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Effect of tree age on chemical compounds of ancient Anatolian black pine (*Pinus nigra* subsp. *pallasiana*) needles in Northwest Turkey

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Plant primary and secondary metabolites are chemical compounds synthesized for essential functions, such as growth and development (primary metabolites), and specific functions, such as pollinator attraction or defense against herbivory (secondary metabolites). Their concentrations in plants are genetically determined, but are also affected by environmental factors. Among these factors, plant age has been reported to influence plant chemical compounds under similar environmental conditions. We aimed to investigate the chemical compounds of ancient Anatolian black pine (*Pinus nigra* subsp. *pallasiana*) needles from trees of different ages. Needles of over 500-, 200-, 100-, 50-, and 25-year-old black pine trees growing under similar environmental conditions were sampled and analyzed for photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoids), proline, total soluble protein, glucose, sucrose, total soluble sugar, peroxidation level (MDA-malondialdehyde), hydrogen peroxide (H_2O_2) and antioxidants such as ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) activities. Significant differences for chemical composition associated with age were found. In general, results showed that over 500-year-old Anatolian black pine had the highest proline, total soluble protein, H_2O_2, sucrose, total soluble carbohydrates, APX, CAT and SOD concentrations, whereas they had the lowest chlorophyll *a*, total chlorophyll, total carotenoid and glucose concentrations. However, 200-year-old trees had the highest glucose, but the lowest chlorophyll *b*, proline, H_2O_2 and total soluble carbohydrates. 50- and 25-year-old trees together showed the highest chlorophyll *a* and *b*, total chlorophyll, total carotenoid and MDA, but lowest total soluble protein and sucrose. In conclusion, these results provide valuable insight into the chemical composition of Anatolian black pine needles in relation to their age, and can be used for complementing studies on tree growth-defence relationships.

Keywords: Ancient Trees, Anatolian Black Pine, Chemical Composition, Turkey

Introduction

Plant primary metabolites are essential for growth and development of cells and tissues, while plant secondary metabolites have many ecological roles, influencing decomposition, flammability and herbivory. Changes in physiological responses and environmental factors affect primary metabolism, such as nitrogen uptake, protein, proline, and carbohydrate contents, antioxidant activity, photosynthetic rate and tree lifespan (Bond et al. 2007). Growth and development in trees occur under the joint influence of environmental conditions and anthropic impacts (Koch et al. 2004, Peichl & Arain 2006).

Tree growth accelerates as canopies develop in young forests and declines substantially soon after the maximum leaf area is attained. The causes of this decline are multiple and may be linked to age- and/or size-related processes. Three stages can be identified in the growth of trees over time. During the formation phase (*i.e.*, before canopy closure) high-rate metabolic synthesis leads to a rapid growth, which is due to the continuous growth and division of meristematic cells, and requires significant amounts of energy (Köstner et al. 2002). In the second phase (maturity), leaf metabolic activity continues but wood anatomy and chemistry start to change, and cambial activity slow downs (Greenwood et al. 2010). In the last phase, leaf area and production are reduced, while branch losses are accelerated, plant injuries are more frequent, as well as attack of pathogens or pests (Gower et al. 1996, Sariyildiz & Anderson 2006).

Anatolian black pine (*Pinus nigra* subsp. *pallasiana*) is the second most widely distributed forest tree species in Turkey. The species covers, either as pure or mixed stands, approximately 4.2 million ha of 21.2 million ha total forest land in Turkey. Climatically, it grows in transitional regions between maritime and continental climates, extending as far as the inner Anatolian steppe (Atalay & Efe 2012). Due to its economic importance in Turkey, the ecology and silviculture of Anatolian black pine have been extensively studied. Neverthe-

