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Guaiacol Peroxidases and Photosynthetic Pigments during Maturation of Spruce Needles*

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Changes in the guaiacol peroxidases activity, electrophoretic pattern, photosynthetic pigments content and photosystem II efficiency in spruce (*Picea abies* L. Karst.) needles of different ages were investigated. Four generations of needles (current-year, one-, two- and three-year-old) were collected from ten Norway spruce trees. Similar F_v/F_m values (the ratio of variable and maximal fluorescence) in all investigated needles indicate that they were healthy with a fully functional photosystem II. There was a significant increase in photosynthetic pigments content during the first year of development. Total guaiacol peroxidases activity was increasing continuously during needles maturation. Significant differences were recorded between the current-year and older needles, as well as between the one-year-old and three-year-old needles. Also, the expression of new isoforms of peroxidases was observed in one-year-old needles compared to current-year needles. No differences in the isoperoxidase pattern were observed between one-, two- and three-year-old needles. The obtained results show that Norway spruce needles were subject to most pronounced biochemical and molecular changes during the first year of development.

Keywords
guaiacol peroxidases
photosynthetic pigments
ageing needles
Picea abies
F_v/F_m

INTRODUCTION

Needles of coniferous trees are long-living plant organs, existing for several years on healthy trees where they are exposed to a variety of stressful influences. Different environmental conditions such as low temperatures,[1] oxygen deficiency[2] or excess light[3] are associated with formation of reactive oxygen species (ROS). Increase in the generation of ROS in plant cells is a common feature of all different stress factors.[4] ROS include superoxide radicals ($O_2^{\cdot -}$), hydroxyl ($^{\cdot}OH$) or peroxyl radicals ($ROO^{\cdot}$) and molecules like singlet oxygen ($^1O_2$), ozone (O_3) or hydrogen peroxide (H_2O_2). High production of ROS causes oxidative damage to the cell or tissue.

In plant cells, chloroplasts are considerable sources of ROS. Three major processes that include participation of oxygen are intermediated by activated oxygen and they occur on PSII, PSI and photorespiratory chain.[4] Photosystem I (PSI) is involved in direct reduction of molecular oxygen. Excess light can cause an increased reduction of PSI. When CO_2 concentration is low, which can be due to environmental conditions or closed stomata, a large amount of O_2 can compete for electrons from PSI. This results in increased generation of superoxide radicals via the Mehler reaction and eventually in H_2O_2 production.[5,6] Some components of photosystem II (PSII) are also capable of transforming molecular oxy-

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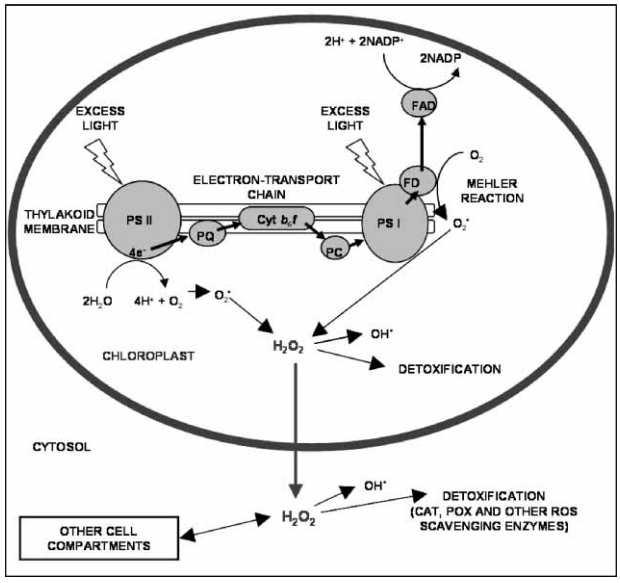

Figure 1. Generation of ROS in chloroplasts and its diffusion into the cytosol and other cell compartments.

gen into singlet oxygen ($^1O_2$), which can be further transformed into H_2O_2 (Figure 1). An increased concentration of H_2O_2 can lead to its diffusion into the cytosol where peroxidases and other ROS scavenging enzymes are employed for its detoxification (Figure 1).

During the ageing of needles and under different stress factors, changes in oxidative metabolism take place in plant tissues. These events increase the concentration of ROS,[5] which can react with proteins, lipids, pigments and other cellular components and cause their dysfunction and degradation. To prevent this effect, plants have developed very efficient enzymatic and non-enzymatic antioxidative defense systems. The main enzymatic components of the defense system are superoxide dismutase, catalase, glutathione reductase and peroxidases.

