dependent H2O2-scavenging enzymes and leaf chlorosis are enhanced in magnesium and potassium deficient leaves, but not in phosphorus deficient leaves. Journal of Experimental Botany 45: 1259-1266

Cakmak I, Engels C. (1999) Role of mineral nutrients in photosynthesis and yield formation, in Rengel, Z.: Mineral Nutrition of Crops: Variations in chemical compounds of walnut (Juglans regia L.) leaves with tree age. The role of potassium in alleviating detrimental effects of abiotic stresses in plants. Journal of Plant Nutrition and Yield Formation, in Rengel, Z.: Mineral Nutrition of Crops:

Del Tredici P (1998) Aging and rejuvenation in trees. Combined Proceedings of the International Plant propagators Society, 48: 637-642

Dickson RE, Tomlinson PT, Isebrands JG (2000) Partitioning of current photosynthate to different chemical fractions in leaves, stems and roots of northern red oak seedlings during episodic growth. Canadian Journal of Forest Research, 30: 1308-1317

Durán C (2017) Local distribution of the temperature and precipitation in Kastamonu province and surrounding. The Journal of International Social Research, 10(52): 509-516

Ercisli S, Sayinç B, Kara M, Yildiz C, Ozturk I (2012) Determination of size and shape features of walnut (Juglans regia L.) cultivars using image processing. Sci. Hortic. 131: 47-55

Evstigneev OI, Korotkov NV (2016) Ontogenetic Stages of Trees. Russian Journal of Ecosystem Ecology, 1(2): 1-31

FAO (2013) Walnut production statistics. Available at: http://faostat3.fao.org. (Accessed on 25.12.2016)

Foyer CH (1988) Feedback inhibition of photosynthesis through source-sink regulation in leaves. Plant Physiology and Bochreist, 26: 483-492

Foyer CH, Shigeoka S (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol 155: 93-100

Genet H, Breda N, Dufrene E (2010) Age-related variation in carbon allocation at tree and stand scales in beech (Fagus sylvatica L) and sessile oak (Quercus petraea (Matt.) Liebl.) using a chronosequence approach. Tree Physiology, 30: 177-192

Green BR (2003) in: Green BR, Parson WW (Eds.) Light-Harvesting Antennas in Photosynthesis. Kluwer Academic Publishers, The Netherlands. pp 129-168

Groover A, Jones AM (1999) Tracehary Element Differentiation Uses a Novel Mechanism Coordinating Programmed Cell Death and Secondary Cell Wall Synthesis. Plant Physiolooy, 119: 375-384

Khanne-Chopra R (2012) Leaf senescence and abiotic stresses share reactive oxygen species-mediated chloroplast degradation. Protoplasma, 249(3): 469-481

Koch GW, Sillett SC, Jennings GM, Davis SD (2004) The limits to tree height. Nature, 428: 851-854

Kropp A, Hofmann B, Schaffer C, Stitt M (1993) Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism of the sink regulation of photosynthesis. The Plant Journal, 3(6):817 - 828

Kumaran A, Karunakaran RJ (2006) Antioxidant and free radical scavenging activity of an aqueous extract of Coleus aromaticus. Food Chemistry, 97: 109-114

Kusunoki M (2011) S1-state Mn4Ca complex of Photosystem II exists in equilibrium between the two most-stable isomeric substrates: XRD and EXAFS evidence. Journal of photochemistry and photobiology, B, Biology, 104(1-2):100-110.

Louis J, Meyer S, Maunoury-Danger F, Fresseau C, Meudec E, Cerovic ZG (2009) Seasonally changes in optically assessed epidermal phenolic compounds and chlorophyll contents in leaves of sessile oak (Quercus petraea): towards signatures of phenological stage. Functional Plant Biology, 36: 732-741

Lutts S, Kinet JM, Bouharmon J (1996) NaCl-Induced senescence in leaves of rice (Oryza sativa L.) cultivars differing in salinity resistance. Annals of Botany, 78: 389-398

Mackensen, J. Ruhiyat, D. & Fölster, H. (2001) Volume-based nutrient content of Acacia mangium, Eucalyptus deglupta and Paraserianthes falcatoria in industrial tree plantations in East Kalimantan, Indonesia. Journal of Tropical Forest Science, 512-526.