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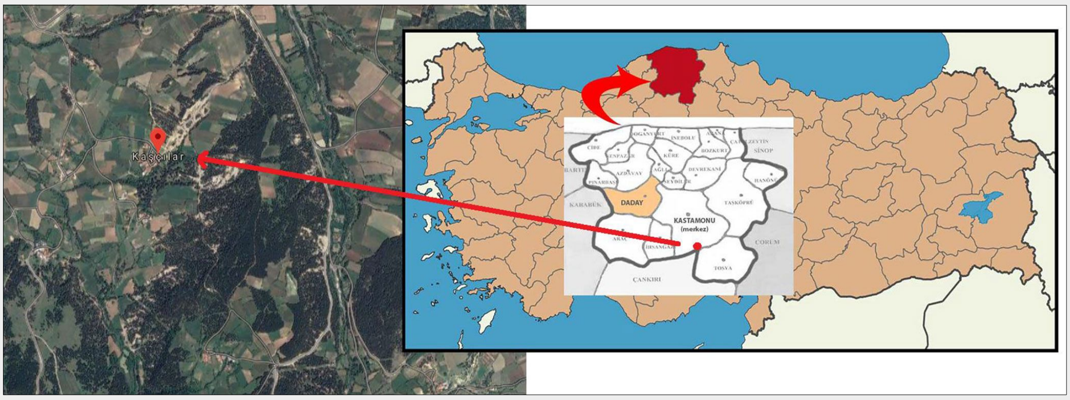

Fig. 1 - Location of the study area (near the Kasçilar village, Kastamonu province, Northern Turkey).

less, no studies have investigated how stand characteristics and tree age influence the chemical composition of Anatolian black pine needles. The chemical composition of plant litter is indicative of its quality as a resource for decomposer organisms. Litter quality has been shown to be a major determinant of litter decomposition rates both within and across terrestrial ecosystems. To better understand litter decomposition dynamics, nutrient cycling, soil organic matter dynamics, and C storage and release in Anatolian black pine forests, factors influencing foliage and litter chemistry of Anatolian black pine needles warrant deep investigation.

We aimed at investigating the chemical composition of Anatolian black pine needles of different ages. Needles of >500-, 200-, 100-, 50-, and 25-year-old black pine trees growing under similar environmental conditions were sampled and analyzed for their contents in photosynthetic pigments, proline, soluble proteins, amino acids, glucose, sucrose, soluble sugar, as well as levels of peroxidation (MDA-malondialdehyde), H_2O_2 and antioxidants (ascorbate peroxidase, APX, catalase, CAT, and superoxide dismutase, SOD).

Materials and methods

Study area and tree sampling

This study was carried out near the Kasçilar village, 18 km from Kastamonu province, Northwest Turkey (41° 11′ 38″ N, 33° 53′ 07″ E, elevation 1350 m a.s.l., NW aspect – Fig. 1). The climate in the area is continental, with long, cold and snowy winters and short and warm summers. The seasonal and daily temperatures show extreme values and precipitation is generally low (Duran 2017). Meteorological data over the period 1975-2016 (Kastamonu Meteorological Station, 800 m a.s.l.) indicate that average annual precipitation is 490 mm, while average monthly temperatures range from -0.8 °C in January to 20.2 °C in July.

Pure Anatolian black forest stands of different age classes grow on a fertile and deep soil. The humus type is moder with moderate litter decomposition rates. The selected Anatolian black pine trees varied in age from 25 to over 500 years and were clearly grouped into five age classes (Tab. 1). All tree age classes were examined within 400 m^2 (20 × 20 m) plot. Canopy cover was rather open for older pine trees over 500-year old (about 0.5) and closer for younger pine trees (about 0.7-0.8). Measurements of diameter at breast height (DBH) and height were made on three sample trees for each tree class in summer 2017. DBH was measured using a diameter tape. Tree age was determined by dendrochronological approach, coring trees at breast height. Tree heights were measured with a Blume-Leiss clinometer.

Fresh needles from the lower parts of the three sample trees for each age class were collected and placed in bags. These samples were then combined to form a mixed sample for each age class and analyzed for photosynthetic pigment amounts (chlorophyll *a*, chlorophyll *b* and carotenoids), proline, protein, lipid peroxidation and hydrogen peroxide (H_2O_2), glucose, sucrose and total soluble carbohydrates, and ascorbate peroxidase, catalase, and superoxide dismutase enzyme activities.

Tab. 1 - Mean characteristics of Anatolian black pine (*Pinus nigra*) donor trees used in this study. Mean attributes are shown for each tree age class. DBH is diameter at breast height.

