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SEASONAL CHANGES OF ANTIOXIDATIVE ENZYMES IN LEAVES OF *QUERCUS CERRIS* L. AND *QUERCUS FRAINETTO* TEN. FORESTS

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Abstract

Effects of seasonal changes on the activity of foliar antioxidative enzymes - superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), in uneven aged field-grown oak trees (*Quercus cerris* L. and *Quercus frainetto* Ten.) were studied. The unfavourable conditions during summer lead to an increasing activity of the enzymes in the leaves of both oak species, alleviating the oxidative stress. Seasonal changes in enzyme activity show ambiguous tendencies between the different studied age groups. Specific variations of both oak species' adaptive capacities to environmental changes were observed as higher activity of the antioxidative enzymes in the leaves of all age groups of *Q. cerris* may be instrumental in more effective protection against oxidative stress. Furthermore, a better stress adaptability of 15-year-old *Q. Cerris* trees can be inferred from the fact that the found interspecific differences are most pronounced for the youngest individuals.

Key words: superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), *Quercus cerris* L., *Quercus frainetto* Ten.

INTRODUCTION

Environmental stress causes an increased formation of reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$). All these forms of oxygen are far more reactive than ground state oxygen. They can undergo complex reactions with each other and with different cellular components (Smirnoff, 1993). Their presence leads to oxidative damage in cellular molecules, such as nucleic acids, proteins and lipids. The cells of all plant organisms have multiple means to remove these activated oxygen species. In the chloroplast and in the cytosol, antioxidative substrates and enzymes interact in a series of reactions and as a result the toxic oxidants are removed and the reduced (i.e., functional) state of antioxidants recovered (Mittler, 2002). $O_2^{\cdot -}$ is disproportionated by superoxide dismutase (SOD) to H_2O_2 and H_2O. H_2O_2 is a potent inhibitor of photosynthesis and can also react with ketoacids. However, most of the toxicity of H_2O_2 is exerted through the hydroxyl radicals ($OH^{\cdot}$) it creates. $OH^{\cdot}$ is generated from H_2O_2 through a metal-

catalyzed reaction, involving mostly Fe(II) or Cu(I). The hydroxyl radical is one of the most reactive molecules known in chemistry (Foyer, 1994). The cellular mechanisms to minimize $OH^{\cdot}$ in glyoxysomes and peroxisomes involves the detoxification of H_2O_2 to H_2O by catalase (CAT), while in chloroplasts it is converted into H_2O by ascorbate peroxidase (APX) in the ascorbate-glutathione cycle (Willekens at al., 1995). This cycle maintains the level of stromal H_2O_2 low enough to prevent thiol-containing enzymes from oxidation. It involves several enzymes such as APX, monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Ascorbate and glutathione function as oxireductants, H_2O_2 as an electron acceptor, and NADPH as an H donor, which is strictly compartmentalized and act in a highly coordinated manner. The ascorbate-glutathione cycle is involved in the full scavenging of H_2O_2 without producing another ROS, and in the dissipation of excess excitation energy in the form of heat (Edreva, 2005).

The tree species must cope with large variations in environmental conditions in their life-span. The antioxidative system has been considered especially important for the acclimatization of wood plants (Polle, Rennenberg, 1994). Several references show an increasing accumulation of ROS under stress conditions leading to a rise in capacity of the antioxidative system. Esterbauer, Grill (1978) have proposed that significantly increased levels of GR during winter play an important role for the hardiness of leaves of evergreen plants. The composition and seasonal fluctuations of antioxidative system in beech leaves have been addressed in several investigations (Polle at al., 1992; Polle, Morawe 1995a, 1995b; Luwe 1996). As an example, the activity of enzyme SOD and GR in the leaves significantly increased in summer (August) as a response to drought stress in comparison to the activity in leaf buds during April (Polle at al., 1992; Polle, Morawe, 1995b). Contrarily, APX activity was higher in the buds than in mature leaves (Polle, Morawe, 1995b). Experiments on the effects of irrigation on a young olive showed that the scavenging functions of SOD were impaired with decreasing leaf water potential (Bacelar at al., 2007).