Peroxidases (EC 1.11.1.7) play an important role in plant defense against harmful effects of ROS. These enzymes are heme-containing glycoproteins and are involved in many physiological processes in plants: auxin catabolism, changes of cell walls (lignification, suberinization), wound healing and defense systems.[7,8] Peroxidases also control the level of H_2O_2 and protect cells affected by some stressful influence. Microclimate and tree age are assumed to be the factors that change the capacity of defense against oxidative stress. Previous investigations showed that large amounts of free radicals are released during the ageing period in peroxisomes of plant cells.[9] The aim of this study was to investigate the changes in guaiacol peroxidases activity and their electrophoretic pattern, photosynthetic pigments content as well as PSII efficiency in spruce needles of different age.

EXPERIMENTAL

The material for study was collected in July 2004 from ten Norway spruce (*Picea abies* L. Karst.) trees grown in the city of Osijek (Croatia). Four generations of needles (CY, current-year; 1-YO, one-year-old; 2-YO, two-year-old; 3-YO, three-year-old) were collected from the lower crown of investigated spruce trees.

Needles were cut into small pieces and powdered in liquid nitrogen. Photosynthetic pigments were extracted with anhydrous acetone (ρ = 0.79 kg L^{-1}). Absorbance was measured at 661.6, 644.8 and 470 nm using an Analytik Jena Specord 40 spectrophotometer. Concentrations of chlorophyll *a, b* and total carotenoids were calculated according to Lichtenthaler.[10] Maximum quantum yield of PSII (F_v/F_m) was determined by measuring the *in vivo* chlorophyll fluorescence.[11] Measurements of chlorophyll fluorescence were performed with a pulse-amplitude-modulated photosynthesis yield analyzer (Mini-PAM, Waltz).

Soluble proteins were extracted in 0.1 M Tris-HCl buffer, pH = 8.0, containing polyvinylpyrrolidone (PVP) at 4 °C. Protein content was determined according to Bradford[12] using bovine serum albumin (BSA) as a standard. Total guaiacol peroxidases (EC 1.11.1.7) activity was determined spectrophotometrically by measuring the absorbance increase at 470 nm. The reaction mixture contained 5×10^{-3} M guaiacol and 5×10^{-3} M H_2O_2 in 0.2 M phosphate buffer, pH = 5.8.[13] The reaction was started by adding 200 μL of protein extract to 800 μL of reaction mixture.

Analysis of the anionic isoperoxidase pattern was performed using vertical native polyacrylamide gel electrophoresis (PAGE). Preparation of 1 mm thick stacking (2.5 %) and separating (12 %) polyacrylamide gels and buffer system was done as described by Laemmli[14] omitting the SDS. The isoperoxidase bands were visualized by immersing the gel in the same reaction mixture as used for peroxidase activity determination. Specific isoform was determined as the coefficient obtained by calculating the ratio of isoform distance to front distance from the start on the gel.

The data were analyzed using the *t*-test modified for small samples.[15] Each needle generation was treated as a single statistical sample; it contained needles from 10 Norway spruce trees processed separately (n = 10). Cluster analysis was made using the program Statistica 6.0.

RESULTS AND DISCUSSION

Mean values of the photosynthetic pigments concentration in four generations of spruce needles are shown in Table I. The present investigation showed an increase in total chlorophylls and carotenoids concentration during the maturation period. The lowest value of total chlorophylls concentration was measured in current-year needles (1.02 mg/g FW; FW, fresh weight). The total chlorophylls concentration in every generation of older needles was significantly higher compared to current-year needles. Differences in chlorophyll pigment concentrations between one-, two- and three-year-old needles

TABLE I. The photosynthetic pigments concentration and photosystem II efficiency in four generations of Norway spruce (*Picea abies* L. Karst.) needles[a],[b]