Marschner H (1995) Mineral Nutrition of Higher Plants. 2nd ed. London, Academic Press

Martinez-Finley EJ, Gavin CE, Aschne M, Gunter TE (2013) Manganese neurotoxicity and the role of reactiveoxygen species. Free Radical Biology, 62: 65-75

Muradoglu F, Balta F (2010) Some physical and chemical characteristics of promising walnuts (Juglans regia L.) genotypes selected from Ahlat (Bitlis)Yuzuncu yil University Journal of Agriculture Sciences, 20(1):41-45 (in Turkish)

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-spesific peroxidase in spinach chloroplasts. Plant Cell and Environment, 22(5): 867-880

Paul MJ, Driscoll SP (1997) Sugar repression of photosynthesis: therole of carbohydrates in signalling nitrogen deficiency through source:sink imbalance. Plant, Cell Environment, 20: 110-116

Pearson D, Melon H, Ronald S (1976) Chemical analysis of Food, 8th edition. Churchill Livingstone. pp: 5-63

Polat M, Okatan V, Güclü F (2015) Determination of some physical and chemical properties of walnut (Juglans regia L.) genotypes grown in the central district of Bitlis/Turkey. - Scientific Papers. Series B. Horticulture, 59: 81-86

Ros Barceló A (1998) Hydrogen peroxide production is a general property of the lignifying xylem from vascular plants. Annals of Botany 82(1): 97-103

Variation in chemical compounds of walnut (Juglans regia L.) leaves with tree age
Schafer C, Simper H, Hofman B (1992) Glucose feeding results in coordinated changes of chlorophyll content, ribulose 1,5-bisphosphate carboxylase/oxygenase activity and photosynthetic potential in photoautotrophic suspension-cultured cells of Chenopodium rubrum. Plant Cell Environment, 15: 343-350
Schaffer AA, Liu KC, Goldschmidt EE, Boyer CD, Goren R (1986) Citrus leaf chlorosis induced by sink removal: starch, nitrogen, and chloroplast ultrastructure. Journal of Plant Physiology, 124(1-2):111-121
Scholes GD, Fleming GR (2005) Energy Transfer and Photosynthetic Light Harvesting. Advances in Chemical Physics, 13: 57-129
Singleton VL, Orthofer R, Lamuela-Raventós RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology, 299: 152-178
Sofo A, Dichio B, Xiloyannis C, Masia A (2004) Effects of different irradiance levels on some antioxidant enzymes and on malondialdehyde content during rewatering in olive tree. Plant Science, 166: 293-302
Spiteller G (2001) Lipid peroxidation in aging and age dependent disease. Experimental Gerontology, 36: 1425-1456
Szabados L, Savoure A (2009) Proline: a multifunctional amino acid. Trends Plant Sci. 2: 89-97
Talon M, Iglesias DJ, Lliso I, Tadeo FR (2002) Regulation of photosynthesis through source: sink imbalance in citrus is mediated by carbohydrate content in leaves. Physiologia Plantarum, 116: 563-572
Thomas H (2013) Senescence, ageing and death of the whole plant. New Phytologist, 197: 696-711
Turfan N, Savaci G, Sarıyıldız T (2016) Uludağ Göknarı ve Sarıçam İbrelerinin Bazı Kimyasal Bileşiklerinin Meşcere Yaşına ve Bazı Toprak Özelliklerine Bağlı Olarak Değişimi. Kastamonu Üni. Orman Fakültesi Dergisi, 16(2): 583-598
Velikova V, Yordanov I, Edrava A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Plant Science, 151: 59-66
Weinstein LH, Robbins WR (1955) The effect of different iron and manganese nutrient levels on the catalase and cytochrome oxidase activities of green and albino sunflower leaf tissues. Plant Physiology, 30: 27-32
Willis RJ (2000) Juglans spp. juglone and allelopathy. Allelopathy Journal, 7: 1-55
Witham FH, Blaydes DF, Devlin RM (1971) Experiments in plant physiology. pp 55-56. Van Nostrand Reinhold Company, New York
Variation in chemical compounds of walnut (Juglans regia L.) leaves with tree age