Investigations on antioxidative enzyme activity in leaves from genus *Quercus* are usually orientated towards the effects of enhanced CO_2 concentration alone or in combination with drought stress (Schwanz at al., 1996; Marabottini at al., 2001; Schwanz, Polle, 1998, 2001). There is currently no information on the seasonal dynamics of the main antioxidative enzymes in leaves of *Quercus cerris* and *Quercus frainetto*. The objective of the present study is therefore to test the hypothesis for both oak species that summer stress conditions lead to an increase in SOD, APX, CAT and GR in the leaves of uneven aged field-grown trees, which is traded off by a decrease in oxidative stress in the leaves. In addition, the question of whether tree age is a significant determinant for the effectiveness of the antioxidative defence system is examined. Furthermore, a comparison of the amount of antioxidative enzymes in the foliages of *Quercus cerris* and *Quercus frainetto* is made.

MATERIALS AND METHODS

Plant material

The study was conducted in Staro Oriahovo, Eastern Balkan in 2006. The plant material for the biochemical analyses was collected from the upper third of the crown of 15-, 35, 140-year-old oak trees of *Quercus cerris* and *Quercus frainetto*, respectively. The leaves were frozen in liquid nitrogen and then preserved and transported in dry ice to the laboratory for analysis.

Extraction and determination of enzyme activities

The extraction and determination of enzyme activities was performed according to Polle et al. (2001). Before working with the material, the leaves were stored at -50 °C. The plant material first was homogenized with 800 mg polyvinylpolypyrrolidone and an extraction buffer tris-HCl (pH 7.8) for determination SOD activity was used. APX, CAT and GR activity was determined by using potassium phosphate buffer (pH 7.0) The samples were centrifuged at 45 000 x g, 4 °C for 25 mins (Beckmann, model L5-50 ultracentrifuge, rotor SW 41). The extract was gel filtrated in Sephadex G 25 columns. Enzyme activities were determined spectrophotometrically (UV/VIS 1601, Shimadzu) at 25 °C according to the following protocols:

SOD (EC 1.15.1.1) activity was assayed by inhibition of photochemical reduction of nitroblue tetrazolium according to the method of Beyer, Fridovich (1987). The reaction mixture contained 50 mM phosphate buffer with pH 7.8, 0.053 mM NBT, 10 mM methionone, 0.0053 riboflavin and an appropriate aliquot of enzyme extract. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT as monitored at 560 nm;

APX (EC 1.11.1.11) activity was assayed by the method of Nakano, Asada, (1987) at the rate of ascorbat oxidation at 290 nm. The reaction mixture contained 50 mM Potassium phosphate buffer with pH 7.0, 0.5 mM ascorbate, 0.1 mM H_2O_2 and 50 µL enzyme extract, having a total volume of 3 mL;

CAT (EC 1.11.16) activity was assayed by depletion of H_2O_2 from a reaction mixture at 240 nm for 30 sec (Aeby, 1984). The reaction mixture contained 100 mM Potassium phosphate buffer with pH 7.0, 15 mM H_2O_2 and 50 µL enzyme extract, yielding a total volume of 3 mL;

GR (EC 1.6.4.2) activity was assayed at the rate of oxidation of HADPH at 340 nm for 1 minute (Foyer, Halliwell, 1976). The reaction mixture contained 100 mM Tris-HCl buffer with pH 7.8, 0.5 mM GSSG, 0.05 mM HADPH, 2 mM EDTA and 100 µL enzyme extract, having a total volume 2.5 mL.

Statistical analysis

The variation in biochemical variables resulting from the effects of age and season was analyzed by a three-way Analysis of Variance (ANOVA). The statistical results represent the mean values of at least 6 determinations with 3 trees from each age. When

significant, it was followed by Bonferroni's multiple range test (at $P<0.05$). The values accompanied by the same letter are not significantly different.