Samples	Pigments conc. / mg g^{-1} FW			
	Chl *a* + Chl *b*	Chl *a* / Chl *b*	Car	F_v/F_m
CY	$(1.02\pm0.19)^{1\text{-YO},2\text{-YO},3\text{-YO}}$	1.97±0.63	$(0.15\pm0.04)^{1\text{-YO},2\text{-YO},3\text{-YO}}$	$(0.82\pm0.01)^{2\text{-YO, }3\text{-YO}}$
1-YO	$(1.55\pm0.27)^{\text{CY}}$	1.90±0.76	$(0.23\pm0.08)^{\text{CY}}$	$(0.82\pm0.01)^{2\text{-YO, }3\text{-YO}}$
2-YO	$(1.55\pm0.20)^{\text{CY}}$	1.81±0.74	$(0.22\pm0.07)^{\text{CY}}$	$(0.80\pm0.02)^{\text{CY, }1\text{-YO}}$
3-YO	$(1.56\pm0.28)^{\text{CY}}$	2.23±0.19	$(0.28\pm0.04)^{\text{CY}}$	$(0.79\pm0.02)^{\text{CY,}1\text{-YO}}$

[a] Mean values ± SD of ten samples (n = 10) for each needle generation are given. Significant differences between investigated samples are marked in superscript.
[b] FW, fresh weight; Chl *a* + Chl *b*, total chlorophyll; Chl *a* / Chl *b*, chlorophyll *a* to *b* ratio; Car, total carotenoids; F_v/F_m, maximum quantum yield of PSII; CY, current-year needles; 1-YO, one-year-old needles; 2-YO, two-year-old needles; 3-YO, three-year-old needles.

were not significant. This may be a consequence of the terminated development of needles. Yooa *et al.*[16] used the leaf chlorophylls content as an indicator of leaf maturity during the development of white clover (*Trifolium repens L.*) leaves. They observed an increase of chlorophylls content during leaf maturation as well as its decrease accompanying leaf senescence. Total chlorophylls content can be changed as a consequence of different influences, such as air pollution,[17–21] disturbed mineral nutrition[22–24] and natural senescence.[25] Photosynthetic pigments associated with the electron transport system are the primary source of singlet oxygen, which is highly toxic and reacts with biological molecules.[4] Differences observed in chlorophyll *a* to chlorophyll *b* ratio between the needles of all generations were not significant. Chlorophyll *b* is synthesized from chlorophyll *a* by a specific oxygenase.[26] It seems that the reaction can also go in opposite direction by the activity of chlorophyll *b* reductase.[27,28] Environmental changes and stressful conditions might cause compensation of chlorophyll *a* content from chlorophyll *b* pool and thereby change the chlorophyll *a* to *b* ratio. Since this was not the case here, we may speculate that older needles were not exposed to elevated stress conditions. To confirm this, we measured the maximum quantum yield of PSII (F_v/F_m, the ratio of variable and maximal fluorescence), which is one of the main parameters that indicate elevated stress in plants.[29] Needles of all generations were shown to have similar F_v/F_m values: 0.79–0.82 (Table I), which indicated that all investigated needles were healthy with a fully functional PSII. Increase in total carotenoids concentration was significant for every generation of older needles compared to current-year needles. There were no significant differences in total carotenoids content between needles of older generations (Table I). Besides light harvesting, carotenoids are known to play an important role in protecting the photosynthetic membranes against destruction at high light intensity,[30] especially the carotenoids involved in the xanthophyll cycle[31].

Peroxidase activity measurement revealed activity changes with the needles age. Total peroxidase activity was increasing continuously during needle ageing (Figure 2). The lowest value was recorded in CY (3.6 ΔA_{470} min^{-1} g^{-1} DW; DW, dry weight), and the values in older needles were: 5.7, 8.1 and 8.9 ΔA_{470} min^{-1} g^{-1} DW in 1-YO, 2-YO and 3-YO, respectively. Statistical significance was determined between the current-year and older needles, as well as between the one-year-old and three-year-old needles. The increase in total peroxidase activity might occur as a result of oxidative damage due to processes that happened during ageing and natural senescence.[7,8,32,33] According to Del Rio *et al.*,[9] ageing causes changes in oxidative metabolism, influencing the activity of other antioxidative enzymes in peroxisomes such as superoxide dismutase and catalase. Ageing induces overproduction of H_2O_2, which can freely diffuse into the cytosol and cause an increase in peroxidase activity (Figure 1). Also, it is known that different environ-