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   | 20   | 21   | 22   | 23   | 24   | 25   | 26   |
|---------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Fe      | 0.974| -0.534| -0.529| -0.600| -0.410| 0.512| -0.581| 0.900| -0.169| -0.829| -0.827| -0.872| 0.405| -0.508| 0.345| 0.341| -0.112| 0.114| -0.019| 0.455| -0.795| 0.609| 0.534| 0.940| -0.563| -0.476| 0.010| 0.418| 0.167| 0.867| 0.883|
|      2  | 0.676| -0.979| -0.979| -0.939| -0.699| 0.329| 0.563| 0.970| -0.312| -0.720| 0.841| -0.109| 0.111| 0.015| 0.459| 0.797| 0.612| -0.531| -1.000| -0.882| 0.309| 0.841| 0.876| -0.877| -0.812| -0.460| -0.037| -0.296| 0.999| -0.578| -0.892| -0.956| 0.575|
|      3  | 0.822| -0.103| 0.105| -0.018| -0.458| -0.797| -0.613| 0.525| 0.996| 0.876| 0.303| -0.996| -0.569| 0.738| 0.984| 0.926| 0.414| 0.410| -0.334| -0.117| -0.526| -0.300| 0.742| -0.898| 0.935| 0.575| 0.897| -0.772| 0.449| 0.934| 0.880|
|      4  | 0.926| 0.414| 0.410| -0.334| -0.117| -0.526| -0.300| 0.742| -0.898| 0.935| 0.575| 0.897| -0.772| 0.449| 0.934| 0.880| 0.540| -0.001| -1.000| -0.976| -0.820| -0.500| -0.708| -0.907| 0.128| -0.577| -0.982| -0.125| -0.884| 0.578| 0.274| -0.120| 0.423|
|      5  | -0.741| -0.952| -0.904| -0.622| -0.229| -0.475| -0.990| -0.409| -0.788| -0.995| -0.406| 0.981| 0.317| 0.540| 0.400| 0.651| 0.957| -0.934| -0.405| -0.407| 0.312| 0.169| 0.576| 0.343| 0.763| -0.954| -0.983| -0.583| 0.953| -0.796| -0.498| 0.988| -0.948| 0.950| -0.120| 0.664|
|      6  | -0.620| -0.267| -0.265| -0.351| -0.754| 0.964| 0.861| -0.180| -0.927| -0.645| -0.066| 0.929| -0.231| -0.935| 0.862| 0.927| 0.717| 0.252| 0.038| -0.773| 0.942| -0.644| -0.646| -0.560| -0.112| -0.325| -0.067| -0.932| -0.994| -0.787| -0.830| -0.933| -0.226| -0.905| -0.825| -0.795| 0.919| 0.657| 0.846| 0.960| 0.565|
|      7  | -0.897| -0.253| -0.255| -0.158| -0.324| -0.700| -0.490| -0.649| -0.990| -0.941| -0.444| -0.889| -0.688| 0.631| -1.000| 0.985| 0.935| -0.268| -0.535| -0.987| -0.865| -0.903| -0.083| -0.778| -0.777| -0.818| -0.991| -0.939| -0.996| -0.427| -0.536| -0.079| -0.634| -0.539| -0.379| -0.966| -0.405| -0.542| -0.218| -0.768| -0.551| -0.258| 0.813| -0.022| -0.411|
|      8  | -0.939| -0.664| -0.666| -0.581| -0.138| 0.300| -0.041| -0.924| -0.817| -0.991| -0.802| 0.815| -0.942| -0.211| 0.894| -0.809| 0.913| -0.676| 0.860| 0.953| -0.543| 1.000| -0.891| -0.048| -0.925| -0.725| -0.726| -0.646| -0.221| -0.218| -0.044| -0.953| -0.766| 0.977| -0.850| -0.763| -0.967| -0.127| 0.853| -0.757| -0.888| -0.736| -0.900| -0.923| -0.470| -0.994| -0.849| -0.132| -0.996|
|      9  | -0.937| -0.672| -0.674| -0.580| -0.149| 0.289| 0.029| -0.928| -0.811| -0.990| -0.809| -0.809| -0.946| -0.200| 0.889| -0.803| -0.910| -0.685| 0.866| 0.949| 0.533| 0.999| -0.886| -0.059| 1.000| -0.997| -0.937| -0.672| -0.674| -0.580| -0.149| 0.289| 0.029| -0.928| -0.811| -0.990| -0.809| -0.809| -0.946| -0.200| 0.889| -0.803| -0.910| -0.685| 0.866| 0.949| 0.533| 0.999| -0.886| -0.059| 1.000| -0.997|

* p<0.05; ** p<0.01; *** p<0.001
1-Chla; 2-Ch b; 3-Chl a:Ch l b; 4-Total Chl; 5-Total Carotenoid; 6-Total phenolic; 7-Flavonoid; 8-Glucose; 9-Sucrose; 10-Total soluble carbohydrate; 11-Starch; 12-Proline; 13-Protein; 14-MDA; 15-H2O2; 16-APX; 17-CAT; 18-SOD; 19-P; 20-K; 21-Ca; 22-Mg; 23-S; 24-Fe; 25-Mn; 26-Si; 27-Zn