Class	No. of Trees	Age at 1.3 m (year)	DBH (cm)	Height (m)
1	3	over 500	320	28
2	3	200	234	27
3	3	100	157	32
4	3	50	78	25
5	3	25	34	21

Chemical analyses of needle samples

For the analysis of photosynthetic pigments, 500 mg of leaf samples were homogenized with 10 ml of 80% acetone and centrifuged at 3000 rpm for 15 minutes. The extract was utilized for chlorophyll estimation (Arnon 1949). Carotenoid amount was estimated using the Jaspars' formula according to the method of Witham et al. (1971). Proline content was determined according to the modified method of Bates et al. (1973). Total soluble protein contents were analyzed according to the method of Bradford (1976) using the Bio-Rad® assay kit with bovine serum albumin as a calibration standard. The level of lipid peroxidation products was determined using the thiobarbituric method and expressed as nmol of MDA-malondialdehyde formed using an extinction coefficient of 155 mM^{-1} cm^{-1} as μmol MDA-malondialdehyde, according to Lutts et al. (1996). Hydrogen peroxide was determined following Velikova et al. (2000). Soluble sugars were assayed according to Pearson's method (Pearson et al. 1976). Antioxidants were determined by using dry needle samples (500 mg), which were ground in powder using liquid nitrogen. The powder was homogenized in 5 mL phosphate potassium (pH 7.6) with 0.1 mM of EDTA. The homogenate was then centrifuged to 15,000×g for 20 min at 4 °C. The supernatant was kept, and 0.8 ml phosphate potassium 0.2 M was added. The homogenate was centrifuged again to 15,000 ×g for 15 min. The combined supernatants were stored on ice and used in order to determine the activity of detoxifying enzymes. The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT (nitroblue tetrazolium), following Cakmak (1994). One unit of SOD was defined as the amount of enzyme necessary to cause 50% inhibition of the rate of NBT reduction at 560 nm. The activity of CAT was determined according to Bergmeyer & Grabl (1983), the decomposition of H_2O_2 was measured by the decline in absorbance at 240 nm. APX was assayed by recording the decrease in absorbance at 290 nm due to the decrease in ascorbic acid content (Nakano & Asada 1981). APX and CAT were expressed per mg protein, and one unit represented 1 μmol of substrate undergoing reaction per mg protein per min.

Statistical analysis

Analysis of variance (ANOVA) was applied for analyzing the differences in the chemical composition of Anatolian black pine needles between five tree age classes using the SPSS program ver. 11.0 for Windows. Following the results of ANOVAs, Tukey's honestly significance difference (HSD) test ($\alpha = 0.05$) was used for testing differences between group means.

Results

Variation in photosynthetic pigments

Mean concentrations of photosynthetic pigments (chlorophyll-*a*, -*b*, total chlorophyll and carotenoids) in needle samples of different age classes are shown in Tab. 2. Concentrations varied significantly among age classes ($p<0.001$). In general, photosynthetic pigments showed a decrease with tree age, being higher in young (25- and 50-year-old) than in old (100-, 200- and over 500-year-old) Anatolian black pine trees (Tab. 2). Mean chlorophyll-*a* concentration was the highest in 50-year-old trees (0.159 mg g^{-1}), followed by 25-year-old (0.146 mg g^{-1}), 100-year-old (0.139 mg g^{-1}), 200-year-old (0.127 mg g^{-1}) and over 500-year-old trees (0.124 mg g^{-1}). Mean chlorophyll-*b* concentration was also the highest in 50-year-old trees, followed by 25-, 200-, over 500-year-old trees, but the lowest concentration was found in 100-year-old trees (Tab. 2). Mean total carotenoid concentration was similar for 25-, 50- and 200-year-old trees (approximately 11.00 mg g^{-1}) but higher than for 100- and over 500-year-old trees, which had a similar concentration (approximately 7.55 mg g^{-1}). Chlorophyll *a*/*b* ratio was the highest in 100-year-old trees (4.89), followed by 200- (4.05), over 500- (3.88), 25- (3.19), and 50-year-old trees (2.84).

Variation in proline, total soluble protein, MDA and H_2O_2

Mean concentrations of proline, total soluble protein, MDA-malondialdehyde and H_2O_2 in Anatolian black pine needles are given in Tab. 3. They all varied significantly among age classes ($p<0.001$). Mean proline, total soluble protein and H_2O_2 concentrations were the highest in over 500-year-old trees (73.46 mg g^{-1}, 28.34 mg g^{-1} and 150.2 μmol, respectively), whereas mean proline and H_2O_2 concentrations were the lowest in 200-year-old trees (51.61 μmol and 100.47 μmol) and mean total soluble protein was the lowest in 25-year-old trees (11.44 mg g^{-1} – Tab. 3). Mean MDA-malondialdehyde concentration was also the lowest in 25-year-old trees (25.97 μmol) but the highest in 50-year-old trees (44.05 μmol).