RESULTS

A significant influence of season on the activity of antioxidative enzymes was observed (Table 1). The highest activity of SOD and GR in all age groups and that of ascorbate peroxidase and catalase in 35- and 140-year-old individuals of both species was reached in summer (Fig. 1a-d). APX and CAT activity decreases in summer only in the leaves of 15-year-old trees (Fig. 1b, c). The activity of all investigated enzymes in the leaves of the two oak species decreased considerably in September (Table 1).

Tree age influenced the activity of SOD, APX, CAT and GR differently in the leaves of *Q. cerris* and *Q. frainetto*. The leaves of the youngest oak trees have the highest activity of APX and CAT. In contrast, the activity of SOD is the highest in the leaves of the oldest trees and GR activity remained unchanged in 15- and 140-year-old trees (Table 2).

Significant interspecific differences with respect to enzyme activities are shown in Table 3. The youngest individuals of *Q. cerris* show an activity of SOD and APX between 2 to 2.6 times higher compared to that in *Q. frainetto* during the whole vegetation period (Fig. 1a-b). The most significant interspecific differences for CAT activity are established again in the leaves of the 15-year-old individuals during the second half of the vegetation – with higher activity in *Q. cerris* leaves (Fig. 1c). The most pronounced differences between the studied species with respect to GR activity was observed in September for 15-year-old trees, where *Q. cerris* leaves have shown higher enzyme activity than those of *Q. frainetto* (Fig. 1d).

Table 1

Mean values of the enzyme activity of oak trees by seasons and results from Bonferroni's multiple range test at $P < 0.05$. The values indicated with the same letter are not significantly different

Variable \ Factor	Season		
	May	**August**	**September**
SOD	9.273 a	20.377 b	5.485 c
APX	0.438 a	0.552 b	0.362 c
CAT	5. 867 a	5. 918 a	5.410 b
GR	0.083 a	0.112 b	0.064 c