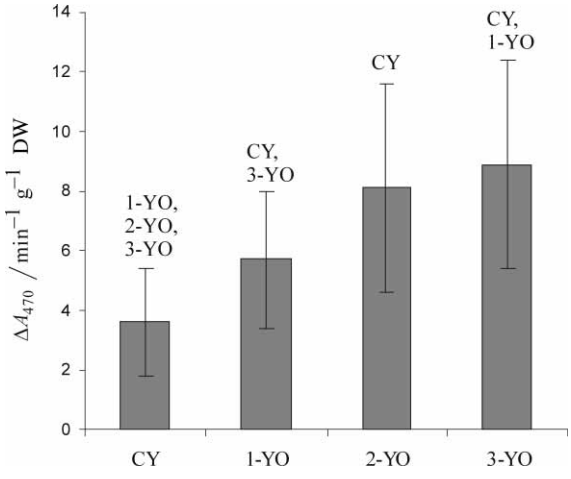

Figure 2. Mean values of total guaiacol peroxidases activity (ΔA_{470} / $min^{-1}mg^{-1}$ DW) in the current-year (CY), one-year-old (1-YO), two-year-old (2-YO) and three-year-old (3-YO) needles of Norway spruce. Bars represent standard deviations (n = 10). Significant differences between investigated samples are marked on top of the bars.

TABLE II. Distribution of anionic isoperoxidase (1 to 10) in the current-year (CY) and older needles (O) of ten examined trees (A to J) of Norway spruce (*Picea abies* L. Karst.)[a],[b]

	A		B		C		D		E		F		G		H		I		J	
	CY	O	CY	O	CY	O	CY	O	CY	O	CY	O	CY	O	CY	O	CY	O	CY	O
1	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
2	+	+	–	–	+	+	–	–	–	–	–	+	+	+	–	–	+	+	–	+
3	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	–	–
4	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	+	+
6	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–
7	–	+	–	–	+	+	+	+	–	+	+	+	+	+	+	+	–	–	+	+
8	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
9	–	–	+	+	–	–	+	+	+	+	–	–	–	–	–	–	+	+	–	–
10	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Within each tree there was no difference in the number of expressed isoperoxidase in ageing period after the first year. Therefore, the data for 1-YO, 2-YO and 3-YO are given together (O).
[b] The presence (+) and absence (–) of specific isoperoxidase are indicated.

mental conditions[7] and air pollutions[34–36] cause an increase in peroxidase activity. The connection between ROS signaling and sensing of the environmental conditions is established by chloroplasts.[37] Changes of environmental variables can affect the photosynthetic electron transport in chloroplasts, resulting in a modified redox state of chloroplasts. This, in turn, regulates the expression of nuclear and chloroplast genes, as well as the activity of ROS scavenging enzymes, such as peroxidases.

However, there is a great variability in peroxidase activity between individual spruce trees (Figure 2).

Electrophoretic analysis revealed differences in the isoperoxidase pattern as well as in the intensity of individual bands between the current-year and older needles.

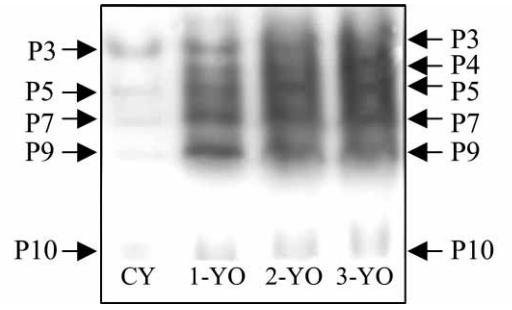

Figure 3. The example showing the isoenzyme profile of anionic guaiacol peroxidases in four needle generations of a single Norway spruce tree. Six isoperoxidases (P3, P4, P5, P7, P9 and P10) were present in the needles. CY, current-year needles; 1-YO, one-year-old needles; 2-YO, two-year-old; 3-YO, three-year-old needles.