Variation in glucose, sucrose and total soluble carbohydrates

Mean concentrations of glucose, sucrose

Tab. 2 - Mean concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids, and ratio of chlorophyll *a*/*b* in Anatolian black pine needles of different ages. Different letters in rows indicate significant differences between means ($p<0.001$) after Tukey's HSD test.

Component	Mean Tree Age (years)					F	Prob
	>500	200	100	50	25		
Chlorophyll *a* (mg g^{-1})	0.124 ± 0.001 ^{a}	0.127 ± 0.001 ^{a}	0.139 ± 0.001 ^{b}	0.159 ± 0.001 ^{d}	0.146 ± 0.002 ^{c}	344.1	<0.001
Chlorophyll *b* (mg g^{-1})	0.0319 ± 0.0003 ^{b}	0.0314 ± 0.0008 ^{b}	0.0283 ± 0.0006 ^{a}	0.0560 ± 0.0004 ^{d}	0.0458 ± 0.0004 ^{c}	541.1	<0.001
Total Chlorophyll (mg g^{-1})	0.156 ± 0.001 ^{a}	0.159 ± 0.001 ^{a}	0.167 ± 0.001 ^{b}	0.215 ± 0.001 ^{d}	0.192 ± 0.002 ^{c}	647.36	<0.001
Total Carotenoid (mg g^{-1})	7.56 ± 0.06 ^{a}	10.91 ± 0.07 ^{b}	7.62 ± 0.02 ^{a}	11.02 ± 0.13 ^{b}	11.15 ± 0.10 ^{b}	510.87	<0.001
Chlorophyll *a*/*b* ratio	3.88 ± 0.04 ^{c}	4.05 ± 0.11 ^{c}	4.89 ± 0.09 ^{d}	2.84 ± 0.02 ^{a}	3.19 ± 0.04 ^{b}	149.78	<0.001

Tab. 3 - Mean concentrations of proline, total soluble protein, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in Anatolian black pine needles of different ages. Different letters in rows indicate significant differences between means ($p<0.001$) after Tukey's HSD test.

Component	Mean Tree Age (years)					F	Prob.
	>500	200	100	50	25		
Proline (mg g^{-1})	73.46 ± 0.16 ^{d}	51.61 ± 0.19 ^{a}	66.70 ± 0.04 ^{c}	67.58 ± 0.08 ^{c}	60.68 ± 0.09 ^{b}	4637.91	<0.001
Total Soluble Protein (mg g^{-1})	28.34 ± 0.24 ^{e}	17.61 ± 0.19 ^{c}	16.51 ± 0.22 ^{b}	19.57 ± 0.16 ^{d}	11.44 ± 0.10 ^{a}	1101.98	<0.001
MDA (μmol g^{-1})	34.82 ± 0.02 ^{c}	29.32 ± 0.02 ^{b}	39.95 ± 0.03 ^{d}	44.05 ± 0.02 ^{e}	25.97 ± 0.01 ^{a}	173879.86	<0.001
H_2O_2 (μmol g^{-1})	150.20 ± 0.05 ^{e}	100.47 ± 0.24 ^{a}	139.21 ± 0.14 ^{c}	140.32 ± 0.07 ^{d}	123.40 ± 0.08 ^{b}	21664.53	<0.001

Tab. 4 - Concentrations of glucose, sucrose and total soluble carbohydrate in Black pine needles of different ages. Different letters in rows indicate significant differences between means ($p<0.001$) after Tukey's HSD test.

Component	Mean Tree Age (years)					F	Prob.
	>500	200	100	50	25		
Glucose (mg g^{-1})	32.30 ± 0.02 ^{a}	47.32 ± 0.02 ^{e}	42.44 ± 0.03 ^{b}	45.31 ± 0.03 ^{d}	45.12 ± 0.03 ^{c}	57926.4	<0.001
Sucrose (mg g^{-1})	106.83 ± 0.07 ^{e}	102.86 ± 0.03 ^{d}	104.28 ± 0.12 ^{c}	100.34 ± 0.07 ^{b}	97.43 ± 0.05 ^{a}	2658.83	<0.001
Total Soluble Carbohydrate (mg g^{-1})	22.94 ± 0.01 ^{e}	22.45 ± 0.02 ^{a}	22.80 ± 0.01 ^{d}	22.56 ± 0.01 ^{b}	22.68 ± 0.01 ^{c}	442.32	<0.001

Tab. 5 - Concentrations of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) in Black pine needles of different ages. Different letters in rows indicate significant differences between means ($p<0.001$) after Tukey's HSD test.