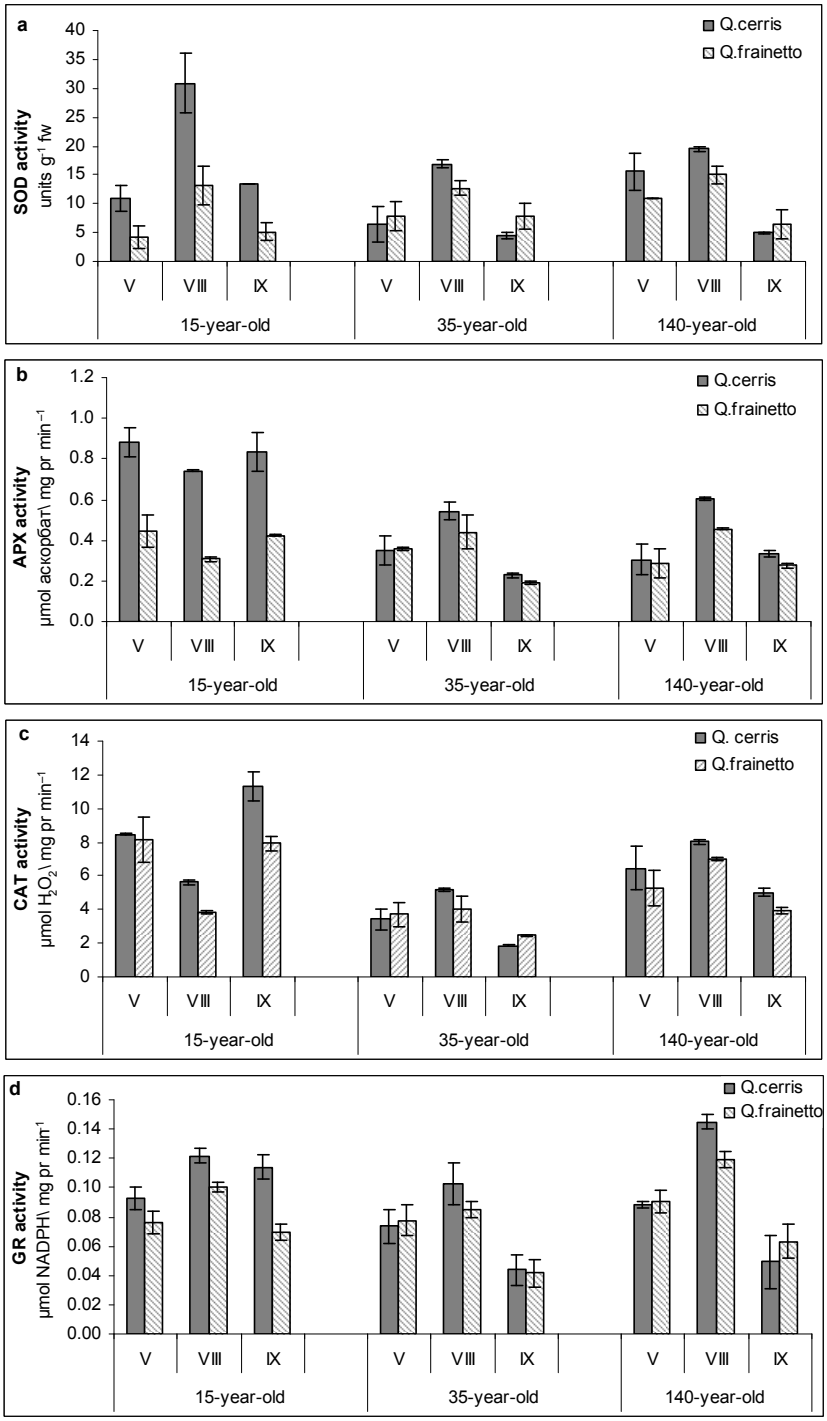

Fig. 1 a-d. Seasonal changes in APX, CAT, SOD and GR activity by age classes in oak leaves. The averages of at least 6 measurements ± SD are shown, $P<0.05$ by tree species

Table 2

Mean values of enzyme activity and results from Bonferroni's multiple range test at P < 0.05 of the oak trees by ages. The values indicated with the same letter are not significantly different

Variable \ Factor	Age		
	15-year-old	**35-year-old**	**140-year-old**
SOD	11.365 a	9.337 a	14.434 b
APX	0.605 a	0.358 b	0.489 b
CAT	7.555 a	3.486 b	6.154 c
GR	0.096 a	0.071 b	0.093 a

Table 3

Mean values of the enzyme activity of the oak trees by species.
Significance of the difference * - P < 0.05

Variable \ Factor	Species		
	Q. cerris	***Q.frainetto***	***Difference***
SOD	12.525	10.899	1.627 *
APX	0.543	0.358	0.186 *
CAT	6.147	5.316	0.831 *
GR	0.091	0.082	0.0087 *

DISCUSSION

Biochemically, the metabolism of plant cells under stress is generally characterized by an increased formation of reactive oxygen species (Mittler, 2002; Edreva, 2005). ROS accumulation could be an 'alarm' signal that initiates pre-emptive defense responses (Shao et al., 2005). The primary toxicity of ROS was considered to reside in their ability to initiate cascade reactions that resulted in the production of the hydroxyl radical and other destructive species such as lipid peroxides. Accordingly, plants evolved an entire set of antioxidative mechanisms of the cell, including non-enzymatic antioxidant mechanisms and ROS-scavenging enzymes such as SOD, CAT, APX and GR (Türkan et al., 2005). SOD can convert the toxic $O_2^{\cdot -}$ to H_2O_2, and CAT can eliminate H_2O_2 by breaking it down into water (H_2O) and molecular oxygen (O_2), thus averting the cellular damage under the unfavourable conditions during summer. Stress factors such as high light intensity, drought and air pollution increase antioxidant enzyme activity, which controls ROS concentration (Foyer, Harbinson, 1994). Confirming this result, in the present study it was found that the activity of SOD, CAT, APX and GR increased in the leaves of 35- and 140-year-old populations (Fig. 1a-d, Table 1) as a reaction to intensive