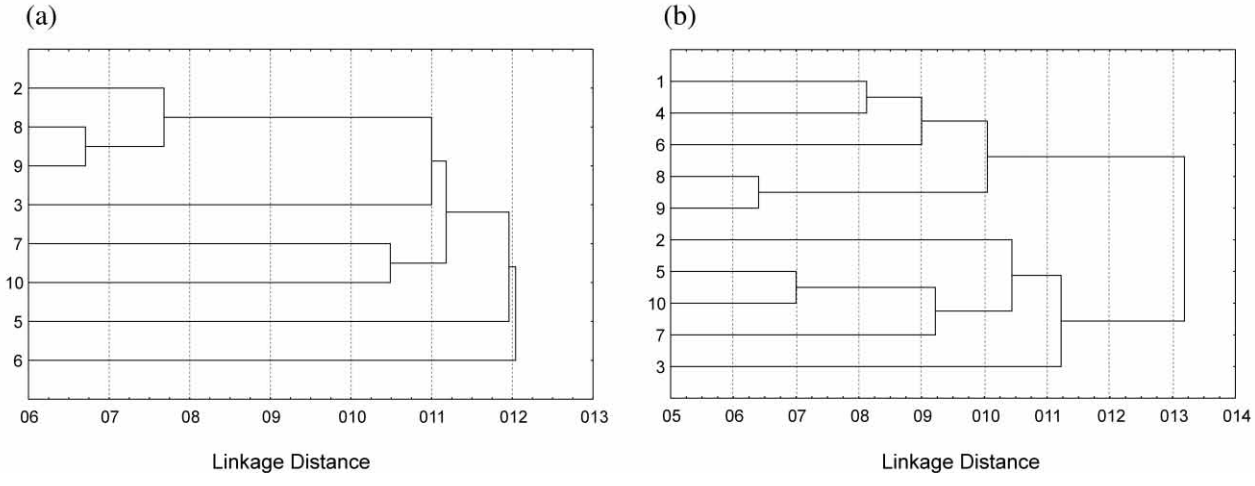

Figure 4. Dendrograms based on the presence of peroxidase isoenzymes (1–10) in the current-year (a) and older needles (b) of Norway spruce. Two main clusters appeared in older needles.

One new isoperoxidase band was observed in each tree, except in tree A (Table II). Isoforms 1 and 4 were not present in current-year needles, nor in older needles taken from trees D and G. Example of the isoperoxidase pattern in four generations of spruce needles taken from the same single tree is shown in Figure 3. There were considerable differences in the electrophoretic pattern of isoperoxidases between trees, probably because of their natural variability.[33] New isoperoxidases were induced due to pollution,[32] different environmental conditions[38] or senescence.[39] There was no difference in the isoperoxidase pattern in one-year-old needles compared to older generations (Figure 3). In older needles, peroxidase isoenzymes were grouped into two main clusters (Figure 4). These two clusters could not be distinguished in the current-year needles.

CONCLUSIONS

Our investigation showed that the most pronounced biochemical changes occurred during the first year of needle development. In that period, total chlorophylls and carotenoids increased significantly. The maximum quantum yield of PSII values around 0.81 indicated that all investigated needles were healthy with a fully functional PSII. Total peroxidase activity was significantly increased between the current-year and one-year-old needles, while differences between older needles were significant only between the one-year-old and three-year-old needles. The expression of new isoperoxidases also occurred in the first year of needle development. Later on, after maturation, no changes in the isoperoxidase pattern were observed. In that period, only changes in the intensity of individual bands were noticed. It can be concluded that the most pronounced changes in biochemical and molecular properties of spruce needles occurred during the first year of development.

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SAŽETAK

Gvajakol-peroksidaze i fotosintetski pigmenti u različito starim iglicama smreke

Hrvoje Lepeduš, Vlatka Gaća i Vera Cesar

Proučavane su promjene u aktivnosti gvajakol-peroksidaza, u elektroforetskome sastavu, u sadržaju fotosintetskih pigmenata i učinkovitosti fotosustava II u iglicama smreke (*Picea abies* L. Karst.) različite starosti. Četiri generacije iglica (ovogodišnje, jedno-, dvo- i trogodišnje) prikupljene su s deset stabala smreke. Slične F_v/F_m vrijednosti u svim ispitivanim iglicama pokazuju da su zdrave s potpuno funkcionalnim fotosustavom II. Značajno povećanje sadržaja fotosintetskih pigmenata se dogodilo tijekom prve godine razvoja. Ukupna aktivnost gvajakol-peroksidaza povećavala se kontinuirano tijekom starenja iglica. Statistička značajnost utvrđena je između ovogodišnjih i starijih iglica te između jednogodišnjih i trogodišnjih iglica. Pojava novih izoformi peroksidaza uočena je u jednogodišnjim iglicama u odnosu na ovogodišnje iglice. Između jednogodišnjih, dvogodišnjih i trogodišnjih iglica nisu uočene promjene u sastavu izoformi peroksidaza. Dobiveni rezultati pokazuju da iglice smreke podliježu najintenzivnijim biokemijskim i molekularnim promjenama tijekom prve godine razvoja.