Component	Mean Tree Age (years)					F	Prob.
	>500	200	100	50	25		
APX (EU mg^{-1} Protein)	0.315 ± 0.003^{d}	0.213 ± 0.003^{b}	0.239 ± 0.002^{c}	0.241 ± 0.002^{c}	0.205 ± 0.002^{a}	411.87	<0.001
CAT (EU mg^{-1} Protein)	0.521 ± 0.001^{e}	0.505 ± 0.002^{d}	0.430 ± 0.001^{c}	0.242 ± 0.121^{a}	0.310 ± 0.001^{b}	5.14	<0.001

and total soluble carbohydrates in Anatolian black pine needles are given in Tab. 4. They all varied significantly among age classes ($p<0.001$). Needles of over 500-year-old trees had the highest mean sucrose (106.8 mg g^{-1}) and total soluble carbohydrate concentrations (22.94 mg g^{-1}), whereas the lowest mean glucose (32.3 mg g^{-1}). Needles of 200-year-old trees, however, had the highest mean glucose concentration (47.3 mg g^{-1}), and the lowest mean total soluble carbohydrate concentrations (22.45 mg g^{-1}). Needles of 25-year old trees had the lowest mean sucrose concentration (97.4 mg g^{-1} – Tab. 4).

Variation in antioxidant enzyme activity

Mean concentrations of APX, CAT and SOD in Anatolian black pine needles are given in Tab. 5. They all varied significantly between age classes ($p<0.001$). Needles of over 500-year-old trees had the highest mean APX (0.315 EU) and SOD (24.6 EU) concentrations, whereas those of 25-year-old trees the lowest (0.205 EU and 11.3 EU). Needles of over 500-year-old trees had the highest CAT concentration (0.521 EU), the lowest being found for 50-year-old trees (0.242 EU – Tab. 5).

Discussion

Effects of tree age on photosynthetic pigment concentration

The photosynthetic pigments play a role in capturing sunlight and converting it into chemical energy (Ito et al. 1994, Mirkovic et al. 2017). In this study, chlorophyll *a*, chlorophyll *b* and total chlorophyll concentrations were generally lower in older Anatolian black pine trees (over 500-, 200- and 100-year-old trees) compared to younger trees (50- and 25-year-old trees – Tab. 2). Chlorophyll *a*/*b* ratio was, however, higher in the needle samples from 100- and 200-year-old trees, as compared with younger trees (Tab. 2). Lower total carotenoid concentration was found in the needles from over 500- and 100-year-old trees, in comparison with 25- and 50-year-old trees (Tab. 2). We hypothesize that the variation in tree canopy height with age could determine such differences. Although we did not measure light conditions in the study area, 100-year-old trees were growing in rather open canopy conditions, in comparison with 50- and 25-year-old trees, while 200- and over 500-year-old trees were growing in intermediate conditions. Similar results were also reported by Ito et al. (1994) and Ohtsuka et al. (1997), who showed that chlorophyll *b* increased under low light conditions, whereas the amount of chlorophyll *a* and the ratio chlorophyll *a*/*b* decreased. The average value of chlorophyll *a*/*b* ratio in 200-year-old trees was thought to be due to low concentrations of MDA-malondialdehyde and H_2O_2. The reason for the presence of highest amount of chlorophyll *a* in this tree age group could be related to adaptation mechanisms for ensuring photoassimilate source/pool ratio. High chlorophyll amounts were found linked with photosynthetic electron transfer, thereby contributing to an increase in the synthesis of ATP, NADPH+ and photoassimilate products (Mirkovic et al. 2017). Low chlorophyll *a*/*b* ratios in 25- and 50-year-old trees were probably associated with low light conditions.