solar radiation and reduced soil moisture in August (Koynarliyska, 2007). This indicates that *Q. cerris* and *Q. frainetto* adopt biochemical adaptive mechanisms under drought stress in order to regulate the redox status in their foliage. As in the present study on two oak species, an excessive activity of SOD and GR in different intracellular organelles was also observed in the leaves of plants and deciduous trees (Perl-Treves, Galun, 1991; Tsang et al., 1991; Polle at al., 1992). Similarly, the enzyme activity has been triggered by unfavourable environmental stresses. Summer is the time with the highest ozone concentrations in the region of Staro Oriahovo (Koynarliyska, Tzvetkova, 2008). Hence, beside the prolonged drought periods, ozone concentration probably contributes to the high activity of the studied enzymes in oak leave tissues in August. An enhanced effect of ozone concentration on the activity of ROS-scavenging enzymes, such as SOD and GR, has also been reported for other tree species (Castillo et al., 1987; Sen Gupta et al., 1991; Polle at al., 1992).

CAT and APX play a key role in the scavenging of H_2O_2, which is produced through dismutation of $O_2^{\cdot -}$, catalized by SOD. The prominently higher level of CAT and APX in 35- and 140-year old oak leaves as found in this work might be interpreted as a higher capacity to decompose H_2O_2 more rapidly under the stressful conditions during the summer.

However, a slightly lower activity of these enzymes was observed in the leaves of the youngest oak trees in August. As has been outlined in the review of Willekens et al., (1995), H_2O_2 is one of the major contributors to oxidative stress and its accumulation could lead to oxidative reactions which provoke an irreversibly damage to cellular components and, as such, contribute to macroscopic effects of stress, including growth retardation, leaf injury and reduction of photosynthetic activity (Quartacci, Navaro-Izzo, 1992). It can inactivate some enzymes in Calvin cycle directly by oxidizing their sulphhydryl groups (Kaiser, 1979). It can be assumed that the high activity of SOD results in an excessive H_2O_2 accumulation in the leaves of 15-year-old oak trees during summer stress conditions in Staro Oriahovo region. However, the H_2O_2 scavenging enzymes succeed to minimize its toxic effect. This can be seen by the high photosynthetic activity in the leaves of the youngest trees measured in August (Koynarliyska, Tzvetkova, 2008), which suggests that CAT and APX maintain the concentration of H_2O_2 at a harmless level. Furthermore, Yang at al. (2008) found that under high light intensity, seedlings subject to drought exhibit a significant enhancement in H_2O_2 level but oxidative damage was not observed.

The higher activity of antioxidative enzymes and non-enzyme antioxidants in the cell tissue in stress conditions is an indicator of the species' hardiness. Our results on seasonal changes in enzyme activity show ambiguous tendencies with respect to tree age. Specific variations of the two oak species' adaptive capacities to environmental changes were observed on a biochemical level. The higher activity of the antioxidative enzymes in the leaves of all age groups of *Q. cerris* may be instrumental in the more effective protection against oxidative stress. Furthermore, a better stress adaptability of 15-year-old *Q. Cerris* trees can be inferred from the fact that the found inter-species differences are most pronounced for the youngest individuals.

CONCLUSION

The present study supports the hypothesis that the stress conditions during summer exceed the scavenging capacity of the antioxidative enzymes. A clearly determinable correlation between tree age and changes of SOD, CAT, APX and GR activity has not been observed. Based on the increased enzyme activity in *Q. cerris* leaves of all age groups it is concluded that this species has a better adaptive capacity in comparison to *Q. frainetto*. In addition, the youngest *Q. cerris* trees have a better antioxidative protection against the effects of oxidative damage on leave tissues.

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