Effects of tree age on proline, total soluble protein, MDA and H_2O_2 concentrations

Proline and total soluble protein contents were lower in younger Anatolian black pine trees. Protein concentration was the lowest in 25-year-old trees, whereas the highest in over 500-year-old trees (Tab. 3). Nitrogen compounds are utilized as nitrogen, carbon and energy source in growth and development (Paungfoo-Lonhienne et al. 2008). Proline and soluble proteins are involved in cell wall relaxation, turgor and osmotic potential (Heuer 1999), activities that can be affected by tree size/age. Concentrations of H_2O_2, proteins, and carbohydrates in older Anatolian black pine tree support this hypothesis. Proline concentration for 100-year-old trees was probably associated with high irradiance. Nitrogenous compounds in 50- and 25-year-old trees were related to low source/pool photoassimilate ratio, these trees being in the juvenile phase.

Cellular components, which undergo the first change during the cell cycle, are plasma and organelle membranes, and cell walls (Spiteller 2003). The concentration of MDA-malondialdehyde was affected by tissue differentiations in younger trees, while MDA-malondialdehyde was affected by tissue deformation in older trees (Renaud & Mauffette 1991). Changes in structural cellular membrane were found to affect MDA-malondialdehyde amounts during tissue ontogenesis and stimulate accumulation of H_2O_2 (Ros Barceló 1998, Spiteller 2003).

H_2O_2 concentration was higher for over 500-, 50- and 100-year-old trees than for 200- and 25-year-old trees (Tab. 3). H_2O_2 concentration varied according to tissue deformation in older trees and tissue differentiation in young trees (Ros Barceló 1998). The lowest level of H_2O_2 for 200-year-old trees could be attributed to their transitional growth phase. The concentration of H_2O_2 in 50- and 100-year-old trees was associated with tree growth and increased branching. H_2O_2 concentration in 25-year-old trees was linked with the juvenile phase.

Effects of tree age on glucose, sucrose and total soluble carbohydrates concentrations

Assimilates, such as glucose, fructose and sucrose, are directly metabolized and used as a carbon and energy source (Peichl & Arain 2006). Glucose concentration was the highest in 200-year-old trees, while sucrose and total soluble carbohydrate concentrations were maximum in over 500-year-old trees. In contrast, glucose was the lowest in over 500-year-old trees, and sucrose and total soluble carbohydrate concentrations were minimum in 25-year-old trees (Tab. 4). Glucose concentration varied according to metabolic needs and size of Anatolian black pine trees. Tree height and sucrose concentration in older plants have been related to hydraulic conductivity (Ritchie & Keeley 1994, Koch et al. 2004). Low glucose and high proline, protein, sucrose and total soluble sugar contents in over 500-year-old trees suggest that glucose was metabolized to sucrose and used for increasing osmotic potential. Chlorophyll *a*/*b*, total soluble carbohydrate and glucose contents in 50- and 25-year-old trees were probably associated to low-light conditions (Valladares & Niinemets 2008).

Effects of tree age on antioxidant enzyme activity

Antioxidant enzymes protect the structure of membranes, proteins, enzymes and other molecules from ROS derivative damage (Cakmak 1994, Teeri & Simola 2002). Morphological and physiological changes in plant organs/tissues affect antioxidant activities (Ray et al. 2012). APX was maximum in over 500- and 50-year-old trees, while CAT and SOD activities were maximum in over 500- and 200-year-old trees. The highest enzyme activity found in these trees could be associated with catabolic reactions for tissue deformation and senescence. It has been reported that leaf area becomes smaller in aging coniferous trees, while lignin content increases (Teeri & Simola 2002). In 100-year-old trees, the low carotenoid and protein content, and high MDA-malondialdehyde and H_2O_2, might affect enzyme activity. Lignin deposition might also affect enzyme activity in 50-year-old trees (Rossi et al. 2008). The lowest enzyme activity noted in 25-year old trees could be related to their juvenile phase (Niinemets 2002).

Conclusions

Our results showed that under similar environmental conditions, the chemical compounds synthesized in Anatolian black pine needles are significantly influenced by tree age. In general, photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid concentrations) and glucose concentrations in the needles from 25-year-old trees were higher than those for needles from the over 500-year-old trees, whereas proline, total soluble protein, MDA-malondialdehyde, H_2O_2, sucrose, and antioxidant (APX, CAT, SOD) concentrations were significantly higher in needles from over 500-year-old trees. All those chemical compounds in plant needles are related to plant defence against attacks by herbivores and litter decomposition. A more detailed understanding of primary and secondary metabolism in Anatolian black pines and variation in primary and secondary metabolites within and among plants and with time are required, and such analyses will be useful for complementing studies on tree growth-defence relationships.

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